TOXICITY OF PETROLEUM HYDROCARBONS IN POLAR SOIL

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By

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ABSTRACT

The objective of this research is to determine the influence of liquid water content on the toxicity of petroleum hydrocarbons (PHC) to soil microorganisms in frozen soil. This research was conducted on soil collected from an aged diesel fuel spill site at Casey Station, East Antarctica, as well as on spiked diesel contaminated soil from Macquarie Island, a sub-Antarctic island.

Suitable soil biogeochemical toxicity endpoints for PHC contamination were identified using sub-Antarctic soil from Macquarie Island spiked with diesel fuel. The sensitivity of nitrification, denitrification, carbohydrate utilization and total soil respiration to diesel fuel was assessed. Potential nitrification activity (PNA) was the most sensitive indicator of contamination assessed for nitrogen cycling, with a PHC concentration effecting microbial activity by 20% of the control response, EC_{20}, of 190 mg PHC kg^{-1} soil.

Petroleum hydrocarbon toxicity in polar soil was assessed by sampling 32 locations at an aged diesel spill site at Casey Station, East Antarctica. Samples were taken nine times throughout an austral summer to encompass frozen, thaw and refreeze periods. Toxicity was assessed using potential activities of substrate induced respiration, total respiration, nitrification, denitrification, and metabolic quotient, as well as microbial community composition and bacterial biomass. The most sensitive indicator was community composition with an EC_{25} of 800 mg kg^{-1}, followed by nitrification (2000 mg kg^{-1}), microbial biomass (2400 mg kg^{-1}) and soil respiration (3500 mg kg^{-1}). Despite changes in potential microbial activities and composition over the frozen/thaw/refreeze period, the sensitivity of these endpoints to PHC did not change with liquid water or temperature.

The influence of liquid water (θ_{liquid}) on nutrient supply rate and gas diffusion, which are important factors in microbial degradation of PHC, was determined using contaminated soil from Casey Station. Freezing reduced nutrient supply rate of both NH_4^+ and NO_3^-. However, an increase in θ_{liquid} was linked to increases in nitrate and ammonia nutrient supply rates in frozen soil. Similarly for gas diffusion, decreases in D_s due to freezing were much more pronounced in soils with low θ_{liquid} compared to soils with higher θ_{liquid} contents. Further research is needed to determine whether bioremediation in cold regions could be enhanced during the period of time where the soil temperature is below 0^oC by controlling factors that increase the amount of liquid water.

The influence of liquid water content on the *in situ* toxicity of PHC to soil microorganisms was evaluated using stable isotope dilution technique to measure gross mineralization and nitrification, which was compared to the toxicity endpoints of potential microbial activities. Liquid water content did not have a significant effect on either gross mineralization or nitrification. Gross nitrification was sensitive to PHC contamination, with toxicity decreasing over time. The EC_{25} value for gross nitrification was 400 mg kg^{-1} for 1 month incubation period. In contrast, gross N mineralization was not sensitive to PHC contamination. Toxic response of gross nitrification to PHC contamination was comparable to PNA with similar EC_{25} values determined by both measurement endpoints (400 mg kg^{-1} for *in situ* nitrification compared to 200 mg kg^{-1} for PNA), indicating that potential microbial activity assays are good surrogates for *in situ* toxicity of PHC contamination in Polar Regions. Based on ecotoxicological data collected, the recommended soil quality guideline for on PHC contamination in polar soils would be 200 mg kg^{-1}.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

θ_{liquid} - liquid water

` - minutes

^{o} – degrees

^{o}C – degrees Celsius

^{o}K – degrees Kelvin

µg – microgram

µL – microliter

µm - micrometre

µs – microsecond

Ω – ohm

AIC – Akaike's information criterion

APE – atom percent excess

AMO – ammonium monooxygenase

As – arsenic

BD – below detection

C – carbon

$CaCl_2$ – calcium chloride

CCME – Canadian Council of Ministers for the Environment

Cd – cadmium

cm - centimetre

CO_2 – carbon dioxide

CO_3 – carbonate

Cr – chromium

Cu - copper

CV – coefficient of variation

d – day

D_s – gas diffusion coefficient in soil

d. wt. – dry weight

E - east

EC_p –concentration that is estimated to cause a toxic response to a certain percentage of test organisms

FID – flame ionization detector

g - gram

GC – gas chromatograph

GHz – giga Hertz

GLM – general linear model

GWC – gravimetric water content

h – hour

HCl – hydrochloric acid

He – helium

H_2O – water

J – joules

K_{air} – dielectric constant of air

K_{ice} – dielectric constant of ice

K_{ow} – octonal-water partition coefficient

K_{soil} – dielectric constant of soil

K_{water} – dielectric constant of water

$KC_2H_3O_2$ – potassium acetate

KCl – potassium chloride

kGy – kilo Grey

KH_2PO_4 - potassium dihydrogen phosphate

KNO_3 – potassium nitrate

kPa – kilo Pascal

L - litre

$LiNO_3$ – lithium nitrate

M – mole per litre

m – metre

MANOVA – multiple analysis of variance

MED – molarity of ethanol droplet

mg - milligram

MHz – mega Hertz

min – minute

mL - millilitre

mM – milli mole per litre

mm – millimetre

mol - mole

MPH – main powerhouse

MRPP – multi response permutation procedure

ms – millisecond

N – nitrogen

NA – not available

$NaClO_3$ – sodium chlorate

$NaNO_3$ – sodium nitrate

nM – nano mole per litre

NaOH – sodium hydroxide

NAPL – non-aqueous phase liquid

NaR – nitrate reductase

ng - nanogram

NH_3 - ammonia

NH_4 - ammonium

$(NH_4)_2SO_4$ – ammonium sulphate

Ni - nickel

NiR – nitrite reductase

nmol - nanomole

NMS – nonmetric multidimensional scaling

NO_2 - nitrite

NO_3 - nitrate

N_2O – nitrous oxide

NOR – nitric oxide reductase

N_2OR – nitrous oxide reductase

NSR – nutrient supply rate

OH - hydroxide

P – phosphorus

PAH – polycyclic aromatic hydrocarbon

Pb - lead

PDA – potential denitrification activity

PHC – petroleum hydrocarbon

PLFA – phospholipid fatty acid

PNA – potential nitrification activity

ppbV – parts per billion volume

ppm - parts per million

ppmV – parts per million volume

PSA – particle size analysis

rpm – rotations per minute

ROS – reactive oxygen species

S – south

s – second

SAB – Special Antarctic Blend

SD – standard deviation

SE – standard error

SIR – substrate induced respiration

SOM – soil organic matter

SSE – sum squared errors

TDR – time domain reflectometry

TiCl – titanium chloride

TNT – 2,4,6-trinitrotoluene

TOC – total organic carbon

TPH – total petroleum hydrocarbons

V – volts

v – volume

CHAPTER 1

Literature Review

1.1. Introduction

Human activity in Polar Regions is reliant on petroleum hydrocarbons (PHC) for power generation, heating and transportation and with its use comes the potential for environmental contamination. Many accidental spills of PHC, as well as improper disposal in the past, have led to numerous contaminated sites in Polar Regions, especially around military bases, oil reserves, and mining operations in the Arctic (AMAP, 1998a; Whyte et al., 1999), as well as scientific bases in the Antarctic (Aislabie et al., 2004; Poland et al., 2003). Petroleum hydrocarbon contamination is the most common type of terrestrial pollution, with over 20 000 documented contaminated sites in Polar Regions (Snape et al., 2008b). These sites span eight circumpolar Arctic nations including Canada, United States (Alaska), Iceland, Sweden, Norway, Finland, Russia, Denmark (Greenland), as well as Antarctica. This number of contaminated sites seems staggering, considering that the Polar Regions are often viewed as one of the last pristine places on earth. However, even this estimate may be low due to the inaccuracy of reporting spills and the actual number of contaminated sites may well exceed 20 000 (Snape et al., 2008b).

Terrestrial PHC contamination can have detrimental effects on the biological, physical, and chemical properties of the soil. Petroleum hydrocarbons spilled onto permafrost can influence the microbial populations (Aislabie et al., 2001; Atlas, 1981), freeze-thaw processes (Grechishchev et al., 2001), thermal and moisture regimes (Balks et al., 2002), as well as the soil pH and nutrient availability (Aislabie et al., 2004; Everett, 1978). Contamination can have a greater impact on ecosystems in Polar Regions than in other environments, as these ecosystems have adapted to harsh conditions in ways that could make them more sensitive (Snape et al.,

2003). Limited liquid water content in polar soil may influence the toxicity of PHC contamination in these ecosystems by affecting availability of nutrients and contaminants.

Many countries have begun the process of cleaning up and monitoring contaminated sites in Polar Regions. This effort is essential to protect the environment including soil, water, plants and animals, as well as the people who live near these sites (Atlas and Cerniglia, 1995). In Polar Regions, most contaminated sites occur in remote isolated locations, making remediation very expensive. Bioremediation is a promising option as it is effective at removing hydrocarbons with limited disturbance to the environment and is a relatively low-cost option (Filler et al., 2006). Bioremediation techniques, which rely on native or introduced soil microorganisms capable of using PHC as a carbon or energy source, have been successfully used in Polar Regions (Horel and Schiewer, 2009; Paudyn et al., 2008; Sanscartier et al., 2009; Snape et al., 2008a).

As the technology for cleaning up contaminated sites in Polar Regions emerges, new questions associated with the clean up of these sites also arise. How clean is clean enough? Are remediation guidelines derived from temperate climates relevant to Polar Regions? What is the most sensitive part of the ecosystem? What biological activity should be monitored to ensure that the ecosystem is being protected? Do the environmental properties associated with Polar Regions increase the sensitivity of the ecosystem? How does the toxicity change over time? These concerns regarding the toxicity of PHC are important to address, as the rate of PHC contamination in these regions is expected to increase in the future.

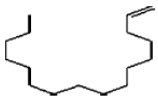
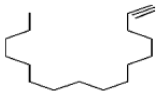
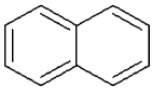
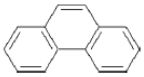
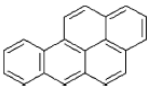
1.2 Properties of petroleum hydrocarbons

Hydrocarbons spilled on polar soils are usually refined petroleum products such as diesel or aviation fuel, comprised of aliphatic and aromatic compounds (Aislabie et al., 2006). Aliphatic hydrocarbons are non-aromatic, non-cyclic organic compounds containing carbon and hydrogen. They can be subdivided into three structurally different groups: (i) alkanes – saturated hydrocarbons with single C-C bonds; (ii) alkenes – unsaturated hydrocarbons containing double C=C bonding; and (iii) alkynes – unsaturated hydrocarbons containing a triple C≡C bond (Table 1.1). Aromatic compounds are conjugated cyclic structures typically composed of one or more benzene rings, and are commonly referred to as polycyclic aromatic hydrocarbons (PAHs). A comparison between the properties of aliphatic and aromatic hydrocarbons is shown in Table 1.1.

Aromatic hydrocarbons generally have higher melting and boiling points than aliphatic compounds, which means they are less volatile. The degree of volatility generally decreases between aliphatic compounds in order of alkane > alkene > alkyne. Therefore, hydrocarbons with single carbon bonds are the most volatile, and are often the first type of compound lost from contaminated soil. The number of C atoms within the molecule also influences the volatility of the compound. The high volatility of low molecular weight compounds, i.e. compounds with a relatively small number of C atoms, results in partitioning of a fraction of these compounds into the gas phase. Thus, low molecular weight aliphatic hydrocarbons are lost from the soil via volatilization.

Most compounds typically have low water solubility, making them less likely to leach from the soil. A fundamental property of these compounds is the octanol-water partition coefficient (K_{ow}), which describes the hydrophobicity of the compound. This property can be used to

Table 1.1 Physiochemical properties of selected hydrocarbons (Howard and Meyln, 1997; Stroud et al., 2007; Verschueren, 1983)

Hydrocarbon group		Name	Formula	Molecular weight ($g\ mol^{-1}$)	Structure	Melting point (^oC)	Boiling point (^oC)	Solubility at 25^oC ($mg\ L^{-1}$)	Log K_{ow}
Aliphatic	Alkane	Hexadecane	$C_{16}H_{34}$	226.44		18	287	0.0009	9.1
	Alkene	Hexadecene	$C_{16}H_{32}$	224.43		3-5	274	0.00123	N/A
	Alkyne	Hexadecyne	$C_{16}H_{30}$	222.42		15	148	N/A	N/A
Aromatic	PAH	Naphthalene	$C_{10}H_8$	128.18		79-83	217.9	30	3.36
	PAH	Phenanthrene	$C_{14}H_{10}$	178.22		97-101	340	1.1	4.16
	PAH	Benzo(a) Pyrene	$C_{20}H_{12}$	252.31		175-179	495	0.0038	6.06

K_{ow}, octonal-water partition coefficient

PAH, polycyclic aromatic hydrocarbon

N/A, Data not available

predict the fate and transport of the compound in the environment, as it affects sequestration and both chemical and biological availability (Reid et al., 2000).

The non-polar nature of these compounds results in partitioning or sorption onto organic matter in soil. The extent to which a chemical partitions into the organic matter is also described by K_{ow} (Bressler and Gray, 2003). When a compound is absorbed strongly to soil organic matter, the bioavailability tends to decline, therefore impeding biodegradation (Yang et al., 2009). Migration is also impeded for PHC compounds bound tightly to the soil organic matter. Therefore, biodegradation rate and migration of PHC compounds is dependent on amount of soil organic matter and chemical properties of the compound itself.

Hydrocarbons differ in their susceptibility to microbial attack. They are generally ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > branched alkenes > low-molecular-weight aromatics > monoaromatics > cyclic alkanes > PAHs > asphaltenes (Perry, 1984; van Hamme et al., 2003). Because aliphatic compounds are the most prone to degradation, the recalcitrant compounds are generally composed of high molecular weight asphaltenes and PAHs (Margesin and Schinner, 1999a).

Temperature affects the physical nature and chemical composition of hydrocarbons. In a polar environment, low ambient temperatures usually result in increased viscosity of oil, reduced evaporation of volatiles, and increased water solubility (Atlas, 1981; Leahy and Colwell, 1990). In polar soil, PHC are excluded from the forming crystalline ice structure as the soil freezes, resulting in an increased concentration of PHC in the remaining liquid pore water (Barnes et al., 2004). However, once the solubility of the PHC compound has been exceeded, PHC excluded from forming ice will be present as non-aqueous phase liquid, NAPL (discussed in

Section 1.4) (Barnes et al., 2004). These changes in the physical nature of hydrocarbons can increase microbial toxicity and delay the onset of biodegradation (Margesin and Schinner, 2001; Whyte et al., 1998).

1.3 Toxicity of petroleum hydrocarbons

Refined petroleum products, such as diesel fuel, aviation fuel, and gasoline, are complex mixtures of compounds with toxicity dependent on the composition of fuel and percentage of each constituent. Some compounds are carcinogenic, mutagenic, or toxic to humans, animals, plants and microorganisms (Yang et al., 2009). The toxicity of PHC contamination is also dependent on the age of the spill and the amount of chemical weathering and biodegradation that has taken place. Even though recalcitrant compounds are generally composed of the more toxic compounds such as PAHs, these compounds are strongly bound to soil and are not bioavailable. Therefore toxicity of PHC contamination generally decreases as the spill ages (Alexander, 2000).

In general, petroleum hydrocarbons have a narcotic mode of action. Narcotic chemicals act via nonspecific disruption of the cell membrane. These chemicals cause changes in membrane fluidity, integrity and functioning (MacLeod et al., 2004). The disruption of the cell membrane can have detrimental effects on soil invertebrates and microorganisms, especially at low moisture and temperature levels as the regulation of cellular membrane composition is critical for survival in these conditions (Holmstrup et al., 2000).

Low molecular weight PHC compounds are generally more toxic, and have lower soil quality guidelines than high molecular weight compounds. For example, the Canadian Council of Ministers for the Environment (CCME) groups PHC compounds into four fractions based on

the number of C atoms, with fraction having its own soil toxicity (Table 1.2). Fraction F1 and F2 would be considered low molecular weight compounds, whereas F3 and F4 would be considered high molecular weight compounds. Toxicity values listed for soil contact of vascular plants and soil invertebrates are based on the effective concentration that causes a 25% reduction from the control response (EC_{25} values) (Table 1.2) on data compiled from both laboratory and field studies (CCME, 2008). The F2 fraction is the most toxic as indicated by the lowest EC_{25} value of 150 mg kg^{-1}.

In addition to narcosis, or baseline toxicity, enzyme-specific toxic effects also exist for specific groups of soil microorganisms. Nitrifying bacteria are one of the most sensitive groups of soil microorganisms to PHC contamination due to the specific mode of toxic action on ammonium monooxygenase, which is the enzyme responsible for ammonium oxidation. Petroleum hydrocarbons, particularly aliphatic compounds, have been shown to inhibit the monooxygenase enzyme through competitive interactions for low molecular weight hydrocarbons and noncompetitive interactions with high molecular weight hydrocarbons (Hyman et al., 1988; Keener and Arp, 1993). Aliphatic hydrocarbons inhibit nitrification by binding reversibly or irreversibly to the AMO catalytic site and alter enzyme turnover rates (Chang et al., 2002; Keener et al., 1998).

Polycyclic aromatic hydrocarbons also display many specific toxicity mechanisms, mostly involving biochemical activation and subsequent induction of oxidative stress which leads to mutagenic and carcinogenic effects (Douben, 2003). In vertebrate organisms, oxidation of PAHs by cytochrome P450 enzymes is an initial step in the activation process to produce polar

Table 1.2 EC_{25} values (mg kg^{-1}) for petroleum hydrocarbon (PHC) toxicity to vascular plants and soil invertebrates (CCME, 2008). Petroleum hydrocarbon compounds are divided into four fractions according to the length of the carbon chain.

Soil Texture	F1 (C_6-C_{10})	F2 (C_{10}-C_{16})	F3 (C_{16}-C_{34})	F4 (>C_{34})
Fine	210	150	1300	5600
Coarse	210	150	300	2800

biochemically reactive electrophilic species, or the ultimate carcinogenic metabolites. These reactive metabolites are capable of interacting with cellular macromolecules, particularly DNA and proteins (Douben, 2003). Evidence of P450 enzymes exists in aquatic and soil invertebrates, as well as algae, which suggests this mechanism of toxic action does occur in lower organisms (de Knecht et al., 2001; James and Boyle, 1998; Kirso and Irha, 1998). Reactive oxygen species (ROS) generated by PAH metabolism, can lead to membrane damage, uncoupling of electrochemical gradients in membranes, cytotoxicity, and other pathological changes (Segner and Braunbeck, 1998; Winston and Di Giulio, 1991).

The toxicity of PAH compounds in soil has been studied more extensively than aliphatic compounds and, in general, PAH compounds are more toxic than aliphatic PHCs (CCME, 2010). There have been numerous toxicological studies on soil invertebrates (Achazi et al., 1995; Crouau et al., 1999; Environment Canada, 1999; Jensen and Sverdrup, 2001; Sverdrup et al., 2002a; Sverdrup et al., 2002b; Sverdrup et al., 2002c; Sverdrup et al., 2002d) and relatively few on soil bacteria (Sverdrup et al., 2002c). Table 1.3 lists toxicological data for PAH toxicity to soil invertebrates and soil microorganisms. The effective concentration is dependent on the PAH

compound, organism tested, the effect measured and the chosen endpoint. For example, the effective concentration can vary for the same compound and organism if different endpoints are chosen (i.e. EC_{10} = 42 mg kg^{-1} and EC_{50} = 250 mg kg^{-1} for phenanthrene toxicity to nitrification). In addition, the toxicity of the same compound can vary between organisms (i.e. EC_{10} for pyrene is 130 mg kg^{-1} for nitrification and 10 mg kg^{-1} for *Folsomia fimetaria* reproduction).

In addition to biological enhancement of PAH toxicity (i.e. degradation by cyctochrome P450 enzymes to more reactive metabolites), PAH toxicity is further enhanced by exposure to sunlight. Due to their multiple aromatic ring structure, PAHs strongly absorb light in the UV-A (320-400 nm) and UV-B (290-320 nm) regions of the solar spectra (Huang et al., 1993). When exposed to sunlight, two different photochemical mechanisms can occur, which include photo-oxidation (generally photo-modification) and photo-sensitization (Greenberg et al., 1993; Lee, 2003). During a photosensitization reaction, absorption of UV-photons causes excitation of the PAH molecule and leads to triplet-state formation (Huang et al., 1993). When excited, PAHs are highly efficient at promoting formation of singlet oxygen ($^1O_2$) and other ROS, which can cause oxidative damage in biological systems (Girotti, 1983). This process only occurs when PAHs are exposed to organisms in the presence of sunlight. In contrast, during photomodifcation, PAHs are structurally altered to a variety of compounds such as aliphatic and aromatic ketones, aldehydes, epoxides, and quinones (El-Alawi et al., 2002; Huang et al., 1995; Lee, 2003). Many of these photoproducts are more toxic than their parent compounds (El-Alawi et al., 2002; Huang et al., 1993; Huang et al., 1995; Mallakin et al., 2000).

Table 1.3 Toxicity of various polycyclic aromatic hydrocarbons (PAH) compounds for soil bacteria and invertebrates (CCME, 2010).

Organisms	Effect	Endpoint	Effective Concentration (mg kg^{-1})	PAH Compound	Reference
Soil bacteria	Nitrification	EC_{10}[a]	130	Pyrene	Sverdrup et al., 2002c
Soil bacteria	Nitrification	NOEC[b]	24	Fluoranthene	Sverdrup et al., 2002c
Soil bacteria	Nitrification	EC_{10}	13	Fluoranthene	Sverdrup et al., 2002c
Soil bacteria	Genetic diversity	NOEC	3000	Fluoranthene	Sverdrup et al., 2002c
Soil bacteria	Nitrification	NOEC	26	Phenanthrene	Sverdrup et al., 2002c
Soil bacteria	Nitrification	EC_{10}	42	Phenanthrene	Sverdrup et al., 2002c
Soil bacteria	Nitrification	EC_{50}	250	Phenanthrene	Sverdrup et al., 2002c
Soil bacteria	Nitrification	EC_{10}	33	Fluorene	Sverdrup et al., 2002c
Folsomia fimetaria (springtail)	Mortality	LC_{50}[c]	107	Acenaphthene	Sverdrup et al., 2002a
Folsomia fimetaria (springtail)	Reproduction	EC_{10}	31	Acenaphthene	Sverdrup et al., 2002a
Folsomia fimetaria (springtail)	Mortality	LC_{50}	> 980	Benz[a]anthracene	Sverdrup et al., 2002a
Eisenia fetida (earthworm)	Mortality	NOEC	260	Benzo[a]pyrene	Environment Canada, 1999
Eisenia fetida (earthworm)	Mortality	LC_{59}	100	Benzo[a]pyrene	Aschazi et al., 1995
Eisenia fetida (earthworm)	Growth	LC_{12}	10	Benzo[a]pyrene	Aschazi et al., 1995
Folsomia fimetaria (springtail)	Mortality	LC_{50}	> 780	Dibenz[a,h]anthracene	Sverdrup et al., 2002a
Folsomia fimetaria (springtail)	Mortality	LC_{50}	> 1030	Chrysene	Sverdrup et al., 2002a
Enchytraeus crypticus (earthworm)	Reproduction	EC_{50}	55	Fluorene	Sverdrup et al., 2002d
Folsomia fimetaria (springtail)	Reproduction	EC_{10}	> 910	Indeno[1,2,3-c,d]pyrene	Sverdrup et al., 2002a
Folsomia fimetaria (springtail)	Reproduction	EC_{10}	20	Naphthalene	Sverdrup et al., 2002a
Enchytraeus crypticus (earthworm)	Reproduction	NOEC	18	Pyrene	Sverdrup et al., 2002d
Folsomia fimetaria (springtail)	Reproduction	EC_{50}	24	Pyrene	Jensen and Sverdrup, 2001
Folsomia fimetaria (springtail)	Reproduction	EC_{10}	10	Pyrene	Sverdrup et al., 2002b

[a] Effective concentration

[b] No observed adverse effect concentration

[c] Lethal concentration

In addition to these biochemical mechanisms of toxicity, PHC can have indirect toxic effects by altering the physical and chemical properties of the soil. The nutrient status of soil is affected, as carbon content increases while nitrate levels decrease and P-levels remain unaffected (Aislabie et al., 2004). The soil often becomes more anaerobic as movement of oxygen into oil-covered soil is restricted (Jae-Young and Day, 2004). Penetration of water can also be restricted in contaminated soil, a consequence of increased hydrophobicity of the soil (Adams et al., 2008). The temperature regime of the contaminated soil often increases by up to 12^{o}C, which is attributed to decreased soil surface albedo and increased absorption of incoming solar radiation (Balks et al., 2002). The increased absorption of solar radiation results in the thaw of permafrost, leading to an increased depth of the active layer and leaching of contaminants to greater depths and possible soil structure instability (Poland et al., 2003). Thawing of the permafrost can result in slumping, which has serious consequences for infrastructure in Polar Regions, resulting in the collapse of buildings, roads and pipelines. These indirect toxic effects often have an important role in the environmental impacts of PHC contamination.

1.4 Fate of spilled petroleum hydrocarbons in a polar environment

When a PHC spill occurs on polar soil, there are many geographical, spatial, and temporal variables that dictate how the contaminant moves through the environment. Although the movement and impact of PHCs in frozen soil has not been studied extensively, research conducted in the Arctic during the early 1970s has greatly contributed to the scientific knowledge of transport of PHC spills in frozen soil and their impacts on Arctic ecosystems (Bliss and Wein, 1972; Collins et al., 1976; Deneke et al., 1975; Everett, 1978; Freeman and Hutchinson, 1976; Mackay and Monhtadi, 1975; Mackay et al., 1974a; Mackay et al., 1974b;

Mackay et al., 1975; McFadden et al., 1977; Walker et al., 1978). Major findings from these studies indicate that the movement of PHC in permafrost soil is dependent on several factors including topography, soil type and dryness, depth of the active layer, time of year, weather, PHC viscosity, and the presence/absence, as well as permeability, of vegetative cover (Biggar, 2004).

The season in which the spill occurs has a major impact on the fate and transport of PHC in a polar environment. If the release of PHC happens during the summer months, when the active layer is thawed, the spill typically penetrates the unfrozen surface layer and is dominated by downward movement of PHC (Mackay et al., 1975). However, in areas that are highly water saturated, downward flow of PHC will be restricted due to low permeability, resulting in increased lateral flow of PHC along the ground and in near surface layers. In the winter when the ground is frozen, a PHC release is dominated by surface runoff, though higher viscosity of PHC at colder temperatures usually inhibits lateral movement (Barnes and Biggar, 2008). At the first thaw following a PHC release in the winter, PHC trapped in pore spaces will become mobile again, with PHC distribution characterized by increasing concentrations at depth and increased lateral spreading (Collins et al., 1994; Mackay et al., 1975).

Although frozen soil is often considered to be an impermeable barrier, contaminant transport through frozen ground is possible due to several physical mechanisms that provide a conduit for transport. Liquid water is present in frozen soil due to salinity, adsorptive, and capillary forces in the soil matrix (Dash et al., 1995), and thus contaminant diffusion may occur through this unfrozen water. However, diffusion is a slow process, and would only result in transport in the magnitude of centimeters per year. Contaminant transport in frozen soil can also occur if a continuous network of air voids exists, as would be the case in a well drained

granular soil. This phenomenon is described by McCarthy et al. (2004) who observed mobile hydrocarbons in a frozen sandy gravel pad near Barrow, Alaska. Soil subject to freezing temperatures will also undergo thermal contraction, which may lead to the development of a network of fissures, especially in the upper regions of the permafrost where temperature changes are the most extreme (Biggar, 2004). This mechanism has been used to describe the development of ice wedges in the Arctic (Williams and Smith, 1991). Such a network of fissures may provide a pathway for free phase non-aqueous phase liquids (NAPL) to move (Biggar, 2004). PHCs have been observed at depths greater than 1.5 m below the permafrost surface at old spill sites at CFS Alert and Isachson High Arctic weather station (Biggar et al., 1998). Contaminant migration through frozen soil is facilitated by air voids in compacted fill or fissures in the native permafrost, depending on the site.

The downward movement of PHC is dictated by permeability and pore structure of the soil, thus variation in these properties can result in extremely heterogeneous distribution of NAPL (Poulsen and Kueper, 1992). Migration of NAPL can be restricted in highly saturated soil, as well as relatively low permeable soils which occur near the permafrost table. In permafrost soil, migration of NAPL can be enhanced by post cryogenic structures, such as ice wedges and fissure networks (Barnes and Biggar, 2008; Biggar, 2004).

Petroleum hydrocarbon migration through permafrost soils is influenced by the formation and presence of ice at all scales (Barnes and Biggar, 2008). At the millimeter scale, ice in pore spaces either interrupts downward migration, causing PHC to move laterally, or impedes movement altogether by blocking open pore space. Segregated ice at the centimeter to meter scale also causes PHC to move laterally in frozen soil and can open channels that enhance PHC

movement when the ice is thawed. At larger scales, discontinuous or continuous permafrost will slow, redirect, or impede PHC migration.

1.5 Cold-adapted, petroleum hydrocarbon degrading soil microorganisms

Bioremediation is a viable remediation strategy for PHC contaminated soil in Polar Regions because of the presence of cold-adapted hydrocarbon degrading microorganisms. Biodegradation of many PHC compounds by indigenous microbial populations have been observed at cold temperatures (Aislabie et al., 2006; Braddock et al., 1997; Margesin and Schinner, 2001; Mohn et al., 2001; Rike et al., 2003; Whyte et al., 1999). However, the polar environment does present multiple challenges to bioremediation including cold and fluctuating soil temperature, limited nutrient and contaminant bioavailability, low moisture/liquid water content, and oxygen status.

Temperature plays a significant role in controlling the degree of microbial PHC metabolism. Metabolic rates in soil are typically slower at lower temperatures (Delille and Coulon, 2008; Nedwell, 1999). Although microbial activity is reduced, many components in crude oil and diesel can still be degraded by psychrophilic and psychrotrophic microorganisms at temperatures below 0^{o}C (Leahy and Colwell, 1990; Whyte et al., 1998; Margesin and Schinner, 1999; Delille, 2000; Gibb et al., 2001; Baraniecki et al., 2002; Eckford et al., 2002).

The nutrient status of a soil directly impacts microbial activity and biodegradation of PHC. Soils of Polar Regions are generally low in nutrients (Tarnocai and Campbell, 2002) and the introduction of high concentrations of hydrocarbons can further deplete available nitrogen and phosphorus when they are assimilated during biodegradation (Roling and van Verseveld, 2002). As with temperate soils, amendment with nitrogen and/or phosphorus can lead to

enhancement of PHC degradation in polar soils (Braddock et al., 1997; Mohn et al., 2001; Whyte et al., 1999). Although N is considered to be the major limiting nutrient, maximal hydrocarbon degradation occurs with supplementation of both N and P (Braddock et al., 1997; Mohn et al., 2001). However, care must be taken not to overfertilize soil as it has been demonstrated that excess nitrogen can depress the rate of microbial activity and petroleum degradation in contaminated soils due to osmotic soil water potential depression (Walworth et al., 2007). The optimum N level for the hydrocarbon degradation depends on soil type. Sand and loamy sand, which have lower water-holding capacities, are much more sensitive to overfertilization with inorganic nitrogen than silt loam or clay (Braddock et al., 1997).

The bioavailability of each PHC compound is dependent on the chemical properties of the contaminant, soil type and amount of soil organic matter, and, in polar soil, soil temperature and liquid water content. In the literature, the bioavailable fraction of organic contaminants in soil is defined as the fraction that may be easily desorbed to or is desorbed from soil and present in the aqueous phase (Alexander, 2000; Reid et al., 2000; Semple et al., 2003) . As described in Section 1.2, aqueous concentration and extent of adsorption to soil organic matter is determined by solubility of the compound and K_{ow}, respectively. In polar soil, low temperatures and limited liquid water content also affect bioavailability of the compounds because as the soil freezes, PHC are excluded from the crystalline ice structure resulting in an increased concentration of PHC in the remaining liquid pore water (Barnes et al., 2004). For PHC compounds with high water solubility, freezing temperatures can increase the aqueous concentration thus leading to enhanced bioavailability.

