Biotreatability studies of oil-contaminated soils from Antarctica

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Abstract

Human activity in Antarctica has resulted in accidental fuel spills on soils. These are broadly damaging to the Antarctic ecosystem, and expensive to clean up completely by physical removal, therefore, alternative remediation techniques, like bioremediation, are required. Hydrocarbon-degrading bacteria have been isolated from Antarctic soils, but few biotreatability studies have been described. On the basis that limiting factors to bioremediation can be manipulated to enhance biodegradation of contaminated soils, this thesis research project aimed to test these limiting factors to contribute to the development of bioremediation protocols for hydrocarbon-contaminated soils from the Ross Sea Region, Antarctica.

Hydrocarbon-contaminated and control uncontaminated soils collected from Scott Base, Marble Point and Wright Valley were chemically analysed for total hydrocarbons and chemical properties, and analysed for total heterotrophic and hydrocarbon-degrading microbes. Both contaminated and uncontaminated control soils were screened for the alkane catabolic genes Pp *alkB,* Rh *alkB1,* Rh *alkB2* and Rh *alkB194* by polymerase chain reaction (PCR) and sequenced to determine the soils' alkane-degrading genetic potential. Additionally, hydrocarbon-degrading bacteria isolated from Scott Base soils were characterised morphologically and screened for *alkB* gene homologues. The degradative ability of microbes was assessed by ^{14}C-hexadecane mineralisation in soil microcosms at 15°C in all untreated soils, water and nitrogen amended Scott Base soils, Scott Base contaminated soil diluted 1:1 with uncontaminated soil, and Bull Pass soil bioaugmented with strains *Rhodococcus* sp. 5/1 and SBO-1, a hydrocarbon-degrading microbial isolate from this study. Molecular analysis of 16S rDNA of SBO-1 showed DNA sequence homology of 99% with *Rhodococcus* sp. 5/14. Changes in microbial populations in the most successful enhancement treatments were detected by denaturant gradient gel electrophoresis (DGGE).

Hydrocarbon-degraders were found only in Marble Point and Scott Base soils (from 10^3 to 10^6 colonies per gram dry weight soil). The most abundant *alk* gene in the soils was Pp *alkB*, followed (in deceasing order of abundance) by Rh *alkB1*, Rh *alkB2* and Rh *alkB194*. This indicated the possible presence of *Pseudomonas* and *Rhodococuss* species in these soils. High rates of mineralisation were detected in Marble Point, *ca.* 60% in 75 days, and low rates in Scott Base soil, *ca.* 4% in 75 days. Nitrogen overfertilisation and water adjustments decreased mineralisation, but addition of 2500 mg N/kg-H_2O-soil combined with 10% moisture enhanced mineralisation *ca.* 4 times in a recent and old spill site in Scott Base soil, indicating that nitrogen was a limiting factor. Dilution of this soil with uncontaminated soil resulted in enhanced mineralisation, 8 times that of unamended soil, reaching *ca.* 40% mineralisation. DGGE showed that the diluted soil was enriched in a *Rhodococcus* species. Dilution of the soil, and consequently of the contaminant, was the most successful treatment for Scott Base soil. This indicated that contaminant concentration was a limiting factor in this soil. Bioaugmentation of Bull Pass soil was partially successful; mineralisation was enhanced after addition of inoculum, but reduction in total hydrocarbons was minimal and attributed to abiotic loss.

Overall, limiting factors to bioremediation can be manipulated to enhance biodegradation by nutrient amendments, dilution of contaminant, and inoculation of hydrocarbon-degraders. The implementation of these treatments may aid in remediation of contaminated soils from the Ross Sea Region.

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List of Abbreviations

μCi = micro Curie

μl = microlitres

$^{14}CO_2$ = radiolabelled ^{14}C-carbon dioxide

a.s.l. = above sea level

alk B = gene encoding for alkane monooxygenase

alkB1 = gene homologue 1 of *alkB*

alkB194 = gene homologue 194 of *alkB*

alkB2 = gene homologue 2 of *alkB*

ATP = adenosine-triphosphate

bp = base pair

BP = Bull Pass

C = carbon

$CaCl_2$ = calcium chloride

CFU= Colony Forming Units

cm = centimeters

cmol = centimoles

CO_2 = carbon dioxide

CoA = Coenzyme A

ddH_2O = double distilled water

DFA = Diesel Fuel Arctic

DFM = Diesel Fuel Marine

DGGE = Denaturant Gradient Gel Electrophoresis

dpm = degradations per minute

dw = dry weight

E = East

g = grams

g dw = grams per dry weight

HCD = hydrocarbon-degraders

HCl = hydrochloric acid

HDB = hydrocarbon-degrading bacteria

JP = Jet Propellant

K = potassium

kb = kilobase

L = litres

m = meters

M = molar

MC = moisture content

mCi = miliCurie

mg = miligrams

ml = mililitres

mm = milimetres

mM = milimolar

MP1 = Marble Point 1 soil sample

MP2 = Marble Point 2 soil sample

MPN= Most Probable Number

MPNES = Most Probable Number Enumeration System

N = nitrogen

NaCl = sodium chloride

NADH = reduced nicotinamide-adenine dinucleotide

NADPH = reduced nicotinamide-adenine dinucleotide phosphate

ng = nanograms

NH_4^+ = ammonium ion

NH_4Cl = ammonium chloride

nm = nanometers

nM = nanomolar

NO_3^- = nitrate ion

P = phosphorus

PCR= polymerase chain reaction

Pp = *Pseudomnas putida*

rcf = relative centrifugal force

rDNA = ribosomal deoxiribonucleic acid

Rh = *Rhodococcus* sp.

rpm = revolutions per minute

rRNA = ribosomal ribonucleic acid

s = second

S = South

SBO = Scott Base old spill

SBR = Scott Base recent spill

sp. = species

TPH= Total Petroleum Hydrocarbon

UCM = Unresolved Complex Mixture

UV = ultraviolet

V = volts

w = weight

W = West

Chapter 1. Introduction and literature review

1.1. Introduction

Contamination with petroleum hydrocarbons is an environmental problem found all over the world and is considered the most widely distributed environmental pollution problem[80]. Antarctica is no exception despite its remoteness and isolation. Hydrocarbons are compounds that naturally occur in the environment, but when derived from industrial extraction and refinery processes, they can be considered contaminants if they "artificially" enter the environment in large quantities. Hydrocarbon contaminants can be found in fresh water systems, seawater, sediments and soils. This type of contamination constitutes a complex environmental problem, because typically, first, the petroleum derivatives are complex mixtures and second, each environment has its own particular biotic and abiotic characteristics that determine the fate and consequences of the contamination event. However, it is *not* a problem that *cannot* be resolved. This literature review studies the problem of hydrocarbon contamination in the soils of the Ross Sea Region, Antarctica. The sources, causes and effects of fuel-contamination and possible remediation strategies to this environmental problem are described.

1.2. Antarctica

1.2.1. The continent

Antarctica is the fifth largest continent on Earth, a continent where man is not indigenous, a place devoted to scientific investigation and international cooperation and often referred to as "the last remaining pristine environment on the planet[20,37,41,43]. It constitutes a unique place on the Earth for several reasons[83]:

- Possesses one of the harshest environments on the planet: extreme and constant cold, a large amount of water in the frozen state, which results in very low humidity and thus arid conditions equivalent to hot deserts.
- Extreme seasonal variations in light.
- Low biodiversity and biomass compared to other the continents.
- The world's highest continent (average elevation is 2,160 m above sea level).
- Limited human access and impact compared to other landmasses; it is almost completely covered with ice (98% of its surface is ice, approximately 30 million km^3 of water).
- A land owned by no one, not exploited economically, operating under an international agreement, a unique situation in today's world.

The combination of all of these factors makes Antarctica a very singular place[83].

1.2.2. The Ross Sea Region

The Ross Sea Region in Antarctica is located approximately between 150° W, 160° E and south of 60° S. It is characterised by the following key landscape aspects[137]: the Transantarctic Mountains, the Ross Ice Shelf, small areas of ice-free ground including the McMurdo Dry Valleys, several active volcanoes, ice-covered ocean and over 4,000 km of coastline (see Figure 3). In this region, the Pacific sector of the Southern Ocean extends into a large embayment, forming the Ross Sea. The Transantarctic Mountains form a 4,000 km long chain across the Western part of the Ross Sea Region from Oates Land in the North to the McMurdo Dry Valleys in the South, and continuing across the continent to the Filchner Ice Shelf. Ice and snow cover the mountains that vary in height from 1,500 to 4,000 m. On the Ross Sea side of the range, glaciers of tens of kilometres long merge with the Ross Ice Shelf. The Ross Ice Shelf is the largest in the world, covering an area of over 530,000 km^2, and measuring from 100 to 1000 m thick. There are several islands along the coastline of the region including: Ross Island, Possession Islands, Coulman Island, Franklin Island and Roosevelt Island. Ross Island is centred by Mount Erebus, the world's most southerly active volcano.

Mount Terror, Mount Terra Nova and Mount Melbourne are other volcanoes in the region. Almost half of the ice-free ground in Antarctica occurs within the Ross Sea Region, including 6,000 km^2 of continuous ice-free ground found in the McMurdo Dry Valleys.

In the Ross Sea Region, there are three permanent scientific stations, which are occupied year-round[137]: New Zealand's Scott Base, United States' McMurdo Station, both in the Ross Island, and Amundsen-Scott South Pole Station (United States), located at the South Pole. Two summer stations in Terra Nova Bay, the Italian Terra Nova Station and the German Gondwana Station have been established. Several summer field camps are operated in the region for scientific support, of which, some are semi-permanent and others are permanent. Permanent camps include the helicopter refuelling facility at Marble Point and the Lake Hoare Camp in the McMurdo Dry Valleys. Smaller camps or refuges exist along the Dry Valleys and Ross Island that are maintained by New Zealand, the United States, Germany and Italy.

1.2.3. The Antarctic Treaty

Antarctica's uniqueness and fragility was recognized internationally during the International Geophysical Year (July 1957 to December 1958), which brought together the research activities of 66 countries to investigate the continent. As a result of this, 12 countries, including New Zealand, established 40 bases in the continent and 20 other bases in the islands of the Southern Ocean. The U.S.A. built the first permanent inland base at the South Pole and its coastal McMurdo Base in 1956 (Figure 1). New Zealand built Scott Base on Ross Island between 1956 and 1957 (Figure 2).

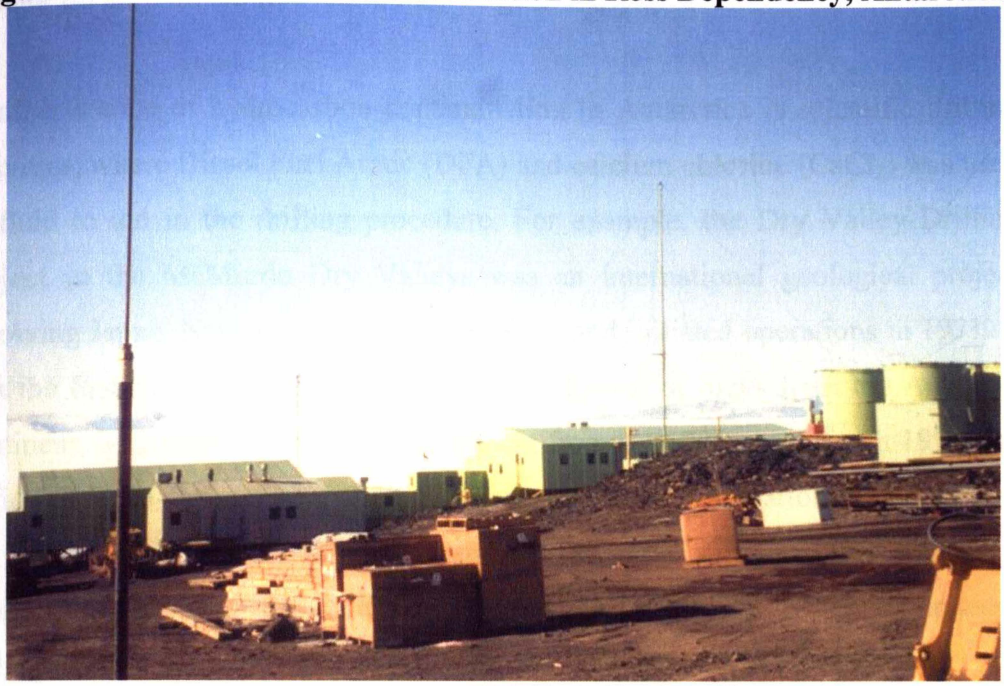
This international effort led directly to the establishment of the **Antarctic Treaty** in 1959, which is the document that dictates the values and principles for management and administration of Antarctica. The Treaty declared Antarctica as a region of cooperative scientific effort where military & nuclear explosions, and waste disposal are currently banned (Articles I.1 and V.1, respectively)[103]. In

1991, the **Protocol on Environmental Protection** to the Antarctic Treaty was developed in Madrid, Spain (entered in force in 1998). This Protocol designates Antarctica as a 'natural reserve, devoted to peace and science', forbids all mineral resource activities, emphasizes responsible environmental management by requiring environmental impact assessments for all activities as well as contingency plans for environmental emergencies, and provides for the establishment of a Committee for Environmental Protection[103]. In this context, and being a signatory to the Antarctic Treaty and its Environmental Protocol, New Zealand is obliged to respond to environmental impacts or damage, such as hydrocarbon contamination, that may result from its activities in the continent.

Figure 1. USA McMurdo Station in the Ross Dependency, Antarctica

(Photo courtesy of Professor R. Farrell)

Figure 2. New Zealand's Scott Base Station in Ross Dependency, Antarctica



(Photo courtesy of Professor R. Farrell)

1.2.4. Human impacts in Antarctica

Ice-free regions in Antarctica constitute less than 1% of the surface and less than 5% of the Antarctic coastline. They are not evenly distributed and are concentrated in the Ross Dependency and Antarctic Peninsula[74]; about half of the total ice-free surface occurs in the Ross Sea Region. Ice-free coastal zones have most of the terrestrial biological activity and also have more intense human activity compared to inland zones, and, thus, are the most likely to be affected by human activity[32]. The environmental consequences of normal procedures and activities in scientific stations and field camps include contamination of soils due to accidental oil spills and leakage of storage tanks and pipelines[38,72], contamination of sea water with diesel or aviation fuels released from grounding of vessels or wreckage (as in the case of the *Bahia Paraiso* wreckage in 1989)[72], deposition of combustion products[72,122], landscape alteration resulting from construction[29], introduction of exotic organisms[54], and disturbance of wildlife[85]. Spillage of fuels has been the most common type of incident and has been recognized as the most environmentally harmful in Antarctica[85,112]. The impact of a major oil spill on ice-free zones may be potentially great[71].

Another source of hydrocarbon contamination in Antarctica is scientific drilling activities, where Diesel Fuel Arctic (DFA) and calcium chloride ($CaCl_2$) was used as fluid to aid in the drilling procedure. For example, the Dry Valley Drilling Project in the McMurdo Dry Valleys was an international geological project involving Japan, New Zealand and the U.S.A., and initiated operations in 1971. It was the first major deep drilling project for retrieval of rocks from the Antarctic continent, with depths of excavation ranging from 4 to 381 meters[82]. In 1973, the Office of Polar Programs of the US National Science Foundation requested that the environmental impact assessment and monitoring of subsequent operations be conducted[93]. Despite their awareness of high probability of contamination, spillage events did occur at the Lake Vida and New Harbour sites (1973-1975), as spillages are almost inevitable during drilling activities[93].

1.3. Hydrocarbon contamination in Antarctica

1.3.1. Fuel usage in the Ross Sea Region, Antarctica

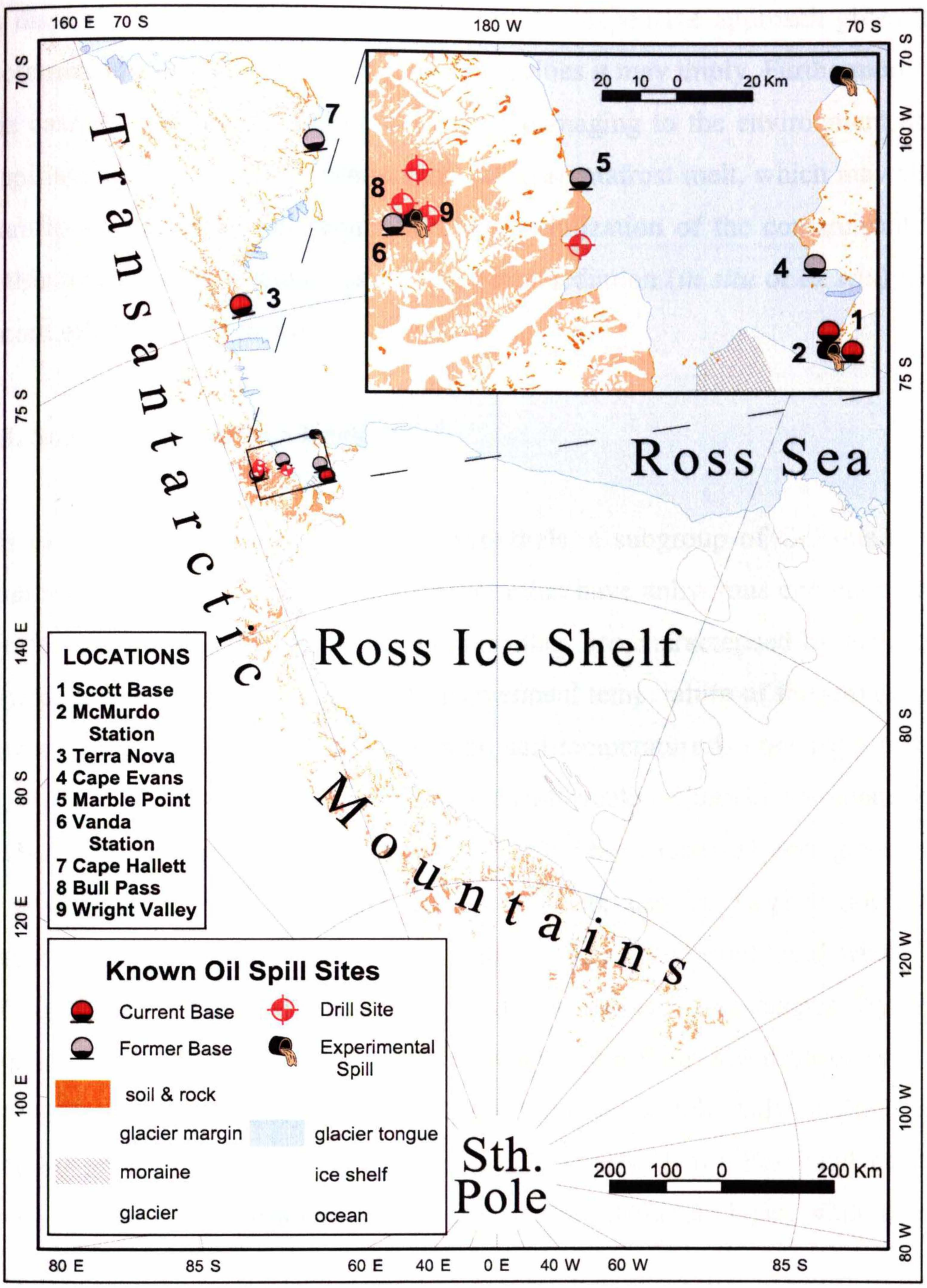
Each year, around 80 million L of fuel are consumed in scientific stations in Antarctica for power generation, heating systems and vehicle and aircraft operations[112]. In the Ross Sea Region, which is most relevant for the present study, there is a total fuel storage capacity of 34,446 million L in all of its bases and camps (McMurdo Station, William's Airfield, Marble Point Camp, Amundsen-Scott South Pole Station, Scott Base and Terra Nova Bay Station). The McMurdo Station has an annual delivery by tanker ship of around 25 million L of fuel that are distributed by pipeline to 18 steel storage tanks, and storage facilities at William's Airfield, and by tanker to Scott Base's storage tank[137]. Fuel is pumped via hose from the tanker to Marble Point (a helicopter refuelling facility) and delivered to the South Pole Station via LC-130 aircraft. Scott Base uses approximately 416,000 L per year of fuel (not including aircraft operations); its storage capacity is 54,000 L, but much of the base's fuel is stored at McMurdo Station[137].

Over the last 3 decades, the type of fuel utilized has varied, but in general the following have been used or are being used to a certain degree in the Ross Sea Region: Jet Propellant (JP)-4 (JP-4), JP-5, JP-8, DFA, Diesel Fuel Marine (DFM), AN-8 and, in lesser degree, Mogas (unleaded gasoline) and kerosene[137]. JP-5 is currently the most used type of fuel in the Ross Sea Region[4], and it is the United States Air Force primary jet fuel. Both JP-5 and JP-8 are kerosene based light aviation turbine fuels composed mostly of C_8 to C_{17} *n*-alkanes (up to 81%) with lesser amounts of aromatic products, mainly naphthalenes, along with low levels of benzene, toluene and xylene[153]. AN-8, is a derivative which contains a different antifreeze, an antioxidant, a corrosion inhibitor and metal deactivator compounds[137]. Other petroleum products used as antifreezing, hydraulic and lubricant agents in Antarctica include glycol and oils.

1.3.2. Oil spills in the Ross Sea Region

With such a large amount of fuel transported and manipulated in this region, spillage events have occurred frequently. At McMurdo Station, the most populated and the largest of the Antarctic bases, there were 323 recorded spill events between 1991 and 1998, a total of 74,004 L, of which the majority (90%) were JP-8/AN-8[137]. Between the years 1994 and 2001, there have been 13 fuel spill events recorded at Scott Base totalling around 1,800 to 2,300 L, the majority of it being JP-8[137]. It is important to mention that spillage events have occurred prior to these dates and have not been documented in detail. Figure 3 shows a map of sites where hydrocarbon spills have occurred in the past in the Ross Sea Region. It is likely that in some of these sites contamination has persisted for more than 40 years[4] and at some of these sites both heavy and light fuels have been the source of contamination. In some contaminated sites at Scott Base, *n*-alkanes predominate and have lesser concentration of monoaromatics and polyaromatic hydrocarbons (PAH)[4].

Figure 3. Fuel-contaminated sites in the Ross Sea Region, Antarctica (From Aislabie *et al.* (2004) [4])



In 1998, the Antarctic Treaty Consultative Meeting agreed on specific guidelines for the handling of fuels in Antarctica with the aim to minimize spills. Upgrades of storage tanks at McMurdo Station and Scott Base were performed during the 1998/1999 and 1999/2000 seasons and spill prevention and contingency plans elaborated[4].

So far, the common practice after a fuel spill event on soil has been the excavation of the contaminated soil with its subsequent shipping to the home country, where it is disposed or remediated[4]. This is however an expensive approach given the remoteness of Antarctica and the logistic difficulties it may imply. Furthermore, in some cases this approach may be even more damaging to the environment than the spillage itself because excavation can cause permafrost melt, which may alter streamflows and cause soil salinisation and mobilization of the contaminants[29]. For this reason, other alternatives such as bioremediation (*in situ* or *ex situ*) may be more effective for Antarctica[4].

1.3.3. Soils in the Ross Sea Region

Soils in Antarctica are classified as **anhyorthels**, a subgroup of Gelisols (with permafrost within 100 cm of the soil surface) that have anhydrous condition, and are referred to as "cold desert soils" because they are characterised by very low temperatures and low moisture[23]. The mean annual temperature of the soil ranges between -15°C and -40°C[14]. During winter, soil temperature is constantly below 0°C, but in the summer there is a short period (6-8 weeks) where soil temperatures are above 0°C. Temperatures of up to +18°C have been reported during summer for several sites in the Ross Sea Region[14]. There can be large temperature fluctuations in the surface layer within a day due to cloud cover and winds, as well as strong temperature gradients between surface and deeper layers[30]. Moisture values in coastal regions are relatively higher than inland areas, such as the McMurdo Dry Valleys, because precipitation is relatively higher. For example, gravimetric soil moisture contents in coastal Scott Base and coastal Marble Point are *ca.* 2% in surface and 10% in the subsurface layer, while inland Dry Valleys moisture values are <1% in some soil profiles[30].

Anyorthel soils are subdivided in several subgroups, but for the soils from the Ross Sea Region relevant to this work the following are the most relevant (descriptions as per Soil Survey Staff (2003)[120]):

a) **Calcic anyorthel**: anyorthels with a calcic horizon with its upper boundary within 100 cm of the mineral soil surface.

b) **Nitric anyorthel:** anyorthels with a horizon 15 cm or more thick that contains 12 cmol/L in 1:5 soil:water nitrate and in which the product of its thickness (in cm) and its nitrate concentration is 3,500 or more.

c) **Typic anyorthel**: other anyorthels.

Antarctic soils in the Ross Sea Region have 3 main layers[24] (Figure 4):

1. A surface pavement layer of gravel, stones or boulders with the absence of fine material that has been removed by wind action.
2. An active layer 17 to 55 cm deep below the pavement surface; its texture is a loamy sand or sand with gravel, stones and boulders and it is seasonally thawed;
3. Permafrost: remains at temperatures of <0°C for at least 2 consecutive years; underlies all ground surface and is ice-cemented or loose as in "dry-permafrost" when the water content is very low (as in the Dry Valleys).

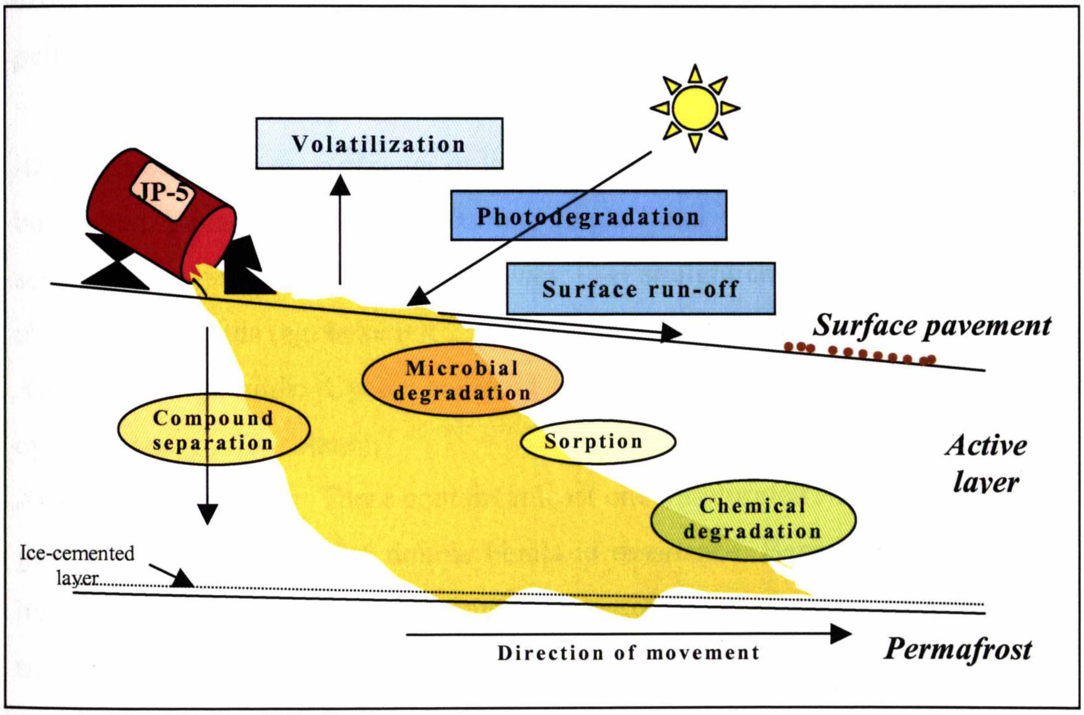
Antarctic soils, except those under penguin colonies ("ornithogenic soils"), are typically mineral soils; they have low levels of organic carbon and nitrogen (for example, 0.07% total carbon and 0.02% total nitrogen in Scott Base soil[7]) and their pH may range from weakly acidic (pH 6 in inland soil) to alkaline (pH 9 in coastal regions)[23,30].

1.3.4. Fate of spilled hydrocarbons in Antarctic soils

The behaviour of hydrocarbons in soil varies greatly depending on the quantity and the physicochemical properties of the mixture of hydrocarbons, the type of soil it impacts, temperature and weather conditions[12,28]. In Antarctic soils, (Figure 4) highly volatile hydrocarbons tend to volatilize, however short-chain alkanes become less volatile with lower temperatures[11,76]. Thus, they become more soluble in water and potentially more toxic to microorganisms. Hydrocarbons that have high affinity for the soil matrix adsorb to the soil particles, and those that are water-soluble dissolve in the vadose zone (unsaturated zone of the soil profile)[101]. In low moisture content soils, such as the Antarctic soils, adsorption onto mineral surfaces is the major mechanism limiting hydrocarbon mobility and vaporisation[16]. At the surface, photodegradation of hydrocarbon compounds and microbial biodegradation[6,73] occurs.

If quantities of the spilled hydrocarbon are large, vertical migration through the active layer occurs until it reaches the ice-cemented layer (permafrost) where hydrocarbons then spread laterally[34] (although infiltration into the permafrost has been observed in Arctic permafrost[21]). Dissolved hydrocarbons that have less affinity for soil particles migrate more rapidly than those with higher affinities; this creates a separation of compounds analogous to a laboratory chromatography[101]. Oils are more viscous and less volatile under Antarctic conditions and are likely to have reduced vertical migration[55,139]. It was reported that Jet A2 lubricating oil had no environmental mobility or degradation in contaminated soils at Bunger Hills, East Antarctica, even at the sediment surface. This has created a long-lived contamination of the terrestrial environment in this site[55].

Figure 4. Fate of hydrocarbon spills on Antarctic soil



During summertime when the active layer of the soil is thawed, further migration and biodegradation may occur. Some hydrocarbons in the surface layer may dissolve in the snowmelt and migrate laterally possibly reaching the marine environment[44,119]. There is still limited knowledge on the fate and transport mechanisms of the hydrocarbons in Antarctic soils, such as hydrocarbon penetration into the permafrost and effects of freeze-thaw processes. Even though these processes have been studied for Arctic soils[34] extrapolations from Arctic to Antarctic environments may not be valid and models specific for Antarctic conditions may be required[4].

1.4. The contaminants

1.4.1 Types of hydrocarbons

Hydrocarbons constitute one of the largest classes of petrochemicals used in the industrialized world. The hydrocarbons deposited accidentally or deliberately in the environment can vary greatly depending on the type of petrochemical spilled. Gasoline, lubricant oils, jet-fuels, kerosene and solvents have varying amounts of different types of hydrocarbons. The following can be found in such petrochemical products in different proportions[79]:

Aliphatic compounds: *n-alkanes* or linear alkanes: straight C-H chains with single bonds (eg. hexane); *branched alkanes*: single bond C-H chains that branch in several directions (eg. pristane); *n-alkenes*: C-H straight chains that contain one or more double bonds (eg. hexene);

Alicyclic compounds: Cyclic compounds that contain single C-H bonds (eg. cyclohexane, cyclopentane);

Aromatic compounds: These contain at least one unsaturated ring system with the general structure C_6H_6 and double bonds in resonance. Benzene is the "parent" hydrocarbon of the unsaturated cyclic compounds. If the molecule contains two or more fused benzenes then it is called a polyaromatic hydrocarbon (PAH). PAH can be carcinogenic to humans[79]. Examples of aromatic compounds are benzene, naphthalene and phenanthrene.

Petroleum contains a mixture of linear and branched aliphatics, unsubstituted and alkyl-substituted alicyclics, and unsubstituted and alkyl-substituted aromatic compounds. There are several hundred individual components in every crude oil, and the composition of each one varies with its origin[11]. Roughly, the gasoline fraction of crude oil is composed of 30-70% aliphatics, 20-70% alicyclics and 10-15% aromatic compounds depending on the source of crude oil[79]. JP-5 and JP-8 are composed of *ca.* 81% C_8-C_{17} *n*-alkanes and ca. 19% aromatics[153].

1.4.2 Hydrocarbon biodegradation

Certain microorganisms have the ability to degrade and mineralise hydrocarbons. The *n*-alkanes are usually considered the most readily degraded compounds in petroleum[79]. Midsize straight-chain aliphatics (*n*-alkanes C_{10} to C_{18} in length) are more readily degraded than *n*-alkanes with shorter or longer chains. However, biodegradation of *n*-alkanes with molecular weights up to C_{44} has been demonstrated[59]. Long-chain *n*-alkanes, cycloalkanes and aromatics have low water solubility, which results in low bioavailability and thus reduced degradation[88]. On the other hand, water solubility of short chain *n*-alkanes (C_5-C_9) is higher, however they are toxic to the cells. Their increased solubility increases their uptake and incorporation into the cell membrane. Given the hydrophobic nature of membranes these alkanes "dissolve" in the membrane, which can disrupt its fluidity and integrity and consequently cause cell damage[79]. However, if short chain alkanes are present in a mixture with other hydrocarbons, the toxicity may be reduced because they may have higher affinity for other components in the mixture and partition into these[79]. In temperate as well as cold climates, aerobic hydrocarbon degradation proceeds with the following order of preference[28]:

n-alkanes (C_{10}-C_{22}) > cycloalkanes (C_{10}-C_{22}) > aromatics (C_{10}-C_{22}) > branched alkanes (C_{10}-C_{22}) > *n*-alkanes, branched alkanes above C_{22} > aromatics, cycloalkanes above C_{22}

Saturated linear alkanes, cycloalkanes and aromatics in the range of C_{10} to C_{22} are most readily biodegradable because they are less toxic than shorter chain hydrocarbons, and because they have higher water solubility compared to compounds above 22 carbons. Branched alkanes and cycloalkanes with 10 to 22 carbons are less biodegradable than their *n*-alkane and aromatic analogs, because branching creates tertiary and quaternary carbon atoms that create hindrance to the enzymes involved in their degradation, blocking the reaction site and contact between enzyme and substrate; consequently, highly branched alkanes are more difficult to degrade.

Gaseous *n*-alkanes (C_1-C_4) are biodegradable but used only by specialized hydrocarbon-degraders[28]. *n*-Alkanes, cycloalkanes and aromatics in the range of C_5 to C_9 may have membrane toxicity, but they are biodegradable at low concentrations by some organisms, however, in most environments they are removed by volatilization[11].

Generally, cycloalkanes require synergistic metabolism (co-metabolism) by 2 or more bacterial species, as was observed by Perry and co-workers (1984)[94]. In this case, one organism oxidises the cycloalkane to its corresponding cyclic alcohol subsequently dehydrogenated to the cyclic ketone, but is unable to open the ring. A second organism, unable to oxidise the cycloalkane, can perform lactonization (conversion of ketone to lactone), ring opening and mineralisation of the remaining aliphatic compound.

Aromatics composed of one, two or three condensed rings are transformed relatively rapidly, whereas aromatics with 4 or more condensed rings are generally more resistant to biodegradation[28]. These PAH have very low solubility in water and adsorb strongly to particle surfaces in soil, which results in reduced bioavailability. However, a number of bacteria have been reported to degrade the PAH chrysene, fluoranthene and pyrene[36,99].

Most fuels can be expected to support microbial growth in the interphase with water, at temperature and pH ranges of –2°C to 55°C and 5.0 to 10.0, respectively[64].

1.4.3 The hydrocarbon-degraders

Hydrocarbon-degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats and are found both in contaminated and non-contaminated environments (reviewed in Rosenberg (1992)[104]). Filamentous fungi and bacteria are the main hydrocarbon-utilizing microbes in soils[12]. The reason they are ubiquitous might be that hydrocarbons are constantly produced in nature by plants and microorganisms[104]. Many species of hydrocarbon-degrading bacteria have been cultured and isolated[28,104] as shown in Table 1, but the most frequently isolated genera are *Pseudomonas, Acinetobacter, Flavobacterium, Corynebacterium* and *Arthrobacter.* Alkane and aromatic degrading bacterial genera isolated from Antarctica include *Pseudomonas*[92], *Acinetobacter*[78], *Arthrobacter*[96], *Sphingomonas*[5] and *Rhodococcus*[17]. Despite the fact that they are found in a variety of environments, it is clear that there is an enrichment of hydrocarbon-degrading bacterial populations in hydrocarbon-contaminated sites compared to non-contaminated sites[104].

Table 1. Genera of bacteria and fungi that include hydrocarbon degraders
(Adapted from Bossert *et al.* (1984)[28] and Rosenberg (1992)[104])

Bacteria		**Fungi**	
Actinomyces	*Pseudomonas*	*Candida*	*Debaryomyces*
Aeromonas	*Achromobacter*	*Rhodotorula*	*Fusarium*
Bacillus	*Arthrobacter*	*Sporobolomyces*	*Geotrichum*
Beneckea	*Micrococcus*	*Phialophora*	*Gliocladium*
Cytophaga	*Nocardia*	*Acremonium*	*Graphium*
Erwinia	*Vibrio*	*Aspergilus*	*Humicola*
Klebsiella	*Acinetobacter*	*Aureobasidium*	*Monilia*
Lactobacillus	*Brevibacterium*	*Beauveria*	*Mortierella*
Leucothrix	*Corynebacterium*	*Botrytis*	*Paecilomyces*
Moraxella	*Flavobacterium*	*Chrysosporium*	*Penicillium*
Nocardia	*Rhodococcus*	*Cladosporium*	*Phoma*
Chromobacterium	*Spirillum*	*Cochliobolus*	*Saccharomyces*
Streptomyces	*Sphaerotilus*	*Cylindrocarpon*	*Scolecobasidium*
Spirillum	*Serratia*	*Sprotrichum*	*Torulopsis*
Vibrio	*Alcaligenes*	*Spicaria*	*Trichoderma*
Xanthomonas	*Mycobacterium*	*Tolypocladium*	*Verticillium*
Sphingomonas	*Peptococcus*		
Alcanivorax	*Proteus*		
Sarcina			

1.4.3.1. Access of microbes to hydrocarbons

Hydrocarbon-degrading bacteria are defined by the production of hydrocarbon-group-specific oxygenases and by their ability to optimize contact with hydrocarbons. Two mechanisms to enhance contact with hydrocarbons have been proposed: specific adhesion mechanisms and emulsification. Some bacteria (eg. *Acinetobacter calcoaceticus)* have the ability to adhere to high molecular weight hydrocarbons (less water soluble) by producing hydrophobic fimbriae that increase their cell surface hydrophobicity[108].

Other bacteria, apart from producing fimbriae and fibrils, produce cell surface molecules such as *gramicidin* and *prodigiosin* or bacterial capsules that increase their surface hydrophobicity and thus allows adherence to hydrocarbon droplets[106,107,109]. *Acinetobacter* sp. HO1-N accumulates extracellular membrane vesicles when grown in hexadecane that partition hydrocarbons forming microemulsions. This is believed to aid in uptake by increasing surface contact[70]. A second strategy for hydrocarbon contact optimization and uptake is the production of surface-active agents or biosurfactants[45]. These are amphiphatic compounds (glycolipids) that reduce the surface tension by accumulating at the interface of a fluid with a solid, or 2 immiscible fluids (eg. hydrocarbon-water interface). It is not uncommon to find that hydrocarbon-degrading bacteria are active producers of biosurfactants in the presence of hydrocarbons[102]. Surfactants help disperse the oil, increase the surface area for uptake and help detach the bacteria from the oil droplet once the degradable compound has been depleted[105]. It has also been suggested that these emulsifiers may act favourably in the degradation of more recalcitrant components typically present in heavily weathered or aged contaminated soils[97]. The hydrocarbon-degrading Antarctic strain *Arthrobacter protophormiae* MTCC 688 has been reported to produce a biosurfactant when grown on *n*-hexadecane[96]. The hydrocarbon emulsification capacity of this biosurfactant was high (60%) when grown on this substrate, which suggests that its production is related to hydrocarbon presence, possibly aiding in its uptake. Production of biosurfactant has also been observed in the hydrocarbon-degrading Antarctic fungi *Candida antarctica*[68], Antarctic bacterial hydrocarbon-degrading strains *Acinetobacter* ADH-1 strain[78] and *Rhodococcus fascians* B11 and B15[149].

In addition, hydrocarbon-degrading bacteria may also produce extracellular polysaccharides that may interact with hydrocarbons facilitating its uptake[146].

1.4.3.2. Microbial physiological adaptations to hydrocarbons

To adapt to the presence of hydrocarbons in their environment, hydrocarbon-degrading bacteria also display changes in the composition of their membrane fatty acids when in contact with hydrocarbons, similar to those changes observed in membranes with changing temperatures. At low temperatures, bacteria reduce the viscosity of their membranes by decreasing the degree of saturation of the fatty acid chains to increase membrane fluidity[62]. They can also achieve this by changing the *cis/trans* ratio and /or increasing the amount of branched fatty acids in the membrane[63]. At high temperatures, bacteria increase the viscosity of the membrane by producing highly saturated fatty acids and *trans* rather than *cis* fatty acids. Using these mechanisms, bacteria are able to survive freezing or melting of their membranes[63]. When the bacterial cell is in contact with hydrocarbons, the physiological response is similar to that at high temperatures, i.e. increased saturation in fatty acids and increased *trans* fatty acids[62,114]. This results in less permeability to hydrocarbons, which is thought to be a protective mechanism against toxic hydrocarbons. The psychrotolerant hydrocarbon-degrading bacteria *Rhodococcus* sp. Q15 decreases its degree of membrane fatty acid saturation as a result of growth at low temperatures, but does it to a lesser degree when it is grown on hydrocarbons at the same temperature[144]. This suggests that this bacterium is able to modulate the fluidity/viscosity of its membrane to accommodate “opposing” situations.

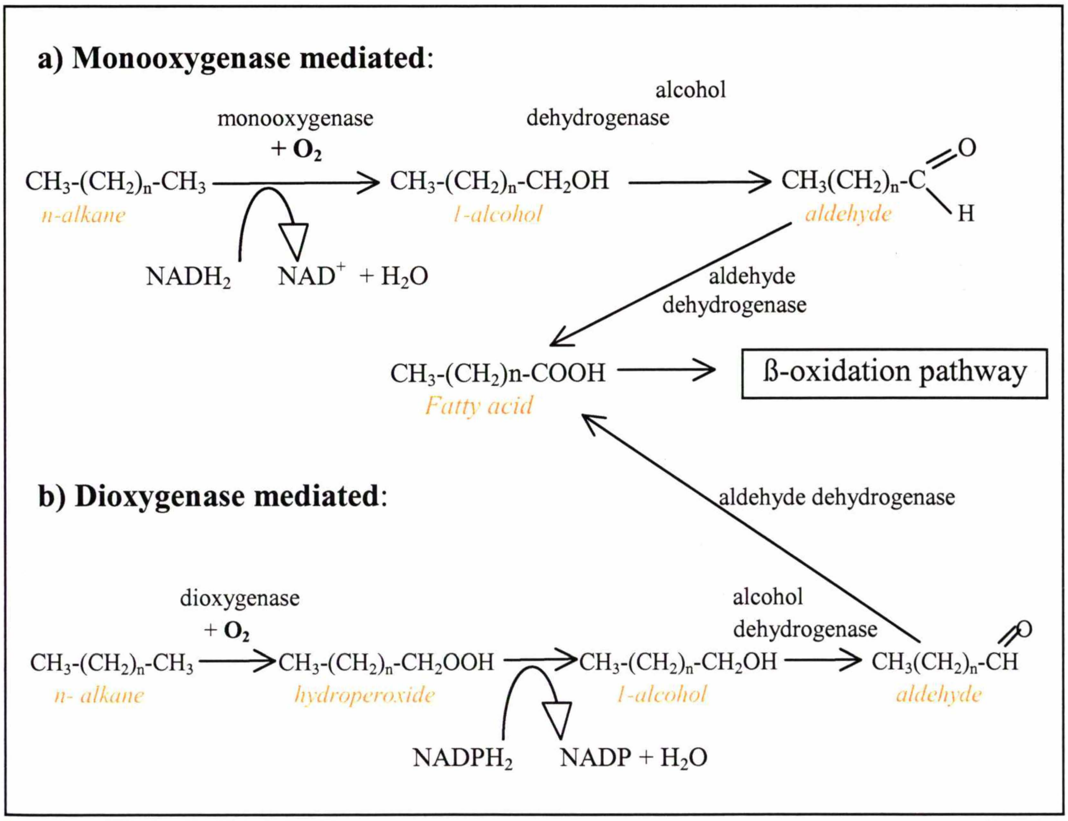
1.4.4. Metabolic pathways and genes involved in alkane biodegradation

For the purpose of this study, only the metabolic pathways of alkane degradation and the genes involved will be reviewed. The biodegradation of alkanes is a common feature to many microorganisms[79], probably because of structural similarities with fatty acids, which are ubiquitous in nature and necessary for membrane structure and function.

As mentioned before, bacteria have a preference for degradation of alkanes with chain lengths C_{10} to C_{18}. Since they have reduced solubility in water, bacteria synthesize specialized lipids (trehalolipids and rhamnolipids) that facilitate the alkanes incorporation into the cell membrane. Once in the membrane, alkanes are oxidised at the cytoplasmic side[86].

Degradation of alkanes can proceed aerobically or anaerobically[58]. The aerobic pathway has a high oxygen demand because the reaction starts with the incorporation of an oxygen molecule into the alkane by an alkane monooxygenase or dioxygenase (Figure 5). Monooxygenases incorporate one oxygen molecule in the terminal carbon to form an alcohol, while dioxygenases incorporate 2 oxygen molecules (terminal C), resulting in the formation of a hydroperoxide intermediate. With monoxygenases, the remaining oxygen molecule from O_2 is reduced to H_2O with $NADH_2$ serving as an electron donor. With dioxygenases, the hydroperoxide is reduced by $NADPH_2$ and hydroperoxide reductase to an intermediate alcohol and H_2O. The intermediate alcohol is then further oxidized to an aldehyde and a fatty acid, which eventually enters the β-oxidation catabolic pathway of fatty acids. This alkane degradation pathway is known as the ***terminal pathway***. The metabolic reactions are summarised in Figure 5.

