Bioremediation of diesel contaminated soils: An investigation into the effects of biosurfactant and organic matter amendments.

By

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Dedication

To my family for their continued support and encouragement
Throughout the course of my education – Thank you.

I would also like to dedicate this thesis to the memory of my mother.

Acknowledgments

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Declaration.

This thesis has not previously been submitted to this, or any other college. With acknowledged exception, it is entirely my own work.

Gary Canny

Abstract

The aim of this study was to investigate the effects of biosurfactants and organic matter amendments on the bioremediation of diesel contaminated soil.

Two strains of *Pseudomonas aeruginosa* with the ability to produce biosurfactant were isolated from a water and soil sample in Co. Sligo. The first strain, Isolate A, produced a biosurfactant which contained four rhamnose containing compounds, when grown in proteose peptone glucose ammonium salts medium with glucose as the carbon source. Two of the components were identified as rhamnolipid 1 and 2 whilst the other two components were unidentified. The second strain, Isolate GO, when grown in similar conditions produced a biosurfactant which contained only rhamnolipid 2.

The type of aeration system used had a significant effect on the abiotic removal of diesel from soil. Forced aeration at a rate of 120L O_2/kg soil/ hour resulted in the greatest removal. Over a 112 day incubation period this type of aeration resulted in the removal of 48% of total hexane extractable material. In relation to bioremediation of the diesel contaminated sandy soil, amending the soil with two inorganic nutrients, KH_2PO_4 and NH_4NO_3, significantly enhanced the removal of diesel, especially the *n*-alkanes, when compared to an unamended control.

The biosurfactant from Isolate A and a biosurfactant produced by *Pseudomonas aeruginosa* NCIMB 8628 (a known biosurfactant producer), when applied at a concentration of three times their critical micelle concentration, had a neutral effect on the biodegradation of diesel contaminated sandy soil, even in the presence of inorganic nutrients. It was deduced that the main reason for this neutral effect was because they were both readily biodegraded by the indigenous microorganisms.

The most significant removal of diesel occurred when the soils were amended with two organic materials plus the inorganic nutrients. Amendment of the diesel contaminated soil with spent brewery grain (SBG) removed significantly more diesel than amendment with dried molassed sugar beet pulp (DMSBP). After a 108 day incubation period, amendment of the diesel contaminated soil with DMSBP plus inorganic nutrients and SBG plus inorganic nutrients resulted in 72 and 89% removal of diesel range organics (DRO), in comparison to 41% removal of DRO in an inorganic nutrient amended control. The first order kinetic model described the degradation of the different diesel components with high correlation and was used to calculate ½ lives. The ½ life, of the total *n*-alkanes in the diesel was reduced from 40 days in the control to 8.5 and 5.1 days in the presence of DMSBP and SBG, respectively. The ½ life of the unresolved complex mixture (UCM) in the diesel contaminated soil was also significantly reduced in the presence of the two organics. DMSBP and SBG addition reduced UCM ½ life to 86 and 43 days, respectively, compared to 153 days in the control. The component of diesel whose removal was enhanced the greatest through the organic material amendments was the isoprenoid, pristane, a compound which until recently was thought to be nonbiodegradable and was used as an inert biomarker in oil degradation studies. The ½ life of pristane was reduced from 533 days in the nutrient amended control to 49.5 and 19.5 days in DMSBP and SBG amended soils. These results indicate that the addition of the DMSBP and SBG to diesel contaminated soil stimulated diesel biodegradation, probably by enhancing the indigenous diesel degrading microbial population to degrade diesel hydrocarbons, whilst the addition of biosurfactants had no enhanced effect on the bioremediation process.

Abbreviations

API	American petroleum institute
CARACAS	Concerted action for risk assessment for contaminated sites
CFU	Colony forming units
CMC	Critical micelle concentration
DMSBP	Dried molassed sugar beet pulp
DRO	Diesel range organics
EC50	Median effective concentration
EPA	Environmental protection agency
FID	Flame ionization detector
GC	Gas chromatography
GEM	Genetically engineered microorganism
GN	Gram negative
GP	Gram positive
HLB	Hydrophile-lipophile balance
IC50	Median inhibitory concentration
LC50	Median lethal concentration
MHC	Moisture holding capacity
n-C9	Normal nonane
n-C10	Normal decane
n-C11	Normal undecane
n-C12	Normal dodecane
n-C13	Normal tridecane
n-C14	Normal tetradecane
n-C15	Normal pentadecane
n-C16	Normal hexadecane
n-C17	Normal heptadecane
n-C18	Normal octadecane
n-C19	Normal nonadecane
n-C20	Normal eicosane

n-C21	Normal heniecosane
n-C22	Normal docosane
n-C23	Noemal tricosane
n-C24	Normal tetracosane
n-C25	Norman pentacosane
NCIMB	National collection of industrial and marine bacteria
NICOLE	Network for industrially contaminated land in Europe
PAH	Polynuclear aromatic hydrocarbons
PCS	Petroleum contaminated soils
PPGAS	Proteose peptone glucose ammonium salts
R_f	Relative mobility
SBG	Spent brewery grain
STD	Standard deviation
THEM	Total hexane extractable material
TLC	Thin layer chromatography
TPH	Total petroleum hydrocarbons
TPHCWG	Total petroleum hydrocarbon criteria working group
UCM	Unresolved complex mixture
UST	Underground storage tanks
WAF	Water accommodated fraction

1.1 Introduction

In recent years the demand for petroleum as a source of energy and as a primary raw material for the chemical industry has resulted in an increase in worldwide consumption from 436 million tons in 1,950 to 3,423 million tons in 1998 (Molitor, 2002). Petroleum hydrocarbon products are used extensively in modern society. Products such as gasoline, petrol, diesel and jet fuels are used in the transportation industries, while the manufacture of chemicals which use petroleum as a base include plastics, pharmaceuticals, pesticides, herbicides and detergents. Along with this increase in production, there has been an increase in incidents of both accidental and deliberate release of petroleum products into our environment, most of which are associated with the manufacture, transport and storage of fuel.

Although tanker disasters, while transporting oil products from the major oil-producing countries to the major oil consuming countries receive the most public attention, such incidents contribute only about 5% of the total petroleum hydrocarbons entering the oceans annually. The major sources of oil pollution are in fact from municipal and industrial wastes and run-offs, leaks in pipes and storage tanks (Wraige, 1997).

The past decade has witnessed a dramatic increase in awareness of the potential problems associated with improper handling and storage of petroleum. In the United States alone, literally hundreds of thousands of incidents of identified soil, ground water and/or surface water contamination have been traced to the storage of petroleum at the millions of tanks across the country. As of September 2001, state and local environment agencies in the US have reported 418,918 confirmed releases from leaking underground storage tanks (UST), with the numbers increasing continuously (USEPA, 2002). At present in Ireland there is no such data available on releases from UST, but due to the large number of both used and unused petrol stations throughout the country it is expected that a certain percentage have developed problems and are releasing petroleum products into the environment.

Domestic fuel oil storage tanks are another area from which large quantities of petroleum products are released into the environment. In Ireland, the comparatively recent trend in change-over from the traditional solid fuel heating systems to the more convenient fuel oil systems has resulted in an increase in the number of these above ground storage tanks. Improper filling and maintenance of there storage tanks results in the release of fuel oil into ground in residential areas.

Releases of petroleum products are not only physically harmful to the environment, but they are also chemically harmful because they contain many toxic compounds in relatively large concentrations (Gold-Bouchot *et al.*, 1997). As a result, soils contaminated with petroleum products must be treated in a proper manner.

Soil treatment technologies are often developed and evaluated in order to conform with regulatory demands, which may require or suggest that residual total petroleum hydrocarbon (TPH) concentrations in soil be reduced to certain levels.

Various technologies exist for remediating soils contaminated with petroleum products, however the treatment selected will depend upon contaminant and site characteristics, regulatory requirements, costs, and time constraints (Ram *et al.*, 1993). Even though there are numerous treatments available, they can all be divided into two broad categories, physical/chemical technologies and biological technologies. Of these two technologies, biological remediation (bioremediation) is emerging as the most cost effective treatment for hydrocarbon polluted soils, especially when contaminated with medium-distillate fuel such as diesel oil, jet fuel, and No.2 heating oil (Wang and Bartha, 1994). Bioremediation has been defined by King *et al.* (1997) as "A treatability technology that uses biological activity to reduce the concentration or toxicity of a pollutant. It commonly uses processes by which microorganisms transform or degrade chemicals in the environment."

Bioremediation options can be further divided into categories according to where the process takes place. *In-situ* bioremediation is the treatment of the contaminant in place,

while *ex-situ* technologies involve the removal of the contaminated material for treatment elsewhere. The main advantage of *in-situ* treatment is that it allows soil to be treated without being excavated and transported, resulting in potentially significant cost savings. However, *in-situ* treatment generally requires longer time periods, and there is less certainty about the uniformity of treatment because of the variability in soil and aquifer characteristics and because the efficacy of the process is more difficult to verify. On the other hand the main advantage of *ex-situ* treatment is that it generally requires shorter time periods than *in-situ* treatment, and there is more certainty about the uniformity of treatment because of the ability to homogenize, screen, and continuously mix the soil. However, *ex-situ* treatment requires excavation of soils, leading to increased costs for engineering and equipment, possibly requirement of permits, and also material handling and worker exposure may need consideration.

Bioremediation of hydrocarbon contaminated sites can be limited by many factors, including nutrient limitation (usually nitrogen and phosphorus), oxygen supply, bioavailability of the diesel, bacterial biomass and the toxicity of the pollutant to the microorganisms degrading the diesel (Piehler *et al.*, 1999)

In the past, numerous studies (Radwan *et al.*, 1995; Venosa *et al.*, 1996; Sikdar and Irvine, 1998; and Taylor and Viraraghavan, 1999) have shown that the addition of nutrients in various forms increased the rates of hydrocarbon bioremediation. In relation to bioavailability of petroleum products, surfactants which are chemicals that contain both a hydrophilic and a hydrophobic portion and lower both the surface and interfacial tension of liquids, are thought to improve bioremediation by increasing the bioavailability of water insoluble compounds, such as diesel hydrocarbons. However, a review of the literature has shown that there are inconsistent reports in relation to surfactant addition, with some authors (Jain *et al.*, 1992; and Herman *et al.*, 1997) suggesting that surfactants enhanced bioremediation processes, while others (Fu and Alexander, 1995; Roch and Alexander, 1995; Deschenes *et al.*, 1996; and Stelmack *et al.*, 1999) reported that surfactants inhibited bioremediation processes. This is why further work on the subject is required.

Review articles on bioremediation of hydrocarbon contamination have mainly focused on environmental factors, i.e. temperature, moisture and oxygen, affecting microbial degradation of organic contaminants (Radwan *et al.*, 1995; Admassu and Korus, 1996; and Rosenberg and Ron, 1996). It is only in the past number of years that the effects of addition of organic material to contaminated soil (more commonly referred to as composting) has been investigated. Composting of yard waste and municipal solid waste has been performed for decades. However, composting of contaminated soils is still an emerging technology. In the composting of contaminated soil, organic amendments such as animal manure, yard wastes and food processing wastes may be added as a means of increasing the amount of nutrients and readily degradable organic matter which are normally deficient in soil (USEPA, 1996a). Other examples of organic amendments used include bark chip (Jorgensen *et al.*, 2000) sewage sludge and compost (Whang, 1999), a mixture of glucose and peptone (Radwan *et al.*, 2000), protein hydrolysate (Harrison *et al.*, 2000) and ground sterile corn-slash (Piehler *el al.*, 1999). The addition of organic material to hydrocarbon contaminated soils is thought to enhance the bioremediation process by increasing the overall microbial activity and also by enhancing the activity of specific hydrocarbon degraders. The use of dried molassed sugar beet pulp and spent grain have huge potential as energy and nutrient sources in the biodegradation of hydrocarbon contaminated soils since these materials are rich in organic matter and inorganic nutrients.

1.2. Selection of target contaminants

In Ireland the major fuel oils in storage tanks are diesel oil, petrol and kerosene. Remediation of soil contaminated with these hydrocarbons is of growing concern due to the ubiquitous contamination by them.

Diesel oil is one of the major fuel oils used world wide. In Ireland, diesel fuels are widely used as fuel for commercial trucks, agricultural machinery, railroad locomotives, industrial engines and domestic heating systems. Diesel is also used as a solvent to clean moving parts because the paraffins in diesel form a thin waxy coat that protects the metal parts from corrosion (Hwang, 1999). Although no information on the volume of soil contaminated with diesel is available in Ireland as of yet, it is anticipated that due to it widespread usage, contamination of environments with diesel fuels is inevitable. For these reasons, diesel oil was selected as the target contaminant for this research.

2. Literature Review

2.1 Composition of diesel

2.1.1 Description of diesel

Diesel fuels are classified as middle distillates and are denser than petrol. They are a complex combination of hydrocarbons produced by the distillation of crude oil. The product definition for diesel oil in the U.S. Chemical Substances Inventory (2002) under the Toxic Substance Control Act is the following:

Diesel Oil (CAS No. 68334-30-5)
A complex combination of hydrocarbons produced by the distillation of crude oil. It consists of hydrocarbons having carbon numbers predominately in the range of C_9-C_{20} and boiling in the range of approximately 163-357°C.

This U.S. definition encompasses both diesel fuel No.1 and diesel fuel No.2. Diesel fuel No.1 is essentially kerosene and can be characterised as a straight-run petroleum distillate with a boiling range of 150° to 400°C and consists predominately of hydrocarbons with carbon numbers in the range of C_9 to C_{16}. Diesel fuel No.2 or automotive and railroad diesel fuel, is defined as the fraction of petroleum that distillates after kerosene. It is generally a blend of straight-run and catalytically cracked streams having a boiling range generally of 160° to 360°C (Millner *et al.*, 1992). A third diesel, referred to as diesel fuel No. 4, or marine diesel, is less volatile and more viscous than diesel fuel No. 2 and is generally classed as a residual fuel. Diesel fuel No. 4 normally contains up to 15% residual oil components (Millner *et al.*, 1992).

Diesel fuels have many commercial uses. Diesel fuel No. 1 is used as rocket and jet engine fuel, domestic heating, solvent, insecticide sprays and as a motor fuel for light motors. Diesel No. 2 is used in drilling fluids and as fuel for trucks, ships, and other heavy automotive equipment. It is also used in large quantities for residential oil burners. Residual fuels oils, such as diesel No. 4 have been used to process steam for electric plants, and in other industries such as maritime industry, plants and factories, and the petroleum industry. Diesel No. 4 has also been used for space and water heating, pipeline pumping, and gas compression.

Due to the complex composition of diesel fuels it is difficult to define their physical and chemical properties. Custance *et al.* (1992) presented physical and chemical properties of diesel oil (see Table 2.1). From the table it can be seen that the solubility of diesel oil was reported as 0.2 mg/L and the density as 0.84 g/ml. Other properties which are used to define diesel are, viscosity of 1.4 to 26.5 (mm^2/s), sulfur content < 1.0 (% by weight), cetane number of 30 to 60 and a flash point of >55 °C.

Table 2.1. Physical and chemical properties of fresh diesel fuel (Custance *et al.*, 1992).

	Value
Density (g/mL)	0.84
Aqueous solubility (mg/L)	0.2
Vapor pressure (mmHg)	0.03
Diffusion coefficient in air (cm^2/sec)	4.63×10^{-2}
Henry's law constant (atm_m^3/mol)	4.2×10^{-2}
Log organic carbon-water partition coefficient (L/Kg)	3.04

2.1.2 Chemical composition of diesel

As diesel No.2 fuel was used in this study this diesel is discussed in greater detail. The chemical composition of diesel No. 2 fuel varies considerably depending on the origin of the crude oil and the manufacturing process used (Millner, 1992). Diesel consists of complex mixtures of aliphatic and aromatic hydrocarbons. The aliphatic alkanes, (paraffins) and cycloalkanes (naphthenes) are hydrogen saturated and compose approximately 64% of the diesel fuel. Aromatic (e.g. benzene) and olefins (e.g. styrene and indene) compose 35% and 1-2%, respectively, of diesel fuel (Agency for Toxic Substances and Disease Registry, 2002). Diesel fuels can also contain small amounts of n-hexane, benzene, toleune, ethylbenzene, and xylene. However, in general the concentrations of these compounds are relatively low compared to other components in diesel (Custance *et al.*, 1992). The typical range of diesel No.2 fuel is C_8-C_{26}, with the majority in the C_{10} to C_{20} range.

Probably the most complete review of the chemical composition of diesel No.2 fuel is that published by the Total Petroleum Hydrocarbon Criteria Working Group (TPHCWG), which as a working group was convened in 1993 to address the large disparity among cleanup requirements being used in the US by different states, at sites contaminated with hydrocarbon material such as fuels, lubricating oils and crude oils. Table 2.2 presents the chemical composition of diesel No. 2 fuel (Potter and Simmons, 1998).

Table 2.2. Summary of the composition of diesel No. 2 fuel oil (Potter and Simon, 1998).

Compound class	Carbon number	Compound	Average weight %
Alkenes		Total alkenes	1.3
Alkyl-Monoaromatics		Total Alkyl-Monoaromatics	6.2
	6	Benzene	0.029
	7	Toluene	0.18
	8	Ethylbenzene	0.068
	8	m+p-Xylenes	0.22
	8	o-Xylenes	0.043

Table 2.2 continued.

Compound class	Carbon number	Compound	Average weight %
	8	Total Xylenes	0.5
	9	1,3,5-Trimethylbenzene	0.18
	9	n-propylbenzene	0.039
	10	1-Methyle-4-isopropylbenzene	0.015
	10	n-Butylbenzene	0.038
Branched Alkanes	12	3-Methylundecane	0.17
	13	2-Methyldodecane	0.28
	14	3-Methyltridecane	0.19
	15	2-Methyltetradecane	0.48
	19	Pristane	0.6
	20	Phytane	0.5
Cycloalkanes		Total Dicycloalkanes	14
		Total Monocycloalkanes	19
		Total Tetracycloalkanes	0.1
		Total Tricycloalkanes	6.2
Diaromatics (except Naphthalenes)		Total Fluorenes	0.56
	12	Biphenyl	0.063
	13	Fluorene	0.082
	13	Total Methlybiphenyls	0.053
	14	Total Methylfluorenes	0.2
	15	Total Dimethylfluorenes	0.42
Inorganics		Total Nitrogen	0.0091
		Total Sulfur	0.072
		Water	0.00052
Metals		Arsenic	0.0000071
		Cadmium	0.000049
		Chromium	0.00017
		Iron	0.0037
		Manganese	0.00032
		Molybdenum	0.000014
		Zinc	0.00031
Monoaromatics		Total Benzocycloparaffins	6.3
		Total Benzodicycloparaffins	3
		Total Diaphthenobenzenes	1.8
		Total Indenes	3.1
	10	Total Indans and Tetralins	5.9
n-Alkanes	8	n-Octane	0.11
	9	n-Nonane	0.38
	10	n-Decane	0.78
	11	n-Undecane	1.4
	12	n-Dodecane	1.7
	13	n-Tridecane	2.1
	14	n-Tetradecane	1.9
	15	n-Pentadecane	2.6
n-Alkanes (continued)	16	n-Hexadecane	2.3
	17	n-Heptadecane	2.2
	18	n-Octadecane	1.6

Table 2.2 continued.