Another obstacle to the bioavailability of PHC in polar soils is the access of hydrocarbons to the cellular enzymatic system (Yang et al., 2009). When ambient temperature is lowered

toward the freezing point, channels in the cell membrane tend to close and cytoplasm is subject to cryogenic stress. If the temperature keeps dropping, growth will diminish considerably and the cell will stop functioning altogether if the cytoplasmic matrix becomes frozen (Finegold, 1996). Therefore, cryogenic stress, which restricts the function of transport channels and may potentially freeze the cytoplasm of soil microbes, may restrict mass transport and limit bioavailability in polar soils.

In polar soil one of the main limiting factors for biological activity, and thus biodegradation of PHC, is the bioavailability of water. Even under freezing temperatures, a portion of water remains in the liquid phase and is available for biological reactions (Ostroumov and Siegert, 1996). This liquid water, θ_{liquid}, occurs as a thin layer surrounding the soil particles, whose thickness is controlled by temperature, salinity, adsorptive, and capillary forces within the soil matrix (Dash et al., 1995; Sparrman et al., 2004). The amount unfrozen water at sub-zero temperatures is determined by the size and shape of the ice crystals, impurity concentrations, and microscopic soil structure (Reed et al., 1979). The thickness of unfrozen water films that surround soil particles and bacteria cells is also dependent on soil temperature (Rivkina et al., 2000), soil organic matter content and composition (Drotz et al., 2009), and particle size distribution (Hillel, 2004). The amount of ice in frozen soil also affects the amount of θ_{liquid} content, with lower ice content associated with higher θ_{liquid} content (Ostroumov and Siegert, 1996). The mass transfer of θ_{liquid} and ions is greatest in frozen soil with low ice content and high chemical potential gradients which are found in localized areas close to ice inclusions (i.e. lenses, veins) (Ostroumov and Siegert, 1996). The presence of liquid water in frozen soil is essential for the mass transport and diffusion of substrate and waste products into and out of soil microorganisms.

Oxygen is also an esstential requirement for aerobic degradation of PHC by soil microorganisms. Oxygen is not likely a limiting factor to the microbial PHC degradation in most coarse textured dry polar soils, especially those of the Antarctic, but could be limiting in waterlogged Arctic soils (Rike et al., 2005). Soils with high water contents typically have low gas diffusivities and associated low oxygen levels which, when coupled with other limitations such as low temperatures and low nutrient availability, can reduce natural biodegradation to nearly negligible rates (Rayner et al., 2007). Under these conditions, aeration may be required to enhance degradation.

Petroleum hydrocarbon degradation also occurs in the absence of oxygen. Anaerobic degradation of PHC in polar soil is also possible and can be stimulated by the addition of fertilizer (Powell et al., 2006). Eriksson et al. (2003) also investigated the potential for anaerobic degradation of PAHs under cold temperatures and found that PAH degradation does occur under these conditions. *In situ* chemical oxidation, a bioremediation technique that involves the addition of reactive chemicals to oxidize PHCs to environmentally inert compounds, has been assessed as a potential remediation strategy in Polar Regions; however this technique has been found to be undesirable in Antarctica as it appeared to hinder biodegradation through the destruction of subsurface microbial communities (Ferguson et al., 2003) . Therefore careful consideration of the main pathway of PHC degradation (i.e. aerobic versus anaerobic) is essential when determining which bioremediation technique is the most suitable for a PHC contaminated site in Polar Regions.

1.7 Remediation guidelines for petroleum hydrocarbon contamination in Polar Regions

Although oil and fuel spills are among the most extensive and environmentally damaging pollution in Polar Regions, there is very limited data on the terrestrial ecotoxicology of PHC in Polar Regions (Poland et al., 2003). It is generally thought that PHC contamination is more damaging in Polar Regions (Snape et al., 2003). The extreme physical environment influences the nature of biota in ways that could increase their susceptibility to contaminants. Many polar species grow slowly and are relatively long-lived. A slow growth rate means that polar species may be in the sensitive, early-development stages for longer, and longevity allows increased time for accumulation of contaminants. Other biological features that may influence the response of polar species to contaminants includes seasonal growth and development, high lipid content, slow recovery rates, short food chains, and geographic concentration of species, however these factors have yet to be investigated (Poland et al., 2003).

Remediation activities have proceeded in Polar Regions; however, due to the lack of ecotoxicological data for terrestrial PHC contamination, relevant remediation guidelines are not available for these regions. Many countries that administer Polar Regions, including Canada, the United States, and Australia, have adapted their own environmental remediation guidelines, which are almost always based on temperate species and environmental conditions. These guidelines provide a mechanism for deriving site-adapted environmental guidelines, which take into account the local conditions of the site (ANZECC/ARMCANZ, 2000; CCME, 1991; US EPA, 1994), yet these procedures are lacking the fundamental information needed to derive site-specific guidelines in Polar Regions. For example, toxicological data for local Arctic or Antarctic species is not available to determine relevant remediation guidelines. In addition, the derivation

of such guidelines is often varied between countries, resulting in an enormous range of values. Table 1.4 lists the soil quality guidelines for several circumpolar countries. There is a vast range of acceptable soil quality guidelines for different countries, with the Scandinavian countries have much more stringent guidelines than in North America. For example, the clean-up criteria for diesel fuel in Norway is 100 mg kg^{-1}, compared to 2000 mg kg^{-1} in Canada.

In addition to the toxicological responses of plants and animals to PHC contamination, other factors, should also be taken into account when deciding whether levels are acceptable (Snape et al., 2003). In the Arctic, cultural factors such as how the land will be used and how the land is perceived are important considerations for soil quality guidelines, as indigenous people, who lead subsistence lifestyles, are tied closely to their environment. The health of indigenous people is directly linked to the health of the environment; therefore the presences of contaminants in the environment, especially contaminants that accumulate in the food chain, pose a risk to human health (AMAP, 1998b). In the Arctic, the primary concern in the development of risk-based environmental standards is the risk to human health, especially when considering remediation of a contaminated site near human settlement or close to important migratory pathways of animals.

In Antarctica, there are no indigenous people to support, therefore the primary concern for contamination shifts from protecting human health to protecting flora and fauna. Most human activity in Antarctica occurs mainly in ice-free coastal oases, which are rare habitats that comprise <0.05% of the land mass of Antarctica (Poland et al., 2003), and are important areas for birds, seals, vegetation, soil invertebrates and microorganisms. As a consequence, the relatively small human presence in Antarctica has the potential to cause disproportionate harm to biodiversity in concentrated areas along the coast (Snape et al., 2003). Moreover, the

Antarctic has been given a unique international role as a symbol of co-operative environmental stewardship and is valued as a place for gauging subtle environmental changes associated with climate change. Both these roles, the symbolic and that of a 'clean laboratory', require that particularly high environmental standards are pertinent for the continent.

To establish relevant soil quality guidelines for Polar Regions, it is essential to consider several factors such as pathways for the transfer of contamination, the environmental impacts, the toxicity of the contaminants, as well as the value of the land. These factors will differ between the Arctic and Antarctica, as well as between locations within each region. However, these two regions have more in common with each other than with the rest of the world, and therefore establishing a set of soil quality guidelines for contaminated sites in Polar Regions is essential to ensure protection of these unique environments.

Table 1.4 Summary of soil quality guidelines for petroleum hydrocarbon contamination by circumpolar countries. The soil quality guidelines units of concentration are mg kg^{-1}. Table adapted from Snape et al. (2008b)

Product	Finland[1]	Norway[2]	Sweden[3]	Canada[4]	Alaska[5]
Gasoline	100	7	5-100	1000	100
Diesel	200	100	100	2000	200
Crude Oil		100	100	2500	200
Residual	600	100	100	2500	2000
Polycyclic Aromatic Hydrocarbon Analytes					
Anthracene	50			2.5[a] (32[c])	3800
Benzo(a)anthracene	40		0.4	6.2[ai]	6.6
Benzo(b)fluoranthene			0.7	6.2[ai]	6.6
Benzo(k)fluoranthene	40		0.4	6.2[ai]	66
Benzo(a)pyrene	40	0.1	0.4	20[a] (72[c])	0.66
Chrysene	40		0.5	6.2[ai]	660
Dibenzo(a,h)anthracene	20			0.23[d]	
Fluorene	20	0.6		50[a] (180[c])	2500
Indeno(1,2,3-c,d)pyrene	40		0.4	2.7[d]	6.6
Naphthalene	100	0.8		8.8[ai]	1900
Pyrene	40	0.1	0.6	7.7[ai]	1900
Petroleum Hydrocarbon Range					
C_6-C_{10} (volatile)	500	7	100	30-230[*]	1400
C_{20}-C_{25} (light extractable)	2000	100	100	150-2200[*]	12500
C_{25}-C_{36} (heavy extractable)	2000	100	100	400-5000[*]	13700

[1] Soil quality guidelines from Finnish Minister of the Environment.

[2] Guidelines from Norway Pollution Control Authority

[3] Soil quality guidelines from Swedish Environmental Protection Agency

[4] Soil quality guidelines for PAH analytes from Canadian Council of Ministers of the Environment (CCME, 2010)

[5] Soil cleanup levels from the Alaska Department of Environmental Conservation (2008)

[a] Agriculture/residential land use

[c] Commercial/industrial land use

[d] Protection of potable drinking water

[i] Soil and food ingestion

[*] Range reflects criteria for four land-use categories (agriculture, parkland/residential, commercial, and industrial)

1.8 Conclusions

Petroleum hydrocarbon contamination in Polar Regions can be degraded by indigenous soil microorganisms when major factors, such as nutrient availability, contaminant bioavailability, temperature and liquid water content are optimized. However, biological communities in Polar Regions may be more sensitive than those in temperate areas because of the harsh environment conditions. The establishment of relevant remediation guidelines for Polar Regions is dependent on international collaboration to collect ecotoxicological data on Arctic and Antarctic species. The urgency of this task increases with the expanding oil and gas activity in the Arctic, as well as increased ecotourism in the Antarctic, which brings with it increased risk of environmental contamination in these relatively pristine, environmentally sensitive areas.

1.9 Research Objectives and Hypothesis

The goal of this thesis is to evaluate the influence of liquid water content on the toxicity of petroleum hydrocarbons to soil microorganisms in polar soil. Research objectives by chapter are provided in Table 1.5. The over-arching hypothesis of this thesis is:

An increase in soil liquid water content will increase the toxicity of petroleum hydrocarbons to soil microorganisms at temperatures below 0^{o}C.

There are four main objectives that will test the over-arching hypothesis that liquid water content influences petroleum hydrocarbon toxicity. The specific research objectives that will be evaluated include:

Objective 1: Determine the most sensitive indicator of PHC contamination in polar soils (Chapters 2, 3, and 5).

Hypothesis: Nitrification, denitrification, soil respiration, and community composition are sensitive indicators of PHC contamination.

Objective 2: Determine how liquid water content influences environmental properties of the soil matrix including gas diffusion coefficient and nutrient status (Chapter 4).

Hypothesis: An increase in liquid water content at temperatures below $0^{o}C$ will increase the nutrient supply rate but has no effect on gas diffusion in frozen soil.

Objective 3: Determine how PHC contamination influences liquid water content in frozen soil (Appendix A).

Hypothesis: PHC contamination increases the amount of liquid water content in frozen soil.

Objective 4: Determine whether potential toxicity assays in unfrozen soil are a good surrogate for *in situ* toxicity (Chapter 5).

Hypothesis: Potential toxicity assays are good surrogates for *in situ* toxicity of petroleum hydrocarbons in frozen soil.

Table 1.5 Summary of research objectives by chapter.

Chapter	Objectives	Chapter Description
1	Literature review	Background information on petroleum hydrocarbon contamination in Polar Regions. Information presented includes the properties, toxicity, and fate and transport of petroleum hydrocarbon in polar soil, as well as bioremediation and development of relevant remediation guidelines for Polar Regions.
2	Soil biogeochemical toxicity endpoints for sub-Antarctic Islands contaminated with petroleum hydrocarbons. *Published in Environmental Toxicology and Chemistry 2007, 26: 890-897.*	Evaluated the use of potential toxicity assays to assess toxicity of PHC contamination in sub-Antarctic soil spiked with diesel fuel.
3	Influence of liquid water and soil temperature on petroleum hydrocarbon toxicity in Antarctic soil. *Published in Environmental Toxicology and Chemistry 2009, 28: 1409-1415.*	• Collection of soil samples from an aged PHC contaminated spill site in Antarctica during periods when soil was frozen, thawed, and refrozen. • Toxicity was assessed using potential toxicity assays and community composition of soil microorganisms.
4	Changes in liquid water alter nutrient supply rate and gas diffusion in frozen Antarctic soils contaminated with petroleum hydrocarbons.	• Ion exchange resins are used to determine the N nutrient supply rate in frozen PHC contaminated soil with different liquid water contents. • Gas diffusivity is measured in frozen contaminated soil with different liquid water contents.
5	Validating potential toxicity assays to assess hydrocarbon toxicity in polar soil.	• *In situ* toxicity of PHC in frozen soil was assessed using stable isotope dilution technique to measure gross N mineralization and gross nitrification rates • *In situ* toxicity was compared to potential toxicity assay assessed for frozen soil
6	Summary and conclusions	Summary of results reported in each chapter and a discussion of how they relate to each other. A comparison of toxicity endpoints from each chapter is made.
Appendix A	Hydrocarbon contamination increases the liquid water content of frozen Antarctic soils. *Published in Environmental Science and Technology 2008, 42: 8324-8329.*	Liquid water content was measured in PHC contaminated polar soil using two different techniques.

CHAPTER 2*

Soil biogeochemical toxicity endpoints for sub-Antarctic Islands contaminated with petroleum hydrocarbons

* This chapter has been published in Environmental Toxicology and Chemistry 2007, 26: 890-897, under joint authorship with Ian Snape (Australian Antarctic Division) and Steven Siciliano (University of Saskatchewan)

2.1 Abstract

Sub-Antarctic islands have been subjected to petroleum hydrocarbon spills and yet no information is available on the toxicity of petroleum hydrocarbons to these sub-polar soils. The purpose of this study was to identify soil biogeochemical toxicity endpoints for petroleum hydrocarbon contamination in sub-Antarctic soil. Soil from Macquarie Island was collected and exposed to ten concentrations of Special Antarctic Blend (SAB) diesel fuel ranging from 0 to 50 000 mg kg^{-1} for a 21 day period. The sensitivity of nitrification, denitrification, substrate induced respiration and total soil respiration to SAB fuel was assessed. Potential nitrification activity was the most sensitive indicator of SAB contamination assessed for nitrogen cycling, with an EC_{20} of 190 mg kg^{-1}. Potential denitrification activity was not as sensitive to SAB contamination, with an EC_{20} of 950 mg kg^{-1} for nitrous oxide production. Nitrous oxide consumption was unaffected by SAB contamination. Substrate induced respiration (SIR, respiration due to sucrose) was a more sensitive indicator (EC_{20} of 16 mg kg^{-1}) of SAB contamination than total respiration (EC_{20} of 220 mg kg^{-1}). However, total soil respiration is a more responsive measurement endpoint, increasing soil respiration over a 72 hour period by 17 mg CO_2, compared to a change of only 2.1 mg CO_2 for SIR. Our results indicate that EC_{20}'s varied between 16 to 950 mg kg^{-1} for Macquarie Island soil spiked with SAB diesel fuel. These results indicate that current cleanup levels derived from temperate zones may be too liberal for soil contamination in sub-Antarctic islands.

2.2 Introduction

Petroleum hydrocarbon contamination is considered to be a significant pollution problem in polar regions (Aislabie et al., 2004; Delille, 2000; Deprez et al., 1999; Roura, 2004), however there is currently no soil toxicity information on petroleum hydrocarbon contamination effects on terrestrial ecosystems in polar or sub-polar regions. Toxicity tests such as plant or earthworm survival tests are commonly used for petroleum hydrocarbon contamination in temperate regions (Environment Canada, 2004; Stephenson et al., 1997). These organisms are not found in polar environments and therefore have limited relevance for use as toxicity endpoints. In the absence of polar and sub-polar soil toxicity data, site managers have relied on guidelines developed for petroleum hydrocarbon contamination in temperate regions. The identification of relevant and reliable soil biogeochemical toxicity endpoints in polar regions will allow site managers to begin incorporating site specific data into site management guidelines. The main objective of this research is to identify and evaluate relevant soil biogeochemical toxicity endpoints for petroleum contamination in polar regions.

Petroleum hydrocarbons have both direct and indirect toxic effects on soil microorganisms. Direct toxic effects are caused by narcosis which is the nonspecific disruption of the cell membrane, causing changes in membrane fluidity, integrity and functioning (MacLeod et al., 2004). This regulation of cellular membrane composition is critical for the survival of soil microorganisms at low moisture levels and temperatures (Holmstrup et al., 2000). The indirect toxic effects of petroleum contamination include the alteration of the physical and chemical properties of soil. The soil often becomes more anaerobic because movement of oxygen into soil is restricted (Jae-Young and Day, 2004). In addition, the temperature regime of contaminated soil increases, up to 10^{o}C, which is attributed to decreased

soil surface albedo (Balks et al., 2002). The nutrient ratio in petroleum hydrocarbon contamination also changes, with C:N and C:P ratios increasing (Aislabie et al., 2004).

Soil microorganisms are a vital component of the terrestrial ecosystem and can be used as a surrogate for soil health (Nielson and Winding, 2002). Soil microbes play a critical role in the cycling of nutrients such as carbon, nitrogen, sulphur and phosphorus and are an important part of the terrestrial food web because plant nutrition is dependent on their metabolic activity (Nielson and Winding, 2002). Soil microorganisms are good indicators of soil health because they live in close contact with the soil and they respond and adapt quickly to changes in environmental conditions (Pereira et al., 2006). Thus microbial activity provides an integrated measure of soil health and can be used to determine toxicity data for contaminated soil.

The activity, biodiversity and abundance of soil microorganisms involved in basic ecosystem processes such as carbon and nitrogen cycling are affected by petroleum contamination. The additional carbon added to the system provides a substrate that only certain microbial groups can utilize. As a result, the composition of the microbial community changes and numbers of hydrocarbon-degrading bacteria, such as *Rhodococcus, Sqhingomonas,* and *Psuedomonas*, become elevated in hydrocarbon contaminated soil (Aislabie et al., 2004). Nitrification activity is a sensitive indicator of toxicity for many contaminants and can be measured using an ammonium oxidizing assay (Gong et al., 1999; Nielson and Winding, 2002; Pereira et al., 2006). A decrease in nitrification activity and an alteration in the diversity of nitrifying bacteria in soil has been observed with petroleum contamination (Christensen et al., 2001; Deni and Penninckx, 1999). Diesel fuel has been shown to inhibit nitrification, but only in soils not previously exposed to diesel (Deni and Penninckx, 1999).

To the best of our knowledge, denitrification activity has not been used as an endpoint for petroleum hydrocarbon toxicity. Denitrification is a term used for the sequential reduction of nitrate to nitrous oxide with some organisms reducing nitrous oxide to nitrogen. Typically, the enzymes involved in this four step reaction are divided into two groups. The first group, (nitrate reductase (NaR), nitrite reductase (NiR) and nitric oxide reductase (NOR)) reduce nitrate to nitrous oxide and the final enzyme (nitrous oxide reductase (N_2OR)) reduces nitrous oxide to nitrogen gas. Denitrification has been used as a sensitive indicator for other contaminants such as heavy metals, 2,4,6-trinitrotoluene and various pesticides(Holtan-Hartwig et al., 2002; Pell et al., 1998; Siciliano et al., 2000). Toluene (a common constituent of gasoline) increases nitrous oxide emissions from soil (Cho and Peirce, 2005). This increase in nitrous oxide emissions is likely due to inhibition of N_2O reductase which is highly sensitive to pollutants (Bollag and Kurek, 1980; Richards and Knowles, 1995).

The purpose of this paper is to provide the first toxicity information for sub-polar terrestrial environments. Our goal was to characterize soil ecosystem response to a common pollutant, petroleum hydrocarbons, in a form likely to be encountered, i.e. Special Antarctic Blend (SAB) diesel fuel.

2.3 Materials and methods

2.3.1 Soil characterization

Soil from Macquarie Island, a sub-Antarctic island south of Australia, was collected on March 19, 2003 from 54^{o} 30.170' S, 158^{o} 55.878' E in a 80.4 cm by 5.4 cm polyvinyl chloride core and stored at 4^{o}C. The top 65 cm of soil, approximately 900 g, from the core was combined. The soil was characterized for soil moisture content (Klute, 1986), particle size (Carter, 1993), and total organic matter (Heiri et al., 2001). The soil characteristics are summarized as follows:

gravimetric water content 22.2% and total organic matter 4.46%. The particle size analysis revealed gravel content (> 2 mm) of 4.16%, coarse sand content (1.0-2.0 mm) of 22.14%, medium sand content (0.25-1.0mm) of 66.35%, fine sand content (0.125-0.25 mm) of 5.65%, and coarse silt content or finer (< 0.063 mm) of 1.70%.

2.3.2 Incubation experiment

Ten different logarithmically increasing concentrations of SAB diesel fuel were created by spiking 220 g soil with 11 g SAB and mixing thoroughly with a clean metal spoon. The remaining treatments were prepared by a 1:1 serial dilution beginning at 50 000 mg kg^{-1}. A logarithmic series of concentrations is used in our experimental design because the response of test organisms to contaminants is related to the proportional increase in concentration (Environment Canada, 2007). Treated soil (110 g) was placed in a 1 L Schott bottle, covered in tinfoil, and incubated at 6 ± 0.02^{o}C for 21 days. A soil respirometer system (N-Con Computer-ox AV4R) maintained an aerobic environment within the microcosms, whilst minimizing volatile losses of hydrocarbons. Soils were sampled weekly for toxicity tests that were performed at 10^{o}C.

The control (0 mg kg^{-1}) was replicated three times while the remaining treatments did not receive replication. Our goal was to estimate an EC_{20} value for SAB fuel using the model of best fit and maximizing dose numbers minimized error associated with our estimates of statistical parameters. As noted by others, replication is not required for point-estimates using regression but would allow subsequent hypothesis testing (Environment Canada, 2007). Although it is customary to use the EC_{50} value to describe the toxicity of a contaminant, the EC_{20} will give a more conservative value, which can then be used as a site-specific remediation guideline. Using a lower p value for EC_p, for example an EC_{10}, can be problematic because the response could fall

within the acceptable range of the control (Environment Canada, 2007). Boric acid at a known EC_{20} value of 540 µg boric acid g^{-1} soil dry weight (Stephenson et al., 1997) was used as a positive control.

2.3.3 Analysis of Special Antarctic Blend fuel

Actual, rather than nominal, SAB concentrations were assessed by gas chromatograph (GC) analysis of a hexane extract of a soil sub-sample (Table 2.I). Sub-samples (10 g) were spiked with 1 mL of an internal standard mixture containing cyclooctane (1 mg mL^{-1}), 1-Bromoeicosane (1.4 mg mL^{-1}) and d_{10}-anthracene (0.1 mg mL^{-1}) in hexane. Following this, additional hexane was added (10 mL) and the samples were made into a slurry with the addition of water (10 mL). Sub-samples were extracted directly by end over end tumbling for 16 hours. The hexane layer was separated (1500 rpm for 30 min), transferred to a GC vial and directly injected onto the GC column.

Hydrocarbons were analyzed by an Agilent 6890 GC fitted with a flame ionization detector (FID). Separation was performed on a 50 m × 0.22 mm inner diameter × 0.25 µm film BP-1 column. Samples were introduced with a 20:1 split into an SGE focus liner at 280°C. The GC oven program was 40°C for 5 min then increasing to 310°C (at 10°C min^{-1}) and holding for 15 min. Helium was used as the carrier gas in ramped flow mode (1.2 mL min^{-1} for 32 min then ramping to 2 mL min^{-1} at 0.16 mL min^{-1}). The FID was heated to 330^{o}C.

Peak area of internal standards were used to compare one sample with another while the area of hydrocarbons in the range *n*-C_9 to *n*-C_{24} was used to calculate the quantity of fuel. Calibration curves were obtained by analyzing 6 different fuel standards prepared gravimetrically with SAB, the internal standard mix and hexane. Applying linear, least squares

Table 2.1 Nominal and measured Special Antarctic Blend (SAB) concentrations.

Calculated SAB value (mg kg^{-1})	Measured SAB value (mg kg^{-1})	St dev of SAB value (mg kg^{-1})
0	<20	20
0	<20	20
0	<20	20
98	86	20
195	139	21
391	314	25
781	730	42
1562	1509	78
3125	3014	152
6250	6487	325
12 500	12 834	642
25 000	26 810	1341
50 000	46 204	2310

regression to the ratio of SAB:internal standard *versus* fuel area (*n*-C_9 to *n*-C_{24}):internal standard area gave the fuel quantity calibration curve. Fuel concentrations in the hexane extract were then calculated automatically from the regression equations. In addition to the calibration curve, quality control steps included running hexane blanks after every 6 samples, running an ongoing calibration standard as well as running repeat sample injection every 6 to 12 samples. Also a solution containing markers from *n*-C_8 to *n*-C_{36} was included at the beginning and end of each analytical run to check the retention times of marker compounds.

2.3.4 Toxicity assays

2.3.4.1 Potential nitrification activity

The potential nitrification assay measures the potential activity of ammonium oxidizing bacteria within the soil (Gong et al., 1999; Torstensson, 1993). In brief, moist soil (5 g) was added to 20 ml of test solution. The test solution contains 4 mM $(NH_4)_2SO_4$, 15 mM $NaClO_3$, and 1 mM KH_2PO_4. Ammonium sulfate provided a growth substrate for the ammonium oxidizing bacteria, while sodium chlorate inhibited the oxidation of nitrite to nitrate, and potassium phosphate was added to buffer the solution (Belser and Mays, 1980). The soil slurry was agitated (70 rpm) at 10 ± 2^oC for up to 40 hours. Samples of 2 mL were taken from the slurry after 16, 28, and 40 hours of incubation. Ammonia oxidation was terminated by adding 2 mL of 4 M KCl. The samples were centrifuged for 3 min at 14000 rpm and filtered through a 0.45 µm syringe filter to remove particulate matter. Aliquots (3.0 ml) were extracted and analyzed for nitrite content using colorimetry (SMEWW, 1995). The production rate of nitrite (ng NO_2^- -N g^{-1} soil h^{-1}), also referred to as the potential nitrification activity (PNA), was calculated from the linear regression of the concentration of nitrite g^{-1} soil over time (h).

2.3.4.2 Potential denitrification activity

The denitrification activity was assessed in a two part experiment. The first part assessed the activity of the NaR, NiR and NOR enzymes by measuring the reduction rate of NO_3^- to N_2O and the second part of this assay assessed the N_2OR enzyme by measuring the consumption rate of N_2O (Siciliano et al., 2000). In brief, 10 g of fresh weight soil was placed in a 120 cm^3 glass serum bottle and 400 μL of 1 M $NaNO_3$ was added to the soil. Each microcosm was purged with dinitrogen, sealed with teflon and aluminum crimps, and incubated at 10°C. Conversion of N_2O to N_2 was blocked by the addition of 10 kPa acetylene (Wrange et al., 2001). Gas samples (5 cm^3) were taken from the headspace of the microcosms on day 2, 7, 11, and 15 and placed in an evacuated tube for analysis of N_2O. The production rate of N_2O was calculated from the linear regression of the amount of N_2O (nmol) produced per g soil by the sampling time (h). After N_2O production in soil samples was exhausted (i.e. all the nitrate source was used up), N_2OR activity was assessed by measuring the disappearance of N_2O (1000 ppmV) added to the sealed anaerobic microcosms (Gong et al., 1999). Following the injection of N_2O, the headspace was sampled on day 2, 4 and 8 and the consumption rate of N_2O was calculated from the linear regression of the amount of N_2O (nmol) consumed per g soil by the sampling time (h).

Nitrous oxide concentrations were determined using a Varian CP3800 GC (Varian Canada Inc., Mississauga, ON) equipped with dual electron capture detectors. Operating conditions for the GC are as follows: injector temperature = 100°C, column temperature = 35°C, detector temperature = 370°C. Separations were carried out using Poraplot Q columns (12.5-m × 0.32-mm inner diameter, fused silica capillary column, DF = 8 μm; includes a 2.5-m particle trap) with UHP He (14.4 mL min^{-1}) as the carrier gas and P5 (95:5 v/v Ar:CH_4 mix) as the make-up gas (12.0 mL min^{-1}). Samples (300 μL) were introduced using a CombiPAL™ auto-sampler (CTC Analytics

AG, Switzerland) with on-column injection, a split ratio of 10:1, and an open-ended 1.0 mL glass syringe. Excess pressure was vented during sample transfer and the syringe flushed with air between injections. The system was calibrated using standard gases (N_2O in N_2) obtained from PraxAir (Mississauga, ON). Data processing was performed using the Varian *Star Chromatography Workstation* (version 6.2) software. Internal calibration curves were obtained by applying linear, least squares regression to the gas concentration (ppbV N_2O) *versus* peak area data; N_2O concentrations in the headspace samples were then calculated automatically from the regression equations. A reference gas (ambient air or a near-ambient gas standard) was included in each analytical run to check the 'within run' precision.

2.3.4.3 Substrate induced respiration

Substrate induced respiration assessed the ability of soil microorganisms to utilize a common form of carbohydrates, sucrose, *versus* the anthropogenic hydrocarbon source, SAB fuel. To measure the amount of soil respiration attributed to the use of sucrose as a carbohydrate source, two soil aliquots of 5 g were taken from each treatment. One of the 5 g soil samples was amended with 7.5 mL of a fertilizer solution containing ammonium nitrate at a concentration of 758 mg N kg^{-1} H_2O. The soil sample amended with this test media assessed the use of SAB fuel and natural sugar as a carbohydrate source (measure of total soil respiration). The other 5 g soil sample was amended with 7.5 mL of a solution containing 1 mg sucrose mL^{-1} H_2O and ammonium nitrate (758 mg N kg^{-1} H_2O). This second treatment assessed soil respiration attributed to SAB fuel, natural carbohydrates, and the additional sucrose added to the system. By difference, the use of sucrose was estimated. Sodium hydroxide traps (5 mL of 1.0 M NaOH) were inserted into each flask to capture the CO_2 produced over the incubation period (72 hours at 10^oC). The flasks were sealed air-tight with rubber stoppers for the

incubation period. The traps were sealed after the incubation period and the amount of trapped CO_2 determined by titrating the OH^-/CO_3^{2-} mixture with a 4.0 M HCl titrant (Metrohm SM Titrino autotitrator). A set endpoint method with two endpoints, pH 8.6 and 4.0 was used to determine the concentration of CO_2 (mg) in the trap (SMEWW, 1995).

2.3.5 Statistical analysis

Regression analysis was used to determine EC_{20} values for exposure and weekly toxicity test data for nitrification activity, denitrification activity (NaR/NiR/NOR and N_2OR), and substrate induced respiration (Environment Canada, 2007). Various models (linear, logistic, gompertz, hormesis, exponential, sigmodial) were applied to all data sets and the fit of the model was assessed by calculating a corrected sum of squared errors (SSE) value. The corrected SSE value was used because some of the models (gompertz and hormesis) have non-unique solutions sets. The $SSE_{corrected}$ uses prior knowledge to constrain the SSE (equation 2.1) and was calculated by applying a correction factor to account for the variance in the EC_{20} value (equation 2.2).

$$SSE = \sum (\hat{y} - y)^2 \qquad [2.1]$$

$$SSE_{corrected} = SSE + \frac{1}{\left(\sigma_{EC_{20}}\right)^2 \left(E\hat{C}_{20} - EC_{20}\right)^2} \qquad [2.2]$$

In which $\hat{y}$ is the predicted response, y is the measured response, $\sigma_{EC_{20}}$ is the variance of the EC_{20} (assumed to be 20%), $E\hat{C}_{20}$ is the eyeballed EC_{20} value, and EC_{20} is the EC_{20} value determined by the model.