Figure 5. Alkane oxidation terminal pathway



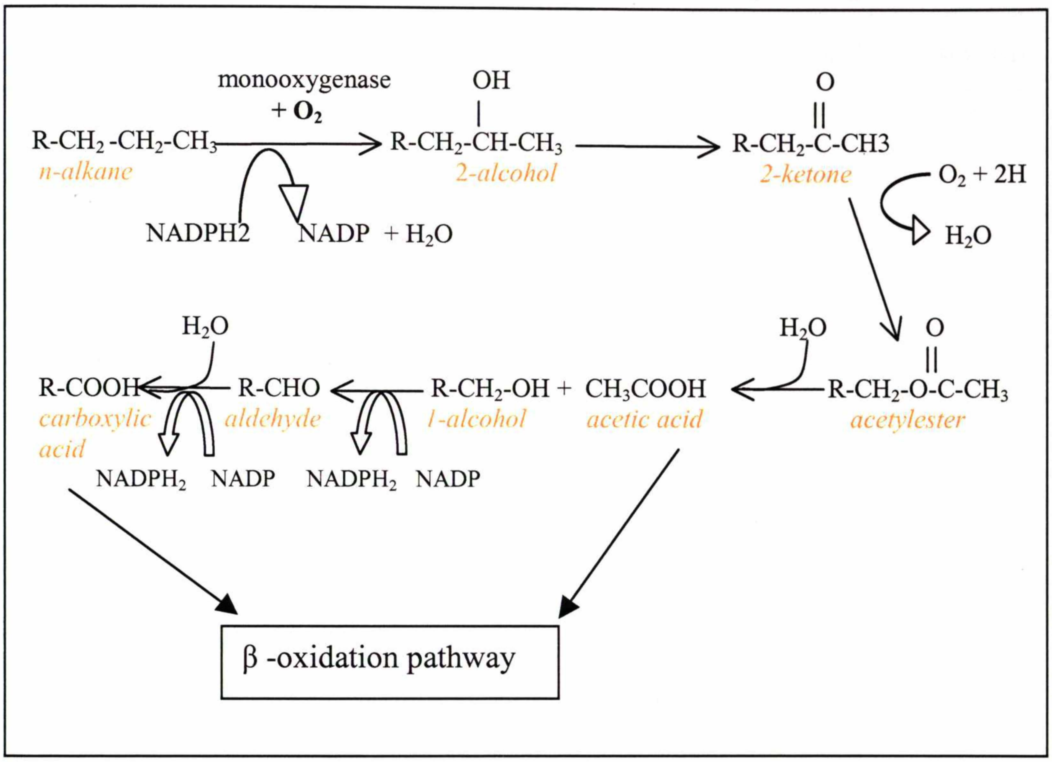
Pathway information from Atlas *et al.* (1987) and Michal (1999) [11,86]

Generally, with both the monooxygenase and dioxygenase-mediated reactions, the initial addition of oxygen to the alkane is the rate-limiting step. Once the fatty acid is formed, it is metabolized rapidly[11]. Frequently, the initial attack occurs on one terminal carbon, however, occasionally both terminal carbons are oxidized resulting in the formation of a dicarboxylic acid[12]. This pathway is known as the ***diterminal*** or ***ω-oxidation pathway***. Branched isoprenoid alkanes, such as pristane (2,6,10,14-tetramethylpentadecane) undergo diterminal oxidation as the major degradative pathway[84]. This metabolic variation bypasses a block to β-oxidation due to branching of the carbon chain[11,84]. As in the terminal pathway, a fatty acid is formed that enters the β-oxidation pathway, which proceeds under anaerobic conditions. The fatty acid is converted to its acyl coenzyme-A form, which undergoes subsequent enzymatic transformations that result in an acetyl-coenzyme A (acetyl-CoA) molecule and the fatty acid shortened by a two-carbon unit. The acetyl-CoA molecule then enters the tricarboxylic acid cycle and is converted to CO_2 and CoA.

Some microorganisms may attack subterminal carbons of alkanes within the chain rather than at its end. In this manner, a secondary alcohol is formed, which is further oxidized to a ketone and finally to an ester. The ester bond is cleaved yielding a primary alcohol and a fatty acid. The alcohol fragment is oxidized through the aldehyde to the fatty acid analogue and both fragments are metabolized further by the β-oxidation pathway. This pathway is known as the ***subterminal pathway***, and is summarized in Figure 6.

Anaerobic biodegradation of hydrocarbons has been observed for >C6 *n*-alkanes, branched alkanes, toluene, alkylbenzenes, benzene, naphthalene and phenanthrene (as reviewed in van Hamme, *et al.* (2003)[129]). The metabolic reactions for anaerobic biodegradation have been studied with more detail for toluene[1] and this has uncovered a unique biochemistry for hydrocarbon degradation[129]. In the proposed pathway[18], a benzylsuccinate synthase adds fumarate to toluene to form benzylsuccinate. Following this addition reaction, ß-oxidation reactions convert benzylsuccinate to benzyl-coenzyme A, a central intermediate in the anaerobic degradation or aromatic compounds[61]. This mechanism may also be involved in the anaerobic metabolism of xylenes, alkylnaphthalenes, *n*-hexadecane and *n*-dodecane (reviewed in van Hamme, *et al.* (2003)[129]). Microbes that degrade hydrocarbons anaerobically have been described[36] and have been grouped as *facultative anaerobes* (nitrate, iron or manganese reducers) or *strict anaerobes* (sulphate-reducers)[58]. Aerobic biodegradation proceeds faster than anaerobic[60].

Figure 6. Alkane oxidation subterminal pathway



Pathway information from Atlas *et al.* (1987) and Michal (1999) [11,86]

The first enzyme of the monooxygenase-mediated alkane degradation pathway is a 3-component complex consisting of: a) **Alkane hydroxylase**: a non-heme-iron integral membrane enzyme that oxidises alkanes in the presence of O_2; b) **Rubredoxin**: a soluble enzyme that transfers electrons from rubredoxin reductase to the alkane hydroxylase; c) **Rubredoxin reductase**: a soluble NADH-dependent flavoprotein that transfers electrons from $NADH_2$ to rubredoxin.

1.4.5. Genes involved in alkane biodegradation

The genes that encode alkane monooxygenases have been characterized for several bacterial species (Table 2). However, only the alkane degradative genes of *Pseudomonas putida* strain GPo1 (formerly *P. oleovorans*), *Acinetobacter* sp. ADP1 and *Rhodococcus* sp. Q15 have been characterized extensively.

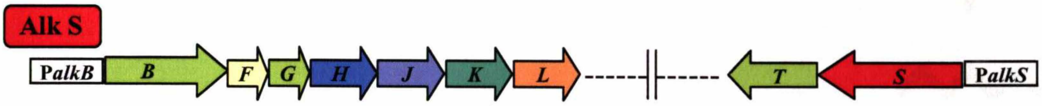
Table 2. Some monooxygenase gene homologues characterized in the literature

Strain	Gene	Gene Location	Isolated from	Reference
P. putida ATCC 29347	Pp *alk B*	OCT plasmid	Enrichment on hexane from soil	33,128
P. aeruginosa PAO1	Pp *alkB1* Pp *alkB2*	Chromosome	Infected wound	118
Acinetobacter sp. ADP1	Ac *alk M*	Chromosome	Soil	98
Rhodococcus sp. Q15	Rh *alkB1*, Rh *alkB2*, Rh *alkB3*, Rh *alkB4*	Chromosome	Sediments from Lake Ontario, Canada	145
Rhodococcus erythropolis NRRL B-16531	Rh *alkB1*, Rh *alkB2*, Rh *alkB3*, Rh *alkB4*	Chromosome	Petroleum-contaminated soil in Japan	145
Alcanivorax borkumensis AP1	*alk B1*	Chromosome	Seawater	116,149
Pseudomonas rugosa	*alk B*	Chromosome	Cow rumen	67,116
Pseudomonas fluorescens CHA0	*alk B*	Chromosome	Soil	116,121
Mycobacterium tuberculosis H37RV	*alk B*	Chromosome	Clinical isolates	116

Pp *alk B* gene encodes for an alkane hydroxylase that is involved in degradation of short chain alkanes only (C_6 to C_{12})[127]. Interestingly, the *alkB* homologous gene in *P. aeruginosa*, which is located in the chromosome rather than the plasmid, encodes for an alkane hydroxylase capable of degrading C_{12}-C_{16} alkanes. *Rhodococcus* strain Q15 and *R. erythropolis* possess each 4 homologues of *alkB* genes and are capable of degrading a vast array of alkanes (C_8 to C_{32})[145]. According to Whyte *et al.* (2002)[145], the most likely explanation for the presence of 4 alkane monooxygenases in one strain is that each one is specific for a certain range of alkanes. Several alkane-monooxygenases with different substrate range may exist in nature and may be scattered in different microbial populations, given the diverse geographic origin of the studied strains (Canada and Japan). Furthermore, Smits *et al.* (2002)[116] demonstrated that alkane hydroxylases that oxidize medium-chain alkanes cluster together in a phylogenetic tree, while the long-chain ones are highly divergent.

In *P. putida* GPo1, the genes for alkane degradation are located in the OCT plasmid, and are clustered in 2 operons separated by 10 kilobases. The gene organization has been well characterised as depicted in Figure 7.

Figure 7. OCT plasmid *alk* genes and regulatory elements



Adapted from Yuste, *et al.* (1998)[151]

P*alkB* is the promoter of the BFGHJKL operon; its expression is activated by the AlkS regulator in the presence of alkanes.

P*alkS* is the promoter for the TS operon, activated by AlkS in the presence of alkanes,

alk S encodes for the transcriptional regulator AlkS,

alk B encodes for alkane hydroxylase (Alk B),

alk F encodes for rubredoxin 1 (Alk F),

alk G encodes for rubredoxin 2 (Alk G),

alk J encodes for alcohol dehydrogenase (Alk J),

alk H encodes for aldehyde dehydrogenase (Alk H),

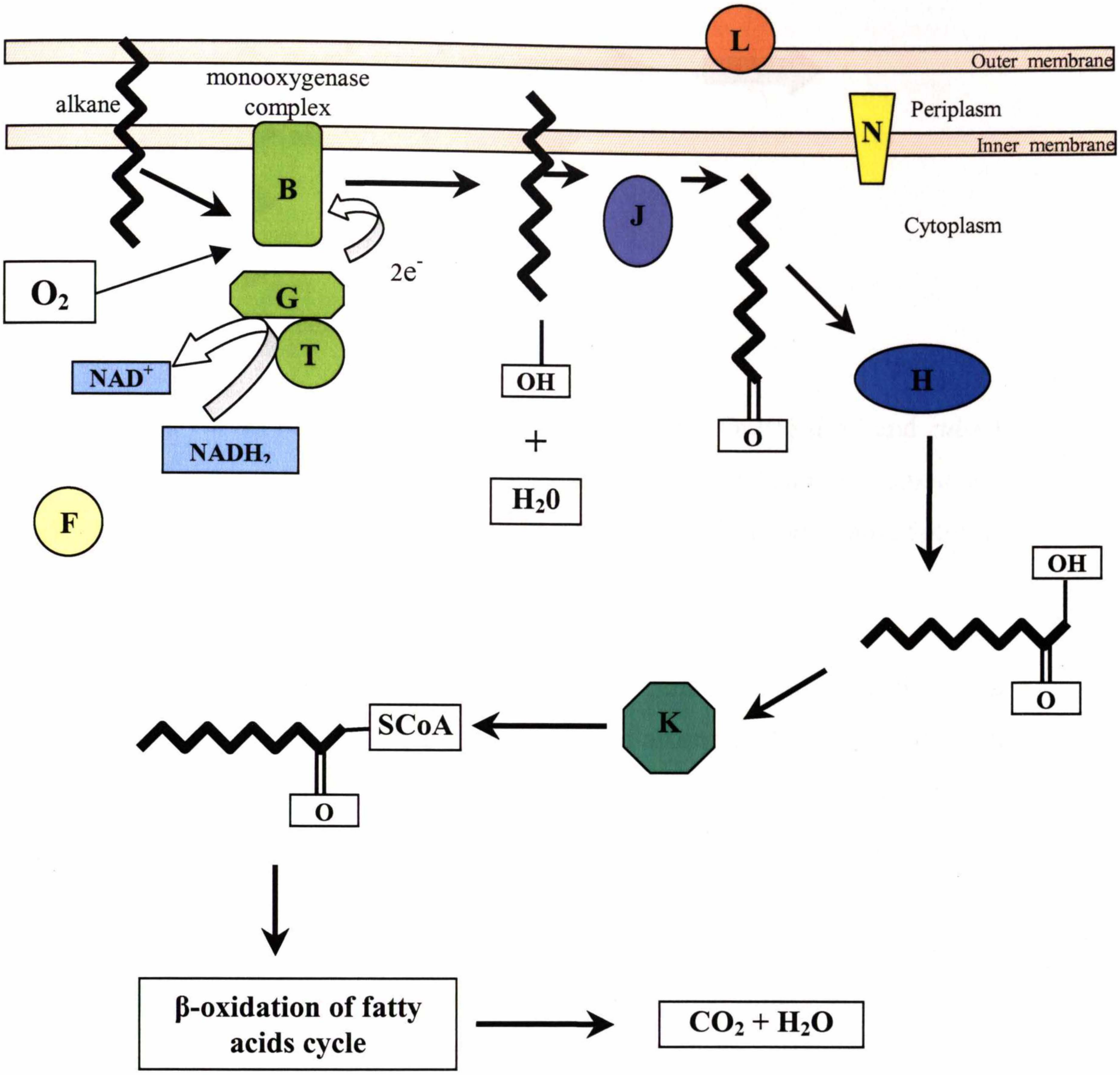
alk K encodes for acyl-CoA synthase (Alk K),

alk L encodes for an outer membrane protein of unknown function (Alk L) that is possibly involved in alkane uptake.

Alk T encodes for rubredoxin reductase (Alk T),

A model of the interaction of these proteins is presented in Figure 8 (adapted from van Beilen, *et al.* 2001[128]):

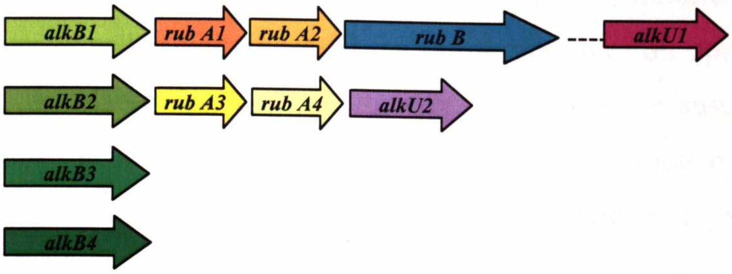
Figure 8. Model of alkane mineralisation degradation in *P. putida*



Adapted from van Beilen, *et al.* (2001)[128]

Whyte *et al.* (2002)[145] found that *Rhodococcus* species have 4 *alkB* gene homologues scattered in the genome. Two of them are in an operon-like structure and the other two exist as separate genes. *Rhodococcus* sp. Q15 can oxidize alkanes via the terminal and the subterminal pathway[142]. The gene organization of *Rhodococcus* sp Q15 and *Rhodococcus erythropolis* is given in Figure 9, as follows[145]:

Figure 9. Alkane gene organization of *Rhodococcus erythropolis* and *Rhodococcus* sp. Q15



adapted from Whyte *et al.* (2002)[145]

rubA1, rubA2, rubA3 and *rubA4* encode for rubredoxins. Only *rubA2* and *rubA4* are functional electron transferases. The function of *rubA1* and *rubA3* is unknown. *rubB* encodes a rubredoxin reductase, and *alkU1* and *alkU2* encode transcriptional regulatory proteins.

The possible explanation for the presence of 4 *alkB* gene homologues is that each alkane hydroxylase is specific for a certain range of alkanes, which would explain why *Rhodococcus* species have broader substrate range (up to C_{36} n-alkanes, branched alkanes and substituted cyclohexane) than *Pseudomonas putida*[145].

In general, the organization of alkane-degrading genes varies among the different alkane-degrading bacteria: some species possess operons, others single isolated genes; some harbour the *alk* genes in plasmids while others in the chromosome; some have multiple homologues of *alkB* genes, others only one. However, in most strains studied so far, the *alk* genes seem to be distributed over the whole genome[116] and none of the rubredoxin reductase gene, except in *Rhodococcus* sp., are located close to an alkane hydroxylase. However, the fact that homologues are widespread in alkane-degrading Gram positive and Gram negative bacteria and that they are quite divergent in their genetic structure[116] is advantageous with regards to molecular studies, because this makes it possible to design specific DNA probes for detection of alkane monooxygenases in the environment[143].

1.4.6. The need for clean-up of hydrocarbon contaminants

The presence of hydrocarbons in aquatic and terrestrial environments represents a threat to human and environmental health. For example, oil spills in land are destructive to plants by contact toxicity, but also because hydrocarbon biodegradation in soil renders root zones anoxic killing the roots of most plants[11]. Hydrocarbons bind strongly to soil surfaces which alter the physicochemical properties of the soil and consequently its ecology[76]. Additionally, the lighter and often toxic hydrocarbon components tend to volatilize into the atmosphere, reducing air quality and threatening human and animal health[28]. Therefore, ever since spillages have occurred, it has been recognised that there is a need to remove these contaminants as soon as possible after the event and to remediate the site trying to restore it to its original condition.

Hydrocarbon contamination in Antarctica is likely to be more detrimental than in more temperate places with respect to far-reaching effects, particularly for microbial and animal life, because spills commonly occur in the rare ice-free coastal regions where the majority of its wildlife exists, including unique ecosystems[51]. Additionally, given the harsh environmental conditions, it is likely that ecological recovery will be slower than elsewhere and hydrocarbons will have a long residence time[52]. Furthermore, the Protocol on Environmental Protection to the Antarctic Treaty requires that human impact on the continent is minimized and old waste disposal sites and abandoned work sites are cleaned up[95]. This includes hydrocarbon-contaminated soils. Therefore, a legal obligation exists for all the Antarctic Treaty signatory countries to clean up contaminated sites. For these reasons, remediation technologies adequate for Antarctica have to be implemented. Several oil-spill remediation strategies have been developed to recover contaminated sites. Of these, not all are applicable to the Antarctic continent because of its remoteness and the harsh environmental conditions that impose logistical constraints that may restrict the implementation of certain remediation technologies.

1.5. Remediation strategies

There are two main approaches to remediate hydrocarbon-contaminated soils: physicochemical strategies and biological strategies, the latter also called bioremediation[129].

1.5.1. Physicochemical strategies

Excavation of the contaminated site and disposal in landfills has been historically the first and most obvious strategy. However, it is often not the most successful or efficient one, because it does not truly remediate the site and just transfers the contamination to another site. **Incineration** of the contaminated soil has been another approach, however, it is not very well accepted due to the great deal of toxic gases it may release and atmospheric contamination that this may cause[79]. A frequent remediation tool developed in the late 1970s and 1980s is **vacuum-enhanced vapour extraction** or soil venting. In this method, a series of wells are constructed around the contaminated zone. To initiate venting, a vacuum is drawn on these wells to force accelerated air movement through the contaminated zone. Some of the pollutants will be absorbed into the wells as air is forced into the system. The disadvantage of this system is that it does not extract the higher molecular weight compounds or lower vapour-pressure hydrocarbons. Another important disadvantage of this system is that it transfers the hydrocarbons from soil to the atmosphere. However, recently this technique was combined with a bioremediation strategy called *biofiltration* where the pumped air is transferred to aboveground soil beds that were activated with microorganisms capable of degrading hydrocarbons[69]. For contaminated water table systems, the initial alternative was groundwater extraction and treatment by filtration and discharge to waste water treatment facilities. The remediation of the vadose zone (the water saturated zone of the soil profile) point contamination became a priority because pollutants remaining in this site successively re-contaminated groundwater systems[101].

***In situ* chemical oxidation** is another technology that can be applied to hydrocarbon-contaminated sites. It involves introducing reactive chemicals

(Fenton's reagent, hydrogen peroxide, hypochlorite) directly into the contaminated soil to oxidise organic contaminants to carbon dioxide and water or to other environmentally harmless compounds[52]. This technology has been successful (for example, Yeh *et al.* (2003)[150] achieved 97% degradation of trichloroethylene in contaminated aquifer sand and Watts *et al.* (2000)[138] observed 95% oxidation of benzene using hydrogen peroxide). However, in some soils, the oxidising reagents may interact with the organic matter rather than the contaminants[152], and some safety and environmental issues may arise with its application (chemical handling, production of volatile organic compounds, toxic by-products, increase of mobility of degraded products)[52]. This technology was tested in Antarctic contaminated soils without; additionally, destruction of the subsurface microbiota was observed, therefore it was not recommended for Antarctic soils[52].

In general, physical methods for decontamination of the environment are very expensive. Over 1 million US dollars a day were spent on physical attempts to clean up pf the oiled shoreline of Prince William Sound, Alaska, following the Exxon Valdez tanker wreckage. Bioremediation technologies may be inexpensive compared to physical methods and therefore the preferred technologies[9]. Because of the remoteness of Antarctica and the logistical problems that physical remediation techniques imply, bioremediation technology may be more adequate for Antarctic contaminated sites[6].

1.5.2. Biological strategies: Bioremediation

Bioremediation has been considered for many years a viable means of hydrocarbon spill remediation[9]. Essentially, bioremediation is a process that utilizes microorganisms to transform toxic chemical contaminating compounds to a less toxic or innocuous form or mineralise them to inorganic species[10]. There are two types of bioremediation strategies: ***in situ***, which are carried out on the site of contamination without removal and ***ex situ*** bioremediation, which involves the removal and transportation of contaminated material to a different location where it is treated biologically.

1.5.2.1. *Ex situ* bioremediation

Ex situ methods include the following:

1.5.2.1.1. Landfarming: Oily wastes from refineries or excavated contaminated soil are dispersed deliberately on soils previously selected to be used as "biological incinerators", which are spread over an impermeable liner. The chosen sites need to undergo preparation to assure that floods, runoff and leaching will not spread the hydrocarbon contamination in an uncontrolled manner[12]. Time and rates of dispersal are controlled as well. This method relies entirely on the hydrocarbon degradation properties of microbial communities in the chosen soil. Commonly, pH is adjusted, fertilisers are applied and ploughing and tilling are periodically performed. It is a method that is in current use[12].

1.5.2.1.2. Biopiling: Contaminated soil is accumulated in piles in confined sites over lined treatment beds where nutrients and water are added, and temperature may be controlled.

1.5.2.1.3. Bioreactor remediation: Contaminated soils are transferred to bioreactors where nutrient and oxygen concentrations and moisture can be controlled to enhance intrinsic biodegradation.

The advantages of *ex situ* approaches are the degree of control achieved over the processes as compared to *in situ* methods. On the other hand, *ex situ* methods can be costly and disruptive of the original ecosystem because they imply excavation of the contaminated soil[101]. For this reason, ex-situ bioremediation methods may not necessarily be the best option for Antarctic contaminated soils.

1.5.2.2. *In situ* bioremediation

In situ technologies are advantageous in that there is no need to remove the impacted soil or water and thus costs may be reduced. However, it is challenging to find the best method for delivering the amendment components usually needed for these methods (oxygen, water and nutrients). *In situ* bioremediation strategies may include one or more of the following:

1.5.2.2.1. Air sparging: In this method, an air sparger well is used to inject air under pressure *below the water table* at a contaminated site. The injected air displaces water in the soil matrix creating a temporary air-filled porosity which causes oxygen levels to increase in the vadose zone[79]. Those molecules that are volatilized into the air stream are generally removed by a vapour extraction well incorporated to the system. This method, as well as bioventing, is based on the fact that by increasing oxygen concentration in this zone, native microbial aerobic hydrocarbon degradation will be enhanced. Addition of oxygen is important because aerobic degradation of hydrocarbons requires less energy of activation and yields more energy than anaerobic degradation[101]. It is the preferred pathway by the microbes and thus it is advantageous to enhance it.

1.5.2.2.2. Bioventing: This method is similar to air sparging in that air is injected into the contaminated system. However, in this case, it is pumped directly *into the vadose zone* below the contaminated soil. Bioventing is a fusion of soil vapour extraction technology and bioremediation[79]; wells are constructed around the contaminated site and a vacuum created through these wells that forces air movement in the zone. This creates an increase of oxygen in the site and thus an increase in biodegradation rates. As with air sparging methods, volatile compounds are removed by an extraction well that subsequently is passed to biofiltration beds.

1.5.2.2.3. Intrinsic bioremediation: This method is also known as "natural attenuation". It refers to the indigenous level of contaminant biodegradation that occurs without any stimulation or treatment. It constitutes the least expensive method and takes advantage of the intrinsic abilities of the soil microbes to degrade contaminants. It is also referred to as the "do nothing" approach.

1.5.2.2.4. Bioaugmentation: Refers to the introduction into polluted soils of microorganisms able to degrade contaminants if either these microorganisms are not present in the soil or if microbial populations have been reduced because of toxicity. The introduction of microbes may fail because of the inability to establish a niche in the environment, poor survival or because there are difficulties in the delivery systems of the introduced organisms. Microorganisms, like the contaminants, adsorb to the soil surfaces[79] and thus migration to the contaminated zone might be difficult. The addition into the soil of "naked" DNA elements or microbial-mediated genetic elements that bear degradative genes is also possible and has been suggested[124]. Soil microorganisms may incorporate these elements, by transformation, and consequently acquire degradation ability. However, this approach may be more controversial in some countries, because it implies the release of genetically modified organisms directly into the environment, which at present, is not publicly accepted or may be legally forbidden in some countries.

Bioaugmentation of Antarctic contaminated soils in laboratory experiments has been recently successful[111]. The hydrocarbon-degrading isolate *Acinetobacter* sp. B-2-2 was used as inoculum for hydrocarbon-contaminated soil. A reduction of 75% of the hydrocarbon after 50 days was observed as a result of bioaugmentation.

1.5.2.2.5. Nutrient addition (biostimulation): Organic wastes and hydrocarbons are rich in carbon but low in nitrogen (N) and phosphorus (P). For this reason the ratio of C:N:P in a contaminated soil is dramatically unbalanced after a spillage event[80]. In order for biodegradation to proceed, N and P may have to be added to counterbalance the excess C. Thus, the goal of nutrient injection into contaminated systems has been to optimize the ratio of C:N:P to allow biodegradation by the native microbial populations.

1.5.2.2.6. Addition of surfactants: Surfactants are molecules that act similarly to detergents; they remove the oily residues or increase their solubility. Contaminant hydrocarbon molecules can be "solubilised" inside surfactant micelles or coat them and emulsify them into solution. This allows increased bioavailability of the contaminant to indigenous microbial populations that are able to degrade it[87].

In general, after a hydrocarbon spillage occurs, ideally the spill is contained and hydrocarbons physically removed as much as possible. Afterwards, other remediation strategies are used. Bioremediation technologies are the methods of choice, because of its low cost when successful, good public acceptance and support, and its less disruptive nature because they do not necessarily imply excavation. However, the success of bioremediation strategies depends on certain key factors: soil type, presence of indigenous microorganisms, environmental conditions, nutrient availability and type and quantity of the pollutants. In a given environment, hydrocarbons can persist for a long period, while under another set of conditions, they can be completely biodegraded in relatively few days[12]. For this reason, it is necessary to consider the limiting factors for bioremediation for a given ecosystem where a spillage of hydrocarbons has occurred.

1.5.3. Limitations to bioremediation of soils

Hydrocarbon degradation in soils is influenced by a number of factors, such as the microorganisms involved, nutrients and oxygen availability, pH, water content, soil type, temperature, and the quality, quantity and bioavailability of contaminants. The pollution history of the site is important, too, because the composition of the contaminant changes with time ("weathering")[80]. Therefore, if natural biodegradation does not occur, or is negligible in a polluted site, or rates are not fast enough, the first thing to be done is to determine the factors limiting biodegradation. Firstly, laboratory tests are used to determine if degrading microbes exist in the contaminated soil. Secondly, environmental factors that may be affecting biodegradation should be identified, for example, low or high pH, lack or low availability of nutrients (N and P), and/or temperatures. The quality and quantity of the contaminant itself may limit its biodegradation because, firstly, pollutants are often mixtures, and one component within the mixture can be toxic to growth and activity of the microbes[64], eg. organic lead[6], and secondly, some pollutants may be toxic at high concentration to the microorganisms that can biodegrade them when present at lower concentrations.

Environmental factors may limit biodegradation as follows:

1.5.3.1. Temperature affects biodegradation in two ways. First, it determines the physical nature and chemical composition of the oil mixture. At higher

temperatures, volatilisation of lower molecular weight alkanes is enhanced and viscosity is reduced. Reduced viscosity increases the surface area available for microbial degradation. However, at temperatures above 30°C, the toxicity of hydrocarbons increases and in general biodegradation slows down[11]. At low temperatures, volatilisation is reduced and viscosity increases. Also, short-chain alkanes are not evaporated and can be toxic to some microorganisms, because they are soluble in the cell membrane and may cause its disruption[11]. Increased viscosity and solidification of compounds reduces bioaccessibility to microbes.
Second, the rates of hydrocarbon metabolism may be reduced at lower temperatures resulting from decreased rates of enzymatic activity[63]. Nonetheless, hydrocarbon-degrading microorganisms occur over a wide range of temperatures and environments, for example, psychrophilic (0°C to 20°C), mesophilic (10°C-45°C) and thermophilic (45°C-70°C) hydrocarbon-degrading microorganisms have been isolated (reviewed in Atlas and Bartha (1992)[12]). Hydrocarbon biodegradation at low temperatures has been documented for Arctic soils, Alaskan aquifer sediments and groundwater, Antarctic soils, seawater and sea ice, and alpine soils and are reviewed in Whyte *et al.* (2002)[143].

1.5.3.2. Oxygen concentration: Oxygen molecules are a key component in the first metabolic reactions of degradation of hydrocarbons. Therefore, availability of oxygen in soil can limit biodegradation. Anaerobic hydrocarbon degradation is possible, but at slower rates. Hambrick (1980)[60] demonstrated that the environmental significance of anaerobic hydrocarbon biodegradation is very low as compared to anaerobic biodegradation. For this reason, several bioremediation techniques stimulate aerobic degradation by the injection of air into the subsurface.

1.5.3.3. Nutrient content: The introduction of large quantities of carbon in soil ecosystems generates an imbalance in the ratios of nutrients and tends to result in a rapid depletion of the available pools of inorganic nutrients like nitrogen and phosphorus[90]. Nitrogen is required for amino acid and protein synthesis, and phosphorus, in general, is involved in energy transport as adenosine-triphosphate (ATP) and NADPH molecules. Several laboratory studies have been conducted where N and P are supplemented to achieve enhanced biodegradation of hydrocarbons and the beneficial effects of fertiliser addition have been demonstrated several times, as reviewed by Atlas and Bartha[11]. In these studies, the questions of how much and which source of N and P should be used is prevalent[135]. The need for the addition of nutrients is not only based on the demand by the organisms, but also on the nutrient levels already present on the ecosystem[134]. This is to be considered firstly in determining how much fertiliser to add.

1.5.3.3.1. Nitrogen

Inorganic nitrogen in soil consists of ammonium ion (NH_4^+) and nitrate ion (NO_3^-) species, either of which may be used as a nitrogen source by most organisms[56]. NH_4^+ is the preferred source given that it is in the reduced form that is biochemically required, while NO_3^- has to be reduced prior to incorporation into metabolic reactions for production of amino acids and nitrogen compounds. Therefore, it is common to find in the literature that fertilisers used for bioremediation studies are generally NH_4^+ based. However, it should be taken into account that these types of fertilisers can reduce the pH of the soil.

Historically, the C:N ratio has been used to determine the nitrogen requirements of a given soil in relation to the amount of available carbon substrate. It has been observed that soil bacteria contain 5:1 C:N ratio in their biomass and that they degrade only 5-10% of organic C present in the soil[132]. This implies that a soil C:N ratio required for total organic C degradation would have to range between 100:1 to 50:1[135] (calculated by dividing the C:N ratio of the biomass by the degradation efficiency)[135]. Further compositional analysis of microbial biomass indicated that a ratio of 106:16:1 C:N:P is prevalent in microorganisms[100]. This "Redfield ratio" has been used as a basis to calculate the amount of nitrogen (N)-containing fertiliser required for bioremediation treatments[50]. C:N ratios have been commonly used despite the fact that these theoretical calculations are based on carbon conversion to biomass and ignore the mineralised carbon converted to CO_2 and lost from the system, which is the basis of hydrocarbon mineralisation experiments. Perhaps this is the reason why there is no consensus in the literature on the optimum C:N or C:N:P ratio to be used for bioremediation, and why there are a wide range of values found in the literature[135].

In soils that have very low nitrogen and water content, as is the case of Antarctic mineral soils, Walworth *et al.* (1997)[135] suggest that rather than using C:N ratios to calculate maximal N application levels in contaminated soils, an appropriate method would be to relate N level to soil water. This is based on the fact that N-fertilisers commonly used are very soluble salts and, as such, they reside largely in the soil solution. This increases the osmotic potential (water potential) of the soil solution and thus potentially generates an osmotic stress to the soil microbial populations. Highly contaminated soils would need a high amount of fertiliser to adjust to traditional C:N ratios. Decrease of microbial activity caused by high levels of N amendments may be related to effects of soluble nitrogen on soil water potential[135]. In their work, Walworth and co-workers (1997)[135] expressed and calculated N as a fraction of the mass of soil water (mg N/ kg-H_2O-soil), determined by dividing soil mass based-N concentration by soil water content:

$$\mathrm{mg\,N}/\mathrm{kg\,H_2O} = \left(\mathrm{mg\,N}/\mathrm{kg\,soil}\right) \times \left(\mathrm{kg\,soil}/\mathrm{kg\,H_2O}\right)$$

In testing different amounts of added N and water contents in a factorial-design experiment for hydrocarbon biodegradation, Walworth and co-workers (1997)[135] found that the maximal activity in the range of soils they used (sand, loamy, silt loam) and conditions tested was obtained when N_{H2O} levels were approximately 2500 mg N/ kg H_2O soil. They also demonstrated that total water potential is very closely related to N level when N is expressed as a function of soil water rather than as a function of soil dry mass (mg N/ kg soil). In their study, they concluded that:

1) C:N ratios may not be a good basis to calculate fertiliser quantities,

2) When N is expressed and calculated on a moisture basis (mg N/ kg H_2O) osmotic and nutritional aspects of N fertilization are taken into account,

3) Soil moisture and nitrogen levels are fundamentally linked, and

4) Coarse-textured soils are more sensitive to overfertilisation than fine textured soils.

Additionally, Ferguson *et al.* (2003)[50] in similar experiments concluded that amendments of nitrogen within the range of 508-1570 mg N/ kg H_2O (which depart greatly from ideal "Redfield" C:N ratios, *eg.* 64:1) yielded a 10-fold increase in mineralisation of hydrocarbons compared to unamended controls, which had 127:1 C:N ratio. In this study, the authors confirmed that calculating amounts of N added as a function of water content was ecologically and perhaps metabolically more relevant than using C:N ratios.

1.5.3.3.2 Phosphorus

In crop plant systems there is a synergistic interaction between N and P[134]. Increasing rates of N fertilization increase P uptake and vice versa. In the case of soil microbial communities, microbial oxidation of NH_4^+ acidifies the soil and may result in decreased P solubility due to formation of aluminium-phosphorus complexes in some soils. Due to these interactions a P optimum is normally hard to determine. Typically, commercial fertilisers that mix nitrogen and phosphorus sources have been used in nutrient amendment bioremediation treatments. Mohn *et al.* (2000)[89] found that by adding P (30 to 210 μg P/ g dw soil) to contaminated soil from the Arctic tundra, the mineralisation of dodecane reached *ca.* 30% after 180 days compared to unamended controls. Walworth *et al.* (1995)[134] found maximum petroleum degradation in diesel-contaminated Arctic soils that were treated with N and P adjusted to ratios of C:P in the range of 38:1 to 33:1.

It is unlikely that phosphorus changes the water potential of the soil solution and creates an osmotic stress for microbial populations because it is sparingly soluble in soil water systems. It is much more difficult to overfertilise soils with phosphorus[50]. For this reason, P is not calculated or reported on a soil water content basis as nitrogen in the literature, but it is calculated using the conventional C:N:P ratios like the Redfield ratio at 16:1 N:P .

1.5.3.4. pH: In general terms, the rate of hydrocarbon degradation in soils is often higher in alkaline conditions than in acidic conditions[79]. Acidic conditions favour growth of fungi, which also degrade hydrocarbons but usually at slower rates than soil bacteria. Dibble and Bartha (1979)[46] showed that raising the original pH of soil from 6 to 7.8 increased the rate of hydrocarbon degradation. Hambrick *et al.* (1980)[60] observed that if pH was reduced to 5.0 and 6.5 from 6.5 and 8.0 values respectively, microbial hydrocarbon degradation exhibited slower rates. Walworth *et al.* (2003)[136] observed that hydrocarbon degradation was greater at pH 6.5 and 7.5 rather than at 5.5 in N-amendment mineralisation studies. On the other hand, Wynn-Williams (1985)[148] concluded that there is no correlation between microbial numbers and pH of sub-maritime Antarctic contaminated soils. Furthermore, Aislabie (unpublished) observed no effect on mineralisation of ^{14}C-hexadecane in hydrocarbon mineralisation studies resulting from reducing pH to 7.4 from 9.4, and mineralisation occurred at a relatively high values pH 9.4. One would expect that microorganisms are adapted to the pH conditions prevalent in the soils where they exist and thus would be expected to be metabolically active at this pH. However, it is important to determine if changes in pH as a result of hydrocarbon contamination affect activity of indigenous microbial communities, to ensure that this factor will not hinder success of bioremediation programmes.

1.5.3.5. Water content: Optimal conditions of water content for soil microorganisms range, between 38% and 81% moisture content[79]. Within this range water and oxygen availability are maximized. Moisture is essential for microbial activity, however too much water interferes with the availability of oxygen. At higher water contents, the oxygen diffusion is reduced and at lower water contents water availability becomes limiting[79]. Water content is also related to bulk density of the soil (mass of soil per unit volume or g/cm^3), so the wide range of optimum water activity is a reflection on the variable mass densities of the soils and its interaction with water. Hydrocarbons present in contaminated soils may change the water-holding capacity of the soil[28], consequently changing water availability for microbes in the soil. By rendering some surfaces hydrophobic, water holding capacity of the soil may be reduced and thus increase the availability of water

Polar desert soils are characterized by having extremely low humidity and precipitation, and water retention in the soil is limited by poor water holding capacities[29]. Therefore, water content in Antarctic soils is likely to be a limiting factor for microbial biodegradation.

1.5.3.6. Soil type

Walworth *et al.* (1997)[135] demonstrated that differences in soil types are an important factor that influence hydrocarbon biodegradation. After testing nitrogen amendments in soils with different textures (sand, loamy sand and silt loam), maximum biodegradation (100%) was reached at different nitrogen levels of 22, 259 and 399 mg N/kg soil, respectively. This indicated that differences in soil texture determined the microbial response to nitrogen amendments. Additionally, sand and loamy sand soils were much more sensitive to overfertilisation than silt loam due to differences in moisture content. Because water holding capacity of the soil varies with different soil textures, and hydrocarbons interact differently with different soil matrices, nutrient concentrations in the soil, and oxygen availability change too. This may lead to differences in the biodegradation capacity of the soils.

1.5.3.7. Bioavailability of the contaminant

Biodegradation of hydrocarbons may be affected by hydrocarbons structure and contamination time due to weathering processed that decrease bioavailability of hydrocarbons. Weathering enhances the sorption of hydrophobic organic contaminants to the soil decreasing the rate and extent of biodegradation[27]. Weathered oils contain a recalcitrant fraction of compounds of high molecular weight ($>C_{25}$) that are not bioavailable[125] due to its structural complexity[57].

The chemical composition of different crude oils and refined products influences bioavailability of the compounds and thus the rate of its biodegradation Walker *et al.* (1976)[133] tested the biodegradation of two crude oils and two fuels, and found increased degradability, by a mixed culture of estuarine bacteria, of a crude oil richer in saturated hydrocarbons and low sulphur content. Delille (2000, 2004)[41,42] has observed that degradation of crude oil is reduced compared to diesel fuel under the same conditions of biostimulation treatments. In general, *n*-alkanes are more readily bioavailable than more complex hydrocarbons (branched, cycloalkanes, aromatics) so fuels with high contents of lighter chain hydrocarbons would be degraded preferentially in the environment than more complex oils[11]. Physical and biological weathering of petroleum compounds tend to decrease in time the bioavailability of its components as weathering results in the accumulation of recalcitrant compounds[53].

1.6. Bioremediation in Antarctica

All the signatory countries to the Antarctic Treaty have the responsibility to manage and dispose of Antarctic station's wastes in a proper manner, to minimize environmental impacts of their activities and to clean up sites that have been contaminated. In accordance with the Environmental Protocol[95], contaminated sites are required to be cleaned-up to avoid adverse impacts in the Antarctic fragile ecosystems. Therefore, there is a need to solve the contamination problem to comply with Antarctic Treaty regulations.

For any bioremediation program to be successful, it is necessary to understand the particular conditions that characterise the system to be analysed and processed. This includes the abiotic limiting factors mentioned earlier (nutrient content, temperature, pH, etc), the characteristics and properties of the impacted soils, the potential of its indigenous microbial communities to degrade hydrocarbons and the nature and concentration of contaminant present in the soil[80]. The fact that psychrotolerant hydrocarbon-degrading bacteria have been isolated repeatedly from Antarctic contaminated soils[2,5,17,73,78,92] fulfils the first and most important requisite for bioremediation: the presence of microorganisms able to degrade the contaminant. Furthermore, given that the Antarctic Treaty's Environmental Protection Protocol forbids the introduction of foreign microorganisms[95], any bioremediation program implemented in the continent would have to make use of the indigenous microorganisms, particularly when using bioaugmentation strategies.

The most limiting factors to bioremediation of Antarctic oil-contaminated soils are the low nutrient content of the soil, prevalent low temperature, low moisture and thaw-freeze cycles[51,81]. However, oil biodegradation may not be hindered by temperature during the summer season, as psychrotolerant hydrocarbon-degrading bacteria may be active in the thawed and relatively warmer soils of the summer[50]. The temperature factor may set a seasonal time frame to biodegradation processes and perhaps to the rates of degradation but does not halt the process at all as demonstrated by mineralisation experiments conducted at 0°C and 7°C[49] and at 5°C [26]. Therefore, the most limiting factors for *in situ* biodegradation in summer may be low nutrient levels, low moisture contents and concentration and types of contaminant in the impacted soil.

To overcome limitations for biodegradation it was recommended to start with treatability studies that allow the identification of particularly limiting factors. Treatability studies determine the potential for biodegradation of a contaminant in a particular site given its particular conditions[89].

Treatability analyses of fuel-contaminated soils from Antarctica are necessary to propose a sound and viable bioremediation programme that suits the conditions of the site. It is also important to consider that there might be logistical limitations to certain bioremediation methodologies due to the remoteness of the continent or the contaminated sites. For this reason, simpler bioremediation strategies would be ideal.

Several *ex situ* biotreatability studies where different nutrient contents, temperature and water content were tested have been performed for fuel-contaminated sites from Old Casey Station[50,51], for contaminated soils of Jubany Station in King George Island, Antarctica[111], and a preliminary study with N-amendment to contaminated soils from Scott Base, Antarctica[6]. *In situ* experimental fuel spill trials in the French Dependency of Terre Adelie, Antarctica have been performed that tested bioremediation potential after several nutrient amendment treatments and temperatures[40,41,43]. Kerry (1993)[73] performed *in situ* biotreatability studies of artificially oil-contaminated soils from Davis Station testing N, P and potassium (K) amendments. However, to date, no major *in situ* remediation institutional programme has been established in any of the stations on the continent.

1.7. Thesis Hypothesis and Objectives

There is a need for more information on biotreatability of contaminated soils in the Ross Sea Region that may contribute to development of guidelines for bioremediation programmes in the zone. This Master of Science thesis research had the general hypothesis that manipulation of the limiting factors to bioremediation can enhance biodegradation of the contaminants in Antarctic soils. The following general objectives were formulated to address the hypothesis:

1. To confirm the presence of alkane-degrading cold-adapted bacteria in contaminated soil samples from Scott Base, Marble Point, and the former Vanda Station and Bull Pass in the Wright Valley;

2. To determine the biodegradation activity in oil-contaminated soil samples under laboratory conditions;

3. To enhance biodegradation in oil-contaminated soils samples using biostimulation (nutrient & water amendments) and bioaugmentation.

Chapter 2. Materials and Methods

2.1. Study soils

Hydrocarbon contaminated soils from Scott Base, Marble Point, Bull Pass and the former Vanda Station, Antarctica were collected aseptically using a sterile trowel, then frozen and transported to New Zealand as described in Balks, *et al.* (2002)[14]. Characteristics of the soils collected, spillage and site description are given in Table 3. Figure 12 shows a map of the sampling sites.

2.1.1. Scott Base soil samples

Two different soil samples were collected from Scott Base. The first sample was soil taken from underneath a former fuel and lubricating oil drum storage tank area, collected in December 1999 (Figure 10). The content of hydrocarbons in the soil is a mixture ranging from C_{10} to C_{14} *n*-alkanes and PAH[3]. The contamination event occurred intermittently over a period of possibly 30 years. For this reason, in this work, this sample has been labelled as "Scott Base old spill site" or SBO. The SBO soil sample used for the laboratory trials in this thesis research constituted a homogenous mixture of soil from surface to 10 cm deep.

The second Scott Base sample was soil taken from underneath a JP-5 fuel storage tank, collected in January 2002 by Dr. Jackie Aislabie. JP-5 fuel was spilt during refuelling activities sometime during 2001. In this thesis research, this sample was referred to as "Scott Base recent spill site" or SBR and is composed of a homogenous mixture of soil from surface to 15 centimetres (cm) deep. Control uncontaminated soils were collected from a flat section on a hill approximately 200 metres (m) North of Scott Base and soil was taken from 2.5-10 cm depth[66].

2.1.2.Bull Pass soil sample

The soil sample from the Bull Pass site was collected in January 1999[14] from a site where drilling activities were carried out in 1985. As a consequence of these activities, DFA was spilt in the soil. The contaminated surface was removed, therefore there was no visible staining of the soil, but contaminated soil was found in the subsurface 10 m downhill from the bore-hole site. It is likely that the fuel moved from the site of contamination to the collection site along the interface between the soil and the underlying bedrock[14]. Soil from Bull Pass used in this thesis research was a homogenous mixture from surface to 60 cm deep. Control uncontaminated soil was collected in the same way as contaminated soil within 400 m of the contaminated site[14] on the same date.

Figure 10. Oil-contaminated soil from underneath fuel tanks, Scott Base

(Photo courtesy of Dr. M. Balks)

2.1.3. Marble Point soil samples

The samples from Marble Point contaminated soil were collected from a pit from two different depths: 0-3 cm and 3-12 cm (Figure 11). In this thesis research, "Marble Point 1" refers to a sample from 0-3 cm depth and "Marble Point 2" to a sample from 3-12 cm depth. These soils were collected in January 1999 from a contaminated site near the old Marble Point camp, which was inhabited from 1957 to about 1963[13]. It was assumed that the fuel spills occurred intermittently in these 6 years as consequence of movement and distribution and, therefore, hydrocarbons were there possibly for over 40 years. The hydrocarbons present in contaminated soils include C_{13}-C_{40} *n*-alkanes, PAH and hopanes, which is indicative of hydraulic and lubricating oils[14]. Control, uncontaminated soils were collected also in 1999 from a pit from 0-3 cm and 3-15 cm deep within 400 m of contaminated soil[14].

Figure 11. Contaminated soil from Marble Point

(Photo courtesy of Dr. M. Balks)

2.1.4. Vanda Station soil samples

Samples from the former Vanda Station site were collected from surface contaminated soil 0 to 2 cm deep. The soil was collected in January 1999 from a contaminated site near to the visible oil stains. Contaminants were possibly lube oil. Uncontaminated, control soil was collected at the same time from the surface (0-2 cm) layer within 400 m of contaminated soil.

Subsequent chemical analysis of all of the control uncontaminated soils (presented in section 3.1) confirmed that these were true controls with <30 mg /kg dry weight of petroleum hydrocarbons.

2.2. Soil analysis

Moisture content, pH, total nitrogen, carbon, and phosphorus, Olsen (available) phosphorus, content of NH_4-N and NO_3-N were performed by the Environmental Chemistry Laboratory from Landcare Research Ltd., Palmerston North, New Zealand, using standard methods[22] given in Appendix 3. Total petroleum hydrocarbon (TPH) analyses were performed by R. J. Hill Laboratories Ltd., Hamilton, New Zealand by extracting the samples in methylene chloride and then analysing the extracts by capillary gas chromatography with a flame ionisation detector as outlined in EPA Method 8015[126].