Compound class	Carbon number	Compound	Average weight %
	19	n-Nonadecane	1
	21	n-Heneieicosane	0.44
	22	n-Docosane	0.31
	24	n-Tetracosane	0.35
Naphthalenes		Total Naphthalenes	3.1
	10	Naphthalene	0.26
	11	1-Methylnaphthalene	0.48
	11	2-Methylnaphthalene	0.89
	11	Total Methylnaphthalenes	0.29
	12	1,3-Dimethylnaphthalene	0.97
	12	1,4- Dimethylnaphthalene	0.18
	12	1,5- Dimethylnaphthalene	0.29
	12	Total Dimethylnaphthalenes	0.69
	13	Total Trimethylnaphtalenes	0.24
Other		2-Azapyrene	0.00014
		Total Thioaromatics	0.3
	10	Ethylhexyl nitrate	0.2
	12	Dibenzoyhiophene	0.015
	13	1-Methylcarbazol	0.0016
	13	2- Methylcarbazol	0.00048
	13	3- Methylcarbazol	0.00038
	13	4- Methylcarbazol	0.00076
	14	1,2-Dimethylcarbazol	0.00058
	14	1,3- Dimethylcarbazol	0.00034
	14	1,4- Dimethylcarbazol	0.001
	14	1,6- Dimethyldibenzothiophene	0.0067
	14	2,6- Dimethyldibenzothiophene	0.02
	14	2-Phenylindole	0.00038
	15	6-Phenylquinoline	0.0007
	16	2-Ethyldibenzothiophene	0.017
	16	Benzo(def)carbazole	0.0003
	18	9-Phenylcarbazole	0.00036
Polynuclear Aromatics		Total Acenaphthenes	1.9
		Total Acenaphthylenes	1.5
		Total Biohenyls and Acenaphthenes	2.6
		Total Phenanthrenes	0.31
		Total Triaromatics	0.5
	14	2-Aminoanthracene	0.0004
	14	2-Aminophenanthrene	0.00024
	14	3-Aminophenanthrene	0.0002
	14	4-Aminophenanthrene	0.00034
	14	Anthracene	0.0058
	14	Phenanthrene	0.088
	15	1-Methylphenanthrene	0.0051
	15	2-Methylanthracene	0.0053
	15	2-Methylphenanthrene	0.16
	15	3-Methylphenanthrene	0.0038

Table 2.2 continued.

Compound class	Carbon number	Compound	Average weight %
	15	4-& 9-Methylphenanthrene	0.0067
	15	9-Cyanoanthracene	0.00064
	15	9-Cyanophenanthrene	0.00068
	15	Total Methylphenanthrenes	0.25
	16	Fluoranthene	0.0059
	16	Pyrene	0.0046
	16	Total Dimethylphenanthrenes	0.057
	17	1-Methylpyrene	0.00029
	17	2-Methylpyrene	0.00028
	17	Benzo(a)Fluorene	0.00028
	18	1-Methyl-7-isopropylphenanthrene	0.00066
	18	Benz(a)anthracene	0.000096
	18	Benzo(g,h,I)fluoranthene	0.000093
	18	Chrysene	0.000015
	18	Chrysene and Triphenylene	0.00012
	18	Triphenylene	0.00033
	20	Benzo(a)pyrene	0.00022
	20	Benzo(b+k)fluoranhene	0.000031
	20	Benzo(e)pyrene	0.000038
	21	Cyclopenta(cd)pyrene	0.000068
	22	Benzo(g,h,I)perylene	0.000012
	22	Indeno(1-2-3-cd)pryene	0.000016
	22	Picene	0.000015
Total Aromatics		Total Aromatics	22
Total Cycloalkanes		Total Cycloalkanes	37
Total Diaromatics		Total Diaromatics	6.3
Total Monoaromatics		Total Monoaromatics	16
Total n-Alkanes		Total n-Alkanes	13
Total Polynuclear Aromatics		Total Polynuclear Aromatics	0.36
Total Straight-Chain and Branched Alkanes		Total Straight-Chain and Branched Alkanes	41

2.2 Release of diesel to the environment.

In the United States alone the total production of distillates, which include diesel fuels, has increased from 16,240 thousand barrels per week in August 1982 to 26,719 thousand barrels in June of 2002 (Energy Information Administration, 2002). At every point in the diesel production, distribution, and consumption process, diesel fuels are invariably

retained in storage tanks. With billions of gallons of diesel being stored throughout the world, the potential for a diesel spill is significant, and the effects of such spills can pose serious threats to the environment.

Diesel may be released to soil and water environments as a result of fuel spills from transport vessels or from facilities located adjacent to surface waters, in runoff from industrial facilities, or from the intentional disposal of excess fuels down drains. Diesel fuels may also enter our environment as a result of leaking aboveground and underground storage tanks and pipelines.

There is no detailed information available on volumes of diesel spilled. However, trends are available on worldwide oil spills, which include diesel spills. Etkin (2001) carried out a historical review and analysis of reported oil spills and found that in the United States the total amount of oil spilled in the 1990s was 134 million gallons, a significant decrease from the previous two decades where 305 million gallons and 206 million gallons of oil were spilled. In the United States most of the oil spilled in the 1990s came from fixed facilities and land pipelines. Vessels contributed only 15%, while Outer Continental Shelf exploration and production facilities contributed only 1.4% of the total oil spillage. The same author estimated that over 943,170,000 gallons of oil was spilled world wide to marine waters between 1990 and 1999, but stated that this figure was an underestimination due to fact that data collected on an international basis was subject to serious reporting errors and omissions, particularly for spills from particular regions, for smaller spills, and from spills from non-vessel sources.

2.3 Environmental fate of released diesel

Due to the fact that this study deals with diesel contaminated soil this section is focused mainly on the fate of diesel after a release to soil. The fate and behaviour of spilled diesel, once it enters soil environments, depends on a number of physicochemical and biological factors including volatilisation, dispersion, advection, sorption and microbial

degradation. The combination of these processes, referred to as "weathering", reduces the concentrations of hydrocarbons in soil and water and alters the chemical composition of the spilled diesel.

The changes in the chemical composition of the spilled diesel have profound effects on its toxicity and biological impact on the environment. These processes are divided into two main categories; those that result only in the reduction of a contaminants concentration but not of the total contaminant mass in the system, which are termed "nondestructive" and those that result in degradation of contaminants which are referred to as "destructive" (USEPA, 1998b).

2.3.1 Nondestructive processes

Nondestructive processes include advection, hydrodynamic dispersion (mechanical dispersion and diffusion), sorption, and volatilisation (USEPA, 1998b).

2.3.1.1 Advection.

Advection is the transport of the contaminant by bulk movement of ground water. It is the most important process driving dissolved contaminant migration in the subsurface. In diesel contaminated sites this process has only a minimal effect due to the fact that diesel has only a water solubility of 0.2mg/L. However some of the more soluble fractions of diesel are transported leaving behind the non-aqueous phase fraction (USEPA, 1998b).

2.3.1.2 Dispersion

Hydrodynamic dispersion is the process whereby a contaminant plume spreads out in directions that are longitudinal and transverse to the direction of the plume migration. It

dilutes the concentration of the contaminant and introduces it to relatively pristine environments. Two very different processes cause hydrodynamic dispersion; mechanical dispersion which is the movement that occurs as a result of local variations in ground water velocity, and molecular diffusion which results from concentration gradients causing the contaminant to migrate from zones of high concentration to zones of lower concentration, even in the absence of ground water flow.

2.3.1.3 Sorption

Sorption, which is the process where contaminants adhere to soil particles, results in the retardation of the contaminant in relation to the ground water flow. Sorption can also influence volatilisation and biodegradation (Riser-Roberts, 1998). Because of their non-polar molecular structure, diesel hydrocarbons most commonly exhibit sorption through the process of hydrophobic bonding (Devinny *et al.*, 1994). Two components of soil particles that have the greatest effect on sorption are organic matter and clay minerals (USEPA, 1998b).

2.3.1.4 Volatilisation

Volatilisation, while not a destructive mechanism, does remove contaminants from soil systems. Volatilisation is not easily quantifiable or predictable, as a number of factors influence the process such as, soil type, depth of contaminant, concentration of the contaminant, temperature, state of the contaminant (i.e. fresh or previously weathered) and the length of time it is exposed (Margesin and Schinner, 1997a).

The particular difficulty with diesel fuel evaporation is that diesel fuels consist of a mixture of hundreds of compounds and this mixture varies from source to source and even over time (Fingas, 1995). Song *et al.* (1990) demonstrated that within 20 weeks of release of diesel oil to a loam soil, 26 to 35% of the added diesel oil was removed due to

volatilisation. In diesel it is only the compounds with low boiling points that are removed due to volatilisation. Sepic *et al.* (1995a) reported that only compounds with boiling points lower than *n*-C13 were removed due to volatilisation over a 48 day period. Volatilization is an important factor to take into account when monitoring diesel removal and, Fingas (1998) stated that evaporation is often considered to be the most important factor included in diesel oil spill models.

2.3.2 Destructive processes

2.3.2.1 Abiotic

Many organic chemicals in soil systems can be degraded through one or more abiotic mechanisms, although the reactions are typically not complete and often result in the formation of intermediates that may be at least as toxic as the original contaminant. In general, five abiotic mechanisms are known to occur in soil systems; hydrolysis, substitution, elimination, oxidation and reduction, all of which are described in greater detail by Dragun (1998). However in general, the rates of these reactions are often quite slow within the normal soil temperatures, with half-lives of days to centuries (Vogel *et al.*, 1987).

2.3.2.2 Biotic

In principle, biotic reactions refer to those reactions that involve biota in soil systems, these include plants (phytoremediation), animals, insects, and microorganisms. Although plants, animals and insects can degrade many hydrocarbons, they do not play a significant role in degrading hydrocarbons in soil. Microorganisms, on the other hand, do play a major role in degrading hydrocarbons in soil and the process where microorganisms breakdown hydrocarbons such as diesel is referred to as bioremediation and is discussed in greater detail in Section 2.7

2.4 Toxicity of diesel

All chemicals have the potential to cause toxicity and harm under the appropriate set of circumstances. Toxicants are defined as chemical agents, which under certain conditions, may produce adverse effects on biological systems, ranging from minor alterations of normal function to death (Millner *et al.*, 1992).

The toxicity of petroleum hydrocarbon mixtures such as diesel fuel is very hard to assess, due to their complex chemical mixture and the fact that once exposed to the environment they under-go significant changes (weathering) that are also difficult to predict.

There have been numerous studies carried out on the toxicity of individual compounds found in diesel fuel, however this data cannot be cross referenced directly to diesel fuel as the toxicity of an individual compound found in a diesel fuel may be quite different from the toxicity reported for the same compound acting alone (Gilbert and Calabrese, 1992).

Diesel fuel, as a whole product, has been studied in a number of *in vitro* and *in vivo* mutagenicity tests. The American Petroleum Institute (API) (1978), Henderson *et al.* (1981) Conaway *et al.* (1984), and Lee and Levy (1989) all evaluated diesel fuel toxicity using the Ames *Salmonella* test. They all concluded that diesel fuel was not mutagenic (with and without metabolic activation) to any of the *Salmonella* tester strains used. American petroleum institute (1978) also reported that diesel fuel did not cause gene mutation in the yeast strain *Saccharomyces cervisiae* D4.

Toxicity testing of diesel fuel using *in vivo* chromosome analysis in rat marrow cells was assessed by American petroleum institute (1978) and Conaway *et al.* (1984). They reported that diesel fuel significantly increased the frequency of chromosomal aberrations in the rat bone marrow after a single intraperitoneal injection of 2 or 6 ml per kg body weight.

Millner *et al.* (1992) reviewed the available epidemiological data and case reports of the carcinogenicity of petroleum hydrocarbons in humans and they found that while several of the epidemiological studies reported involved subjects or occupational groups with mixed exposures, particularly to gasoline and diesel fuel, there were no studies on diesel fuel itself. Since there was no adequate human data regarding the carcinogenic potential of diesel alone they used the conclusions of the International Agency for Research on Cancer (IARC) concerning the carcinogenicity of petroleum refining in general, and concluded that there is limited evidence that working in the petroleum refineries entails a carcinogenic risk. This limited evidence applies only to skin cancer and leukemia.

While the toxicity of diesel fuel to humans is of great concern after spillages, it is not the humans that are at greatest risk, but the indigenous species of the environment in the immediate area of the spill.

Lytle and Peckarsky (2001) evaluated the spatial and temporal effect of a diesel spill on stream invertebrates in a river in New York. They reported that the diesel fuel spill significantly reduced the density of invertebrates by 90% and the taxonomic richness by 50% at least 5km downstream of the spillage. They found that densities recovered within a year, however, the fauna immediately below the spill was species poor and significantly over represented by a single dominant taxon, suggesting that 15 months was not sufficient for full community recovery from the diesel spill.

Acute aquatic tests with *Daphina magna* showed that diesel fuel had a median lethal concentration (LC50) of 1.43 ppm, while Britvic *et al.* (1993) reported that carp (*Cyprinus carpio)* exposed for three days to 50ppb diesel fuel exhibited an increase of bile fluorescene to a level 5.8 fold higher than unexposed carp.

To date there have been no studies available on ecotoxicological testing of diesel fuel to terrestrial test organisms, however limited work has been carried out on other petroleum mixtures. Stephenson *et al.* (2000) assessed the toxicity of four fractions of a crude oil

mixture, F1 (C6-C10), F2 (>C10-C16), F3 (>C16-C34) and F4 (>C34), to a battery of terrestrial test organisms using methods developed by Environment Canada. The test organisms included 3 plant species, barley (*Hordeum vulgare* var. Chapais), alfalfa (*Medicago sativa*), and northern wheat-grass (*Agropyron dasystachyum*), earthworm (*Eisenia andrei*) and springtail (*Onychiurus folsomi*). The two fractions, which are relevant to diesel fuel, are fractions F2 and F3 as these are the fractions present in most diesel fuels. They reported that the toxicity of the different fractions varied considerably and was dependent on the plant species. In general the order of increasing toxicity was F4 <F3 <F2, with F2 having median inhibition concentration (IC50) of 2.91, 3.10 and 3.10 mg/g for alfalfa, barley, and northern wheat-grass respectively. They found that overall, invertebrates were more susceptible to adverse chronic exposure than plants, but the order of increasing toxicity was the same with F2 being most toxic. F2 negatively affected reproduction of both the springtail and earthworm at median effective concentration (EC50) of 1.47 and 0.49 mg per g, respectively.

All the above reviewed reports have carried out their toxicity testing on fresh diesel fuel samples, however once diesel is exposed to environmental conditions (Section 2.3) it immediately starts to weather, altering its chemical composition and toxicity properties. Neff *et al.* (2000) studied the effect of weathering on the toxicity of a diesel fuel to marine animals. They artificially weathered the diesel by distillation. Distillation temperatures of 150, 200 and 250°C were used to stimulate on-the-water weathering equivalent to 1 to 3 hours, 0.5 to 1 days, and 2 to 5 days, respectively, at 13°C air and water temperature with moderate wind conditions (Storm-Kristiansen *et al.*, 1993). Toxicity testing was carried out on the water accommodated fraction (WAF) of the diesel and its weathered fractions. The WAF was accomplished by a low energy mixing technique in which the oil was layered on top of seawater and gently mixed for 60 hours. They reported that the toxicity of the diesel fuel fluctuated with increased weathering and was dependent on species type. Table 2.3 is a summary of the results.

The exact toxic effect of diesel fuels and their weathered products to human and other forms of life is still unclear, but there is significant data available demonstrating that

they are toxic, and that environments contaminated by such products need to be remediated in order to render them "safe".

Table 2.3. The acute toxicity (96 hour LC50 for fish and crustaceans), expressed as percent water-accommodated fraction (WAF), of the WAFs of an Australian diesel fuel and its weathered fractions to five species of marine animals.

Oil fraction	**Clownfish** (%WAF)	**Sliverside minnow** (%WAF)	**Mysid** (%WAF)	**Shrimp** (%WAF)	**Sea Urchin** (%WAF)
Australian Diesel	>100	54	30	31	>100
Australian 200°C+	>100	79	32	31	>100
Australian 250°C+	>100	68	32	34	>100

2.5 Legislation in relation to diesel contaminated sites

With the increasing expertise in the area of contaminated land management, the perception of the problem has changed. In the early 1980s contaminated sites caught politicians and the general public by surprise. Up until then contaminated sites were perceived as, a few, very severe incidents with poorly known but possibly disastrous consequence for human health and the environment. The perceived risks led to policies aimed at maximum risk control, pollution should be removed or contained completely (Ferguson and Kasamas, 1999).

Today contaminated land is no longer perceived as a few severe incidents but as a widespread infrastructural problem of varying intensity and significance. It is now recognised that drastic risk control of cleaning up all sites to background levels or to levels suitable for the most sensitive possible land use, is technically and financially not feasible. For example, in the Netherlands in 1981 the number of sites thought to be contaminated and in need of cleanup was 350 with an estimated cost of 500 million

Euro. By 1995 this number had risen to 300,000 sites with an estimated cost of 13 billion Euros. Realities such as these have led to a revision of strategies on contaminated land management towards approaches based on the concept of *fitness-for-use*. At the same time the need is recognised for policies to protect soil and ground water from future pollution (Ferguson and Kasamas, 1999).

To date there is no legislation relating specifically to diesel contaminated sites. Instead diesel contaminated sites come under the heading of petroleum contaminated sites (PCS). In relation to policies on PCS, they vary greatly from country to country, and even within the one country they often vary significantly and are usually land-use dependent.

In the United States it was not until the 1980s that legislation in relation to PCS started to appear. In 1985, Kostecki *et al.* (1988) carried out a survey of the 50 states in America and found that of the 22 states that had established levels for cleanup of PCS, only 5 states considered the level formal. A follow up survey in 1987 (Bell *et al.*, 1989) indicated rapid development of numerous different cleanup standards between and within the states, including action levels, remediation goals, and cleanup levels. In 1990, due to the confusion in the United States regarding policies, rules and regulations for the clean-up of PCS, the Association of the Environmental Health of Soils (AEHS) conducted a state-by-state survey of environmental regulatory agencies in order to determine cleanup standards for PCS for use by regulated communities. This survey was completed in 2000 and the results were published by Kostecki *et al.* (2001) and it found that all 50 states had established some level of standards for PCS. Most states had established laboratory test protocols, detection levels, notification levels, action levels and clean up levels for the different products in PCS. However due to the complexicity of PCS and the fact that cleanup requirements differ greatly depending on the site location and sensitivity of the area, no one standard protocol was agreed amongst the different states. The survey also deduced that no one method of analysis satisfied all products in PCS, and even for individual products such as diesel fuel no one standard method of measurement prevailed throughout the different states.

The level of cleanup required varied greatly from state to state with numerous states taking the "site specific" approach for the determination of the cleanup level required. The states which had a defined numerical standard were not conclusive on one particular cleanup level, for example the cleanup level for diesel varied from 10mg Diesel range organics (DRO)/kg soil in Maine, to 100mg DRO/kg soil in District Columbia, while the state of Massachusetts had a clean up level of 1000 to 5000mg/kg soil for C_9-C_{18} aliphatics in diesel (Kostecki *et al.*, 2001).