Comparing the fit of various models to select the most appropriate model for the data set is important because the model selection can greatly influence the EC_{20} value. If the candidate

models have the same degree of complexity (i.e. same number of parameters) then the log likelihood statistics generated by most statistical software packages can be used to compare different models (Newman and McCloskey, 1996). To compare models of varying complexity, the number of parameters must be accounted for, which can be done using Akaike's information criterion (AIC) (Akaike, 1987). The AIC provides a measure of the relative fit of the candidate models by adjusting the log likelihood statistic for the number of floating parameters (equation 2.3) (Burnham and Anderson, 2002).

$$AIC = 2L + 2m = n\left[\ln(2\pi) + \ln\left(\frac{SSE}{(n-m)+1}\right)\right] + 2(m+1) \qquad [2.3]$$

Where L is the log likelihood statistic, m is the number of floating parameters in the model and n is the number of observations. The model of best fit is one that minimizes the AIC value.

2.4 Results

There is a clear dose response relationship for SAB exposure and potential nitrification activity (Figure 2.1). Linear (equation 2.4) and logistic (equation 2.5) (Environment Canada, 2007) models are both shown in Figure 2.1 because based on AIC values, both models fit the data equally well (31.3 for linear, 31.9 for logistic).

Linear

$$y = \frac{(-b \times p)}{EC_p} \times x + b \qquad [2.4]$$

Logistic

$$y = \frac{b}{\left[1 + \frac{p}{(1-p)}\right] \times \left(\frac{x}{EC_p}\right)^a} \qquad [2.5]$$

in which x is the dose (PHC concentration on a logarithmic scale) or exposure (PHC concentration x time of exposure, days), b is the control response, p is the chosen percentage of deviation from the control (i.e. 20%), EC_p is the concentration of TPH where the microbial activity is effected by a chosen percentage (p), and a is a fitting parameter. The parameters for the linear equation are: b = 15.11, and x = 1.391, with an $SSE_{corrected}$ of 1.334 and the parameters for the logistic equation are: b=10.588, x=4.14, and a=13.406, with an $SSE_{corrected}$ of 0.052. However, the EC_{20} values for these two models differ drastically, with 25 mg fuel kg^{-1} soil × day for the linear model and 13 800 mg fuel kg^{-1} soil × day for the logistic model. It is difficult to comprehend the units of mg fuel kg^{-1} soil × day so we divided the EC_{20} value for exposure by the maximum incubation period (21 days) to convert the units back to mg fuel kg^{-1} soil, resulting in EC_{20} values of 1.2 mg fuel kg^{-1} soil (linear) and 660 mg fuel kg^{-1} soil (logistic). The logistic model appears to be the best model because it calculates an EC_{20} value than is much closer to the eyeballed EC_{20} value.

Standardizing the incubation period and expressing the EC_{20} value as a concentration of the contaminant with units of mg kg^{-1} for the specified incubation time is an alternative to the expressing the EC_{20} values in relation to exposure. For example, standard operating procedures for traditional toxicity assays such as the 48-hour *Daphnia magma* acute toxicity test or the 21-day *Hexagenia limbata* make use of standardized incubation times(Burton et al., 2003). Based on the exposure-activity relationship of Figure 2.1 in which activity steadily decreased as dose

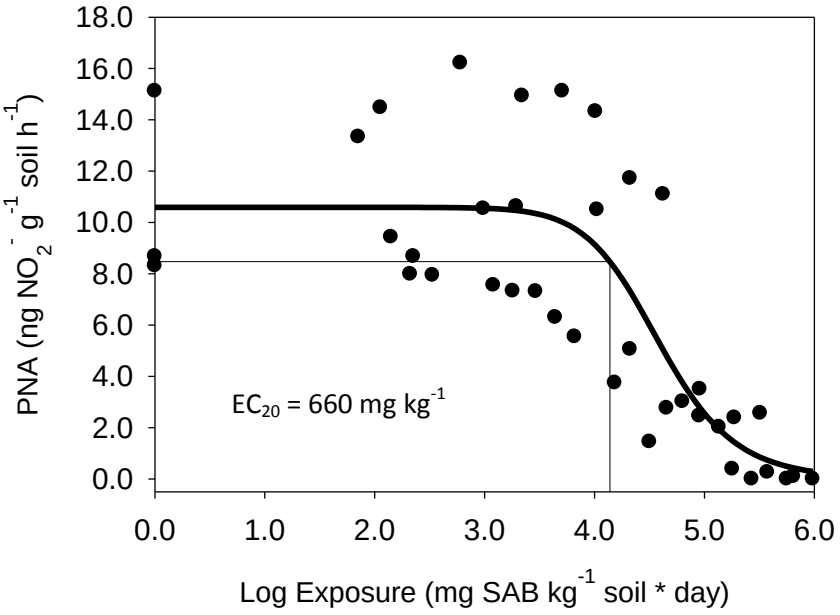


Figure 2.1 Effect of Special Antarctic Blend (SAB) diesel fuel exposure (log mg fuel kg^{-1} soil × d) on potential nitrification activity. The logistic (solid line) model is shown and the EC_{20} value is indicated with drop lines. The exposure (x-axis) was calculated by multiplying the concentration and incubation time (mg fuel kg^{-1} soil × d) and expressed on a $\log_{10}$ scale. The potential nitrification activity (PNA) of each treatment was calculated by applying a linear regression to the amount of nitrite measured in a prepared soil slurry over time.

increased, we have chosen to present the data from soil samples taken after three weeks incubation with SAB fuel for the remaining toxicity assays.

The potential nitrification activity for soil incubated for 21 days is the most sensitive indicator of petroleum hydrocarbon toxicity (Figure 2.2, top panel). As seen with the nitrification exposure data, the linear and logistic models fit the 21-day incubation data equally well, with AIC values of 40.5 (linear) and 40.4 (logistic) and EC_{20} values of 8.1 mg fuel kg^{-1} soil (linear) and 190 mg fuel kg^{-1} soil (logistic). The parameters for the linear model are: b=10.33, x=0.91 with an $SSE_{corrected}$=13.9; and for the logistic model the parameters are: b=8.116, x=2.281, a=6.609, with an $SSE_{corrected}$=9.30. Once again the linear model (equation 2.4) does not provide a reasonable value for the EC_{20} value, as the calculated EC_{20} (8.1 mg fuel kg^{-1} soil) is much lower than the eyeballed EC_{20} (approximately 200 mg fuel kg^{-1} soil). The logistic model (equation 2.5) is the best fit for this data set, with an EC_{20} of 190 mg fuel kg^{-1} soil. Although week 3 data behaves very similar to the exposure data for potential nitrification activity, the EC_{20} values differ, with week 3 data being more sensitive than the exposure data (i.e. lower EC_{20}). The exposure data has a higher EC_{20} (660 mg fuel kg^{-1} soil for exposure data compared to 190 mg fuel kg^{-1} soil for week 3 data). For the exposure data, the inclusion of earlier exposure times that were not as sensitive to the contamination resulted in a higher EC_{20} for the exposure data.

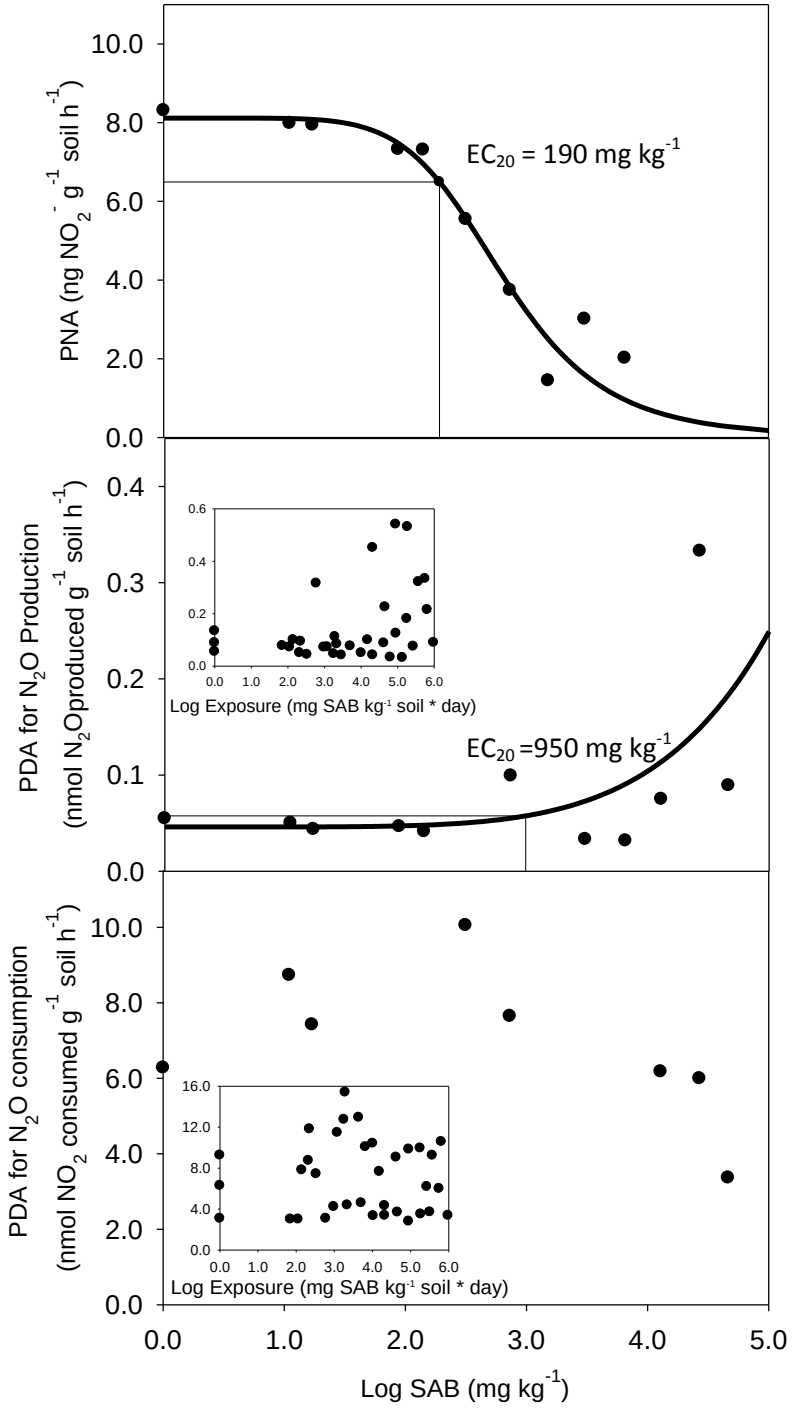


Figure 2.2 Effect of Special Antarctic Blend (SAB) diesel fuel (log mg kg^{-1}) on the nitrogen cycle after 21 d incubation at 6°C. Potential nitrification activity (PNA, top panel) and potential denitrification (PDA) was measured (N_2O production, middle panel and N_2O consumption, bottom panel. Potential nitrification activity is modelled by a logistic equation (solid line, top panel) PDA for N_2O production is modelled by an exponential equation (solid line, middle panel). The EC_{20} value for each model is indicated with drop lines. The effect of SAB exposure (log mg fuel kg^{-1} soil × d) on potential N_2O production and consumption are shown in the insets of the middle and bottom panel, respectively.

The denitrification pathways were not sensitive indicators of petroleum hydrocarbon contamination (Figure 2.2, middle and bottom panels). After 21 days of incubating the soil with SAB fuel, the potential N_2O production displayed a slight increase at very high levels of SAB concentration (Figure 2.2, middle panel). At a concentration of 25 000 mg fuel kg^{-1} soil the activity level of NaR/NiR/NOR increases to 0.33 nmol N_2O produced g^{-1} soil h^{-1}. A general increase in potential NaR/NiR/NOR activity was observed with an increased exposure to SAB fuel (Figure 2.2, inset of middle panel). Because of this overall increase in N_2O production, we have decided that the N_2O production response at 25 000 mg fuel kg^{-1} soil is not an outlier. The model of best fit was a logistic, 4 parameter (equation 2.6) (Brain et al., 2006) (AIC, 32.5) with an EC_{20} value of 950 mg fuel kg^{-1} soil.

Logistic, 4 parameter $$y = w + \left\{ \frac{a}{\left[1 + \left(\frac{x}{EC_p} \right)^b \times \left(\left(\frac{a}{(1+p)\times(w+a) - w} \right) \right) - 1 \right]} \right\} \quad [2.6]$$

in which a is the control response, p is the chosen percentage of deviation from the control, EC_p is the concentration of petroleum where the microbial activity is effected by a chosen percentage (p), b and w are fitting parameters. The parameters for the logistic, 4 parameter up equation are as follows: a = 13.9, b = 5.55, and w = -13.9, with an $SSE_{corrected}$ = 0.443.

The potential N_2O consumption was also used as a measurement endpoint for SAB contamination and was found to be an insensitive indicator of petroleum hydrocarbon contamination (Figure 2.2, bottom panel). The activity level for nitrous oxide consumption appears to be decreasing at very high levels of SAB concentration, however, the exposure data

for potential nitrous oxide consumption activity indicates there is no trend, and that nitrous oxide consumption is not affected by petroleum contamination (Figure 2, inset of bottom panel). The nitrous oxide consumption data was not modeled because there was no trend in the exposure of SAB to this activity level.

An increase in total respiration was observed with an increase in SAB fuel (Figure 2.3, closed circles and inset). Total soil respiration (measured over a 72 hour period) for the control was very low (0.226 mg CO_2) but starts to increase once levels of petroleum hydrocarbons in the soil reach approximately 200 mg fuel kg^{-1} soil and continues to increase exponentially. The exponential increase in total soil respiration is observed when nitrogen is not a limiting factor (recall that ammonium nitrate was added to the test media to ensure that the system was not N-limited). The highest amount of soil respiration over a 72 hour period was 17.0 mg CO_2 which occurred in the highest treatment of SAB fuel (50 000 mg fuel kg^{-1} soil). An exponential model (equation 2.7) (SigmaPlot 9.0, 2004)fits this data well (R^2 = 0.9963) and an EC_{20} of 220 mg TPH kg^{-1} was determined algebraically from the observed control response.

Exponential, 2 parameter $$y = a \times e^{(b \times x)} \qquad [2.7]$$

in which a is the control response and b is a scale parameter. The parameters for the exponential, 2 parameter are a = 0.0043 and b = 1.77. The amount of total respiration *versus* dose (concentration × time) is shown in the inset of Figure 2.3. An exponential increase is also observed for the exposure data with an EC_{20} of 7300 mg fuel kg^{-1}soil × day, and when divided by the maximum incubation period, the EC_{20} for the exposure data is 350 mg fuel kg^{-1} soil. The total respiration of the positive control for a 72 hour period (540 µg boric acid g^{-1} soil) was extremely low, 0.04 mg CO_2. In addition to total soil respiration, the amount of respiration

attributed to sucrose (measured over a 72 hour period) also increased with an increase in SAB fuel (Figure 3, open circles). The results of this assay show that soil respiration due to sucrose is induced by the addition of SAB fuel up to approximately 12 500 mg fuel kg^{-1} soil. At high concentrations of SAB fuel (25 000 and 50 000 mg fuel kg^{-1} soil), the respiration attributed to sucrose decreased slightly (5.2 and 4.6 mg CO_2), but was still higher than the control response (2.5 mg CO_2). The model of best fit for this data set was the sigmoidal, 3 parameter model (equation 2.8) (SigmaPlot 9.0, 2004).

Sigmoidal, 3 parameter $$y = \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}} \qquad [2.8]$$

in which a is the control response, x_o is the minimum x value, and b is a fitting parameter. The parameters for the sigmoidal, 3 parameter equation are a = 6.85, b = 0.627, x_o = 1.37. The EC_{20} value was determined algebraically from the observed response of the controls and the value of the EC_{20} is 16 mg fuel kg^{-1} soil. The amount of CO_2 respired from sucrose for the positive control (540 µg boric acid g^{-1} soil) was 0.57 mg CO_2, and was much lower than the respiration due to sucrose for the SAB treated soil.

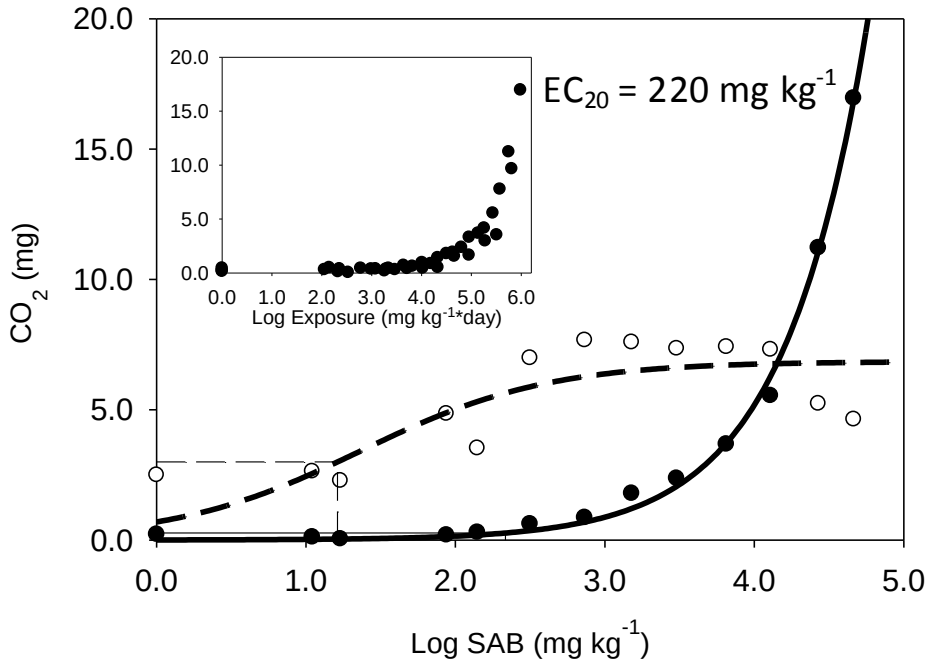


Figure 2.3. Effect of Special Antarctic Blend (SAB) diesel fuel (log mg fuel kg^{-1} soil) on total soil respiration (closed circles) and substrate induced respiration (open circles) for soil incubated for 21 d at various concentrations of SAB fuel. An exponential model (solid line) was fit to the total respiration after 21 d incubation and the resulting EC_{20} value is 216 mg kg^{-1} (drop lines). A sigmoidal, 3 parameter equation (dashed line) was fitted to the substrate induced respiration after 21 d incubation and EC_{20} value for this data is 16 mg fuel kg^{-1} soil (drop lines). The effect of SAB exposure (log mg fuel kg^{-1} soil × d) on total respiration is shown in the inset of this graph.

2.5 Discussion

From the suite of toxicity assays applied to Macquarie Island soil spiked with SAB diesel fuel, we determined that potential nitrification activity is the most sensitive measurement endpoint for the native microbial community, with an EC_{20} of 190 mg fuel kg^{-1} soil. Other authors have also shown nitrification activity to be the most sensitive indicator of toxicity when numerous measurement endpoints were used (Pereira et al., 2006; Sverdrup et al., 2002c). Many other researchers have found that nitrification activity (ammonium-oxidizing bacteria) is inhibited by petroleum hydrocarbon contamination (Christensen et al., 2001; Deni and Penninckx, 1999; Miller et al., 2007; Weissmann and Kunze, 1994). For example, Weissman and Knuze (1994) found that nitrification rates in petroleum hydrocarbon contaminated soil drop to less than 10% of control samples, leading to nitrogen imbalances within the soil. Nitrification activity may depend more on the availability of ammonium rather than the presence of petroleum hydrocarbons. However, our soil toxicity assay addresses this issue by assessing the potential activity by providing an adequate substrate for the ammonium-oxidizing bacteria to utilize. Potential nitrification activity is also a sensitive measurement endpoint for other contaminants, such as 2,4,6-Trinitrotoluene (TNT) (Gong et al., 1999), heavy metals such as As, Cd, Cr, Cu, Ni and Pb (Pereira et al., 2006), and solvents such as methanol, acetonitrile, trichloromethane, and dichloromethane (Miller et al., 1997). In contrast to our results, Deni and Penninckx (1999) found that ammonium oxidation is inhibited in non-contaminated agricultural soil briefly exposed to diesel fuel, but that long-term diesel fuel contamination does not inhibit ammonium oxidation. These authors also found that the nitrite-oxidizing activity in soil is not inhibited in soil with a long history of diesel contamination (Deni and Penninckx, 2004).

An increase in potential nitrous oxide production with increased exposure to SAB fuel was observed in our experiments. Our results agree with the results of Cho and Pierce (2005) who observed an increase in nitrous oxide emissions from soil that has been exposed to toluene. The increase in nitrous oxide production in field soils may be due to alterations in the oxygen content of the soil. Petroleum hydrocarbon contaminated soil is often more anaerobic because hydrocarbons impede the movement of oxygen into soil pores (Jae-Young and Day, 2004). Denitrifying bacteria are more suited to anaerobic conditions, and as such, their activity increases in a hydrocarbon contaminated soil.

Nitrous oxide consumption (potential N_2OR enzyme activity) was insensitive to petroleum hydrocarbon contamination. Other researchers have used this enzyme as a sensitive indicator for other pollutants such as pesticides and heavy metals (Bollag and Kurek, 1980; Richards and Knowles, 1995). Not all denitrifying bacteria contain nitrous oxide reductase and our results suggest that this subset of denitrifiers may be less susceptible to hydrocarbon contamination compared to the entire denitrifying communities.

The carbon cycle was sensitive to SAB contamination. Soil respiration is often used as a biological indicator for bioremediation of petroleum hydrocarbon contaminated sites and has been shown to be positively correlated with residual hydrocarbons (Margesin et al., 2000a). Thus, while increased respiration may not necessarily be a negative impact on an ecosystem, it is a clear indication that the hydrocarbons are bioavailable to the soil microbial community.

Even though substrate induced respiration was a more sensitive endpoint than total respiration, we believe that total respiration is a better assessment for the toxicity of petroleum hydrocarbons to soil microorganisms. For substrate induced respiration, there was not much

difference between the amount of CO_2 respired by the control and the various treatments of SAB. For example, the amount of CO_2 respired by the control was 2.50 mg, for the 50 000 mg kg^{-1} SAB treatment 4.60 mg CO_2 was respired, and the maximum amount of CO_2 respired from sucrose was 7.67 mg which occurred in the 781 mg kg^{-1} SAB treatment. In contrast, there was a large difference between the treatments for total respiration, with 0.226 mg CO_2 respired by the control and 17.0 mg CO_2 respired in the 50 000 mg kg^{-1} SAB treatment. Based on the EC_{20} values of 16 mg fuel kg^{-1} soil for sucrose respiration and 220 mg fuel kg^{-1} soil for total respiration, substrate induced respiration is a more sensitive endpoint, however total respiration is more responsive to increasing hydrocarbon contamination and has an EC_{20} value that is very similar to other biogeochemical endpoints.

In Canada, petroleum hydrocarbon contamination is regulated by the Canadian Council of Ministers of the Environment (CCME). The remediation criteria established by the CCME is based on land use (agricultural, residential/parkland, commercial, or industrial), soil texture (fine or coarse grained soil), exposure pathway, and the fraction of petroleum hydrocarbons (CCME, 2003). The generic level for petroleum hydrocarbon contamination (fraction 2, which encompasses C_{10} to C_{16}) in coarse soil used for commercial or industrial purposes with eco soil contact as the exposure pathway is 2000 mg fuel kg^{-1} soil. Nutrient cycling is one of the exposure pathways identified by the CCME, however, there is currently no information pertaining to the level of petroleum hydrocarbons that affects nutrient cycling. The application of toxicity assays used in this study would help to provide this critical information for the protection of the soil ecosystem.

The spiked soil used in our experiments is meant to simulate an oil and gas spill in the field, however there are different properties associated with soil spiked in a laboratory setting

versus an environmental spill. The spiked soil would be representative of a recent spill in the field, but as the spill ages, the chemical, physical, and biological properties of the soil change over time (Aislabie et al., 2004) and therefore the sensitivity of the nitrifying and denitrifying bacteria may change over time as well. The EC_{20} values derived using spiked soil may not be relevant to an aged fuel spill in the field. For example, Millar *et al.* studied the effect of organic contaminants (fuel and pesticides) on the nitrification activity level within soil and observed a decrease in nitrification activity with exposure to various solvents including methanol, acetonitrile, trichloromethane, and dichloromethane; however the inhibition of nitrification was not permanent and nitrification was eventually restored in all treatments (Miller et al., 1997). Deni and Penninckx have also observed differences in the effect of diesel on microbial activities in long-term *versus* short-term diesel contamination of soil and have shown that long-term exposure to diesel hydrocarbons does not inhibit nitrite-oxidizing activity (Deni and Penninckx, 1999; Deni and Penninckx, 2004). Therefore, our results for the inhibition of potential nitrification activity in polar soil must further be tested using a variety soils collected from aged fuel spill sites.

CHAPTER 3*

Influence of liquid water and soil temperature on petroleum hydrocarbon toxicity in Antarctic soil

* This chapter has been published in Environmental Toxicology and Chemistry 2009, 28: 1409-1415, under joint authorship with Ian Snape (Australian Antarctic Division) and Steven Siciliano (University of Saskatchewan)

3.1 Abstract

Fuel spills in Antarctica typically occur in rare ice-free oases along the coast, which are areas of extreme seasonal freezing. Spills often occur at sub-zero temperatures but we know little of ecosystem sensitivity to pollutants, in particular the influence that soil liquid water and low temperature has on toxicity of petroleum hydrocarbons (PHC) in Antarctic soil. To evaluate PHC toxicity, 32 locations at an aged diesel spill site in Antarctica were sampled nine times to encompass frozen, thaw and refreeze periods. Toxicity was assessed using potential activities of substrate-induced respiration, basal respiration, nitrification, denitrification, and metabolic quotient, as well as microbial community composition and bacterial biomass. The most sensitive indicator was community composition with a PHC concentration effecting 25% of the population (EC_{25}) of 800 mg kg^{-1}, followed by nitrification (2000 mg kg^{-1}), microbial biomass (2400 mg kg^{-1}) and soil respiration (3500 mg kg^{-1}). Despite changes in potential microbial activities and composition over the frozen/thaw/refreeze period, the sensitivity of these endpoints to PHC did not change with liquid water or temperature. However, the variability associated with ecotoxicity data increased at low liquid water contents. As a consequence of this variability, highly replicated (n=50) experiments are needed to quantify a 25% ecological impairment by PHCs in Antarctic soils at a 95% level of significance. Increases in biomass and respiration associated with changes in community composition suggest that PHC contamination in Antarctic soils may have irrevocable effects on the ecosystem.

3.2 Introduction

Petroleum hydrocarbons (PHC) are the most common type of contamination in cold regions (Snape et al., 2008b), but there are no environment specific ecotoxicology data available for this region (Snape et al., 2003). Most contamination in Antarctica is attributed to human activity associated with research stations, which are located mainly in ice-free coastal oases. These rare habitats comprise <0.05% of the land mass of Antarctica (Poland et al., 2003) and are important areas for birds, seals, vegetation, soil invertebrates and microorganisms. Yet, there is no information on the sensitivity of these areas to PHC contamination and there are currently no remediation guidelines for Antarctica.

Toxicity guidelines for PHCs might differ in polar environments due to unique environmental characteristics such as sub-zero temperatures and low liquid water content. In polar ecosystems, soil organisms remain active at temperatures below 0^{o}C but are limited by liquid water content in frozen soil (Bargagli, 2005; Panikov et al., 2006). Liquid water is present in soil at temperatures below 0^{o}C due to freezing point depression, in which salinity, capillary, and adsorptive forces lower the freezing point of water (Williams and Smith, 1991).

Toxicity of PHC is mainly attributed to a narcotic mode of action, which is characterized by the disruption of the cellular membrane(MacLeod et al., 2004). Petroleum hydrocarbons are widely reported to disrupt key soil ecosystem functions such as respiration and nitrogen cycling (Aislabie et al., 2004; Deni and Penninckx, 1999; Margesin et al., 2000b; Shi et al., 2002). Soil functions, such as microbial activity and community composition, can be used to assess the toxicity of contaminants in a soil ecosystem (Dawson et al., 2007; McMillian et al., 2007; Shi et al., 2002). Nitrification and soil respiration are two important enzymatic activities carried out by soil microorganisms that are affected by PHC contamination (Dawson et al., 2007; Hyman et al.,

1988; Schafer et al., 2007). Phospholipid fatty acid (PLFA) analysis provides information on microbial community composition and has been used by many researchers to show that soil microbial community composition is sensitive to PHC contamination (Bundy et al., 2004; Frostegard et al., 1993; Margesin et al., 2007; Shi et al., 2002). PLFA analysis is useful in ecotoxicity evaluations because it offers a technique that reflects the physiological stress, nutritional status, and viable biomass of the microbial population (Margesin et al., 2007).

The purpose of this study is to estimate the PHC toxicity to key biogeochemical cycles for a fuel spill at an Antarctic research station. We hypothesized that decreased liquid water content will increase PHC toxicity to soil biogeochemical cycles because the organisms responsible for these processes will be under significant osmotic and thermal stress.

3.3 Experimental Section

3.3.1 Site Description

Soils were sampled at a six year old PHC contaminated spill site at Casey Station, East Antarctica (66^{o} 17'S, 110^{o} 32'E) during the austral summer of 2005-2006. The spill occurred during the 1999 austral winter when approximately 5000 L of Special Antarctic Blend (SAB) diesel fuel leaked from a storage tank near the main powerhouse (MPH) (Snape et al., 2006). SAB fuel is a diesel fuel that contains a higher portion of the lighter fractions of PHCs (Snape et al., 2005). Six years following the initial spill event, the contamination levels still exceeded 25,000 mg kg^{-1}.

A sampling grid was established at the MPH site using a Nikon total station (Figure 3.1). Thirty-two sampling locations were selected along a transect orthogonal to the diesel plume and identified by easting (m) and northing (m) based on a reference point (northeast corner of the

boom surrounding the fuel tank on the north side of the MPH) which was given the coordinates (0, 0). Sampling locations encompassed a range of PHC contamination, from uncontaminated (0 mg fuel kg^{-1}) to approximately 25,000 mg fuel kg^{-1}. The concentration of fuel at each location was analyzed from a hexane extract (details in Section 2.3.3) at the beginning, middle and end of the field season using a gas chromatograph fitted with a flame ionization detector (GC-FID)(Snape et al., 2005) (Table 3.1). Soil from each location was also characterized for particle size distribution (Horiba LA-950, Laser Scattering Particle Size Distribution Analyzer), total organic carbon (TOC) (loss on combustion at 550°C), and gravimetric water content (Table 3.1). At each site, thermocouples and time-domain reflectometry (TDR) probes were installed vertically in the soil at a depth of 8.5 cm to measure temperature and liquid water content.

Soil was sampled nine times throughout the field season to encompass frozen, thaw and refreeze periods. Soil was sampled at a depth of 0-15 cm and passed through a 4.75 mm sieve to remove large rocks. Soil temperature and volumetric water content were measured at time of sampling for each location (Figure 3.2). Temperature was measured using Type K thermocouples (Cole-Parmer, Anjou, Quebec, Canada) and a hand-held Digi-Sense Dual JTEK thermocouple thermometer (Cole-Parmer, Anjou, Quebec, Canada). Liquid water content was measured using TDR probes (constructed by researchers) with a TDR100, SDMX50 50 Ω multiplexer, CR10X datalogger, and PS100 12.0 V power supply (all from Campbell Scientific, Canada).

Table 3.1 Soil characteristics for each sampling location at the Main Powerhouse (MPH) spill site.