Figure 12. Sampling sites in the Ross Sea Region, Antarctica
(Map from Balks *et al.* (2002)[14])

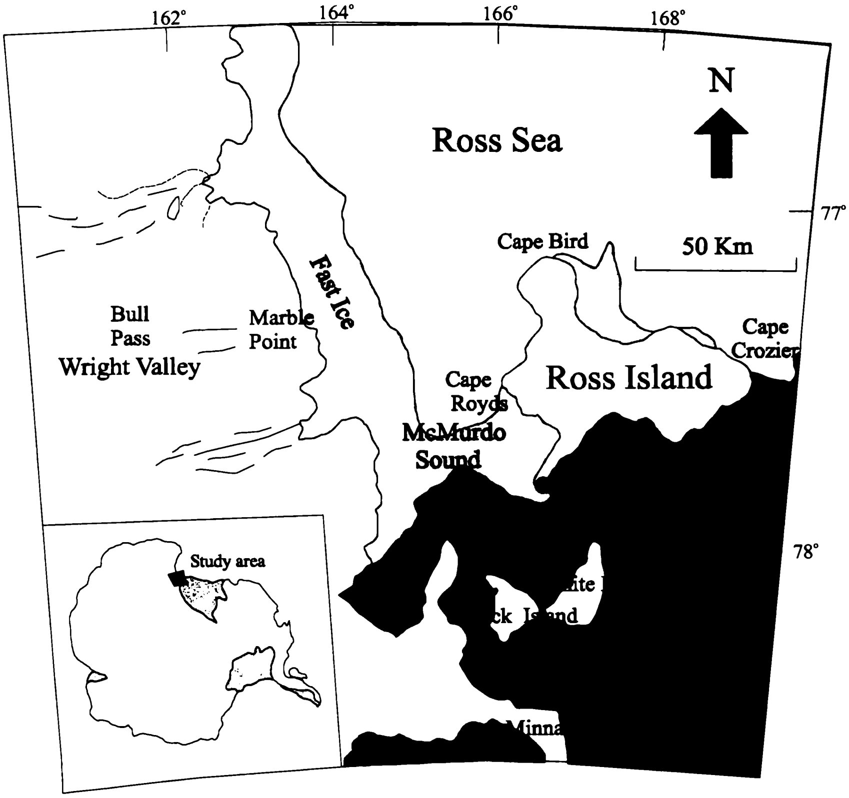


Table 3. Characteristics of the sites, spillage and soils used in this study (Data from Balks *et al.* (1998, 2002)[15,16])

Soil sample	Description of site	Soil collection date	Spillage date	Age of spillage	Type of contaminant	Soil classification	Soil description
Scott Base old spill	Former storage area for drums and lubricating oils. Two hundred m directly uphill from Scott Base. Slope of 6 degrees to SE, 60 m a.s.l. Less than 100m² of contaminated soil	December 1999	Intermittently from *ca* 1980 until 1998 (when storage area was decommissioned), as consequence of movement and distribution	Possibly over 20 years	C_{10}-C_{14} *n*-alkanes, PAH	Typic anhyorthel	Scoriaceous basalt, Stony, gravely sands derived mainly from basalt with ice-cemented permafrost below c. 30 cm
Bull Pass	Seismic borehole drilling site in the Wright Valley. Contaminated surface soil was removed, but fuel flowed downhill along soil-bedrock interface. Four hundred m E of Bull Pass camp. Slope of 4 degrees to S, 250 m a.s.l. Less than 100m² of contaminated soil	January 1999	1985 during seismic borehole drilling activities	20 years	Diesel Fuel Arctic (C_9-C_{15} *n*-alkanes)	Nitric anhyorthel	Siliceous, silt dominated glacial till Two cm of gravel desert pavement over 1+ m of silt with a few large rocks. Visible salt accumulation of profile face. No ice-cement (less water)
Marble Point	Oil stains on soil surface at site of Old Marble Point Camp. Sixty m E of lake edge. Slope of 3 degrees to E, 60 m a.s.l. Less than 100m² of contaminated soil	January 1999	Intermittently between 1957 and 1963, as consequence of movement and distribution	Possibly over 40 years	Possibly hydraulic and lubricating oils (C_{13}-C_{40} *n*-alkanes, PAH and hopanes)	Calcic anhyorthel	Two cm of desert pavement gravels over sandy gravel with > 30% boulders/stones. Ice-dominated horizon from 87-100 cm.
Scott Base recent spill	Soil collected from underneath a fuel storage tank in Scott Base	January 2002	2001during refuelling activities	Less than one year	JP-5	Typic anhyorthel	Scoriaceous basalt, Stony, gravely sands derived mainly from basalt with ice-cemented permafrost below c. 30 cm.
Vanda Station	Oil stains on surface at site of former Vanda Station	January 1999	Intermittently from 1969 until 1993, when the station was in operation	Between 12 and 34 years	Possibly lube-oil	Typic anhyorthel	Gravely sand over bedrock slabs with sandy material in cm joints

2.3. Microbial enumeration methods

2.3.1. Culturable heterotrophs enumeration by plate counting

Five grams of wet weight soil were mixed in 45 mililitres (ml) of 0.1% sodium pyrophosphate in sterile double distilled water solution containing 15 g sterile glass beads. This suspension was homogenised by shaking at 220 rpm at 4°C. Ten-fold serial dilutions in 4/2 buffer (see Appendix 1) were made, and 100 microlitres (µl) of each one plated in R2A agar (see Appendix 1) with three replicates per dilution. The initial soil-buffer suspension was considered as the first ten-fold dilution. Plates were sealed and incubated at 15°C for 3-4 weeks. After incubation, individual colonies were counted. Plates with 30 to 300 Colony Forming Units (CFU) were selected and final numbers were calculated using the equation:

$$\text{CFU}\big/_{\text{g dw}} = (\text{No.CFU in plate}) \times (\text{dilution})^{-1} \times (\text{dry weight correction factor})$$

dw= dry weight
dry weight correction factor= $\text{g ww}/\text{g dw}$
ww = wet weight

2.3.2. Enumeration of hydrocarbon degraders by respiroradiometric most-probable-number (MPN) method

Five grams of soil were suspended in 45 ml of 0.1% (w/v) sodium pyrophosphate sterile solution containing 15 g glass sterile beads. The suspension was homogenised by shaking at 220 revolutions per minute (rpm) for 1 hour at 4°C. Ten-fold serial dilutions in 4/2 buffer were made from this suspension. One ml of each dilution was dispensed in 160 ml serum bottles containing 10 ml of Bushnell Haas (BH) liquid media (see Appendix 1). Six vials were inoculated per dilution. Of the six vials, one was sterilised by autoclaving for 1 hour at 121°C, and taken as a sterile control. Additionally, 0.5 ml concentrated HCl was added to each sterile control serum bottle. Each serum bottle was then spiked with 15,000 degradations per minute (dpm) of *n*-

hexadecane-1-^{14}C (1.0 mCi/ml); SIGMA) dissolved in 50μl of JP-5, and sealed with a Subaseal suspending a small plastic cup (Kontes well) containing 300μl 1M potassium hydroxide (KOH) as the CO_2 trapping agent. Serum bottles were statically incubated at 15°C for 6 weeks. After incubation, 200 μl of trapping solution were collected from each bottle, mixed with 10 ml liquid scintillation cocktail ("Ultima Gold" Perkin Elmer) and radioactivity was measured in a Packard Tri-Carb 2900 TR liquid scintillation analyzer. Those traps containing at least double the radioactivity of the autoclaved controls were regarded as positive. The Most Probable Number (MPN) for hydrocarbon degraders was calculated using the "MPNES" program[147].

Alternatively, a non-respiroradiometric MPN method was used where the same steps were followed except for the addition of radioactive label and KOH as a CO_2 trapping agent (KOH). The presence of hydrocarbon degraders was determined by their ability to grow on the media supplemented with JP-5. Growth was visually detected as turbidity in the media compared with the sterile controls and disruption of the hydrocarbon layer. The MPN for hydrocarbon degraders was calculated using the MPNES program[147].

2.4. Polymerase chain reaction (PCR) of alkane degrading genes from soil

The study soils were screened for the presence of alkane-degrading genes by polymerase chain reaction (PCR). This method was used to amplify *alkB* gene homologues known to be involved in the bacterial degradation of alkanes. *Rhodococcus* strain 7/1[17] was used as a positive control for *alkB1*, *alkB2* and *alkB194* genes. This strain was isolated from oil-contaminated soils from around Scott Base, Antarctica. Strain *Pseudomonas putida* ATCC 29347 (PpG6 = *P. oleovorans* GPo1) was used as a positive control for *alkB* gene[127]. This strain was isolated from a hexane enrichment culture by Baptist *et al.* (1963)[15]. Primers for the 16S ribosomal deoxyribonucleic acid (rDNA) genes were used as internal controls to ensure that there was no inhibition of PCR reaction due to components of the soil samples.

2.4.1. DNA extraction method

The method used for extraction of genomic DNA from soil was the PSC-B (Phosphate, SDS, Chloroform-Bead beater) method. Soil (0.5g) was aseptically weighed and added to a sterile polypropylene bead-beater vial containing 0.5 g each of 0.1 mm and 3.0 mm silica-zirconium beads and 300 μl of phosphate buffer (100 mM NaH_2PO_4 pH 8.0). Three hundred μl of SDS lysis buffer (100 mM NaCl, 500 mM Tris pH 8.0, 10% SDS-see Appendix 1) were added and mixed gently. In the same vial, 300 μl of chloroform-isoamyl (24:1) alcohol were added.

Vials were shaken in a FastPrep machine at 4.5 m/s for 40 seconds, then spun in an Eppendorf microfuge at full speed (13,400 rpm or 12,100 rcf) for 5 minutes to pellet cell debris. Supernatant was transferred to a new sterile 1.5 ml tube and mixed with enough 7M ammonium acetate to achieve final concentration of 2.5 M. Tubes were centrifuged in a MiniSpin Eppendorf microfuge at 13,400 rpm for 5 minutes. Supernatant was transferred to new sterile tube and 0.54 volumes of isopropanol were added. Tubes were incubated at room temperature for 15 minutes and then spun at 13,400 rpm for 5 minutes. Supernatant was discarded and pellet washed with 1 ml 70% ethanol. Once more, tubes were spun at 13,400 rpm for 5 minutes. Supernatant was discarded and pellet was air dried for 15-45 minutes. Pellet was then resuspended in 50 μl of sterile distilled water and kept at 4°C if used next day or -20°C for longer-term storage.

2.4.2. PCR protocol

Primer sequences for gene homologues *alkB*, *alkB1*, *alkB2* and *alkB194* were obtained from Whyte *et al.* (2002)[145] and are shown in Table 4. Primers were synthesized by Invitrogen New Zealand Limited.

Table 4. Primer sequences used in this study

Gene	Fragment size (base pair)	Primer	Sequence (5'-3')	Reference strain
alk B	870	*alk B* forward	TGGCCGGCTACTCCGATGATCGGAATCTGG	*Pseudomonas putida* ATCC 29347
		alkB reverse	CGCGTGGTGATCCGAGTGCCGCTGAAGGTG	
alk B1	629	*alk B1* forward	ATCTGGGCGCGTTGGGATTTGAGCG	*Rhodococcus sp.* strain Q15
		alk B1 reverse	CGCATGGTGATCGCTGTGCCGCTGC	
alk B2	552	*alkB2* forward	ACTCTGGCGCAGTCGTTTTACGGCC	*Rhodococcus sp.* strain Q15
		alkB2 reverse	CCCACTGGGCAGGTTGGGCGCACCG	
alk B194	194	*alk B194* forward	CACAGYTGGAACAGYGATCRC	*Rhodococcus sp.* strain Q15
		alk B194 reverse	TCCATCACYTTKCGCCACAG	
Universal 16S rDNA	511	8-F	AGAGTTTGATCCTGGTCCAG	Eubacteria domain
		519-R	GTATTACCGCGGCTGCTGG	

PCR amplification was performed using an Eppendorf Mastercycler gradient thermocycler as follows:

Initial denaturation: 94°C for 2 minutes
30 amplification cycles :
    Denaturation: 94°C for 1 minute
    Annealing: 55°C for 1 minute
    Extension: 72°C for 2 minutes
Final extension: 72°C for 7 minutes

Each amplification reaction was carried out using Platinum Supermix (Invitrogen) (see Appendix 1), and each reaction tube contained 45 μl of Supermix, 1 μl of 10 μM primer solution (forward and reverse-200 nM final concentration), 3 μl genomic DNA (previously adjusted to 10 ng/μl). PCR products were separated by electrophoresis in 2% agarose gels in TBE buffer @ 90 Volts for 2 hours. A 100 base pair (bp) DNA ladder (Invitrogen) or 1 kilobase (kb) PLUS ladder (GIBCO) was used to estimate the size of bands in the gels. PCR products were visualised under ultraviolet (UV) light after being exposed to ethidium bromide solution (1 mg/L) for 10 minutes (shaking) and rinsed with distilled water for 10 minutes.

2.4.3. Sequencing

PCR products were sequenced using a MegaBACE DNA Analysis System (Amersham Biosciences) by the Waikato DNA Sequencing Facility at the University of Waikato. The sequences obtained were then computer analysed ("blasted") on the National Centre for Biotechnology Information (NCBI) GenBank databases using the BLASTN 2.2.10 program, which performs a nucleotide-nucleotide blast. The best match was used to confirm the identity of the PCR product obtained using *alkB* gene homologue primers.

2.5. Hydrocarbon-degrading isolates

2.5.1. Isolation of hydrocarbon-degrading bacteria

The most dominant colony types from the R2A plates for culturable heterotrophs enumeration were selected. Individual colonies were picked and re-streaked on BH plates with JP-5 supplemented in the headspace as a source of carbon and incubated for 4 weeks at 15°C. Additionally, these colonies were also grown on 10 ml of liquid BH media supplemented with 50μl of JP-5 jet fuel, henceforth called BH-JP-5. Those that grew under these selective conditions were kept for further analysis.

2.5.2. Gram staining and substrate specificity

Each isolate that grew in BH-JP-5 was screened for its Gram stain and ability to degrade alkanes as a source of carbon. Gram staining was carried out with a Becton-Dickinson Gram Stain kit (4 step procedure).

For substrate specificity assays, liquid sterile BH medium (10 ml) in a vial of approximately 60 ml, was supplemented with 50μl of one the following substrates as sole source of carbon: hexane, undecane, dodecane, hexadecane and pristane. Prior to addition into the sterile BH medium, the carbon source was filter sterilized with a nylon 0.2 μm membrane Acrodisc filter (PALL). Each isolate was inoculated to each one of these vials and incubated statically at 15°C for 4 weeks. Inoculated carbon-free medium was used as a negative control. Microbial growth in liquid medium was both visually determined by comparing with control turbidity and hydrocarbon layer disruption, and by measuring light scattering at 600 nm with Shimadzu UV-160A spectrophotometer. Those vials with an optical density above 0.2 were scored as positive.

2.5.3 PCR of *alk* genes in hydrocarbon-degrading bacterial isolates

Bacteria isolated from Scott Base soils able to grow on JP-5 were screened for the presence of *alk* genes by PCR. The primers used were the same as those used for the soil screening. DNA was extracted as described previously, using 0.1 g of cell biomass from BH-JP-5 plates. PCR conditions were the same as described previously, and PCR products were sequenced as described in Section 2.4.4 to confirm identity of the amplified DNA.

2.6. Mineralisation assays

Mineralisation of ^{14}C-hexadecane (1.0 mCi/ml SIGMA) was used as an indication of hydrocarbon degradation in all the study soils and experimental treatments.

2.6.1. Microcosms description

Ten grams (dry weight equivalent) of soil were aseptically placed in sterile 70 ml plastic pottles and spiked with 0.5 μCi (1.1 x 10^6 dpm) of ^{14}C-hexadecane dissolved in 10 μl filter sterilized JP-5. The soils were placed inside glass jars that contained 10 ml 1M KOH solution in a small beaker as CO_2 trap and 5 ml sterile water in a glass vial to maintain moisture. The jars were sealed and incubated statically in the dark at 15°C for at least 60 days. At each sampling time, 0.5 ml of the KOH trap were taken and mixed with 10 ml liquid scintillation cocktail ("Ultima Gold" Perkin Elmer, Boston, MA, USA). Radioactivity was determined using a Packard Tri-Carb 2900 TR model liquid scintillation analyzer. The amount of $^{14}CO_2$ trapped from the soil was taken as a measure of mineralisation.

The KOH trap was removed and replenished at each sampling date. In all the assays performed, three replicates per treatment were prepared and one sterile control. Sterile controls were prepared by autoclaving soils for one hour and adding 0.5 ml of concentrated HCl to the soil.

2.6.2. Unamended soils mineralisation assay

The following soils were tested for mineralisation of ^{14}C-hexadecane to screen for their intrinsic microbial ability to degrade alkanes without any amendments:

- Scott Base old spill
- Scott Base recent spill
- Marble Point 1 (0-3 cm)
- Marble Point 2 (3-12 cm)
- Bull Pass
- Vanda Station

Moisture content was not adjusted and no nutrient amendments were made.

2.6.3. Amendment treatments

Given that nitrogen availability and moisture contents in Scott Base soils may be limiting factors for microbial degradation of hydrocarbons, treatments (also referred to as 'microcosms') with supplementation of nitrogen and/or adjustment of moisture content were prepared for both old spill and recent spill Scott Base sites.

Previous to the set up of the microcosms, ten grams of soil were supplemented with enough ammonium chloride (NH_4Cl) to reach 2500 mg N/kg-water-soil (as per Walworth, 1997)[135]. Moisture content was adjusted by adding sterile distilled water to reach 10%, 20% or 25% moisture content, depending on the treatment. A set of treatments with excess nitrogen was also prepared. The treatments set up for SBO are described in Table 5, SBR treatments are described in Table 7, and the characteristics of these treatments are summarised in Tables 6 and 8, respectively.

2.6.3.1. Scott Base old spill site (SBO)

Table 5. Amendment treatments in SBO soil

Moisture content	No N amendment	+ N	
		2500 mg N/ kg-H_2O-soil	2 x 10^5 mg N/ kg-H_2O-soil (excess)
No moisture adjustment	1	NT	2
10%	3	4	5
20%	6	7	8
25%	9	10	11

NT= not tested. This treatment was not established because it was not possible to adjust to 2500 mg N/ kg-water-soil without adding any extra water.

Table 6. Characteristics of treatments in SBO amendment experiments

Treatment number	Total N in soil (mg N/ kg soil dw)	Amount of N added per 10 g soil (mg)	Amount of NH_4Cl added per 10 g soil (g)	Amount of water added per 10 g soil (ml)	Final N_{H2O} (mg N/ kg H_2O-soil)	C:N
1	166	0.00	0.0000	0.00	6640	241:1
2	5166	50.00	0.1900	0.00	206,640	8:1
3	166	0.00	0.0000	0.75	1660	241:1
4	250	0.84	0.0032	0.75	2500	160:1
5	5166	50.00	0.1900	0.75	51,660	8:1
6	166	0.00	0.0000	1.75	830	241:1
7	500	3.34	0.0127	1.75	2500	80:1
8	5166	50.00	0.1900	1.75	25,830	8:1
9	166	0.00	0.0000	2.25	664	241:1
10	625	4.59	0.0174	2.25	2500	64:1
11	5166	50.00	0.1900	2.25	20,664	8:1

2.6.3.2. Scott Base recent spill site (SBR)

Table 7. Amendment treatments in SBR soil

% Moisture content		**+ N**	
	No N amendment	**2500 mg N/ kg-H_2O-soil**	**2 x 10^5 mg N/ kg H_2O-soil (excess)**
No adjustment	1	NT	2
10%	3	4	5
20%	6	7	8

NT= not tested. This treatment was not established because it was not possible to adjust to 2500 mg N/ kg-water-soil without adding any extra water.

Table 8. Characteristics of treatments in SBR amendment experiments

Treatment number	**Amount of N in soil (mg N/ kg soil dw)**	**Amount of N added per 10 g soil (mg)**	**Amount of NH_4Cl added per 10 g soil (g)**	**Amount of water added per 10 g soil (ml)**	**Final N_{H2O} (mg N/ kg H_2O-soil)**	**C:N**
1	200	0.00	0.000	0.000	2695	37:1
2	5200	50.00	0.190	0.000	70,080	1.4:1
3	200	0.00	0.000	0.258	2000	37:1
4	250	0.50	0.002	0.258	2500	30:1
5	5200	50.00	0.190	0.258	52,000	1.4:1
6	200	0.00	0.000	1.258	1000	37:1
7	500	3.00	0.011	1.258	2500	15:1
8	5200	50.00	0.190	1.258	26,000	1.4:1

2.6.4. SBO dilution treatment

SBO soil was mixed in a 1:1 proportion with non-contaminated soil from Scott Base with the same moisture content (2.5%). This mixture was amended with enough nitrogen (NH_4Cl) to reach 2500 mg N/kg-H_2O-soil, and moisture content was adjusted to 10% or 20%. Non-contaminated soil was used as a control. Mineralisation assays were run on the following treatments, with the following characteristics as given in Table 9.

Table 9. Characteristics of treatments in dilution experiment

Treatment number	Moisture content (%)	Amount of N added per 10 g soil (mg)	Amount of NH_4Cl added per 10 g soil (g)	Amount of N in soil (mgN/kg soil dw)	Final N_{H2O} (mgN/ kg H_2O-soil)	C:N
1	2.5	0.0	0.0000	120	4800	177:1
2	10	0.0	0.0000	120	1300	177:1
3	10	1.7	0.0065	290	2900	77:1
4	20	0.0	0.0000	120	650	177:1
5	20	4.2	0.0160	540	2700	42:1
6	2.5	0.0	0.0000	100	4000	9.7:1
7	10	0.0	0.0000	100	1000	9.7:1
8	20	0.0	0.0000	100	500	9.7:1

2.6.5. Bull Pass soil bioaugmentation experiment

2.6.5.1. Mineralisation assays

As no hydrocarbon-degraders were detected in Bull Pass soil, 2 bacterial strains (*Rhodococcus* 5/1[17] and SBO-1 isolate (this thesis, see Section 3.4)) isolated from the Scott Base contaminated soil were inoculated separately and independently into soil samples. The strains were added to the soil to test the hypothesis that only by bioaugmenting the soil with hydrocarbon degraders, would biodegradation of hydrocarbons (alkanes) occur. Mineralisation of ^{14}C-hexadecane, as described for the previous mineralisation assays, was monitored in the following treatments, as given in Table 10:

Table 10. Characteristics of bioaugmentation treatments

Treatment	Strain added	Moisture content (%)	N_{H20} (mg N/ kg-H_2O-soil)
BP1	None	10	2630
BP2	*Rhodococcus* 5/1	10	2630
BP3	SBO-1	10	2630
BP4	None	20	1315
BP5	*Rhodococcus* 5/1	20	1315
BP6	SBO-1	20	1315

Each microcosm contained 20 g dry weight equivalent (20.94 g wet weight) and was supplemented with sterile double distilled water (ddH_2O.)

Bioaugmentation bacterial inoculum was prepared as follows:

Each of the bacterial strains was grown on BH agar plates supplemented with JP-5 in the headspace and incubated for at least 3 weeks at 15°C. All biomass was taken from the surface of the agar plate with a sterile loop and suspended in 5 ml 4/2 dilution buffer. Cells were washed 2 times with 4/2 buffer and pellet redissolved in sterile water to obtain an inoculum of approximately 10^8 CFU per ml. To enumerate the number of bacteria inoculated, serial dilutions of this inoculum were made for plate counting on R2A media. Each treatment replicate (20 g dry weight of soil) was inoculated with 0.2 ml of cell suspension, and enough ddH_2O was added to adjust to 10% or 20% moisture content. The treatments with no strain added were only adjusted to 10% and 20% moisture with sterile distilled water.

Once the soil was inoculated, a subsample of 1 g was taken to enumerate culturable heterotrophs on R2A media and hydrocarbon degraders by non-respiroradiometric MPN method. A subsample of 1 g was also taken at the end of the incubation period for the same enumerations to compare numbers with initial inoculation.

2.6.5.2. Total Petroleum Hydrocarbon (TPH) analysis

Parallel to the mineralisation assays of Bull Pass bioaugmented soil, another set of treatment replicates were prepared exactly the same as for the mineralisation assay, but lacking the radioactive label. These were set to monitor the decrease of alkanes in the soil as a result of bioagumentation. TPH analyses were done at the beginning and end of the experiment for each treatment.

2.6.6. Kinetic analysis

Kinetic models generated by regression analysis using the SigmaPlot 8.0 program were used to describe the behaviour of the mineralisation data for the soils in this work. These models predict the maximum value of mineralisation for each treatment. The most relevant mineralisation curves, *eg*. those that respond positively to the treatments tested here, were analysed with kinetic models.

2.7. Denaturant gradient gel electrophoresis (DGGE) analysis

The most successful amendment treatments of Scott Base soil and Bull Pass bioaugmented soil were analysed by DNA extraction and denaturant gradient gel elctrophoresis (DGGE) to monitor the changes in microbial populations. Two hydrocarbon-degrading bacteria isolated from Scott Base soil were used as positive controls for Scott Base analysis, and the inoculated strains were used as positive controls for Bull Pass bioaugmented soil.

2.7.1. DNA extraction method

Template genomic DNA was extracted from 0.5 g soil samples by the bead-beater method described in 2.5.1.

2.7.2. PCR protocol

Bacterial primers for partial 16S rDNA gene were used for the PCR reaction. Ten μM solutions of forward 338Fgc primer and reverse 519RC primer were prepared. Primer sequences where obtained from Muyzer *et al.* (1993) [91] and are as follows:

338Fgc – 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC TCC TAC GGG AGG CAG CAG 3'

519RC – 5' ATT ACC GCG GCT GCT GG 3'

Each amplification reaction was carried out using Platinum Supermix, and each reaction tube contained 45 μl of Supermix, 1μl of each primer solution (200 nM final concentration) and 3 μl genomic DNA (previously adjusted to 10 ng/μl). PCR amplification was performed using an Eppendorf Mastercycler gradient thermocycler as given in Table 11.

Table 11. PCR protocol for DGGE

Step	Temperature	Time
1	94°C	5 minutes
2	94°C	30 seconds
3	65°C decreasing 0.5°C per cycle	1 minute
4	72°C	1 minute
5	21 times to step 2	
6	94°C	1 minute
7	55°C	1 minute
8	72°C	1 minute
9	8 times to step 6	
10	72°C	5 minutes
11	4°C HOLD	

2.7.3. Denaturant gradient gel electrophoresis protocol

DGGE was performed using a DCode™ Universal Mutation Detection System (BIORAD). Gels were poured and cast using a Gradient Delivery System (Model 475, BIORAD) according to Section 4 of the BIORAD Instruction Guide. Twenty μl PCR product and 20 μl of 2X DGGE loading buffer (see Appendix 1) were loaded into an 8% polyacrylamide gel that contained a urea denaturant gradient from 25% to 60% denaturant (see Appendix 1). Electrophoresis was performed at 130 volts for 6 hours at a constant temperature of 60°C in 7 L 1X Tris Acetate EDTA (TAE) buffer (see appendix 1). After electrophoresis, the gel was stained in 500 ml ethidium bromide solution (1 μg/ml) for 20 minutes and de-stained in ddH$_2$O for 20 minutes. Gels were visualised with UV transilluminator and photographed.

Chapter 3. Results

3.1. Soil analysis

Chemical analysis of soils from Scott Base, Marble Point, Vanda Station and Bull Pass and control soils was completed, as well as moisture content and pH determination. The C:N ratio was calculated using total N and total C values. These and the N_{H2O} value for contaminated and control soils are presented in Table 13.

3.2. Soil microbial enumeration

Given that the presence of microorganisms is essential for microbial biodegradation, total culturable heterotrophs and hydrocarbon-degraders (HCD) were enumerated in all the study soils. The numbers of microbes detected in each soil are presented in Table 12 and Figure 13 presents a graph of the logarithmic transformation of these numbers. All soils contain culturable heterotrophic bacteria, with Bull Pass and Vanda Station soils harbouring lower numbers ($<10^5$ CFU/g dry weight (dw)), and Scott Base and Marble Point soils harbouring similar numbers ($>10^6$ CFU/g dw). No HCD were detected in Bull Pass and Vanda station with this method. HCD are present in the highest numbers in Scott Base old spill soils followed by Scott Base recent spill and both Marble Point soils, which have equivalent numbers

Table 12. Bacterial numbers in the study soils

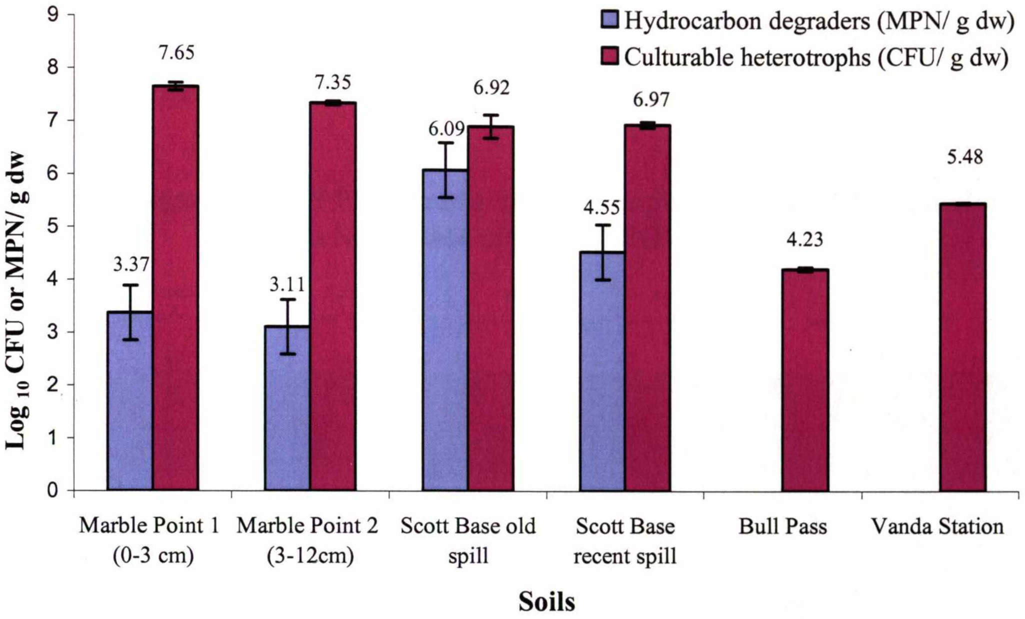
Soil	Culturable heterotrophs (CFU/ g dw)	Hydrocarbon-degraders (MPN / g dw)
Marble Point 1	$4.40 \times 10^7 \pm 7.79 \times 10^6$	$2.34 \times 10^3 \pm 3.30$
Marble Point 2	$2.23 \times 10^7 \pm 1.84 \times 10^6$	$1.28 \times 10^3 \pm 3.30$
Scott Base old spill	$8.38 \times 10^6 \pm 3.88 \times 10^6$	$1.23 \times 10^6 \pm 3.30$
Scott Base recent spill	$9.29 \times 10^6 \pm 1.14 \times 10^6$	$3.54 \times 10^4 \pm 3.30$
Bull Pass	$1.68 \times 10^4 \pm 1.82 \times 10^3$	<10
Vanda Station	$3.00 \times 10^5 \pm 7.02 \times 10^3$	<10

Table 13. Chemical analysis of contaminated and uncontaminated (control) soils

	Scott Base old spill	Scott Base recent spill	Scott Base control[c]	Bull Pass	Bull Pass control	Marble Point 1 0-3 cm	Marble Point 1 control 0-3 cm	Marble Point 2 3-12 cm	Marble Point 2 control 3-15 cm	Vanda Station	Vanda Station control
TPH[a] (mg/kg dw)	40,200	7490	<30	1040	<30	29,100	<20	18,300	<20	2410	<30
Total C (mg/kg dw)	45,000	3120	966	7,600	200	53,300	2800	33,600	1100	3000	600
Total N (mg/kg dw)	160	70	100	200	100	180	200	110	70	6.9	2.9
NH_4-N (mg/kg dw)	0.6	3.10	1.7	3.6	1.2	3.5	3.4	2.2	2.3	3.6	1.6
NO_3-N (mg/kg dw)	0.1	0.83	14.8	263	2.9	0.5	2.2	0.5	5.9	3.3	1.3
Total P (mg/kg dw)	2157.6	2200	1993	254	200	600	700	700	700	200	0.02
Olsen P (mg/kg dw)	5.6	12	9.5	1	2.7	3	2	3	2	27	0.00
Moisture content (%)	2.5	7.4	2.5	4.7	1.0	1.9	2.4	6.4	5.3	0.3	0.2
pH	8.48	9.5	9.05	7.7	7.7	8.3	9.6	9.2	9.0	7.4	9.1
C:N [b]	281:1	43:1	9.6:1	38:1	2:1	296:1	14:1	306:1	1.5:1	435:1	206:1
N_{H2O} b (mg N/kg H_2O-soil)	6400	946	4000	4255	10,000	9474	8333	1719	13,208	2300	1450

a = See Appendix 2 for the chromatograms of TPH analysis of the soils; b = calculated values; c = data from Holmes (2002)[69]

Figure 13. Microbial numbers in the study soils

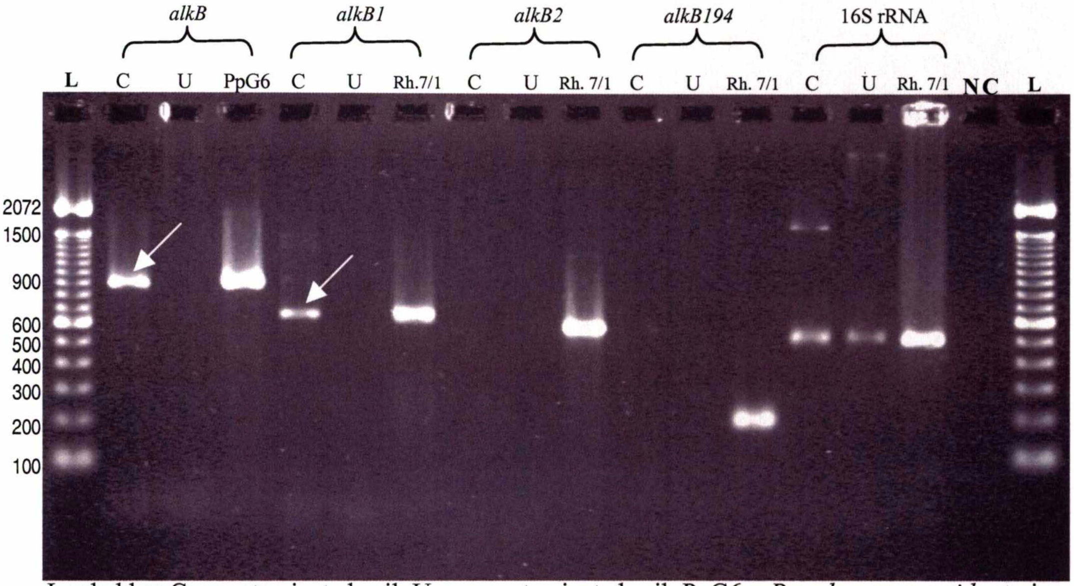


3.3. PCR of *alkB* gene homologues in soil

The soils were screened for the presence of alkane monooxygenase gene *alkB* and its homologues *alkB1*, *alkB2* and *alkB194* by PCR amplification. Figures 14 to 19 show photographs of the agarose gels where the PCR products were visualized by ethidium bromide fluorescence. In these gels, the presumptive *alk* gene content, as indicated by a band of the appropriate size in the gel matrix, of the contaminated soil (labelled C in the Figures) was compared with uncontaminated control soil (labelled U in the Figures). A PCR was done to amplify the *alkB* gene homologue of strain *Pseudomonas putida* PpG6 (ATCC 29347) and the *alkB1, alkB2* and *alkB194* homologues of the strain *Rhodococcus* 7/1. These PCR products were run in the gels as positive controls. The appropriate size band was obtained for each one of the positive control bands. The PCR product resulting from a primer for the16S rRNA gene was used in all cases as a control to ensure that amplification was not hindered by any soil component (as DNA was extracted directly from soil samples). The size marker used was a 100 base pair ladder. The negative control (NC) was the PCR mixture only.

All the positive PCR products were sequenced to confirm that the product obtained was indeed the amplification of an *alkB* gene homologue.

Figure 14. Agarose gel of PCR products from amplification of *alkB* homologues from Scott Base old spill (SBO) site soil

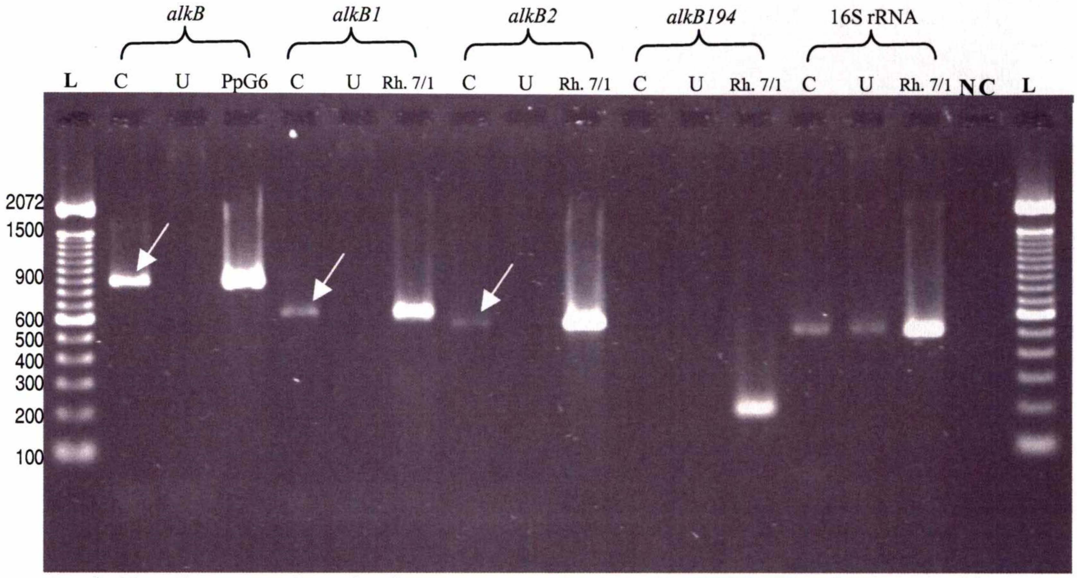


L = ladder; C = contaminated soil; U = uncontaminated soil; PpG6 = *Pseudomonas putida* strain ATCC 29347; Rh.7/1 = *Rhodococcus* sp. strain 7/1; NC = negative control

As shown in Figure 14, the gene amplification products of *alkB* and *alkB1* in SBO soil (indicated by arrows in the Figure) were positive and had the same band size as the positive control. This result was confirmed by sequence analysis: *alkB* gene PCR product showed a 99% identity with *Pseudomonas aureofaciens alk B* gene[131], and *alkB1* gene showed 94% sequence identity with the putative *alkB1* gene from *Rhodococcus erythropolis*[117]. The PCR reaction for genes *alkB2* and *alkB194* was negative in SBO contaminated soil. Thus, the old spill soil in Scott Base was positive for *alkB* and *alkB1* gene homologues, but not for *alkB2* and *alkB194*. No positive reactions were found in control uncontaminated soil from this site. Amplification of

16S rRNA gene from soil confirmed that there was no inhibition of the PCR reaction resulting from soil components (at least for this, and presumptively any primer).

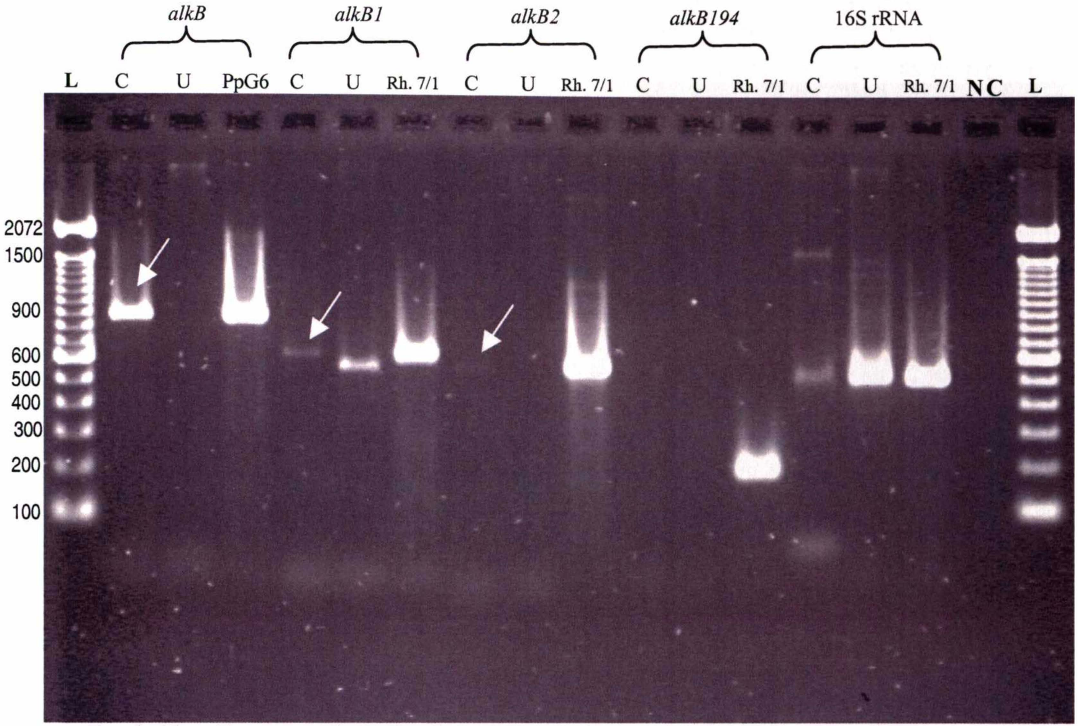
Figure 15. Agarose gel of PCR products from amplification of *alkB* homologues from Scott Base recent spill (SBR) site soil



L = ladder; C = contaminated soil; U = uncontaminated soil; PpG6 = *Pseudomonas putida* strain ATCC 29347; Rh.7/1 = *Rhodococcus* sp. strain 7/1; NC = negative control

Figure 15 shows that positive correct-sized bands were obtained for genes *alkB* (870 bp), *alkB1* (629 bp) and *alkB2* (552 bp) (indicated by arrows in the Figure). Sequence analysis of these products revealed a 95% identity with *Pseudomonas aureofaciens alkB* gene, 95% identity with *Rhodococcus* sp. Q15[145] *alkB1* gene and 96% identity with *alkB2* gene from *Rhodococcus erythropolis,* respectively. No positive amplification was observed for *alkB194* gene. Therefore, the recent spill site in Scott Base was positive for *alkB*, *alkB1* and *alkB2* gene homologues, but not for *alkB194*. No positive reactions were found in control uncontaminated soil from this site. Amplification of 16S rRNA gene from soil confirmed that there was no inhibition of the PCR reaction resulting from soil components.

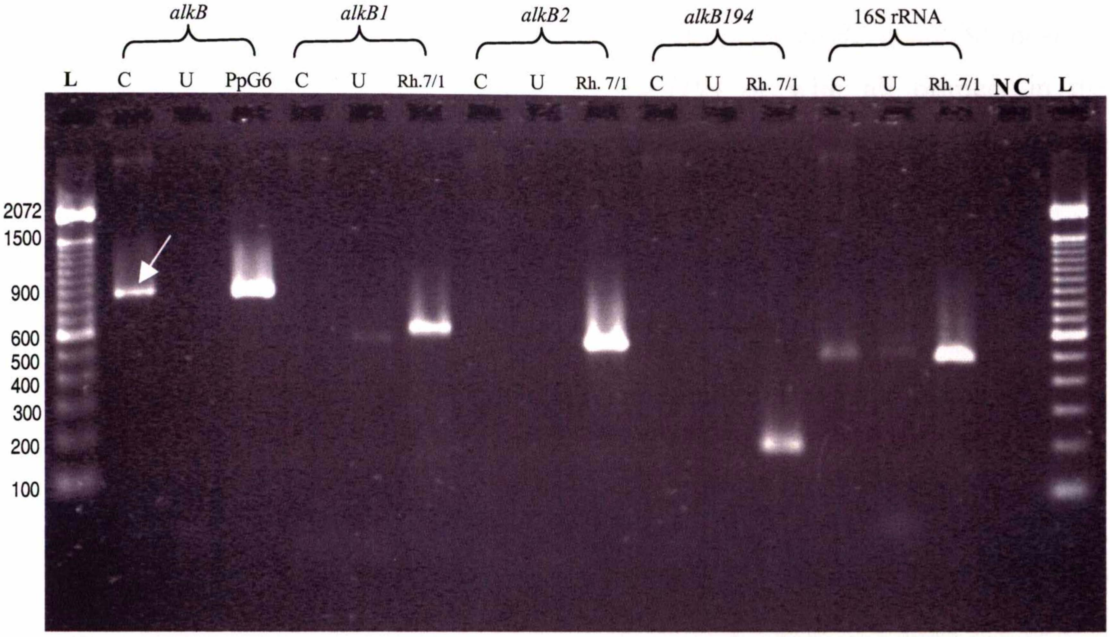
Figure 16. Agarose gel of PCR products from amplification of *alkB* homologues from Marble Point 1 soil



L = ladder; C = contaminated soil; U = uncontaminated soil; PpG6 = *Pseudomonas putida* strain ATCC 29347; Rh.7/1 = *Rhodococcus* sp. strain 7/1; NC = negative control

Figure 16 shows that PCR products were positive and showed the same band size as controls for *alkB* (870 bp), *alkB1*(629 bp) and *alkB2* (552 bp) genes in contaminated soils (indicated by arrows in the Figure). Non-contaminated soil showed a positive band of slightly smaller size for *alkB1* gene. Sequencing results could only confirm the presence of *alkB* gene in the contaminated soil. The blast of this PCR product had a 97% match to *Pseudomonas aureofaciens alkB* gene. No match in the GenBank was found for the PCR products obtained with primers for *alkB1* and *alkB2* in Marble Point 1 contaminated soil or with the primer for *alkB1* in uncontaminated soil. Therefore, Marble Point 1 soil was positive for *alkB*, but not for *alkB1*, *alkB2* or *alkB194.* Amplification of 16S rRNA gene from soil confirmed that there was presumptively no inhibition of the PCR reaction resulting from soil components.