At a European level a new dimension of international cooperation was started by the European Commission DG XII in 1996. Two Concentrated Actions on scientific aspects of contaminated soil and groundwater, Concerted Action on Risk Assessment for Contaminated Sites in the European Union (CARACAS) and Network for Industrially Contaminated Land in Europe (NICOLE), were initiated within the Environment and Climate Program to tackle the problem of contaminated sites. They bring together the combined knowledge of academics, government experts, consultants, industrial landowners and technology developers (Ferguson and Kasamas, 1999).

CARACAS was started in February 1996 after the agreement by environmental regulators from 16 European countries that such a scientific cooperation was required. Its main objectives were,

- Evaluation of the practical state-of-the-art of contaminated land risk assessment;
- Summary of current risk assessment approaches in European countries,
- Recommendations on scientific priorities for future R&D programs;
- Identification and compilation of ongoing R&D projects in Europe;
- Establishment of an information network on risk assessment issues;
- Stimulation of multinational cooperation on scientific aspects;
- Cooperation with other major international initiatives on contaminated land (Bardos *et al.*, 1999).

NICOLE, a forum established in 1996, is an alliance of industrial problem holders, research performers, technology developers, service providers and research planners. Its objectives were very similar to CARACAS except it is more relevant to industrial and commercial activities.

Irelands relatively late arrival into the industrial age means that its contaminated land problems are smaller than those of most other European countries, and significantly smaller that the U.S. At present Ireland lacks specific legislation for dealing with PCS and other contaminated land. However, some existing legislation does provide a considerable range of powers to the Environmental protection agency (EPA) and Local authorities to deal with the issue of contaminated land, these include;

- The Waste Management Act 1996
- The Environmental Protection Agency Act 1992
- The Local Government (Water pollution) Acts 1977, 1990
- The Building Control Act 1990
- The Air Pollution Act 1987

In line with other European countries, Ireland's approach to contaminated land encompasses pollution prevention, the polluter pays principle, the precautionary principle and the use of risk assessment to identify and prioritise sites requiring remedial action (Vik *et al.*, 2001). At present the EPA is considering setting non-statutory guideline values for contaminants, both in soil and groundwater. It is proposed that these values will be derived from existing risk-based generic guideline values adopted in other European countries. The values will be tailored to meet Irish conditions and policies through a process of consultation with relevant bodies.

In relation to petroleum contaminated sites in Ireland, currently the policy is that the local authority must be notified if a site becomes contaminated with a petroleum product and it is then up to the authority to decide on the appropriate action. The appropriate action is different from site to site and can vary from excavation and disposal of the

contaminated soil to landfill, to remediation of the contaminated soil either *in-situ* or *ex-situ*. In Ireland, the cleanup standard for PCS is land use specific, but in some cases the Dutch standard is used, which states a clean up level of 100mg Oil (total petroleum hydrocarbons) per kg soil. However, this is only used as a guideline not a definite endpoint.

2.6 Current Treatment Technologies

There are several treatment technologies available for the remediation of soils contaminated with diesel fuel, however the selection of treatment depends on a number of factors such as, the type of fuel, site characteristics, regulatory requirements, costs, and time constraints.

This section gives an overview of the different treatments available. However, as this study focused on biological treatments, more emphasis will be directed towards biological treatments.

2.6.1 *Ex-situ* processes

Excavation is a common approach to dealing with contaminated soil. Once the soil is excavated it can be treated on-site, off-site or in some cases it can be disposed of in landfill sites without treatment, all of which are referred to as *ex-situ* treatments.

Once the contaminated soil has been excavated, a number of physical, chemical and biological treatments can be employed to treat the soil. Some of the physical and chemical treatments, which have being applied successfully to treat hydrocarbon contaminated soils, include thermal treatment, incineration, soil washing, chemical treatment, chemical extraction, superficial fluid oxidation, volatilisation, and steam extraction, all of which are discussed in greater detail by Riser-Roberts (1998).

Biological treatments are emerging as the most cost effective treatment for hydrocarbon polluted soils, especially when the contaminant is a medium-distillate fuel such as diesel oil, jet fuel, and No.2 heating oil (Wang and Bartha, 1994). There are several ways in which excavated soils can be biologically remediated, including landtreatment/landfarming, numerous types of bioreactors, biopiles, and composting all of which involve microorganisms, and phytoremediation where plants are used to treat the contaminated soil (Riser-Roberts, 1998).

Advantages of excavation of contaminated soil are that it is easy to perform, and it rapidly removes the contamination from the site in a matter of hours, as opposed to other remediation methods which may require several months or even years to complete. Excavation is often used when urgent and immediate action is needed in order to stop the contaminant migrating and causing a health problem. However, there are a number of problems associated with excavation. It allows uncontrolled release of contaminant vapors to the atmosphere and nearby buildings, also buried utility lines, sewers, and water mains can interrupt the excavation process. Aboveground treatments also tend to be more expensive than *in-situ* methods (Riser-Roberts, 1998). The excavated contaminated soil may also be considered hazardous waste and disposal of such wastes are becoming increasingly restricted by regulations.

2.6.2 In-situ processes

In-situ treatment of contaminated soils is where the contaminated material is treated in its original location and offers an alternative to the traditional approach of excavation and re-disposal or on-site treatment. Numerous processes and systems have been proposed for *in-situ* treatment and, again, they can be broken down into biological and physical/chemical processes.

Some of the *in-situ* physical/chemical processes which have been successfully applied to remediate hydrocarbon contaminated soils include oxidation/reduction (Lee and Ward, 1985), hydrolysis, neutralisation, soil flushing/washing/extraction followed by pump and treat (USEPA, 1985), air striping (Dragun, 1998), soil venting (Dupont, 1993), and soil heating (Ram *et al.*, 1993). While all of these processes are relatively efficient in the clean-up of hydrocarbon contaminated soils, they are, however, expensive and in most cases unaffordable for government and private industries. *In-situ* bioremediation, on the other hand is relatively inexpensive and is now being considered as a viable alternative, but like *ex-situ* bioremediation, enhancements have to be carried out in order to make the process viable.

One of the drawbacks of bioremediation, or any other *in-situ* remediation effort, is the numerous uncertainties as a result of dealing with a complex and inaccessible material. Evaluation of remediation requires integrating tools and concepts from various diverse disciplines, which may not be standardised for cross-referencing (Riser-Roberts, 1998).

2.7 Bioremediation of diesel

Environmental contamination by petroleum products such as diesel is widespread and development of remediation efforts must accommodate the fact that there are vast numbers of sites that need to be treated and that there will be individual requirements for each site. Also, appropriate measures must be available for those areas of the world that cannot afford expensive treatments. Therefore, remediation must be simple and cost effective for both industrialised nations and underdeveloped countries in order to confront and resolve this enormous problem (Riser-Roberts, 1998).

Atlas (1991) reported that after the oil spill at Prince William Sound, Alaska, more than $1 million a day was spent in a partially successful attempt to clean it up. Neither the government nor the private sector can afford the cost of physically cleaning up all the

known toxic waste sites and, for this reason, bioremediation is now being considered as a viable alternative (Riser-Roberts, 1998).

As mentioned previously (Section 1.1) bioremediation is the processes by which microorganisms transform or degrade chemicals in the environment. The biodegradation of hydrocarbon compounds results in the modification or decomposition of the compound by soil microorganisms, to produce ultimately microbial cells, water and carbon dioxide. Microorganisms degrade compounds through the use of enzymes. These enzymes can be found either within cell membranes (intracellular) or outside the microorganisms cell membrane (extracellular). Whether or not an organic compound is transformed depends on (a) the chemical binding to the enzyme and (b) conformational changes at the enzyme active site. Degradable compounds will produce the ideal alignment of the enzyme and the organic compound, while persistent chemicals will produce a less favorable alignment. Compounds that are recalcitrant to microbial degradation either do not bind at all or fail to produce an alignment that leads to reaction (Dragun, 1998).

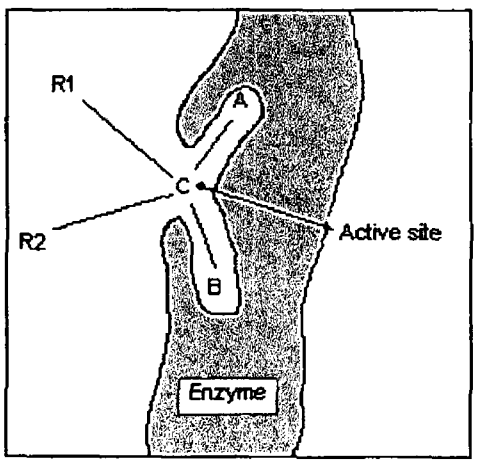


Figure 2.1 A hypothetical complex between an organic chemical and an enzyme within a soil microorganism. The molecular fragment A-C-B (but not the rest of the molecule R1 and R2) is transformed. Reproduced with permission from Dragun, 1998.

Bioremediation is currently receiving considerable attention as a remediation option for soils contaminated with diesel and other organic compounds. There is an enormous amount of both field application and laboratory tests being conducted to elucidate and refine the technology. In the United States alone, the amount of sites utilising bioremediation has increased from 3 in 1982 to over 135 in 1992 (Agency for Toxic Substances and Disease Registry, 2002). In order for a bioremediation process to be successful, microorganisms with the ability to degrade the target contaminant must be present.

2.7.1 Hydrocarbon degrading microorganisms

During recent years, a tremendous amount of research effort has concentrated on the isolation and characterisation of microorganisms that have the ability to degrade and metabolise hazardous organic chemicals, such as diesel fuel. The diverse group of bacteria, fungi and yeasts capable of degrading petroleum hydrocarbons are collectively called *hydrocarbonoclastic microorganisms* (Atlas, 1992a).

The ability to utilise hydrocarbon is widely distributed among diverse microbial populations. At least 30 genera containing over 100 species of microbes have been identified with the capacity for degradation of hydrocarbons (Arthur *et al.*, 1995). Their distribution in the environment is wide spread and they have been isolated from marine, freshwater and soil habitats. The distribution of hydrocarbonoclastic microorganisms reflects the historical exposure of the environment to hydrocarbons. Sizable increases in populations of hydrocarbonoclastic microorganisms occur when environmental samples are exposed to petroleum hydrocarbons. In unpolluted ecosystems, hydrocarbon utilisers generally constitute <0.1% of the microbial community while in oil-polluted ecosystems they can constitute up to 100% of the viable microorganisms (Atlas and Cerniglia, 1995).

The most commonly isolated organisms which have the ability to utilise hydrocarbons are heterotrophic bacteria of the genera *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Vibrio, Bacillus, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium* (Atlas, 1992) *Sphingomonas,* (Dagher *et al.*, 1997; and Shin *et al.*, 1999), *Mycobacterium* (Ermolenko *et al.*, 1997), *Alcaligenes* (Greene *et al., 2002*), *Aeromonas, Erwinia, Klebsiella, Lactobacillus, Leucothrix, Moraxella, Streptomyces* and *Xanthomonas* (Atlas and Cerniglia, 1995), and *Rhodococcus* (Kretschmer *et al.*, 1982; Guzev *et al.*, 1997 and Whyte *et al.*, 1998)

The genera of hydrocarbonoclastic fungi most frequently isolated are *Penicillum* and *Verticillium* (Davis and Westlake, 1979), *Aspergillus* and *Cladosporium* (Atlas and Cerniglia, 1995). Other less frequently isolated hydrocarbon degrading strains include *Beauveria, Mortieriella, Phoma, Scolecobasidium* and *Tolypocladium* spp.(Riser-Roberts, 1992)

Komagata *et al.* (1964) carried out a study of 500 yeasts and found that 56 were able to degrade hydrocarbons. Almost all of these belonged to the genus *Candida.* Hydrocarbonoclastic strains of *Rhodosporidium, Rhodotorula, Saccharomyces, Sporobolmyces* have been isolated from soil by Ahearn *et al.* (1971), Bento and Gaylarde (1996) and Chang *et al.* (2001).

Some moulds have also been isolated with the ability to utilise specific hydrocarbons, including four strains of *Pseudallescheria boydii* (April *et al.*, 1998) and two strains of *Graphium* (Lowery *et al.*, 1951; and Curry *et al.*, 1996). These strains were isolated from oil-contaminated soils which had the ability to degrade linear crude oil aliphatics.

It is only in more recent years that studies have focused on the isolation of microorganisms that have the ability to utilise diesel fuel as their sole carbon source. Wilson and Bradely (1997) and Barathi and Vasudevan (2001) isolated strains of *Pseudomonas fluorescens* which were able to utilise diesel fuel, while Gao *et al.* (2000)

and Grishchenkov *et al.* (2000) isolated other *Pseudomonas* sp. which were able to degrade diesel fuel as the sole carbon source.

Rhodococcus species, with the ability to degrade a wide range of aliphatic hydrocarbons present in diesel fuel, were isolated by Guzev *et al.* (1997) and Whyte *et al.* (1998). Grishchenkov *et al.* (2000) isolated a *Brevibacillus* sp. BS2202 which was able to utilise diesel hydrocarbons under both aerobic and anaerobic conditions, while *Acinetobacter calcaocetius* and *Acinetobacter venetiamus* were both shown to have the ability to utilise diesel fuel (Marin *et al.*, 1995; Baldi *et al*,. 1999, and Greene *et al.*, 2002). Ekundaya and Obuekwe (2000) isolated 35 yeasts (27 strains of *Candida* and four strains of *Endomycopsis*), which were able to grow on numerous hydrocarbons including diesel fuel.

The biodegradation of individual hydrocarbon compounds by pure hydrocarbonoclastic microorganisms has been studied extensively (Reham and Reiff, 1981; Gibson and Subramanian, 1984; and Heitkamp *et al.*, 1988) and the number of strains are increasing constantly. The problem associated with these pure cultures is that they can only utilise certain individual or groups of hydrocarbons. It is now generally agreed that there is no one "hero" microbial species that is capable of attacking all the components of complex hydrocarbon mixtures such as diesel. Wardley-Smith (1976) reported that a single species cannot even degrade all the members within a homologous series. For example, specificity has been found within the alkanes and for aromatics such as benzene and naphthalene. Some hydrocarbons like alkanes can be easily degraded by a large number of microorganisms which use them as sources of carbon and energy, while on the other hand other hydrocarbons such as polycyclic aromatic and cyclo-alkanes are mainly degraded by cometabolism (Bouchez *et al.*, 1995).

Because of the large diversity of molecules contained in petroleum products, the cometabolism phenomenon is favoured (Richard and Vogal, 1999). The importance of cometabolic transformation of organic contaminants in the environment was first realised when the isolation of the responsible organism(s) by traditional microbiological enrichment techniques proved unsuccessful, despite the fact that transformation of the

specific contaminant was shown to be taking place in the environmental niche under investigation (Colleran, 1997). Cometabolism is defined as the process whereby microorganisms involved in the metabolism of growth promoting substrate also transform other organic contaminants (co-substrates) that are not growth supporting if supplied as a sole carbon and energy source (Colleran, 1997). For example, Ko and Lebeault (1999) reported that a bacterial strain, *Rhodococcues equi* P1, was unable to grow on decaline (a cyclo-alkane) as a sole carbon source. However, the addition of hexadecane as a growth substrate resulted in complete degradation of 1% (v/v) decaline. Transformation of trichloroethylene under aerobic conditions was catalyzed by methanotrophic bacteria, which utilised methane as their growth substrate (Little *et al.*, 1988). A *Pseudomonas fluorescens* sp. was isolated by Richard and Vogal (1999) and was shown to cometabolise phenanthrene with Tween 80 as the growth substrate.

A mixed microbial community of all hydrocarbon-degrading microorganisms is not always the best solution. The importance of "secondary" strains of non-hydrocarbon degrading bacteria has been demonstrated as playing a major role in optimisation of hydrocarbon degradation. Hubert *et al.* (1999) found that incubation of a soil sample with 0.1% (w/w) toluene resulted in a community that was dominated by *Pseudomonas* strain Q7. However, this strain was not able to grow by itself in an atmosphere equilibrated with 0.1% (w/w) toluene, whereas it grew efficiently in co-culture with another *Pseudomonas* sp., suggesting that it is toluene metabolites that are utilised by strain Q7. Richard and Vogel (1999) isolated a diesel-degrading consortium consisting of seven members. Only three of the seven strains were able to grow on diesel fuel as the sole carbon source. In experiments with the combined consortium the appearance of the "secondary" strains only occurred after growth of the three "primary" strains, demonstrating that the "secondary" strains were maintained in the community because of their capacity to utilise metabolites produced by the other strains. At the end of the experiment the involvement of the secondary strains resulted in better overall minerilisation efficiency.

It is true that genetic engineering and artificial selection for microbes that are specific pollutant destructors are coming of age in the laboratory. However, the effective field application of these "hero" species is still very skeptical (King *et al.*, 1997).

2.7.2 Metabolic degradation pathways of hydrocarbons in diesel oil

From reviewing the literature describing the pathways involved in the degradation of aliphatic and aromatic hydrocarbons, the compounds that make up most of diesel fuel, a number of generalisations can be made (Altas, 1982a, 1982b; Dragun, 1998; and Hwang 1999)

- Aliphatic hydrocarbons are generally easier to degrade than aromatic hydrocarbons, with straight chain aliphatic hydrocarbons being easier to degrade than branched chain aliphatic hydrocarbons.
- The introduction of branching into hydrocarbons decreases the biodegradation rate, for example methyl branching interferes with beta-oxidation.
- Long chain aliphatic hydrocarbons are easier to degrade than short chain hydrocarbons, with chain lengths of less than 9 carbons being toxic to microorganisms.
- Saturated hydrocarbons are easier to degrade than unsaturated hydrocarbons. The presence of carbon-carbon double or triple bonds hinders degradation.

2.7.2.1 Aliphatic hydrocarbon degradation

Aliphatic hydrocarbons in diesel oil are mainly comprised of *n*-alkanes and branched alkanes. The *n*-alkanes are generally considered the most readily degradable components in petroleum mixtures (Atlas, 1982). The first step in the aerobic degradation of

hydrocarbons is the incorporation of molecular oxygen which is catalysed by either monooxygenase or dioxygenase enzymes (Rittmann *et al.*, 1994). In most cases, the transformation of aliphatic hydrocarbons involves the use of monooxygenases and the well-known process of β-oxidation (Atlas, 1982a; Singer and Finnerty, 1984; and Dragun, 1998). The aliphatic hydrocarbon is converted in successive reactions into alcohol, then an aldehyde, and then into an aliphatic acid as shown in Figure 2.2 (Dragun, 1998). During beta-oxidation, two carbon atoms are cleaved from the aliphatic acid to form a new alcohol and acetic acid. The new alcohol can be furthered degraded by reacting with dehydrogenase, monohydrogenase, and beta-oxidation enzymes to form aliphatic hydrocarbons with fewer atoms.