Sampling Location		Special Antarctic Blend Diesel Fuel		Total Organic Carbon	Soil Texture			Soil Classification
Easting (m)	Northing (m)	Concentration (mg kg^{-1})	Uncertainty (mg kg^{-1})	%	% Sand	% Silt	% Clay	
-6	28	BD[1]	NA[2]	0.28	31.1	58.4	10.5	Silt Clay Loam
-2	28	BD	NA	0.13	17.1	75.6	7.3	Silt Loam
0	26	BD	NA	0.52	28.3	61.1	10.6	Silt Clay Loam
2	18	1960	130	0.46	15.4	42.6	42.0	Loam
2	24	BD	NA	0.45	34.5	61.5	4.0	Silt Clay Loam
2	26	BD	NA	0.50	33.9	61.5	4.6	Silt Clay Loam
4	18	4155	250	0.40	31.1	40.6	28.3	Clay Loam
4	20	7930	455	0.43	29.2	63.2	7.5	Silt Clay Loam
4	26	BD	NA	0.34	18.3	73.7	8.0	Silt Loam
4	28	160	45	0.40	36.1	60.4	3.5	Silt Clay Loam
6	16	2825	265	0.32	17.6	63.0	19.0	Silt Loam
6	18	7640	455	0.37	36.5	54.8	6.8	Silt Clay Loam
6	26	100	45	0.24	11.0	73.4	15.6	Silt Loam
8	16	4035	250	0.53	22.9	64.8	12.3	Silt Loam
8	18	890	205	0.22	4.8	40.9	54.3	Sandy Loam
8	20	2645	270	0.59	36.4	55.2	8.4	Silt Clay Loam
8	22	4350	285	0.42	22.1	63.2	14.7	Silt Loam
10	14	1640	205	1.25	35.1	59.6	5.3	Silt Clay Loam
10	18	1245	180	0.52	34.7	61.5	3.9	Silt Clay Loam
10	20	5265	485	0.40	27.2	63.1	9.6	Silt Clay Loam
12	14	9685	245	1.19	33.7	61.1	5.3	Silt Clay Loam
12	16	2612	245	0.35	26.3	69.5	4.3	Silt Loam
12	18	26460	2680	NA	NA	NA	NA	NA
12	20	8445	625	1.11	31.2	60.8	7.9	Silt Clay Loam
12	22	432	65	0.51	37.1	56.7	6.2	Silt Clay Loam
14	14	7110	520	0.68	28.1	66.9	5.0	Silt Clay Loam
14	16	7235	525	0.62	28.4	61.8	9.7	Silt Clay Loam
14	20	9185	490	0.51	36.3	59.3	4.4	Silt Clay Loam
14	22	4235	210	0.67	27.0	63.7	9.2	Silt Loam
16	14	2570	260	0.48	26.8	66.9	6.3	Silt Loam
18	14	BD	NA	0.70	36.1	54.0	9.0	Silt Clay Loam
18	17	3330	295	0.50	33.1	60.0	4.6	Silt Clay Loam

[1]Below detection limit (BD)

[2]Not available (NA)

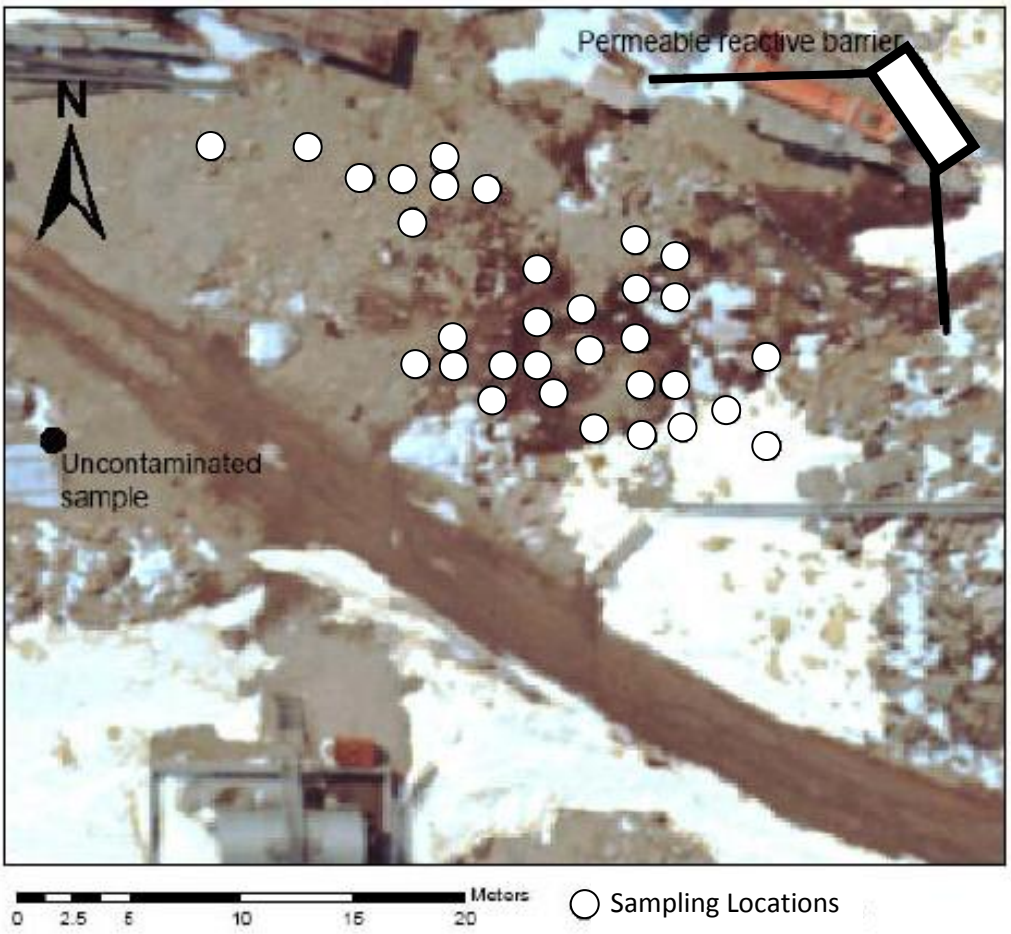


Figure 3.1 Aerial view of contaminated site at Casey Station, East Antarctica. Sampling locations are indicated by the white circles and the uncontaminated sample location is indicated by the black circle.

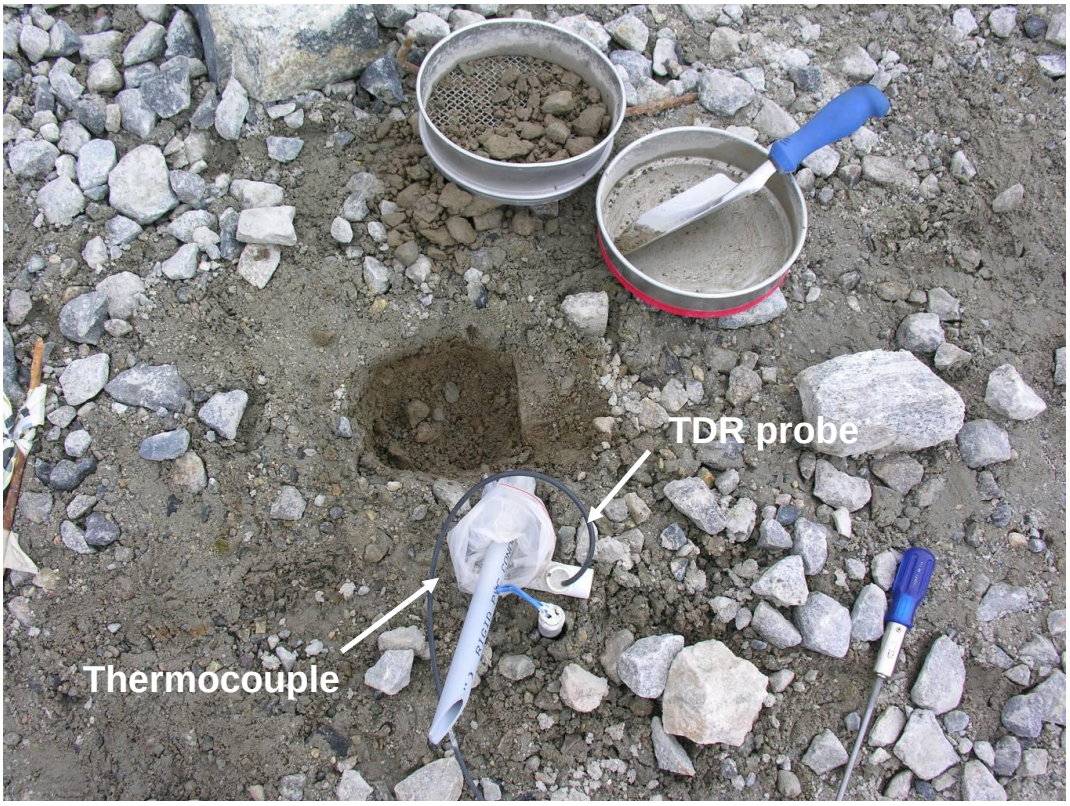


Figure 3.2 Thermocouple and time domain reflectometry (TDR) probes were installed at each sampling location to collect soil temperature and liquid water content at each sampling time.

3.3.2 Toxicity Assays

For each sampling period, soil from each location was analyzed for the following potential microbial activities: nitrification (Torstensson, 1993) , denitrification (production and consumption of nitrous oxide) (Siciliano et al., 2000), basal respiration (Schafer et al., 2007), substrate-induced respiration (Schafer et al., 2007), and metabolic quotient (Joergensen and Emmerling, 2006). These assays are described in detail in Section 2.3.4. It is should be emphasized that these assays are a measure of the potential activity under optimal conditions (nutrients, temperature, and liquid water).

3.3.3 Phospholipid fatty acid analysis

Microbial community composition was assessed spatially (32 locations) and temporally (frozen, thaw, and refreeze periods) using phospholipid fatty acid (PLFA) analysis (Frostegard et al., 1993; Margesin et al., 2007) . Lipids were extracted from freeze-dried soil (4.0 g) and combined with 15 ml methanol:chloroform:phosphate buffer solution (2:1:0.8 v/v/v) for 2 h on a shaker table at room temperature. Samples were centrifuged (10 min, 1000 rpm). Supernatant was transferred to another tube. The pellet was rinsed with a chloroform:phosphate buffer solution (4:5 v/v) and combined with the supernatant. The supernatants were allowed to separate into two phases. The bottom layer was transferred to another tube and evaporated under a stream of N_2. Samples were washed twice with methanol, evaporated with N_2, and stored at -20^{o}C. The lipid material was resuspended in chloroform (1.0 mL) and fractioned into neutral-, glycol-, and polar lipids on Bond Elute Si cartridges (Varian, Canada) by elution with chloroform, acetone, and methanol, respectively. The polar lipid containing fraction was evaporated under a N_2 stream. The samples were methlyated with 19:0 methyl ester internal

standard after redissolving the dried polar lipids in 1 mL 1:1 methanol:toluene and 1 mL methanolic KOH, and incubated (15 min, 35^{o}C). Two mL 4:1 hexane:chloroform and 2 mL milliQ water were added, samples were centrifuged (5 min, 1000 rpm), and upper layer (hexane) was transferred to a vial containing 10 µL 0.1 µg µL^{-1} 19:0 methyl ester. Samples were rinsed once more with 2 mL 4:1 hexane:chloroform, centrifuged and upper layer combined with previous hexane layer. Samples were evaporated under N_2, suspended in 200 µL hexane, transferred to GC vials and analyzed on a Hewlett Packard 5890 GC-FID (Agilent Technologies, USA). Phospholipid fatty acid concentrations (nmol g^{-1} soil) were calculated on a freeze-dried mass basis.

Fatty acids nomenclature uses the total number of carbon atoms: number of double bonds, followed by the position of the double bond from the methyl end (ω) of the molecule. Fatty acids can be either *cis* or *tran* configurations which are denoted by *c* and *t*, respectively. Prefixes *a* and *i* indicate anteiso- and isobranching, respectively; *cy* indicates cyclopropane fatty acids. The fatty acids chosen to represent bacterial PLFA include i15:0, a15:0, 15:0, i16:0, 16:1 ω7c, 17:0, i17:0, cy17:0, 18:1 ω7c, cy19:0 and were further broken down into Gram-positive (i15:0, a15:0, i16:0, i17:0) and Gram-negative (cy17:0, 18:1 ω7c, cy19:0) groups (Margesin et al., 2007). Fungal biomarkers include 18:2 ω6,9c as well as 16:1 ω5c and 18:1 ω9c (Joergensen and Emmerling, 2006).

3.3.4 Statistical Analysis

Ecotoxicity data was analyzed for spatial and temporal differences in potential microbial activity and community composition. Regression analysis was performed on dose-response relationships between PHC concentration and potential microbial activities using SigmaPlot 2004, Version 9.01. Best-fit relationship was chosen from reparameterized equations for linear,

logistic and exponential models based on optimal r^2 value (Environment Canada, 2007). EC_{25} values were derived from model of best-fit, and represent the concentration that causes a 25% difference from the control response. The EC_{25} value represents a concentration value that will protect 75% of the soil ecosystem function and thus can be considered as a site-specific remediation guideline for the particular endpoint the calculation has been based upon. Significant differences in EC_{25} values between sampling periods were tested for using Zajdlik's ad hoc method No. 2 (Environment Canada, 2007).

Phospholipid fatty acid data was analyzed using nonmetric multidimensional scaling (NMS), an ordination technique suited to data that are non-normal, or on arbitrary or discontinuous scales (McCune and Grace, 2002), using PC-Ord, Version 5.10. The parameters used for the NMS analysis were: Sorensen (Bray-Curtis) distance, random starting configurations, 250 runs with real and randomized data. The PLFA content was transformed on a logarithmic scale prior to NMS analysis. The correlation between environmental/soil variables (liquid water content, soil temperature, soil organic matter, PHC concentration) and NMS scores were determined. To compare between sampling times (frozen, thaw, and refreeze), a multi-response permutation procedure (MRPP), a nonparametric procedure that allows for testing the hypothesis of no difference between groups, was preformed on the data (McCune and Grace, 2002).

3.4 Results

Petroleum hydrocarbon contamination altered PNA and soil respiration activities (Figure 3.3), indicating that these activities are good indicators of PHC toxicity. Petroleum hydrocarbons decreased PNA in an exponential fashion with an EC_{25} of 2000 mg kg^{-1} observed for the entire season, modeled by the equation,

$$y = 5.731 \times 0.25^{x/3.302} \quad [3.1]$$

where y is PNA and x is log TPH concentration (r^2 = 0.618). In contrast, PHC increased soil respiration in a logistic fashion with an EC_{25} of 3500 µg g^{-1} observed for the entire season, modeled by the equation

$$y = 0.589 / \left(1 + \left((x/3.55)^{5.912}\right) \times \left(-0.25/1.25\right)\right) \quad [3.2]$$

where y is soil respiration and x is log TPH concentration (r^2 =0.780). The other potential microbial activities, including denitrification (N_2O production and consumption), SIR, and metabolic quotient, were not sensitive to PHC contamination (Figure 3.3). The median value for each potential microbial activity were as follows: 1.3 nmol NO_2 g^{-1} soil d. wt. hr^{-1} for PNA; 0.5 mg CO_2 for basal respiration; 0.01 mg CO_2 for SIR, 0.3 nmol N_2O produced g^{-1} soil d. wt. hr^{-1} for NaR/NiR/NOR enzyme activity, 0.2 nmol N_2O consumed g^{-1} soil d. wt. hr^{-1} for N_2OR enzyme activity; and 0.2 mg CO_2 μg^{-1} C g^{-1} soil d. wt. for metabolic quotient.

Similar to PNA and soil respiration, microbial community composition and microbial biomass were altered by PHC contamination during the frozen, thawed and refreeze periods (Figure 3.4). Approximately 83% of the variation in PLFA data was explained by axis 1 (23%) and axis 2 (60%). Axis 1 was correlated with TPH, r = 0.408, and r = 0.216 for axis 2. The decrease in Axis 1 scores was linearly related to TPH concentration as modeled by the equation,

$$y = 0.153 - \left(\left(0.25 \times 0.153x\right)/800\right) \quad [3.3]$$

where y is Axis 1 scores and x TPH concentration (r^2 = 0.202). The TPH dependence on community composition is seen in all three sampling periods (frozen, thawed, and refreeze). When the community composition between sampling periods are compared by MRPP analysis, a

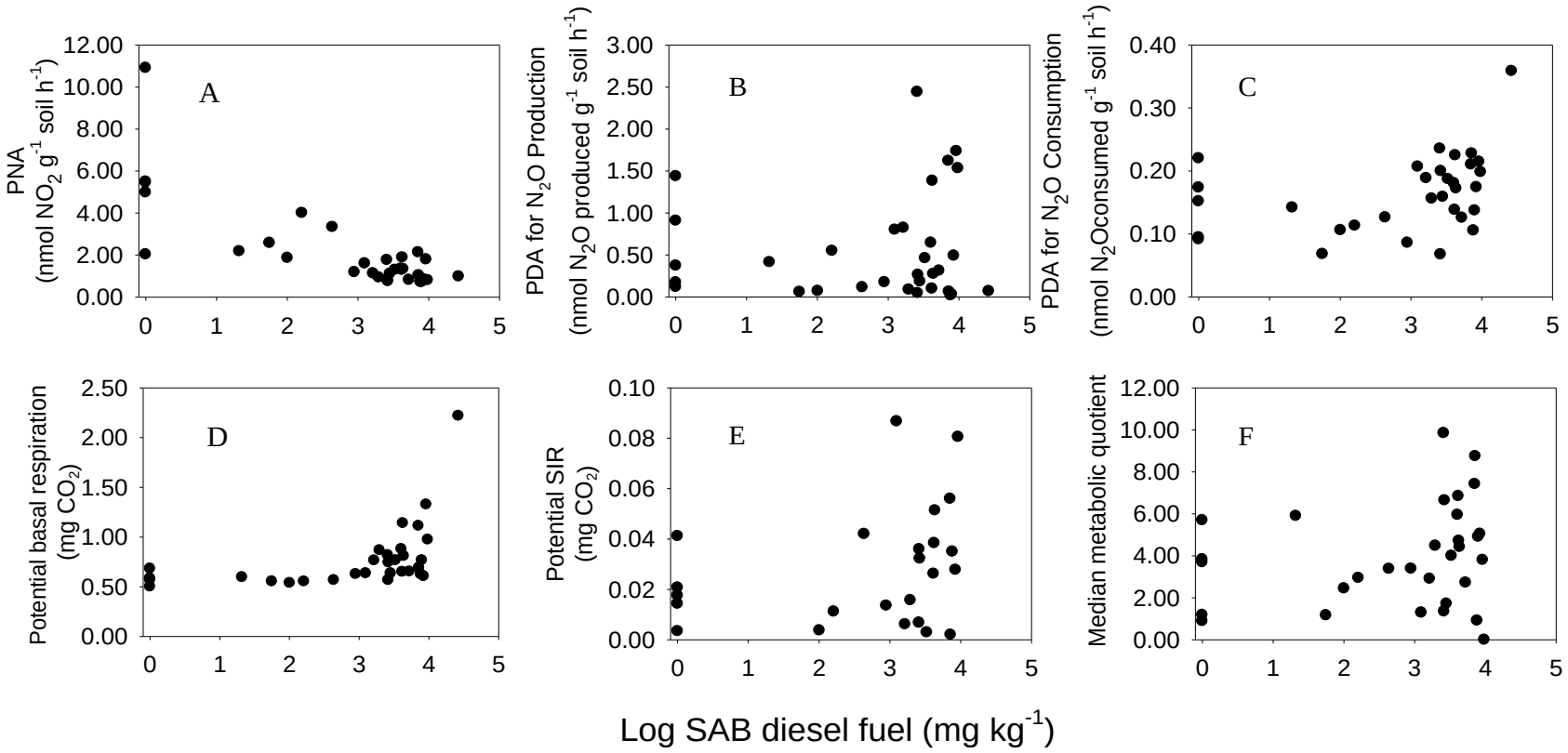


Figure 3.3 Dose-response curves for potential microbial activities and Special Antarctic Blend (SAB) diesel fuel contamination at the main powerhouse (MPH) spill site at Casey Station. The median response (n=9) at each sampling location is graphed for each ecotoxicological measurement endpoint (potential nitrification activity (PNA) (Panel A), potential denitrification (PDA) for N_2O production (Panel B), PDA for N_2O consumption (Panel C), potential basal respiration (Panel D), potential substrate induced respiration (SIR) (Panel E), and metabolic quotient (Panel F) and are given on the y-axis with SAB (Special Antarctic Blend) diesel fuel concentration (mg fuel kg^{-1}) on a log scale on the x-axis.

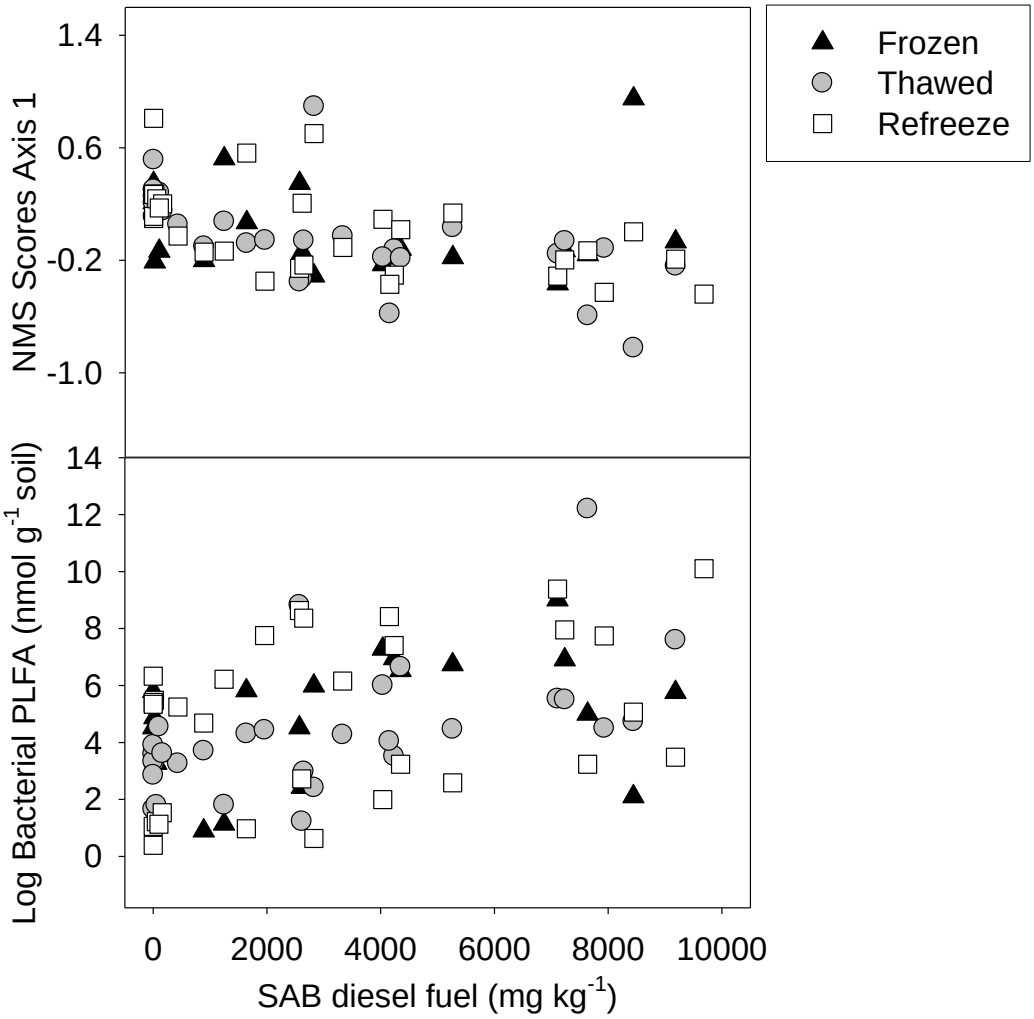


Figure 3.4 Microbial community composition changes for frozen, thawed, and refreeze periods. Nonmetric multidimensional scaling (NMS) scores for axis 1 are shown as a function of Special Antarctic Blend (SAB) diesel fuel concentration (r = 0.408) (top panel). Log of bacterial phospholipid fatty acid (PLFA) content is shown as a function of SAB diesel fuel concentration (bottom panel).

difference is observed between frozen and thawed soil (p=0.07), as well as between thaw and refreeze samples (p=0.02), but not between frozen and refreeze sampling periods (p=0.27). The MRPP analysis indicates that a shift in microbial community occurs when the soil goes from a frozen to thawed state, but then returns to the same condition when the soil refreezes.

Microbial biomass increased linearly as PHC contents in soil increased (Figure 3.4, bottom panel) with an EC_{25} value of 2400 mg kg^{-1} for the combined season, modeled by the equation

$$y = 3.5 - ((-0.25 \times 3.5x)/2400) \quad [3.4]$$

where y is bacteria biomass and x is TPH concentration (r^2 =0.202). While general bacterial PLFA biomarkers indicated an increase in bacterial population with an increase in PHC concentration, the biomarkers assigned specifically to Gram-negative and Gram-positive bacteria did not show a change with PHC contamination. Similarly, the fungal population did not show a response to PHC contamination.

Potential nitrification activity, soil respiration, microbial community composition, and bacterial biomass were sensitive indicators of PHC toxicity with EC_{25} values of 2000, 3500, 800 and 2400 mg kg^{-1} soil, respectively for combined sampling periods (Table 3.2). The EC_{25} values were not calculated for the remaining potential microbial activities because of poor dose-response curves generated by these endpoints. Over the frozen-thawed-refreeze period, EC_{25}'s fluctuated but did not significantly change, with the exception of the refreeze period for PNA (Table 3.2). However, the dose-response relationship obtained for PNA during the refreeze sampling period was difficult to model, as EC_{25} values vary greatly depending on the type of regression model applied (20 mg kg^{-1} determined by linear model and 24,000 mg kg^{-1} determined by exponential model, with similar r^2 values of 0.443 and 0.420, respectively).

Table 3.2 EC_{25} values and regression parameters for select measurement endpoints.

	Frozen	**Thawed**	**Refreeze**	**Combined**[1]
Potential Nitrification Activity				
Model	exponential	exponential	linear / exponential	exponential
EC_{25} (mg kg^{-1})[2]	200[ab]	1000[ac]	20[b] / 24000[c]	2000[ac]
SE[3]	400	1600	6.7 / 62000	2500
(r^2)[5]	0.353	0.42	0.420/0.443	0.618
Potential Basal Respiration				
Model	logistic	logistic	logistic	logistic
EC_{25} (mg kg^{-1})[2]	5400[d]	2400[d]	3000[d]	3500[d]
SE	9600	3100	3500	1700
r^2	0.447	0.284	0.276	0.780
Microbial Community Composition				
Model	linear	linear	linear	linear
EC_{25} (mg kg^{-1})[2]	NA[4]	670[e]	880[e]	800[e]
SE	NA	175	220	170
r^2	NA	0.385	0.306	0.202
Bacterial Biomass				
Model	linear	linear	linear	linear
EC_{25} (mg kg^{-1})[2]	3800[f]	1700[f]	2500[f]	2400[f]
SE	2700	680	1500	730
r^2	0.145	0.338	0.151	0.202

[a] "Combined" refers to the all nine sampling periods.

[2] Different letters represent significant differences between sampling periods at the 95% level of significance. Statistical significance in EC_{25} was only tested between sampling period and not between measurement endpoint.

[3]SE = standard error

[4]NA = not available

[5]r-squared

Therefore, we are reluctant to interpret the significant differences in EC_{25} values for PNA as real differences. For the remaining endpoints, confidence limits for EC_{25} values overlapped between sampling periods, indicating that there is no difference in toxicity between sampling periods.

Despite liquid water content and temperature ranging from 0.04 to 0.56 cm^3 water cm^{-3} soil and -11.2 to 12.1^{o}C, there were no associated changes with potential microbial activities (Figure 3.5). However, variability of the potential activities increases at low liquid water content, especially for PNA (Figure 3.5). The PNA variability was much higher than that observed for basal respiration with a maximum CV of 174% for PNA compared to 56% for basal respiration. Coefficient of variation for PNA was linked to liquid water content which was modeled by the equation:

$$y = -0.2557x + 2.0542 \quad [3.5]$$

where y is the coefficient of variation and x is liquid water content (r^2 = 0.677). Coefficient of variation for basal respiration was also linked to liquid water, modeled by:

$$y = -0.0219x + 0.5018 \quad [3.6]$$

where y is the coefficient of variation and x is liquid water content (r^2 = 0.345). Potential nitrification activity and basal soil respiration are the only microbial activities shown in the graph because of their PHC dependence; however the variance of all microbial activities increased at low liquid water contents. For example, maximum CV for N_2O consumption and N_2O consumption were both observed at 0.10 – 0.19 cm^3 water cm^{-3} soil with values of 340% and 320%, respectively.

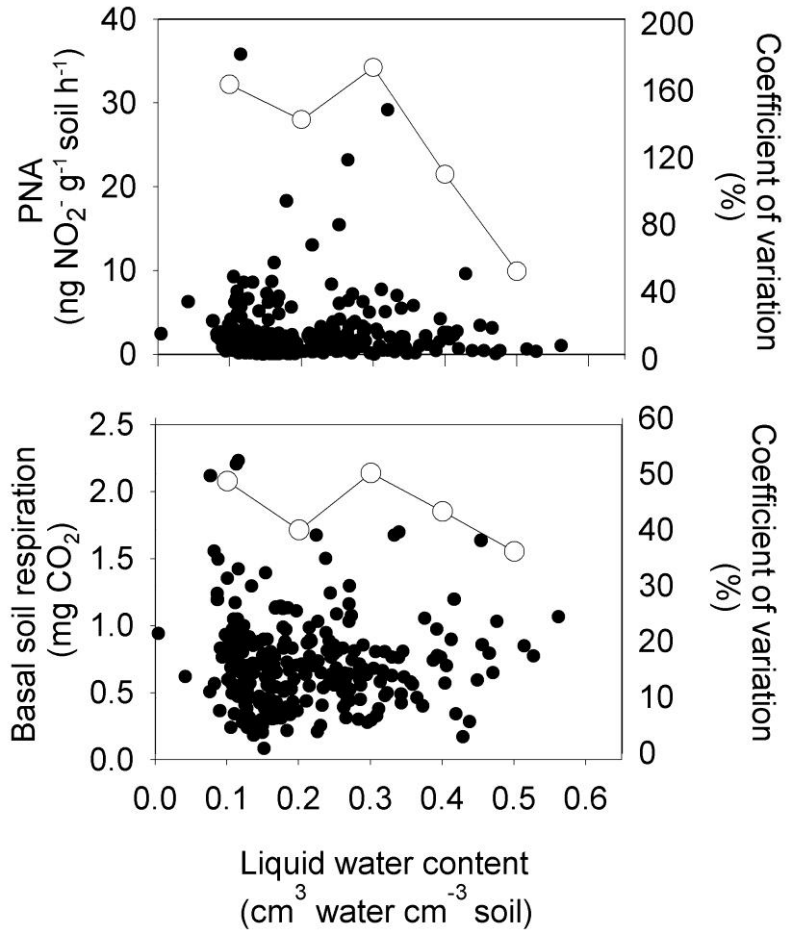


Figure 3.5 Potential nitrification activity (PNA) and basal soil respiration as a function of liquid water content. Coefficient of variation (CV) increases at low liquid water content, especially for PNA. To calculate CV, volumetric liquid water content was binned into six equal bins, ranging from 0 to 0.59 cm^3 water cm^{-3} soil, with minimum bin size of 40.

3.5 Discussion

To the best of our knowledge, this study is the first of its kind to study the effect of liquid water content and soil temperature on the toxicity of PHC. Despite large shifts in liquid water,

from 0.04 to 0.56 cm^3 water cm^{-3} soil, and temperature, from -11.2 to 12.1^oC, there was relatively little change in PHC toxicity to potential microbial activity or community composition. It should be noted that this does not imply that *in situ* activities are insensitive to temperature or liquid water, rather that toxicity assays used to assess soil functioning in response to toxicant impacts, are not confounded by large temperature or liquid water changes at the study site. Thus, potential soil functioning tests may be an effective means for toxicologists to reduce the confounding effects of variable climatic and moisture parameters when assessing PHC impact at a contaminated site.

Although little evidence was found for either liquid water content or soil temperature having an influence on the toxicity of PHC to potential microbial activities, we did observe an increase in variability of microbial activity at low liquid water contents. High variability in soil properties is often associated with soil in polar regions due to a process known as cyroturbation, where soil from the sub-surface is mixed with surface soil by frost heave action (Bockheim and Tarnocai, 1998). Here we observed high variability in biological parameters as a function of liquid water content at sub-zero temperatures. Other researchers have shown phytotoxicity tests results to be exceptionally variable in Arctic soils compared to temperate soils (Anaka et al., 2008). High variability leads to large minimum detectable differences between treatments (Kraufvelin, 1998).