Figure 17. Agarose gel of PCR products from amplification of *alkB* homologues from Marble Point 2 soil

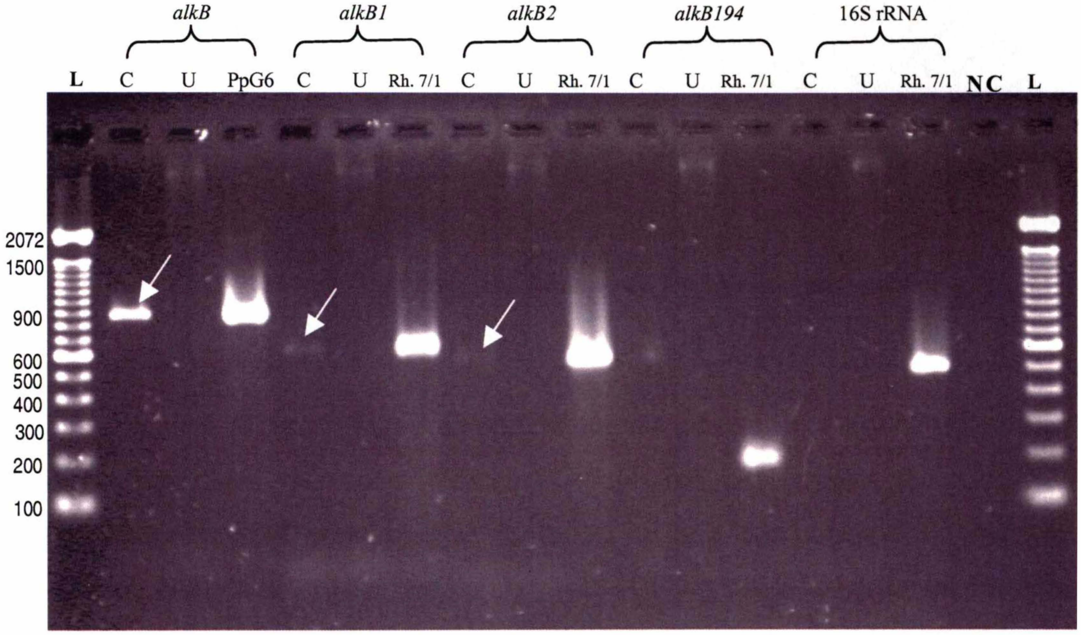


L = ladder; C = contaminated soil; U = uncontaminated soil; PpG6 = *Pseudomonas putida* strain ATCC 29347; Rh.7/1 = *Rhodococcus* sp. strain 7/1; NC = negative control

When visualised in the agarose gel by fluorescence of intercalated ethidium bromide (Figure 17), PCR products in Marble Point 2 soil showed a positive band (indicated by an arrow in Figure 17) of the same size (870 bp) as control for *alkB* gene in the contaminated soil. A band of smaller size was also faintly visible for the *alkB1* PCR product in the uncontaminated soil, however, sequence analysis of this product did not show homology with sequence of the *alkB1* gene. On the other hand, sequence analysis confirmed the identity of *alkB* gene by a match of 97% with *P. aureofaciens alkB* gene. No positive PCR reaction was obtained with primers for *alkB1*, *alkB2* and *alkB194* genes in either contaminated or uncontaminated soil. Thus, Marble Point 2 contaminated soil was positive for *alkB* gene, but not *alkB1, alkB2* and *alkB194.*

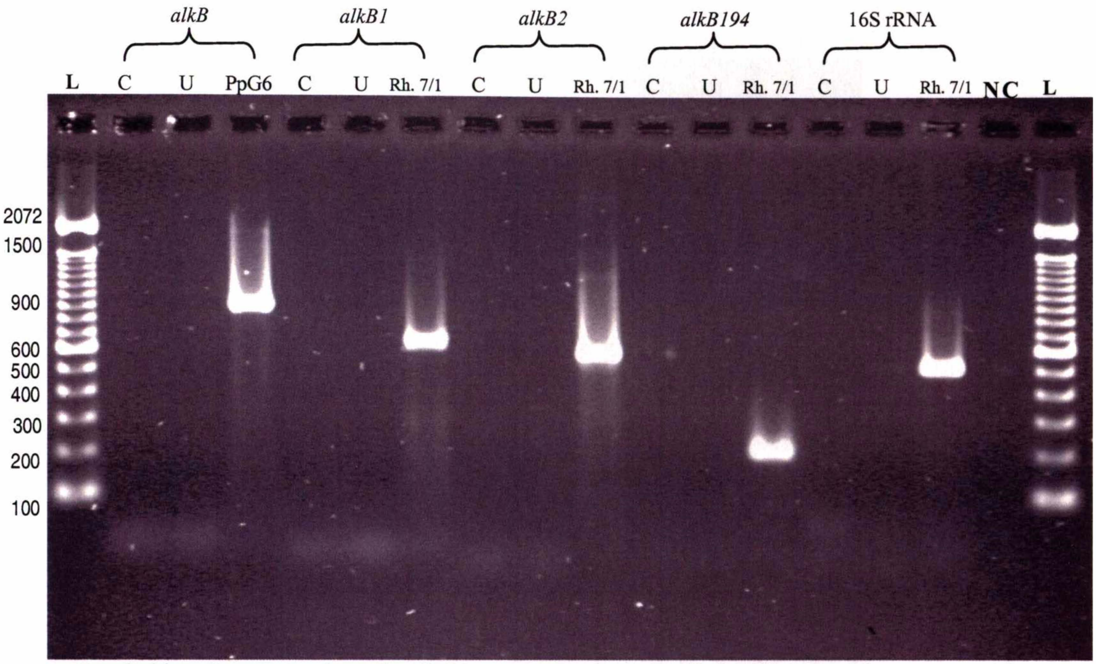
PCR products from Vanda Station (Figure 18) soil were positive for *alkB*, *alkB1* and *alkB2* (indicated by arrows in the Figure), as visualised by fluorescence of intercalated ethidium bromide in the agarose gel. However, sequence analysis revealed that only *alkB* and *alkB2* corresponded to correct amplification of *alk* genes. The *alkB* PCR product had a match of 98% with *P. aureofaciens alkB* gene and *alkB2* had a 97% identity match with *Rhodococcus erythropolis alkB2* gene. No positive amplification was found with primers for *alkB194* or with all primers in the uncontaminated soil. Thus, Vanda Station contaminated soil was positive for *alkB* and *alkB2* genes, but not for *alkB1* and *alkB194*.

Figure 18. Agarose gel of PCR products from amplification of *alkB* homologues from Vanda Station soil



L = ladder; C = contaminated soil; U = uncontaminated soil; PpG6 = *Pseudomonas putida* strain ATCC 29347; Rh.7/1 = *Rhodococcus* sp. strain 7/1; NC = negative control

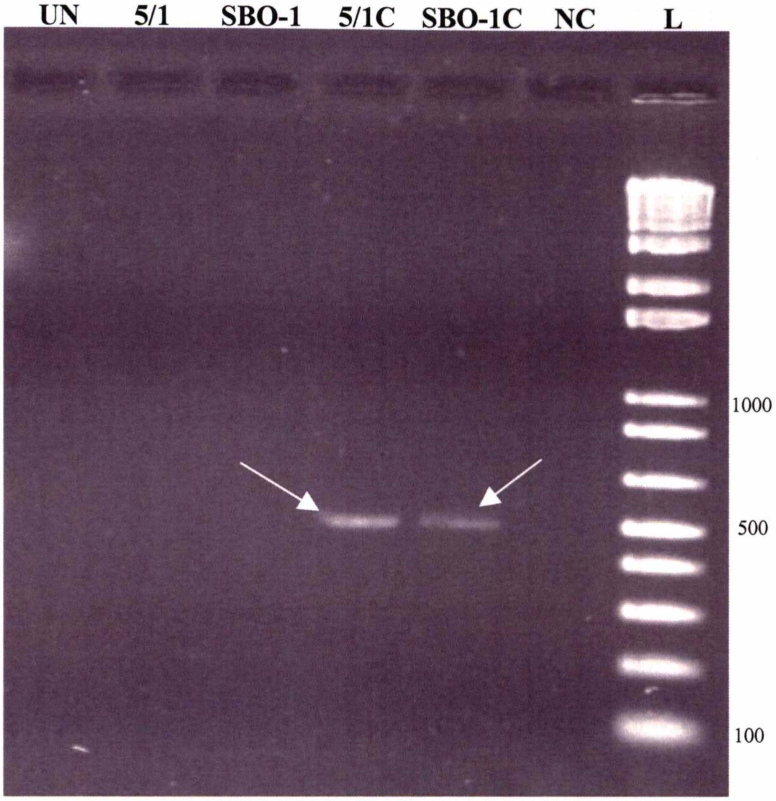
Figure 19. Agarose gel of PCR products from amplification of *alkB* gene homologues in Bull Pass soil



L = ladder; C = contaminated soil; U = uncontaminated soil; PpG6 = *Pseudomonas putida* strain ATCC 29347; Rh.7/1 = *Rhodococcus* sp. strain 7/1; NC = negative control

In Bull Pass soil (Figure 19), no amplification was detected for any of the primers used. Because there was no amplification of the 16S rRNA gene, the possibility existed that the PCR reaction was inhibited by soil components. To test this hypothesis, Bull Pass soil, inoculated with 10^7 CFU/g dw of 2 different bacterial strains *Rhodococcus* sp. 5/1 and isolate SBO-1, was used for DNA extraction and subsequent analysis with 16S rRNA primers. These products were electrophoresed in a gel using the amplification product of the 16S rRNA gene of the strains inoculated as positive controls. As Figure 20 shows, no product was amplified from the soil, even after this inoculation, but amplification was positive for the strains with primers for the16S rRNA gene (indicated by arrows in the Figure 20).Therefore, it is probable that soil components inhibited the PCR reaction. Thus, this experiment could not demonstrate the presence or absence of *alkB* gene homologues in Bull Pass.

Figure 20. Agarose gel of PCR products from inoculated Bull Pass soil amplified with 16S rRNA primers



UN= uninoculated soil; 5/1= soil inoculated with strain 5/1; SBO-1 = soil inoculated with isolate SBO-1; 5/1C = 5/1 strain as positive control; SBO-1C = SBO-1 strain as positive control; NC= PCR mix as negative control; L= ladder

Table 14 shows a summary of the results from the screening of *alkB* gene homologues in the studied soils. When indicated (++) PCR product identity was confirmed by sequencing and blast on the GenBank. One + in the table means that the gene was possibly amplified as visualised in agarose gel, but either no match was found in the GenBank, or PCR product was not sequenced successfully.

Table 14. Summary of presence of *alkB* gene homologues in study soils as indicated by PCR and DNA sequence analysis

Soil	Gene			
	Pp alkB	***Rh alkB1***	***Rh alkB2***	***Rh alkB194***
SBO	++	++	-	-
SBO control	-	-	-	-
SBR	++	++	++	-
SBR control	-	-	-	-
Marble Point 1	++	+	+	-
Marble Point 1 control	-	-	-	-
Marble Point 2	++	-	-	-
Marble Point 2 control	-	+	-	-
Vanda Station	++	++	++	-
Vanda Station control	-	-	-	-
Bull Pass	-	-	-	-
Bull Pass control	-	-	-	-

3.4. Hydrocarbon-degrading bacterial isolates

Soil from Scott Base was selected for isolation of hydrocarbon-degrading bacteria. A selection of the most dominant colonies was made from the culturable heterotrophs plates and these were tested for their ability to degrade JP-5 jet fuel. Eleven hydrocarbon-degrading bacterial isolates were obtained from the old spill site (SBO) and 6 from the recent spill site (SBR).

3.4.1. Morphological characteristics and substrate use

The ability of the bacterial isolates to utilize different alkanes as a carbon source (C-source) was tested. A summary of the isolates characteristics including Gram stain, colony morphology and their ability to utilize the C-sources provided is given in Table 15. Isolates are identified according to their site of origin, eg. SBO = Scott Base old spill site and SBR= Scott Base recent spill site. The majority of the isolates were Gram-positive rods with orange and round colonies, were able to degrade *n*-

alkanes from C_{11} to C_{16} and all of them degraded the branched alkane pristane. Only one isolate, SBO-9, degraded hexane when provided as a sole C-source.

Table 15. Characteristics of isolates from Scott Base soils

Isolate	Colony colour*	Colony shape*	Cell shape	Gram stain	Carbon sources				
					Hexane (C_6)	Undecane (C_{11})	Dodecane (C_{12})	Hexadecane (C_{16})	Pristane (branched C_{19})
SBO-1	Orange	Round	Rods	+	-	+	+	+	+
SBO-2	Orange	Round	Rods	+	-	+	+	+	+
SBO-4	Orange	Round	Rods	+	-	+	+	+	+
SBO-6	Orange	Round	Rods	+	-	+	+	+	+
SBO-9	Cream	Irregular, slimy	Coccus	+	+	+	+	+	+
SBO-10	Cream	Irregular, slimy	Coccus	+	-	+	+	+	+
SBO-11	Orange	Round	Rods	+	-	+	+	+	+
SBO-12	Orange	Round	Rods	+	-	+	+	+	+
SBO-13	Orange	Round	Rods	+	-	+	+	+	+
SBO-14	Orange	Round	Rods	+	-	+	+	+	+
SBO-19	Orange	Round	Rods	+	-	-	+	+	+
SBR-1	Orange	Round	Rods	+	-	+	+	+	+
SBR-6	Cream	Round	Coccus	-	-	+	+	+	+
SBR-7	Orange	Round	Rods	+	-	+	+	+	+
SBR-8	Orange	Round	Rods	+	-	+	+	+	+
SBR-10	Orange	Round	Rods	+	-	+	+	+	+
SBR-11	Cream	Irregular	Coccus	+	-	+	+	+	+

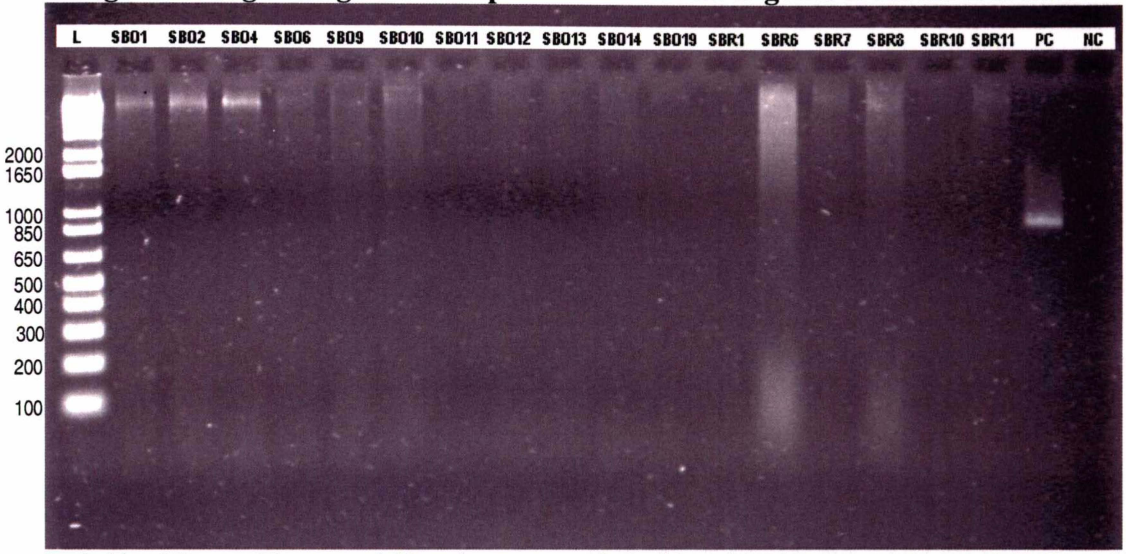
*colony colour and shape in R2A media

Both SBO-9 and SBO-10 isolates produced vast amounts of white slime when grown in RNA media and colonies displayed an irregular shape presumably because of this slime.

3.4.2. PCR of *alkB* genes in hydrocarbon-degrading bacterial isolates

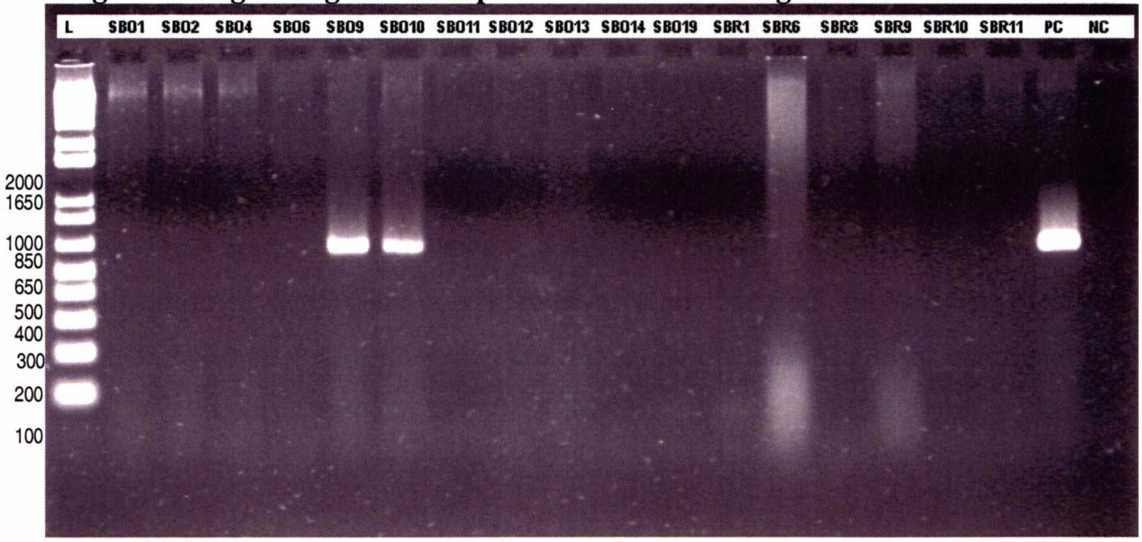
The hydrocarbon-degrading bacterial (HDB) isolates were screened for the presence of *alkB* gene homologues, *alkB*, *alkB1*, *alkB2* and *alkB194,* by PCR amplification with an appropriate gene homologue primer. Sequencing of positive PCR products was performed and sequence analysed by blastN program in the GenBank to confirm the identity of the product. Figures 21-24 show the agarose gels where the PCR products of the different gene homologues were detected by ethidium bromide fluorescence. The positive controls in these PCRs were the strain *Pseudomonas* *putida* PpG6, positive for *alkB*, and the strain *Rhodococcus* sp. 7/1, positive for *alkB1, alkB2, alkB194*. PCR mix was used as a negative control (NC). The size marker used was a 1kb PLUS ladder.

Figure 21. Agarose gel of PCR products from *alk B* genes from HDB isolates



L= ladder; PC = positive control (*Pseudomonas putida* PgG6 strain), NC = negative control (PCR mix)

Figure 22. Agarose gel of PCR products from *alk B1* genes from HDB isolates

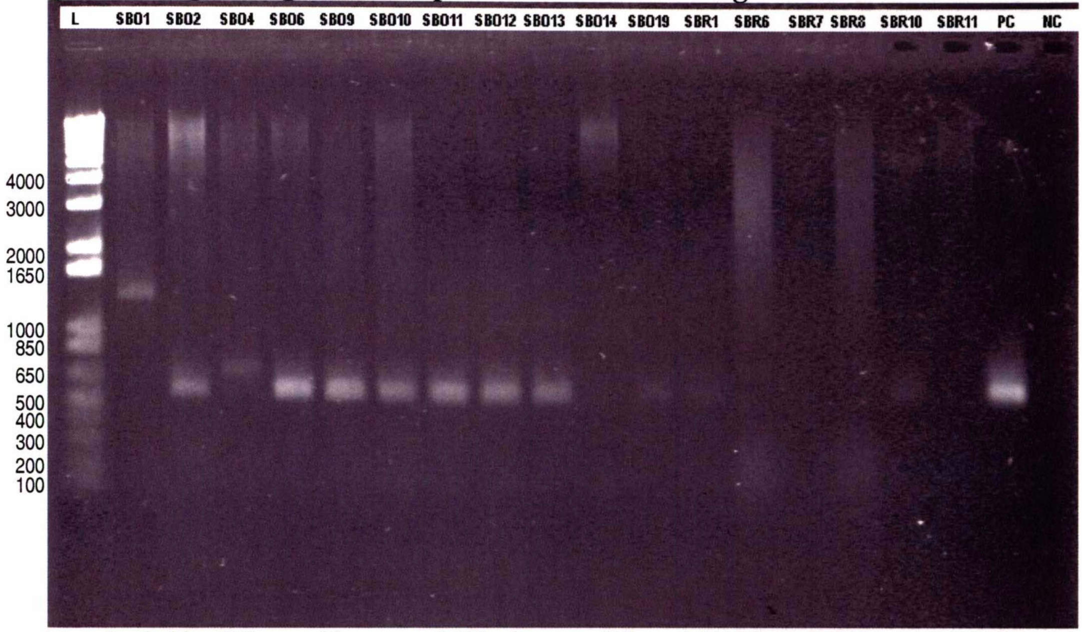


L= ladder; PC = positive control (*Rhodococcus* 7/1 strain), NG = negative control (PCR mix)

As shown in Figure 21, none of the PCR products were positive for the *alkB* gene. Figure 22 shows that only isolates SBO-9 and SBO-10 were positive for *alkB1* gene.

The sequence analysis of the PCR products of both isolates with *alkB1* primers, revealed a 97% identity match with putative *alkB1* gene of *Rhodococcus erythropolis*[117]. Therefore, isolates SBO-9 and SBO-10 are the only ones positive for *alkB1* gene homologue.

Figure 23. Agarose gel of PCR products from *alk B2* genes from HDB isolates

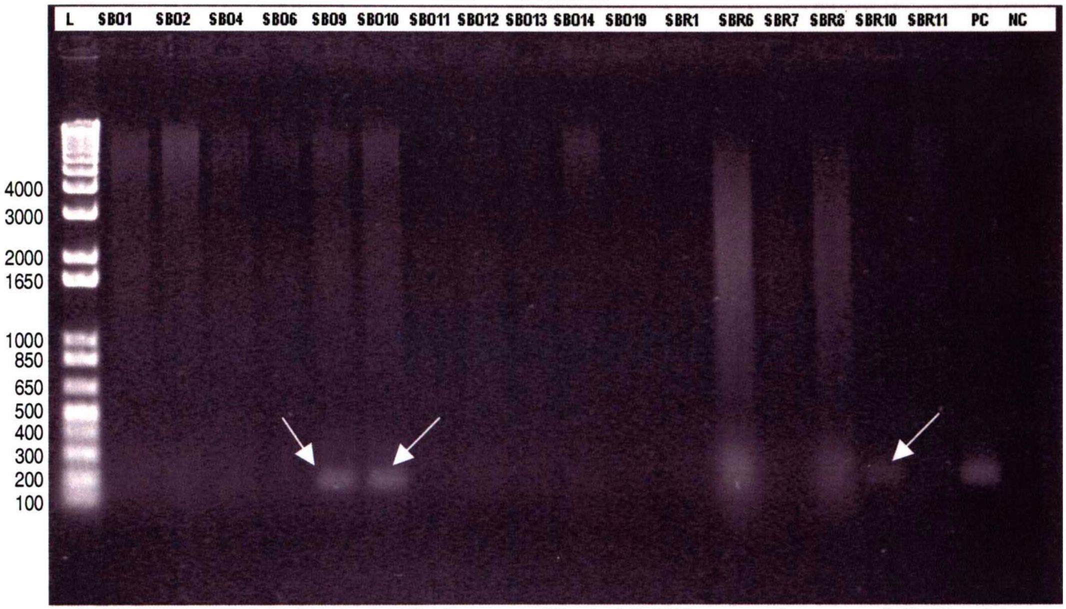


L= ladder; PC = positive control (*Rhodococcus* 7/1 strain), NG = negative control (PCR mix)

The gel in Figure 23 shows that the majority of the isolates showed a positive amplification of *alkB2* gene as evidenced by a positive PCR product of the correct size (552 bp), and visualised by ethidium bromide fluorescence in the gel. However, after DNA sequence analysis, only the following PCR products had a good match and thus were presumptively confirmed to be positive for the *alkB2* gene: SBO-2, SBO-6. SBO-9, SBO-10, SBO-11, SBO-12, SBO-13, SBO-19, SBR-1 and SBR-8. SBO-1 and SBO-4 isolates amplified a different sized band, which did not match with *alkB* genes in the GenBank, indicating either spurious PCR fragments were amplified, or the presence of another homologue of the *alkB* gene with low sequence homology to the *alkB* genes annotated in the GenBank databases.

All the isolates positive for *alkB2* except SBO-9 and SBO-10, had between 83-84% match with *alkB2* gene of *Rhodococcus* sp. strain 1BN, a strain isolated from soil in Italy possibly contaminated with hydrocarbons[8]. DNA sequences from PCR products of SBO-9 and SBO-10 with *alkB2* primers had a 95% match with putative *alkB2* from *Rhodococcus erythropolis* Q15[145].

Figure 24. Agarose gel of PCR products from *alk B194* genes from HDB isolates



L= ladder; PC = positive control (*Rhodococcus* 7/1 strain), NG = negative control (PCR mix)

Figure 24 shows that a positive band in the gel was obtained for PCR products from SBO-9, SBO-10 and SBR-10, as indicated by an arrow. DNA sequence analysis and blast analysis confirmed that only SBO-9 and SBO-10 were positive for *alkB194*. Both strains had a match of 96% with putative alkane-1-oxygenase gene from *Rhodococcus* sp. Q15[117].

Table 16 summarizes the PCR results for *alkB* gene homologues in the HDB isolates from Scott Base based on the sequencing analysis of PCR products. None of the isolates were positive for *alkB; alkB1* was present in only 2 isolates, SBO-9 and SBO-10; the majority of the isolates from the old spill site were positive for *alkB2* and only some were positive for *alkB194.*

Table 16. *alk B* gene profile for HDB isolates based on DNA sequence analysis of positive PCR products

Site of isolation	Isolate number	*alkB*	*alk B1*	*alk B2*	*alk B194*
Scott Base old spill site	SBO-1	-	-	-	-
	SBO-2	-	-	+	-
	SBO-4	-	-	-	-
	SBO-6	-	-	+	-
	SBO-9	-	+	+	+
	SBO-10	-	+	+	+
	SBO-11	-	-	+	-
	SBO-12	-	-	+	-
	SBO-13	-	-	+	-
	SBO-14	-	-	-	-
	SBO-19	-	-	+	-
Scott Base recent spill site	SBR-1	-	-	+	-
	SBR-6	-	-	-	-
	SBR-7	-	-	-	-
	SBR-8	-	-	-	+
	SBR-10	-	-	-	-
	SBR-11	-	-	-	-
Positive controls	*Rhodococcus* 7/1		+	+	+
	Pp G6	+			

3.5 Mineralisation assays

This Section presents the results for several mineralisation assays. Firstly, mineralisation results of the study soils without any amendments are presented; these experiments were conducted to determine the natural or intrinsic ability of the soils to degrade hydrocarbons (indicated by mineralisation of ^{14}C-hexadecane to ^{14}C-CO$_2$). Following this, results of water and nitrogen amendment treatments in Scott Base soils are presented, as well as the "dilution" experiment where contaminated soil was mixed with uncontaminated soil. Finally, results are presented for the bacterial bioaugmentation experiment for Bull Pass soil.

3.5.1 Unamended soils ^{14}C-hexadecane mineralisation assays

All the soils used in this thesis research were tested for their ability to degrade radiolabelled hexadecane under no amendment conditions, that is, no nitrogen and water addition. Produced $^{14}CO_2$ in the mineralisation experiments (methodology as described in Section 2.6) conducted for 75 days was plotted against time. Mineralisation of each soil was compared to a sterile control to ensure $^{14}CO_2$ production was due to microbial activity. Figure 25 shows the results obtained for this group of soils. Figure 26 shows a graph where the y-axis was adjusted to a maximum of 5% $^{14}CO_2$ to visualise with more detail the set of curves with results below 5%.

Figure 25. Mineralisation of ^{14}C-hexadecane in oil-contaminated soils from Ross Sea Region, Antarctica

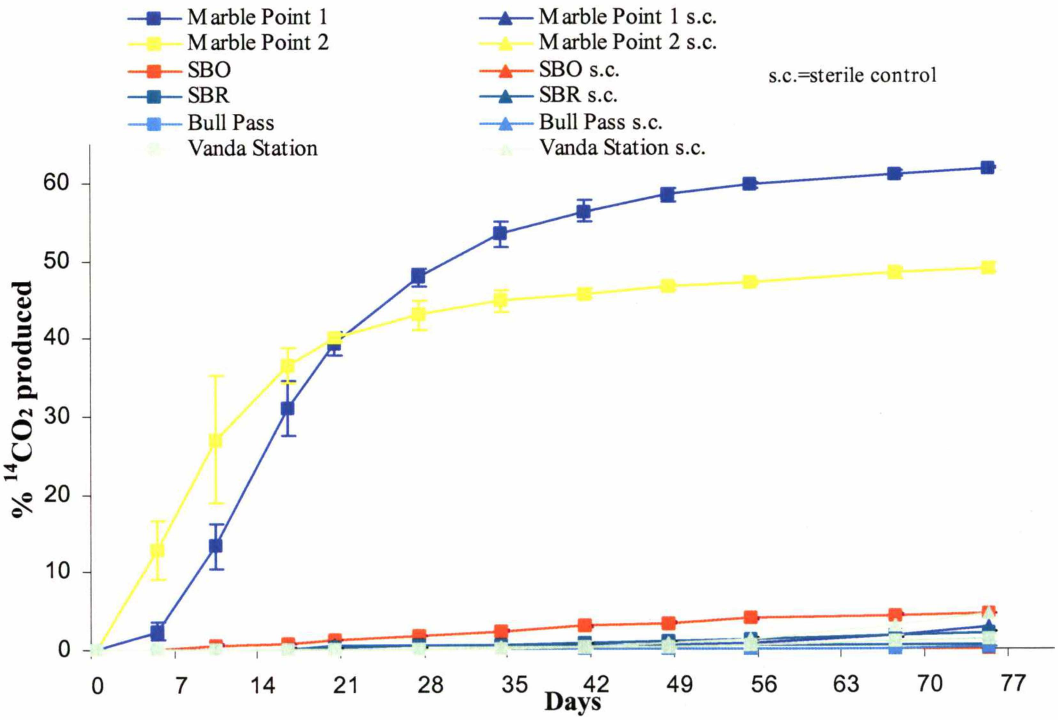
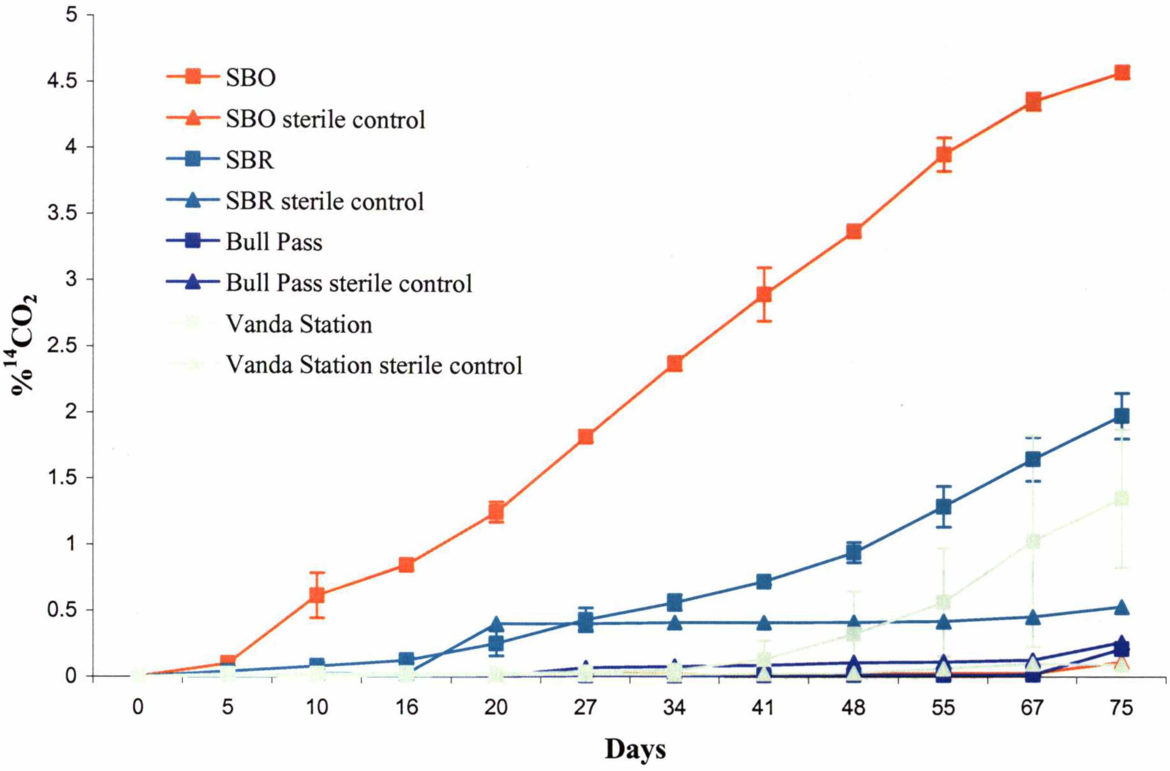


Figure 26. Mineralisation of ^{14}C-hexadecane in oil-contaminated soils from Scott Base, Bull Pass and Vanda Station



Mineralisation was highest in Marble Point 1 soil (60% $^{14}CO_2$ produced in 75 days), followed by Marble Point 2 (*ca.* 50% in 75 days). Because the sterile controls had negligible mineralisation, the results indicated that the $^{14}CO_2$ produced was from microbial respiration. All the other contaminated soils yielded very low (<5%) mineralisation amounts compared to Marble Point. As shown in the graph in Figure 26 and data in Table 17, Scott Base old spill site had increased mineralisation compared to the recent spill site (approximately 2 fold), Vanda Station (3.5 fold) and Bull Pass (22 fold). Vanda Station and Bull Pass soils had negligible mineralisation (<2% after 75 days), which were similar to sterile controls. This indicates that there was no detectable microbial mineralisation of hexadecane in Bull Pass and Vanda Station soils.

Table 17. Maximum mineralisation values obtained in study soils after 75 days

	Soil					
	Marble Point 1	Marble Point 2	Scott Base old spill	Scott Base recent spill	Bull Pass	Vanda Station
$\%^{14}CO_2$	61.78%	48.95%	4.89%	1.98%	1.35%	0.21%

The mineralisation data observed for Marble Point and Scott Base old spill soils conform to a sigmoidal logistic model described by the following equation:

$$y = \frac{a}{1 + \left(\frac{x}{x_o} \right)^b}$$

Where y is the percentage of mineralisation as measured by the $^{14}CO_2$ production, a is the maximum percentage of mineralisation, b is a regression coefficient, and x_o is the initial mineralisation percentage. The values for the parameters described here are summarised in Table 18. These results will be discussed in Section 4.3

Table 18. Kinetic parameters of hexadecane mineralisation in Marble Point and Scott Base old spill site soils

Soil	Mo (%)	Mp (%)	a (%)	b	r^2
Marble Point 1	61.78	61.18	62.57	-2.49	0.99
Marble Point 2	48.95	48.19	49.32	-1.77	0.99
Scott Base old spill	4.89	4.60	6.69	-1.69	0.99

Mo = maximum mineralisation observed; Mp =maximum mineralisation predicted by the model on day 87; a = maximum mineralisation achievable under this model; b = regression coefficient

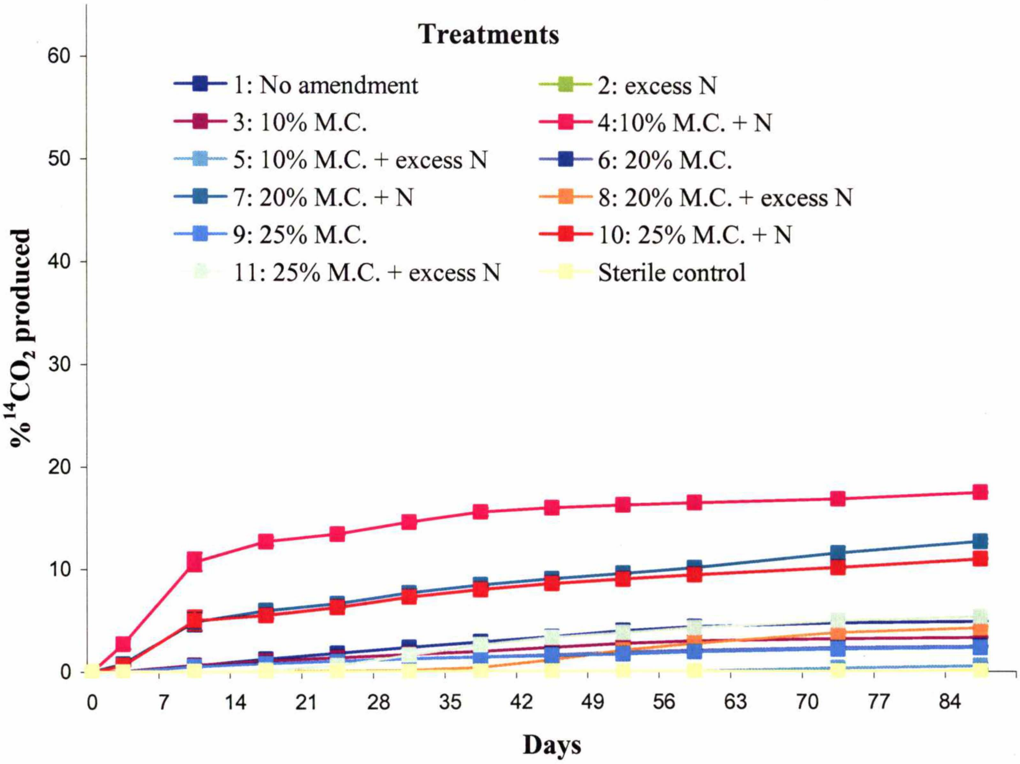
3.5.2. Nitrogen and water amendments in SBO soil

3.5.2.1. Mineralisation results

Given that SBO soil showed reduced mineralisation of hexadecane and that its moisture content and nitrogen content might be limiting microbial biodegradation

(Table 12), factorial treatments were set up where nitrogen and water were added. Water was adjusted to 10, 20 and 25% moisture content (MC) and nitrogen was added in amounts sufficient to reach 2500 mg N/ kg$H_2$0-soil or in excess (5000 mg N/kg soil). This experiment tested the hypothesis that these amendments can overcome some of the limitations to biodegradation. The results for the amendment treatments are presented in Figure 27.

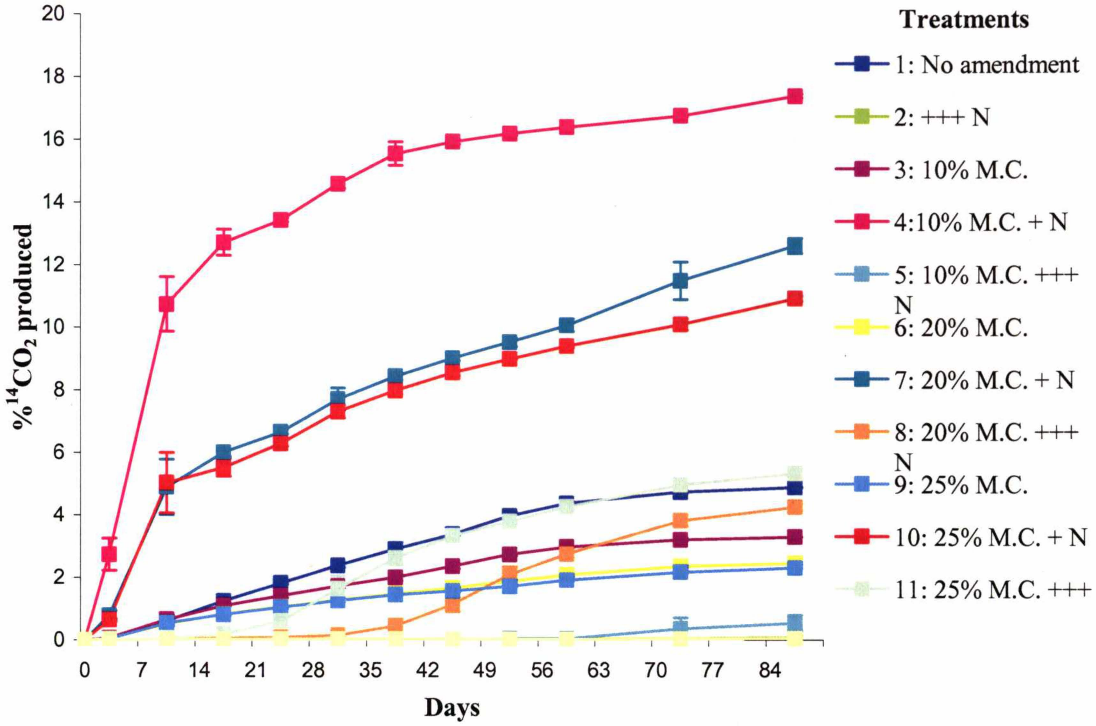
Figure 27. Mineralisation of ^{14}C-hexadecane in SBO soils amended with nitrogen and water



Note: Error bars are present in this figure, but are mostly unresolvable from the symbols; see Figure 28 for a better representation of the error.

There is an increase in mineralisation in SBO soil as a result of the N and water amendment treatments of up to *ca*. 20% after 87 days in treatment 4. The scale of the *y*-axis in the graph was adjusted to a maximum of 20% in Figure 28 to visualise these results with more detail.

Figure 28. Mineralisation of ^{14}C-hexadecane in SBO soils after amendment treatments (graph with adjusted *y*-axis)



+++N = excess nitrogen

Compared to the unamended soil, the most successful treatment was 10% M.C. and nitrogen adjusted to 2500 mg N /kg H_2O-soil (treatment 4). This treatment showed 17.5% mineralisation in 87 days, an increase of 3.5 times the mineralisation of the unamended soil (4.89%). The treatments where water was amended to 20% and 25% MC plus nitrogen adjusted to 2500 mg N/kg H_2O-soil (treatments 7 and 10), showed increased mineralisation too. Treatment 7 showed 12.7% mineralisation and treatment 10 showed 10.9% mineralisation in 87 days. This means an increase of 2.6 and 2.2 times compared to unamended soil, respectively. Treatments where only water content was adjusted (treatments 3, 6 and 9) showed decreased mineralisation compared to unamended control. The treatment where nitrogen was added in excess, treatment 2, showed no mineralisation. Treatments where nitrogen was added in an excess combined with water adjusted to 20 and 25% MC (treatments 8 and 11, respectively) yielded no mineralisation for the first 24 and 31 days, respectively, but gradually increased to levels of unamended soil. Treatment 5 (excess nitrogen, 10% M.C.) showed reduced mineralisation below the baseline of unamended soil.

Table 19 shows a summary of the mineralisation values for all the treatments after 87 days of incubation and the increase in mineralisation compared to the unamended control (if applicable).

Table 19. Summary of mineralisation values for SBO treatments

Treatment number	Treatment	% hexadecane mineralised after 87 days	Increase compared to unamended soil
1	No amendment	4.9	NA
2	Excess nitrogen (5000 mg N/kg)	0.0	none
3	10% M.C.	1.9	none
4	10% M.C. + N (84 mg/kg soil)	17.5	3.5 times
5	10% M.C. + Excess N (5000 mg/kg)	0.0	none
6	20% M.C.	1.2	none
7	20% M.C. + N (334 mg/kg)	12.7	2.6 times
8	20% M.C. + Excess N (5000 mg/ kg)	3.0	none
9	25% M.C.	2.2	none
10	25% + N (459 mg N/ kg)	10.9	2.2 times
11	25% + Excess N (5000 mg/kg)	5.2	none

NA = not applicable

The mineralisation data obtained from treatments 4 and 7 fit a hyperbolic kinetic model described in the following equation:

$$y = \frac{ax}{b+x}$$

where y is the percentage of mineralisation as measured by the $^{14}CO_2$ production, a is the maximum percentage of mineralisation, and b is a regression coefficient. Values for the parameters obtained with this equation are presented in Table 20.

Table 20. Kinetic parameters of mineralisation in SBO amendment treatments

Treatment number	Treatment	Mo (%)	Mp (%)	a (%)	b	r^2
4	10% MC +N	17.47	17.69	19.489	10.16	0.98
7	20% MC +N	12.66	12.35	16.55	33.96	0.98
10	25% MC +N	10.90	10.54	13.612	25.35	0.98

Mo = maximum mineralisation observed on day 87; Mp =maximum mineralisation predicted by the model on day 87; a = maximum mineralisation achievable; b = regression coefficient

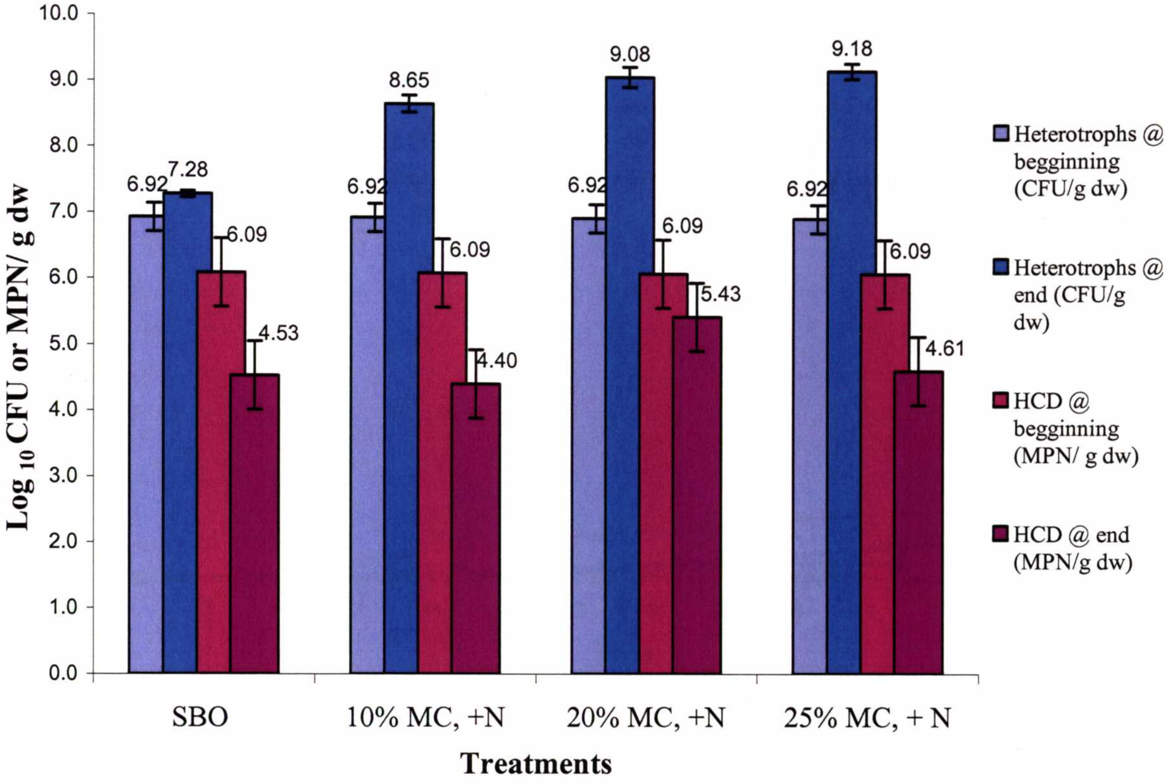
3.5.2.2. Microbial numbers in SBO soil amended with water and nitrogen

The culturable heterotrophs and hydrocarbon-degraders from soils of the most successful amendment treatments for SBO soil (eg. treatments 4, 7 and 10) were enumerated at the beginning and end of mineralisation assays. Results are presented in Table 21 and the logarithms of these numbers are presented in Figure 29. The results show that the heterotrophic population increased in numbers at the end of all treatments by 2 to 3 orders of magnitude. The population of hydrocarbon-degraders decreased in numbers in the unamended soil and in treatments 4 (10% MC +N) and 10 (25% MC +N) by 2 orders of magnitude, while it did not change in treatment 7 (20% MC +N). These results will be further discussed in section 4.3.3.