Branched aliphatic hydrocarbons are generally more resistant to biodegradation than their straight chain alkane counterparts (Dragun, 1998), and in hydrocarbon mixtures it is commonly observed that the degradation of branched alkanes is repressed by the presence of *n*-alkanes (Singer and Finnety, 1982). Oxidation of branched alkanes is initiated in the same manner as oxidation of *n*-alkanes: monooxygenase catalyzed hydroxylation of a terminal methyl group, followed by successive dehydrogenations that ultimately produce a branched alkanoic acid (Rittmann *et al.*, 1994).

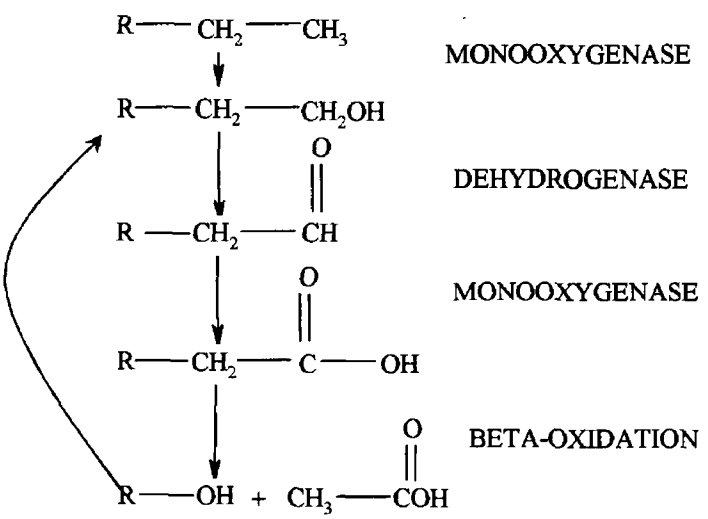


Figure 2.2. Metabolic pathway involved in the degradation of aliphatic hydrocarbons (Dragun, 1998).

Highly branched isoprenoid alkanes, such as pristane and phytane, have been found to undergo a dichotomous oxidation pathway (Figure 2.3). The metabolism of pristane was shown to proceed through pristanic acid with subsequent oxidation to either pristandioic acid followed by sequential beta-oxidation or 4,8,12-trimethyltridecanoic acid, followed by beta -oxidation (Singer and Finnerty, 1984; and Morgan and Watkinson, 1989).

2.7.2.2 Cyclic aliphatic hydrocarbon degradation

The transformation of cyclic hydrocarbons involves a sequence of reactions similar to those of aliphatic hydrocarbons. They are oxidised and converted to an aliphatic hydrocarbon which is then degraded to other products by β-oxidation.

2.7.2.3 Aromatic Hydrocarbon degradation

The transformation of benzene rings involves the class of enzyme known as dioxygenases. Molecular oxygen is incorporated into the aromatic ring structure to form a dihydroxybenzene degradation product. The aromatic ring is destroyed by cleavage, and new aliphatic degradation products are formed (see Figure 2.4) (Dragun, 1998).

The biodegradation of aromatic chemicals can also occur in anaerobic systems, where the pathway usually involves the reduction of the aromatic ring to form a cyclic hydrocarbon, which is then degraded by other pathways (Dragun, 1998).

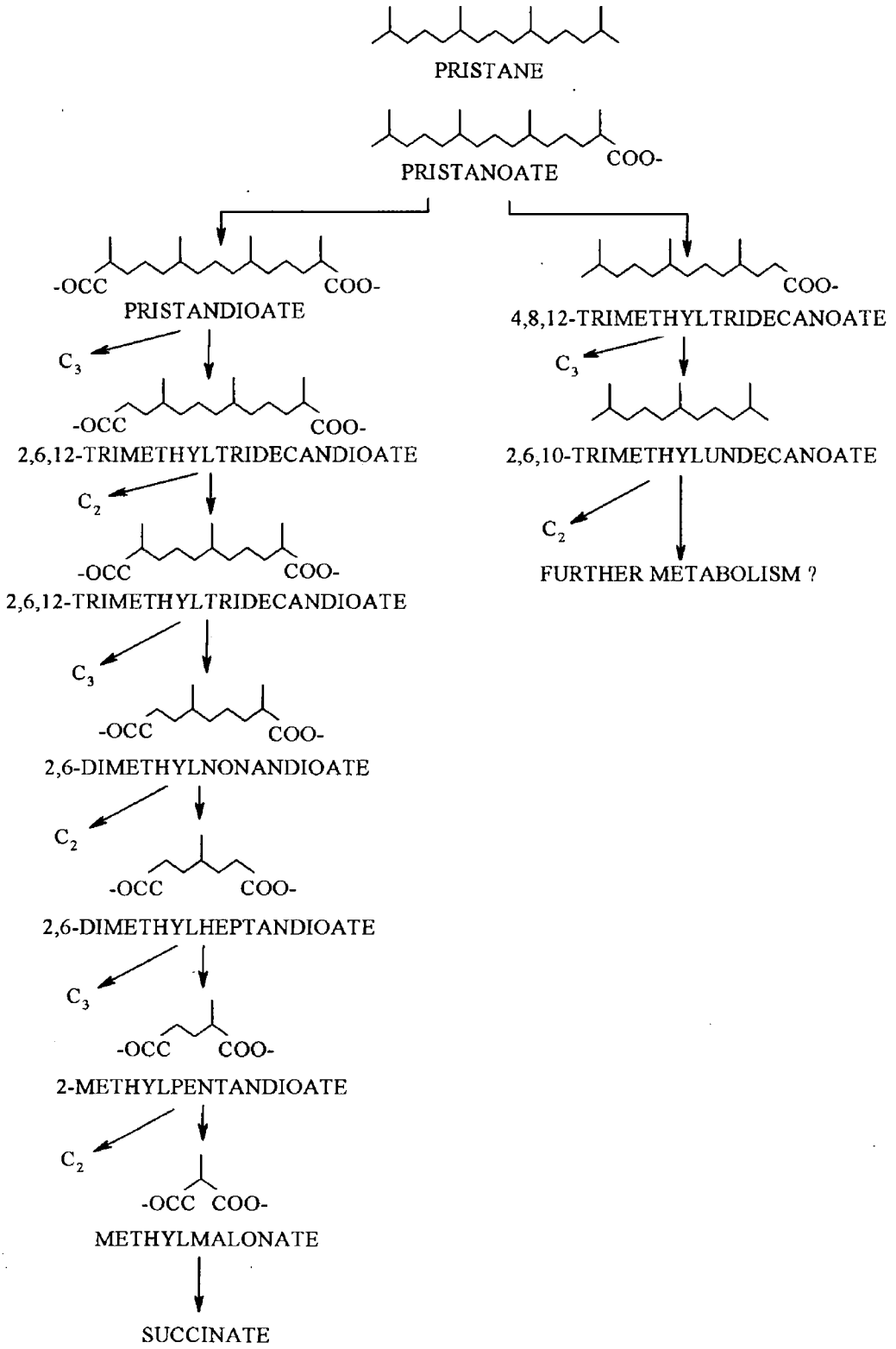


Figure 2.3. Metabolic pathway involved in the degradation of pristane (Hwang, 1999).

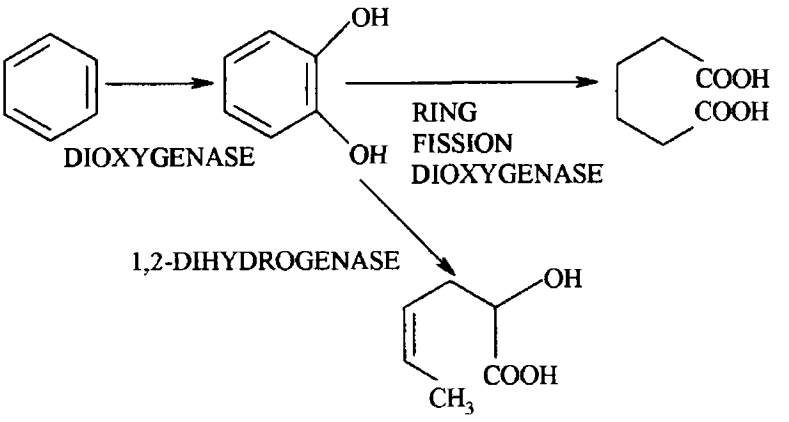


Figure 2.4. Proposed metabolic pathway involved in the degradation of aromatic hydrocarbons (Dragun, 1998).

2.7.3 Factors affecting the rate of bioremediation

The following section discusses some of the important factors affecting the biodegradation of diesel fuels or any other organic compounds. These factors affect biodegradation processes whether they take place *in-situ* or *ex-situ*.

2.7.3.1 Composition and size of microbial populations

The bioremediation of petroleum hydrocarbons is generally dependent upon the presence of soil microorganisms capable of degrading petroleum hydrocarbons and the number of these microorganisms in the soil system. The relationship between

degradation rates and population size is, the greater the number of microorganisms capable of degrading the compounds, the faster the degradation. If the indigenous microorganisms (i.e. the naturally-occurring microorganisms) are insufficient in numbers or ability to degrade the pollutants involved then independently cultured microorganisms which are known to metabolise the pollutants can be introduced into the environment. This type of amendment is more often referred to as seeding or bioaugmentation (Section 2.8.4).

2.7.3.2 Microbial nutrients

Over the past number of decades researchers have shown that certain elements are necessary for the normal growth and nutrition of biota, including microorganisms. In relation to microorganisms these essential elements are divided into two groups, macronutrients and trace nutrients. Macronutrients includes nitrogen (as ammonia) and phosphorus (as orthophosphate) while the trace nutrients include potassium, calcium, magnesium, sulfur, iron, manganese, copper, zinc, boron, molybdenum, chloride and cobalt (Dragun, 1998). Optimum growth of microorganisms depends upon all of these essential elements, and any one of them, if out of balance, can reduce or entirely prevent growth or metabolism. In order for optimal growth, these elements must also be available to the microorganisms in a useable form, appropriate concentrations, and proper ratios. It is the macronutrients (N and P) that are of major concern in field application of bioremediation, because the minor and trace nutrients are usually present in sufficient amounts in the natural environment and are almost never the limiting factors in the field (King *et al.*, 1997). However, when an organic contaminant enters the field system in bulk quantities, the soil's supply of the macronutrients is usually always inadequate to support desirable microbial growth (Dragun, 1998).

2.7.3.3 Oxygen

In order for biodegradation of petroleum hydrocarbon to occur, microorganisms must utilise an electron acceptor, such as oxygen. Although there is some evidence that certain hydrocarbons appear to require anaerobic metabolism for decomposition, most biodegradation of hydrocarbon compounds is aerobic, since hydrocarbon oxidation processes generally require the oxygenase enzymes (Atlas and Bartha, 1987). The amount of oxygen required will depend on the concentration of organics in the soil system. Dragun (1998) published an equation that could be used to calculate the theoretical amount of oxygen required to degrade 1mg/L of a hydrocarbon substrate;

$$C_xH_y + [(x + (y/4)]O_2 \rightarrow xCO_2 + (y/2)\,H_2O$$

Usually, about 3 to 4 mg/L of oxygen is required to degrade 1mg/L of a medium-length hydrocarbon compound. If 50 % of the organic material is converted to bacterial cell matter and the other half oxidised to carbon dioxide, only 4 to 6mg/l of organic material can be converted and oxidised under oxygen saturation conditions (Dragun, 1998).

2.7.3.4 Temperature

Soil temperature is one of the most important factors controlling microbiological activity and the rate of biodegradation of organic matter (Sims and Bass, 1984). Temperature not only affects the rate of microbial metabolism but also the physical nature and composition of the petroleum and the composition of the microbial community. Rising temperatures can decrease adsorption of certain contaminants, which can make more of the organic material available for microorganisms to degrade. On the other hand, higher temperatures increase evaporation of short-chain alkanes and other low-molecular-

weight hydrocarbons which usually cause solvent-type membrane toxicity to microorganisms (Atlas, 1994).

Microbial utilisation of hydrocarbon compounds can occur at temperatures ranging from -2°C (Delille *et al.*, 1997) to 70°C, with the majority of hydrocarbonoclastic microorganisms exhibiting maximum growth in the range of 20 to 35°C (Dragun, 1998).

In general, raising the temperature increases the rate of degradation of petroleum compounds in soil, with microbial growth usually doubling for every 10°C increase (Thibault and Elliott, 1980). Rates of microbial hydrocarbon degradation in soil increased as temperatures were increased by various amounts from 4°C to 30°C (Westlake *et al.*, 1974), from 1°C to 18°C (Sandvik *et al.*, 1986), from 17°C to 27°C (Song *et al.*, 1990), from 5°C to 37°C (Dibble and Bartha, 1979) and from 5°C to 20°C (Frankenberger *et al.*, 1991). Walker and Colwell (1974) found slower, but more extensive biodegradation of a model petroleum occurred at 0°C than at higher temperatures (5 and 10°C) due to a decrease in toxicity at lower temperatures, as a consequence of the lower solubility of some hydrocarbons.

The data indicated that, in most cases, temperature limits hydrocarbon biodegradation, except in soils that are permanently exposed to low temperatures in which the microorganisms have become adapted to the cold environment.

2.7.3.5 Soil moisture content

Biodegradation of organic contaminants requires water for microbial growth and for the dispersal of nutrients and by-products during the breakdown. Extremes of very wet or very dry soil markedly reduce biodegradation rates. Aerobic hydrocarbon biodegradation is diminished under saturated conditions due to the lack of oxygen

supply and in dry conditions microbial activity is hindered due to insufficient moisture levels necessary for metabolism.

In general, soils consist of approximately 50% pore space and 50% solid matter. As water enters a soil system it moves down into the subsurface, displacing air as it goes and starts to fill the pore spaces. When all the pores are full with water the soil is said to be saturated, this value is often referred to as the moisture holding capacity of the soil. Several authors have reported ranges of moisture content for optimum biodegradation. King *et al.* (1997) reported a range of 20 to 50% of the soils moisture holding capacity. Pramer and Bartha, (1972) indicated that a range of 50 to 70%, while Bossert and Bartha (1984) found that 30 to 90% of the moisture holding capacity was required for optimum biodegradation of hydrocarbons. Inhibition can occur at levels below 30% due to inadequate water activity (Riser-Roberts, 1998), whilst values above approximately 85% can interfere with soil aeration.

2.7.3.6 Soil pH

Biological activity in the soil is seriously affected by pH, through the availability of nutrients and toxicants and the tolerance of organisms to pH variation (Riser-Roberts, 1998). The optimum pH for microbial metabolism is usually between 6.6 and 8.5. If the soil is acidic bacteria tend to be out-competed by soil fungi for the available nutrients. The pH of the soil also influences the solubility or bioavailability of macro- and micronutrients, especially phosphorus (Parr *et al.*, 1983).

2.7.3.7 Adsorption

Adsorption is another parameter which can have a major influence on the biodegradation of an organic chemical. Before a compound can be biodegraded, it must be available, generally as a free solute (Mihelcic *et al.*, 1993), and in aqueous phase (Ogram *et al.*,

1985). Due to their hydrophobic nature and their low water solubility, petroleum hydrocarbon compounds tend to adsorb strongly to soil particles. Numerous authors have reported that the adsorption of hydrophobic compounds such as diesel removes them from solution lowering their bioavailability and in-turn decreasing their rate of biodegradation. Berg *et al.* (1998), Olivera *et al.* (1998), Riser-Roberts (1998) and Bardi *et al.* (2000) reported than phenanthrene was biodegraded to a lesser extent when adsorbed to organic soil constituents than when in liquid culture.

The adsorption of hydrocarbon compounds can be influenced by, the type and quantity of clay minerals, the type of cation that is saturating the clay, the exchange capacity and the specific surface area of the clays, the amount of organic matter in the soil, pH, temperature and the type and concentration of the hydrocarbon contaminant (Dragun, 1998)

Other authors have indicated that the degradation of certain organic chemicals can in fact be increased by their adsorption to soil particles due to the fact that there is a greater population density of microorganisms on or near soil particle surfaces than in the water phase. As a result, adsorption of an organic chemical increases the concentration of the chemical in areas where microorganisms flourish, and the potential for the microorganism to attack the chemical is enhanced (Dragun, 1998). If the compound is toxic, adsorption can also improve biodegradation by lowering its concentration in solution (Van Loosdrecht *et al.*, 1990). A variety of models that combine sorption and biodegradation are described by Mihelcic *et al.* (1993).

2.8 Optimisation of bioremediation

Bioremediation can be expressed as either engineered or natural. Any modification of the bioremediation process is considered engineered bioremediation, and the lack of intervention is natural bioremediation. Microbial populations capable of biodegrading

diesel or other petroleum hydrocarbons may commonly be found in subsurface soils, however, natural breakdown of the compounds will occur too slowly without intervention to prevent accumulation of the pollutants to unacceptable levels. Over the past number of decades numerous studies have being carried out to try to establish the most favorable conditions required to achieve optimum bioremediation rates.

2.8.1 Oxygen

One of the most common and successful methods for enhancing bioremediation processes includes increasing oxygen availability. Increasing oxygen availability may be accomplished by air sparging (bioventing), the addition of hydrogen peroxide or ozone, the addition of oxygen saturated water, adding nitrate, tilling in land farm style operations, turning of compost piles, or amending with bulking agents. All the above methods of increasing the amount of oxygen available have been reported to have had varying success in the bioremediation of petroleum contaminated soils and are discussed in detail by Riser-Roberts (1998).

2.8.2 Nutrients

Microbiological studies in laboratories, experimental field trials, and cleanup operations following oil spill incidents have demonstrated that a bioremediation strategy based on the enhancement of oil biodegradation rates through nutrient addition was effective (Frankenberger *et al.*, 1989; Morgan and Watkinson, 1989; Rosenberg *et al.*, 1992; Radwan *et al.*, 1995; Demque *et al.*, 1997; Rhykerd *et al.*, 1999; Taylor and Viraraghavan, 1999; and Margesin *et al.*, 2000b). It is generally thought that this technology can be applied without hazard to the environment if the applications are conducted within approved safety limits (Oh *et al.*, 2001).

As already mentioned, the microbial degradation of hydrocarbon compounds requires the presence of nitrogen, phosphorus and potassium in addition to smaller amounts of zinc, calcium, manganese, magnesium, iron, sodium and sulfur for optimum biological growth (Arora *et al.*, 1982). Soluble inorganic fertilisers, slow-release fertilisers (SRFs), and oleophilic fertilizers have been applied as nutrients, and their abilities to optimise and sustain indigenous bacterial oil-degradation activity have been evaluated under natural conditions (Lee, 2001). Ideally, nutrient concentration in oil contaminated environments should be sufficient to support the maximal growth rate of oil-degrading microorganisms, and this concentration should be maintained throughout the bioremediation operations. Various authors provide ratios for these nutrients. There is some disagreement on the exact ratios, but in general they are not too dissimilar. The carbon, nitrogen, and phosphorus content of bacterial cells in general is in the ratio of 100 parts carbon to 15 parts nitrogen to 3 parts phosphorus (Zitrides, 1983). Therefore, theoretically 150mg nitrogen and 30mg of phosphorus are required to convert 1 g of hydrocarbon to cellular material (Rosenberg *et al.*, 1992). By the amount of carbon in a spilled substrate that ends up as bacterial cells, it is possible to calculate the amount of nitrogen and phosphorus necessary to equal this ratio for optimum bacterial growth (Thibault and Elliott, 1980). The optimal carbon/nitrogen (C/N) and carbon/phosphorus (C/P) ratios generally used in bioremediation studies have been shown to be 10:1 and 10:0.3, respectively (Oh *et al.*, 2001).