Experimental designs for ecotoxicological studies in polar soils should consider taking large sample sizes in order to improve the ability to detect toxicological effects in these systems. Otherwise there is a significant risk of investigators detecting false negatives during their investigations of polar sites. From our results, we suggest that when liquid water content is low, a minimum of 50 samples be taken from both contaminated and reference sites in order to

detect a 25% toxicological impact from contamination. When abundant liquid water is present, the minimum number of samples for soil respiration can be reduced to 20, however for PNA minimum sample number should be kept at 50.

A study conducted on sub-Antarctic soil spiked with diesel fuel, shows similar dose-response relationships for potential microbial activity to PHC contamination (Schafer et al., 2007). In the previous study, PNA decreased, while basal respiration and SIR increased, and denitrification activity remained unchanged by diesel fuel contamination. However, PHC toxicity was an order of magnitude greater in the study with spiked diesel fuel. The EC_{20} values determined by the laboratory study were ~200 mg kg^{-1} for PNA and basal soil respiration; whereas the EC_{25} values for the same measurement endpoints in this study were ~2000 and 3500 mg kg^{-1}, respectively. This difference in toxicity may be due to the age of the fuel spill or chemical composition of the fuel as it ages. The spiked soil contains a higher portion of light, volatile, short-chained hydrocarbons, which are generally more toxic than the long-chained hydrocarbons that dominate an aged fuel spill. In an aged fuel spill many of the short-chained hydrocarbons would be lost via volatilization, therefore soil microbiota may be able to tolerate higher concentrations of PHC in an aged spill. Although we cannot directly compare the EC_x values of these two studies because of physical, chemical, and biological differences between the two soils, this study is indicative of a decrease in toxicity as a fuel spill ages. Bioremediation studies have also demonstrated a decrease in PHC toxicity over time, especially when biostimulation (addition of nutrients) or landfarming (tilling of contaminated soil) techniques are used, but also when natural attenuation is used as a remediation technique (Chaineau et al., 2003; Salson et al., 2004).

Potential nitrification activity decreased as the PHC contamination of an aged diesel spill increased. In contrast, other researchers found that over a prolonged period of time, nitrification activity recovered at a PHC contaminated site (Deni and Penninckx, 2004). In Antarctica, nitrification activity was still impacted in areas of high contamination six years after the initial diesel fuel spill. Petroleum hydrocarbons have a specific mode of toxic action on the ammonium monooxygenase enzyme, with alkanes, alkenes, and alkynes having an inhibitory effect on this enzyme via competitive and non-competitive binding (Hyman et al., 1988; Keener and Arp, 1993). Thus, it appears that PHC contamination prevents nitrifiers from metabolizing, and consequently their populations decline and have not yet recovered. In a study using these same soils from Casey station, the prevalence of the gene encoding ammonium monooxygenase declined in response to PHC concentration (Pisz et al., 2007). Using that data, we can calculate an EC_{25} for ammonium monoxygenase prevalence of 2400 mg kg^{-1}, modeled by

$$y = 2.96 + \left[\frac{a}{1 + \left[\left(x/2400 \right)^{5.37} \times \left[13.6/\left(0.75 \times \left(2.96 + 13.6 \right) - 2.96 \right) \right] - 1 \right]} \right] \qquad [3.7]$$

where y is the prevalence of the gene, x is the concentration of diesel fuel and a is a fitting parameter (r^2 = 0.212). The difference in environmental conditions or the nitrifying community itself may be responsible for the incapability of the Antarctic nitrifying bacteria to recover from the PHC contamination. It remains to be seen if this effect is only seen in Antarctic PHC contaminated sites or is also seen in Arctic sites.

Community composition was the most sensitive endpoint to PHC contamination. While community composition changed, microbial biomass and respiration increased. This suggests that these terrestrial microbial ecosystems are severely carbon limited and PHC contamination

stimulates the microbial community. Comparable results have been reported by other researchers (Macnaughton et al., 1999; Margesin et al., 2007; Shi et al., 2002); however, these other studies have also observed an increase in Gram-negative bacteria, which was not observed in this study. These results are troubling because they suggest that once Antarctic soil is contaminated, the microbial ecosystem may be irrevocably altered with little hope of returning Antarctic soil microbial ecosystems to their pristine condition.

In conclusion, we demonstrated that potential microbial activities, such as nitrification and soil respiration, can be used as sensitive indicators of PHC contamination in polar soil. PHC contamination is associated with a decrease in PNA and an increase in potential soil respiration. In a polar environment, where N is very limited, a decrease in nitrification coupled with an increase in soil respiration (which places extra demand on available nutrients) may place additional stress on the soil ecosystem because of N limitation. This nutrient stress, in addition to PHC toxicity, likely explains the shift in the microbial community composition in response to PHC contamination. This interaction between N limitation and PHC toxicity suggests that remediation systems that use extensive fertilization may only temporarily reduce toxicity to the overall ecosystem. Nitrification tests should be used to confirm that reductions in toxicity associated with fertilization remediation schemes in polar systems have resulted in a sustainable ecosystem. It is doubtful if current remediation systems will be able to return microbial ecosystems to their pre-impact state.

CHAPTER 4

Changes in liquid water alter nutrient supply rate and gas diffusion in frozen Antarctic soils contaminated with petroleum hydrocarbons

4.1 Abstract

Bioremediation has been used to remediate petroleum hydrocarbon (PHC) contaminated sites in Polar Regions, however limited knowledge exists in understanding how frozen conditions influence factors that regulate microbial activity. One such factor is the amount of liquid water (θ_{liquid}) in frozen soil. We hypothesized that increased θ_{liquid} would affect nutrient supply rates and gas diffusion under frozen conditions as a result of changes in θ_{liquid}. If true, management practices that increase θ_{liquid} should also increase bioremediation in polar soils by reducing nutrient and oxygen limitations. The influence of θ_{liquid} on nutrient supply rate in PHC contaminated soil was determined using contaminated soil from Casey Station, East Antarctica. The θ_{liquid} was altered between 0.007 and 0.035 cm^3 cm^{-3} by packing soil cores at different bulk densities (1.4, 1.7, and 2.0 g cm^{-3}) and also by using soils from the same site with different PHC concentrations (0 – 8000 mg kg^{-1}). Nutrient supply rate of NH_4^+ and NO_3^-, as well as gas diffusion coefficient, D_s, were measured at two temperatures, 21°C and -5°C to correct for bulk density effects. Not surprisingly, freezing reduced nutrient supply of both NH_4^+ and NO_3^-. However, θ_{liquid} was linked to nitrate and ammonia nutrient supply rates in frozen soil. Supporting this, PHC contamination, which is known to alter θ_{liquid}, increased nutrient supply rates but only under frozen conditions. Similarly for gas diffusion, decreases in D_s due to freezing were much more pronounced in soils with low θ_{liquid} compared to soils with higher θ_{liquid} contents. For example, in soils with 0.016 cm^3 cm^{-3} θ_{liquid} freezing decreased D_s by 60% whereas in soils with 0.028 cm^3 cm^{-3} θ_{liquid} freezing increased D_s in soils. The results of this study indicate that bioremediation in cold regions could be enhanced during the period of time where the soil temperature is below 0°C by controlling factors that increase the amount of liquid water,

however additional studies are needed to determine the relationship between degradation rates and liquid water content under frozen conditions.

4.2 Introduction

Petroleum hydrocarbon contamination is the most common terrestrial pollutant in polar regions (Snape et al., 2008b). Clean up of contaminated sites in polar regions is very expensive because of the remote isolated locations. Bioremediation techniques, which rely on native or introduced soil microorganisms capable of using PHCs as a carbon or energy source, are commonly used in polar regions (Horel and Schiewer, 2009; Paudyn et al., 2008; Sanscartier et al., 2009). The addition of nutrients, as well as oxygen sparging, are often used to enhance bioremediation and reduce clean up time. However, harsh environmental conditions of polar regions can reduce the efficacy of bioremediation, thus requiring a longer time for clean-up (Horel and Schiewer, 2009). Understanding how the environmental conditions change some of the physical factors controlling bioremediation, such as nutrient availability and oxygen penetration, will aid in the management of bioremediation projects in polar regions.

Liquid water (θ_{liquid}) is one of the limiting factors for microbial activity in frozen soil. The amount of θ_{liquid} in frozen soil is important for microbial survival at temperatures below $0^{o}C$ because the unfrozen water allows the diffusion of microbial substrates and waste products (Ostroumov and Siegert, 1996). A substantial amount of θ_{liquid}, exists between 0 and $-10^{o}C$ (Dash et al., 1995). The occurrence of θ_{liquid} below $0^{o}C$ is attributed to three forces – salinity, adsorptive, and capillary forces (Dash et al., 1995). Salinity increases θ_{liquid} by lowering the chemical potential of pore water thus causing the melting point to decrease by approximately $0.1^{o}C$; adsorptive forces increase θ_{liquid} by wetting solid surfaces with a melted layer thus

reducing the free energy; and capillary forces increase θ_{liquid} by changing the Gibbs free energy of the pore water at curved surfaces (Williams and Smith, 1991). In addition to these forces, the presence of PHC in soil also increases θ_{liquid} in frozen soil by changing the partial molar free energy of the soil pore water (Siciliano et al., 2008).

The role of oxygen in the breakdown on PHC has been well established. In passive bioremediation of PHC contaminated soils, oxygen diffusion is the primary mechanism for supplying the oxygen required for microbial hydrocarbon biodegradation processes (Huesemann and Truex, 1996). The extent of oxygen penetration is strongly dependent on the gas diffusion coefficient (D_s) in soil, with increases in D_s associated with increased oxygen penetration and decreased cleanup times (Rike et al., 2005). The magnitude of D_s is inversely related to the soil moisture content and bulk density (Fujikawa and Miyazaki, 2005). In regards to bioremediation in cold regions, it is imperative to maintain adequate oxygen levels in the soil, especially during the summer season when microbial activity is at its greatest. Oxygen status of the soil is important during the winter months as well, as microbial degradation of PHC continues in frozen soil (Margesin and Schinner, 1999b), therefore understanding the magnitude D_s at temperature below $0^{o}C$ is important to maximize bioremediation in polar regions.

Microbial activity and, hence, the rate of intrinsic bioremediation, is dependent on the bioavailability of substrates, electron acceptors and nutrients, which are related to environmental conditions (Yang et al., 2009). In polar regions, nitrogen, and to a lesser extent, phosphorus are in low concentrations in the soil and often limit hydrocarbon degradation (Roling and van Verseveld, 2002) and these nutrients are often added to stimulate biodegradation of PHC (Thomassin-Lacroix et al., 2002). Nutrient limitation to microbial activity

in polar soil is further exacerbated in the winter, as nutrient availability becomes even more reduced during the winter months (Giblin et al., 1994; MacKenzie and Quideau, 2010). These winter months comprise the majority of the annual cycle in polar regions and thus, understanding nutrient supply rates during winter months will allow us to optimize polar bioremediation systems for the late winter/early spring period when fertilizers can not yet be added to contaminated soil.

We hypothesized that changes in θ_{liquid} would alter gas and nutrient diffusion rates, which in turn are the primary controls of bioremediation in polar soils. We used ion exchange resins to quantify the nutrient supply rate of NH_4^+ and NO_3^- in frozen soils contaminated with PHC. We altered θ_{liquid} in frozen soil by altering the bulk density of soil columns. However, since nutrient and gas diffusion constants are also known to vary with bulk density, we compared diffusion at room and frozen temperatures which allows us to remove the 'bulk density' effect and investigate if θ_{liquid} was linked to changes in diffusion.

4.3 Materials and Methods

4.3.1 Soil

Soil collected from a contaminated site at Casey Station, East Antarctica (66° 17'S, 110° 32'E) was used in this study. The spill occurred during the 1999 austral winter when approximately 6000 L of Special Antarctic Blend (SAB) diesel fuel leaked from a storage tank near the main powerhouse (MPH). Six years following the initial spill event, contamination levels still exceeded 25,000 mg kg^{-1}. For this study, soils from nine sampling locations are divided into three groups of PHC contaminated soil. The PHC groups are 0 – 200, 1000 – 2000, and 7000 – 8000 mg kg^{-1}. Sterilized soil is used in all experiments. Soil was sterilized at the Canadian

Irradiation Centre (Laval, PQ, Canada) via gamma irradiation with a minimum dose of 25.0 kGy (Trevors, 1996).

4.3.2 Liquid Water Content

Liquid water content (θ_{liquid}) was manipulated by changing the bulk density of the repacked soil cores (ρ_{bulk}). Three bulk densities, 1.4, 1.7, and 2.0 g cm^{-3}, are used in this experiment corresponding to loose, medium, and firmly packed soil, respectively. The porosity (ϕ) of the soil cores were calculated by the following equation,

$$\phi = 1 - \frac{\rho_{bulk}}{\rho_{particle}} \qquad [4.1]$$

where $\rho_{particle}$ is the density of the particle, which is assumed to be 2.65 g cm^{-3}. The porosity of the repacked soil cores are 47%, 36%, and 25% for bulk densities of 1.4, 1.7, and 2.0 g cm^{-3}, respectively. Time domain reflectometry (TDR) probes are used to measure θ_{liquid} of frozen soil (Spaans and Baker, 1995). The TDR technique is able to differentiate between the frozen and unfrozen fraction of water based on the difference in the dielectric constant of these two phases of water (K_{water} = 80.36, K_{ice} = 3.2) and between the other soil constituents (K_{air} = 1; $3 \leq K_{soil} \leq 7$) in the frequency range of 1 MHz to 1 GHz (Topp et al., 1980). Additional information on TDR probe construction and calibration is available from Appendix A.

4.3.3 Nutrient Supply Rate

The nutrient supply rates (NSR) for NH_4^+ and NO_3^- are measured using cation and anion exchange resins, respectively. The resins were obtained from Western Ag Innovation Inc. (Saskatoon, SK, Canada). The “sandwich test”, a technique whereby an ion-exchange resin is sandwiched between soil, is used to measure NSR in contaminated soil (Qian et al., 2008). In

brief, the resins are regenerated by soaking in 0.5 M HCl (cation) or 0.5 M $NaHCO_3$ (anion). The charged resins are sandwiched between two Snapcap® 7 Dram vial lids packed with soil and wrapped in Parafilm®. Prior to sandwich assembly, nutrients, either NH_4^+ or NO_3^-, are added at a rate of 125 mg kg^{-1}, the optimal rate for bioremediation in sub-Antarctic soil (Walworth et al., 2007). Following 24 hour incubation, the sandwiches are dissembled; the resins rinsed with deionized water and eluted in 20 mL 0.5 M HCl shaken at 200 rpm for 1 hour. NH_4^+ and NO_3^- concentrations are measured colorimetrically using Smart Chem™ 200 from Westco Scientific Instruments Inc. (Brookfield, CT, USA). NSR is calculated as

$$NSR = (C \times V)/S \qquad [4.2]$$

where *C* is the concentration of the adsorbed cation or anion (μg mL^{-1}) in HCl eluent, *V* is the volume of eluent (mL), and *S* is the surface area of the membrane strip (cm^2).

4.3.4 Measurement of nutrient flux at frozen temperatures

The NSR for NH_4^+ and NO_3^- are measured at 21°C and -5°C for three PHC treatments packed at three bulk densities. The room temperature nutrient flux measurements were made using sandwiches assembled immediately after the addition of nutrients to the soil. In contrast, for frozen temperatures, nutrients were added to soils, soils are frozen at -5°C and then assembled into sandwiches. By measuring nutrient supply rates at frozen and room temperatures for multiple bulk densities, we can correct for bulk density influences on nutrient supply rates due to changes in porosity, independent of changes in θ_{liquid} content caused by changing bulk densities. Multiple analysis of variance was used to determine significant differences between TPH, bulk density and temperature, at a 95% significant level. If a

significant difference was detected between treatments, a multiple comparison of the means was used to determine which treatments are significantly different from each other.

4.3.5 Gas Diffusion Experiment

Gas diffusion was measured using the Currie Method (Rolston and Moldrup, 2002). One end of a soil core with gas concentration, C_s, is placed in contact with a chamber with initial gas concentration C_0. A tracer gas is added to the chamber at t = 0 s which diffuses through the soil core over time. The rate of change in concentration in the chamber over time is related to the soil gas diffusion coefficient, D_s.

The unsteady diffusion of a nonreactive (physically, chemically, biologically) gas is described by the combination of Fick's first law and the continuity equation

$$\varepsilon\left(\partial C_g / \partial t\right) = D_s\left(\partial^2 C_g / \partial x^2\right) \qquad [4.3]$$

where ε is the soil air content (m^3 air m^{-3} soil) calculated from total porosity minus the volume fraction of water (liquid and ice), C_g is the concentration in the gaseous phase (g gas m^{-3}soil air), t is time (s), D_s is the soil gas diffusion coefficient (m^3 soil air m^{-1} soil s^{-1}), and x is distance (m soil). If we assume that the soil is uniform with respect to the diffusion coefficient and that ε is constant in space and time, then the solution for the relative concentration in the chamber C_r is

$$C_r = \frac{C_g - C_s}{C_0 - C_s} = \sum_{n=1}^{\infty} \frac{2h \exp\left(-D_s \alpha_n{}^2 t / \varepsilon\right)}{L\left(\alpha_n{}^2 + h^2\right) + h} \qquad [4.4]$$

where C_s is the atmospheric concentration of the gas, C_0 is the initial concentration of gas in the chamber, $h = \varepsilon / \left(a \varepsilon_c\right)$, ε_c = 1 is the air content of the chamber (m^3 air m^{-3} chamber) and a is the volume of the chamber (m^3) per cross-sectional area of the soil core (m^2); L is the length of the

chamber (m) and α_n, with n = 1,2,…., are the positive roots of (αL)tan(αL)=hL (from lookup table p. 1118 Rolston and Moldrup, 2002). At some time greater than zero, the terms for $n \geq 2$ are negligible with respect to the first term and the equation 2 simplifies to

$$C_r = \frac{2h\exp\left(-D_s{\alpha_1}^2 t/\varepsilon\right)}{L({\alpha_1}^2+h^2)+h} \qquad [4.5]$$

The value used for α_1 is 0.5932 (Rolston and Moldrup, 2002). Thus, the diffusion coefficient, D_s, can be determined from the plot of ln C_r vs. t which becomes linear with slope $-D_s{\alpha_1}^2/\varepsilon$ for sufficiently large t.

The tracer gas used in this experiment was N_2O and analyzed using a Gasmet™ DX4015 Fourier Transform Infrared (FTIR) portable gas analyzer (Gasmet Technologies Inc., La Prairie, QC, Canada). It is noted that sterile soil was used in these experiments therefore N_2O production or consumption via microbial processes is negligible. N_2O was added at approximately 20 ppm and analyzed every 20 s for 2 hours.

The gas diffusion co-efficient was measured at 21°C and -5°C for three PHC treatments packed at three bulk densities. Multiple analysis of variance was used to determine significant differences between TPH, bulk density and temperature, at a 95% significant level. If a significant difference was detected between treatments, a multiple comparison of the means was used to determine which treatments are significantly different from each other.

4.4 Results

As expected, increasing bulk density increased the amount of θ_{liquid} in frozen soil, (Figure 4.1). An increase in θ_{liquid} is also observed with an increase in PHC contamination. Bulk density effect on θ_{liquid} content was greater for uncontaminated soil, increasing by nearly 2% from

lowest bulk density to the highest bulk density (0.007 to 0.025 cm^3 water cm^{-3} soil) compared to contaminated soil (range for 1000 – 2000 mg kg^{-1} treatment is 0.015 to 0.023 cm^3 water cm^{-3} soil; 7000 – 8000 mg kg^{-1} is 0.022 to 0.035 cm^3 water cm^{-3}). At the lowest bulk density of 1.4 g cm^{-3}, θ_{liquid} increased linearly with PHC contamination:

$$y = 0.0767x - 0.0073 \quad [4.6]$$

where y is liquid water content and x is PHC concentration ($r^2 = 1$).

Freezing reduced the nutrient supply rate of NH_4^+ between 32 – 40%, with the exception of the lowest bulk density where NH_4^+ bioavailability increased (Figure 4.2). At a bulk density of 1.4 g cm^{-3}, NH_4^+ NSR in the uncontaminated soil increased by 60% under frozen conditions. The NH_4^+ NSR decreases with bulk density in frozen soil, especially at high PHC contamination levels. In contrast, PHC contamination had a minimal effect on NH_4^+ NSR under frozen conditions ($p = 0.224$) with NH_4^+ NSR increasing from 72 μg cm^{-2} in uncontaminated soil to 113 μg cm^{-2} in soil contaminated with 7000 – 8000 mg kg^{-1} PHC.

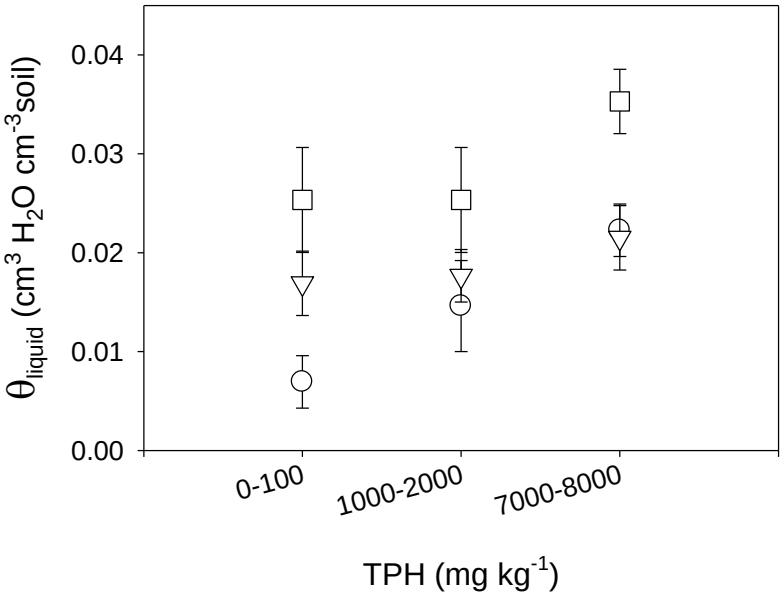


Figure 4.1 Liquid water content (θ_{liquid}) at -5°C in soil collected from Casey Station, East Antarctica contaminated with a range of total petroleum hydrocarbons (TPH). Soil (n=3), with error bars representing standard deviation, was packed at three bulk densities, 1.4 g cm^{-3} (O), 1.7 g cm^{-3} (∇), 2.0 g cm^{-3} (□).

The effect of freezing on NO_3^- bioavailability was greater than that observed for NH_4^+, with reduction ranging from 20 – 56% (Figure 4.3). Even though the interaction between temperature and TPH treatment is not significant (p = 0.149), a difference in nutrient supply rate was observed at -5°C, with NO_3^- NSR increasing with a rise in TPH concentration. Bulk density has a significant effect on NO_3^- NSR (p = 0.01). Under both room temperature and frozen conditions, bioavailability of NO_3^- was approximately 10 times higher than that of NH_4^+ with NO_3^- NSR ranging from 510 – 2500 µg cm^{-2}, whereas NH_4^+ NSR ranged from 45 – 130 µg cm^{-2}. Thus, despite the greater influence of freezing on NO_3^-, under frozen conditions the dominant source of N supply is nitrate, not ammonium.

There is a large increase in nutrient supply rate with an increase in θ_{liquid} under frozen conditions (Figure 4.4). A dramatic increase in NH_4^+ bioavailability is observed at low θ_{liquid} content of less than 0.01 cm water cm^{-3} soil, which then plateaus around 0.02 cm^3 water cm^{-3} soil. The bioavailability of NO_3^- similarly increases exponentially at low θ_{liquid} content, although at a slower rate than NH_4^+, and reaches a plateau around 0.03 cm^3 water cm^{-3} soil. The relationship between nutrient supply rate and θ_{liquid} content for NH_4^+ and NO_3^- in frozen soil is nonlinear and is best described by an exponential rise to maximum equation,

$$y = y_o + a\left(1 - \exp^{-bx}\right) \qquad [4.7]$$

where y is the dependent variable (NH_4^+ or NO_3^- NSR); x is the independent variable (θ_{liquid}); y_0, a and b are model parameters.

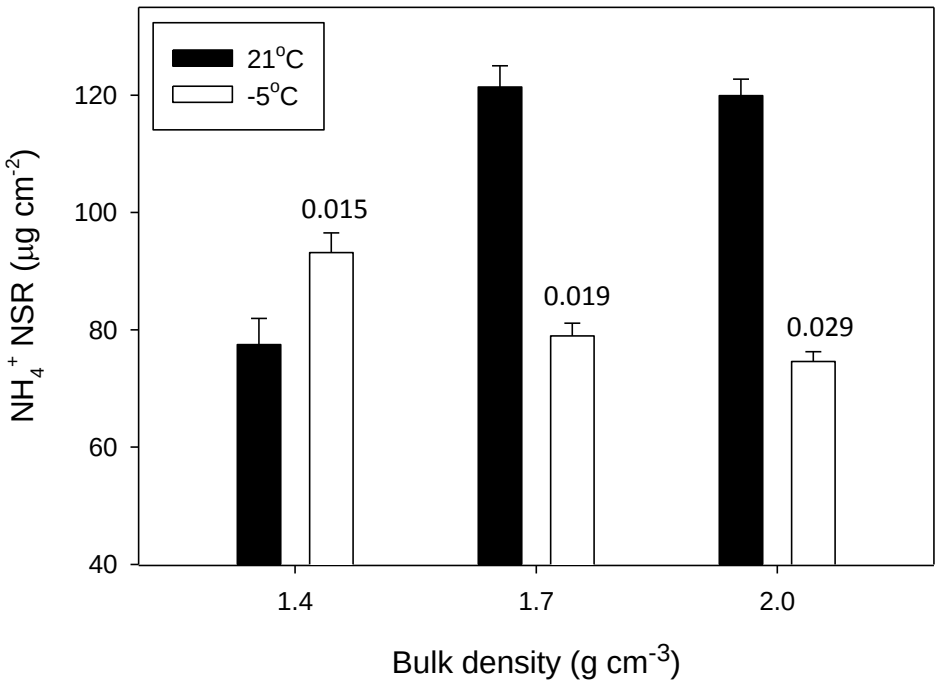


Figure 4.2 Temperature and bulk density effects on NH_4^+ nutrient supply rate (NSR) for contaminated soil. Each bar is the average NH_4^+ NSR (n=9) with error bars representing the standard error. Average liquid water content (n=3) at -5°C (values above white bars) are 0.015, 0.019, and 0.029 cm^3 H_2O cm^{-3} soil for bulk densities of 1.4, 1.7, and 2.0 g cm^{-3}, respectively.

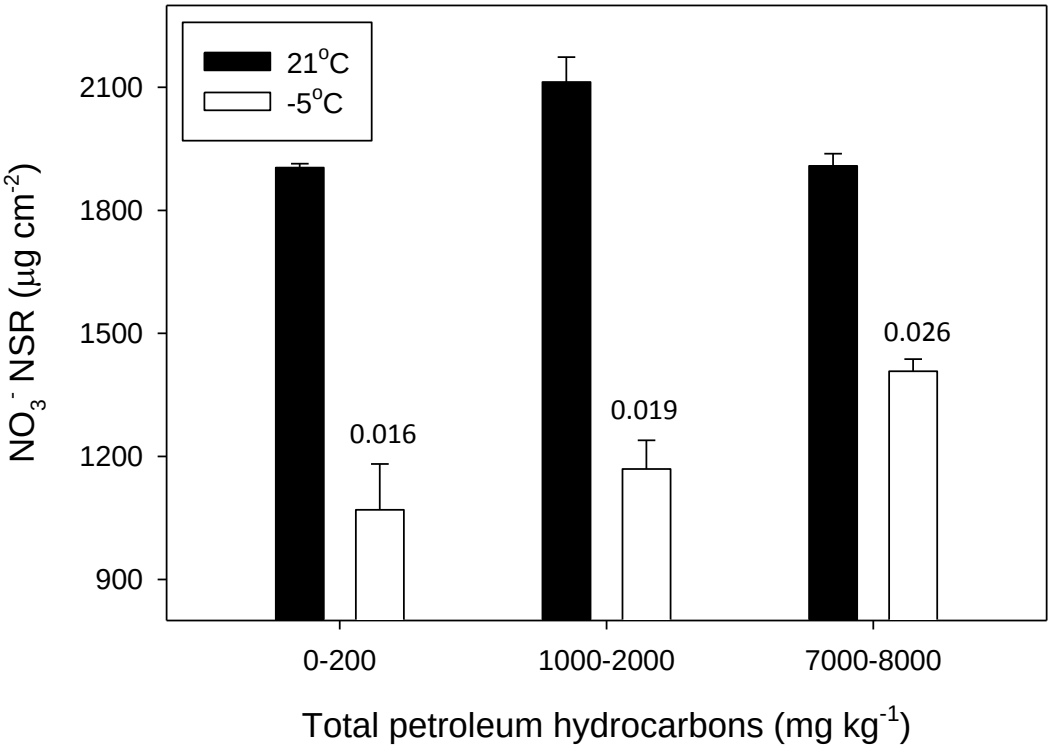


Figure 4.3 Effect of temperature and total petroleum hydrocarbon contamination on NO_3^- nutrient supply rate (NSR). Each bar is the average NO_3^- NSR (n=3) with error bars representing the standard error. Average liquid water content (n=3) at -5°C (values above white bars) are 0.016, 0.019, and 0.029 cm^3 H_2O cm^{-3} soil for petroleum hydrocarbon concentrations of 0-200, 1000-2000, and 7000-8000 mg kg^{-1}, respectively.

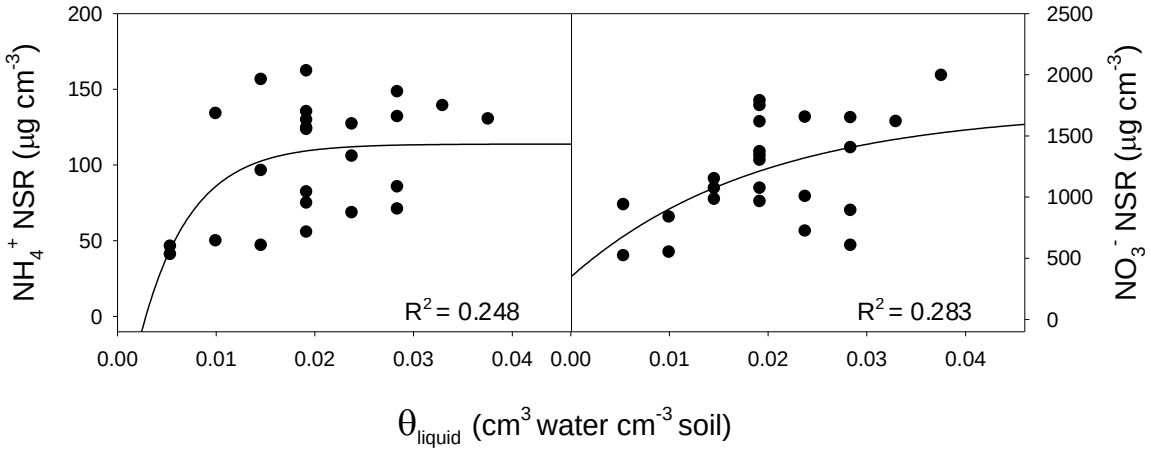


Figure 4.4 Relationship between nutrient supply rate (NSR) and liquid water content for NH_4^+ (left panel) and NO_3^- (right panel) at -5°C. Nonlinear regression analysis was applied to the data, which was modeled by exponential rise to maximum, $y = y_0 + a\left(1 - \exp^{-bx}\right)$.