Table 21. Microbial numbers in SBO amended soils at the beginning and end of incubation period

Treatment number	Treatment	Culturable heterotrophs (CFU/g dw)		Hydrocarbon-degraders (MPN/ g dw)	
		Beginning of treatment	End of treatment	Beginning of treatment	End of treatment
4	10% MC + N	$8.38 \times 10^6 \pm 3.88 \times 10^6$	$4.47 \times 10^8 \pm 9.16 \times 10^7$	$1.23 \times 10^6 \pm 3.302$	$2.52 \times 10^4 \pm 3.302$
7	20% MC + N		$1.16 \times 10^9 \pm 2.75 \times 10^8$		$2.70 \times 10^5 \pm 3.302$
10	25% MC + N		$1.41 \times 10^9 \pm 2.63 \times 10^8$		$4.10 \times 10^4 \pm 3.302$

Figure 29. Culturable heterotrophs and hydrocarbon-degraders in SBO soil at beginning and end of incubation period

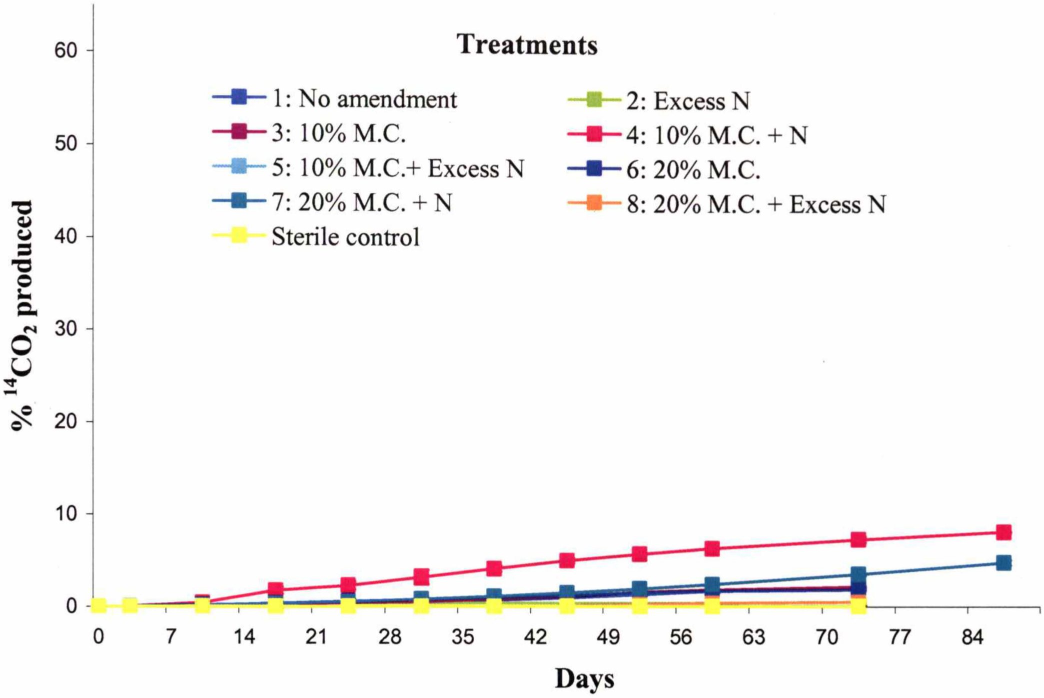


3.5.3. SBR soil amended with nitrogen and water

3.5.3.1. Mineralisation results

SBR soil showed very low natural mineralisation rates compared to Marble Point soils. The same rationale used for the previous amendment treatments in SBO soil was applied to the recent spill soil; specifically, the same nitrogen and water amendments were added (excluding the 25% MC treatment). Mineralisation results for these amendment treatments are presented in Figure 30.

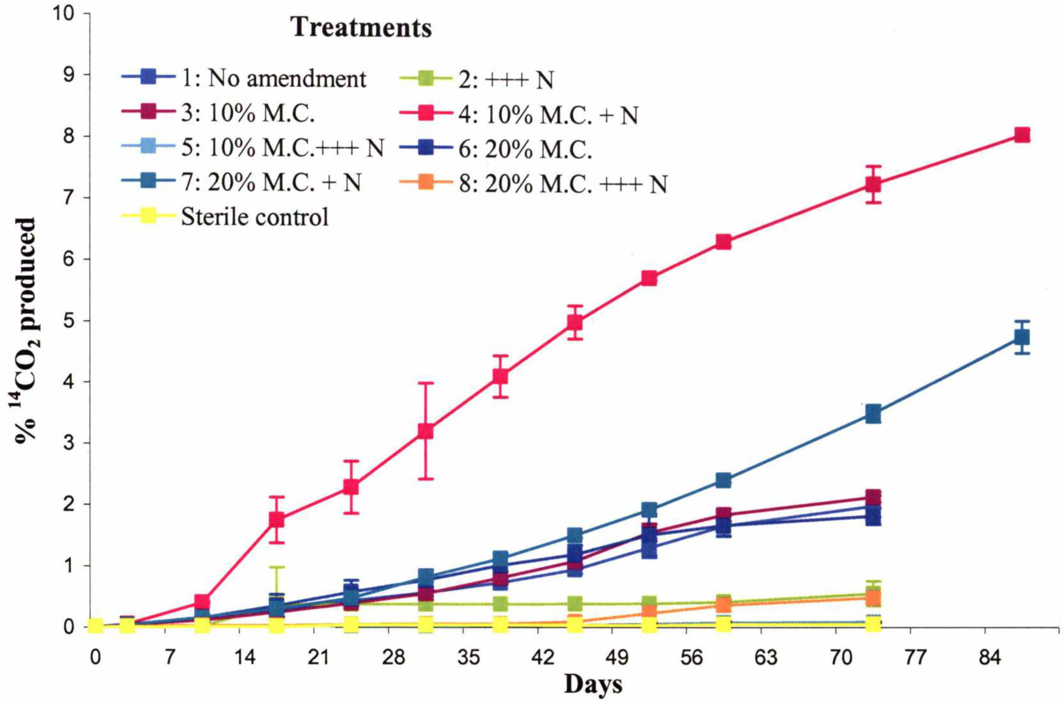
Figure 30. Mineralisation of ^{14}C-hexadecane in SBR soil amended with nitrogen and water



Note: Error bars are present in this figure, but are mostly unresolvable from the symbols; see Figure 31 for a better representation of the error.

There is an increase in mineralisation in SBR soil as a result of the N and water amendment treatments of up to *ca.*10% after 87 days in treatment 4. This was less (as expressed by percentage $^{14}CO_2$ produced) as compared to the value obtained in SBO soil with the same treatment. However, it was a significant increase compared with the unamended control, which had a total 1.98% of $^{14}CO_2$ production. The scale of the *y*-axis in the graph was adjusted to a maximum of 10% in Figure 31 to visualise these results with more detail.

Figure 31. Mineralisation of ^{14}C-hexadecane in SBR soil amended with nitrogen and water (graph with adjusted *y*-axis)



Treatments where nitrogen combined with water was added (treatments 4 with 10% MC and 7 with 20% MC) showed increased mineralisation compared with unamended control. Treatment 4 yielded a better result than 7, that is, 7.2% compared to 3.4% of isotope recovery as $^{14}CO_2$. These two treatments were the most successful ones compared to any other amendment in SBR soil. When soils were amended with water but no nitrogen (treatments 3 with 10% MC and 6 with 20% MC), no increased mineralisation was observed compared to the unamended control (baseline). Treatments with excess nitrogen showed decreased mineralisation compared with unamended control. Table 22 summarises the mineralisation values of all SBR treatments after 73 or 87 days of incubation and increases in mineralisation compared to baseline values. As treatments 4 and 7 had increasing mineralisation curves compared to other treatments after day 73, these were not stopped on day 73 as the rest of the treatments, but were kept further to obtain more data for mineralisation modelling analysis.

Table 22. Summary of mineralisation values for SBR treatments

Treatment number	Treatment	% hexadecane mineralised in 73 or 87* days	Increase compared to unamended soil
1	No amendment	1.5	NA
2	Excess nitrogen (5000 mg N/kg)	0.5	NA
3	10% MC.	0.4	NA
4	10% M.C. + N (50 mg/kg soil)	7.2	4.8 times
5	10% M.C. + Excess N (5000 mg/kg)	0.0	NA
6	20% MC.	1.7	NA
7	20% M.C. + N (300 mg/kg)	3.4	2.3 times
8	20% M.C. + Excess N (5000 mg/ kg)	0.4	NA

NA = not applicable; * treatments 4 and 7 only

The mineralisation data obtained from treatments 4 and 7 conformed to a sigmoidal logistic model described by the following equation:

$$y = \frac{a}{1+\left(\frac{x}{x_o}\right)^b}$$

Where y is the percentage of mineralisation as measured by $^{14}CO_2$, a is the maximum percentage of mineralisation, b is a regression coefficient, and x_0 is the initial mineralisation percentage. The values for the parameters described here are summarised in Table 23.

Table 23. Kinetic parameters of mineralisation in SBR amendment treatments

Treatment number	Treatment	Mo (%)	Mp (%)	a (%)	b	r^2
4	10% MC +N	8.06	8.06	11.43	-1.72	0.99
7	20% MC +N	4.75	4.68	6.19	17.86	0.99

Mo = maximum mineralisation observed; Mp =maximum mineralisation predicted by the model on day 87; a = maximum mineralisation achievable under this model; b = regression coefficient

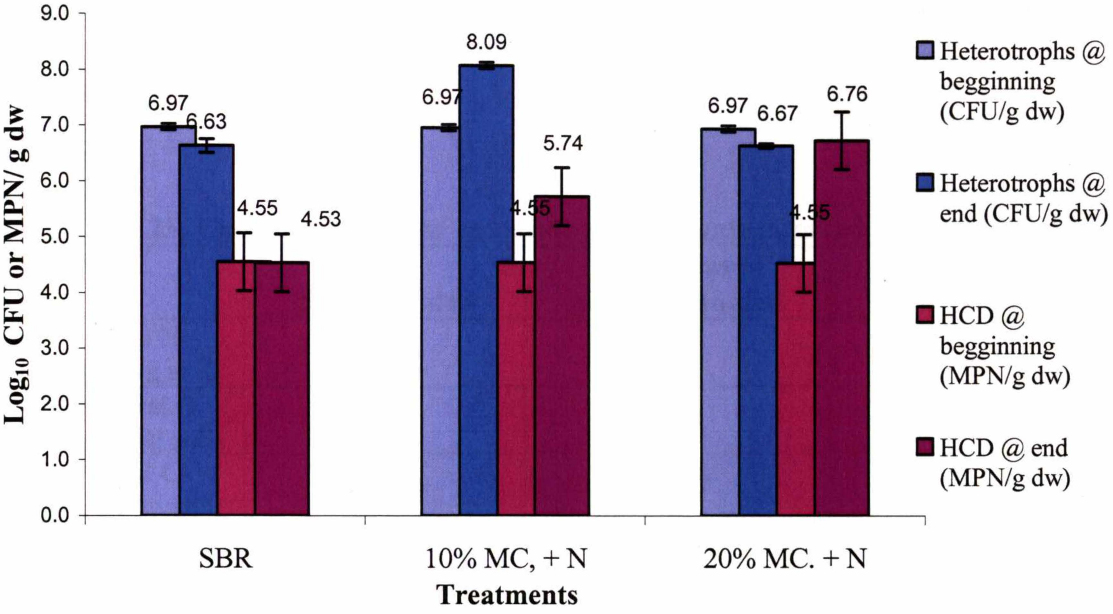
3.5.3.2. Microbial numbers in SBR soil amended with nitrogen and water

Culturable heterotrophs and HCD were enumerated at beginning and end of the incubation period in mineralisation microcosms. Results are presented in Table 24. Numbers from this Table were transformed to logarithms in order to present them in the graph shown in Figure 32. The heterotrophic population increased in treatment 4 (10% MC, +N) by two orders of magnitude, but decreased slightly in treatment 7 (20% MC, +N) and the unamended soil. This decrease was negligible because values were still found within the same order of magnitude. In terms of hydrocarbon-degraders, numbers did not change in the non-amended soil, while they increased in both of the soils amended with nitrogen and water. HCD increased by one order of magnitude in treatment 4 and by 2 orders of magnitude in treatment 7.

Table 24. Microbial numbers in SBR amended soils at beginning and end of incubation period

Treatment number	Treatment	Culturable heterotrophs (CFU/g dw)		Hydrocarbon-degraders (MPN/ g dw)	
		Beginning of treatment	**End of treatment**	**Beginning of treatment**	**End of treatment**
1	**Unamended**	9.29×10^6 $\pm 1.14 \times 10^6$	4.28×10^6 $\pm 1.18 \times 10^6$	3.54×10^4 ± 3.30	3.42×10^4 ± 3.30
4	**10% MC, + N**	9.29×10^6 $\pm 1.14 \times 10^6$	1.22×10^8 1.50×10^7	3.54×10^4 ± 3.30	1.23×10^8 ± 3.30
10	**20% MC, + N**	9.29×10^6 $\pm 1.14 \times 10^6$	4.44×10^8 $\pm 4.33 \times 10^7$	3.54×10^4 ± 3.30	4.63×10^6 ± 3.30

Figure 32. Culturable heterotrophs and hydrocarbon-degraders in SBR soil at beginning and end of incubation period



3.5.4. Dilution experiment

Given that the best amendment treatment of SBO soil resulted in a 3.5 times increase in mineralisation compared to the unamended control, it was considered that other limiting factors were acting against a more efficient mineralisation, assuming that higher mineralisation could be reached. One hypothesis was that the nature of the contaminant mixture did not allow an optimal microbial activity. For this reason, an experiment where SBO soil was mixed in a 1:1 proportion with uncontaminated soil was designed. The aim of this experiment was to test if the "dilution" of the soil (and thus of the contaminant) would stimulate microbial degradation. Further analysis included nitrogen and water amendments to test how these factors would interact with each other.

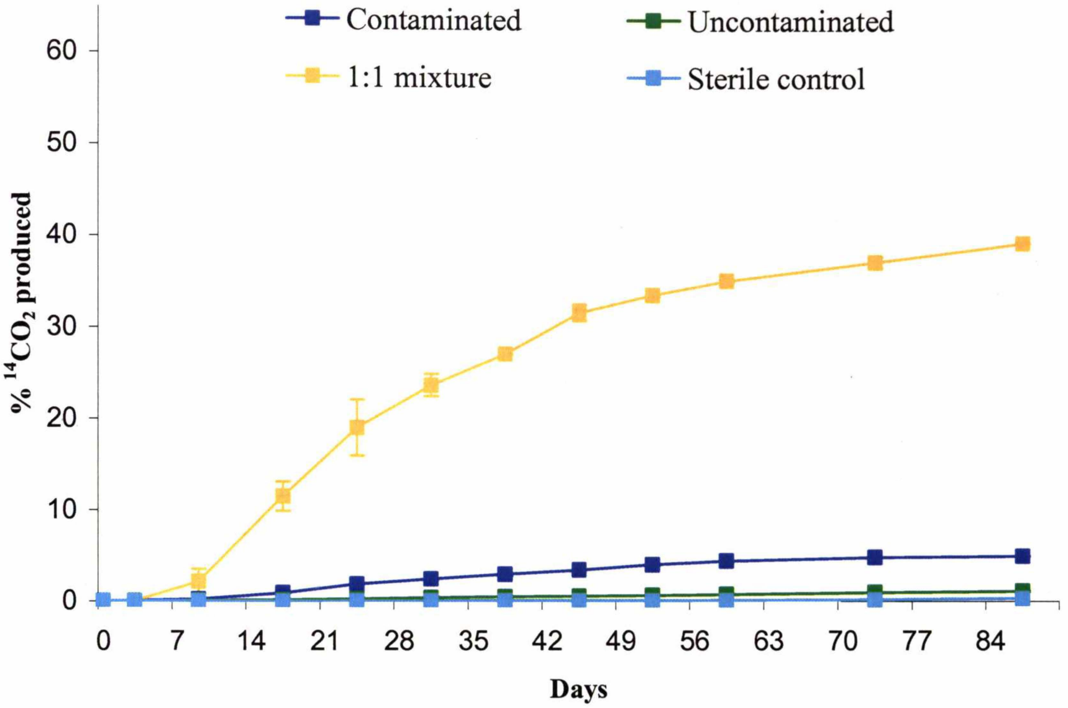
3.5.4.1 Contaminated vs. uncontaminated vs. 1:1 Mixture

The mineralisation of hexadecane was compared in SBO contaminated soil with uncontaminated soil and its 1:1 mixture. Values of the nutrient contents in the three soils are given in Table 25. The results of this experiment are presented in Figure 33.

Table 25. Chemical values of contaminated:uncontaminated mixed SBO soil

	SBO contaminated	**Scott Base uncontaminated**	**1:1 mixed unamended**
Total N (mg N/kg soil)	160	100	120
Mineral N (mg N/ kg soil)	0.7	14.8	3.93
Total C (mg C/ kg soil)	45,000	966.7	20,000
Total P (mg P/ kg soil)	2157.6	1993	2230
Available P (mg P/ kg soil)	5.6	9.5	8
Moisture content (%)	2.5	2.5	2.5
C:N	281:1	10:1	167:1
N_{H2O} (mg N/kg H_2O)	6400	4000	4800

Figure 33. Mineralisation of ^{14}C-hexadecane from contaminated, uncontaminated, and 1:1 mixture SBO soil



Note: Error bars are present in this figure, but are mostly unresolvable from the symbols given that error values are small.

The effect of mixing the two soils notably increased mineralisation. After 87 days of incubation, the mixed soil showed 38.9% of hexadecane mineralised. This was an increase of 8 times compared with contaminated soil. There was a marginal mineralisation of 1% observed in the uncontaminated soil, however this was negligible compared to the other 2 soils and the sterile control.

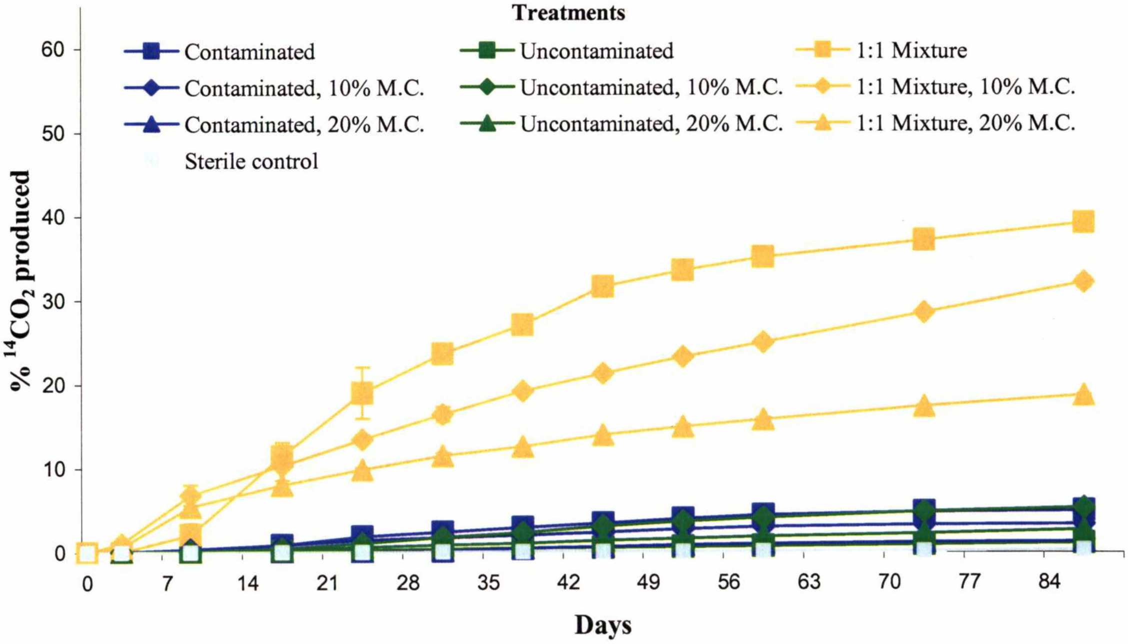
3.5.4.2. Water amendments

Moisture content was adjusted to 10% and 20% in contaminated, uncontaminated and 1:1 mixed soil to test if the addition of water further increased the mineralisation of hexadecane in the mixed soil. Contaminated and uncontaminated soils were included in these assays as controls to ensure that any possible increase in mineralisation was truly a result of the mixture of soils. Results of these assays are presented in Figure 34. The scale of the *y*-axis in the graph was adjusted to a maximum of 10% in Figure 35 to visualise these results with more detail. N_{H2O} values of the treatments are presented in Table 26.

Table 26. Nitrogen values in water amended dilution treatments

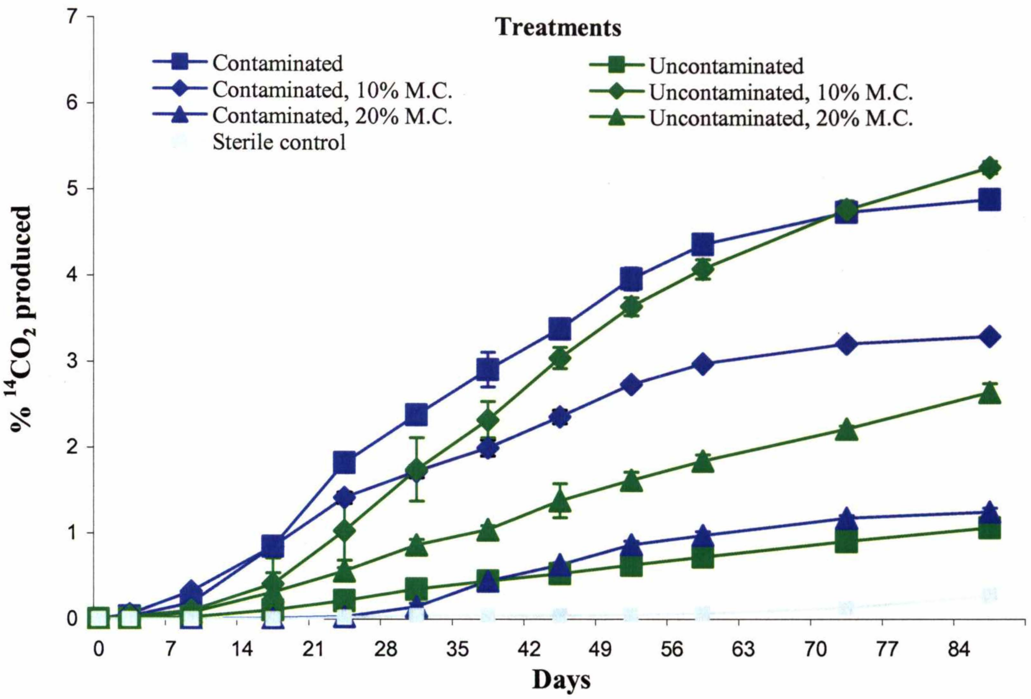
Treatment	Total N (mg N/ kg dw)	N_{H2O} (mg N/ kg-H_2O-soil)
Contaminated, 10% MC	160	1600
Uncontaminated, 10%MC	100	1000
Mixed, 10% MC	120	1200
Contaminated, 20% MC	160	800
Uncontaminated, 20%MC	100	500
Mixed, 20% MC	120	600

Figure 34. Water amendments in contaminated, uncontaminated and mixed SBO soil



Note: Error bars are present in this figure, but are mostly unresolvable from the symbols given that error values are small.

Figure 35. Water amendments in contaminated, uncontaminated and mixed SBO soil (with *y*-axis adjusted)



Note: Error bars are present in this figure, but are mostly unresolvable from the symbols given that error values are small.

The data from the graphs in Figures 34 and 35 clearly demonstrated that the addition of water to the mixed soil had no further enhancing effect in the mineralisation of hexadecane on top of the observed enhancement that resulted solely from mixing the 2 soils (as shown in Figure 33). It is also evident that as more water was added, less mineralisation was observed in both the diluted and the contaminated soils. Low mineralisation was observed in the uncontaminated soil (0.9%), and surprisingly, addition of water increased mineralisation in this soil to *ca*. 5.0%.

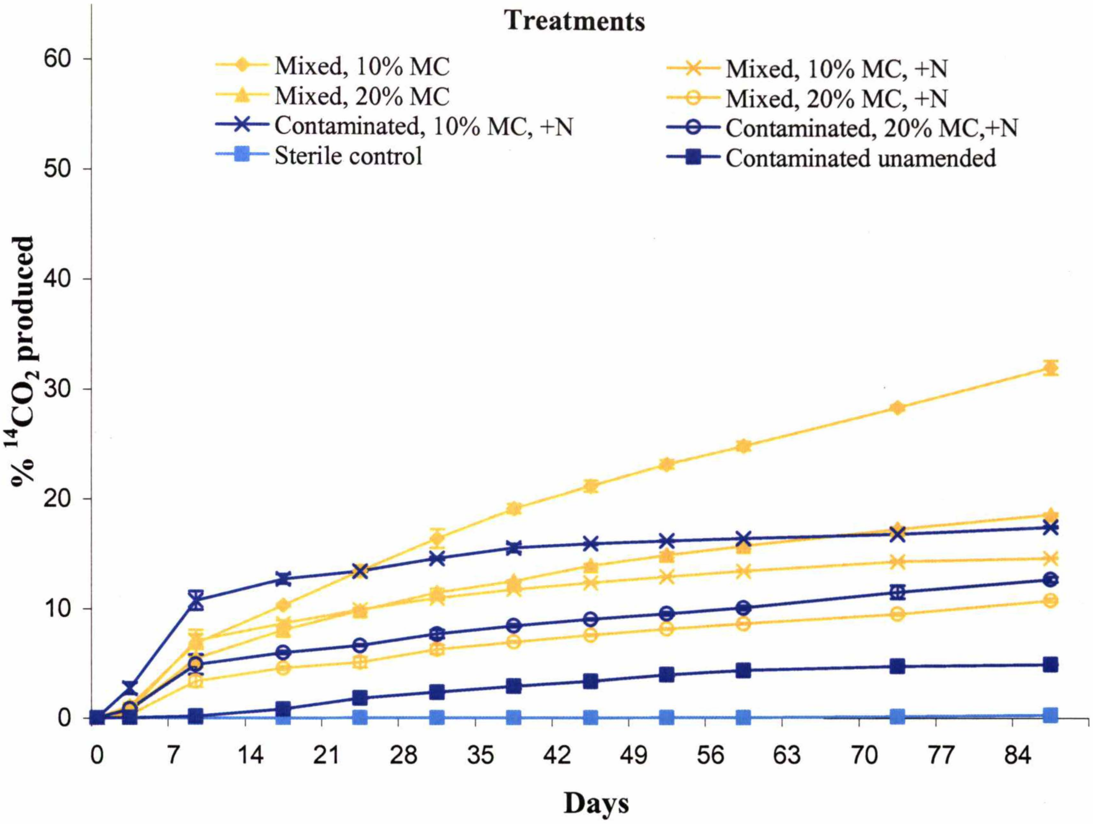
3.5.4.3. Water and nitrogen amendments in 1:1 mixed soil

Amendments of water and nitrogen adjusting to *ca.* 2500 mg N/ kg-H_2O-soil were made to SBO contaminated:uncontaminated (1:1) mixed soil to test if these treatments further enhanced mineralisation. Results of these mineralisation assays are presented in graphical form in Figure 36. Values of N_{H2O} and total nitrogen in these fertilized soils are presented in Table 27. Contaminated soil data from the SBO amendment treatments was included in this graph to compare values obtained in non-mixed soil with the same amendment treatments. In the graph in Figure 36, the data from mixed soil treatments are indicated in yellow, and the data from contaminated non-mixed soil in blue.

Table 27. Nitrogen values in water and nitrogen amended dilution treatments

Treatment	**Total N (mg N/ kg dw)**	**N_{H2O} (mg N/ kg-H_2O-soil)**
Contaminated, 10% MC +N	250	2500
Mixed, 10% MC + N	290	2900
Contaminated, 20% MC +N	500	2500
Mixed, 20% MC +N	540	2700

Figure 36. Mineralisation of ^{14}C-hexadecane in mixed and contaminated SBO soil amended with water and nitrogen



For the mixed soil, the addition of nitrogen and water, both 10% MC +N and 20% MC +N, reduced mineralisation compared to the treatments where only water was added (10% and 20% MC treatments). Therefore, unlike the contaminated soil, the addition of nitrogen did not have an enhancing effect on the mixed soil.

All the treatments tested in the "dilution" experiments enhanced mineralisation of hexadecane to a certain degree compared to the unamended SBO soil (taken as a baseline).

Similar treatments in mixed and non-mixed soils did not have the same effects. While in SBO non-mixed soil, nitrogen and water addition enhanced mineralisation more than water amendments. In mixed soil, it was the addition of water only that enhanced mineralisation substantially, and supplementation with nitrogen did not enhance mineralisation further, even when total nitrogen was adjusted to *ca.* 2500 mg N/ kg H_2O-soil.

Comparing all the treatments tested for SBO soils (N-water amendments & dilution), mineralisation of hexadecane was enhanced the most by diluting contaminated with uncontaminated soil. Table 28 shows a list of the treatments that enhanced mineralisation, in decreasing order of total percentage $^{14}CO_2$ produced for the last day of incubation, and the increase that this implies as compared to the unamended contaminated soil (SBO soil's intrinsic mineralisation).

Table 28. Mineralisation results for dilution-amendment treatments

Treatment	% hexadecane mineralised after 87 days	Increase respective to unamended control
Mixed, unamended	38.9	8.0 times
Mixed, 10% MC	31.4	6.4 times
Mixed, 20% MC	18.1	3.7 times
Contaminated 10% M.C. + N (84 mg/kg soil)	17.0	3.5 times
Mixed, 10% MC, +N (170 mg/kg)	14.1	2.9 times
Contaminated 20% M.C. + N (334 mg/kg)	12.5	2.6 times
Contaminated 25% MC + N (459 mg N/ kg)	10.9	2.2 times
Mixed, 20% MC, + N (420 mgN/kg)	10.4	2.1 times
Contaminated, unamended (CONTROL)	4.9	NA

NA = not applicable

The mineralisation data of the 1:1 dilution treatment and 1:1 dilution + 10% MC fit a sigmoidal logistic model described by the following equation:

$$y = \frac{a}{1 + \left(\frac{x}{x_o} \right)^b}$$

Where y is the percentage of mineralisation as measured by $^{14}CO_2$, a is the maximum percentage of mineralisation, b is the regression coefficient, and x_0 is the initial mineralisation percentage.

The data from the 1:1 dilution + 20% MC treatment fit a hyperbolic model described by the following equation:

$$y = \frac{ax}{b + x}$$

Where y is the percentage of mineralisation as measured by the $^{14}CO_2$ production, a is the maximum percentage of mineralisation, and b is the regression coefficient. Values of kinetic parameters are summarised in Table 29 for the three treatments. The implications of these results will be discussed in section 4.3.4.

Table 29. Kinetic parameters of mineralisation in SBO dilution-amendment treatments

Treatment	**Mo (%)**	**Mp (%)**	**a (%)**	**b**	**r^2**
1:1 dilution	39.15	38.64	41.74	-2.17	0.99
1:1 dilution +10%MC	32.08	31.66	74.06	-0.93	0.99
1:1 dilution + 20% MC	18.63	18.39	43.14	43.14	0.99

Mo = maximum mineralisation observed; Mp =maximum mineralisation predicted by the model on day 87; a = maximum mineralisation achievable under this model; b = regression coefficient

3.5.4.4. Microbial numbers in dilution experiments

To investigate the behaviour (quantitatively) of the bacteria in the soil as a result of the dilution-amendment treatments, enumerations of culturable heterotrophs and hydrocarbon-degraders in the soils from the most successful treatments were made at the beginning and end of the incubation period, and the results are given in Table 30 and Figure 37.

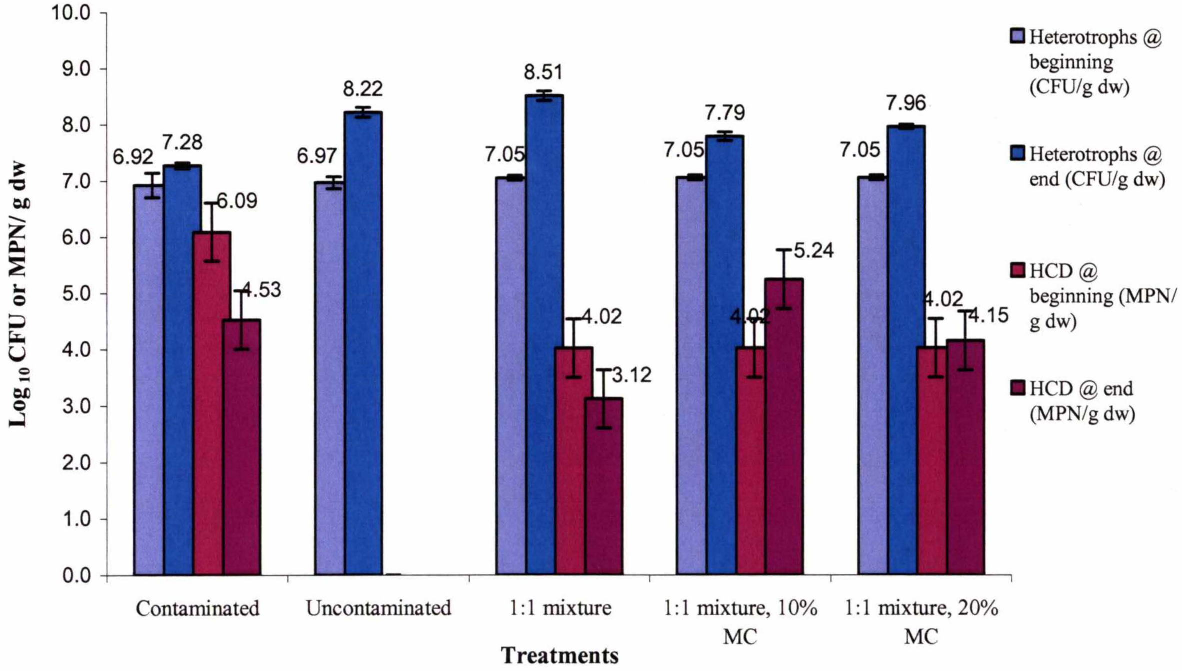
At the beginning of the mineralisation assays with the mixing of contaminated with uncontaminated soil (1:1 mixture), no increase in numbers of heterotrophs was observed. On the other hand, numbers of HCD decreased from 10^6 to 10^4 CFU/g dw as a result of mixing the 2 soils.

At the end of the mineralisation assays, a general small increase in heterotrophs was observed in all the treatments, including the uncontaminated soil. In terms of hydrocarbon-degraders, the unamended soil decreased in two orders of magnitude, the mixed soil did not change, the mixed soil amended to 10% MC increased in one order of magnitude and the mixed soil amended to 20% MC did not vary. Thus, there is no general tendency in HCD numbers in the treatments tested here.

Table 30. Microbial numbers in SBO diluted-amended soil

Treatment	Culturable heterotrophs (CFU/g dw)		Hydrocarbon-degraders (MPN/ g dw)	
	Beginning of treatment	End of treatment	Beginning of treatment	End of treatment
SBO contaminated	8.38×10^6 $\pm 3.88 \times 10^6$	1.89×10^7 $\pm 2.16 \times 10^6$	1.23×10^6 ± 3.30	3.36×10^4 ± 3.30
SBO uncontaminated	1.17×10^6 $\pm 2.79 \times 10^5$	1.66×10^8 $\pm 3.22 \times 10^7$	<10	<10
1:1 mixture	1.13×10^7 $\pm 9.7 \times 10^5$	3.25×10^8 $\pm 4.15 \times 10^6$	1.04×10^4 ± 3.30	1.3×10^3 ± 3.30
1:1 mixture + 10% MC	1.13×10^7 $\pm 9.7 \times 10^5$	6.05×10^7 $\pm 1.01 \times 10^7$	1.04×10^4 ± 3.30	1.73×10^5 ± 3.30
1:1 mixture + 20% MC	1.13×10^7 $\pm 9.7 \times 10^5$	8.76×10^7 $\pm 8.20 \times 10^6$	1.04×10^4 ± 3.30	1.40×10^4 ± 3.30

Figure 37. Culturable heterotrophs and hydrocarbon-degraders in SBO soil at the beginning and end of dilution-amendment treatments



3.5.5. Bull Pass bioaugmentation experiment

3.5.5.1. Mineralisation results

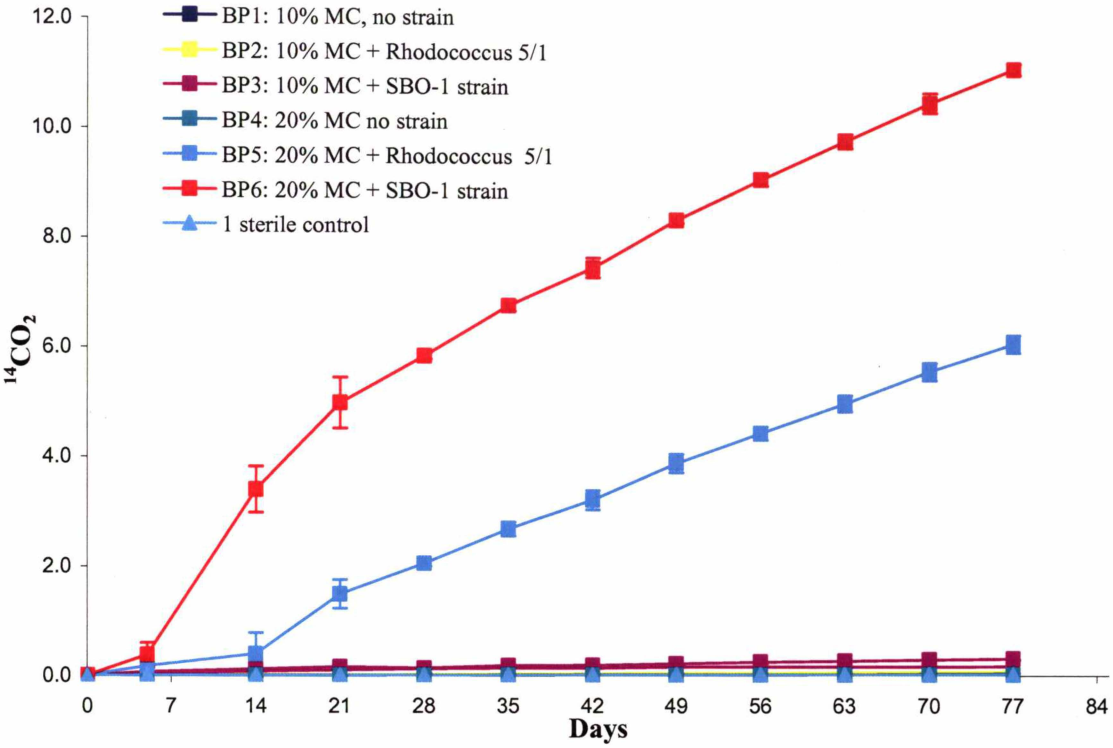
As previously shown in Section 3.2 with results given in Table 13, no hydrocarbon-degrading bacteria were detected in Bull Pass soil. The hydrocarbon contamination in this site was mainly C_9 to C_{15} *n*-alkanes (see Appendix 2). Therefore, an experiment was designed where this soil was bioaugmented with 2 strains known to degrade alkanes; *Rhodococcus* 5/1[17] and SBO-1, an isolate from this thesis research (described in Table 15). The inoculum used to bioaugment the soil was enumerated by the dilution-plating method in R2A plates and the numbers of bacteria inoculated are presented in Table 31.

Table 31. Counts in R2A media of inoculum added

Inoculum	CFU/ml	In 20 g soil	CFU/ g dw
SBO-1	2.32×10^9	4.64×10^8	2.32×10^7
Rhodococcus 5/1	1.13×10^9	2.26×10^8	1.13×10^7

Treatments in this experiment included moisture content adjustments. There were no nitrogen amendments. Given that the mineral nitrogen of this soil is relatively high (263 mg NO_3-N /kg), it was assumed that nitrogen was not a limiting factor for this soil. The hexadecane mineralisation results of this bioaugmentation assays are given in Figure 38.

Figure 38. Mineralisation of ^{14}C-hexadecane from Bull Pass bioaugmented soil



Treatments BP5 and BP6 resulted in positive mineralisation, with 11% and 6% of isotope recovery, respectively. This was a substantial increase compared to uninoculated soil (0.02%) after 77 days. No other treatment enhanced mineralisation.

The kinetic behaviour of the mineralisation data for treatment BP5 fit a sigmoidal model described by the following equation:

$$y = \frac{a}{1 + \left(\frac{x}{x_o}\right)^b}$$

Where y is the percentage of mineralisation as measured by $^{14}CO_2$, a is the maximum percentage of mineralisation, b is the regression coefficient, and x_0 is the initial mineralisation percentage.

Treatment BP6 fits a hyperbolic model described by the following equation:

$$y = \frac{ax}{b+x}$$

Where y is the percentage of mineralisation as measured by the $^{14}CO_2$ production, a is the maximum percentage of mineralisation, and b is the regression coefficient. Values of kinetic parameters are summarised in Table 32 for the two treatments. The models predict that the maximum mineralisation that can be reached, if the data follow the same behaviour for treatments BP5 and BP6, are 10.84% and 22.6%, respectively.

Table 32. Kinetic parameters of mineralisation in Bull Pass bioaugmentation treatments

Treatment number	**Treatment**	**Mo (%)**	**Mp (%)**	**a (%)**	**b**	**r^2**
BP5	**5/1 + 20% MC**	5.5	5.48	10.84	-1.68	0.99
BP6	**SBO-1 + 20% MC**	10.5	10.30	22.6	82.57	0.99

Mo = maximum mineralisation observed; Mp =maximum mineralisation predicted by the model on day 87; a = maximum mineralisation achievable under this model; b = regression coefficient

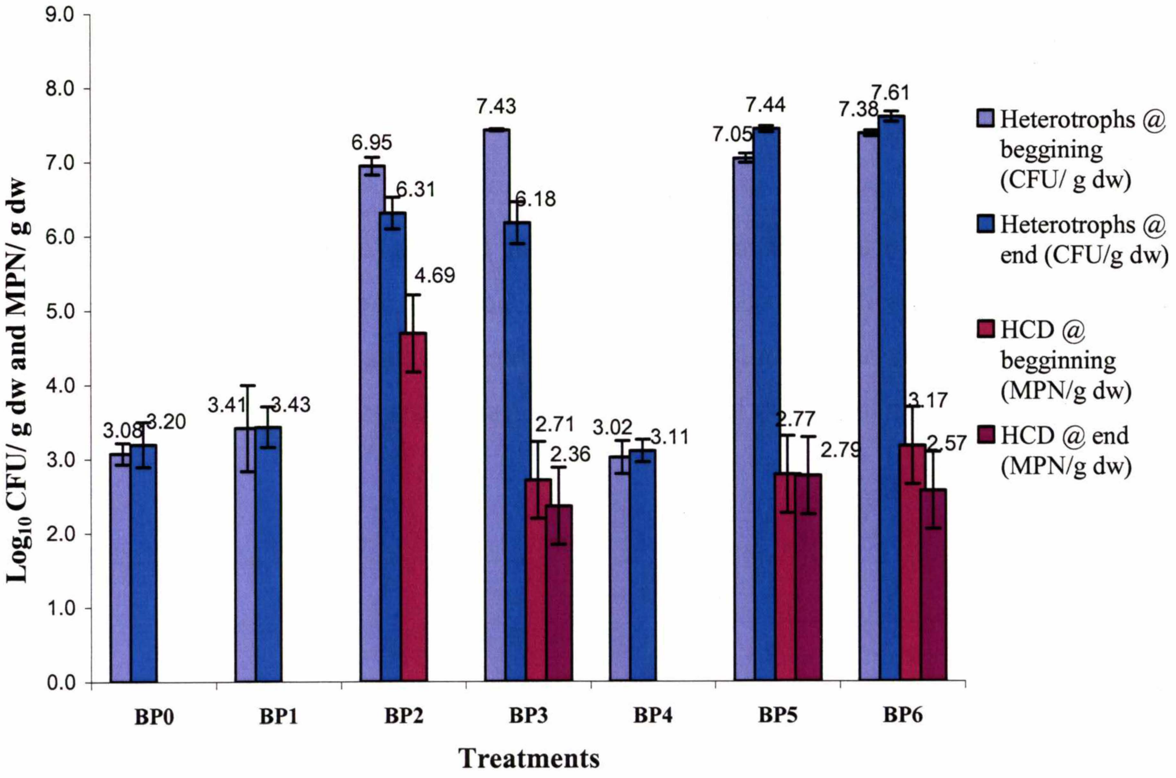
3.5.5.2. Microbial numbers in Bull Pass bioaugmented soil

Numbers of culturable heterotrophs and HCD were also monitored in the soils of this mineralisation experiment at the beginning and end of the incubation period. Numbers of hydrocarbon-degraders in the soil were expected to reflect the amount of bacteria inoculated (10^8 CFU/g dw) given that no HCD were detected previously in this soil in this work (see Table 13). However, as shown in Table 33 and Figure 39, the numbers of hydrocarbon-degraders enumerated in the inoculated soils immediately after inoculation (beginning of treatment) are between 3 and 5 orders of magnitude less than the bacteria inoculated (as given in Table 29). From 10^7 CFU/g dw inoculated bacteria, only 10^3 to 10^4 CFU/ g dw survived in the soil after inoculation.

Table 33. Microbial numbers in bioaugmentend Bull Pass soils

Treatment	Culturable heterotrophs (CFU/g dw)		Hydrocarbon-degraders (MPN/ g dw)	
	Beginning of treatment	End of treatment	Beginning of treatment	End of treatment
Bull Pass uninoculated soil	1.19×10^3 $\pm 2.64 \times 10^2$	1.57×10^3 $\pm 7.33 \times 10^2$	<10	<10
BP1 (10% MC)	2.59×10^3 $\pm 2.03 \times 10^3$	2.71×10^3 $\pm 1.16 \times 10^3$	<10	<10
BP2 (5/1 + 10% MC)	8.89×10^6 $\pm 1.68 \times 10^6$	2.06×10^6 $\pm 6.83 \times 10^5$	4.95×10^4 ± 3.30	<10
BP3 (SBO-1 +10% MC)	2.71×10^7 $\pm 6.79 \times 10^5$	1.51×10^6 $\pm 6.61 \times 10^5$	5.15×10^2 ± 3.30	2.30×10^2 ± 3.30
BP4 (20% MC)	1.04×10^3 $\pm 3.61 \times 10^2$	1.28×10^3 $\pm 3.02 \times 10^2$	<10	<10
BP5 (5/1 + 20% MC)	1.12×10^7 $\pm 1.05 \times 10^6$	2.78×10^7 $\pm 1.72 \times 10^6$	6.12×10^2 ± 3.30	5.9×10^2 ± 3.30
BP6 (SBO-1 + 20% MC)	2.41×10^7 $\pm 1.52 \times 10^6$	4.04×10^7 $\pm 4.16 \times 10^6$	1.48×10^3 ± 3.30	3.70×10^2 ± 3.30

Figure 39. Culturable heterotrophs and hydrocarbon-degraders in BP soil at beginning and end of incubation period



Enumerations at the end of the incubation time showed that numbers of culturable heterotrophs did not change in the unamended soil and in soils with 10% and 20% moisture content (BP1 and BP4 respectively). Heterotrophic populations decreased in inoculated treatments where water was adjusted to 10% MC (BP2 and BP3), while they increased in inoculated soils adjusted to 20% MC (BP5 and BP6).