2.8.3 Organic matter

The amendment of contaminated soil with organic material has been used for numerous decades, especially in the area of composting. In the past, organic material has been added as bulking agent in order to condition the soil. Bulking agents usually consist of inexpensive material such as straw, sawdust, bark or wood chip, mulch, cotton hulls or other organic materials (Rhykerd *et al.*, 1999; and Jorgensen *et al.*, 2000). If it is an organic material that is added then the technology is termed composting (Jorgensen *et al.*, 2000).

Organic matter is very important to the microbial ecology and activity of the soil (Sims and Bass, 1984). Its high cation exchange capacity and high density of reactive functional groups help bind both organic and inorganic compounds that are added to the soil. These properties also help retain the soil bacteria which can then attack contaminants (Riser-Roberts, 1998). Bacteria have been reported as being able to survive better in the presence of organic matter, especially in dry weather (Gobdout *et al.*, 1995). Generally the presence of solid organic matrix appears to be essential for enhanced degradation of organic contaminants (Kaestner and Mahro, 1996).

Organic material that is added to hydrocarbon contaminated soil as a bulking agent conditions the soil by breaking up and mixing soil clumps, which allows for increased soil porosity, resulting in better soil aeration (Riser-Roberts, 1998; and Rhykerd *et al.*, 1999). Bulking agents can also control the moisture content in soils by providing for better drainage when there is excess water (Huesemann, 1994) and in other soils it can increase water-holding capacity by swelling when wet to absorb two to three times its weight in water (Hornick, 1983). This conditioning of soil results in improved environmental conditions for microbes, which in turn increases their rate of metabolism. Rhykerd *et al.* (1999) carried out a study on the effect of two organic bulking agents (chopped Bermudagrass hay and sawdust from *Populus tremnloides* wood) and found that they significantly enhanced the disappearance of total petroleum hydrocarbons (TPH) in crude oil contaminated soil. Chang and Weaver (1998) also carried out an investigation into the use of bermudagrass and another grass alfalfa as bulking agents and found that they enhanced the biodegradation of crude oil in soil. Bulking of contaminated soil with chopped wheat straw, hay, wood chips, pine bark, peat, and loam enhanced remediation of 3,4-dichloroanaline and benzo(a)pyrene (Morgan *et al.*, 1993).

Addition of organic material can also have a more direct effect on microorganisms. It can act as a supplementary carbon and energy source, which can be used to stimulate the metabolism of even recalcitrant xenobiotics, either through co-metabolism (Alexander, 1981; and Harrison *et al.*, 2000) or simply because of the presence of additional carbon and energy source (Yagi and Soudo, 1980). Guzev *et al.* (1997) studied the effect of

addition of D-glucose to diesel contaminated soil and found that concentrations of glucose up to 1mg per g of soil augments the population of hydrocarbon-oxidising microorganisms about tenfold and increases biodegradation of diesel, whereas greater amounts of glucose inhibit diesel utilisation. Similarly, this inhibitory effect of glucose was reported by Baryshnikova *et al.* (1994) when using chemostat culture. They found that the degradation of diesel by *Rhodococcus minmus* was inhibited when glucose was added at concentrations greater than 660 mg/L.

Harrison *et al.* (2000) found that the addition of protein hydrolysate (derived from hoof and horn material) increased the number of hydrocarbon-degrading organisms in soils, even when there was no hydrocarbon present. This illustrates that hydrocarbonoclastic microbes are present in uncontaminated soils and that their numbers can be increased by other carbon sources apart from hydrocarbons. The same author also found that the addition of protein hydrolysate to a diesel contaminated soil significantly enhanced the removal of diesel, with 21% more diesel removal in the amended soil after a 21 day period.

The addition of particulate organic material to a water system, in the form of ground, sterile corn-slash (post-harvest leafs and stems) was found to enhance the diesel fuel degradation (Piehler *el al.*, 1999). This enhancement was attributed to, increased bacterial biomass through the stimulation of bacterial growth in the presence of greater surface area for microbial attachment, and the presence of some readily available carbon from the corn.

An organic carbon source which has received little attention in the past as a possible amendment is molasses. Molasses is the by product of the sugar industry and is the final syrup spun off after repeated crystallisation in the extraction of sugar. It is a dark heavy viscous liquid from which no further sugar can be crystallised by normal methods. The material contains a rich source of organic carbon, as well as a high mineral and vitamin content (Al-Hadhrami *et al.*, 1996). Sugar cane molasses was used by Al-Hadhrami *et al.* (1996) as a novel approach to enhance bacterial respiration and *n*-alkane

biodegradation of Omani crude oil. They reported that there was a statistically significant enhanced degradation of a number of the *n*-alkanes found in the crude oil. They found that the *n*-alkanes C_{14}, C_{15}, C_{16}, C_{17}, C_{25}, C_{29} and C_{30} were degraded to a greater extent in the presence of molasses, than in its absence. A further study by the same author (Al-Hadhrami *et al.*, 1997) confirmed these results by demonstrating that the presence of molasses significantly enhanced the biodegradation of pure *n*-alkanes ranging from *n*-C_{14} to *n*-C_{25}.

Spent grain, which is a by-product from the brewing industry, is an organic material which to the best of the author's knowledge has never been used in laboratory studies to amend soils contaminated with hydrocarbon compounds. This material is rich in protein, fat and fibre which make it ideal for use as an organic amendment. Apart from its chemical and physical attributes, this material has other advantages in that it is produced in large quantities (Guinness Ireland produce ~80,000 tonnes per year), is relatively cheap and is readily available in most countries.

One factor that should be noted, is that, when organic carbon is added in order to manage treatment of hydrocarbon contaminated soils, addition of nitrogen and phosphorous will be required to bring the C:N:P ratio close to that of the bacterial biomass (Riser-Roberts, 1998), which incur additional expenditure.

2.8.4 Bioaugmentation

It is unknown what is required for a microorganism to become "indigenous" to a particular environment. However, such microorganisms have become the stable members of a community and have a selective competitive advantage in occupying available niches in that environment (Atlas and Bartha, 1993). Biological treatment methods, generally, rely upon the stimulation and natural selection of indigenous microorganisms in the soil or groundwater (Sims and Bass, 1984). However, there may be scenarios where the natural microflora may not have the metabolic capacity to

degrade certain compounds or on the other hand, may have that ability but not the biomass necessary to degrade the compounds rapidly enough to meet the treatment criteria (Riser-Roberts, 1998).

When a contamination incident occurs that requires immediate treatment or when the indigenous microorganisms at the site lack sufficient numbers or capacity to degrade the pollutants involved, bioremediation design specialists often turn to the possibility of employing cultures of "specialised" microbes that are artificially introduced into the soil. This introduction of microbial inoculants into an environment in order to promote and/or enhance bioremediation is referred to as bioaugmentation or seeding (King *et al.*, 1997).

This technique involves independently culturing either natural isolates or genetically engineered microorganisms (GEMs), selected for their ability to degrade or transform specific hazardous pollutants, and then adding them to the contaminated material, in order to increase the rate and/or the extent of biodegradation of the pollutant. Seeding microorganisms into soil was first tried around 1968 (Gutnick and Rosemberg, 1977) when an inoclum of *Cellumonas* sp. and nutrients were shown to degrade hydrocarbon contaminants more effectively than nutrients alone (Schwendinger, 1968). Since then bioaugmentation has been applied in a number of different cases with varying success.

From reviewing the literature, the best results from bioaugmentation experiments have been reported in studies in which the environment is controlled to some extent, such as in laboratory fermentors and chemostats where competition with the indigenous microflora is reduced or nonexistent and system parameters can be optimised to achieve the highest rates of biodegradation (Leahy and Colwell, 1990). Another area where seeding with hydrocarbonoclastic microorganisms appears to be a promising treatment is for severely environmentally distressed soil, such as the Artic climates, where the activity and growth rate of the indigenous organisms may be limited (Hunt *et al.*, 1973).

Terrestrial ecosystems, typically, contain large numbers of microorganisms, including a significant proportion of hydrocarbon-utilising microorganisms, which readily increase

in response to hydrocarbon contamination (Atlas, 1977; and Lee and Levy, 1987). The presence of these indigenous microbial populations which are highly adapted to a particular soil environment would be expected to negatively influence the ability of introduced microorganisms to compete successfully and survive. Other potential problems associated with bioaugmentation soil environments are reviewed by Goldstein *et al.* (1985) and Riser-Roberts (1998) and include, insufficient movement of introduced microbes through the soil system to the areas of contamination, predation by higher organisms, extreme weather conditions, presence of inhibitory substances and the preferential metabolism of competing organic substances.

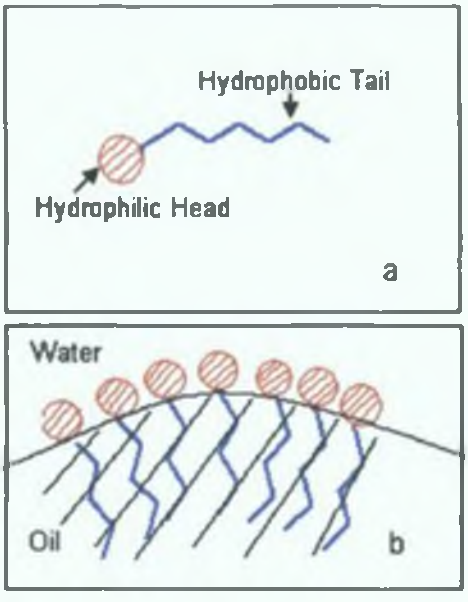
2.8.5 Contaminant alteration

As mentioned before, oil-water interfacial tension and adsorption of petroleum hydrocarbons are the two most important factors affecting their biodegradation (Setti *et al.*, 1995). In the past number of decades a great deal of work has been carried out on attempting to decrease interfacial tension and adsorption of petroleum products through the addition of surfactants. Surfactants are surface–active compounds that promote the wetting, solubilisation and emulsification of various compounds, including hydrocarbons, (Amdurer *et al.*, 1985) and it is hypothesised that they can increase the bioavailability and in-turn biodegradation rates of hydrophobic contaminants. The following sections introduce surfactants and their application in bioremediation processes.

2.9 Surfactants

Surfactants are amphipathic molecules which contain both a hydrophilic ("water-loving") and a hydrophobic/lipophilic ("oil-loving") moiety within the same molecule (Figure 2.5a). Since both the hydrophobic and hydrophilic groups reside within the same molecule, surfactants tend to partition at the interfaces between fluid phases with a

different degree of polarity and hydrogen bonding, like that of oil/water or air/ water interfaces (Figure 2.5b). They also have the ability to partition at water/solid interfaces, acting as a wetting agent (Fiechter, 1992).



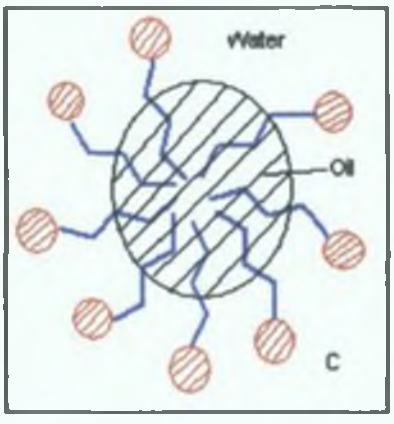


Figure 2.5. (a) surfactant molecule denoted by a circle representing the hydrophilic head attached to a hydrocarbon tail; (b) partioning at a water/oil interface; (c) circular micelle formation

A property that all surfactants share is that they are surface active and reduce surface and interfacial tension of fluids. For example, distilled water has a surface tension of 73mNm^{-1} at 20°C. Through the addition of an effective surfactant this value can be lowered to <30mNm^{-1}. However surfactant addition will only lower the surface tension of liquids to a certain level after which additional surfactant will not significantly lower the surface tension any further (See Figure 2.6). The concentration of surfactant required to achieve the lowest possible surface tension is defined as the critical micelle concentration (CMC). Alternatively, the CMC is defined as the surfactant concentration at which the concentration of free monomers ceases to increase and any further monomers added will form micellar structures (Zhang and Miller, 1992). In the process of micelle formation, the surfactant molecules that aggregate to form micelles have the ability to surround slightly soluble molecules (Figure 2.5c), a process that effectively

disperses or emulsifies them into the aqueous phase (Singer and Finnerty, 1984). It is the minimal surface tension value and the CMC of a surfactant that is used to give a measure of the efficiency of the surfactants (Fiechter, 1992).

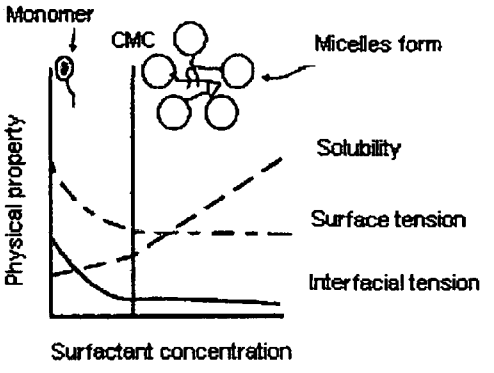


Figure 2.6. Schematic diagram of the variation of surface tension, interfacial and contaminant solubility with surfactant concentration. Reproduced with permission from Mulligan *et al.* (2001).

Surfactants can be divided up into different groups depending on the charge of the hydrophilic portion of the molecule. The hydrophile achieves its water solubility either by its ionic nature or by hydrogen bonding. Thus, we have ionic or nonionic surfactants depending on the nature of the hydrophile. The ionic surfactants are divided into anionic (negatively charged) and cationic (positively charged). Surfactants having the ability to exhibit both positive and negative sites are called amphoteric. Those surfactants which do not have any ionic charges are called nonionic.

At present, almost all surfactants currently in use are chemically synthesised, with petroleum as the raw material and are referred to as synthetic surfactants (Banat *et al.*, 2000). However, in the last number of decades another group of surfactants which have received increased attention are microbially produced surfactants, more commonly known as biosurfactants.

2.9.1 Biosurfactants

The term "biosurfactant" has been used very loosely and refers to any usable and isolatable compound obtained from microorganisms that have some influences in interfaces. Thus, it is also used for emulsifying and dispersing agents that do not significantly lower the surface tension of water or exhibit other properties of a classical surfactant (Desai and Desai, 1993).

Biosurfactants are a structurally diverse group of surface-active molecules synthesised by living cells. Like synthetic surfactants, biosurfactants are chemicals which have both clearly defined hydrophobic and hydrophilic groups. They have the ability to reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures (Banat, 1995). Because of their structural diversity, their possible production by fermentation and many potential areas of use (environmental protection, petroleum production, cosmetics, crop protection and drugs), biosurfactants are very attractive alternatives to chemically synthesised surfactants (Lang and Wullbrandt, 1999). It is because of the above and the fact that they have the ability to meet most synthetic surfactant requirements that world-wide interest in biosurfactants has increased dramatically (Morkes, 1993).

Biosurfactants have a number of advantages over synthetic surfactants. Owing to the diverse biosynthetic capability of microbes, novel compounds can be produced, which may be different and more effective for specific purposes than chemical surfactants (Van Dyke *et al.*, 1991). In addition, the chemical structure and physical properties of biosurfactants can be modified by either genetic, biological, or chemical manipulations allowing one to tailor biosurfactants for specific needs (Johnson and Schroeder, 1995). For example, Wild *et al.* (1997) reported the selective isolation of a rhamnolipid deficient mutant bacterium which did not produce mono-rhamnolipid, one of two rhamnolipids normally produced by the non-mutated species.

Biosurfactants are also very stable in extreme environments. Kretschmer *et al.* (1982) reported that a trehalose lipid produced by *Rhodococcus erythropolis* was stable at extreme pH values from pH 1.0 to pH 14.0 at 40°C. Gurjar *et al.* (1995) isolated a bioemulsifier produced by *Bacillus stearothermopilus* VR-8 which was stable at pH values of 2.0 to 8.0.

It has also been reported that biosurfactants are also stable at extreme temperatures. Banat (1993) isolated a biosurfactant from a thermophilic *Bacillus sp.* which was stable after heat sterilisation at 120°C for 10 minutes. A bioemulsifier isolated by Gurjar *et al.* (1995) was stable over a temperature range of 50 to 80°C, whilst the surface activity of the biosurfactant isolated by Kretschmer *et al.* (1982) was not reduced at temperatures exceeding 90°C. At the other extreme Whyte *et al.* (1999) isolated a biosurfactant from a *Rhodococcus* sp. which was found to be stable at temperatures as low as 5°C.

Another advantage of biosurfactants is that they may be synthesised from renewable feed stocks such as glucose, sucrose, lactose, natural oils such as Soya oil, Olive oil, Corn oil (Mercade *et al.*, 1993; and Sim *et al.*, 1997) as well as molasses and cornsteep liquor (Patel and Desai, 1997).

However, probably the most important advantage of biosurfactants over chemical surfactants is their ecological acceptability (Zhou and Kosaric, 1995). Biosurfactants are naturally occurring, biodegradable products and have low toxicity, while chemically synthesised surfactants cause ecological problems owing to their resistance to degradation, toxicity and accumulation in natural ecosystems. Thus, the release of biosurfactants to the environment may be easier to justify to appropriate regulatory agencies than the release of synthetic surfactants (Joong-Hoon *et al.*, 1998)

2.9.1.1 Biosurfactant origin and classification

Unlike chemically synthesised surfactants, which are classified according to the nature of their polar groupings, biosurfactants are categorised mainly by their chemical composition and their microbial origin (Desai and Banat, 1997). In general, their structure includes a hydrophile moiety consisting of amino acids or peptides, anions or cations; mono-, di-, polysaccharides, and a hydrophobic moiety consisting of unsaturated or saturated fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopetides, lipoproteins, phospholipids, fatty acids, polymeric surfactants, and particulate surfactants. Structural types of these classes are shown in Figure 2.7.