Gas diffusion coefficient, $D_{s,}$ was influenced by bulk density under both temperature regimes (Figure 4.5). Bulk density had a significant effect on D_s at room temperature (p=0.000) and -5°C (p=0.004). At room temperature, D_s for all bulk densities were significantly different from each other. As expected, under room temperature soil cores with the lowest bulk density had the highest D_s, with an average value of 1.68 m^3 soil air m^{-1} soil s^{-1} (SE=0.06). There is a 76% decrease in D_s at bulk density of 2.0 g cm^{-3}, with average D_s=0.39 m^3 soil air m^{-1} soil s^{-1} (SE=0.01). Under frozen conditions, gas diffusion coefficients for bulk densities of 1.4 g cm^{-3} and 2.0 g cm^{-3} were significantly different from one another. Average values for gas diffusion coefficients under frozen conditions are 0.70 (SE=0.01), 0.61 (SE=0.01), and 0.55 (SE=0.005) m^3 soil air m^{-1} soil s^{-1} for bulk densities of 1.4, 1.7, and 2.0 g cm^{-3}, respectively. At the lowest bulk density, there is a larger relative difference between gas diffusion coefficients at 21°C and -5°C. A difference of 58% is observed in D_s between the two temperatures at 1.4 g cm^{-3}, compared to a 22% difference at bulk density of 1.7 g cm^{-3}, and 0% difference at bulk density 2.0 g cm^{-3}. In contrast to bulk density, TPH did not have a significant effect on D_s at either temperature (p=0.108 for room temperature, p=0.560 for -5°C).

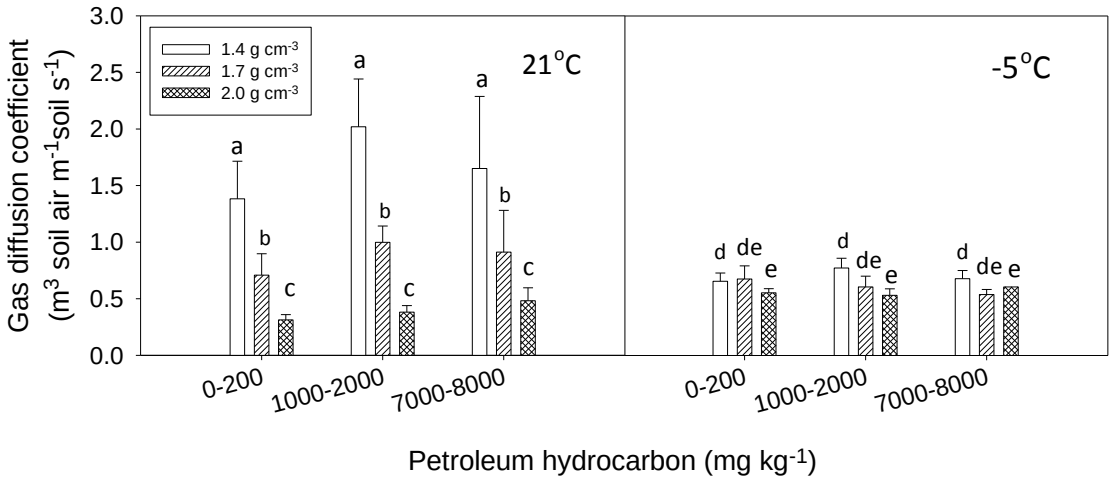


Figure 4.5. Gas diffusion coefficient for contaminated soil packed at three different bulk densities (1.4, 1.7, and 2.0 g cm^{-3}) measured at room temperature (left panel) and -5°C right panel). Each bar is the average gas diffusion coefficient (n=3) with error bars representing the standard deviation. Different lower case letters indicate a significant difference between treatments at 95% confidence level.

4.5 Discussion

Nutrient supply rates in frozen contaminated soil are linked to θ_{liquid} content. To the best of our knowledge, this is the first study to quantify nutrient supply rates in contaminated soil under frozen conditions using ion exchange resins. Not surprisingly, the nutrient supply rate for both NH_4^+ and NO_3^- were lower in frozen soil. Despite the reduction, our results show there are sufficient quantities of nutrients available for microbial activity in frozen soil, which supports other research that has shown bioremediation as a viable remediation strategy for polar regions even under frozen conditions (Rike et al., 2003).

Ion exchange resins have been used previously to assess nutrient availability in frozen soils. Giblin et al. (1994) were the first to show that ion exchange resins could be used to measure *in situ* nutrient availability in arctic soils during the winter. Researchers commonly use ion exchange resins in climate change studies to examine changes in soil nutrient availability due to warming in Arctic soils (Chapin et al., 1995; Hobbie and Chapin, 1998). Freeze-thaw cycles do not affect the physical integrity of ion exchange resins and have no effect on N and P adsorption and desorption characteristics of the resins (Mamo et al., 2004).

In this study, the bioavailability of NO_3^- was approximately ten times higher than NH_4^+, under both room temperature and frozen conditions. This difference in bioavailability between ions can be explained by differential ion mobilities. The NO_3^- mobility exceeds NH_4^+ mobility by about 30-fold, resulting in more NO_3^- ions adsorbing to the ion exchange resin (Binkley, 1984).

The nutrient supply rate measured in this study ranged from 40 – 162 µg NH_4^+ cm^{-2} and 517 – 2530 µg NO_3^- cm^{-2} for a 24 hour incubation, which is higher compared to ambient nutrient rates of other soils. Nutrient supply rate in a boreal aspen forest ranged from 2.7 – 13.7 µg 10

cm^{-2} 2 wk^{-1} for NH_4^+ and 1.6 – 31.7 µg 10 cm^{-2} 2 wk^{-1} for NO_3^- (Huang and Schoenau, 1997). Typical nutrient supply rates for agricultural range from 200–550, 550–1100, and >1100 µg NO_3^- 10 cm^{-2} 2 wk^{-1} for brown, dark brown, and black soils in Saskatchewan, respectively (Qian and Schoenau, 2005). The higher NSR is likely due to the addition of nutrients at a rate of 125 mg N kg^{-1} soil, which was added because it is the optimal rate of N for bioremediation in sub-Antarctic soil (Walworth et al., 2007).

Few studies have used ion exchange resins to measure nutrient supply rates in PHC bioremediation projects. Ion exchange resins were used to measure nutrient availability in oil sands reclaimed boreal forest soil. Soil nutrient availability was predominantly affected by site, season and year of sampling, with high NH_4^+ availability in the spring on vegetated/fertilized sites and high NO_3^- availability year round on the non-vegetated site (MacKenzie and Quideau, 2010). Ion exchange resins were also used in a well-site rehabilitation project, and where available N was found to be higher as a result of soil disturbance and lower in seeded treatments due to plant uptake (Hammermeister et al., 2003).

This study shows that the amount of θ_{liquid} is affected by bulk density and amount of PHC contamination. The increase in θ_{liquid} with an increase in bulk density can be attributed to an increase in adsorptive and capillary forces. Increasing bulk density decreases the pore space in the soil matrix, thus causing an increase in the adsorptive and capillary forces as the liquid water is confined to smaller spaces. An increase in θ_{liquid} with PHC contamination has been previously reported (Siciliano et al., 2008) and is described in Appendix A. The mechanism associated with this increase is still unknown; however it has been suggested that the increase in θ_{liquid} is due to a decrease in partial molar free energy of the pore water (Siciliano et al., 2008).

Our results indicate that gas diffusion in frozen soil is influenced by θ_{liquid} content. The relative difference between D_s at 21°C and -5°C were drastically different between bulk densities. These differences can be attributed to differences in θ_{liquid} content between the bulk densities. The largest decrease in gas diffusion between the two temperatures occurred at in soil with the lowest bulk density, and thus the lowest θ_{liquid} content. At the highest bulk density, corresponding to the highest θ_{liquid} content, there was virtually no difference in gas diffusion between a thawed and frozen soil core. Frozen soil with high θ_{liquid} content likely contains a continuous film of unfrozen water that allows gas to diffuse through it. This continuous film is unlikely to occur in soils with low θ_{liquid}, with gas diffusion limited by the presence of ice in the soil pore space. In regards to bioremediation in Polar Regions, soil aeration could be enhanced in frozen soil by the presence of liquid water in the soil matrix. However, manipulating θ_{liquid} content by compacting the soil is not an effective technique to enhance soil aeration, and perhaps manipulating other factors that control θ_{liquid} content may be beneficial to bioremediation in Polar Regions.

Other factors that influence the amount θ_{liquid} in frozen soil include temperature, the amount and composition of organic matter, amount of ice, and chemical potential gradients. Soil temperature influences the thickness of unfrozen films of water that surround soil particles and bacteria cells. The thickness of these films decreased threefold when temperature dropped approximately 10°C (15 nm at -1.5°C to 5 nm at -10°C) (Rivkina et al., 2000). Soil organic matter (SOM) content and composition influences the amount of θ_{liquid} via effects on both the osmotic and matric potential (Drotz et al., 2009). The osmotic potential is determined by the presence of solutes in the soil water and the exchanges between solid and soluble organic compounds, and therefore influences the freezing point depression caused by salinity of the pore water. The

matric potential is determined by the pore size distribution and the interaction of water molecules with surfaces of soil particles and colloids (Hillel, 2004). The matric potential is largely determined by the water-holding properties of SOM (Drotz et al., 2009). The amount of ice in frozen soil also affects the amount of θ_{liquid} content, with lower ice content associated with higher θ_{liquid} content. The mass transfer of θ_{liquid} and ions is greatest in frozen soil with low ice content and high chemical potential gradients which are found in localized areas close to ice inclusions (i.e. lenses, veins) (Ostroumov and Siegert, 1996).

In the past, research on bioremediation in cold regions has focused on maximizing microbial activity during the summer months, when the active layer of soil is above 0^{o}C (Braddock and McCarthy, 1996; Braddock et al., 1997; Margesin and Schinner, 1997; Mohn et al., 2001). This period is extremely important for biological activity; however, the summer season is very short in polar regions, with the active layer above the permafrost only thawing out for a period of 1-2 months (Mohn et al., 2001). Therefore, even though rates of microbial activity are low during winter months, cumulatively this activity could account for a substantial amount of biodegradation in polar regions, especially if factors regulating microbial activity under frozen conditions were optimized (Rike et al., 2003).

Bioremediation in cold regions could be enhanced during the period of time where the soil temperature is below 0^{o}C by controlling the factors that increase the amount of liquid water. Other researchers have shown that microbial activity continues during the winter months in polar soils (Edwards et al., 2006; Rike et al., 2003) and that this activity is largely controlled by the mobility of nutrients and gases under frozen conditions (Rivkina et al., 2000). *In situ* biodegradation of PHCs in arctic soil during winter months is known to occur, especially in soils with high concentrations of PHCs and high nutrients (Rike et al., 2003). Knowledge gained

from our study and the work of others, suggests that it may be possible to enhance biodegradation in the winter months by controlling bulk density, soil organic matter, and ice content to optimize liquid water contents which in turn, would optimize nutrient supply for PHC degradation.

CHAPTER 5

Validating potential toxicity assays to assess petroleum hydrocarbon toxicity in polar soil

5.1 Abstract

Potential microbial activities are commonly used to assess soil toxicity of petroleum hydrocarbons (PHC) and are assumed to be a surrogate for what is actually happening in the environment. However the relevance of this assumption needs to be evaluated for frozen soil where microbial activity is limited by the amount of liquid water. In this study, we evaluated the influence of liquid water content on the *in situ* toxicity of PHC to soil microorganisms using stable isotope dilution technique and compared the *in situ* toxicity to the toxicity endpoints of potential microbial activities. The toxicity of PHC contaminated soil from an aged diesel fuel spill at Casey Station, East Antarctica was assessed. To determine *in situ* toxicity, gross mineralization and nitrification rates were determined by the stable isotope dilution technique. PHC contaminated soil, ranging from 0 – 8000 mg kg^{-1}, was packed at bulk densities of 1.4, 1.7, and 2.0 g cm^{-3} to manipulate liquid water content, and incubated at -5^{o}C for periods of 1, 2, and 3 months. Liquid water content did not have a significant effect on either *in situ* mineralization or nitrification. *In situ* nitrification was sensitive to PHC contamination, with toxicity decreasing over time. The EC_{25} value (a point estimate of the toxicant concentration that causes a 25% inhibition from the control response) for *in situ* nitrification was 400 mg kg^{-1} for 1 month incubation period. In contrast, *in situ* N mineralization was not sensitive to PHC contamination. Toxic response of *in situ* nitrification to PHC contamination was comparable to potential nitrification activity with similar EC_{25} values determined by both measurement endpoints (400 mg kg^{-1} for *in situ* nitrification compared to 200 mg kg^{-1} for potential nitrification activity), indicating that potential microbial activity assays are good surrogates for *in situ* toxicity of PHC contamination in Polar Regions.

5.2 Introduction

Establishing relevant remediation guidelines for petroleum hydrocarbon (PHC) contamination in Polar Regions has been identified as a key issue in the field of environmental pollution. The number of contaminated sites in Polar Regions is estimated to exceed 20,000 sites, with the vast majority located in ecologically sensitive areas or close to human habitation (Snape et al., 2008b). Management decisions, including whether or not to remediate, which sites are ranked highest priority, and at what stage remedial action is considered to have reached its goal, are all based on soil quality guidelines (Snape et al., 2003). Because of the current lack of information needed to derive relevant remediation guidelines for Polar Regions, these decisions are often based on data collected from temperate and tropical ecosystems.

Soil toxicity tests are powerful tools that can be used to derive generic or site-specific soil quality guidelines. The selection of relevant toxicological measurement endpoints for protection of the soil ecosystem is a critical step in the development of remediation guidelines, and may vary between regions. For example, commonly used measurement endpoints for PHC contamination in temperate regions are the root and shoot length of northern wheatgrass (*Elymus lanceolatus*) (Environment Canada, 2005). While this measurement endpoint is relevant to agricultural land use in temperate regions, it holds little significance for Antarctic regions where plant life is limited to moss, lichens and algae.

Evaluating ecotoxicity of PHC contamination in Polar Regions requires selecting measurement endpoints for organisms that are native to the Arctic and Antarctic. Ideally, these indictors will be abundant and ubiquitous in all polar ecosystems, allowing a standardized method to be developed. Indicators must also be sensitive to PHC contamination. Soil microorganisms meet all the above criteria and are good indicators of soil toxicity for several

reasons: 1) they are vital to the function and structure of the soil ecosystem; 2) they are ubiquitous and abundant, which allows comparisons to be make across different sites and ecoregions; 3) they live in close contact with the soil and therefore any contaminants that exist in the soil; 4) they have a high surface area to volume ratio which aids in absorption of contaminants; 5) they respond and adapt quickly to changes in environmental conditions allowing rapid assessment of toxic effects (Nielson and Winding, 2002).

Petroleum hydrocarbons can have several different effects on soil microorganisms. Hydrocarbons can act as a carbon source for certain groups of microorganisms capable of degrading these compounds (Atlas, 1995; Atlas and Cerniglia, 1995; Chakraborty and Coates, 2004; Leahy and Colwell, 1990; Spomann and Widdel, 2000), thus an increase in soil respiration is often observed in PHC contaminated soil. The microbial community composition often shifts, with hydrocarbon-degrading microorganisms increasing from less than 0.1% of total bacterial population in pristine environments to up to 100% of the total microbial population in PHC contaminated soil (Lindstrom et al., 1999). Petroleum hydrocarbons also exert toxic effects on soil microorganisms, primarily through narcosis which disrupts the function, integrity and fluidity of cellular membranes. Regulation of the cellular membrane is critical for survival of soil microorganisms, especially in polar environments where organisms are exposed to low temperature and moisture levels (Holmstrup et al., 2000). In addition to narcosis, enzyme-specific toxic effects for nitrifying bacteria have been observed, with PHC compounds inhibiting ammonium oxidation via competitive and non-competitive interactions (Hyman et al., 1988; Keener and Arp, 1993). There are also indirect effects of PHC contamination caused by changes to the chemical and physical properties of the soil, such as increased hydrophobicity, reduced nutrient status, and increased temperature (Aislabie et al., 2004).

Limited soil toxicological data is available for PHC toxicity to soil microorganisms in polar soils (Schafer et al., 2007; Schafer et al., 2009). These studies have used potential toxicity assays, a measure of microbial activity under optimal conditions (nutrients, temperature, and water), to assess toxicity of PHC to native soil microorganisms. Sensitive measurement endpoints for PHC contamination include potential nitrification activity, soil respiration and microbial community composition. Theses studies report effective concentrations that cause a certain percentage, *p*, of reduction in activity from the control response, EC_p, for the selected measurement endpoints. For sub-Antarctic soil spiked with diesel fuel, the EC_{20} for potential nitrification activity is 190 mg kg^{-1} and 220 mg kg^{-1} for soil respiration (Schafer et al., 2007). In comparison, toxicity assessed for an aged fuel spill in polar soil was approximately ten times more tolerant, with EC_{25} values of 2000 mg kg^{-1} for potential nitrification activity, 3500 mg kg^{-1} for soil respiration, and 800 mg kg^{-1} for microbial community composition (Schafer et al., 2009).

Potential toxicity assays, which are a quick, easy, and relatively inexpensive way to assess soil toxicity, are assumed to be a surrogate for what is actually happening in the field; however the relevance of this assumption needs to be evaluated for polar soils. *In situ* measurements allow an accurate description of what is happening to the nutrient cycles in an ecosystem. One of the best methods available for measuring *in situ* nitrogen cycling is the stable ^{15}N isotope dilution technique, where the product pool is labeled with ^{15}N and activity is measured by dilution of the product pool. This technique has been successful at estimating gross mineralization and nitrification rates in a number of different ecosystems (Bedard-Haughn et al., 2004; Bustamante et al., 2006; Danso et al., 1992; Ross et al., 2004); however, to the best of our knowledge this technique has yet to be applied to frozen polar soil.

In a polar environment, *in situ* toxicity is thought to be influenced by harsh environmental conditions, making these ecosystems more sensitive to environmental contaminants (Snape et al., 2003). One environmental factor thought to contribute to the increased sensitivity is the limited liquid water content at temperatures below 0^{o}C. Liquid water is present in frozen soil due to salinity, adsorptive and capillary forces that lower the freezing point of water, and can be manipulated by changing the bulk density of soil (Williams and Smith, 1991). Soil microorganisms are able to remain active at sub-zero temperatures because the unfrozen water allows the diffusion of microbial substrates and waste products (Ostroumov and Siegert, 1996). By using the stable isotope dilution technique in soil packed at different bulk densities, we will be able to investigate how microbial activity changes in contaminated soil under different liquid water regimes.

The purpose of this study was to validate the use of potential toxicity assays to assess the toxicity of PHC contamination in polar soil by comparing it to *in situ* measurements of toxicity. Toxicity will be assessed on contaminated soil from Casey Station, East Antarctica, using both potential toxicity assays and stable isotope dilution techniques. The influence of liquid water content on the toxicity of PHC contamination will also be examined via an *in situ* toxicity assessment. The ecotoxicity data generated will be useful in the development of relevant remediation guidelines for PHC contamination in polar ecosystems.

5.3 Materials and Methods

5.3.1 Soil

Soils were collected from a six-year-old PHC-contaminated spill site at Casey Station, East Antarctica (66^{o}17′ S, 110^{o}32′ E) during the austral summer of 2006. The spill event occurred in the austral winter of 1999 when approximately 5,000 L of Special Antarctic Blend (SAB) diesel

fuel leaked from a storage tank near the main powerhouse (MPH) (Snape et al., 2006). Special Antarctic Blend diesel fuel contains a higher percentage of the lighter fractions of PHCs (Snape et al., 2005). At the time of sampling, contamination levels still exceeded 25,000 mg kg^{-1}. Additional information on the contaminated site is available from Schafer et al. (2009). Soils were transported to the University of Saskatchewan and stored at -20^{o}C until further analysis.

Soils, sampled at a depth of 0 – 15 cm and passed through a 4.75-mm sieve to remove large rocks, were characterized for particle size distribution (PSA), total organic carbon (TOC), gravimetric water content (GWC), and total petroleum hydrocarbons (TPH) (Table 5.1). For this study, soils were divided into three groups of PHC contaminated soil of 0 – 200, 1000 – 2000, and 7000 – 8000 mg kg^{-1} (Table 5.1). Texture was estimated using a laser scattering particle size distribution analyzer (Horiba LA-950, Horiba Instruments, Oakville). Total organic carbon and gravimetric water content were determined by standard methods (Skjemstad and Baldock, 2008; Topp et al., 2008). The concentration of SAB fuel was analyzed from a hexane extract using a gas chromatograph fitted with a flame ionization detector. Details on SAB analysis are given in Section 2.3.3. Uncertainty in SAB concentration is calculated from the square root of the sum of squares of the measured SAB concentrations multiplied by the uncertainty in the calibration (5%) and the amount of SAB measured in the blank (20 mg kg^{-1}).

Soils from Casey Station were used to determine toxicity of SAB diesel fuel to *in situ* mineralization and nitrification under frozen conditions. For each TPH treatment, soil cores (6.35 mm diameter and 20 mm height) were packed at three different bulk densities and incubated at -5^{o}C for time periods of 1, 2, and 3 months. After each time period, soil cores were labeled with $^{15}NH_4$ and $^{15}NO_3$ to determine gross mineralization and nitrification rates, respectively.

Table 5.1 Soil characteristics for contaminated soil collected from Casey Station, East Antarctica.

	SAB[1] Diesel		Soil Texture			TOC[2]	GWC[3]
	Concentration (mg kg^{-1})	Uncertainty (mg kg^{-1})	% Sand	% Silt	% Clay	(%)	(g H_2O g^{-1} soil)
Group 1	55	BD[4]	18.3	73.7	8	0.339	0.161
	20	BD	28.3	61.1	10.6	0.523	0.255
	100	45	11	73.4	15.6	0.242	0.184
Group 2	1960	130	15.4	42.6	42	0.455	0.317
	1640	205	35.1	59.6	5.3	1.252	0.265
	1245	180	34.7	61.5	3.9	0.518	0.334
Group 3	7235	525	28.4	61.8	9.7	0.617	0.571
	7640	455	36.5	54.8	6.8	0.37	0.246
	7930	250	29.2	63.2	7.5	0.427	0.201

[1]Special Antarctic Blend (SAB) diesel fuel
[2]Total organic carbon (TOC) content
[3]Gravimetric water content (GWC)
[4]Below detection (BD) limit

Positive and negative control values were also established for each bulk density and incubation period. Boric acid was used as a reference toxicant for the positive control sample at a concentration of 110 mg kg^{-1}.

To determine the influence of liquid water on *in situ* toxicity of PHC, liquid water content (θ_{liquid}) was manipulated by changing the bulk density of the repacked soil cores. Three bulk densities, 1.4, 1.7, and 2.0 g cm^{-3}, were used in this experiment corresponding to loose, medium, and firmly packed soil, respectively. Time domain reflectometry (TDR) probes were used to measure θ_{liquid} of frozen soil (Spaans and Baker, 1995). The TDR technique is able to differentiate between the frozen and unfrozen fraction of water based on the difference in the dielectric constant of these two phases of water (K_{water} = 80.36, K_{ice} = 3.2) and between the other soil constituents (K_{air} = 1; $3 \leq K_{soil} \leq 7$) in the frequency range of 1 MHz to 1 GHz (Topp et al., 1980). Additional information on TDR probe construction and calibration is described in Appendix A.

5.3.2 ^{15}N Isotope Dilution Technique

Four isotope-dilution cores were prepared for each TPH treatment (n=3), bulk density (n=3) and time period (n=3). One pair was labeled with $(^{15}NH_4)_2SO_4$ (for gross N mineralization) and the other pair was labeled with $K^{15}NO_3$ (for gross nitrification); each solution contained 15 mg N ml^{-1} at 98% ^{15}N enrichment. Frozen soil cores that had incubated for 1, 2 or 3 months (see above) were manipulated in a cold room (held at -5^{o}C) to insure that cores remained frozen. A total of 400 µL of label was added to each core, with 200 µL applied to top and bottom. This small volume of labeling solution in conjunction with the core size insured that soils cores remained frozen while the label was added in order to minimize changes in microbial activity. A rubber stopper was used to force the label to penetrate the frozen soil.

One core of each pair was extracted immediately using 2 M KCl for the time 0 measurement. The other core was incubated at -5^{o}C for 48 hours before extraction. The KCl extracts were filtered using Whatman No.1 filter paper and analyzed for inorganic N concentration colorimetrically using the Smart Chem^{TM} 200 from Westco Scientific Instruments Inc. (Brookfield, CT, USA).

The concentration of ^{15}N was analyzed from the KCl extracts using the acidified diffusion disk method described by Brooks et al. (1989). The original method was modified by encasing the acid disk with Teflon (polytetrafluoroethylene [PTFE]) tape (Sorensen and Jensen, 1991). $^{15}NH_4$ was captured on the disk by adding MgO to make the extract alkaline, causing $^{15}NH_4$ to be converted to $^{15}NH_3$ vapour which was captured on the disk. The $^{15}NH_4$ disk was removed from solution and a new disk added to capture $^{15}NO_3$ by adding $TiCl_3$, which reduces $^{15}NO_3$ to $^{15}NH_3$. Acid disks were analyzed for N concentration and atom percent ^{15}N excess (APE) using an ANCA–

GSL elemental analyzer coupled to a continuous flow Tracer/20 mass spectrometer (Europa Scientific, SerCon Ltd., Cheshire, UK).

The difference in N concentration and ^{15}N excess (APE) between the time 0 and 48 h samples were used to calculate gross mineralization and nitrification according to Davidson et al (1991). The equation is as follows:

$$m = \frac{\left[NH_4^+\right]_0 - \left[NH_4^+\right]_t}{t} \times \frac{\log((APE_0 \times \left[NH_4^+\right]_t)/(APE_t \times \left[NH_4^+\right]_0)}{\log(\left[NH_4^+\right]_0 / \left[NH_4^+\right]_t)} \qquad [5.1]$$

where m is gross N mineralization rate (mg kg^{-1} d^{-1}), $[NH_4^+]_0$ is the initial $^{14+15}NH_4^+$ concentration, $[NH_4^+]_t$ is the concentration of $^{14+15}NH_4^+$ at time, t (d), APE_0 is the atom percent ^{15}N excess at time 0, APE_t is the atom percent ^{15}N excess at time t. Gross nitrification rate is calculated in a similar manner by substituting NO_3^- concentrations and atom percent ^{15}N excesses from the NO^3 diffusion disks in the above equation. Raw data for mineralization and nitrification are shown in Table 5.2 and 5.3, respectively.

Table 5.2 Concentration of NH_4 and atom percent excess used in the calculation of gross mineralization rates.

Incubation Period	TPH Group	Bulk Density (g cm^{-3})	Initial NH_4 (µg g^{-1})		Final NH_4 (µg g^{-1})		Initial APE		Final APE	
			Average	SD	Average	SD	Average	SD	Average	SD
1 month	Group 1	1.4	5.24	0.05	5.22	0.05	7.60	0.18	7.06	0.32
		1.7	4.33	0.05	4.31	0.03	7.45	0.32	6.73	0.12
		2.0	3.69	0.06	3.67	0.01	6.82	0.99	6.60	0.24
	Group 2	1.4	5.56	0.30	5.56	0.30	6.15	1.18	5.62	0.59
		1.7	4.66	0.27	4.57	0.21	5.84	1.11	5.36	0.58
		2.0	3.99	0.27	3.95	0.26	5.24	0.44	4.55	0.54
	Group 3	1.4	5.34	0.14	5.22	0.25	7.33	0.74	6.37	0.23
		1.7	4.37	0.10	4.28	0.19	7.17	0.68	6.54	0.37
		2.0	3.75	0.10	3.65	0.13	6.83	0.82	5.92	0.40
2 month	Group 1	1.4	5.24	0.05	5.28	0.07	7.60	0.18	6.79	1.22
		1.7	4.33	0.05	4.37	0.04	7.45	0.32	7.13	0.16
		2.0	3.69	0.06	3.73	0.04	6.82	0.99	6.98	0.28
	Group 2	1.4	5.56	0.30	5.65	0.30	6.15	1.18	6.14	0.84
		1.7	4.66	0.27	4.70	0.27	5.84	1.11	5.45	1.52
		2.0	3.99	0.27	4.07	0.27	5.24	0.44	4.91	1.76
	Group 3	1.4	5.34	0.14	5.46	0.21	7.33	0.74	6.56	0.85
		1.7	4.37	0.10	4.44	0.11	7.17	0.68	6.91	0.53
		2.0	3.75	0.10	3.80	0.07	6.83	0.82	6.79	0.24
3 month	Group 1	1.4	5.39	0.02	5.38	0.06	8.06	0.22	7.25	0.20
		1.7	4.37	0.05	4.35	0.05	7.90	0.33	6.97	0.43
		2.0	3.78	0.09	3.74	0.09	7.63	0.14	7.08	0.40
	Group 2	1.4	5.58	0.38	5.52	0.43	6.71	1.34	6.17	0.83
		1.7	4.66	0.19	4.61	0.23	6.31	1.24	5.97	0.94
		2.0	4.02	0.22	3.95	0.21	6.04	1.41	5.51	0.84
	Group 3	1.4	5.65	0.38	5.57	0.39	6.47	1.78	7.20	0.55
		1.7	4.42	0.11	4.39	0.12	7.85	0.63	7.02	0.52
		2.0	3.78	0.11	3.73	0.10	7.61	0.67	6.79	0.64

APE - Atom % ^{15}N Excess
SD – Standard deviation

Table 5.3 Concentration of NO_3 and atom percent excess used in the calculation of gross nitrification rates.

Incubation Period	TPH Group	Bulk Density (g cm^{-3})	Initial NO_3 (µg g^{-1})		Final NO_3 (µg g^{-1})		Initial APE		Final APE	
			Average	SD	Average	SD	Average	SD	Average	SD
1 month	Group 1	1.4	12.42	0.64	8.19	2.92	8.66	3.56	11.52	0.51
		1.7	10.30	0.92	6.59	0.35	10.42	0.51	10.44	0.75
		2.0	8.32	0.22	11.92	10.88	9.49	0.02	10.23	0.67
	Group 2	1.4	10.13	2.77	27.07	7.72	10.46	0.43	10.76	0.79
		1.7	10.07	1.41	23.27	7.10	6.37	5.86	11.11	0.81
		2.0	9.00	0.73	14.56	6.06	7.61	3.62	10.84	0.88
	Group 3	1.4	11.27	1.70	16.66	2.42	6.60	6.13	12.31	0.45
		1.7	10.77	0.27	10.07	2.92	9.64	0.41	12.43	0.32
		2.0	9.77	0.04	10.76	0.85	7.29	3.10	9.59	2.31
2 month	Group 1	1.4	12.42	0.64	5.93	3.04	8.66	3.56	14.43	0.90
		1.7	10.30	0.92	5.25	0.42	10.42	0.51	13.36	2.48
		2.0	8.32	0.22	2.73	2.75	9.49	0.02	13.98	1.66
	Group 2	1.4	10.13	2.77	5.96	0.82	10.46	0.43	14.42	0.40
		1.7	10.07	1.41	4.62	1.31	6.37	5.86	14.98	0.29
		2.0	9.00	0.73	4.35	0.45	7.61	3.62	13.71	1.35
	Group 3	1.4	11.27	1.70	3.67	2.85	6.60	6.13	14.89	0.33
		1.7	10.77	0.27	5.07	1.25	9.64	0.41	14.36	1.40
		2.0	9.77	0.04	3.60	0.71	7.29	3.10	14.35	1.03
3 month	Group 1	1.4	10.09	1.66	8.09	2.95	12.80	0.04	12.69	0.53
		1.7	6.54	2.89	8.51	2.41	12.65	0.13	12.64	0.22
		2.0	6.77	0.81	6.58	0.64	12.76	0.09	9.39	5.80
	Group 2	1.4	12.23	5.97	14.68	6.45	12.35	0.11	12.41	0.83
		1.7	11.56	1.19	11.20	5.58	12.20	0.57	12.40	0.46
		2.0	9.22	1.39	6.61	5.66	12.14	0.07	12.21	0.17
	Group 3	1.4	12.72	4.11	3.06	1.84	11.76	1.58	12.74	0.28
		1.7	8.68	1.60	1.29	3.02	12.63	0.28	12.82	0.26
		2.0	6.36	2.00	2.99	0.72	12.47	0.67	10.62	3.30

APE - Atom % ^{15}N Excess
SD – Standard deviation

5.3.3 Potential Nitrification Activity

Contaminated soil from Casey Station was collected when the soil was frozen and immediately assessed for PHC toxicity using potential nitrification activity (PNA). PNA is a measure of the potential activity of ammonium oxidizing bacteria within the soil (Gong et al., 1999). Soil, 5.0 g wet weight, was added to 20.0 mL test solution containing 4 mM $(NH_4)_2SO_4$, 15 mM $NaClO_3$, and 1 mM KH_2PO_4. The slurry was incubated at 10^{o}C, agitated at 70 rpm, and sub-sampled (2.0 ml) at 16, 28, and 40 h of incubation. Ammonia oxidation was terminated in the sub-samples with 4 M KCl (2.0 ml), centrifuged (14 000 rpm) for 3 minutes, filtered (0.45 µm syringe filter) and analyzed for NO_2^- content colorimetrically. The production rate of NO_2^-, or potential nitrification activity, was calculated from the slope of linear regression of the concentration of NO_2^- g^{-1} soil versus sampling time (h).