The numbers of hydrocarbon-degraders did not change at the end of this experiment for any of the treatments, except in the soil inoculated with strain 5/1 and adjusted to 10% MC, where no hydrocarbon-degraders were recovered when the experiment was ended (nor were they present in the soil when the experiment started).

3.5.5.3. Total Petroleum Hydrocarbon (TPH) analysis

The content of total petroleum hydrocarbons in all treatments was measured at the beginning and end of the mineralisation assays in Bull Pass bioaugmented soil to detect any possible decrease in hydrocarbons resulting from biodegradation. Results of TPH analysis are presented in Table 34 and Figure 40; chromatograms are presented in Appendix 2.

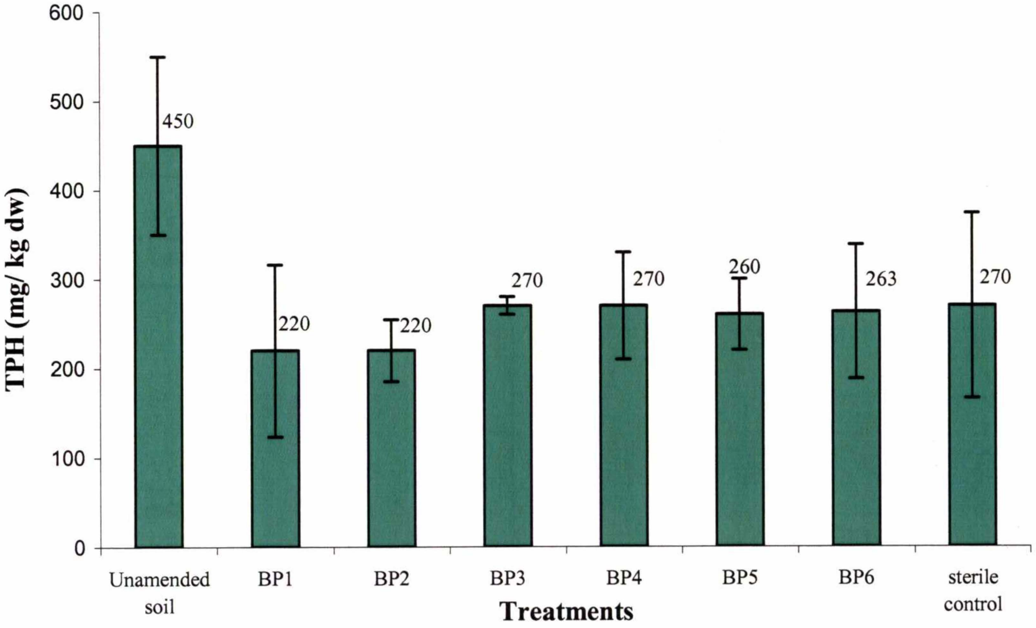
A reduction in TPH was observed in all treatments at the end of the incubation period, including the sterile control. Given that similar reductions in TPH were observed in all treatments and the sterile control, this reduction can only be explained by abiotic loss of the hydrocarbons rather than biological degradation.

Table 34. Amounts of total petroleum hydrocarbons in Bull Pass bioaugmentation treatments at beginning and end of incubation period

Treatment	TPH*				
	1	2	3	Average	Standard deviation
Unamended soil (beginning)	550	350	450	450	100
BP1	330	180	150	220	96
BP2	180	240	240	220	34
BP3	280	260	270	270	10
BP4	210	270	330	270	60
BP5	220	300	260	260	40
BP6	220	220	350	263	75
Sterile control	210	390	210	270	104

* 3 replicates were analysed per treatment

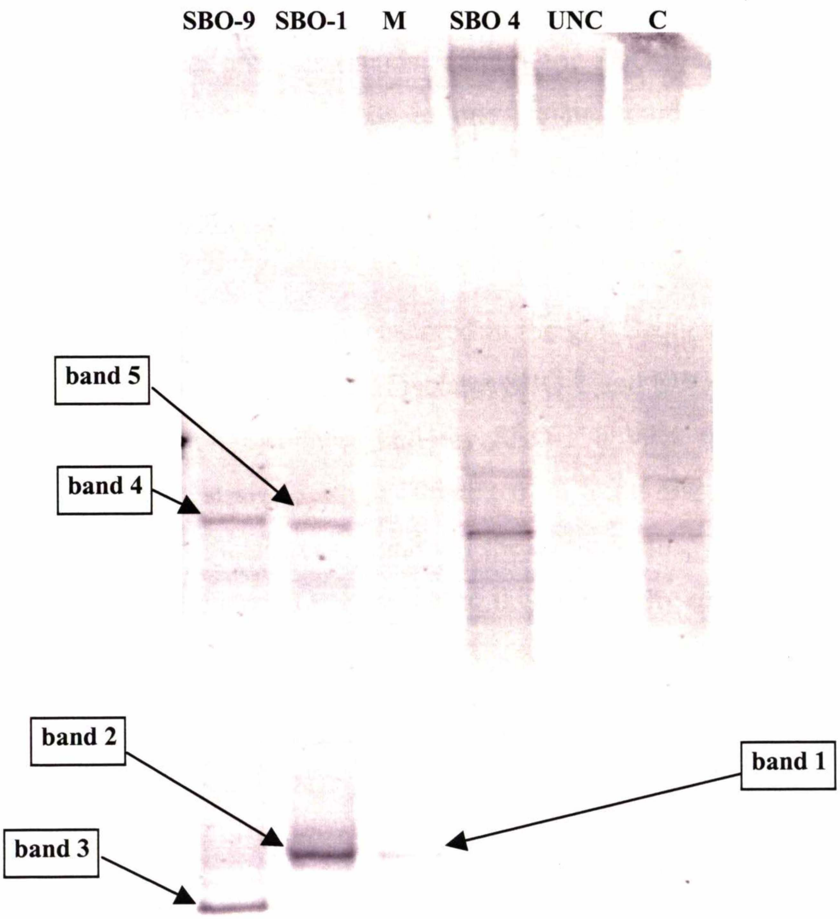
Figure 40. Average amount of total petroleum hydrocarbons in Bull Pass bioaugmentation treatments



3.6. DGGE analysis of successful treatments

DGGE was used to analyse the microbial communities of SBO soil after its most successful mineralisation treatments, which were treatments 4 with 10% MC + N amendment (SBO 4) and the "dilution" (M for "mixed") treatments. Figure 41 illustrates the results obtained for the DGGE in the acrylamide gel. The 16S rRNA gene profile of the contaminated Scott Base soil (C) was compared to uncontaminated control soil (UNC) and to the 2 mentioned treatments. Two hydrocarbon-degrading bacteria isolated from the SBO contaminated soil (in this thesis research), SBO-1 and SBO-9 were used as positive controls, as these were expected to be present at least in the contaminated soil, since they were isolated from the contaminated soil.

Figure 41. DGGE acrylamide gel of some SBO treatments



SBO-9 = HDB isolate; **SBO-1** = HDB isolate; **M** = 1:1 mixed soil from Dilution mineralisation treatment; **SBO4** = SBO soil amended with 10% MC + N; **UNC** = uncontaminated SBO soil; **C** = contaminated SBO soil

The general 16S rRNA gene profile observed in the contaminated SBO soil does not change in the SBO #4 treatment, while it does in the 1:1 mixture treatment. In the 1:1 mixture, one particular band can be observed (band 1 in Figure 41), which is the same size as a band observed in the SBO-1 isolate (band 2). For this soil, no other bands were detected in this DGGE gel. The uncontaminated soil has faintly the same pattern as the contaminated one. The intense bands observed for the hydrocarbon-degrading bacterial isolates (bands 2 and 3) are not found in either contaminated or uncontaminated soils, however the less intense bands (bands 4 and 5) seem to be present in both SBO #4 treatment soil and in the contaminated unamended soil (labelled C in figure 41). Isolates SBO-9 and SBO-1 showed 2 different size bands each one in this DGGE analysis (bands 3 and 4 for SBO-9 and bands 2 and 5 for SBO-1). The presence of two bands in one isolate could mean that the isolate possesses 2 copies of the 16S rRNA gene that are slightly different in sequence, and, therefore, separated differently in the denaturant gradient in the gel.

No sequencing was done for bands observed in the gel. Sequencing was done of the PCR product of the 16S rRNA gene of isolates SBO-1 and SBO-9. The sequence was blasted in the GenBank databases and for SBO-1 a 99% match was found for *Rhodococcus* sp. 5/14 strain, a strain isolated from Scott Base by Bej *et al.*[17]. For SBO-9 isolate, a 98% match was found for the same strain. Therefore, it is highly likely that both of these strains correspond to this previously described *Rhodococcus* strain. These DGGE results will be further discussed in Section 4.3.6.

Chapter 4. Discussion

4.1 Antarctic soils of this thesis research

4.1.1. Chemical properties

The chemical properties of the soils used in this thesis research were characteristic of typical Antarctic soils[14,30]. They were generally low in moisture content (ranging from 0.3% to 7.4%), low in total nitrogen (2.9 - 200 mg N/kg dw), mineral nitrogen (0.7 – 266.6 mg N/ kg dw), and low in available phosphorus content (0-27 mg P/ kg dw) (as shown in Table 12). In contrast, agricultural loamy soils may contain 713-2553 mg N/ kg dw of total nitrogen and 3-153 mg N/ kg dw of mineral nitrogen (including NH_4-N and NO_3-N)[113]; agricultural clay soils may contain 723 – 3765 mg N/ kg dw[113] and shrub-steppe soils 1500 – 3300 mg N/ kg dw total nitrogen and 3.71 to 7.00 mg N/ kg dw of mineral nitrogen[25].

Bull Pass soil had higher nitrate and moisture content compared to the rest of the studied soils. Soils with a high content of nitrate salts have been recorded previously in the inland edge of the Transantarctic Mountains, neighbouring the Bull Pass collection site[35]. The presence of nitrate salts are thought to be derived from the ocean, transported to Antarctica in the upper atmosphere, deposited with snowfall and accumulated over time. The reason for the relatively high moisture content observed in contaminated Bull Pass soil may be explained by the methodology used to determine this value: in moisture gravimetric measurements, the soils were dried at 105°C and the difference between wet and dry weight was assumed to be attributed to moisture content. This method does not account for the loss of volatile hydrocarbons in contaminated soil when it is heated, thus introducing a potential methodological error. In this case, given that the moisture value of Bull Pass uncontaminated soil was significantly lower (1.0%) than the contaminated one (7.4%), it was possible that the relatively high value of moisture detected in this soil was a function of weight loss as a result of hydrocarbon volatilisation. Furthermore, previous studies found typically low values of soil moisture at Bull Pass (eg. 0.4%, 1.4% and 4.0%)[7], confirming the high moisture content of contaminated Bull Pass soils likely to be attributed to hydrocarbon loss.

The pH values of these soils range between neutral and slightly alkaline, confirming previous studies[6,14].

The content of TPH of the soils varied depending on the amount and type of hydrocarbons spilled, and on the time since the spillage event. Scott Base Old (SBO) spill site had the highest TPH content (as shown in Table 12) and a high proportion of unresolved complex mixture (UCM) of hydrocarbons (see chromatogram in Appendix 2). "UCM" is a term used to describe a raised baseline hump often observed in gas chromatograms of petroleum, reflecting a chromatographic overlap of thousands of compounds whose chemical composition cannot be described by any existing analytical technology[53]. Weathered (degraded) petroleum, where evaporation, dissolution and chemical and biological degradation have occurred, normally contains less resolvable compounds and a higher proportion of UCM. Lubricating oils are also enriched in UCM[57]. The soil collected from the old spill site in Scott Base came from beneath a former drum storage area where lubricating oils, engine oils, and fuels were stored. The fact that this soil contains a high proportion of UCM may be a result of contamination with these lubricating oil compounds, and/or weathering of the hydrocarbons, given that this soil was contaminated for over 20 years.

The TPH contents in Scott Base Recent (SBR) spill site contained a resolvable series of *n*-alkanes ranging from C_8 to C_{15} (see Appendix 2) derived from a JP-5 spillage. Unlike the old spill site, SBR soil did not contain a significant proportion of UCM. This may be a result of the relatively short period of time following the spill event (compared with SBO) and also the type of contaminant spilled. SBR contaminated soil was a result of an acute, single JP-5 spillage event, as opposed to SBO soil, where several spillages have occurred over time (chronically), and the contaminants are a mixture of different oils and fuels.

TPH content in Bull Pass soil showed a resolvable series of *n*-alkanes ranging from C_8 to C_{14} derived from a spillage of Diesel Fuel Arctic (DFA), 20 years prior to collection and lacked a significant proportion of UCM. The contaminant in Bull Pass soil was found in the subsurface, which has probably resulted in reduced weathering of the hydrocarbons, and possibly accounts for the lack of UCM. Furthermore, the lack of microbial degradation, as observed in the mineralisation assay for unamended Bull Pass soil in this study (discussed in Section 4.3.6), would also account for the lack of UCM.

4.1.2 Microbial numbers in the soils

As discussed in Chapter 1 of this thesis, the predominant limiting factors for biodegradation of hydrocarbon compounds in the environment included the availability of nutrients, water, and the presence of hydrocarbon-degrading (HCD) microbes. The Protocol on Environmental Protection to the Antarctic Treaty (Annex on conservation of flora and fauna)[95] forbids the introduction of any foreign organism into the continent, therefore only those that already exist in the soil and are capable of biodegrading hydrocarbons can be used in any bioremediation programme. For this reason, the study soils were screened for the presence of hydrocarbon-degraders as well as total culturable heterotrophic bacteria.

It is important to note that in Bull Pass and Vanda Station soils no hydrocarbon-degraders were detected and, compared to the other soils, these contained a slightly reduced number of culturable heterotrophs (1.68×10^4 and 3.00×10^5 CFU g^{-1} respectively, compared to $10^6 - 10^7$ CFU g^{-1} in the other soils studied in this thesis research).

It has been previously reported that Dry Valley soils are low in biomass[7], and that microbial growth may be limited by their extremely low soil moisture content[31]. In coastal areas, like Marble Point, moisture content of soils are higher due to greater snowfall. At inland dry valley sites, snowfall is less frequent and abundant and evaporation rates are high due to low humidity and persistent winds[31]. As such, soil moisture content is dramatically lower in the Dry Valleys. This extreme aridity may be one of the causes for the observed limited microbial biomass in both Bull Pass and Vanda Station soils. Additionally, the high content of nitrate in Bull Pass soil may also contribute to an increased osmotic pressure, which may be limiting for microbial growth in Vanda Station soil, the extremely low nitrogen contents (2.9 mg N/kg dw) are likely to be limiting factors to microbial growth.

In contrast to Bull Pass and Vanda Station, both Marble Point and Scott Base contaminated soils contained hydrocarbon-degraders and higher numbers of culturable heterotrophs (as shown in Table 13). Both Scott Base old spill and recent spill sites contained higher numbers of hydrocarbon-degraders than Marble Point soils. This does not necessarily mean that Marble Point soils have reduced hydrocarbon biodegradation potential than Scott Base soils. It may mean instead that the distinct environmental conditions prevalent in Marble Point (including different soil contaminants to Scott Base) and a longer time since the spillage of hydrocarbons (see Table 3) determined and sustained different microbial populations (including possibly hydrocarbon-degraders). Future experiments with an analysis of microbial biodiversity comparing both soils would address this issue.

Scott Base old spill site has the highest numbers of hydrocarbon-degraders of all the soils studied (1.23×10^6 MPN g^{-1}) and this number is in accordance with previous results for this soil[6]. This may be a result of the prolonged exposure of microbial populations to the contaminant, which has allowed hydrocarbon-degraders to establish and proliferate with time.

The microbial results presented here have implications for bioremediation in Antarctica. The fact that Marble Point and Scott Base soils contain hydrocarbon-degraders is favourable, as it means that these microbes could possibly be used for bioremediation programmes for these sites without having to remove the contaminated soil from Antarctica for remediation, or introduce hydrocarbon-degrading bacteria or fungi. These microbes could also become a source of microorganisms to bioaugment other sites, such as, Bull Pass or Vanda Station soils, where no hydrocarbon-degraders were detected. However, it should be taken into account that the differences in soil type, chemistry and biology of the different sites are not equivalent and may limit the survival, and therefore success, of introduced strains for bioremediation purposes.

Given that the method used to detect hydrocarbon-degraders in this work was based on the degradation of JP-5, the possibility exists that the enumeration method may be selecting for those bacteria able to degrade compounds found in JP-5 (mainly *n*-alkanes) and other hydrocarbon-degraders present in the soil may not be enumerated.

From these observations, it was possible to conclude that numbers of culturable heterotrophs in contaminated sites from the Antarctic region studied here varied greatly from site to site, ranging from 10^4 to 10^7 CFU/g dw, and that not all contaminated sites possess hydrocarbon-degrader populations that are detectable with the methods used. Hydrocarbon-degrader populations ranged between below detection to 10^6 MPN/ g dw.

4.1.3 *alk B* gene content in the soils

To further analyse the hydrocarbon degradation potential of the study soils, a genetic screening of alkane monooxygenase-encoding gene *alk B* and some of its homologues was performed by PCR amplification, sequencing and comparison ("blast") of the sequence to previously reported sequences on the GenBank.

Alkane monoxygenases are enzymes essential for the degradation of alkanes. They catalyse the initial oxidation of the alkane to a 1-alkanol. Different types of alkane hydroxylases have been described[145], and may be needed by bacteria to degrade different chain-length *n*-alkanes through different biochemical pathways (monoterminal, diterminal or subterminal oxidation). Therefore, for detection of the alkane-degraders' catabolic *alkB* gene in the environment, it is better to use different genetic probes rather than just one for a more comprehensive *alkB* gene detection[131]. For this reason, in this thesis research 4 different 'probes' (oligonucleotide primers) were used for genes *Pseudomonas putida* (Pp) *alkB*, *Rhododoccus* sp. (Rh) *alkB1*, Rh *alkB2* and Rh *alkB2*. Specific primers for these gene homologues were used as 'probes' for PCR reactions with genomic DNA directly isolated from the soil. It was assumed that if the *alkB* gene and/or its homologues were present in the soil, then the soil microbes have alkane-degradation potential.

In contaminated soils, *alkB* and *alkB1* were demonstrated to be present in both Scott Base old spill site (SBO) and recent spill site (SBR) soils, according to positive reaction with PCR primers for the *alkB* and *alkB1* genes and a high homology score match with previously described *alkB* gene from *Pseudomonas aureofaciens*[131] and *Rhodococcus erythropolis* putative *alkB1* gene[117]. Only SBR soil was positive for *alkB2,* as shown by a 96% homology match with *alkB2* gene of *Rhodococcus erythropolis*[117]. This demonstrated the presence of an additional strain of *Rhodococcus* in SBR soil that either possesses only this gene homologue, or both *alk*B1 and *alk*B2. The possible presence of an additional bacterial species or strain does not necessarily imply higher numbers of hydrocarbon-degraders, given that SBO soil has higher numbers of these. Differences in hydrocarbon-degrader population in the recent spill site may be explained by the different type of contaminant in both soils: while in the old spill site there was a mixture of weathered ("aged") complex hydrocarbons, in the recent spill site, there was mainly JP-5, that was probably not highly weathered compared to SBO spoil because the spillage event occurred 21 years later than in SBO (as shown in Table 3). Thus, different fuels possibly favoured the growth of different hydrocarbon-degraders. Another possible explanation was that the fuel type resulted in a selective enrichment of fewer genotypes in SBO than in SBR soils. However, this is just a preliminary observation and further population microbial studies are required to confirm this.

In Scott Base uncontaminated soils, no *alkB* gene homologues were found. As no hydrocarbon-degraders were detected by the most probable number (MPN) method in this soil either, it was possible that no alkane-degraders were present in Scott Base uncontaminated soils. As previously shown in Figure 25, marginal hexadecane mineralisation, more than the sterile control, was observed in Scott Base uncontaminated soil in a microcosms assay. This demonstrated that alkane-degraders existed in pristine soils in Scott Base, but in extremely low numbers that were not detected by the MPN method used (as shown in Table 30) or by PCR amplification of the *alkB* homologue genes.

As observed in Scott Base soils, *alkB* gene was also detected in both Marble Point contaminated soils. The sequence of the PCR products amplified with *alkB* primers gave 97% homology match with decane-degrader *Pseudomonas aureofaciens* RWTH 529 isolated by Vomberg *et al.* (2000) [131]. As these two soil samples corresponded to different depths (0-3 cm and 3-12 cm), this result indicated that alkane-degrading *Pseudomonas* species were present both in surface and subsurface of the soil profile, which contributed to degradation of the alkanes present in these soils.

In Vanda Station soils, *alkB* and *alkB2* gene homologues were present in the contaminated soil, as confirmed by the sequencing results, but all homologues were absent from control soils. As observed with Scott Base soils, this may mean that hydrocarbons were necessary to enrich the populations to levels detectable by PCR. Vanda Station soils had the genetic potential for alkane degradation despite the fact that no hydrocarbon-degraders were detected by the MPN method, probably because their numbers in this soil were very low. Although no mineralisation enhancing treatments were tested in this soil, it would be worthwhile to test this in the future given the genetic potential for hydrocarbon-degradation. The extremely low water and nutrient content of this soil (0.3% MC and 6.9 mg N/ kg soil) may be seriously limiting hydrocarbon microbial degradation *in situ*.

In Scott Base uncontaminated soil, *alkB* genes were not found by PCR, hydrocarbon-degraders were not enumerated by MPN method, but mineralisation of ^{14}C-hexadecane was observed. In contrast, in Vanda Station soil, *alkB* genes were found by PCR, no hydrocarbon-degraders were enumerated by MPN, and mineralisation, albeit low (<2%), was observed (as shown in Figure 26). These results imply that mineralisation assays were more sensitive to the presence and activity of hydrocarbon-degraders, followed by PCR and then MPN analyses. It should be noted that PCR, MPN and mineralisation assays are only indirectly comparable in terms of presence or absence of hydrocarbon-degraders. Additionally, when an *alkB* gene is detected by PCR in the soil, it does not necessarily mean that the organism containing the gene is capable of degrading alkanes or that the gene is being expressed.

In Bull Pass soils, no *alkB* genes were detected. Given that no amplification was observed when universal bacterial primers for the 16S rRNA gene were used for this soil, two bacterial strains (*Rhodococcus* 5/1 and SBO-1 isolate from this work) were used to inoculate the soil and repeat the amplification procedure. As no amplification with 16S rRNA primers in the inoculated soil was thereafter achieved, it was possible that the PCR reaction was inhibited by soil compounds, including the contaminant diesel fuel itself (although not specifically tested for).

By comparing *alkB* gene screening results in all 6 soil samples, the following observations arise:

- None of the soils contained all 4 gene homologues;
- All soils possessed the Pp *alkB* gene, except Bull Pass;
- The highest diversity of *alkB* genes (*alkB, alkB1* and *alkB2*) was detected in soils from SBR and Vanda Station;

- Marble Point soils had the least diversity of *alkB* gene homologues (only *alkB*) and,
- None of the control uncontaminated soils were positive for any of the *alkB* primers.

Unlike the findings of Whyte *et al.* (2002)[143] in a similar screening of *alkB* genes in Antarctic soils, in this study Pp *alkB* gene, rather than Rh *alkB1* or *alkB2* was the most widespread of all. Whyte *et al.* (2002)[143] found that Rh *alkB1* was detected in 90% of the contaminated polar soils that they analysed, followed by Rh *alkB2* at 65%. In the present study, Rh *alkB2* was slightly less abundant than *alkB1* too, but here only 6 different soil samples were tested compared to the 30 studied by Whyte *et al.* (2002)[143], so a larger number of samples from the Ross Sea Region would be required to compare this study with that of Whyte and co-workers (2002)[143].

Interestingly, the presence of *alkB* genes in the soil does not correlate in this study with numbers of hydrocarbon-degraders or with mineralisation ability. For example, Marble Point soils had the highest mineralisation values of the soils tested, but numbers of hydrocarbon degraders were lower than in Scott Base soils (by 1 to 3 orders of magnitude) and numbers of *alkB* gene variants were lower than Scott Base soils, as well. These results possibly imply that in Marble Point soils an additional microbial population was not detected in these analyses, such as fungi, which may be playing an important role in achieving the high hexadecane degradation observed in this site. Further studies addressing this issue are needed.

The fact that no *alkB* genes were detected in any of the uncontaminated soils suggests that either their numbers are extremely low, and therefore undetectable by the PCR method used here. Alternatively, the hydrocarbon-degraders may not have existed in these soils previous to the contamination event and were introduced by humans as a result of activities related to handling of fuels. Similar observations were made by Whyte *et.al* (1999)[140], where no *alkB* genes were detected in pristine soils from the high Arctic, but were found in all neighbouring contaminated soils. However, an anthropogenic origin of the hydrocarbon-degraders present in the soils studied here may not be valid because by using the same oligonucleotide primers from this work, Whyte *et al* (2002)[143] detected the Pp *alkB*, Rh *alkB1* and Rh *alkB2* genes in both pristine and contaminated soils from the Brazilian Antarctic Station; Comandante Ferraz. Therefore, it is likely very low numbers of hydrocarbon-degraders existed in the uncontaminated soils and were not detectable by PCR under the conditions used here. Furthermore, Laurie *et al.* (2000)[75] demonstrated that a laboratory enrichment of polyaromatic-degrading populations in pristine soils was necessary to detect these microbes by quantitative PCR. They found a PCR detection limit of 2 x 10^5 copies of gene per g soil dry weight. Therefore, it is possible that the abundance of microbes (indirectly indicated by a low number of genes) in uncontaminated soils were below detectable limits in this study.

In general, all of the contaminated soils, except Bull Pass, proved to have the genetic potential to sustain alkane degradation. Although this was also demonstrated by isolation and enumeration of hydrocarbon-degraders (see Section 3), the genetic analysis shows that specifically bacteria from the genus *Pseudomonas* and *Rhodococcus* are prevalent in contaminated soils from the Ross Sea Region in Antarctica, and may play an important role in alkane-degradation in these soils.

4.2 Bacterial isolates

Given that Scott Base sites had the highest numbers of hydrocarbon-degraders, these soils were selected for the isolation of hydrocarbon-degrading bacteria (HDB). The hydrocarbon contaminants in these sites contain *n*-alkanes ranging from C_{10} to C_{17}, therefore the ability of the 17 isolated HDB to utilise the different alkanes as a C source was tested. All isolates, except SBO-19, were able to degrade *n*-alkanes ranging from C_{11} to C_{16} and the branched alkane pristane. All the isolates, except one (SBO-9), did not degrade hexane, which could reflect a higher toxicity of this compound given its shorter carbon chain[28] The majority of the isolates (12 out of 17) shared the same morphology: orange round colonies in R2A and Gram positive rods, and ability to utilise C_{11}- C_{16} *n*-alkanes and pristane as C sources.

None of the isolates were positive for the *alkB* gene (see Table 16). This was not consistent with the results obtained for the gene screening of total DNA extracted from the Scott Base soils where the isolates where obtained, since DNA directed extracted from both old and recent spill sites were positive in a PCR reaction for *alkB*. Because the same primers were used for the soils and the isolates to screen for the *alkB* gene, it was expected that at least some of the isolates would have been positive for this gene. This contradictory result shows a bias in the selection of strains, which implies that the isolates obtained were not necessarily representative of the bacterial populations in these soils, but rather those favoured by the culturing methods. A further bias attributed to the molecular procedures (*eg* poor genomic DNA recovery from soil, low homology of primers, inaccurate annealing temperatures during PCR) could also contribute to this discrepancy.

Isolates SBO-14, SBR-7 and SBR-11 were negative for all the 4 *alkB* gene homologues, however they were able to degrade *n*-alkanes ranging from C_{11} to C_{40} and pristane. This implies that they may possess other *alkB* homologues not detectable with the primers used in this analysis.

The majority of the isolates were positive for *alkB2* and/or *alkB194,* and were able to degrade *n*-alkanes C_{11} to C_{16} and pristane. SBO-9 was the only isolate able to degrade hexane, one of the 2 isolates positive for *alkB1.* These results imply the possibility that *alkB2* and *alkB194* conferred the ability to degrade *n*-alkanes within the C_{11} to C_{16} range, and that *alkB1* was needed for the degradation of hexane and possibly lower molecular weight *n*-alkanes. However, further investigation, like mutation analysis, will be needed to confirm these observations. For example, if a hexane mutant of SBO-9 could recover its ability to degrade hexane by complementation with a plasmid carrying the *alkB1* gene, then the alkane hydroxylase encoded by this gene would confer the ability to metabolise short chain alkanes such as hexane.

It is interesting that all the isolates are able to degrade both *n*-alkanes and the branched alkane (pristane). Unlike *n*-alkanes, which are mainly degraded by terminal oxidation, highly branched alkanes undergo diterminal oxidation to avoid blocking ß-oxidation due to branching[11]. Given that several *alkB* homologues may exist for degradation of different chain *n*-alkanes, it is possible that an alternative or additional *alkB* gene is involved in the diterminal oxidation of branched alkanes in the isolates studied here that enables degradation of pristane. The possibility that different alkane hydroxylases confer the ability to degrade different ranges of *n*-alkanes has been previously suggested[145]. The results obtained in this thesis research support this hypothesis.

Given that SBO-9 isolate produced vast amounts of slime (on R2A media) and was the only isolate able to degrade hexane, the possibility exists that the slime could be acting to reduce the membrane toxicity of the compound and aiding in its uptake and degradation. However, further studies and characterisation of the slime will be needed to test this hypothesis.

Bacteria with the same characteristics (eg. colony shape, colour and C-source utilisation) to those found in these isolates were observed in bacteria isolated from Scott Base, therefore it is possible that those found here correspond to the *Rhodococcus* sp. previously described[17]. Additionally, all the isolates that were positive for *alkB1*, *alkB2* or *alkB194* had high sequence matches with alkane genes from either *Rhodococcus erythropolis*, *Rhodococcus* sp. Q15 or *Rhodococcus* sp. 1BN. Therefore, it is very likely that the isolates are *Rhodococcus* species.

4.3 Mineralisation assays

^{14}C-labelled hexadecane was used to measure the mineralisation of hydrocarbons in the contaminated soils. After determining the natural mineralisation capacity of each soil (unamended soils), laboratory trials were set to enhance mineralisation of SBO and SBR soils by nitrogen and water addition, and Bull Pass soil by bacterial bioaugmentation.

4.3.1. Unamended soils: testing natural attenuation

Intrinsic biodegradation or natural attenuation of ^{14}C-hexadecane in Scott Base soils was low compared to Marble Point soils (*ca.* 2% for the recent spill, *ca.* 4.6% for the old spill compared to *ca.* 49% and 60% for Marble Point soils). In Vanda Station soils, mineralisation activity was very low (1.4%) and in Bull Pass it was practically non-existent (0.2%). The results of these mineralisation experiments imply that the environmental conditions in Scott Base, Bull Pass and Vanda Station soils do not favour biodegradation of contaminants. Biodegradation may be impeded by one or a number of limiting factors, each of which may be affecting the soils differently. The reduced or nil microbial degradation observed in the soils may partially account for contaminant persistence in the environment.

4.3.1.1. Marble Point

Although Marble Point soils are subject to similar limiting environmental factors to the rest of the soils studied here (low nutrients, low moisture, low temperature) (as shown in Table 13), these soils possess a high ability to mineralise hydrocarbons (*ca.* 49 - 60% $^{14}CO_2$ in 75 days). It is possible that the high mineralisation capacity in Marble Point soils results from differences in microbial population composition. Free-living nitrogen-fixing bacteria were isolated from fuel-contaminated Marble Point soils by Eckford and co-workers (2002)[47] from enrichments on N-deficient semisolid malate medium. Eckford and co-workers (2002)[47] proposed the hypothesis that these nitrogen fixers may exist in association with hydrocarbon-degraders, the former providing fixed nitrogen to the hydrocarbon degraders, and the latter providing carbon sources for the nitrogen fixers. Five isolates were obtained from Marble Point and the Wright Valley, but no nitrogen–fixing bacteria were found in Scott Base soils[47], which is indicative of the differences in the microbial ecology of the soils. The presence and synergistic activity of nitrogen-fixers in Marble Point may be a possible explanation of the successful mineralisation results observed in this site in this thesis research. Further tests are needed to verify this hypothesis.

Additionally, differences in the soil texture of the studied sites may had influenced the microbial degradation activity[135]. Different soil types interact differently with hydrocarbons, allowing more or less availability of water, nutrients, contaminant and oxygen diffusion. It is possible that the particular soil characteristics of Marble Point soil favoured mineralisation of hexadecane over those characteristics of Scott Base, Bull Pass and Vanda Station.

The kinetic model that best fitted the mineralisation data of the unamended soils from Marble Point and Scott Base predicted the maximum achievable mineralisation of these soils under the conditions tested. The fit of the data to the sigmoidal model reached for these curves was highly significant (r^2=0.99). In this case, the predicted maximum mineralisation for both Marble Point soils (62.6% for MP1 and 49.3% for MP2) was much higher than for Scott Base old spill soil (6.96%). This indicated that Marble Point soils had significantly more degradative potential than Scott Base under the conditions tested. Thus, it was worthwhile to try enhancing mineralisation for Scott Base soils in this research. It is important to mention that while hexadecane mineralisation is a good indicator of total hydrocarbon mineralisation, the kinetics of hexadecane mineralisation cannot be extrapolated to other hydrocarbons[89].

Overall, in Marble Point soils, natural attenuation plays an important role in contaminant degradation, being more active in the surface layer (MP1) than in the subsurface (MP2). An ideal situation would be to achieve 100% of contaminant loss and complete soil recovery. This could be achieved by a combination of biodegradation and abiotic loss combined. Further studies would be required to quantify mineralisation versus abiotic loss in Marble Point soils, and how mineralisation can be further increased.

4.3.1.2. Scott Base, Bull Pass and Vanda Station

In Scott Base, Bull Pass and Vanda Station soils, natural attenuation is either negligible or does not occur under the conditions tested. Reduced intrinsic hydrocarbon mineralisation rates have also been observed for unamended contaminated soils from Casey Station under laboratory conditions[50]. Soil samples from long-term fuel-contaminated sites, yielded 1.46 –1.85% recovery of ^{14}C-octadecane as $^{14}CO_2$ after 95 days of incubation at 10°C. Natural attenuation in these Antarctic soils was also negligible under the conditions measured. Aislabie *et al.* (1998)[6] also found relatively low intrinsic mineralisation of *ca.* 6.0% $^{14}CO_2$ produced from ^{14}C-hexadecane after 90 days of incubation in unamended Scott Base contaminated soil at 8°C.

Although exceptions exist, as in the case of Marble Point, it is probable that natural attenuation is unlikely to yield satisfactory biodegradation of fuel-contaminated soils in Antarctica.

4.3.2. Comparison between an old spill and a recent spill

The main differences between the two Scott Base soils studied were the time of the spillage event, the number of spills which contributed to the contamination and the type of hydrocarbons present in the soils. Given that the SBO contamination events occurred more than 20 years ago, it is possible that the oil has been weathered to a greater extent than the recent spill site. "Weathering" refers to the result of biological, chemical and physical processes that can affect the type of hydrocarbons that remain in the soil[77]. As discussed in Section 4.1.1., weathering processes and the contribution of different hydrocarbon sources (including lubricating oils) may explain the presence of a UCM in the old spill site and the lack of it in the recent spill site.

The results obtained in this research showed that the old (SBO) and recent (SBR) spill soils also differed in their numbers of hydrocarbon-degraders. Hydrocarbon-degraders were higher in the SBO soil by two orders of magnitude (1.23×10^6 hydrocarbon-degraders g^{-1}) compared to SBR (3.54×10^4 hydrocarbon-degraders g^{-1}). It was previously demonstrated that there is an enrichment of hydrocarbon-degraders in contaminated soils compared to uncontaminated soils[6,9]. It is possible that this enrichment effect is also observed in long-term contaminated sites, where certain bacterial populations have been selected and are well adapted to the conditions. Baseline mineralisation values for SBO and SBR were 4.89% and 1.98%, respectively, after 75 days of incubation under the same conditions. It is tempting to relate the lower mineralisation observed in the SBR site to the reduced numbers of hydrocarbon-degraders enumerated at this site. However, this contradicts the results observed in the dilution experiments that showed increased mineralisation (*ca.* 39% $^{14}CO_2$ after 87 days, as shown in Figure 33) in the mixed soil, which had reduced numbers of hydrocarbon-degraders (1.3×10^3 hydrocarbon-degraders g^{-1}, as in Table 30) compared to the SBO soil (1.23×10^6 hydrocarbon-degraders g^{-1}). Given the result obtained for SBR soil, it would perhaps be more appropriate to think that a difference in type of hydrocarbon-degraders, rather than their numbers, determined the mineralisation ability of the soil. This could be understood by more in-depth molecular experiments concerning microbial biodiversity in the soils. It is also possible that the different types of hydrocarbons present in the two sites influenced the numbers of hydrocarbon-degraders and their rates of ^{14}C-hexadecane mineralisation.

The *alkB* profile of both SBO and SBR soils were different, demonstrating two possibilities: 1) genetic potentials of these soils were different even at the same geographical location (Scott Base) and 2) microbial populations were different in the two soils. Further microbial population analyses are needed to confirm these observations. The fact that different mineralisation curves (adjusted to different kinetic models) were obtained may be an indication of the different microbial populations in both soils. It is possible that the difference in mineralisation ability resulted from a population of hydrocarbon-degraders that adapted in time to the selective conditions prevalent in the old spill site. It is important to mention that hydrocarbons in recently contaminated soils are potentially more toxic to microorganisms because volatilisation of toxic short chain alkanes or photodegradation processes have not occurred to the same extent as in aged contamination[125]. This could be a limiting factor for biodegradation in recently contaminated soils constituting an initial selective pressure.

It is interesting to note that the same amendment treatments (10% and 20% moisture content (MC) and 2500 mg N/ kg-H_2O-soil) had similar effects in both SBO and SBR soils, resulting in increased mineralisation. Although total mineralisation was lower in the recent spill site with the same nitrogen amendment treatment (7.2% compared to 17.0% in SBO after 87 days), this treatment was indeed successful in SBR soil because it increased mineralisation 4.8 times more than unamended soils (as shown in Table 22). The fact that the same treatments in both soils resulted in similar outcomes suggests that the treatments tested here may be successful in any spill within the Scott Base area, providing same soil composition. The higher total mineralisation observed in SBO soils may be a result of better-adapted microbial populations.

Trindade *et al.* (2005)[125] compared biodegradation of crude oil on a weathered contaminated soil with a newly contaminated (spiked) one and found that the recently contaminated soil showed lower biodegradation efficiency and TPH removal than the aged soil. Similar to the results with the amendment treatments in Scott Base soils in this research, Trindade *et al.* (2005)[125] found increased mineralisation after nutrient supplementation in both soils, however better results were obtained in the aged soil. Additionally, these researchers found reduced numbers of hydrocarbon-degraders in the recently contaminated soil compared to the weathered one. Trindade *et al.* (2005)[125] concluded that a well-adapted native microbial consortium was responsible for a better decontamination, and that hydrocarbon-degraders may need acclimation to be able to degrade optimally. Their results support the hypothesis that the type of hydrocarbon-degraders (well-adapted communities with efficient metabolic potential) is important for optimal biodegradation of hydrocarbons.

4.3.3. Amendment treatments in Scott Base soils: testing biostimulation

Because the intrinsic mineralisation of hexadecane was low in Scott Base soils, the aim of the amendment treatments was to enhance the natural mineralisation baseline by manipulating possible limiting factors, in this case, nitrogen and water content.

4.3.3.1 Water amendments

For both old and recent spill soils, when water only was added to 10% and 20% MC, mineralisation was not enhanced. In SBO soil, mineralisation dropped below the baseline of 4.89% $^{14}CO_2$ (as shown in Figure 28) and in SBR soil no difference was observed from the baseline mineralisation of 1.98% $^{14}CO_2$ (as shown in Figure 31). This result implied the possibility that water was not a limiting factor for biodegradation in these soils. However, because only two moisture contents were tested, one cannot exclude the possibility that small increases in water content (*eg.* less than 10%) could enhance mineralisation. Further studies are needed to clarify this point.

As soil water potential approaches zero, microbial respiration may be reduced as a result of decreased concentration or lack of dissolved nitrogen and other nutrients[135]. This could explain why there was a reduction of microbial degradation of hexadecane with increasing water content in the soils studied in this thesis research. A similar tendency was observed by Ferguson *et al.* (2003)[50] when they adjusted Antarctic oil-contaminated soils to different moisture contents. Moisture adjustments of *ca.* 40% and 10% yielded 8.4% and 9.2% $^{14}CO_2$, respectively, after 95 days incubation at 10°C. Unlike results in this research, Ferguson *et al.* (2003)[50] did observe increased mineralisation in treatments with moisture amendments compared to unamended controls (1.5% $^{14}CO_2$ after 95 days), however, the same tendency of reduced mineralisation with increased moisture content above 10% was also observed. Since the addition of water reduced mineralisation instead of enhancing it in this study, moisture may not be a limiting factor for biodegradation of hydrocarbons in Scott

Base soils. Furthermore, increasing amounts of water may limit degradation ability of the microbes, possibly by a reduction in the soil water potential.

4.3.3.2. Nitrogen and water amendments

In their investigations to find the optimum levels of nitrogen in biostimulation assays of Arctic oil-contaminated soils (loamy sands), Walworth and collaborators (1997)[135] found that for soils with low water and nitrogen content, optimum values of hydrocarbon mineralisation (measured as oxygen consumption) were obtained when nitrogen was adjusted to 2500 mg N/ kg-H_2O-soil. The soils from Scott Base used in this study were low in nitrogen and water content, therefore, nitrogen and water amendment were carried out to adjust to this 'optimal' value. When nitrogen and water were added to Scott Base contaminated soils in sufficient amount to reach Walworth's recommended value, mineralisation was enhanced above the baseline by 3.5 times in SBO soil and by 4.8 times in SBR soil after 87 days of incubation, reaching 17% of $^{14}CO_2$ in SBO soil and 7.2% in SBR soil. This implies that the addition of both water and nitrogen was necessary to enhance mineralisation in these soils. These experiments demonstrated that the Walworth recommended value of 2500 mg N/ kg H_2O resulted in enhanced mineralisation in contaminated soils from Scott Base. However, mineralisation levels above 17% were not reached with these treatments. There is a possibility that mineralisation can be further enhanced, but the Walworth value might not necessarily be optimum for Scott Base soils. It could be worthwhile to test a broader range of nitrogen additions in Scott Base soils, and measure water potential, as per Walworth *et al.* (1997)[135], to achieve a real "optimum" mineralisation value. However, it is important to keep in mind that there are other potential factors, apart from low nitrogen content, such as type and concentration of the contaminant or low temperature that could be limiting biodegradation in these soils.

The mineralisation results obtained with the nitrogen amendment treatments in this research coincide with those obtained by Aislabie *et al.* (1998)[9] where enhanced mineralisation of ^{14}C-hexadecane (*ca.* 20% total mineralisation after 90 days) was observed after addition of 160 mg N/ kg soil (2921 mg N/ kg H_2O-soil) to Scott Base contaminated soil at 8°C. As observed in this thesis research, maximum values did not exceed 20 % under the conditions tested. This confirms that different nitrogen amendments may be needed to find an optimum level.

Ferguson *et al.* (2003)[50] tested a range of nitrogen amendments in fuel-contaminated soils from Casey Station following the recommended Walworth value, and observed an enhancement of 7.5 times more mineralisation in soil amended with 1570 mg N/ kg-H_2O-soil compared to unamended soil. These results suggested that values slightly lower to the suggested 'optimum' were indeed more successful for fuel-contaminated soils at Casey Station.

4.3.3.3.Kinetic model

The effect on mineralisation following addition of nitrogen and water to the Scott Base soils was also reflected in the kinetic behaviour of the mineralisation data. Amended soils data from the old spill site do not fit the model for unamended soils and display a different behaviour that adjusts to a hyperbolic curve rather than a sigmoidal model. This could be a reflection on the kinetics of the metabolic activity of the hydrocarbon-degraders as a result of the stimulation treatments. The values predicted for maximum mineralisation achievable in these treatments (19.5% for 10%MC + N; 16.6% for 20%MC + N and 13.6% for 25%MC +N) (given in Table 20) were between 2 and 3 times those predicted for unamended soils (6.7 %), which indicates an important positive effect as a result of biostimulation (nutrient and water addition).

4.3.3.4. Microbial numbers in biostimulation treatments

In SBO soils, and to a lesser degree in SBR soils, numbers of culturable heterotrophs increased in soils amended with nitrogen and water. Since nitrogen and water are available to all microorganisms present in the soil, it is not surprising to find an increase in numbers of culturable bacteria as a result of this stimulation. It is surprising, however, to find that hydrocarbon-degrader numbers were reduced from 10^6 to 10^4 MPN/ g dw in the treatment that enhanced mineralisation in microcosms assays for SBO soil (10% MC + 2500 mg N/ kg-H_2O-soil). These contradictory results confirm that enhanced mineralisation does not necessarily reflect an increase in population numbers, but rather an increase in metabolic efficiency of the established microbial population. A more efficient biodegradation of the hydrocarbons may generate more intermediary compounds that may be made available to the total heterotrophic community and, as a result, culturable heterotrophs in general may increase in numbers. However, there is still no evidence that confirms this last observation. To test this hypothesis, soil culturable heterotrophs could be enumerated before and after inoculation in pristine soils of intermediary compounds of alkane degradation pathways, such as alcohols and aldehydes derived from alkanes (as shown in Figure 5).

Unlike SBO, SBR soils amended with nitrogen and water to 10% and 20% MC, increased numbers of hydrocarbon-degraders (10^4 to 10^6 MPN/ g dw). This may mean that the carbon sources in the SBR soil, the majority being *n*-alkanes, are more readily biodegradable than those in SBO soil. Therefore, different carbon sources may have stimulated or restricted hydrocarbon-degrader populations.

Bento and co-workers (2005)[19] found similar results to those observed here. A decrease rather than an increase in diesel-degraders was observed at the end of nutrient biostimulation treatments in diesel-contaminated soil microcosms, but an increase in biodegradation of diesel was detected as a result of these treatments. The authors observed a reduction from 10^7 to 10^3 diesel degraders ml^{-1} after 12 weeks of incubation in nitrogen-amended soils, but a total degradation of *ca.* 60% of C_{12}-C_{23} hydrocarbons from diesel fuel and an increase in microbial activity (measured by dehydrogenase activity) in the same treatment following the second week of treatment. The authors suggested that these contradictory results could indicate that "specialized" microorganisms were adjusting to changing conditions, increasing their metabolic activity and thus limiting growth of the microbial population[19].