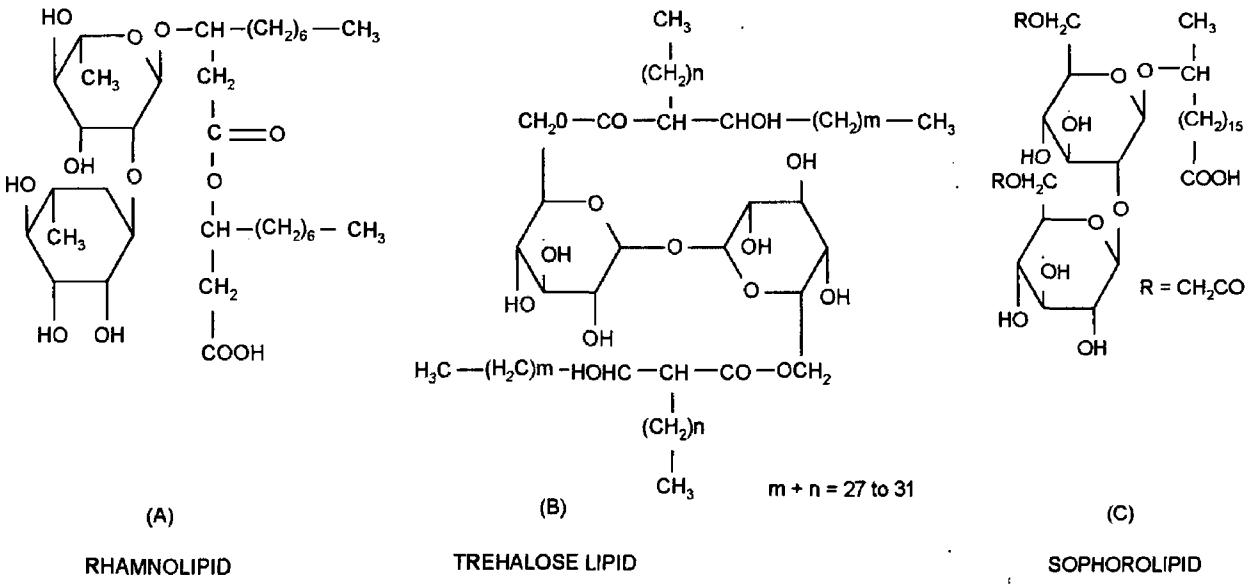


Figure 2.7. Structure of three common glycolipid surfactants. (A) Rhamnolipid type 1 from *Pseudomonas aeruginosa* in which two rhamnose subunits are linked to two β-hydroxydecanoic acids in a side chain. (B) Trehalose lipid from *Rhodococcus erythropolis*, in which the disaccharide trehalose is linked to two long-chain α-branched β-hydroxy fatty acids. (C) Sophorolipids from *Torulopsis bombicola* in which dimeric sophorose is linked to a long-chain (C18) hydroxy fatty acid (Desai and Banat, 1997).

Owing to their diverse biosynthetic capabilities, microbes are among the most promising candidates in the search for enlarging the present range of surfactants. Over the last century numerous microbes have been isolated with the ability to produce surface active compounds and even today many isolates are being discovered with the ability to produce surface active compounds. The biosurfactant-producing microbes are not limited to one particular species but are distributed among a wide variety of genera, with the majority of biosurfactants being produced by bacteria. However, fungi and yeasts have also been reported to produce surface-active compounds. The major types of biosurfactants and their microbial origin are listed in Table 2.4.

Table 2.4. Biosurfactant producing microorganisms and the types of biosurfactants they produce.

Group	**Biosurfactant**	**Microorganism**	**Reference**
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*	Zhang and Miller 1992; Ochsner *et al.*, 1994; and Haba *et al.*, 2000.
		Pseudomonas sp.	Hisatsuka *et al.*, 1971
		Pseudomonas fluorescens	Wilson and Bradely, 1996
		Bacillus sp.	Banat, 1993
	Trehalose lipids	*Rhodococcus erythropolis*	Rapp *et al.*, 1979
		Arthrobacter paraffineus	Duvnjak *et al.*, 1982
		Rhodococcus sp.	Page *et al.*, 1999
	Sophorolipids	*Torulopsis bombicola*	Kosaric *et al.*, 1984
		Torulopsis petrophilum	Cooper and Paddock, 1983
		Arthrobacter sp.	Suzuki *et al.*, 1974
		Candida bombicola	Zhou and Kosaric, 1995
	Glucose lipid	*Alcaligenes sp.*	Schulz *et al.*, 1991
Lipopeptides and lipoproteins	Lichenysin	*Bacillus licheniformis*	Yakimov *et al.*, 1995
	Surfactin	*Bacillus subtilis*	Cooper *et al.*, 1981
		Bacillus pumilus	Morikawa *et al.*, 1992
		Bacillus licheniformis	Lin *et al.*,1994; Jenny *et al.*, 1991.
	Arthrofactin	*Arthrobacter sp.*	Morikawa *et al.*, 1993
	Surlactin	*Lactobacillus acidophilus*	Velraeds *et al.*, 1997

Table 2.4 continued.			
Group	**Group**	**Group**	**Group**
	Gramicidins	*Bacillus brevis*	Marahiel *et al.*, 1977
	Viscosin	*Pseudomonas fluorescens*	Neu *et al.*, 1990a
Fatty acids and phospholipids	Fatty acids	*Corynebacterium lepus*	Cooper *et al.*, 1979
	Neutral lipid	*Nocardia erythropolis*	MacDonald *et al.*, 1981.
		Bacillus cereus	Cooper and Goldenberg, 1987
	Phospholipids	*Thiobacillus thiooxidans.*	Beeba and Umbreit, 1971
Polymeric	Liposan	*Candida lipolytica*	Cirigliano and Carman 1984
	Emulsan	*Acinetbactor calcoaceticus*	Rosenberg, 1993a, 1993b
	Carbohydrate-protein-lipid	*Corynebacterium hydrocarboclastus*	Zajic *et al.*, 1977
		Bacillus stearothermophilus	Gurjar *et al.*, 1995
	Alasan	*Acinetobacter radioresistens*	Barkay *et al.*, 1999
Particulate biosurfactants	Whole cell	Variety of bacteria	Rosenberg, 1993a.

2.9.2 Application of surfactants in bioremediation

Although most petroleum hydrocarbons are biodegradable, rates of biodegradation in the environment are limited due to numerous factors. It is generally believed that sorbed and solid phase hydrocarbons are not available to microorganisms and it is the limited rate of mass transfer of hydrocarbons from the sorbed and solid phase to the aqueous phase that is responsible for slow biodegradation according to Zhang *et al.* (1997). Addition of surfactants to hydrophobic contaminants have the potential to overcome this problem as a result of their ability to lower surface/interfacial tensions and increase the aqueous solubility and in turn increase the bioavailability of these materials to hydrocarbon degrading microorganisms. This potential has been widely recognised and many investigators have evaluated the effects of adding surfactants to environments to promote biodegradation.

The use of surface active compounds in the clean up of petroleum hydrocarbon releases to the environment is not a new concept. In 1967 oil-dispersing agents were used in the clean up of the *Torrey Canyon* oil spill (Al-Hadhrami *et al.*, 1996). However, these first dispersing agents were generally solvent-based and highly toxic and, therefore, not acceptable for long term use in the environment (Al-Hadhrami *et al.*, 1996). In response to this, a second generation of dispersing agents was formulated that incorporated less toxic constituents.

A variety of experimental systems and initial conditions have been used to study the effects of surfactants on biodegradation, making direct comparisons between findings of these studies difficult. So far, most experimental approaches described in the literature have been empirical. No mechanistic model of the influences of surfactants on the biodegradation of hydrophobic substrates has been developed, and any recent attempts to do so in well defined systems have not provided a satisfactory quantitative model. The majority of studies have been carried out in laboratory scale systems, with most of the investigators concentrating on either individual or homologous series of hydrocarbon compounds, rather than the more complex fuel mixtures.

To date, there are contradictory reports as to whether surfactants are beneficial or not to the biodegradation of petroleum hydrocarbons. Some investigators have reported stimulation of the biodegradation of hydrocarbons through the addition of surfactants, some have reported that surfactants have no effect on biodegradation processes, whilst other investigators have found that surfactants actually inhibit the biodegradation of hydrocarbons.

2.9.2.1 Effect of synthetic surfactants on bioremediation

Several investigators have shown that synthetic surfactants can improve physical removal of aromatic and aliphatic hydrocarbons from soil in laboratory columns (USEPA, 1985; Abdul and Gibson, 1990, 1991; Abdul *et al.* 1992; and Ang and Abdul,

1991) and in slurries (Aronstein *et al.*, 1991; Laha and Luthy, 1991, 1992; and Aronstein and Alexander, 1992). In order for surfactants, either synthetic or biological, to be effective at the physical removal of entrapped hydrocarbon compounds, a concentration above their CMC is required to mobilise the compounds (Herman *et al.*,1997). However, physical removal only removes the hydrocarbon from the soil, and there is still the problem of treatment of this contaminated aqueous phase which can be just as difficult as the original problem.

Several authors have investigated the use of synthetic surfactants in enhancing the bioremediation of hydrocarbon contaminated soil and have shown that certain synthetic surfactants enhance the biodegradation of different hydrocarbons in soil slurries (Table 2.5) and in soil microcosms (Table 2.6).

In contrast, numerous other studies have shown that synthetic surfactants do not enhance hydrocarbon biodegradation, but instead, inhibit the biodegradation of hydrocarbons in soil slurries (Table 2.7) and in soil microcosms (Table 2.8).

The majority of surfactants investigated in these studies were nonionic with only a few anionic surfactants been examined. Cationic surfactants have not been used in this area of research because soils have a negative charge and thus retain cationic compounds and also because they often possess high antimicrobial activity against a broad range of Gram positive and Gram negative bacteria (Irkhin *et al.*, 1989).

Table 2.5. List of surfactants that enhance the bioremediation of hydrocarbons in soil-slurry systems.

Surfactant	**Surfactant type**	**Hydrocarbon**	**Reference**
Alfonic 810-60	Nonionic	Phenanthrene, Hexadecane	Fu and Alexander, 1995
Brij 35	Nonionic	Phenanthrene	Jahan *et al.*, 1999
Corexit 0600	Nonionic	Phenanthrene	Jahan *et al.*,1997
Triton X-100	Nonionic	Phenanthrene	Fu and Alexander., 1995
Tween 40	Nonionic	Phenanthrene	Jahan *et al.*, 1997

Table 2.6. List of surfactants that enhance the bioremediation of different hydrocarbons in soil systems.

Surfactant	Surfactant type	Hydrocarbon	Reference
Witconol SN70	Nonionic	Pyrene	Thibault *et al.*, 1996
Triton X-100	Nonionic	Cresote	Carriere and Mesania, 1995

Table 2.7. List of surfactants that inhibit the bioremediation of hydrocarbons in soil-slurry systems.

Surfactant	Surfactant type	Hydrocarbon used	Reference
Witconol SN70	Nonionic	Pyrene	Thibault *et al.*, 1996
Triton X-100	Nonionic	anthracene	Stelmack *et al.*, 1999
Dowfax 8390	Anionic	anthracene	Stelmack *et al.*, 1999
Neodol 25-3S	Anionic	phenthrane	Fu and Alexander, 1995

Table 2.8. List of surfactants that inhibit the bioremediation of different hydrocarbons in soil systems.

Surfactant	Surfactant type	Hydrocarbon used	Reference
SDS	Anionic	13 of the 16 USEPA priority PAH, diesel	Deschenes *et al*, 1996; Margesin and Schinner, 1999
Triton X-100	Nonionic	Weathered Diesel fuel	Bregnard *et al*, 1998

2.9.2.2 Effects of biosurfactants on bioremediation

Growing interest in biosurfactant application for treating petroleum hydrocarbon contamination has developed recently (Van Dyke *et al.*, 1993; and Banat *et al.*, 2000). As with synthetic surfactants, inconsistent results exist as to the effectiveness of using biosurfactants to augment hydrocarbon bioremediation processes in soil.

Biosurfactants have been used to enhance physical removal of hydrophobic compounds from soil systems, and have been well documented by Van Dyke *et al.* (1993), Scheibenbogen *et al.* (1994), Roy *et al.* (1997) and Bai *et al.* (1997). They all reported that certain biosurfactants are as effective as, if not better, than synthetic surfactants at removing hydrophobic compounds from soil particles. Shreve *et al.* (1995) described a rhamnolipid biosurfactant, produced by a *Pseudomonas aeruginosa,* that was approximately nine times more effective at solubilising hydrocarbon into aqueous phase, than the structurally similar synthetic anionic surfactant alky benzene sulfonate (ABS).

As regards the use of biosurfactants to enhance biodegradation of hydrocarbon compounds, results in the literature are just as conflicting as those for synthetic surfactants. For example, Jain *et al.* (1992) used a non-sterile silt loam soil to assess the biodegradation of a model hydrocarbon mixture and reported that the addition of 100 μg of UG2 biosurfactant per g of soil, a rhamnolipid surfactant produced by *Pseudomonas aeruginosa* UG2, significantly enhanced the biodegradation of the aliphatic tetradecane, pristane and hexadecane, but not of the aromatic 2-methylnaphthalene, the more water-soluble of the four compounds. In the absence of biosurfactant, 71%, 60%, and <2% of tetradecane, hexadecane and pristane, respectively, was degraded in a 2 month period, while in the presence of the biosurfactant these figures were increased to 82%, 72% and 33% respectively. Other authors who reported stimulation of biodegradation upon biosurfactant addition are listed in Table 2.9.

The affect biosurfactant addition has on hydrocarbon biodegradation is not always advantageous with a number of authors reporting a decrease in hydrocarbon degradation through the addition of certain biosurfactants (Table 2.10). For example, Zhang and Miller (1994) reported that rhamnolipid biosurfactant (0.6mM), produced by *Pseudomonas aeruginosa* ATCC 9027, inhibited the biodegradation of octadecane by four different strains tested.

Table 2.9. List of biosurfactants that enhance the bioremediation of hydrocarbons.

Biosurfactant type	System used	Hydrocarbon used	Reference
Rhamnolipid	Soil-Slurry	Phenanthrene, Crude oil	Providenti *et al.*, 1995, Rocha and Infante, 1997.
Rhamnolipid	Soil	Tetradecane, Hexadecane, Pristane, Phenanthrene	Jain *et al.*, 1992; Herman *et al.*, 1997
Sophorolipids	Soil-slurry	Tetradecane, Pentadecane, Hexadecane, Pristane, Naphthalene, Phenyldecane	Oberbremer *et al.*, 1990

Table 2.10. List of biosurfactants that inhibit the bioremediation of hydrocarbons.

Biosurfactant type	System used	Hydrocarbon used	Reference
Rhamnolipid	Soil	13 EPA priority PAH's	Deschenes *et al.*, 1996
Rhamnolipid	Soil-slurry	Phenanthrene	Providenti *et al.*, 1995

2.9.2.3 Mode of surfactant enhancement

The main way in which surfactants enhance hydrocarbon biodegradation is understood to be by increasing the bioavailability of hydrocarbons. The means by which surfactants enhance bioavailability of hydrocarbon compounds can be explained by three main mechanisms: (i) dispersion of nonaqueous-phase liquid hydrocarbons, leading to an increase in contact area, which is caused by the reduction in the interfacial tension between the aqueous phase and the nonaqueous phase; (ii) increased solubility of the pollutant, caused by the presence of micelles which may contain high concentrations of hydrophobic organic compounds and (iii) "facilitated transport" of the pollutant from the solid phase to the aqueous phase, which can be caused by a number of phenomena, such as lowering of the surface tension of pore water in soil particles, interaction of the surfactant with solid interfaces, and the interaction of the pollutant with single surfactant molecules (Volkering *et al.*, 1995)

It has previously been reported that the addition of surfactants to soil and aqueous systems, can affect microbial localisation, promoting localisation at fluid interfaces involving hydrophobic phases, which in-turn can enhance biodegradation (Shreve *et al.*, 1995). Another method by which surfactants enhance biodegradation of hydrophobic compounds is that they may act as inducers of enzymes necessary for hydrocarbon breakdown, or may be incorporated into the cell membrane, altering its properties so that hydrocarbons can more readily enter the cell (Brueil and Kushner, 1980).

2.9.2.4 Mode of surfactant inhibition

Even though there are numerous scenarios where surfactants enhance the biodegradation of hydrocarbon compounds there are as many, if not more, reporting that surfactants have been either a neutral or inhibitory effect on hydrocarbon biodegradation. The mechanism by which surfactants inhibit biodegradation processes appear to be very

complex and to date not fully understood. A number of possible explanations have been hypothesised by different authors.

Surfactants when applied at concentrations above their CMC, aggregate to form micelles which incorporate fine droplets of the hydrophobic substrate into the micelle which subsequently enters the aqueous phase. Then the problem arises as to whether the degrading organisms can utilise this micelle bound substrate. Volkering *et al.* (1995) reported that the biodegradation of naphthalene by *Pseudomonas* sp. 8908N was inhibited by Triton X-100 and Tergitol NPX when applied at concentrations above their CMC, due to the naphthalene in the micellar phase not being readily available to the microorganisms.

Other authors have demonstrated that the preferential utilisation of certain surfactants by the degrading microorganisms was responsible for the inhibition observed in the biodegradation of different hydrocarbons (Providenti *et al.*, 1995; and Deschenes *et al.*, 1996)

The toxic effect of actual surfactants towards the degrading microorganisms can also be responsible for inhibiting the biodegradation process (Lupton and Marshall, 1979; Tiehm, 1994; Roch and Alexander, 1995; and Jahan *et al.*, 1997), while high concentrations of the actual solubilised hydrocarbons may also be toxic to the degrading populations (Carriere and Mesania, 1995).

Foght *et al.* (1989), Shreve *et al.* (1995), Bruhem *et al.* (1997), Churchill and Churchill, 1997, Herman et al. (1997), and Stelmack *et al.* (1999) found that attachment of certain hydrocarbon degrading bacteria, such as *Rhodococcus* sp., *Mycobacterium* sp. and strains of *Pseudomonas*, to the oil phase is essential for their growth on hydrophobic compounds and that the presence of surfactants can decrease their attachment to the hydrocarbons which consequently reduces their ability to utilise the substrate.

Despite these general trends, the effects of surfactants and biosurfactants on the biodegradation of organic compounds are difficult to predict.

2.10 Objectives of present study

- To screen for and isolate microorganisms with the ability to produce biosurfactant, from local environment samples.
- To characterise the biosurfactants and to assess their effectiveness in enhancing the bioremediation of diesel contaminated soil.
- To evaluate the effect of amendment of diesel contaminated soil with two novel organic materials, dried molassed sugar beet pulp and spent brewery grain, on the bioremediation of diesel contaminated sandy soil.
- To investigate the degradation rates of different components of diesel; *n*-alkanes, unresolved complex mixtures and pristane.

3. Materials and Methods

3.1 Chemicals and Reagents

Calcium chloride, di-potassium hydrogen phosphate, ammonium nitrate, ferric chloride, potassium dihydrogen phosphate, magnesium sulphate, ammonium chloride, sodium chloride, di-sodium hydrogen phosphate, potassium chloride, sodium chloride, ammonium dihydrogen phosphate, ferric sulphate, methanol, ethanol, crystal violet, naphthalene and mercuric chloride (all general purpose reagents) were purchased from BDH Chemicals, Poole, Dorset, U.K. Nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, octadecane, nonadecane, eicosane, heneicosane, docosane, tricosane, tetracosane, pentacosane, 1-phenyltridecane (all 99%+ pure), 1-phenylhexane (97%+), acetic acid, sulphuric acid (Analar grade), Tris-HCl (reagent grade), Safranin, and anthrone were purchased from Sigma Aldrich, Tallaght, Dublin. Hexane (HPLC grade) was purchased from Lab Scan, Stillorgan, Dublin. Glucose and urea (general purpose grade) were purchased from Lennox, John F. Kennedy Drive, Nass Road, Dublin 12. Chloroform was purchased from Alkem Chemicals Ltd., Little Island Industrial Estate, Cork. Proteose peptone, nutrient agar, yeast extract and anaerogen (all Oxoid) were purchased from Browns Medical and Laboratory supplies, Sandyford Industrial Estate, Foxrock, Dublin. Ringers (Lab-M) was purchased from Cruinn Diagnostics Ltd., Unit 6, Phase 2 Western Industrial Estate, Knockmitten Lane, Dublin 12.