5.3.4 Statistical Analysis

In situ mineralization and nitrification were analyzed using the general linear model (GLM) for multivariate analysis of variance (MANOVA) to detect differences between treatments of incubation time, bulk density, and TPH. Minitab® 16.1.0 was used to perform statistical analyses.

Regression analysis was used to determine the effective concentration (EC_{25}) that causes a 25% reduction in activity from the control response (Environment Canada, 2007). This point estimate is a standard endpoint used by Environment Canada to report toxicity for quantitative sub-lethal tests. The TPH concentrations are transformed to a logarithmic scale prior to regression analysis. Regression analysis was performed according to Environment Canada (2007), using Sigmaplot® 10.0.

5.4 Results

Petroleum hydrocarbon contamination has a negative effect on both *in situ* nitrification rate and potential nitrification activity (PNA) (Figure 5.1). The EC_{25} values were similar between these two measurement endpoints, with a value of 400 mg kg^{-1} for *in situ* nitrification and 200 mg kg^{-1} for PNA. The model of best fit for both measurement endpoints is the exponential model

$$y = a \times 0.25^{(x/\ c)} \qquad [5.2]$$

where *y* is the nitrification activity, *x* is the logarithmic value of TPH, *a* is a fitting parameter and *c* is the logarithmic value of the EC_{25}. The model for *in situ* nitrification is $y = 2.66 \times 0.25^{(x/\ 2.60)}$, r^2 = 0.474 and for PNA the model is $y = 11.9 \times 0.25^{(x/\ 2.31)}$, r^2 =0.353. These two measures of nitrification activity are moderately correlated, with a Pearson correlation coefficient of 0.562.

Liquid water, which was manipulated by changing the bulk densities of the soil cores, did not have a significant effect on *in situ* nitrification rate (p = 0.597) or mineralization rate (p = 0.427) (Figure 5.2). Variation in both nitrification and mineralization activity was observed at different bulk densities, but there was no consistent trend across the three time points. Liquid water has the largest influence on nitrification rate after 1 month incubation in soil with 7000 – 8000 mg TPH kg^{-1} soil. Nitrification at θ_{liquid} of 0.022 $cm^3\ cm^{-3}$ was only 0.52 mg $kg^{-1}\ d^{-1}$ but as liquid water increased to 0.035 $cm^3\ cm^{-3}$, nitrification increased to 1.5 mg $kg^{-1}\ d^{-1}$.

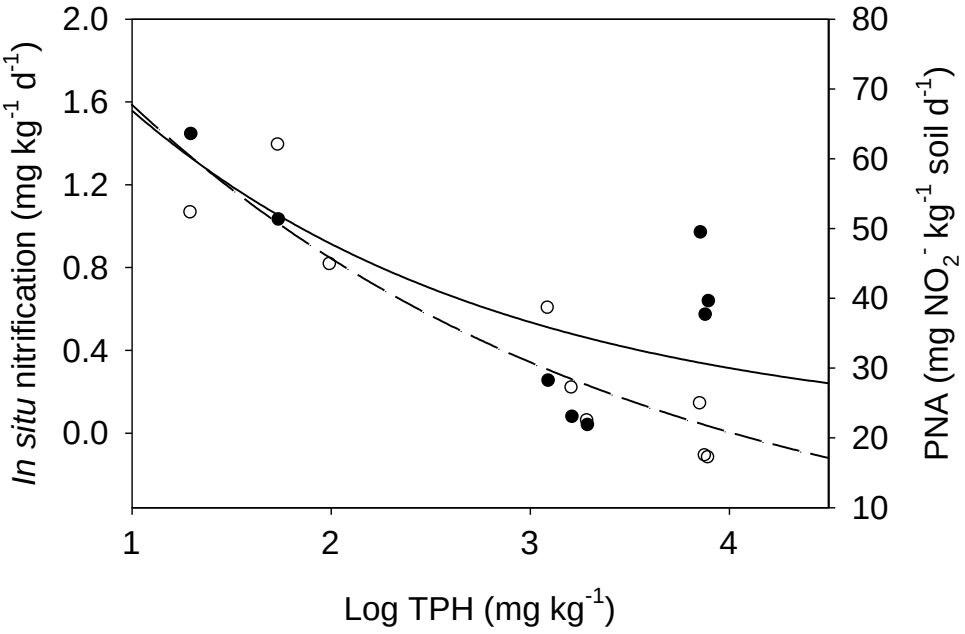


Figure 5.1 Dose-response relationship for in situ nitrification activity (closed circles) and potential nitrification activity (PNA, open circles) in soil contaminated with diesel fuel, expressed on a logarithmic scale as total petroleum hydrocarbons (TPH). Regression analysis was used to determine the effective concentration that caused a 25% decrease from the control response (EC_{25}) using an exponential model (solid line for *in situ* nitrification, dashed line for PNA).

Low but positive values were observed for both *in situ* mineralization and *in situ* nitrification during all three incubation periods (Figure 5.2). Incubation had a significant effect on *in situ* nitrification (p=0.000) with nitrification decreasing with incubation time with an average nitrification at two months of 1.6 (SE=0.13) mg kg^{-1} d^{-1} decreasing to 1.06 (SE=0.09) after three months at -5^{o}C. Similarly, mineralization also decreased with time, decreasing from 0.31 (SE=0.03) mg kg^{-1} d^{-1} after 1 month to 0.23 (SE=0.02) after 3 months. In contrast to incubation time, bulk density and TPH did not have a significant effect on *in situ* nitrification (p=0.847 and p=0.254, respectively). The highest average nitrification activity was 2.16 mg kg^{-1} day^{-1}, observed at 2 months. Minimum *in situ* nitrification was 0.01 mg kg^{-1} day^{-1}, which occurred at 1 month in the soil contaminated with 1000 – 2000 mg kg^{-1} TPH. The average nitrification activity for the positive and negative controls was 1.62 mg kg^{-1} day^{-1} and 1.44 mg kg^{-1} day^{-1}, respectively. None of the treatments had a significant effect on mineralization (p=0.552 for incubation; p=0.427 for bulk density; p=0.790 for TPH). Maximum *in situ* mineralization rate of 0.51 mg kg^{-1} day^{-1} was observed at 2 month incubation in the treatment with the lowest TPH concentration and bulk density (lowest liquid water content). Minimum *in situ* mineralization occurred in the highest TPH and bulk density (highest liquid water) treatment at 2 month incubation, with a rate of 0.09 mg kg^{-1} day^{-1}. The average *in situ* mineralization activity for the positive and negative control groups was 0.238 and 0.123 mg kg^{-1} day^{-1}, respectively.

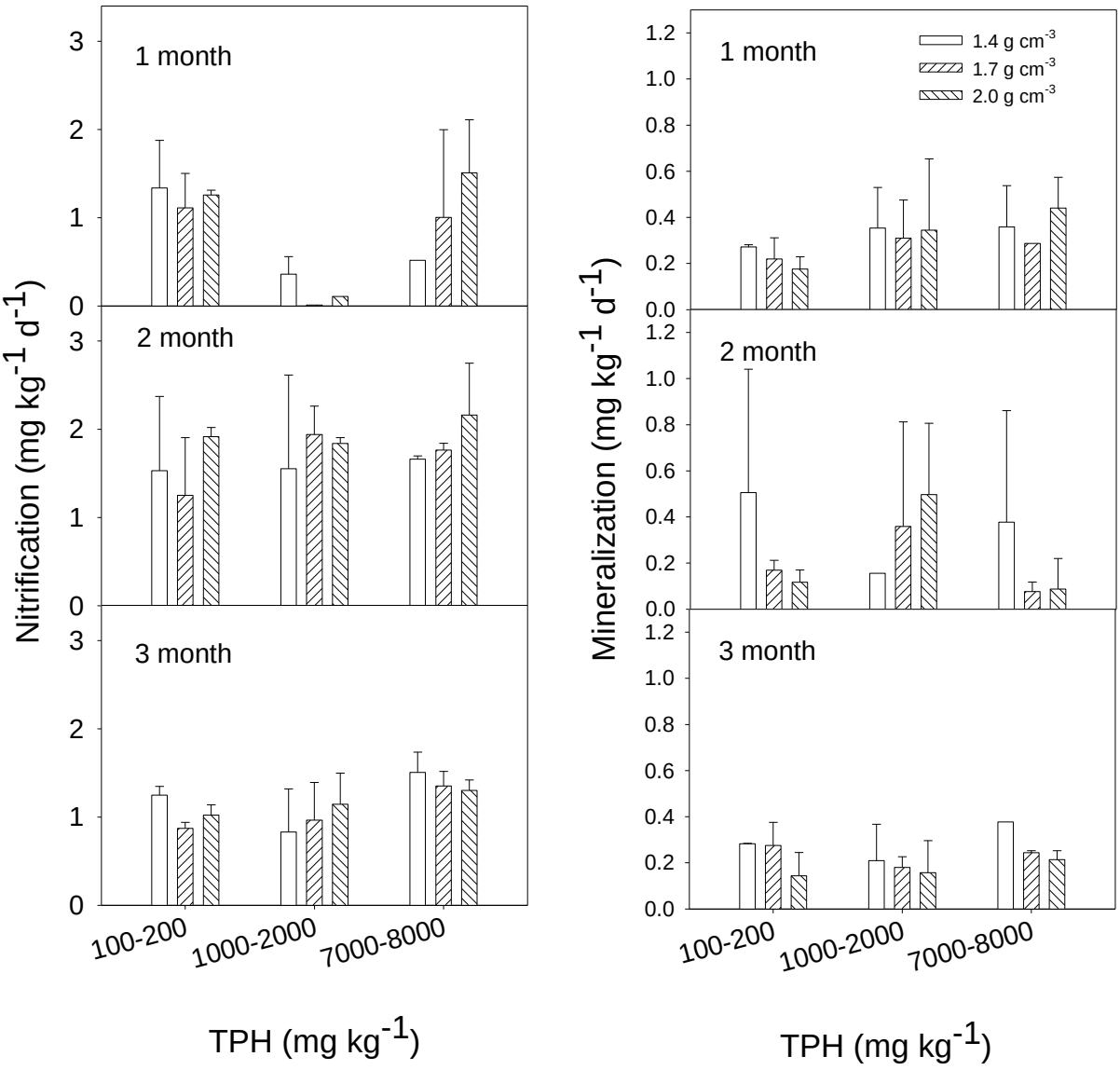


Figure 5.2 Nitrification and mineralization activity in Antarctic soil as a function of aged hydrocarbon and liquid water. Soils were grouped into three distinct total petroleum hydrocarbon (TPH) contents (n=3) and liquid water content manipulated by altering the bulk density of the cores (n=3). Soils were incubated at -5°C for three months and activity assessed using ^{15}N isotope dilution techniques.

5.5 Discussion

Our results indicate that potential toxicity assays, particularly for nitrification, are good indicators of *in situ* toxicity of PHC contamination in polar soils. In addition to a strong correlation between *in situ* and potential nitrification, both measurement endpoints were equally sensitive to PHC contamination, with similar EC_{25} values of 400 mg kg^{-1} for *in situ* nitrification, and 200 mg kg^{-1} for PNA. This observation validates the assumption that potential toxicity assays are good surrogates for *in situ* toxicity.

The EC_{25} values obtained in this study are similar to the EC_{25} values found in other studies. Schafer et al. (2007) used PNA to assess toxicity of sub-Antarctic soil spiked with diesel fuel, thus simulating a fresh spill, and found an EC_{20} value of 190 mg kg^{-1}. Schafer et al. (2009) used PNA to assess the toxicity of an aged diesel fuel spill at Casey Station and obtained an EC_{25} of 2000 mg kg^{-1}. In comparison, the EC_{25} values in this study ranged from 200 – 400 mg kg^{-1}. One explanation for the differences between EC_{25} values is that as the spill ages, the toxicity of PHC usually decreases, as the recalcitrant compounds are bound more tightly to the soil particles, thus reducing bioavailability of the contaminants (Alexander, 2000). The decrease in toxicity is reflected by the higher EC_{25} value for the aged fuel spill at Casey.

A decrease in toxicity over time was observed with the *in situ* nitrification experiment. At two and three month incubation periods, *in situ* nitrification activity was no longer inhibited by PHC contamination. This decrease in toxicity over time may indicate that ammonium oxidizing bacteria are capable recovering from a TPH spill. Similarly, Deni and Penninckx (1999) observed that diesel fuel applied to soil previously contaminated with diesel did not inhibit nitrification activity in the soil, as the ammonia-oxidizing bacteria in the polluted soil had acquired a

resistance to the hydrocarbon, possibly by increasing the affinity of nitrifying bacteria for ammonium in the soil. Alternatively, the decrease in nitrification activity with incubation time may reflect the decreasing availability of ammonia due to decreased mineralization activity with time. This basal nitrification activity was then unaffected by TPH which supports the proposed mechanism of hydrocarbon action on nitrifiers being linked to reduced enzyme turnover (Keener et al., 1998). Reduced enzyme turnover would have little impact at very low activity rates.

In situ mineralization was not a sensitive indicator of PHC toxicity in this study. Other researchers have observed an initial inhibition of N-mineralization in soil contaminated with diesel fuel, but the inhibition was completely reversed within a 2 to 3 week time period (Kandeler et al., 1994; Margesin et al., 2000b). The incubation time periods in this study were 1, 2, and 3 months and therefore may have been too long between the start of the experiment and time of first sampling to capture the initial inhibition of N mineralization. Other researchers have observed an increase in N mineralization for soils with a high concentration of diesel, which can be attributed to an increase in nitrogenous organic compounds from dead microorganisms (Pena et al., 2007). Accumulation of NH_4^+ may occur in hydrocarbon contaminated soil due to an increase in N mineralization and a decrease in nitrification (Joergensen et al., 1995; Pena et al., 2007).

One explanation for N mineralization being less sensitive to PHC contamination than nitrification is the large diversity of bacteria capable of N mineralization compared to nitrifying bacteria. Of the many species of soil microorganisms capable of carrying out the N mineralization process, some are capable of degrading PHCs, utilizing them as a carbon or energy source (Pena et al., 2007). These hydrocarbon-degrading N mineralizing bacteria thrive in PHC contaminated soil, increasing in number exponentially, and thereby generate more NH_4^+ in the soil (Lindstrom et al., 1999). In contrast, the process of nitrification is only carried out by a

small number of nitrifying bacteria. Among some of the most important nitrifying bacterial species are *Nitrosomonas* and *Nitrobacter*, both of which are sensitive to hydrocarbon toxicity (Weissmann and Kunze, 1994). In diesel-contaminated soils, nitrification becomes inhibited because of the reduction in the number of nitrifying bacteria (Deni and Penninckx, 1999). This enhanced sensitivity is thought to be due to the specific inhibition of ammonia monoxygenase (AMO) by hydrocarbons. Hydrocarbons can either: (1) reversibly compete with ammonia at the AMO catalytic site, (2) bind to the enzyme and alter enzyme turnover or (3) irreversibly inhibit AMO (Chang et al., 2002; Keener et al., 1998).

Liquid water content did not influence the toxicity of PHC. We hypothesized that liquid water would increase the sensitivity of soil microorganisms to PHC because of changes in the bioavailability of the contaminant. Our results indicated that liquid water does not influence the toxicity of PHC to either *in situ* nitrification or mineralization. In the past, the bioavailable fraction of organic contaminants in soil was defined as the fraction that is easily desorbed from soil and present in the aqueous phase (Alexander, 2000; Reid et al., 2000; Semple et al., 2003). This definition leads us to believe that microbes are only able to utilize dissolved hydrocarbons (Ogram et al., 1985; Wodzinski and Coyle, 1974). However, research has shown that micro-organisms are capable of degrading both dissolved and sorbed fraction of hydrocarbons (Tang et al., 1998). Many studies have found that the degradation rate of PHC compounds exceeds the rate at which they are desorbed from soil into aqueous phase (Huesemann et al., 2003; Huesemann et al., 2004; Thomas et al., 1986). Our study supports this conclusion from a toxicological point of view, i.e. the lack of influence of liquid water on the toxicity of PHC is likely due to the ability of microorganisms to access both the dissolved and sorbed fraction of PHC.

In conclusion, this study shows that ecotoxicological data generated from potential nitrification activities are good surrogates for *in situ* toxicity for petroleum hydrocarbons in Polar Regions. This finding will allow site managers to use a fast, easy, and reliable technique to generate toxicity data across the polar regions.

CHAPTER 6

Summary and Conclusions

6.1 Influence of liquid water content on toxicity of petroleum hydrocarbons

The overall goal of the research described in this thesis was to determine the influence of liquid water content on the toxicity of petroleum hydrocarbons to soil microorganisms in frozen polar soil. It was hypothesized that an increase in liquid water content would increase the toxicity of petroleum hydrocarbons. It was thought that an increase in liquid water content would increase the bioavailable fraction of PHCs, as more hydrocarbons would be dissolved, and thus available for microbial uptake.

It was initially thought that the influence of liquid water content could be detected by measuring the toxicity of PHCs throughout the course of an austral summer (Chapter 3). Samples were taken multiple times throughout the summer to encompass periods of time when the soil was frozen, thawed and refrozen. Contaminated soil was assessed for toxicity using potential toxicity assays, which involved the addition of water and nutrients. The toxicity did vary throughout the course of the season, as indicated by changes in EC_{25} values (Table 3.2). In general, soil microorganisms were more sensitive to PHC contamination when the soils were thawed, i.e. lower EC_{25} values for thawed soil. However, it is difficult to determine the influence of liquid water from this data because the nature of the potential toxicity assays involves the creation of a soil slurry, thereby changing the liquid water content of the assay.

The influence of liquid water content on the toxicity of PHCs to soil microorganisms was then assessed using stable isotope dilution technique in soil cores packed at different bulk densities (Chapter 5). Liquid water content was successfully manipulated by changing the soil bulk density; however no significant effect of liquid water content on the toxicity of PHCs was

found. Therefore, this research shows that liquid water content does not influence the toxicity of PHCs to soil microorganisms in frozen contaminated soil.

6.2 Influence of liquid water on soil properties that control microbial activity

Liquid water content influences the bioavailability of nitrogen in frozen soil (Chapter 4). This finding significantly contributes to the area of bioremediation in cold climates as it suggests that biodegradation may be enhanced during the winter months by increasing the liquid water content of frozen soil. Methods of increasing liquid water content in frozen soil include manipulating bulk density, increasing the soil organic matter, or decreasing the amount of ice present in frozen soil. This research also showed that liquid water content increases in frozen soil contaminated with PHC (Appendix A). By increasing the liquid water content in frozen soil, PHC contamination inherently provides conditions suitable for enhanced bioremediation during the winter months. The research contained in this thesis suggests that additional management techniques that increase liquid water content in frozen soils may also be effective in increasing degradation rates.

6.3 Sensitivity of selected measurement endpoints to petroleum hydrocarbons

We investigated the toxicity of PHC on several different endpoints in both C and N cycles (Chapters 2, 3, and 5), as well as microbial community composition (Chapter 3). Table 6.1 summarizes the toxicity endpoints of each study. The toxicity estimates varied depending on the selected measurement endpoint and age of the spill. In general, nitrification was the most sensitive measurement endpoint for petroleum hydrocarbon contamination, with EC_{25} values

ranging from approximately 200 mg kg^{-1} up to 2000 mg kg^{-1}. This difference in toxicity can be attributed to the age of the spill. However, there are exceptions to nitrification being the most sensitive endpoint.

For example, in Chapter 2 the most sensitive indicator was substrate induced respiration (SIR); however because SIR activity actually increases in the presence of petroleum hydrocarbons, this was not viewed as the most sensitive indicator of toxicity. The other exception is from Chapter 3, where community composition was found to be more sensitive to PHC contamination than potential nitrification. The increased sensitivity of community composition indicates that this endpoint might be the most suitable for assessing toxicity to ensure the polar soil ecosystem is protected. However, there are drawbacks for using community composition as an endpoint for routine toxicity assessments as the analysis is expensive, time consuming, and requires highly qualified personnel to perform the analysis.

In addition to being a sensitive indicator of PHC contamination, the potential nitrification activity assay has many advantages to using it in routine toxicity assessment. This method is fast, easy, and relatively inexpensive to perform; therefore it is possible to process many samples quickly and easily. Nitrification is a common microbial process in all ecosystems, and thus it would be easy to compare the toxicity between ecoregions if a standardized protocol was developed. When the PNA assay is used for frozen soil samples, the microorganisms respond and adapt quickly to the changes in temperature, nutrients, and water (Chapter 3), thus eliminating the effect of environmental parameters on the outcome of the toxicity assay. This research has also demonstrated that PNA can be used to assess toxicity of both spiked and aged fuel spills (Chapters 2 and 3).

Table 6.1 Comparing EC_{25} values of all studies.

Section of Thesis	Age of Spill	Measurement Endpoint	Model	r^2	Toxicity Estimate
Chapter 2	3 weeks	PNA	Logistic	0.957	EC_{20} = 190 mg kg^{-1}
		PDA - N_2O production	Logistic	0.013	EC_{20} = 950 mg kg^{-1}
		PDA - N_2O consumption	NA	NA	Not sensitive
		Soil respiration	Exponential	0.996	EC_{20} = 220 mg kg^{-1}
		Substrate induced respiration	Sigmodial	0.5589	EC_{20} = 16 mg kg^{-1}
Chapter 3	6 years	PNA	Exponential	0.618	EC_{25} = 2000 mg kg^{-1}
		PDA - N_2O production	NA	NA	Not sensitive
		PDA - N_2O consumption	NA	NA	Not sensitive
		Soil respiration	Logistic	0.78	EC_{25} = 3500 mg kg^{-1}
		Substrate induced respiration	NA	NA	Not sensitive
		Metabolic quotient	NA	NA	Not sensitive
		Microbial community composition	Linear	0.202	EC_{25} = 800 mg kg^{-1}
		Bacterial biomass	Linear	0.202	EC_{25} = 2400 mg kg^{-1}
Chapter 5	6 years	In situ mineralization	NA	NA	Not sensitive
		In situ nitrification	Exponential	0.474	EC_{25} = 400 mg kg^{-1}
		PNA frozen soil	Exponential	0.353	EC_{25} = 200 mg kg^{-1}

PNA, potential nitrification activity

PDA, potential denitrification activity

NA, not applicable

Prior to this research, it was assumed that potential toxicity assays were a good surrogate for *in situ* toxicity. This assumption was validated by comparing the toxicity values of potential nitrification activity to gross nitrification rates (Chapter 5). The EC_{25} values generated by these two measurement endpoints are very similar to each other (Table 6.1), indicating that this assumption is valid. However, caution must be used when extending this assumption to other endpoints, as we only validated this assumption for nitrification. Further studies are required to determine whether the assumption holds true for other microbial processes.

6.4 Future Research

From the research presented in this thesis, we have learned that liquid water content in frozen soil does not influence toxicity of PHC but likely has an effect on degradation rates. Further research to investigate the relationship between liquid water content and degradation rates is needed to determine whether this is a viable remediation strategy for contaminated sites in Polar Regions. If manipulation of liquid water content can be used to enhance biodegradation rates in Polar Regions, this would be a significant advancement to bioremediation technology and its application to cold environments.

Further research is also required to identify quick, easy, and inexpensive methods to measure sensitive effects of petroleum hydrocarbon contamination. In this research, we validated the use of potential nitrification activity as a surrogate for gross nitrification rates. The potential nitrification activity is a cheap, easy, and fast method to generate soil toxicity data from a large number of samples. However, in addition to nitrification, there are other sensitive indicators of PHC toxicity to soil microorganisms including community composition and soil respiration. There is a need to determine quick and inexpensive methods for these indicators and to validate their use in polar soils.

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Appendix A

Hydrocarbon Contamination Increases the Liquid Water Content of Frozen Antarctic Soils

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A.1. Abstract

We do not yet understand why fuel spills can cause greater damage in polar soils than in temperate soils. The role of water in the freezing environment may partly be responsible for why polar soils are more sensitive to pollution. We hypothesized that hydrocarbons alter the liquid water in frozen soil, and we evaluated this hypothesis by conducting laboratory and field experiments at Casey Station, Antarctica. Liquid water content in frozen soils (θ_{liquid}) was estimated by time domain reflectometry in laboratory, field collected soils, and in situ field measurements. Our results demonstrate an increase in liquid water associated with hydrocarbon contamination in frozen soils. The dependence of θ_{liquid} on aged fuel and spiked fuel were almost identical, with a slope of 2.6×10^{-6}mg TPH (total petroleum hydrocarbons) kg^{-1} for aged fuel and 3.1×10^{-6} mg TPH kg^{-1} for spiked fuel. *In situ* measurements found θ_{liquid} depends ($r^2 = 0.75$), on fuel for silt loam soils (θ_{liquid}) $0.094 + 7.8 \times 10^{-6}$ mg TPH kg^{-1}) but not on fuel for silt clay loam soils. In our study, θ_{liquid} doubled in field soils and quadrupled in laboratory soils contaminated with diesel which may have profound implications on frost heave models in contaminated soils.

A.2. Introduction

Petroleum hydrocarbon spills are a common form of localized pollution in cold regions, with contamination most often caused by failed infrastructure, human error, sabotage, or natural hazards (Snape et al., 2008). Fuel spills often occur in permafrost soils. These spills pose a risk to human health and to terrestrial and aquatic ecosystems. Generally, spills are considered to be capable of causing greater damage in soils that undergo seasonal freezing (AMAP, 1998) than in soils that do not. The impact of fuel spills on permafrost integrity, tundra communities, and bacteria have been studied; however, the risks of fuel spills in polar regions as compared to

temperate regions have not been quantitatively established (Chapman and Riddle, 2005; Schafer et al., 2007). Likewise, we do not yet fully understand why fuel spills can cause greater damage in polar soils than in temperate soils (Snape et al., 2008).

The dynamics between solid and liquid water in the freezing environment may partly be responsible for why polar soils are more sensitive to pollution and more susceptible to fuel spills. A portion of soil-water exists in the liquid phase at temperatures below $0^{o}C$ (θ_{liquid}). One consequence of θ_{liquid} is secondary frost heave, a process in which water moves to a freezing front though a zone of partially frozen water known as the freezing fringe (Gilpin, 190; Padilla and Villeneuve, 1992). Secondary frost heave leads to an accumulation of segregated seams of ice in the soil near the surface that can damage the foundations of buildings, pipelines, and storage tanks (Fowler and Noon, 1993; Rempel et al., 2004). The presence of θ_{liquid} also affects other soil processes. For example, it influences the dispersal of water and contaminants (Barnes and Biggar, 2008) and may enable bacteria to survive for relatively long periods in permafrost (Rivkina et al., 2000). This survival is linked to the recent observation that petroleum biodegradation by bacteria occurs in permafrost at temperatures < $0^{o}C$ (Rike et al., 2005), when many supposed the process effectively stops at $0^{o}C$ (Ferguson et al., 2003).

This θ_{liquid} in soil is thought to arise primarily from three phenomena: surface wetting, solute freezing point depression, and particle curvature (Dash et al., 1995; Sparrman et al., 2004). Liquid water only converts to ice when the partial molar free energy of ice is lower than that of liquid water. Surface wetting depresses the partial molar free energy of liquid water, thus decreasing the temperature at which liquid water converts to ice. Solute freezing point depression occurs due to the exclusion of ions from ice, thus increasing the concentration of ions in the remaining liquid water. The increase in soil solution salinity decreases the temperature at which soil solution freezes. In soil, the concentration of dissolved salts within the

soil solution is usually low and only lowers the freezing point by 0.1°C (Williams and Smith, 1991). On curved particles, the free energy of the surface liquid is dependent on the sign and magnitude of the curvature. Consequently, a combination of surface wetting and curvature result in the Gibbs-Thomson effect in which the amount of liquid water present at a given temperature is increased if the curvature is viewed as concave from the solid side (Dash et al., 1995). Combined, these three phenomena reduce the freezing point of soil liquid water to between -0.1 and -1.5°C (Williams and Smith, 1991) and allow substantial (up to 0.5 g liquid H_2O g^{-1} soil) liquid water to exist between -2 and -10°C (Dash et al., 1995).

As part of a multidisciplinary study into the fate and effects of fuel spills in Antarctica, we are interested in the role of liquid water in frozen, petroleum-contaminated soils. We hypothesized that hydrocarbons alter the θ_{liquid} in frozen soil, and we evaluated this hypothesis by conducting laboratory and field experiments at Casey Station, Antarctica. Specifically, we evaluated if time domain reflectometry would work in the presence of diesel contamination, and if so, would diesel increase liquid water content. Finally, we also investigated if the increase in liquid water content at frozen temperatures was the consequence of changes in the Gibb's free energy of the adsorbed liquid water under these conditions. To the best of our knowledge, no peer-reviewed reports investigating the influence of diesel on θ_{liquid} in frozen soils have been published. At very high concentrations, diesel depresses the freezing point, with 0.1 mL diesel g^{-1} soil reducing the freezing point by 0.05°C (Grechishchev et al., 2001), but no mention is made of liquid water content below the freezing point. A recent conference proceeding from Purdue University indicates that freshly spiked diesel at concentrations of 20 g kg^{-1} increased the θ_{liquid} of a soil between -1 and -5°C (Chenaf et al., 2006).

A.3. Materials and Methods

A.3.3. Study Site

Our study site is immediately below the Main Power House at Australia's Casey Station in Antarctica. The contamination was caused by a spill that occurred in September 1999, which is near the end of the Austral winter (Snape et al., 2006). Approximately 5000 L of a light diesel blend flowed from the fuel storage area adjacent to the power house toward the north.

A.3.4. Measuring Liquid Water Content in Frozen Soils

Time domain reflectometry (TDR) determines θ_{liquid} by measuring the dielectric constant of the medium and is capable measuring as little as 0.05 m^3 H_2O m^{-3} soil (Topp et al., 1980). However, probes must be calibrated because variables such as soil solution salinity (Topp et al., 1988), temperature and soil type (Patterson and Smith, 1980; Patterson and Smith, 1985; Yoshikawa and Overduin, 2005), and soil freezing (Spaans and Baker, 1995; Yoshikawa and Overduin, 2005) can influence TDR response. The θ_{liquid} can be measured by TDR because the dielectric constant of liquid water (K_{water} = 80.36 at 20^{o}C) is much higher than that of the other soil constituents (K_{air} = 1; 3 ≤ K_{soil} ≤ 7), including ice (K_{ice} = 3.2), in the frequency range of 1 MHz to 1 GHz (Topp et al., 1980).

The use of TDR in frozen soil can be problematic because TDR waveforms attenuate as soil solution salinity increases (Topp et al., 1988) but TDR reflection improves as temperature decreases (Patterson and Smith, 1985). TDR measurements of θ_{liquid} for ice-water mixtures are within ±2.5% of the liquid water content (Patterson and Smith, 1980). To improve the accuracy, extensive calibrations specific for frozen soil are required (Spaans and Baker, 1995). In our case, we did not calibrate the probes at frozen temperatures because expected variation in the field would be much greater than 2.5% and thus, comparisons between treatments would still be

valid. We compared our calibrated TDR responses to those obtained using NMR analysis of deuterium in soil to assess the accuracy of the TDR probes and the influence of diesel fuel on TDR performance.