Other biostimulation studies in Antarctic oil-contaminated soils have been carried out illustrating that oil biodegradation can be stimulated by nitrogen amendments under in situ conditions in Antarctic contaminated soils. Delille *et al.* (2004)[41], for example, observed enhanced biodegradation by addition of Inipol EAP-22 commercial fertiliser (C:N:P of 62:7.4:0.7) following *in-situ* nutrient-amendment experiments in artificially oil-contaminated Antarctic soil. The addition of fertiliser resulted in a reduction of total hydrocarbon content from *ca.* 55% to *ca.* 2.5%. Unlike the results observed in the present thesis research, the authors found an increase of hydrocarbon-degraders from 10^4 to 10^8 MPN ml^{-1} after 330 days of treatment in soils from the contaminated plots. In a previous study, the same authors[43] also observed an increase in hydrocarbon-degraders in oil contaminated ornithogenic soils from Terre Adelie, Antarctica. The reason for this difference in hydrocarbon-degrader numbers may result from the fact the Delille's studies were done *in situ*, while the present thesis research was done *ex situ*, and also under different environmental conditions, with different soil types and contaminant types. Kerry (1993)[73] tested the effect of adding nutrients (N, P, and potassium) in artificially fuel-contaminated Antarctic soils (using Special Antarctic Blend (SAB) as contaminant) near Davis Station. Nitrogen fertiliser (NH_4NO_3) was added as a 1% solution in a C:N ratio of 61:1 to soils low in total N (3.1 mg kg^{-1}). Moisture was adjusted to 45-59%. This treatment resulted in a significant reduction of hydrocarbons, from 20,000 mg kg^{-1} to *ca.* 1000 mg kg^{-1} after 1 year of treatment, compared to unamended controls. This treatment was also correlated to highest microbial activity (measured by fluorescein diacetate hydrolysis assays). Moisture adjustments used by Kerry (1993)[73] were higher than those used in fertilisation treatments in the present study, but nitrogen concentration and C:N ratios were similar (*ca.* 500 mg kg^{-1}, and C:N ratio of 60:1 used by Kerry and 80:1 used in this study, as shown in Table 6). Similar to the results obtained in SBO fertilisation treatments, the addition of N solutions was a successful treatment in stimulating hydrocarbon degradation in these soils.

4.3.3.5. Nitrogen overfertilisation

As discussed previously, Walworth and collaborators (1997)[135] described that highly contaminated soils would need high amounts of nitrogen to adjust to a narrow C:N ratio (*eg*. 10:1), leading to an increase in the osmotic potential of the soil solution and consequently a decrease in microbial degradation (respiration). Therefore, Walworth and collaborators (1997)[135] concluded that the C:N ratio in the soil was not a good parameter for the calculation of fertilizer amounts for stimulation of bioremediation. In the amendment treatments in this thesis research, when excess nitrogen was added (5000 mg N/ kg soil) to SBO and SBR soils in C:N ratios close to the 6:1 Redfield ratio (8:1 and 1.4:1 respectively) (see Tables 6 and 8), mineralisation values either remained unchanged or dropped below the baseline. These results demonstrated that, even though nitrogen and water additions enhanced mineralisation, large amounts of N had the opposite effect of decreasing it. The results obtained in this thesis research confirm the observations of Walworth and collaborators (1997)[135], and showed that the higher the N_{H2O} value, the less mineralisation observed. The results obtained in this thesis research also supported the hypothesis that it is more appropriate to calculate the amount of nitrogen used for fertilisation based on the amount of water in the soil rather than using fixed values of C:N ratios. This allowed a more realistic estimation of the amounts of nitrogen needed and avoided overfertilisation which had a negative effect in biodegradation, perhaps as a result of osmotic stress[134].

Ferguson *et al.* (2003)[50] observed that when soil was fertilised with nitrogen to near Redfield ratios (4:1 C:N, equivalent to 27,944 mg N/ kg-H_2O-soil), hydrocarbon mineralisation was severely inhibited. However, after an extended lag phase of over 42 days, an increase in mineralisation rates was observed similar to the rate in the unamended soil. Ferguson *et al.* (2003)[50] suggested that psychrophilic hydrocarbon-degraders able to tolerate high concentrations of nitrate salts (halotolerants) were present in low numbers in the initial microbial population, and took several days to reach high numbers that could yield measurable mineralisation and resist overfertilisation. Similar results were obtained in this thesis research, where soils amended with 25,830 and 20,664 mg N/ kg H_2O-soil in SBO soil showed a long lag phase of 24 and 38 days respectively (as shown in Figure 28), after which, mineralisation increased to levels close to unamended soils. These results support Ferguson's hypothesis of the presence of psychrophilic halotolerant hydrocarbon-degrading microbes in the soil microbial communities. Psychrophilic hydrocarbon-degrading *Rhodococcus* sp. have been found to tolerate up to 6% NaCl[97] and 2.5% NaCl[39] concentrations. *Rhodococcus* sp. have been isolated previously from Scott Base contaminated soil[17] and in this research, too, specifically isolate SBO-9 (from sequence analysis of the 16S rDNA gene). The development of a halotolerant hydrocarbon-degrader microbial population in Scott Base soils explaining the mineralisation observed in the excess-nitrogen treatments is an attractive hypothesis, however, further tests will be needed to prove this.

4.3.4.The effect of diluting contaminated soil

Another possible limiting factor in SBO soil could be the concentration of hydrocarbons (40,200 mg/ kg soil) and the nature of the contaminant itself. The high proportion of UCM could have a negative effect on mineralisation or availability of degradable compounds. Given that the UCM contains thousands of different compounds, some of these compounds may have a toxic effect in soil microbes that slows down ^{14}C-hexadecane mineralisation. Thomas *et al.* (1995)[123] observed that chemically oxidised lubricating oil with a large fraction of UCM had a toxic effect in mussels. This chemical oxidation was shown to yield products similar to those produced by photodegradation[48], such as carboxylic acids, ketones and lactones, which are still hydrophobic but have a greater solubility than UCM hydrocarbons and are narcotic toxicants for mussels[123]. Other studies by Smith *et al.* (2001)[115] and Rowland *et al.* (2001)[110] have demonstrated toxicity of some compounds in UCMs derived from biodegraded oils. Additionally, Mohn *et al.* (2000)[89] found that high concentrations of dodecane, a suspected component of the UCM, (100,000 mg/kg) added to soil in microcosms experiments inhibited mineralisation. Thus, the possibility existed that either a high concentration of hydrocarbons or compounds present in SBO soil's UCM were limiting biodegradation, with possibly toxic effects.

The aim of the "dilution" experiment was to test if a dilution of the contaminant with uncontaminated soil would enhance the intrinsic mineralisation observed in the SBO spill site. A 1:1 mixture of contaminated:uncontaminated soil resulted in a substantial enhancement of mineralisation, obtaining *ca.* 40% $^{14}CO_2$ after 87 days of incubation at 15°C (as shown in Figure 33). This equates to an increase of 8 times the unamended control and double the mineralisation obtained with the nitrogen-water amendment treatments. It was also superior to mineralisation obtained in nitrogen biostimulation experiments by Aislabie *et al.* (1998)[6] at 8°C (20% $^{14}CO_2$), by Ferguson (2003)[50] at 15°C (*ca.* 7% $^{14}CO_2$) and Whyte (2001)[141] (*ca.* 20% $^{14}CO_2$ at 5°C), and similar to highest mineralisation values reached by Mohn (2000)[89] at 7°C (*ca.* 60% $^{14}CO_2$) and Ferguson (2003)[51] at 42°C (*ca.* 35% $^{14}CO_2$). This showed that, in this case, a simple 1:1 mixture of these two soils resulted in equally successful, if not better, mineralisation of other biostimulation treatments in oil-contaminated polar environments. Given this result, it was clear that by diluting the soil and, consequently, the contaminant it was possible to substantially enhance hexadecane mineralisation in SBO soil at 15°C.

Considering that nitrogen and water contents are typically limiting factors for mineralisation of hydrocarbons[11,134], it was hypothesized that the manipulation of these factors could further enhance mineralisation in this mixed soil. For this reason, water and nitrogen amendments were carried out independently, in an attempt to further enhance biodegradation. Relative to the mixed unamended soil, addition of water did not enhance mineralisation, but, in fact, reduced it. As previously observed in SBO and SBR soil, the more water added (2.5%, 10% and 20% MC), the less mineralisation observed (*ca.* 39%, 31% and 18% $^{14}CO_2$, respectively). This indicated, once again, that water was not a limiting factor for biodegradation in Scott Base soil, even when it was mixed with uncontaminated soil.

In an attempt to 'optimise' mineralisation in the mixed soil, nitrogen was added to achieve Walworth's theoretical 'optimal' value of N_{H2O} = 2500 mg N/ kg-H_2O-soil for hydrocarbon degradation in polar soils. Results from these mineralisation experiments (as in Figure 36) showed that nitrogen amendments did not enhance mineralisation, and, contrary to the expected outcome, reduced mineralisation to 10% and 14% $^{14}CO_2$ when amended to 10% and 20% MC, respectively. Thus, neither water nor nitrogen addition to the mixed soil enhanced mineralisation above the 40% obtained by only mixing contaminated with uncontaminated soil. This is surprising, as similar studies in the literature mentioned above showed that addition of both parameters often enhanced mineralisation[50,51,134,135]. This result indicated that the N_{H2O} value obtained by mixing the two soils (4800 mg N/kg-H_2O-soil) was sufficient to sustain active mineralisation, and that this value is possibly closer to an optimal for this soil than the 2500 mg N/ kg soil suggested by Walworth. A finer analysis of small nitrogen amendments, adjusting to values slightly above and below 4800 mg N/ kg-H_2O-soil will be needed to find an optimal N_{H2O} concentration that could possibly enhance mineralisation even further in this "diluted" soil. The results of nitrogen and water amendment treatments in diluted soils showed that the dilution *per se* of the contaminant plays a more important role in enhancing mineralisation than addition of nitrogen and/or water in SBO soil. No studies on the effect of diluting contaminated soil with uncontaminated soil has been reported for Antarctic soils, thus this thesis research reports the first results in this respect for this type of experiment.

According to the kinetic model to which the data in this treatment was adjusted in this treatment, the maximum mineralisation achievable would be 41.74%, which is considerably higher than that observed in the unamended soil. This value of maximum mineralisation also implies that there is room for further enhancement and that possibly by the manipulation of other limiting factors like phosphorus content or increased temperature higher mineralisation values may be achieved.

4.3.5.Microbial numbers in diluted soils

The mixture of contaminated with uncontaminated soil implied not only a dilution of the contaminant, but also of nutrient contents (see results in Table 25), and changes in the bacterial populations.

Immediately after mixing the contaminated with the uncontaminated soil, Enumerations of culturable heterotrophs and hydrocarbon-degraders were carried out at the beginning and end of the experiment. Immediately following mixing of the soils, numbers of hydrocarbon degraders were reduced from 10^6 MPN/ g dw in the contaminated soil to 10^4 MPN/ g dw in the mixed. At the end of the incubation period, numbers of hydrocarbon-degraders were the same order of magnitude (10^4) in both the unamended mixed soil and the mixed soil amended with 20% moisture, while a small increase was observed in the mixed soil amended with 10% moisture content. It is clear that the reduced number of hydrocarbon-degraders in the mixed soil did not negatively affect mineralisation of hexadecane. This is assumed because mineralisation in this treatment reached the highest levels observed in this research work and the numbers of hydrocarbon-degraders at the end of the incubation period did not change significantly from those at the beginning of the treatment (Figure 37). The numbers of hydrocarbon-degraders did not correlate with enhanced mineralisation. This confirms the result observed in SBO fertilisation treatments, implying enhanced mineralisation was a result of increased metabolic efficiency rather than an increase in numbers of hydrocarbon-degraders.

The heterotrophic population increased slightly in all dilution treatments. Certain compounds in the unresolved complex mixture of SBO soil may have had a toxic effect in both the hydrocarbon-degrader and the heterotrophic populations. However, when their concentration was reduced by dilution with uncontaminated soil, the toxic effect may have reduced or even disappeared, resulting in better environmental conditions for microbial proliferation. It is also possible that the incubation temperature used for these experiments (15°C) had an enhancing effect on microbial growth, because numbers of culturable heterotrophs increased in 2 orders of magnitude in the uncontaminated soil as well.

4.3.6. Microbial population analysis in diluted soils

To further investigate the bacterial populations in the contaminated:uncontaminated mixed soil from Scott Base, a Denaturant Gradient Gel Electrophoresis (DGGE) analysis was performed comparing contaminated, uncontaminated and mixed soils. Two hydrocarbon-degrader SBO isolates (SBO-1 and SBO-9) were used as positive controls. Soil from SBO treatment #4 (10% MC + N) (see Figure 28) was also included to compare "best-result" treatments. The results of this analysis indicated that while there was no evident change in the bacterial 16S rRNA profile of the SBO amendment treatment #4 compared to the contaminated soil, there was a change in the DGGE produced pattern of the diluted soil (see Figure 41). One band in particular was evident. It is possible that given the new conditions of the diluted soil, a shift in the contents of the population occurred, and one particular hydrocarbon-degrader species dominated. Given the enhanced mineralisation observed in this treatment, it was possible that this dominating band represented a bacterial species that had a more efficient hydrocarbon-degrading ability. Interestingly, the dominating band corresponded to that observed in the SBO-1 isolate used as a control. The possibility existed that these bacteria were favoured under the dilution conditions and proliferated reaching equilibrium after 87 days of incubation. It was also possible that bacteria similar to SBO-1 were responsible for the enhanced mineralisation observed in the dilution treatment.

A sequence analysis of the PCR product generated from isolate SBO-1 with primers for the 16SrRNA was carried out. A sequence homology search (nucleotide by nucleotide "blastN") in the GenBank revealed that this sequence had 99% match with *Rhodococcus* sp. 5/14 isolated and characterised by Bej *et al.* (2000)[17] from Scott Base contaminated soil. As this strain was isolated from the same location and morphological characteristics were the same in both strains (orange colonies in R2A media, Gram positive rods, use of C_{11}-C_{16} alkanes and pristane as a carbon source) (as shown in Table 15), it is very likely that the SBO-1 isolate may be closely related to *Rhodococcus* sp. 5/14. Thus, the DGGE analysis showed that the dilution treatment may have favoured conditions for an enrichment of *Rhodococcus* species that was probably responsible for the high levels of hexadecane mineralisation observed in this treatment.

On the basis of these results, SBO-1 isolate was chosen as an inoculant to test bioaugmentation in Bull Pass soils. In conjunction, the previously described *Rhodococcus* strain (5/1 ICMP No. 13755)[17] isolated from Scott Base contaminated soils was also used as an inoculum.

4.3.7. Bioaugmentation in Bull Pass soil

Bull Pass soil is contaminated with *n*-alkanes ranging from C_9 to C_{14} with a total TPH content of 1040 mg/kg dw. However, no hydrocarbon-degraders were detected in this soil. The combination of these two factors made it an ideal candidate for bioaugmentation. No nitrogen amendments were made as this soil has a relatively high content of mineral (nitrate) nitrogen (263 mg N/kg dw) equivalent to 4255 mg N/ kg H_2O-soil. For this reason, nitrogen was not considered the most limiting factor for biodegradation in this case. Given that the moisture content of the soil was low (4.7%), water amendments were considered necessary and adjusted to 10% and 20%.

In terms of hexadecane mineralisation, inoculation with the 2 bacterial strains was successful compared to uninoculated soils, only when the water content was adjusted to 20% for both strains. Mineralisation reached *ca.*11% of isotope recovery with SBO-1 isolate and *ca.* 6% with 5/1 strain after 77 days of incubation. In contrast, hexadecane mineralisation was not enhanced with only water adjustments or with inoculum plus water adjustments to 10% MC. This indicates that indeed lack of hydrocarbon-degraders and possibly low water content constituted limiting factors for Bull Pass soil and that they were overcome to some extent with these treatments.

The mineralisation data was fitted to 2 different kinetic models in this experiment. A hyperbolic model best described data from SBO-1 (+ 20% MC), while a sigmoidal model best described data from 5/1 (+20% MC) inoculation. This could perhaps be an indication of the different metabolic abilities of the 2 strains. Higher values of maximum mineralisation achievable were predicted by the kinetic model (22.6%) for SBO-1 strain compared to strain 5/1 (10.8%). This could mean that strain SBO-1 adapted better to the conditions in Bull Pass, however this could also be explained by the fact that SBO-1 inoculum had double the amount of bacteria than 5/1 inoculum at the start of the bioaugmentation experiment.

Bacteria were inoculated in numbers approximately of 10^8 CFU in 20 g soil in each microcosms, as enumerated by the dilution-plating method described in section 2.6.5.1. Immediately after bacteria were inoculated in the soil, a subsample of soil was taken to enumerate both total heterotrophs and hydrocarbon-degraders in all the treatments. This screening showed an increase of culturable heterotrophs at the beginning of the treatments of 3 to 4 orders of magnitude in the soils that where inoculated (from 10^3 to 10^6 and 10^7 CFU/ g dw). The numbers of hydrocarbon-degraders from the inoculated soils were around 10^2-10^3 MPN/ g dw, while in the non-inoculated soils, irrespective of their moisture content, numbers of hydrocarbon-degraders were <10 MPN/ g dw. Comparing the numbers of total heterotrophs in non-inoculated (10^3 CFU/g dw) with those of inoculated soils (10^7 CFU/ g dw) and numbers of hydrocarbon-degraders in inoculated soils (*ca.* 10^3 MPN/ g dw), it is evident that the inoculated bacteria did not have a 100% of survival or did not retain their hydrocarbon-degrading ability, as 10^7 CFU/ g dw were inoculated. Only 1 of every 100,000 bacteria inoculated survived and/or became metabolically active. Despite this, the bacterial population growth following inoculation (10^3 MPN/g dw) was sufficient to mineralise the hydrocarbons present in the soil, as indicated by a significant isotope recovery in the mineralisation assays. Numbers of hydrocarbon-degraders were the same at the beginning and end of the experiment, which means that they possibly stabilised at these numbers early in the experiment.

The reduction in numbers of inoculated bacteria may possibly have reflected their struggle to compete with indigenous bacteria and the new environmental conditions encountered. The decline in bacterial numbers following their introduction into soil systems is a commonly observed phenomenon in bioaugmentation experiments, with growth of the introduced bacteria being a rare event (reviewed in van Veen (1997)[130]). This inhibitory effect in the soil has been called "microbiostasis"[65]. Microbiostasis was apparently observed in the bioaugmented Bull Pass soil because only low numbers of inoculated bacteria survived and no growth was detected following inoculation. Microbes, however, were actively degrading hexadecane throughout the incubation period.

The analysis of TPH at the end of the treatments showed that there was a decrease in amount of TPH of *ca.* 2 times in all of the treatments (from 450 mg/kg dw to *ca.* 250 mg/kg dw), including the sterile control. This means that there was a significant decrease in hydrocarbons attributed to abiotic loss, probably volatilization. This result indicated that the mineralisation observed after inoculation was marginal and did not signify an important loss of total hydrocarbons in the experimental systems used here. Given that the numbers of bacteria that survived after inoculation were much lower than the numbers inoculated, it is possible that a higher initial number of bacteria are required to achieve a significant biological loss of hydrocarbons. Further analyses, with higher concentrations of bacteria in the initial inoculum are necessary to test this hypothesis. Another possibility is that the duration of the experiment did not allow a more significant reduction in TPH. Thus, a longer incubation period may be necessary to detect significant changes in TPH content.

The isolate that was thought to be responsible for enhanced mineralisation in the dilution experiment (SBO-1) did not show the same levels of mineralisation as those observed in the mixed soil treatment (shown in Figure 33). Given the different physical, chemical and biological characteristics of the soil and contaminant present in Bull Pass, one cannot realistically expect the same results.

Successful bacterial bioaugmentation in Antarctic contaminated soils was observed by Ruberto *et al.* (2003)[111] in laboratory microcosms studies. Inoculation of gas-oil contaminated soil from Jubany Station with psychrophilic hydrocarbon-degrading *Acinetobacter* B2-2 strain, isolated from a contaminated river, decreased total hydrocarbon content in 65% compared to abiotic controls and more efficiently than fertilized soils. Numbers of inoculum were 2.7 x 10^7 CFU/ g dw, similar to the ones used in this work, but in contrast survival of the inoculum was high, detected at 10^6 CFU/ g dw at the end of the experiment. The strain used by Ruberto *et al.* 2003)[111], was not indigenous to Antarctica and therefore, it would not be possible to use it for inoculation experiments *in situ*. Thus, there is still a need to further investigate bioaugmentation with Antarctic indigenous strains in contaminated soils from this continent.

Mohn *et al.* (2000)[89] found that inoculation with *ca.*10^9 cells g dw^{-1} indigenous and non-indigenous hydrocarbon-degrading microbes increased dodecane mineralisation in oil-contaminated Arctic soils. In this case, inoculated bacteria reduced the lag time prior to dodecane mineralisation observed with fertilization treatments. Despite these two successful examples it is rare to find evidence indicating that bioaugmentation stimulated hydrocarbon-degradation in soils[89].

4.3.8. Final remark

The general hypothesis that drove this thesis research was that by manipulating the possible limiting factors to hydrocarbon biodegradation it was possible to enhance biodegradation. The experiments conducted here tested this hypothesis in several ways, and the results demonstrated that it is indeed possible to enhance biodegradation, particularly mineralisation of hexadecane, by manipulating nitrogen, water and contaminant content in the studied soils. Therefore, the general hypothesis was proven to be correct.

Chapter 5. Conclusions and suggested future research

This Chapter summarises the conclusions from this thesis research and describes suggested future research, in short statements, as indicated from the Results, given in Chapter 3, and general discussion in Chapter 4.

5.1. Conclusions

- The catabolic gene *alkB* and its homologues were detected in Marble Point, Scott Base and Vanda Station soils. The Pp *alkB* genotype was the most prevalent one in all the studied soils, followed by Rh *alkB1*. Bacteria from the genera *Pseudomonas* and *Rhodococcus* may be responsible for alkane degradation in these soils.

- Contaminated soils from Marble Point can be remediated by natural attenuation given the high intrinsic mineralisation capacity observed.

- Contaminated soils from Scott Base have low intrinsic mineralisation ability, but mineralisation can be enhanced by the enhanced by the addition of nitrogen to values around 2500 mg N/ kg-H_2O-soil.

- Calculations of the amount of nitrogen added for fertilisation of hydrocarbon contaminated soils in Antarctica should take into account the water content of the soil rather than C:N ratios of soil, because, as shown in this thesis, this can result in an excess of nitrogen (overfertilisation) that does not enhance mineralisation.

- The sole addition of water to contaminated soils from Scott Base did not enhance biodegradation of the contaminants, and it reduced intrinsic mineralisation possibly as a result of reduced nutrient concentration or oxygen availability.

- For weathered Scott Base contaminated soil, dilution of the soil with uncontaminated soil proved to be the most successful treatment in enhancing biodegradation. Mixing of the contaminated with uncontaminated soils may have reduced the concentration of toxic compounds allowing better conditions for mineralisation.

- Hydrocarbon-degraders were enriched in contaminated soils compared to uncontaminated soils, as shown by MPN enumerations and PCR screening of the *alkB* genes in soils from Marble Point and Scott Base. However, no increase in numbers of HCD was observed as a result of the treatments used in this work. This indicates that the effect of enhancing mineralisation is an increase in biodegradation efficiency rather than an increase in numbers of hydrocarbon-degraders.

- Increased biodegradation efficiency in SBO diluted soil could be related to the favourable selection of a dominant hydrocarbon-degrading *Rhodococcus* strain similar to *Rhodococcus* sp. strain 5/14.

- Bioaugmentation of Bull Pass soil with hydrocarbon-degrading strains from Scott Base combined with water amendments resulted in increased hexadecane mineralisation, but no significant loss in total hydrocarbons. A bacterial inoculum larger than 10^8 HDB/g dw and longer incubation might be needed to achieve a significant hydrocarbon loss.

In summary, this work concludes that it is possible to manipulate the limiting factors to biodegradation in soils from Antarctica to enhance mineralisation of hydrocarbons. Limiting factors proved to be low nitrogen content in Scott Base soil, as well as nature and/or amount of the contaminant in Scott Base old spill site, and lack of hydrocarbon-degraders in Bull Pass soil. Bioremediation is a viable restoration option for Antarctic contaminated soils, using natural attenuation at Marble Point, biostimulation and dilution for Scott Base and possibly through bioaugmentation for Bull Pass soils.

5.2. Suggested future research

5.2.1. For Scott Base soils:

- Analysis of *alkB* genes in the studied soils showed that SBR soil has more homologues than SBO soil. A microbial population analysis by DGGE of Scott Base soils contaminated with different hydrocarbon sources would address the questions of which soil has more bacterial diversity and what are the effects in microbial populations of different sources of contamination in soil from the same site and/or location.

- A substantial enhancement in mineralisation was observed when SBO soil was mixed in a 1:1 proportion with uncontaminated soil. It would be interesting to investigate if the same effect was obtained with the recent spill soil. If it does, then another factor apart from high content of UCM in SBO soil may be limiting biodegradation in SBO soil.

- It is important to test toxicity of hydrocarbons in SBO and SBR soils and investigate if there is any correlation between toxicity, numbers of hydrocarbon-degraders and mineralisation ability. This would clearly define a possible limiting factor for biodegradation in these soils. Toxicity of hydrocarbons could be measured using the Microtox assay of toxicity, which is based on toxicity-associated changes in the light emission of *Vibrio fisherei*.

- In an effort to determine optimal nitrogen fertilisation levels to further enhance mineralisation in Scott Base soils, it would be worthwhile to test a broad range of nitrogen amendments using the N_{H2O} value and measuring water potential in non-diluted and diluted (contaminated:uncontaminated) soil.

- Water amendments in these soils did not enhance mineralisation but instead reduced it. Only two moisture adjustments were tested, so to determine if water is a true limiting factor in Scott Base soils, a range of small water amendments between 2.5% and possibly 30% would need to be tested.

- Experiments to test the hypothesis of the presence of halotolerant hydrocarbon-degraders in Scott Base soils suggested by Ferguson *et al.* (2003), would further characterise bioremediation capability of soils under high fertilisation regimes.

5.2.2. For Marble Point soils:

- High intrinsic mineralisation values were observed in these soils, but low numbers of hydrocarbon-degraders and low abundance of bacterial alkane degradation genes. For these reasons, it would be important to investigate the role of fungi in hydrocarbon-degradation in these soils.

- Research is required to analyse is the contribution of mineralisation to hydrocarbon loss in Marble Point soils, how much is being lost by abiotic processes and how could mineralisation be further increased.

- The good intrinsic mineralisation ability of these soils may be related to the presence of nitrogen fixers. It would be interesting to characterise the interaction of nitrogen fixers and hydrocarbon-degraders in Marble Point soils and investigate what is their role in this ecosystem.

5.2.3. For Vanda Station soils:

- Soils from Vanda Station have extremely low moisture content. It would be interesting to determine what is the threshold of moisture content that is limiting for microbial growth and hydrocarbon-degradation.

- This soil has the genetic potential for alkane degradation as shown by the alkB gene screening analysis. It is thus worthwhile to test water and nitrogen amendment treatments to stimulate hydrocarbon biodegradation.

- As this soil has very low biomass and possibly extremely low numbers of hydrocarbon-degraders, it would be interesting to test bioaugmentation of this soil with hydrocarbon-degrader strains isolated from either Marble Point or Scott Base soil.

5.2.4. For Bull Pass soils:

- Improvements in the methodology of bioaugmentation of Bull Pass are needed. Given that the survival of the inoculum was reduced, a number of bacteria higher than 10^7 CFU/g dw in the inoculum may be needed to achieve a better survival. A longer incubation time would possibly be needed to determine if biological loss of TPH is significant after bioaugmentation.

5.2.5. For all the soils:

- In all the soils studied here, it would be interesting to know what are the effects of temperature and phosphorus supplementation in biodegradation of hydrocarbons, and determine if these are limiting factors.

- It is important to mention that the measurement of $^{14}CO_2$ evolved from a ^{14}C-labelled tracer compound in microcosms assays, does not account for the proportion of ^{14}C incorporated as biomass in the soils. Therefore, when only $^{14}CO_2$ values are reported, the real values of mineralisation may be underestimated. It would be more accurate to measure both values in any further work dealing with biodegradation of hydrocarbons in Antarctic contaminated soils.

5.2.6. For the isolates:

- Characterise the slime in SBO-9 to determine if it is a surfactant or an exopolysaccharide and determine if it is involved in the uptake of hexane and short chain alkanes that are typically toxic to other hydrocarbon-degraders.

- Further characterise SBO-1 isolate and verify its identity with molecular tools.

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Appendix 1

Media,
buffers
and solutions

Appendix 1. Media, buffers and solutions

R2A media

Base for enumeration of heterotrophic organisms in treated potable water.
Formula per litre:

Yeast extract	0.5 g
Protease peptone No.3	0.5 g
Casamino Acids	0.5 g
Dextrose	0.5 g
Soluble starch	0.5 g
Sodium pyruvate	0 .3 g
Dipotassium phosphate	0.3 g
Magnesium sulphate	0.05 g
Agar	15 g

Final pH = 7.2 ± 0.2

Bushnell Hass media

For studying microbial utilization of hydrocarbons.
Formula per litre:

Magnesium sulphate	0.2 g
Calcium chloride	0.02 g
Monopotassium phosphate	1 g
Ammonium phosphate dibasic	1 g
Potassium nitrate	1 g
Ferric chloride	0.05 g

Tris buffer 1M pH = 8.0

Dissolve 121 g Tris base in 800 ml distilled water. Adjust to pH 8.0 by adding 42 ml of concentrated HCl. Adjust volume to 1 litre. Sterilize by autoclaving.

4/2 dilution buffer

Formula per litre:

Na_2HPO_4	4 g
KH_2PO_4	2 g

SDS lysis buffer

Add 8 ml H_2O to 3 g SDS, then add 15 ml 1.0 M Tris pH 8.0 and 0.6 ml 5 M NaCl. Heat gently to dissolve and adjust final volume to 30 ml with H_2O.

Platinum PCR Supermix

Complexed recombinant Taq DNA polymerase with Platinum® Taq Antibody	22 U/ml
Tris HCl pH 8.4	22 mM
KCl	55 mM
MgCl2	1.65 mM
dGTP	220 µM
dATP	220 µM
dTTP	220 µM
dCTP	220 µM

Solutions for 8% acrylamide-denaturant gel (for DGGE)

TAE buffer 50X

Tris base	242 g
Acetic acid, glacial	57.1 ml
0.5 M EDTA pH 8.0	100 ml
Milli-Q water	to 1000 ml

Polyacrylamide solution

40% wt/vol Acrylamide/Bis Acrylamide (37.5:1) solution.

0% denaturant solution

40% Acrylamide/BisAcrylamide	20 ml (8% final concentration)
50X TAE buffer	2 ml (1X final concentration)
Milli Q water	78 ml

100% denaturant solution

40% Acrylamide/Bis Acrylamide	20 ml (8% final concentration)

50X TAE buffer	2 ml (1X final concentration)
Formamide	40 ml (40% v/v final concentration)
Urea	42 g (7M final concentration)
Milli Q water	To 100 ml

LOW (25%) denaturant solution

Mix 12.0 ml of 0% denaturant solution with 4.0 ml 100% denaturant solution. Keep on ice.

HIGH (60%) denaturant solution

Mix 6.4 ml 0% denaturant solution with 9.6 ml 100% denaturant solution. Keep on ice.

Immediately before mixing/pouring the gel in the dispenser system, the following is added (always keeping on ice):

	High solution	**Low solution**
DCode dye solution	100 µl	---
10% fresh Ammonium persulphate	144.4 µl	144.4 µl
TEMED*	14.4 µl	14.4 µl

* N,N,N^1,N^1-tetramethylethylenediamine

The gel takes 30 minutes to polymerise.

DCode dye solution

Bromophenol Blue	0.05 g
Xylene cyanol	0.05 g
1X TAE buffer	10.0 ml

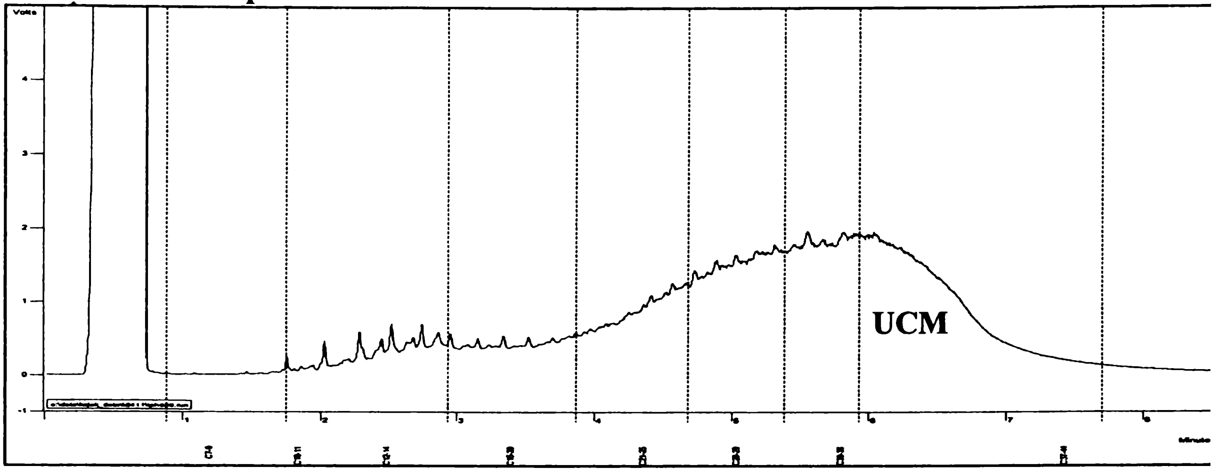
2X DGGE Loading buffer

0.05% bromophenol blue	0.25 ml
0.05% xylene cyanol	0.25 ml
70% glycerol	7.00 ml
Milli-Q water	2.50 ml
Total volume	10.00 ml