3.2 Apparatus

Torsion balance, Model OS, from White Elec. Inst. Co. LTD., Spring Lane North, Worchetershire, WR14 1BL, U.K. Air pump, Model B100 SE, from Charles Austin Pumps Ltd., 100 Royston Road, Byfleet, Surry, U.K. Ultrasonic bath from Ultrawave Ltd. Oxford St. Cardiff CF2 1YY, U.K. BI-2000 electrolytic respirometer from Bioscience, Inc., 1550 Valley Center Parkway, Suite 140, Bethleham, PA 18017, USA.

Hitachi U-1100 spectrophotometer UV, Shimadzu Gas Chromatograph, Model GC-17A, Shimadzu Class VP (Version 4.2) software and a Chrompac CP-Sil 8 CB capillary column from Masons, Greenville Hall, 228 South Circular Road, Dublin 8. Rotary evaporator (Labo-Roto 300), orbital laboratory shaker KS 125, Whatmann filter papers and Watmann K5F silica gel 150A TLC plates all from Alkem Chemicals Ltd., Little Island Industrial Estate, Cork. The BIOLOG identification system including the Biolog turbidimeter, the BIOLOG microplate reader, and the Microlog 3 software as well as GN, GP, and YP plates were all purchased from AGB Scientific Ltd., Dublin Industrial Estate, Dublin 11, Dublin. Sigma 3-15 centrifuge, sterile 1ml & 10ml pipettes, 0.22μm filters, petri dishes from Sigma Aldrich, Tallaght, Dublin. Microbank from Pro-lab Diagnostics, 7 Westwood Court, Neston, South Wirral, Cheshire, U.K.

3.3 Materials.

3.3.1. Soil

The soil used throughout this study was a sandy soil collected from the top 15cm of the soil surface from a location in Co. Sligo, Ireland (grid G69 37 from ½ inch map, sheet 7, Ordnance Survey of Ireland, 1980). The site was a sand dune ecosystem isolated from any industrial or human activity, with no historical record of contamination and it could be reasonably assumed that no selection of specialised microorganisms had taken place. The collected soil was air-dried, passed through a 2mm aperture sieve, and stored at room temperature prior to use. The soil was classified as fine sand as it had greater than 50% particles between 0.5 and 0.1mm (Day, 1965). The main physical – chemical characteristics of the soil which included organic matter, organic carbon, total Kjeldahal nitrogen, available phosphorus and available potassium were determined by laboratory analysis using standard methods (Black, 1965). The results are summarised in Table 3.1.

Table 3.1. Physical-chemical properties of the soil.

Parameter	Concentration (per dry weight)
pH	7.7
Organic Matter	9.89 % (w/w)
Organic Carbon	0.41 % (w/w)
Available phosphorus	12 mg/kg
Total Kjeldahal nitrogen	150 mg/kg
Available potassium	30 mg/kg
NH_4-N	*2.4 mg/kg
NO_3-N	*3 mg/kg
Moisture holding capacity	36.57 % (w/w)

* = Analysis carried out by Teagasc laboratories.
Results are an average of 3 samples.

3.3.2. Dried molassed sugar beet pulp

Dried molassed sugar beet pulp (DMSPB) is a by-product of the Irish sugar industry. It consists of the residues left after the extraction of juices from the topped and tailed roots of sugar beet mixed with molasses. The final product contains between 22 and 30% molasses (Ryan, 2002). The other main characteristics of DMSBP are presented in Table 3.2.

3.3.3. Spent brewery grain

Spent brewery grain is a by product of beer production from Guinness Ireland, Dublin, Ireland. This material, rich in protein, fat and fibre, is currently used as a low grade animal feed ingredient. Table 3.3 shows the main characteristics of this material.

Table 3.2. Physical-chemical properties of the dried molassed sugar beet pulp.

Parameter	Concentration (per dry weight)
Moisture	12.5% (w/w)
pH	5.8
Organic Matter	94.4% (w/w)
Organic Carbon	31.18%(w/w)
Total Kjeldahal nitrogen	1.42 %
NH_4-N	*197mg/kg
NO_3-N	*205 mg/kg
Total protein	[a]9.5%
Fats	[a]0.7%
Sugars	[a]22%
Fibre	[a]12.5%
Ash	[a]8.9%

[a] = Results obtained from manufacturers.
* = Analysis carried out by Teagasc laboratories.
Results are an average of 3 samples.

Table 3.3. Physical-chemical properties of the spent brewery grain.

Parameter	Concentration (per dry weight)
Moisture	<1% (w/w)
pH	5.7
Organic Matter	94.9% (w/w)
Total Kjeldahal nitrogen	13.88%
NH_4-N	*78mg/kg
NO_3-N	*<9mg/kg
Total protein	[a]29.7%
Fats	[a]8.2%
Sugars	[a]4.4%
Fibre	[a]51.8%
Mineral matter	[a]3.7%

[a] = Results obtained from manufacturers.
* = Analysis carried out by Teagasc laboratories.
Results are an average of 3 samples.

3.3.4 Diesel

The diesel used in this study was agricultural grade diesel and was obtained from a Statoil service station in Sligo. The exact composition of the diesel used in this study is not known as it depends on its origin and, is also batch specific. The specifications for diesel produced by Statoil are presented in Table 3.4 and the diesel used in this study falls within these limits.

Table 3.4. Specifications for diesel produced by Statoil Ireland Ltd.

Property	**Units**	**Specification**	**Typical**
Appearance at 20°C		Clear & Bright	Clear & Bright
Water	ppm-wt	<=200	40
Density at 15°C	kg/m^3	820.0 - 845.0	844.5
Recovered at 250°C	%-vol	<=64	27
Recovered at 350°C	%-vol	>=85	94
50% recovered	°C	240 - 300	278
95% recovered	°C	<=380	355
Color, ASTM		<=1.5	<1.0
Flash point	°C	>=60	67
Total sulphur	mg/kg	<=50	45
PAH	%-wt	<=11	8
Copper strip corrosion 3h at 50°C		No. 1	No. 1
Oxidation stability	g/m^3	<=25	<=15
Viscosity at 40°C	mm^2/s	2.00 – 4.50	3.05
Lubricity at 60°C	µm	<=460	<=460
Cloud point	°C	<=3	-4
Cold filter plugging point	°C	<=-5	-16
Carbon residue on 10% residue	%-wt	<=0.20	<=0.15
Ash	%-wt	<=0.0.	<=0.01
Particulate matter	mg/L	<=24.0	6
Total acid number	mg_KOH/g	<=0.2	<=0.2
Cetane index, four variable		>=46.0	51.10
Cetane number		>=51.0	51.6
Conducivity	pS/m	>=50	73

3.4. Isolation of biosurfactant producing microorganisms

The first stage in the isolation of a biosurfactant producing microorganism involved screening for hydrocarbon degrading microorganisms, as it has been well documented that biosurfactants are predominantly synthesized by hydrocarbon degrading microorganisms.

3.4.1 Isolation of diesel utilising microorganisms

Isolation of hydrocarbon degrading microorganisms was carried by the standard enrichment isolation technique. Microorganisms were collected from two domestic sewage treatment facilities, one river water sample, one marine sediment, two pristine soil samples and one soil sample previously exposed to petroleum contamination. From each location approximately 100g or 100 ml of sample was collected and stored at 4°C until further use. For enrichment, bulk inoculum (1g or 1ml of sample) was added separately to shake flasks (250ml Erlenmeyer flasks) containing 50ml Bushnell-Hass medium supplemented with diesel as the sole carbon source (Brown *et al.*, 1991). This medium consisted of per litre; $MgSO_4$ (0.2g), $CaCl_2$ (0.02g), K_2HPO_4 (1.0g), NH_4NO_3 (1g), $FeCl_3$ (0.05g). After autoclaving and cooling, sterile diesel (cold sterilized by passing through a 0.45µm filter) was added to give a final concentration of 5% (v/v). Flasks were shaken at 180rpm at room temperature for two weeks. Ten ml of the liquid portion was sub-cultured at biweekly intervals thereafter into fresh medium. After four sub-cultures, samples from flasks showing visible growth were diluted in Ringers (1ml in 9ml of sterile ringers) and streaked onto nutrient agar and incubated at 22°C for 72 hours. Single colonies were sub-cultured onto fresh agar plates in order to continue the isolation and purification.

Bushnell-Hass-Diesel medium was inoculated with the isolated strains to ensure they retained their capacity to grow on diesel as the sole carbon source. Only the strains which showed good visible growth indicating diesel utilization were selected for further investigations.

3.4.2 Characterisation of isolated microorganisms

3.4.2.1 Gram stain

The Gram stain was carried out following a protocol by Cappuccino and Sherman (1998). Briefly, purified cultures were smeared onto a clean glass slide, allowed to air dry and then heat fixed. The smear was then stained with crystal violet for one minute, washed with tap water and then gram's iodine used as a mordant (one minute). The stain was decolorized with 95% ethanol and counterstained for 30 seconds with safranin. The stain was then washed with tap water and dried with blotting paper, before examination under an oil emersion lenses. Gram positive (GP) appeared purple/blue, gram negative (GN) appeared red/pink.

3.4.2.2 Microorganism identification

Isolated microorganisms were identified using the BIOLOG identification system. Biolog Microplates test the ability of a microorganism to utilize or oxidize a pre-selected panel of different carbon sources. The test yields a characteristic pattern of purple wells which constitutes a "metabolic Fingerprint" of the capabilities of the inoculated organism.

Cultures were streaked onto nutrient agar and incubated for 6-12 hours at 30°C. Growth was rolled onto a sterile cotton swab and suspended in a sterile 20mm diameter test tube containing approximately 30ml of sterile saline solution (0.85% NaCl). The inoculum density of the solution was adjusted to give a transmittance level of 53% to 59% when using the Biolog turbidimeter (first the transmittance of the turbidimeter was set to 100% using uninoulated saline solution). This transmittance level gave an approximate cell density of approximately 3×10^8 cells/ml.

The cell suspension was then placed in a sterile petri-dish and using an 8-channel repeating pipetter, 150µl of suspension was dispensed into the 96 wells of the appropriate plates (i.e. GN plates for gram negative bacteria, GP plates for gram positive bacteria and YP for yeast). Care was taken not to carry over chemicals or splash from one well into another. The plates were then incubated at the temperatures and time period recommended by the manufacturers. After incubation, plates were read using a Microplate reader attached to a computer containing Microlog 3 software, which performed automatic readings of the plates at a wavelength of 590nm. The pattern was then cross-referenced to an extensive library of species and if an adequate match was found, an identification of the isolate was made.

3.4.2.3 Cell hydrophobicity

The relative hydrophobicity of bacterial cells was measured by the BATH assay (Rosenberg and Rosenberg, 1980). Bacterial cells to be assayed were prepared by the following procedure. The bacteria were grown up for 24 hours in Bushnell-Hass-Diesel medium. The cultures were then centrifuged at 10,000 x *g* for 10 minutes in order to concentrate the cells. The pellets of cells were washed twice with Ringers solution to remove any interfering solutes. The cells were then re-suspended in buffer salts solution (pH 7.0) containing per litre; K_2HPO_4 (16.9g), KH_2PO_4 (7.3g), urea (1.8g), and $MgSO_4.7H_2O$ (0.2g), to give an optical density (OD) at 400nm of 1.0. Cells (4.0ml) and hexadecane (1ml) were mixed in a screw-top test tube (1.5 by 8cm), and the test tube vortexed for 1min. After vortexing, the solution was allowed to stand for 30min. The aqueous phase was then carefully removed using a Pasteur pipette, and the turbidity of the aqueous phase measured at 400nm. Hydrophobicity is expressed as the percentage of adherence to hexadecane which is calculated as follows:

$$Hydrophobicity = 100\ x\ \frac{1 - OD\ of\ the\ aqueous\ phase}{OD\ of\ the\ cell\ suspension}$$

3.4.3 Culture maintenance

Strains were maintained short term on nutrient agar slants with transfers every two months to fresh slants. Each slant was incubated for 12 to 18 hours at 22°C before storage at 4°C. Long term storage was at -70°C using Microbank. In brief, a heavy turbid suspension of the organism is made in the cryo-solution, contained in the microbank vial. The vial is then mixed to coat the beads which are also contained in the vial. Excess cryo-solution is then removed using a sterile pipette and the vials were frozen at -70°C or stored in liquid nitrogen. Organisms were recovered when necessary by simply culturing one of the beads in either nutrient broth or on nutrient agar plates.

3.4.4 Screening for biosurfactant producing microorganisms

Four different media were used to screen the isolated diesel degrading microorganisms for their ability to produce surface active agents.

* Bushnell Hass broth containing 5% diesel as the sole carbon source (see section 3.2.1);

* Minimal medium consisting of per litre: NH_4Cl (0.5g), $MgSO_4.7H_2O$ (0.5g), NaCl (0.4g), 0.5ml of KH_2PO_4 solution and 0.5ml of $Na_2HPO_4.H_2O$ solution. Each of the last 2 solutions contained 10g of the chemical per 100ml of H_2O and were prepared separately, autoclaved, and added to the medium at the end, along with 5% diesel which had been cold sterilized.

* Proteose-peptone-glucose-ammonium salts medium (PPGAS), consisting of per litre; NH_4Cl (1.07g). KCl (1.50g), Tris-HCl (18.9g), Glucose (5.0g), proteose peptone (10.0g), $MgSO_4$ (0.39g), pH adjusted to 7.2 with NaOH.

* Basal fermentation medium containing per litre; Glucose (30g), yeast extract (5g), K_2HPO_4 (1g) and $MgSO_4.7H_2O$ (0.5g).

The inoculum used in all cases was a 24 h culture of each microorganism under test, after growth on nutrient agar. A loopful of culture was used to inoculate 200ml of sterile medium in 500ml Erlenmeyer flasks. The flasks were incubated at room temperature on an orbital shaker at 180rpm. Every 24 hours, 20ml was removed and analysed for biosurfactant production. This was carried out by measuring the surface tension of the culture broth (see Section 3.6.3). Only the strains with the highest surface tension reduction were selected for further investigations.

3.5 Biosurfactant production

3.5.1 Biosurfactant production on glucose

Production of surfactant by the two bacteria selected from the screening procedure and by a known producer *Pseudomonas aeruginosa* NCIMB 8626 (obtained from the National Collection of Industrial and Marine Bacteria, UK) was carried out according to a procedure by Zhang and Miller (1992). The inoculum was prepared from a 24 h culture of the test bacterium grown at 37°C in flask culture at 200 rpm in Kay's minimal medium (25ml), composed of per litre; $NH_4H_2PO_4$ (3.0g), K_2HPO_4 (2.0g), Glucose (2.0g), $FeSO_4$ (0.5 mg of Fe per liter), $MgSO_4$ (1.0g), in Erlenmeyer flask. Two ml of this was used to inoculate 200 ml of phosphate-limited proteose peptone-glucose-ammonium salts (PPGAS) medium in a 1 L Erlenmeyer flask. The composition of this medium is described in section 3.4.4. These cultures were incubated at 37°C, shaken at 180rpm and the production of biosurfactants was monitored at regular intervals (every 2 hours) by sub-sampling the culture broth and measuring the surface tension.

3.5.2 Biosurfactant production on DMSBP

The three biosurfactant producing bacteria were investigated for their ability to produce biosurfactants when grown on DMSBP as the sole carbon source. For this, the glucose in the PPGAS medium was substituted with 5g of DMSBP. The incubation and sampling conditions were the same as those outlined in section 3.5.1.

3.6 Recovery and characterisation of biosurfactants

3.6.1 Partial purification of biosurfactants

Partially purification of the biosurfactants was carried out according to a protocol by Zhang and Miller (1992). The culture broth was centrifuged at 10,000 x *g* for 10 minutes in order to remove cells. The pH of the culture supernatant was lowered to <2 using 5M HCl, at which point the biosurfactant precipitated, observed by the presence of a turbid suspension. The surface-active compounds were then separated by liquid-liquid extraction with a mixed solvent, chloroform:ethanol (2:1). An equal amount of the solvent was added to the acidified supernatant in a separation funnel and the contents shaken well. The solution was allowed to settle for ten minutes and the organic layer removed. This extraction procedure was carried out a further two times. The different portions of solvent were then pooled together and the organic solvent evaporated off using a rotary evaporator, operating at 50°C. The yellowish oily residue (partially purified biosurfactant) was dissolved in 20mM phosphate buffer (pH 7.0) (Appendix 1) and used for further characterization.

3.6.2 Qualitative analysis of biosurfactants

Rhamnolipids produced by the different bacteria were separated by thin layer chromatography (TLC) on silica gel plates (Banat *et al.*, 1991). The plates used were

Whatman K5F silica gel 150A plates (50mm x 200mm). Approximately 100µL of sample (either culture supernatant or partially purified biosurfactant) was applied to the glass plates with a 25-µL glass capillary tube. The components were separated using a chloroform:methanol:acetic acid solvent system (65:15:2 v/v/v) in a fume hood operated at room temperature. The rhamnolipid components were developed by spraying the plates with an anthrone solution (2% anthrone in concentrated sulphuric acid), and then baking them in an oven at 110°C for 10 minutes. The rhamnolipids appeared as blue spots at first (when hot) and then turned brown on cooling. The different rhamnolipids were identified according to their retardation factor or relative mobility (R_f). The R_f value is the distance moved by the solute/spot (measured to center of the spot) in comparison to the solvent front.

3.6.3 Surface and interfacial tension

The surface tension and interfacial tension (against hexane) of the cell free culture broth (cells removed by centrifuging for 10 minutes at 10,000 x *g*), partially purified biosurfactant and synthetic surfactant solutions, were determined according to the Du Nuoy ring method using a torsion balance. A volume of the solution to be measured, (10-20ml) was placed in a clean glass beaker and the platinum ring placed in the solution. The force required to pull the ring through the surface of the liquid (or through one liquid to another in the case of interfacial tension) was measured using the above apparatus and was reported in milli-newtons per meter (mN/m). The samples were tested in increasing concentrations and the ring was rinsed with deionised water between samples. The ring was cleaned in acidic solution between measurements of different surfactants or measurements of a high concentration sample followed by a low concentration sample. A series of consistent readings were averaged to yield the surface tension value for each sample. The critical micelle concentration (CMC) of the solution is defined as the concentration of surfactant beyond which no further reduction in surface tension is observed, and was measured by plotting a graph of the surface tension of the solution versus the log of surfactant concentration (Bregnard *et al.*, 1998), The

critical micelle dilution, a parameter used as an indirect measurement of surfactant concentration, is the dilution beyond which no further reduction in surface tension is observed (Abu-Ruwaida *et al.*, 1991).