Deuterium NMR experiments were carried out on a Bruker Avance spectrometer (B_0 = 8.46 T, $\bullet_L(^2H)$ = 55.280 MHz) using a 7 mm double resonance magic-angle spinning probe on soils prepared using 99.9% D_2O and packed into 7mm outer diameter zirconia rotors. ^2H chemical shift referencing ($\delta(^2H)_{D2O(l)}$ = 4.80 ppm) and pulse calibration were carried out using 99.9% D_2O (Sen et al., 1992). ^2H NMR spectra of soil samples were recorded under stationary conditions using the quadrupolar echo pulse sequence $\left[(\pi/2)_x - \tau_1 - (\pi/2)_y - \tau - ACQ\right]$ (Sen et al., 1992). A sweep width of 500 kHz, acquisition time of 8 ms, $\pi/2$ pulse widths of 8.3 μs, interpulse delays of 30 μs, and pulse delays of 1 s were used to acquire 2000 scans for soil samples with mass densities of 1.3, 1.7, and 2.0 g cm^{-3}. Variable-temperature ^2H NMR experiments were carried out using the BVT-2000 temperature control unit and a $N_2(l)$ dewar fitted with a heat exchanger. Acquisition of data commenced at room temperature and the temperature was lowered until 268 K. After each adjustment, the temperature was allowed to stabilize for a minimum period of 20 min and the probe was tuned. To determine the signal intensity for each experiment, the total integrated peak area was calculated.

A.3.5. Time Domain Reflectometry Probe Construction and Calibration

The constructed TDR probes had 15 mm of stainless steel rod housed in an epoxy filled T-joint, leaving 85 mm of probe exposed to the surrounding media. Soil liquid water content was measured using a TDR100, SDMX50 50Ω multiplexer, CR10X datalogger, and PS100 12.0 V power supply (Campbell Scientific, Edmonton, Alberta). Each TDR probe (n = 32) was calibrated against eight volumetric water contents ranging from 0.005 to 0.26 g cm^{-3} at 18°C. Uncontaminated site

soil was collected, sieved to pass 5.6 mm, and air-dried. The gravimetric water content of this soil was assessed by oven drying (48 h, 110°C) three 10 g aliquots of the soil. Air-dried soil was packed into a 2 L cylinder to a bulk density of 1.30 g cm^{-3}. To take a replicate reading, the probe was inserted into the soil, a waveform collected, and the probe removed from soil. This was repeated three times for six different probes. After assessing the TDR response at specific water content, soil was removed from the 2 L cylinder, mixed with a specified amount of water, and repacked to a bulk density of 1.35 g cm^{-3}. To evaluate whether TDR probes were responding to fuel concentrations, we spiked air-dried clean soil with eight concentrations of Special Antarctic Blend (SAB) diesel fuel and then, using seven different probes, repeated the above procedure with the exception that we used a poly vinyl chloride (PVC) pipe (27.4 cm long, 8.1 cm diameter) to pack cores to a bulk density of 1.38 g cm^{-3} and measured moisture contents ranging from 0.038 to 0.20 g cm^{-3} at 18°C.

We assessed the interaction of freezing and fuel on TDR probe response at -18 and 22°C in dry, clean soil and in dry, contaminated soil. Clean soil was oven-dried for 48 h at 110°C. Readings (n = 19) were taken using two probes at -18 and 22°C. We then added 5790 (SD = 470) mg TPH kg^{-1} SAB diesel fuel to the soils, mixed the soils, and repeated the experiment.

A.3.6. Liquid Water Content of Frozen Contaminated Soils

The effect of aged hydrocarbons on θ_{liquid} was evaluated by collecting a site soil (10 580 mg TPH kg^{-1}) and diluting it with nearby reference soil. Soil was brought to 0.208 g H_2O cm^{-3}, mixed, and packed into a PVC pipe to a bulk density of 1.35 g cm^{-3}. Soil cores were sealed, and over the course of 8 h frozen to -15°C. During this freezing process, temperature and θ_{liquid} were measured every 60 s. This process was repeated for soils that had been spiked with eight concentrations of SAB diesel fuel. Gravimetric soil moisture was set to 0.208 g H_2O cm^{-3}.

We surveyed fuel concentrations from 40 locations that transect the plume perpendicular to the flow direction. Approximately 10 g of soil sample was collected and sieved, and fuel concentration was assessed (Snape et al., 2006). From this initial survey, a subset of 32 locations was selected to provide a range of fuel concentrations. Approximately 1.5 m of snow was removed. Probes (15 cm) and thermocouples were installed to a depth of 15 cm using a portable hand drill. After probe installation, a block of hard insulation was wedged into place above the probes and the holes were refilled with snow. Field TDR readings were confirmed by collecting field soil, sieving to pass a 5.6 mm sieve, and packing the soil to a bulk density of 1.30 g cm^{-3}. TDR probes were installed, the soil was frozen to -5^{o}C for a 24 h period, and TDR response assessed. To test the effect of probe installation on TDR readings, we inserted TDR probes into duplicate 800 g portions of site soil and cooled the soil to-18^{o}C. The response of this soil with TDR probes frozen in place was compared to the response of soils with TDR probes inserted by a hand drill.

A.3.7. Soil Texture and Water Repellency

Particle size distribution was assessed by a Horiba LA-950, Laser Scattering Particle Size Distribution Analyzer and soil textures calculated (SSSA, 1997). Soil-water repellency on air-dried soil was assessed using the molarity of ethanol droplet (MED) test (King, 1981; Litvina et al., 2003). Soils with a MED between 0 and 1 are slightly water repellent, and soils with a MED >2.2 are severely water repellent.

A.3.8. Chemical Potential of Soil-Water

The chemical energy of soil-water can be assessed using the Groenevelt-Parlange potential (*G**) (Groenevelt and Parlange, 1974), in which the temperature (*T*), pressure (*P*), mass of soil solid (m_s), mass of soil-water (m_w), and the chemical potential of soil air are controlled. In this scenario

$$dG^* = -SdT + VdP + \mu_s dm_s + \mu_w dm_w - m_a d\mu_a \quad [A1]$$

in which *S* is entropy, *V* is volume, μ_s is chemical potential of soil solids, μ_w is chemical potential of soil-water, m_a is mass of air, and μ_a is the chemical potential of air. To use this relationship, one must use an experimental system that is permeable to air. With the Groenevelt-Parlange potential, one can use the soil drying curve to estimate the work required to alter the water content of soil (Sposito, 1981):

$$dG^* = \mu_w dm_w \quad [A2]$$

This is transfer of chemical energy due to changes in soil moisture. If we consider a primary drying curve,

$$\Delta G_d^* = \int_{\theta_f}^{\theta_i} \mu_w (p.d.c.) dm_w \quad [A3]$$

in which θ_f and θ_i are the final and initial gravimetric water content respectively and $\mu_w(p.d.c)$ is the chemical potential along the primary drying curve. In its integrated form this is

$$\Delta G_d^* = m_s g \times (area\ below\ drying\ curve) \quad [A4]$$

The subscript (*d*) in ΔG_d^* refers to the Groenevelt-Parlange potential along the drying curve and has units of J. The area below-drying-curve is the chemical potential of soil-water along this curve. We divide by the mass of soil analyzed for final units of J g^{-1} soil. However, the partial molar free energy of water calculated in this laboratory method does not directly reflect field

conditions because changes in temperature, salt, and geometrical arrangement will occur between field and laboratory samples altering the free energy (Bolt and Frissel, 1960).

The moisture-holding capacity and water vapour surface area of soil at low moisture potential were assessed by allowing air-dried soil to come to equilibrium with an atmosphere in equilibrium with a saturated salt solution. Approximately 5 g of air-dried soil was placed over saturated solutions of $KC_2H_3O_2$, $CaCl_2 \cdot 6H_2O$, and $LiNO_3$ at room temperature in a sealed container (de Jong, 1999). The relative vapour pressures (p/p_o) for each of these are $KC_2H_3O_2$ = 0.20, $CaCl_2 \cdot 6H_2O$ = 0.323, and $LiNO_3$ = 0.47 (CRC, 1979; Newman, 1983). These vapour pressures can be converted to water potential (φ) by the following relationship (Hillel, 2004):

$$\varphi = RT \ln \frac{p}{p_o} \qquad [A5]$$

where R is the specific gas constant for water vapour, p is the vapour pressure of the soil-water, p_o is the vapour pressure of the pure free water, and T is the ambient temperature in Kelvin. Soil was weighed until the weights were constant, and then the soils were oven-dried at 105^{o}C for 48 h and weighed once more. This method can also be used to calculate the water vapour surface area of soil, assuming a water monolayer covers 3600 $m^2\ g^{-1}$ water (de Jong, 1999).

A.4. Results

The individual TDR probes calibrated (average r^2 = 0.992) across a range of θ_{liquid}, from 0.005 g cm^{-3} to 0.260 g cm^{-3}. At these low values of θ_{liquid}, TDR response was described using a quadratic equation. Thus, we solved the quadratic calibration equation using the Solver function of Microsoft Excel for field soils and for our spiked and aged soil experiments. At soil moistures of 0.038 – 0.20 g cm^{-3} fuel concentrations up to 20 250 mg TPH kg^{-1} did not influence TDR response to soil moistures (Figure A.1).

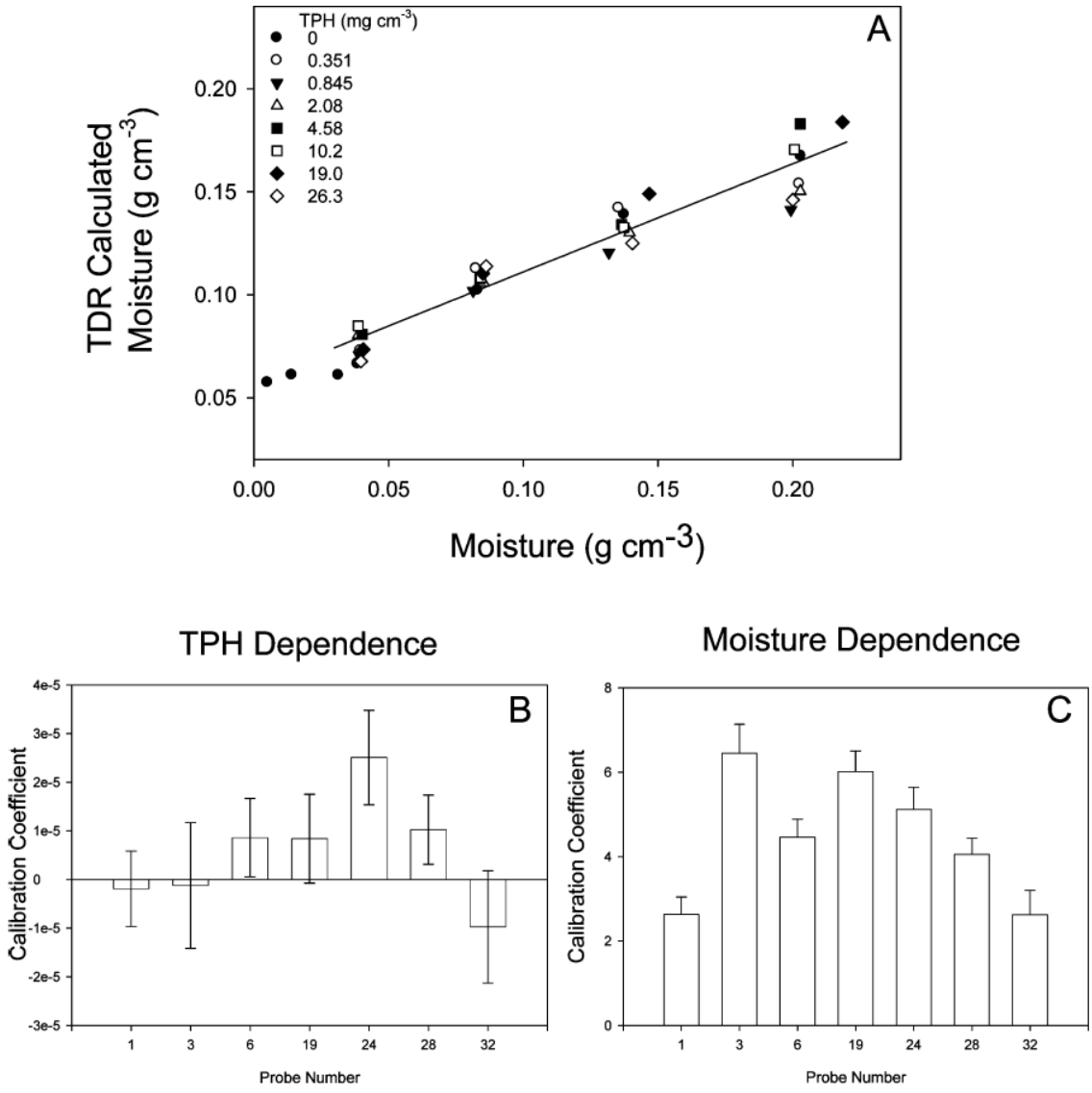

Figure A.1 Calibration of time domain reflectometry probes as a function of moisture and fuel (TPH). Soil from Casey station was air-dried, sieved (5.6 mm), amended with Special Antarctic Blend diesel, and packed into cores to a bulk density of 1.38 g cm^{-3} at 18°C. Panel A is the calibration of seven different probes across four different moistures and eight fuel concentrations. Panel B is the fuel calibration coefficient for each of these probes. Panel C is the moisture calibration coefficient for each of these probes. Error bars on the bar graphs represent the standard deviation of the estimated slope for each probe.

At these moisture contents, we used a linear calibration relationship for this experiment to allow us to directly compare calibration coefficients and perform multiple linear regression on the data set. In soil containing fuel and water, probes calibrated to a wide range of volumetric soil contents and did not respond to fuel levels. For example, for probe 6, the TDR calibration coefficient for fuel was only 1.023×10^{-5} (TDR response per mg TPH kg^{-1}) which is equivalent to a TDR volumetric calibration coefficient of 1.33×10^{-8} TDR response per mg TPH cm^{-3} in a soil with a bulk density of 1.3 g cm^{-3}. This volumetric response to TPH can be directly compared to the water calibration coefficient of 4.05 TDR response per g cm^{-3} θ_{liquid}). In a soil with 0.1 θ_{liquid} g cm^{-3} with 5000 mg TPH kg^{-1} (or 6.5 mg TPH cm^{-3}), the TDR response to θ_{liquid} will be 0.405 and to TPH it will be 8.6×10^{-8}. None of the fuel calibration coefficients for individual probes were significantly different from zero (Figure A.1B). Best subsets linear regression confirmed this interpretation, with only θ_{liquid} being selected as the most significant predictor. As expected, individual probes varied substantially in their response to θ_{liquid} (Figure A.1C), requiring that each probe be calibrated against water content for the soil of interest. Further at low liquid water contents, nonlinear calibration was essential.

There was little observed difference between the results obtained with TDR probes and the results obtained with deuterium NMR analysis across four soils with TPH concentrations ranging from 0 to 7640 mg TPH kg^{-1} soil. TDR and NMR results were almost identical with an $r^2 = 0.985$ ($p < 0.06$) and the slope of the relationship between TDR and NMR being 0.98 with a standard error of 0.067. If we consider NMR and TDR as duplicate determinations of water content, the average percent deviation of duplicates was 6.2%. We would note that the absolute difference in water content between NMR and TDR analysis was well within the range of error expected in TDR estimates.

In oven-dry (48 h, 110°C) soil, freezing and fuel influences did not interact to alter TDR response in dry contaminated soils ($P \leq 0.485$). When frozen, dry contaminated soil had an average TDR response of 0.141 at -18°C compared to 0.141 at 22°C. Similarly, there was no TDR artifact due to freezing of dry soil ($P \leq 0.077$), with an average TDR response of 0.134 at -18°C compared to 0.136 at 22°C in clean soil.

Liquid water content (θ_{liquid}) between -2 and -10°C was closely related to aged hydrocarbon concentrations in soil (r^2=0.94). The θ_{liquid} doubled as hydrocarbon concentration increased from 2180 mg TPH kg^{-1} to 10 580 mg TPH kg^{-1} (Figure A.2A). We selected the temperature range of -2 to -10°C because θ_{liquid} did not change appreciably in this temperature range. For example, between -2 and -10°C, θ_{liquid} changed on average (n = 8) 0.004 g H_2O cm^{-3} over a temperature range of 8°C, or 1.13% of the θ_{liquid} present in soil. The change in θ_{liquid} at this temperature is miniscule compared to the large change in θ_{liquid} we observed between 10 580 and 970 mg TPH kg^{-1} of 0.021 g H_2O cm^{-3}. The results presented in Figure A.2A should be interpreted with caution because the range of fuel concentrations were created by diluting contaminated site soil with nearby reference soil. The contaminated and reference sites are both situated below the Main Power House and are of similar texture but are separated by a road. Thus, it is possible that the relationship graphed in Figure A.2A is due to our diluting the contaminated soil with reference soil and not due to decreasing fuel concentrations. One would expect this dilution to cause a change in soil texture, but as the overlay in Figure A.2A demonstrates, there was no discernible change in soil texture caused by dilution.

Reference soil was amended with SAB diesel fuel to directly determine the influence of fuel concentration on θ_{liquid} with no need for soil dilutions (Figure A.2B). A θ_{liquid} trend similar to that observed in aged hydrocarbon soil was observed in soil freshly spiked with SAB diesel up to approximately 4000 mg TPH kg^{-1}. Frozen θ_{liquid} tripled from 0.005 to above 0.015 g cm^{-3} over this

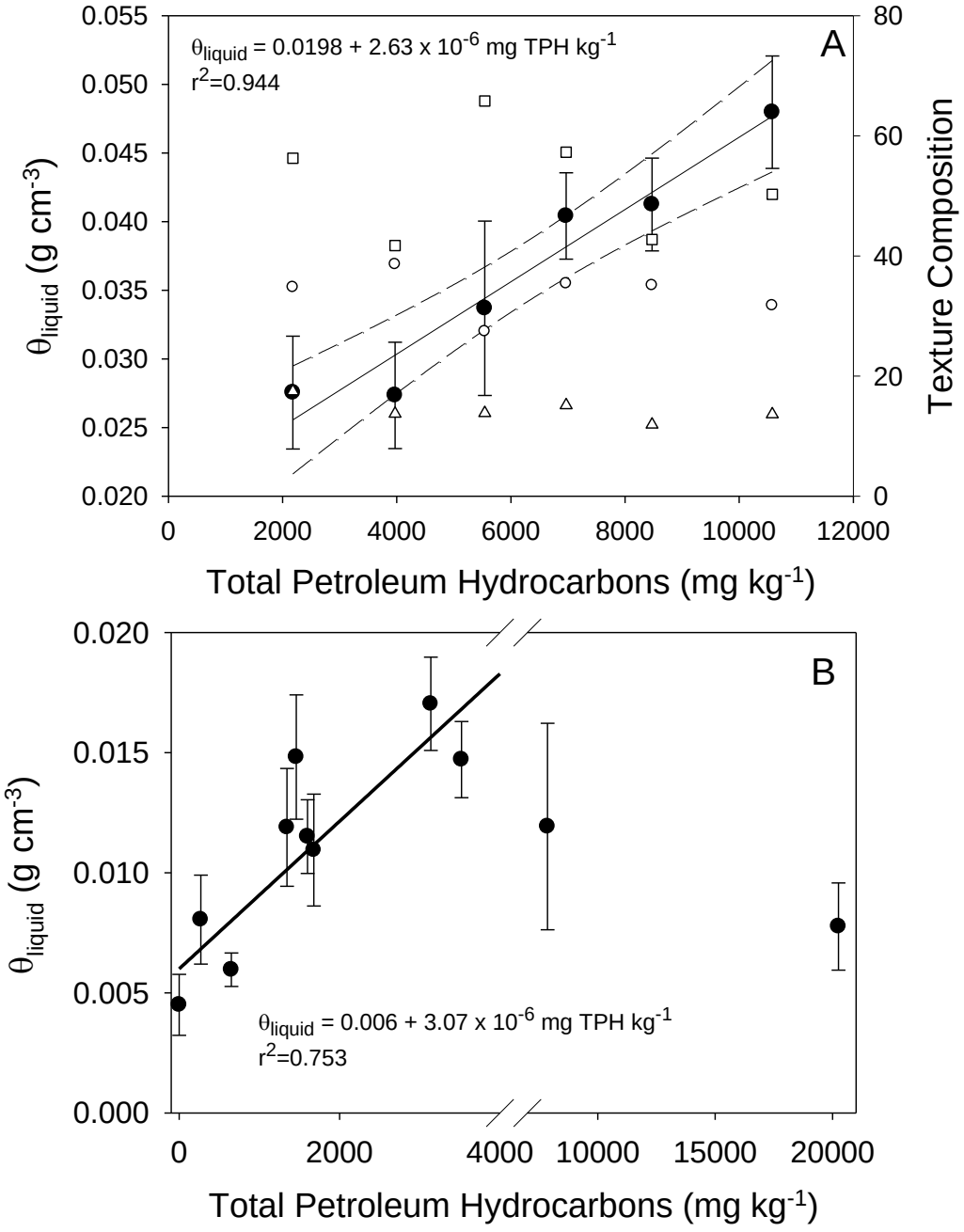

Figure A.2 Liquid water remaining in aged (Panel A) and spiked (Panel B) hydrocarbon-contaminated Antarctic soil between -2 and -10°C. The percent sand (□), silt (O), and clay (Δ) for each dilution are plotted on the right axis. Error bars, representing standard deviations, are obscured by the symbols.

range of diesel contamination in a linear fashion (θ_{liquid}) 0.006 + 3.07 × 10^{-6} mg TPH kg^{-1}, r^2=0.75. The dependence of θ_{liquid} on aged fuel and spiked fuel was similar (Student's t test, P ≤ 0.161), with an aged fuel slope of 2.64 × 10^{-6} mg TPH kg^{-1} (SD = 0.32 × 10^{-6} mg TPH kg^{-1}) and spiked fuel slope of 3.07 ×10^{-6} mg TPH kg^{-1} (SD=0.67×10^{-6} mg TPH kg^{-1}). However, for the spiked soils this relationship did not continue past 4000 mg kg^{-1}; rather, θ_{liquid} decreased at the last two fuel concentrations of 7820 and 20 250 mg TPH kg^{-1}. This may be an experimental artefact caused by first wetting the soil, adding large amounts of diesel fuel, mixing the soil, and then packing it into PVC cores. At these total moisture capacities, 0.208 g cm^{-3}, the existence of a non-aqueous phase is likely and may explain why liquid water did not increase at these higher freshly spiked diesel concentrations.

Liquid water in site soil at approximately -7.2°C varied between 0.08 and 0.13 g cm^{-3} at sites containing between 0 and 27 000 mg TPH kg^{-1} (Figure A.3). Installation of TDR probes into frozen ground did not influence the measured θ_{liquid} of soil below 0°C (data not shown), and the reproducibility of TDR readings between subsequent weeks and laboratory readings was approximately 3%. However, not all TDR probes gave reasonable waveforms after installation, with only 27 of the 32 probes working successfully after installation. Similar to the laboratory observations, θ_{liquid} increased with increasing TPH; however there was substantial variability at low TPH concentrations.

In contrast to the laboratory observations, there was not a clear linear trend of θ_{liquid} increasing with TPH levels in soil for the majority of the site. There was no apparent association between MED and θ_{liquid}. The dependence of θ_{liquid} on diesel was linked to soil texture: the majority of the sites were classified as silt clay loam (n = 17) and silt loam (n = 8), and the remaining sites were classified as clay loam (n = 1) or loam (n = 1). If the extreme point of 26 640

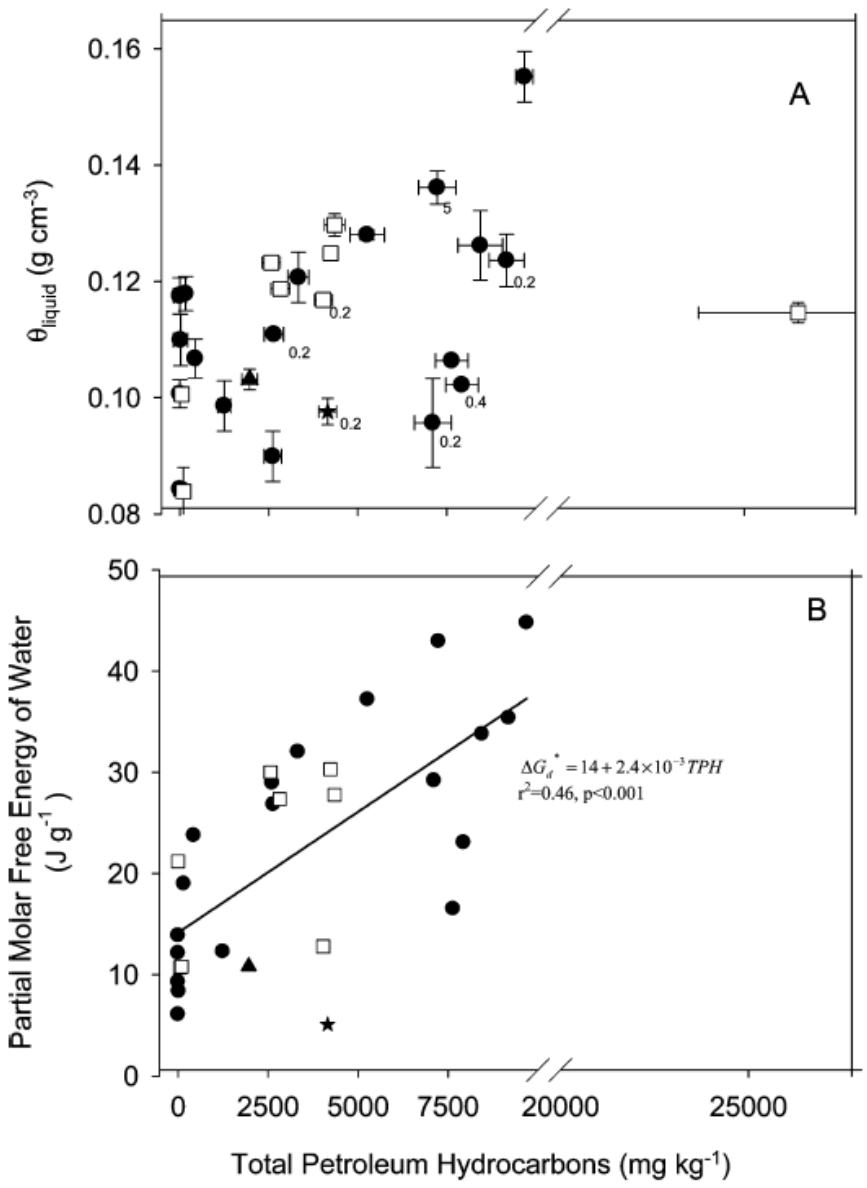

Figure A.3 Liquid water remaining (Panel A) in contaminated soil below the Main Power House between -2 and -10°C. Panel B depicts the chemical energy $\left(\Delta G_d^*\right)$ required to dry approximately 5 g of soil from -220 to -102 J g^{-1} at 22°C. Silt clay loam is represented by ●, silt loam by □, loam by ▲, and clay loam is represented by ☆. If the molarity of ethanol droplet (MED) value was greater than 0, it is indicated to the lower right of a data point. Each point in Panel A is the average of two sampling times, November 19 and 27, 2005, and a laboratory confirmation. The field observations had an observed soil (5 cm depth) temperature of-7.2°C (±1.5 °C) for both dates, and the laboratory assessment was performed at -5.0°C. The line in Panel B indicates the model ii regression relationship between ΔG_d^* and TPH. Error bars indicate the uncertainty associated with each data point.

mg TPH kg^{-1} is excluded, there was a strong dependence of θ_{liquid} on fuel for silt loam soils (θ_{liquid}) 0.094 + 7.75 × 10^{-6} mg TPH kg^{-1}, r^2 = 0.75 (p = 0.008). The dependence of θ_{liquid} on fuel in field soils was more than double (i.e., 7.75 × 10^{-6} mg TPH kg^{-1}; SD = 1.79 × 10^{-6} mg TPH kg^{-1}) that observed with the laboratory samples (3.07×10^{-6} mg TPH kg^{-1}), which were typically sandy loam soils. In contrast, there was no relationship between θ_{liquid} and fuel in silt clay loam soils (r^2 = 0.04, p = 0.224).

The partial molar free energy of the soil-water correlated (r = 0.68) with the fuel content of the soil and was dependent on TPH concentration (ΔG_d^*, r^2 = 0.46, p < 0.001) (Figure A.3B). The sample point from grid location 12, 18 with a fuel concentration of 26 640 mg TPH kg^{-1} was lost in transit back from Antarctica and was unavailable for this laboratory analysis. The correlation of the partial molar free energy of soil-water with fuel concentrations was stronger (r = 0.75) for the silt clay loam compared to the silt loam (r = 0.58). The partial molar free energy of soil-water correlated (r = 0.59) with the θ_{liquid}.

A.5. Discussion

Our results clearly demonstrate an increase in liquid water associated with hydrocarbon contamination in some cold soils (i.e., -7.2^{o}C). This increase in liquid water was determined using TDR probes that were demonstrated to reliably assess liquid water in the presence of hydrocarbons. The TDR probes reported this increase in liquid water in diluted site soil, spiked reference soil and an in situ aged fuel spill. The field results obtained largely from a clay loam did not display as clear a trend as those obtained in the laboratory which was a sandy loam. Despite this, liquid water contents in site soils increased by approximately 50% or 0.04 g cm^{-3} at circa -7.2^{o}C by hydrocarbon contamination. This increase in θ_{liquid} of frozen soils due to hydrocarbon contamination has not been previously reported.

We considered the interactions of fuel with solute freezing point depression, surface wetting and particle curvature that influence θ_{liquid}. Although we did not specifically determine the relative roles of each mechanism, others have noted only a minimal freezing point depression due to diesel (Balks et al., 2002). The effect that surface wetting and curvature have on the Gibbs-Thomson effect, however, is supported by correlations between calculated partial molar free energy in soil and contaminant concentrations. Fuel decreases the partial molar free energy of soil-water, which may explain the observed increase in θ_{liquid} in fuel-contaminated soils. A decrease in partial molar free energy means that less liquid water will change to ice at a given field temperature. We speculate that fuel may decrease the partial molar free energy through the dissolution of hydrocarbons into the water medium or through changes to the soil geometry that influence surface melting.

Balks et al. (2002) noted an increase in soil temperature in Antarctic soils contaminated with hydrocarbons. This increase in soil temperature only occurred during the summer months and was linked to an increased soil surface albedo. These investigators postulated that fuel contamination may have altered the moisture holding capacities of these Antarctic soils and thereby led to increased summer soil temperatures (Balks et al., 2002). They observed no differences in moisture-holding capacities between contaminated and uncontaminated soils; however, they assessed moisture holding capacities using pressure plates at much lower moisture tensions than we used in our investigation. In addition, they investigated a wide variety of soil types in western Antarctica and a small range of hydrocarbons for each soil. As can be seen from our data, even on a fairly uniform site such as the one at Casey Station, there is substantial variability which may mask θ_{liquid} trends. Additional work is required to assess if the θ_{liquid} –TPH dependence observed in Eastern Antarctica also occurs in other Antarctic and Arctic soil types.

Liquid water in soil is widely accepted as being a requirement of microbial activity in the subsurface (Panikov et al., 2006), and investigators have recently found that microbial activity in Arctic hydrocarbon-contaminated soil was initiated at temperatures greater than -6^{o}C (Rike et al., 2005). This temperature is significant because above this temperature the proportion of soil-water available as a liquid rises exponentially (Williams and Smith, 1991), suggesting that liquid water may be a controlling feature of in situ bioremediation in cold regions. Our results suggest that microbial activation, if it is indeed dependent on the liquid water film, will occur at lower temperatures in contaminated soils due to an increase in θ_{liquid} as compared to uncontaminated soils.

Our observations suggest that infrastructure instability may occur near major hydrocarbon spills. It is commonly assumed that a source of liquid water is required for substantial frost heave to occur in polar soils. However, studies conducted in the absence of a water table indicate that even with complete removal of an external water supply, significant frost heave rates can still occur due to the upward redistribution of water normally existing in frost-susceptible soil prior to freezing (Hermansson and Spencer Guthrie, 2005). The increase in θ_{liquid} associated with fuel observed here may alter frost-heave dynamics by increasing the available pool of water, which can redistribute toward the freezing front. The redistribution of water observed in that previous study (Hermansson and Spencer Guthrie, 2005) appeared to be approximately 0.07 cm^3 cm^{-3} which is well within the 0.08 maximal increase we observed in this study. In our study, θ_{liquid} doubled in field soils and quadrupled in laboratory soils. The effects of this increase in θ_{liquid} on existing frost heave models are unclear, but could be profound.

APPENDIX REFERENCES

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