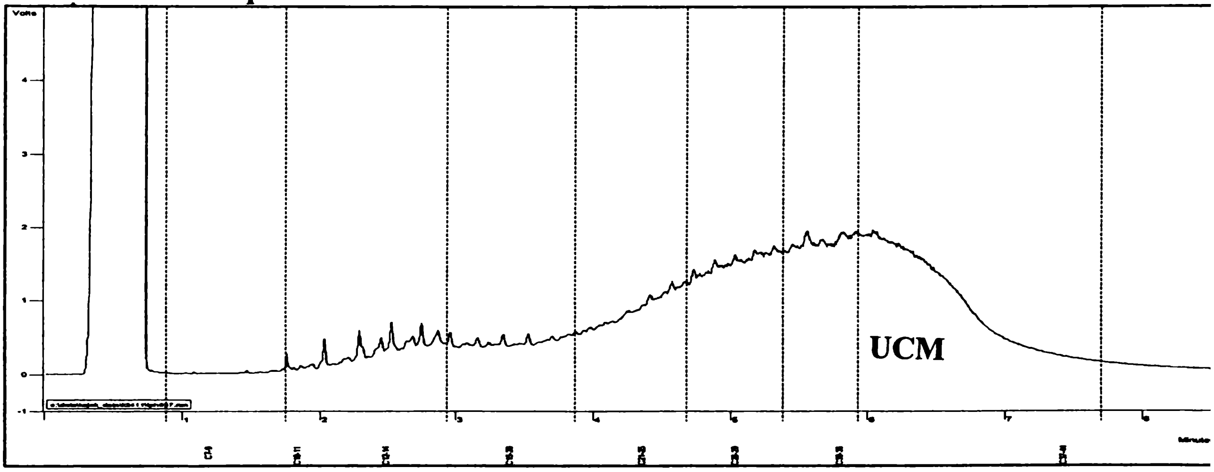
Appendix 2

Total Petroleum Hydrocarbon Chromatograms

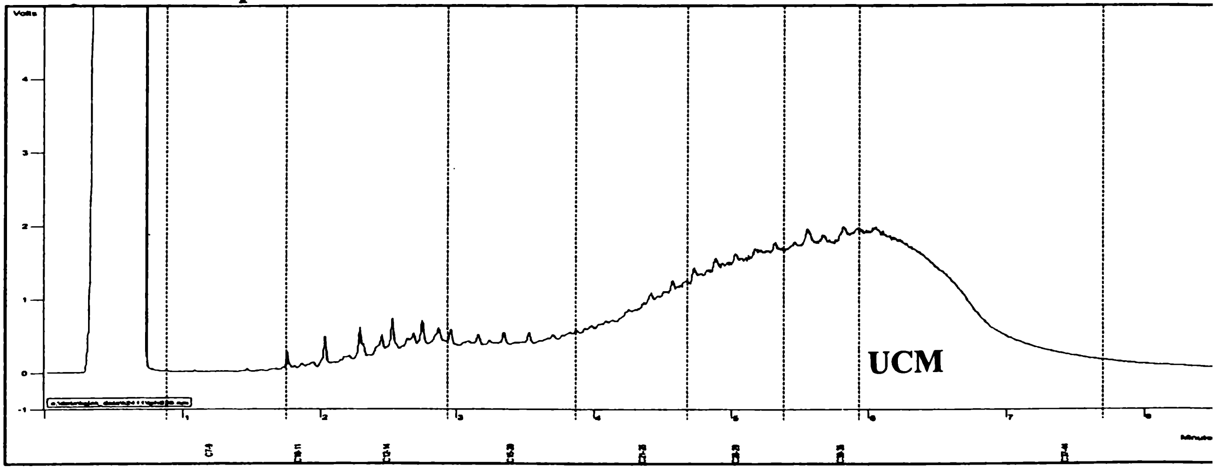
Scott Base Old Spill site-1



Scott Base Old Spill site-2

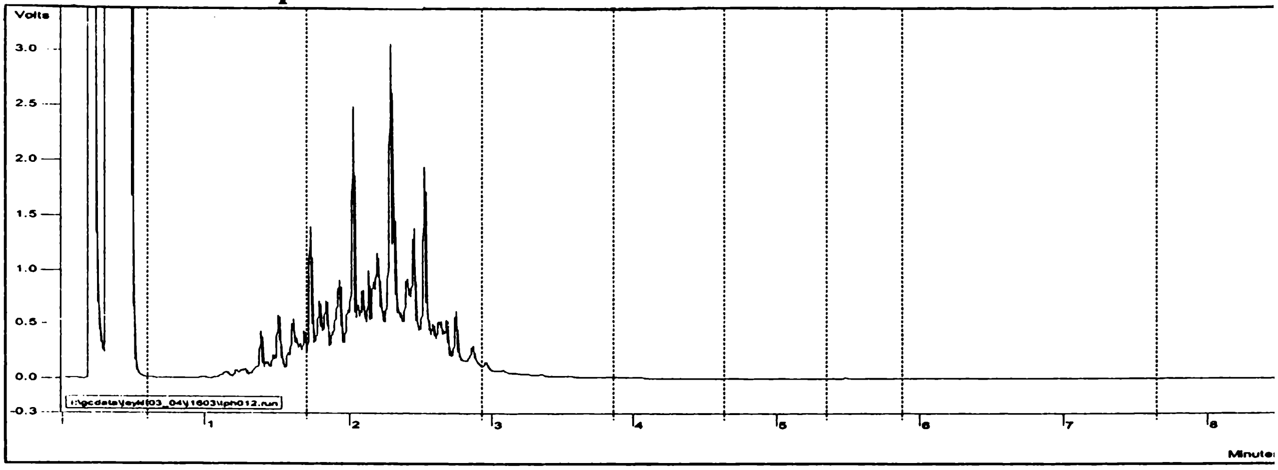


Scott Base Old Spill site-3

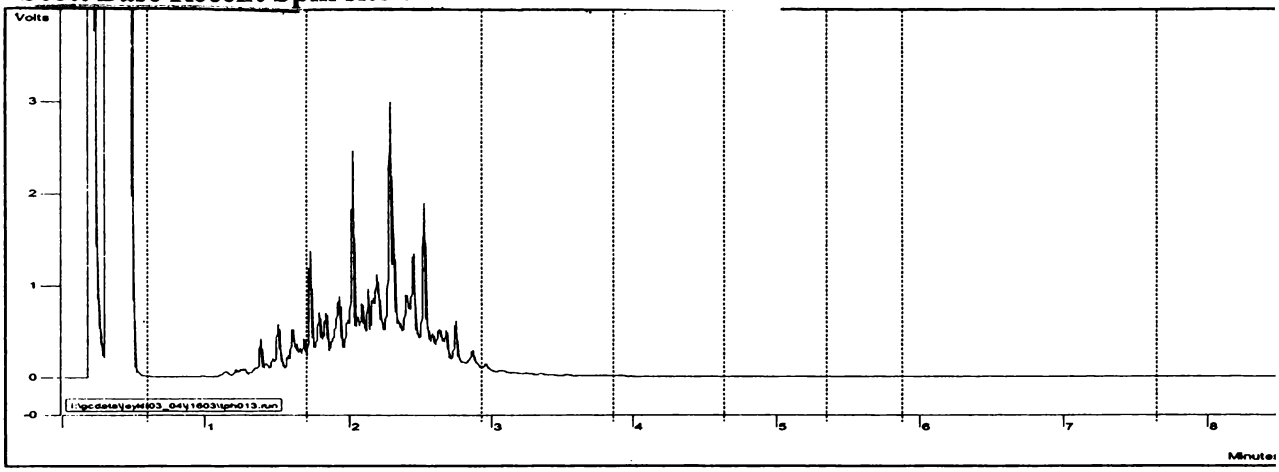


C7 C10 C15 C20 C25 C30 C34 C44

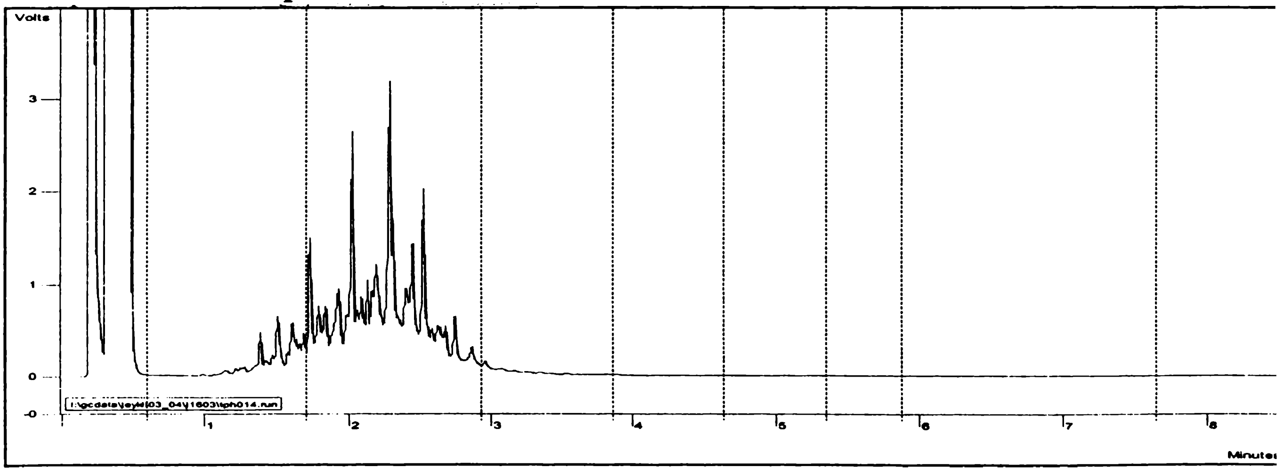
Scott Base Recent Spill Site-1

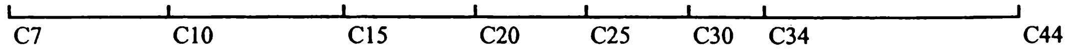


Scott Base Recent Spill site-3

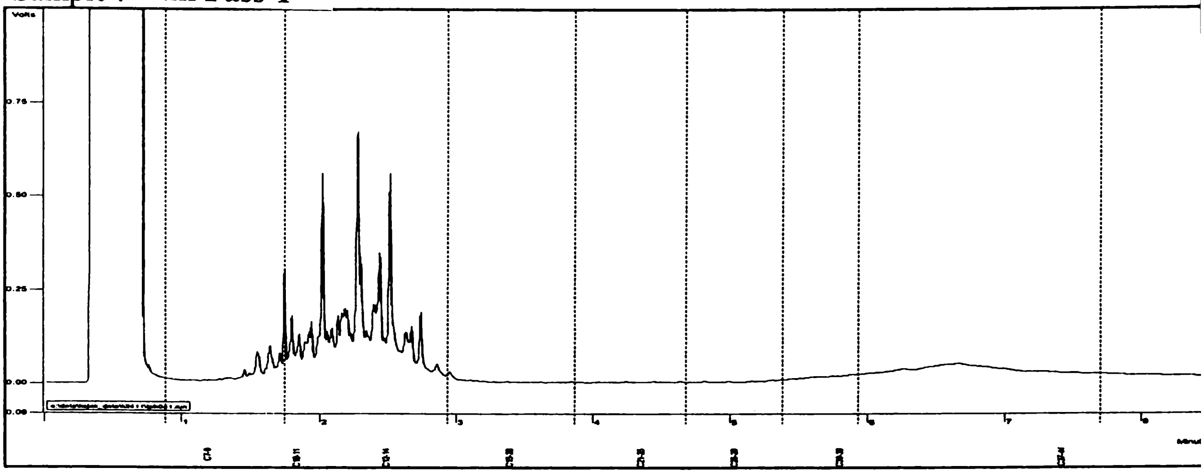


Scott Base recent Spill site-2

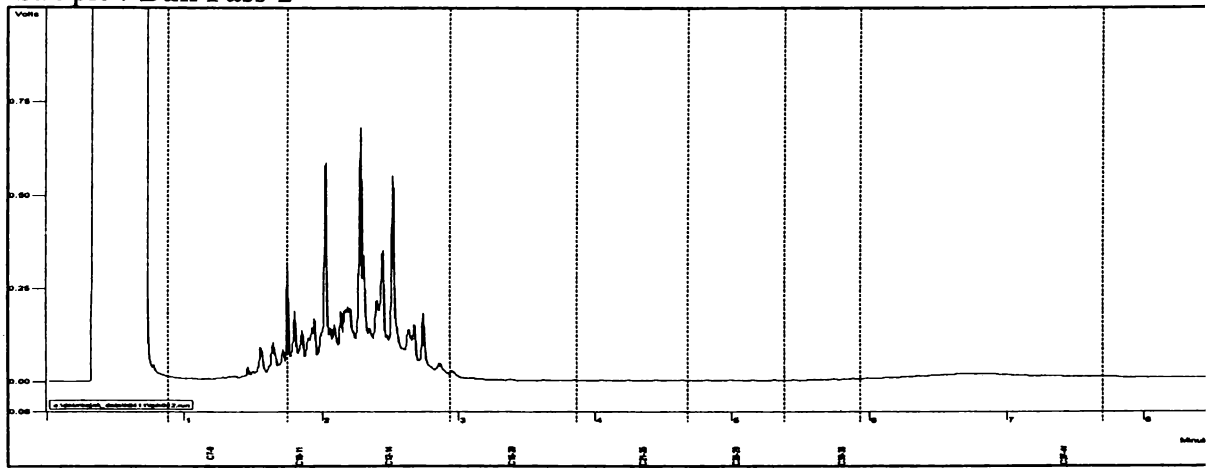




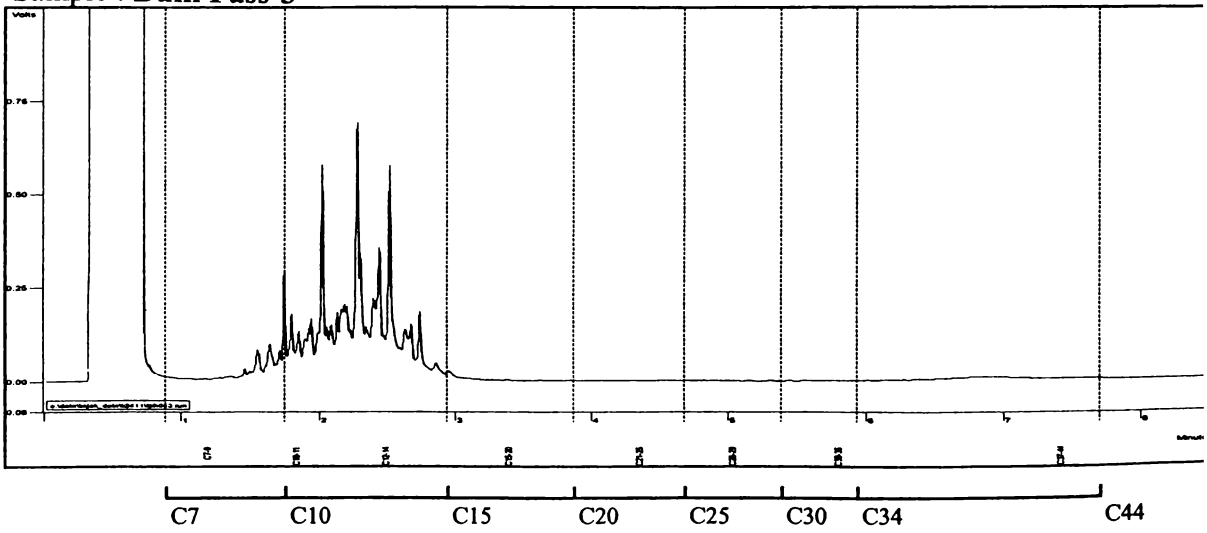
Sample :. **Bull Pass-1**



Sample : **Bull Pass-2**

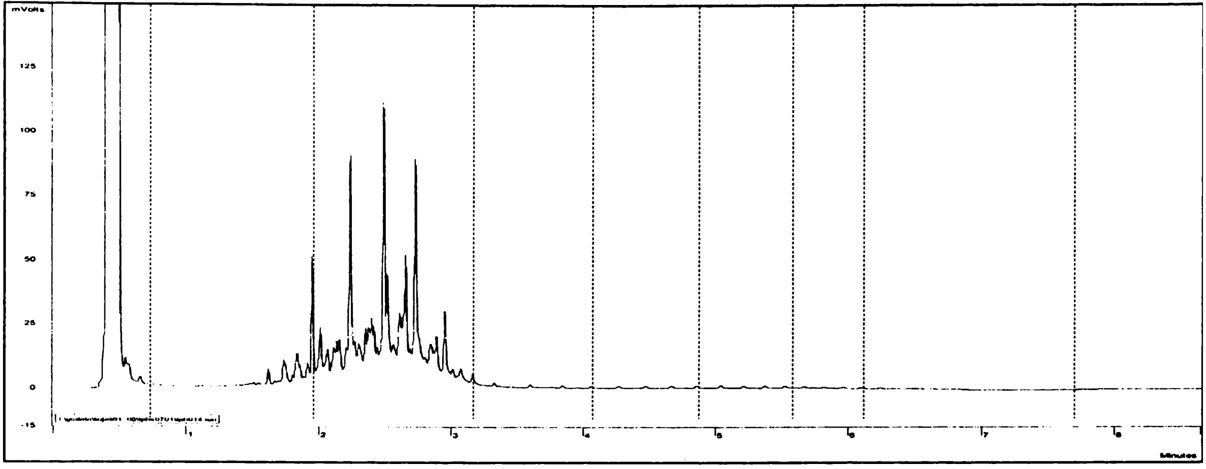


Sample : **Bulll Pass-3**

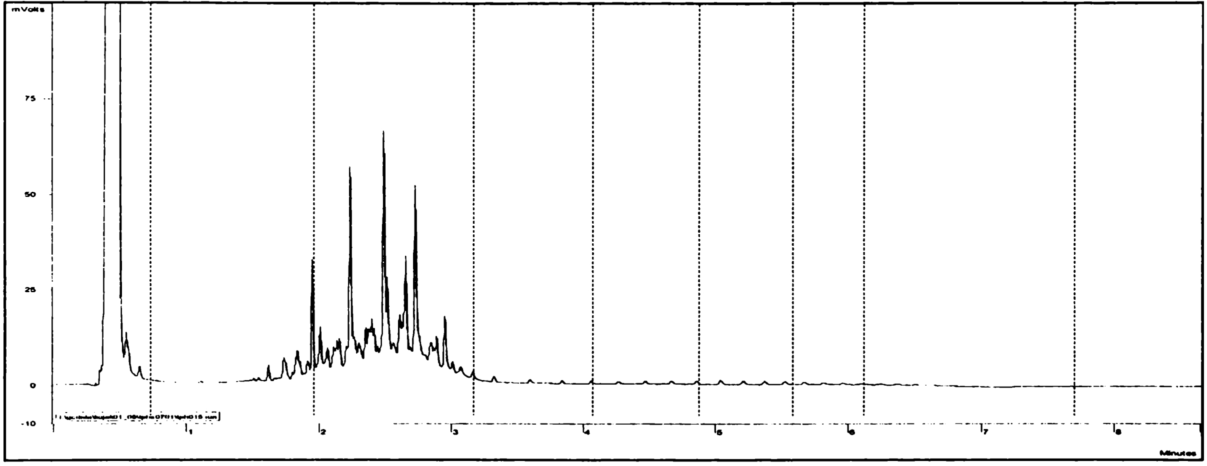


Bull Pass Bioaugmentation Experiment

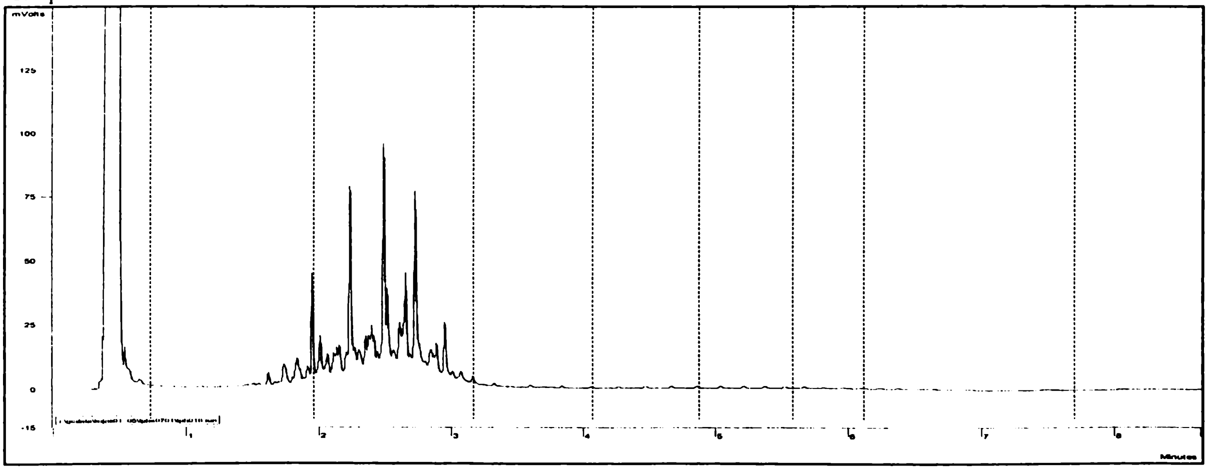
Sample : **Time cero –1**

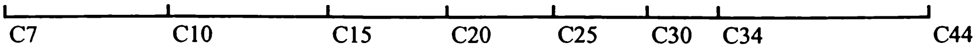


Sample : **Time cero –2**

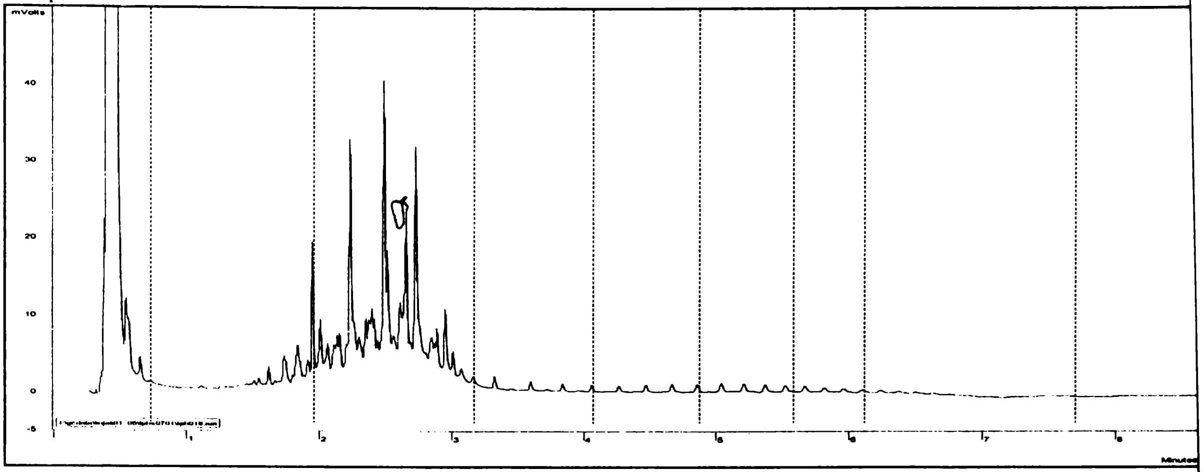


Sample : **Time cero – 3**

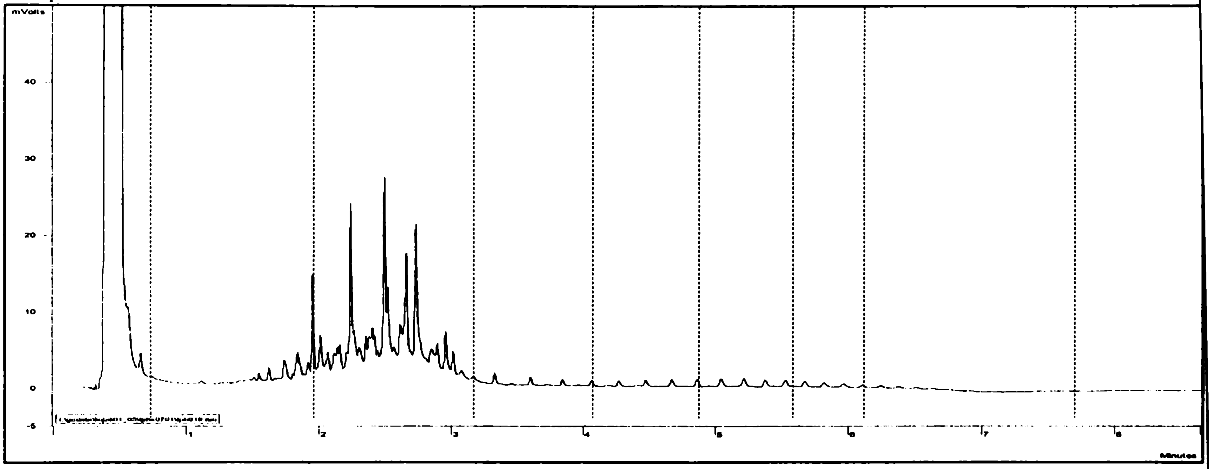




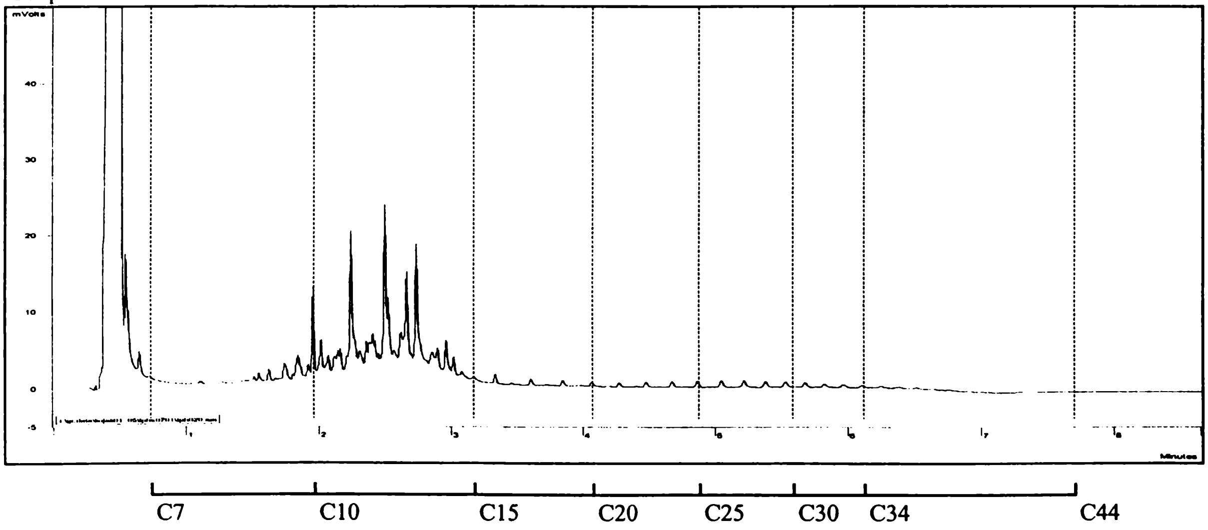
Sample : **BP1 - 1**



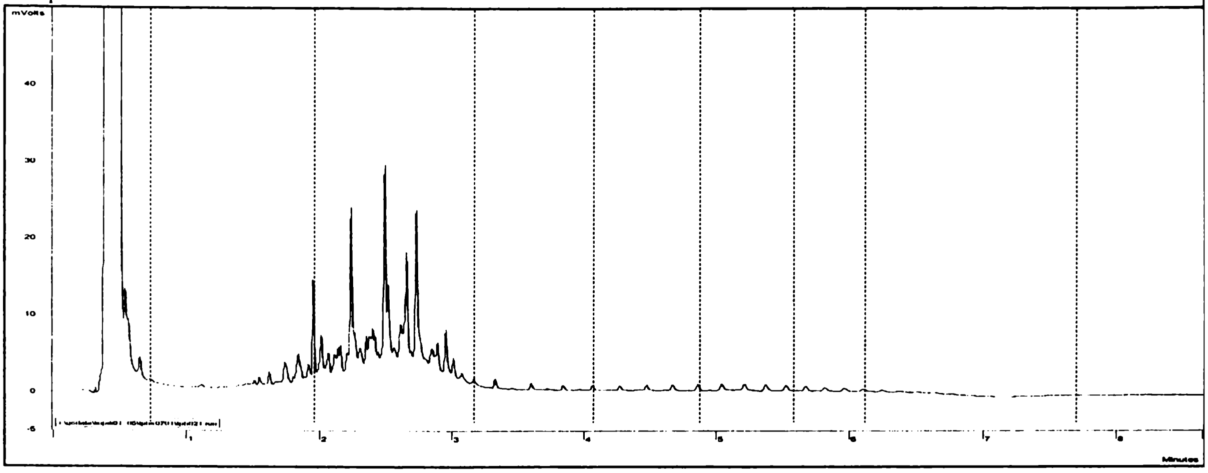
Sample : **BP1 – 2**



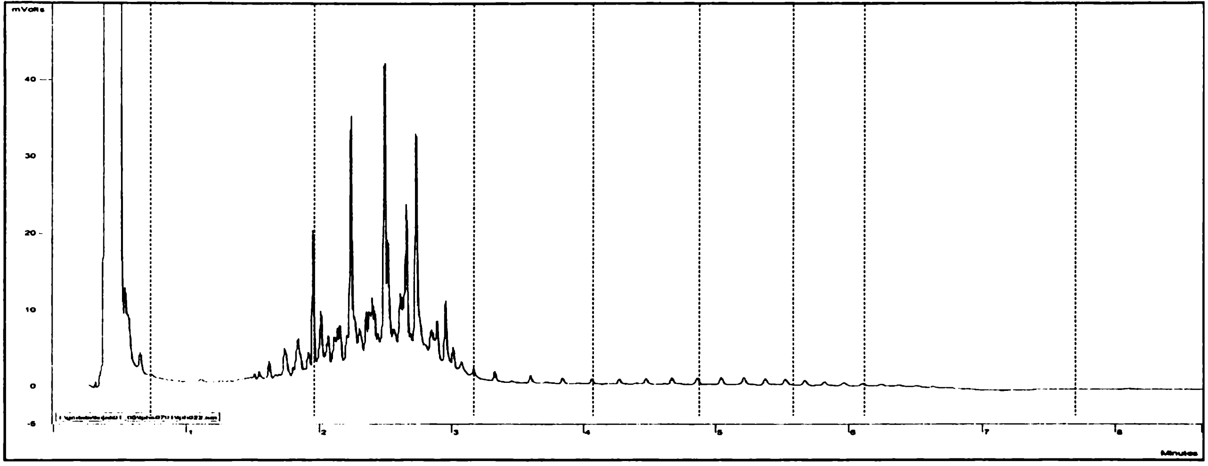
Sample : **BP1 – 3**



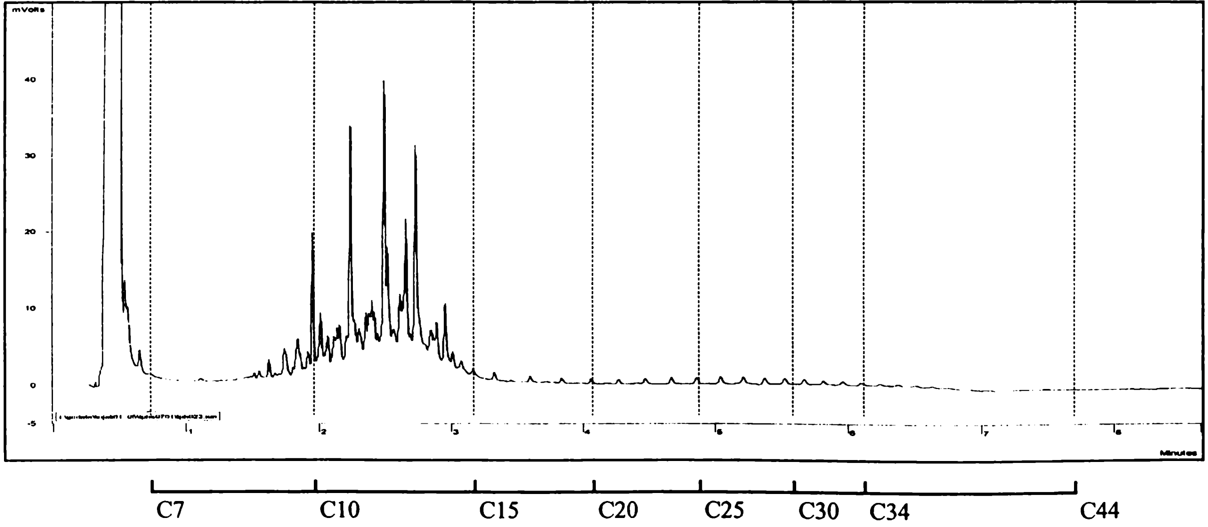
Sample : **BP2 – 1**



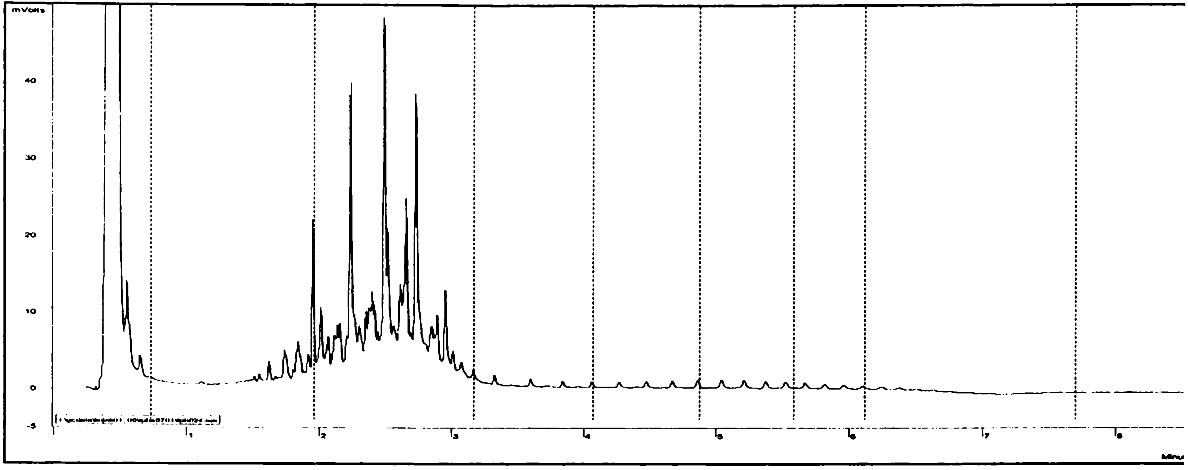
Sample : **BP2 – 2**



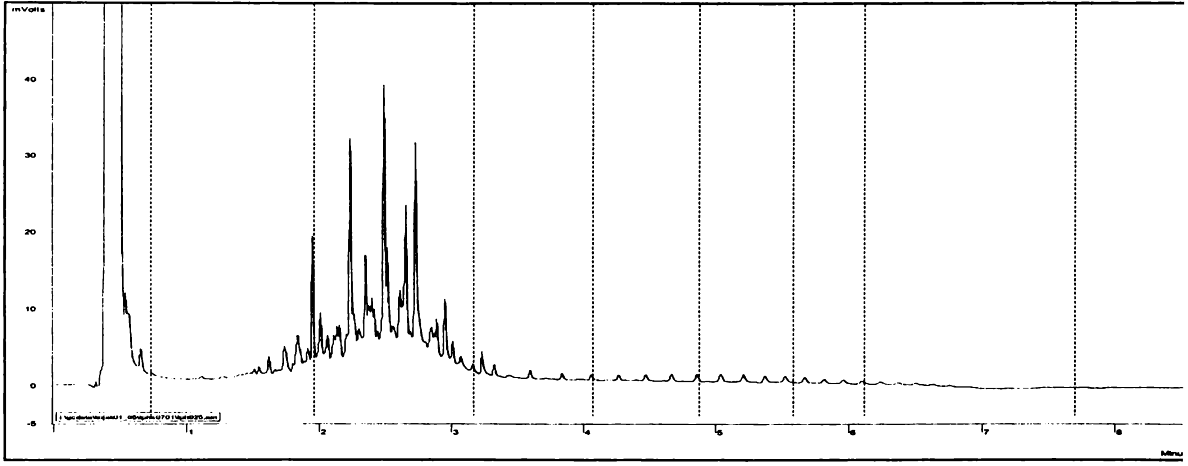
Sample : **BP2 – 3**



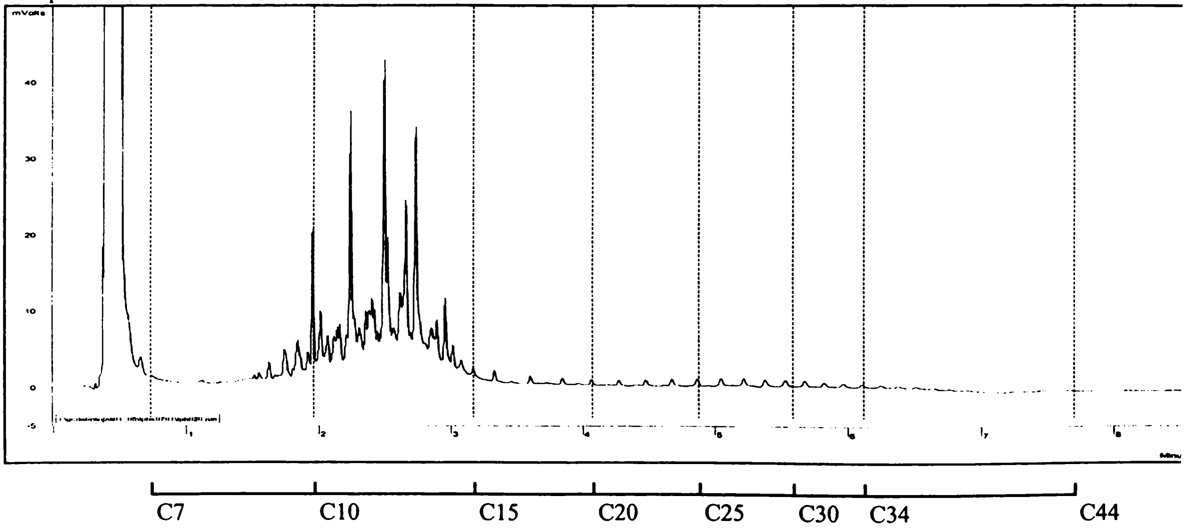
Sample : **BP3 – 1**



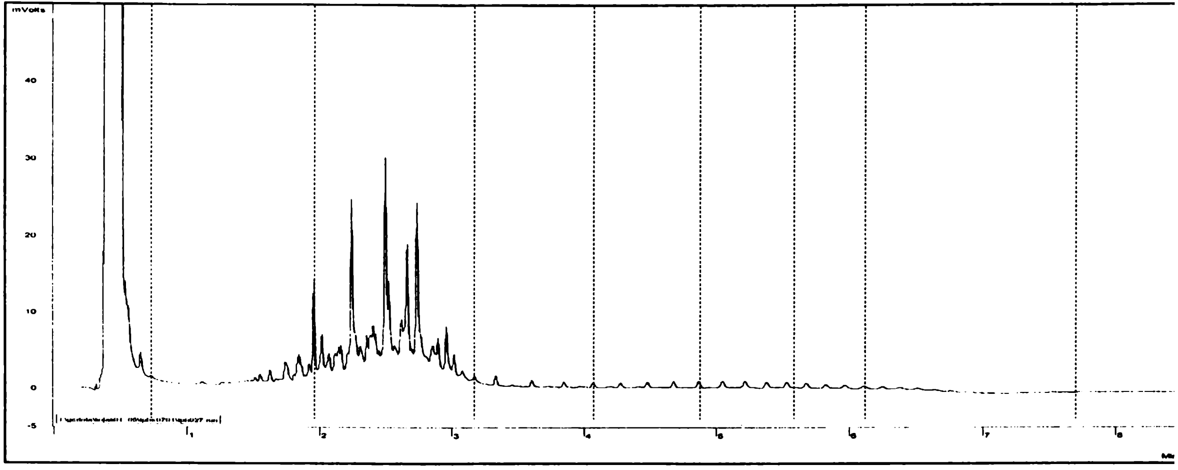
Sample : **BP3 – 2**



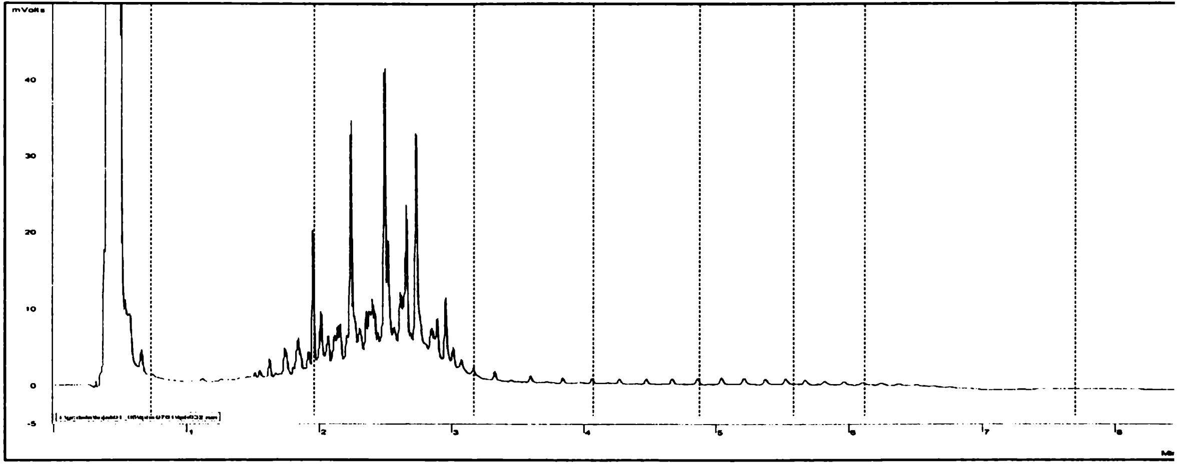
Sample : **BP3 – 3**



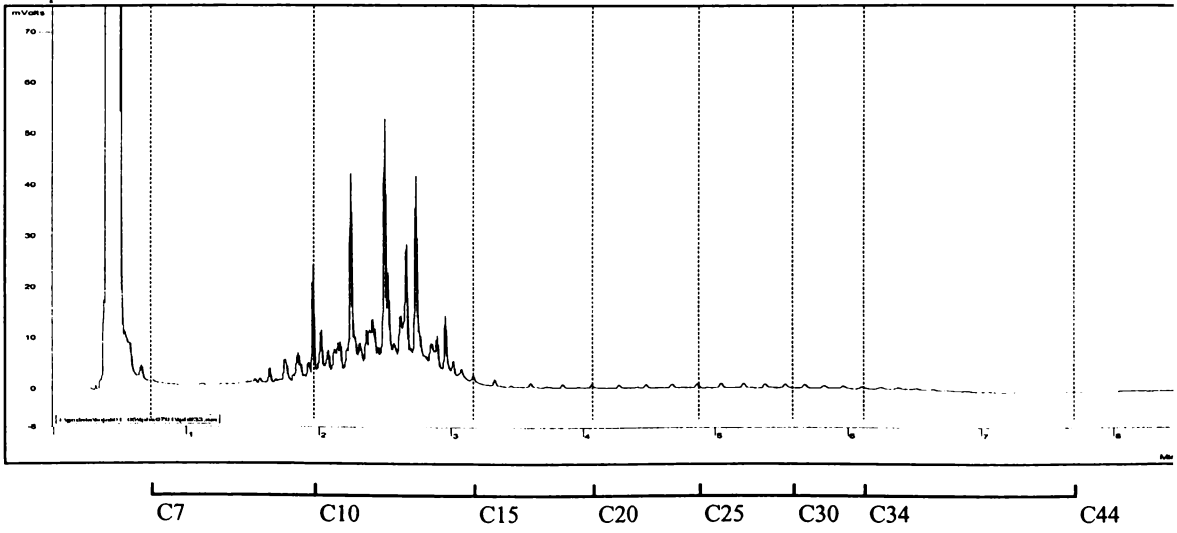
Sample : **BP4 – 1**



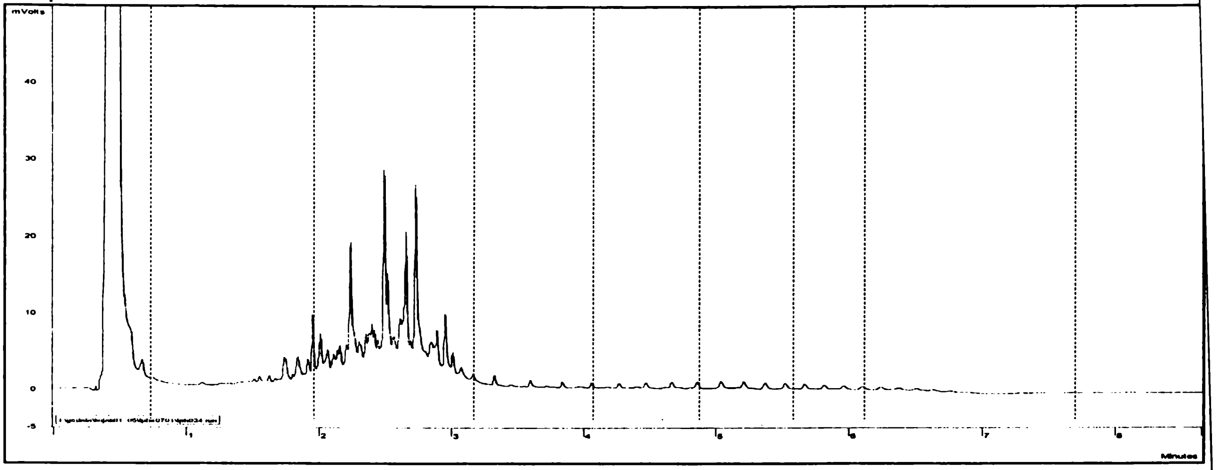
Sample : **BP4 – 2**



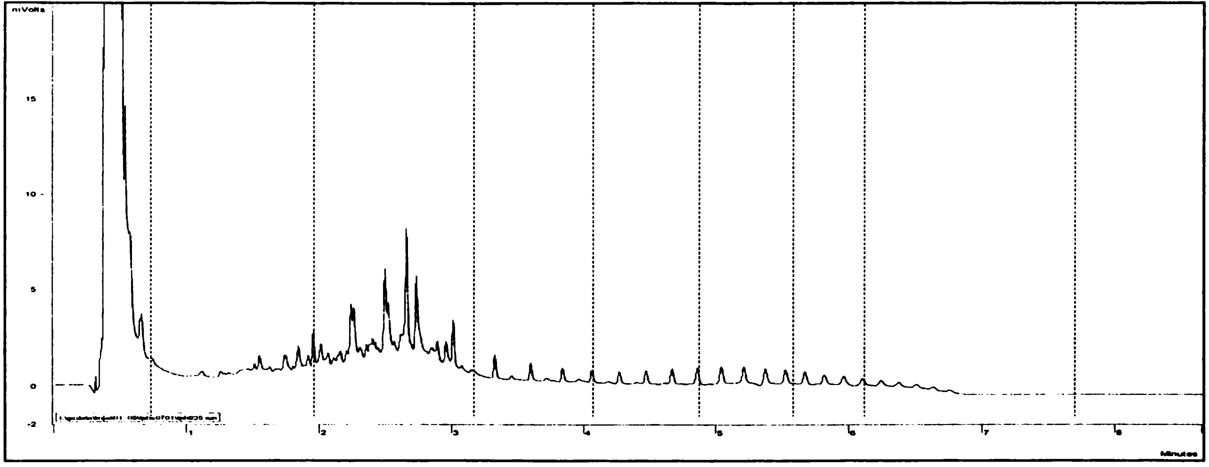
Sample : **BP4 – 3**



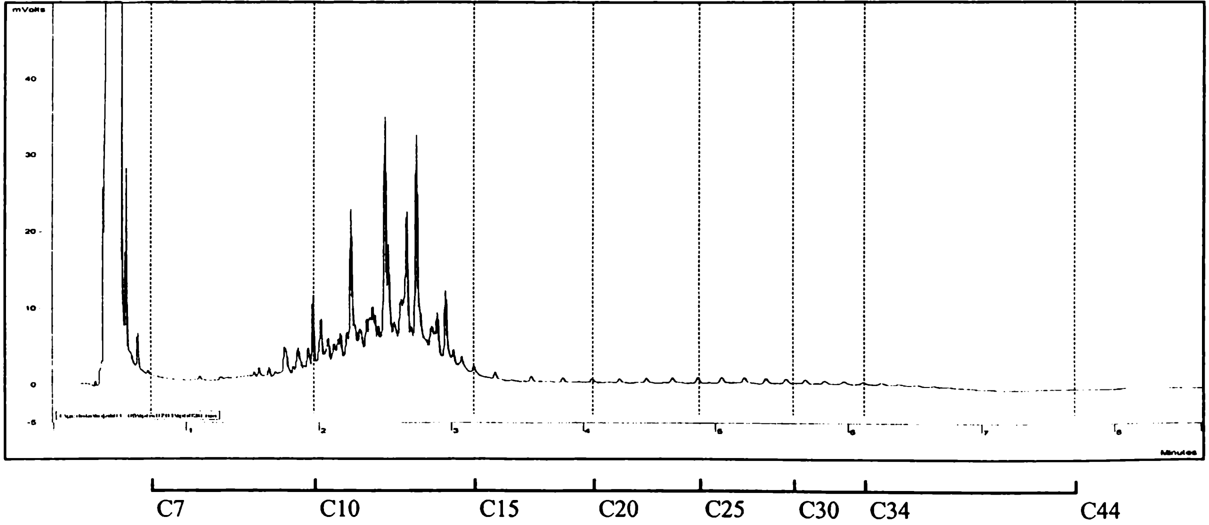
Sample : **BP5 –1**



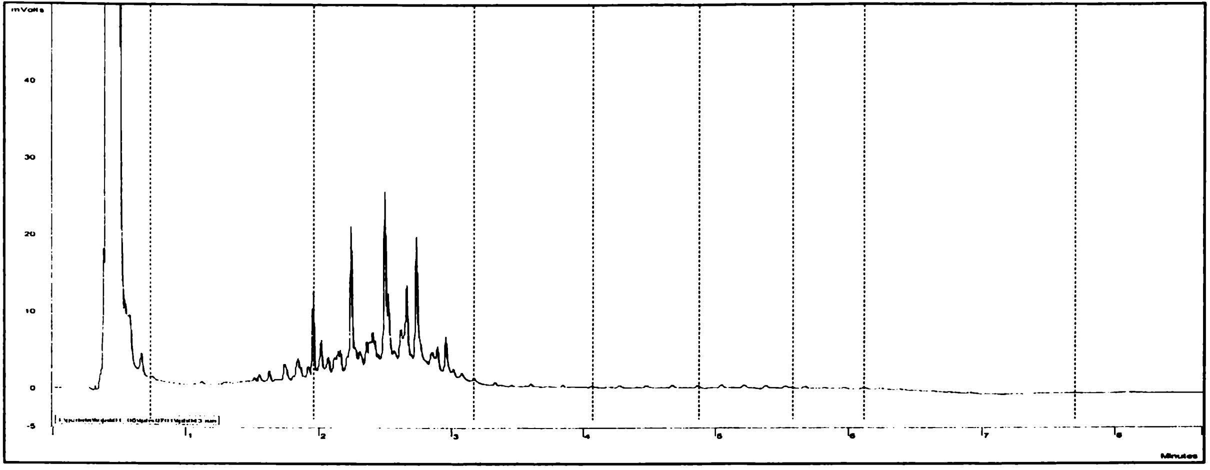
Sample : **BP5 – 2**



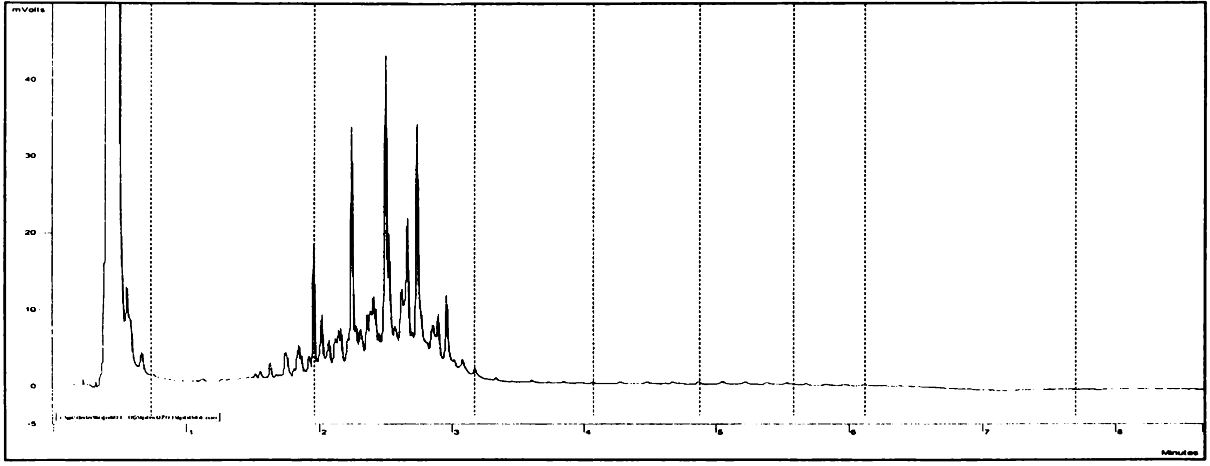
Sample : **BP5 – 3**



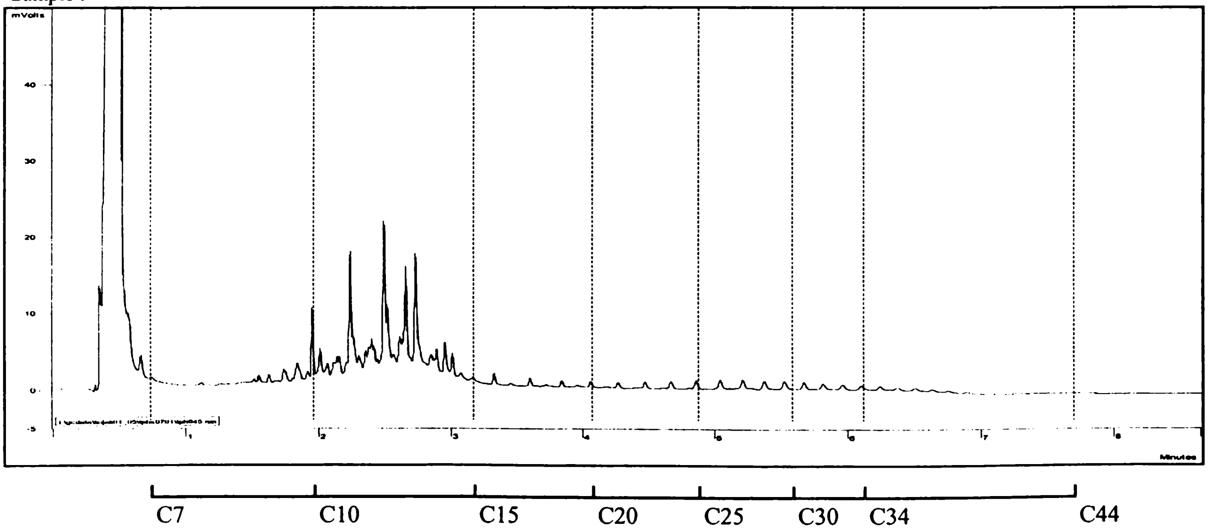
Sample : **Sterile control –1**



Sample : **Sterile control –2**



Sample : **Sterile control –3**



Appendix 3

Standard Methods for soil chemical analysis used by the Environmental Chemistry Laboratory from Landcare Research Ltd., Palemrston North, New Zealand

Method 114 Total carbon and nitrogen

Soil is heated in a stream of high purity oxygen in a Leco furnace to produce CO_2, N_2 and NO_x. A subsample of the combustion gases is passed through a heated copper catalyst that further reduces the NO_x to N_2, which is then measured by thermal conductivity. The CO_2 is measured with an infrared detector.

Method 118 Mineral Nitrogen

Mineral nitrogen is the nitrate- and ammonium- nitrogen that is extracted from the soil using 2M KCl (1:10 soil:extractant, 1 hour shaking.) The ammonium and nitrate are determined colorimetrically on a Lachat flow injection analyser.

Ammonia reacts with hypochlorite ions that are generated in situ by alkaline hydrolysis of sodium dichloroisocyanurate. This reaction forms monochloramine, which then reacts with salicylate ions in the presence of sodium nitroprusside to form a blue indophenol-type compound that absorbs strongly at 660 nm.

Nitrate is reduced to nitrite on a copperised cadmium column then diazotised with sulphanilamide followed by coupling with N-(1-naphthyl) ethylene diamine dihydrochloride. The method is adapted from that described by Blakemore *et al.* (1987) and is useful in nitrogen balance and leaching studies, and also for assessing plant-available nitrogen.

Blakemore, L.C., Searle, P.L., Daly, B.K. 1987. Methods for chemical analysis of soils. NZ Soil Bureau Scientific Report 80. 103p.

QuikChem Methods Manual. Lachat Instruments Division, Zellweger AnalyticsInc. Milwaukee, WI, USA.

Method 122 Total Kjeldahl Phosphorus

Soil is digested with concentrated sulphuric acid, plus sodium sulphate to raise the boiling point and copper as a catalyst, to convert phosphorus in the sample to orthophosphate that is then determined by a colorimetric method on a Lachat flow injection analyser. The colorimetric determination is based on the reaction of orthophosphate with ammonium molybdate and antimony potassium tartrate under acidic conditions. The complex formed is then reduced with ascorbic acid to form a blue complex that absorbs light at 880 nm.

Method 124 Olsen-Available Phosphorus

This method is based on the phosphorus extraction method of Olsen *et al.* (1954), as described by Blakemore *et al.* (1987), and uses an extraction with bicarbonate to estimate plant available phosphorus in soil (0.5M sodium bicarbonate, pH 8.5, 1:20 soil:extractant, 30 minutes shaking.) It is very similar to that used by commercial laboratories to produce soil P test values for New Zealand soils (Cornforth, 1980).

Phosphate in the extracts is determined by a colorimetric method on a Lachat flow injection analyser. The colorimetric determination is based on the reaction of orthophosphate with ammonium molybdate and antimony potassium tartrate under acidic conditions. The complex formed is then reduced with ascorbic acid to form a blue complex that absorbs light at 880 nm.

Blakemore, L.C.; Searle, P.L.; Daly, B.K. 1987. Methods for Chemical Analysis of Soils. New Zealand Soil Bureau Scientific Report 80. 103p.

Cornforth, I.S. 1980. Soils and fertilisers: Soil Analysis: Interpretation. Ag Link FPP 556. NZ Ministry of Agriculture and Fisheries.

Olsen, S.R.; Cole, C.V.; Watanabe, F.S.; Dean, L.A. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. USDA Department Circular 939.

Quikchem Methods Manual. Lachat Instruments Division, Zellweger Analytics Inc. Milwaukee, WI, USA

Method 104 Moisture Content

Samples are dried for 16 hours in a forced air convection oven at 105 °C then re-weighed. As analyses are carried out on air-dry material, the moisture factor is used to correct results to an oven-dry basis.

Method 106 pH

8g of soil is mixed to a slurry with 20 mL of deionised water (or 0.01M $CaCl_2$ or M KCl) and left to stand ovenight, then the pH is measured using a combination electrode. Use of $CaCl_2$ as a suspension medium is advocated by Peech (1965) because it is similar in electrolyte composition to soil solutions at optimum moisture conditions for plant growth in non-saline soils. Results with $CaCl_2$ are about 0.5 to 1 pH unit lower than with water. When 1M KCl is used, extensive ion exchange takes place, including the release of aluminium. Proton donors are in turn brought into solution, lowering the measured pH. It has been suggested by Black (1968) that pH values obtained with 1M KCl, which can be more than 1 pH unit lower than with water, may approach the pH values in the ion atmospheres of the original soil.

Black, C.A. 1968. Soil-Plant Relationships. 2nd ed. Wiley, New York.

Blakemore, L.C., Searle, P.L., Daly, B.K. 1987. Methods for chemical analysis of soils. NZ Soil Bureau Scientific Report 80. 103p.

Peech, M. 1965. Hydrogen-ion activity. Agronomy 9: 914-926.