3.6.4 Soil washing with surfactants

The effectiveness of different surfactants on the removal of sorbed diesel contaminants was carried out as follows. Air dried sandy soil was contaminated with diesel (5%). Ten grams of the contaminated soil was placed in a screw-top centrifuge tube. Twenty ml of surfactant solutions at various concentrations (0.01 to 5 times their CMC) was added to the tube. The tubes were then placed in an orbital shaker for 10 minutes at 200rpm. After treatment the tubes were centrifuged for ten minutes at 10,000 x g in order to remove any particles. The supernatant was then transferred to a separatory funnel and the diesel extracted according to section 3.10.2.

3.6.5 Surfactant toxicity

The toxicity of the different surfactants used in the soil remediation experiments was investigated. A sample of the sandy soil used throughout this study was utilized as a source of indigenous soil microorganisms. Each surfactant was added to 200ml of nutrient broth in 500ml flasks, to give final concentrations of five times their CMC, i.e. the highest concentration employed in the soil remediation experiments. Two grams of sandy soil was added into each flask. The flasks were incubated at room temperature on an orbital shaker at 180rpm. The growth rates in each flask were monitored by measuring the optical density of the culture broths, at 600nm, at regular time intervals. The effects of the surfactants were compared to a control which had nutrient broth inoculated with the soil and had no surfactants present.

3.6.6 Measurement of surfactants in soil

The measurement of surfactants present in soil samples was carried out by extracting the soil with water followed by surface tension measurement of the aqueous phase. Approximately 10g (to 4 decimal places) of soil to be analysed was placed in a screw-top centrifuge tube and 10ml of distilled water added. Tubes were mixed well and placed in an ultrasonic bath for 15min. The tubes were then centrifuged for 10min at 10,000 x *g*. The aqueous phase was carefully removed using a pipette and the surface tension measured using the method in Section 3.6.3.

3.7 Soil microcosm setup

Two types of microcosms were used in the soil bioremediation experiments. The first type of microcosm was used for static and forced aerated trials. This microcosm consisted of a PVC pot with a capacity of 3 kg. Actual amount of added contaminated soil was about 2 kg. The bottom of the pot was layered with glass wool in order to retain the sample. The top of each pot was covered with tinfoil in order to minimize evaporation (Figure 3.1A). The forced aeration experiments took place in a microcosm similar to the static system except that an air-stone diffuser (150mm x 10mm x 10mm) was placed 10 mm from the bottom (Figure 3.1B), and was connected to an aeration pump (Model B100 SE).

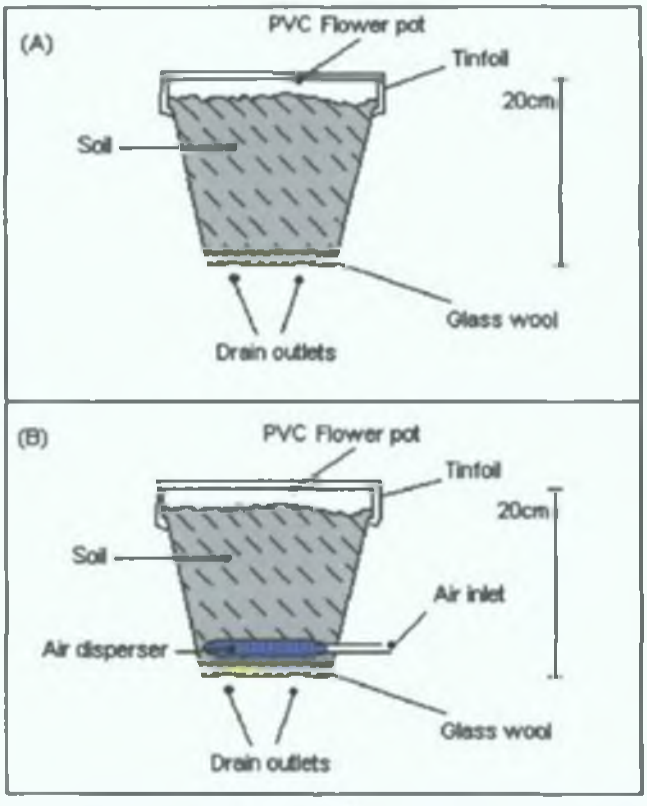


Figure 3.1. A schematic of the experimental microcosms used for (A) static and (b) forced aeration soil bioremediation experiments.

The second type of microcosm attempted to imitate a pilot scale cylindrical composting reactor (Figure 3.2). This type of reactor consisted of a PVC tube 25cm in diameter and 40cm in length and had a capacity of approximately 8 kg. In this microcosm the actual weight of added contaminated soil was about 3 kg. Both ends of the reactor were covered with PVC caps which were removable for extracting sample. Each reactor contained two openings ~2cm in diameter to allow for movement of air and addition of amendments. These openings were plugged with rubber bungs when mixing took place. Samples were mixed manually which involved rotation of the reactors 10 to 15 times in both a vertical and horizontal motion.

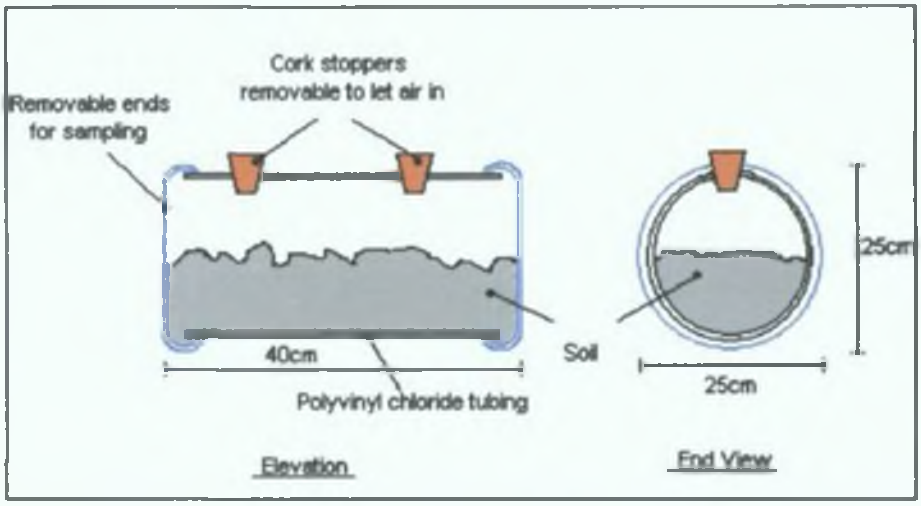


Figure 3.2. A schematic of the pilot scale compost reactor used in the manually aerated experiments.

3.8 Experimental conditions for soil bioremediation

3.8.1 General conditions

In this research the sandy soil was artificially contaminated with fresh diesel. The diesel was spiked at 5% v/w on a dry weight basis. In order to maximize distribution of diesel, an appropriate amount of diesel was spiked to every 200 – 300 g of dry sample. The diesel was distributed at five points on the surface of the sample and then mixed thoroughly to give a well homogenized sample.

Moisture content of the samples was adjusted to approximately 60-70% of their moisture holding capacity. This was based on numerous reports in the literature suggesting that biodegradation of simple or complex organic material in soil is commonly greatest between 50-70% of the soil moisture holding capacity (for example, Pramer and Bartha, 1972; and Riser-Roberts, 1998). Moisture content was controlled using either tap water

or, in the case of biosurfactant amended soils, with biosurfactant solution. Weekly appropriate volumes were added in order to maintain moisture levels. Throughout this study all treatments were carried out in duplicate and microcosms were incubated at room temperature.

3.8.2 Abiotic losses

Three different methods of aeration were examined for their effect on the abiotic removal of diesel from the sandy soil. They were; (1) static aeration, which involved the natural diffusion of air from the atmosphere through the soil surface; (2) manual aeration, whereby the sample was manually turned on a regular basis in order to distribute oxygen through out the sample; (3) forced aeration, where a pump delivered air at a rate equivalent to approximately 120 L O_2/kg soil/hour. In all three cases, the soil samples were sterilized by treating with 0.15% $HgCl_2$ (Walworth and Reynolds, 1995)

3.8.3 Inorganic nutrient amended experiments

Inorganic nutrients in the form of NH_4NO_3 and KH_2PO_4 were added to the samples on day zero to adjust the C:N:P of the contaminated soil to approximately 1000:10:1.5. Ten ml of a stock nutrient solution containing 78g/L NH_4NO_3 and 18g/kg KH_2PO_4 was added per kg of soil. Biweekly, similar amounts of nutrients were added to ensure that nutrients did not become limiting.

3.8.4 Biosurfactant amended experiments

Biosurfactants produced by *Pseudomonas aeruginosa* NCIMB 8626 and a culture isolated in the laboratory known as Isolate A were investigated for their effects on the biodegradation of diesel contaminated soil. Stock biosurfactant solutions were obtained

by culturing each microorganism in PPGAS. After 80 hours of growth the culture broth was centrifuged at 8,000 x *g* for 10 minutes in order to remove cells. These culture broths contained biosurfactants at concentrations equivalent to approximately 15 times their CMC and were used as stock solution for amending soils.

The effect of biosurfactant on diesel biodegradation was investigated in two stages. The first stage investigated whether a single application of biosurfactant solution on its own would enhance diesel biodegradation, while the second stage investigated whether multiple additions of biosurfactant plus inorganic nutrients would enhance diesel biodegradation. Various volumes of the stock biosurfactant solutions were added to achieve different final concentrations See Table 3.5. The biosurfactant solutions were added slowly at various points and mixed vigorously to ensure uniform distribution. Controls without biosurfactants were run concurrently in order to compare results.

Table 3.5. Biosurfactant concentrations used.

Biosurfactant type	**Final concentration per kg soil (x CMC)**	**Volume of culture broth required (ml/kg dry sample)**
NCIMB 8626 biosurfactant	3.0	200
	1.0	67
	0.1	6.7
Isolate A biosurfactant	3.0	200
	1.0	67
	0.1	6.7

3.8.5 Organic matter amended experiments

The two organic materials used in this study to amend diesel contaminated soil were DMSBP and SBG. Both organics were added to give a final concentration of 5% on a dry weight basis, and in all cases, inorganic nutrients were added as in Section 3.8.2. In order to maximize distribution, the organics were added to the dry soil prior to the addition of diesel and were mixed thoroughly using the cone-and-quarter method. Nutrient and moisture content were adjusted biweekly. A control with only inorganic

nutrient amendment was used to compare the effectiveness of each amendment. The effect of both manual and forced aeration was examined.

The effect SBG had on the anaerobic degradation of diesel was also examined. Samples were prepared as above, except the microcosms were placed in anaerobic jars for the duration of the experiment. Anaerobic conditions were achieved using Oxoid Anaerogen sachets. An OXOID anaerobic indicator was used as a visual check that anaerobic conditions had been achieved.

In order to investigate the consequence of adding the water extractable material from SBG on the biodegradation of diesel contaminated soil, the SBG was soaked in boiling water for 6 hours, to remove extractable material. The solids were then removed by filtering the solution through a Whatman No. 41 filter. The extracted material was added to the contaminated soil at a concentration equivalent to 5% SBG. Samples were also supplemented with inorganic nutrients

3.8.6 Respirometer experimental conditions

Soil microorganism respiration was studied using the BI-2000 electrolytic respirometer (Bioscience, Inc., USA). This instrument gives direct measurement of oxygen uptake in mg/L by absorbing the CO_2 produced during respiration in 45% KOH solution contained in a central well of each reactor bottle.

Known amounts of sample (100-500g, weighed to 4 decimal places) amended with all the necessary treatments were placed in reactor bottles. Ten ml of the 45% KOH was placed in the internal trap and the reactor vessel was then assembled according to the manufacturer. The amount of O_2 consumed in each reactor vessel was recorded at regular intervals (0.01 to 6 hours) using the BI-2000 software package.

3.9 Sampling and analytical methods

3.9.1 Sampling

In order to minimize sampling error, duplicate samples were taken from two locations within each microcosm using a sterile corer (100mm x 12mm diameter). The two sub-samples were then integrated to one sample by mixing. The combined sample was then analysed for moisture content, total mesophilic bacteria count and residual hydrocarbon content. The moisture content was measured at each sampling point and used to convert analytical data from wet weight basis to dry weight basis. This measurement was also used to calculate the amount of water required in order to maintain the require moisture level (i.e. 60-70%) moisture holding capacity. After each sampling, the moisture level and nutrient content of the soils were re-adjusted.

3.9.2 Moisture Holding Capacity

The moisture holding capacity (MHC) of the soil was determined by placing a known amount of air-dried soil (approximately 100g) in a plastic column, with one end sealed with a wire mesh and glass wool, in order to retain the sample. The column was then allowed to stand in water for up to five hours until it was saturated with water through capillary flow. Excess water droplets were then removed and the increase in weight was used to calculate the MHC.

$$Moisture\ holding\ capacity = \frac{increase\ in\ weight\ (through\ wetting)}{dry\ weight\ of\ sample} x100$$

3.9.3 Moisture content analysis

The moisture content was measured by the direct gravimetric loss in weight through oven drying. In brief, a known amount of sample (5 to 50g recorded to four decimal places) was placed in a conditioned crucible (conditioned at 101°C for 24 hours and allowed to cooled in a desiccator) and placed in a drying oven at 101°C for 24 hours. The sample was then removed and placed in a desiccator until cool. The sample was then reweighed and the percentage moisture content was calculated as follows;

$$\%\ Moisture\ content = \frac{weight\ of\ wet\ sample - weight\ of\ dry\ sample}{weight\ of\ wet\ sample} x\ 100$$

3.9.4 Total mesophilic bacteria counts

Total mesophilic bacteria count in solid-phase samples was carried out by the standard pour plate method. Results were converted to dry-weight of sample. Therefore, immediately after weighing the sample for microbial analysis, a separate portion of sample was analysed for moisture content according to the method in Section 3.9.3. A known amount of wet sample (approximately 10g weighed to four decimal places) was transferred to 90ml sterile Ringers, which was then mechanically shaken for 1 hour before serial dilutions was carried out in 9ml aliquots of Ringers. One ml of each serial dilution was plated in triplicate with Nutrient agar. The plates were incubated at 22°C for 72 hours and colony forming units (CFU) were counted and total mesophilic bacteria counts were calculated and results reported as CFU/g dry soil at 22°C for 72hours.

3.10 Diesel analysis

All glassware used in diesel analysis including, extraction flasks, round bottomed flasks, measuring vessels and volumetric flasks were washed with detergent, rinsed with tap water, then solvent rinsed with n-hexane and dried in an oven at 100°C. Glassware was then cooled to room temperature and in the case of the round bottomed flasks were cooled and stored in a desiccator until further use. Diesel analysis for solid-phase materials was carried out by solvent extraction with n-hexane followed by either gravimetric analysis reported as total hexane extractable material, (THEM) and/or gas chromatography analysis, diesel range organics (DRO), total *n*-alkanes, pristane and unresolved complex mixture (UCM)) with results being reported per g dry soil.

Diesel Range Organics (DRO) is all chromatographic peaks, both resolved and unresolved, eluting between the peak start of *n*-nonane (*n*-C_9) and the peak end of n-pentacosane (*n*-C_{25}), using forced baseline-baseline integration. Total *n*-alkanes is the sum of all the straight chain alkanes between *n*-C_9 to *n*-C_{25}. Pristane is the isopernoid which elutes out immediately after *n*-C_{17}, whilst the UCM is the unresolvable fraction eluting within the same range as DRO.

3.10.1. Diesel extraction from soil

Diesel extraction was carried out using solvent extraction with *n*-hexane as the solvent and an ultrasonic extraction procedure adapted from EPA method 3550b (USEPA, 1996b). A weighed sample of soil (5-10g, weighed to four decimal places) was transferred to a 100ml Erlenmeyer flask for extraction. $MgSO_4$ (dried overnight at 102°C) was added to the sample in order to remove water (Approximately 3g of $MgSO_4$ was added to every 10g soil). The contents were mixed with a spatula to form a free-flowing powder. At this stage the surrogate standard was added (1ml naphthalene stock standard to give a final concentration of 0.05% w/v) along with 20ml of *n*-hexane, the

contents of the flask were mixed, placed in an Ultrasonic water bath and extracted for 10 minutes. The extract was decanted and filtered through Whatmann No.41 filter paper (or equivalent), into a pre-weighed round bottom flask. The extraction procedure was repeated twice with 20ml portions of *n*-hexane. The extracts were combined together in one flask, which was then stored at 4°C until ready for solvent evaporation. Sample blanks were carried out by extracting clean soil (sandy soil prior to contamination with the diesel) according to the above procedure.

3.10.2 Diesel extraction from aqueous solution

Diesel extraction from aqueous solutions was carried out using solvent extraction in a separating funnel. A known volume of sample was placed in a separating funnel and acidified to pH <2 using 1M HCL. Serial extraction was carried out three times with *n*-hexane. The extracts were combined and were passed through a Whatman No. 41 filter containing dried $MgSO_4$ in order to remove water. The filtered extract was collected in a round bottomed flask and stored at 4°C until ready for solvent evaporation. Sample blanks were carried out by extracting distilled water according to the above procedure.

3.10.3 Solvent evaporation

Solvent evaporation was carried out in a round bottomed flask connected to a rotary evaporator and the solvent evaporated off by immersing the lower half of the flask in a water bath operating at 50°C. When the flask appeared almost dry, it was removed from the rotary evaporator and was swept out with air for 15-20 seconds to remove solvent vapor. This was carried out by inserting a glass tube connected to a vacuum source. The outside of the flask was wiped dry and placed in a desiccator and allowed to cool to room temperature. The extract was then ready for further analysis.

3.10.4 Gravimetric analysis of diesel

The flasks containing extract were removed with a tongs and re-weighted on a balance to four decimal places. The weight of diesel was determined by subtracting the tare weight of the flask from the total weight of the flask. If surrogate standard had been added prior to extraction then its weight was subtracted at this stage. These results were reported as THEM mg/kg dry weight.

3.10.5 Gas Chromatography analysis

3.10.5.1 GC conditions

The Gas Chromatograph used in this study was a Shimadzu GC-17A, fitted with a CP-Sil 8 CB column 30m length × 0.25mm internal diameter × 1µm film thickness, and equipped with a flame ionization detector. The instrument was connected to a Shimadzu Class VP chromatograph data system. The operating conditions used are presented in Table 3.6. The initial temperature started at 60°C and increased at 10°C/minute to 300°C, and maintained at that temperature for 15 minutes. The injection port and detector temperature were set at 300°C each. The pressure was automatically programmed using the Shimadzu Class VP system. The pressure of hydrogen gas and air for the flame ionization detector was 60 and 50 kPa, respectively, and nitrogen carrier gas was delivered at a rate of 1.3ml/minute. A 1µL sample was manually injected into a split injector operating at a split ratio of 15:1.

A typical chromatogram of diesel generated from the GC-FID according to the conditions mentioned above is presented in Figure 3.3.

3.10.5.2 GC sample preparation

Hydrocarbon extracts recovered from soil were re-dissolved in approximately 3ml of *n*-hexane and transferred to a 10ml volumetric flask. The sample container was rinsed a further two times with 1-2 ml of *n*-hexane and the solvent mixture transferred to the volumetric flask. One ml of both internal standards, 1-phenylhexane and 1-phenyltridecane, was added to give final concentrations of 0.05% and 0.1% v/v, respectively. Volume was made up to the mark with n-hexane and stored at 4°C until analysis was carried out.

Table 3.6. Operation conditions for the gas chromatography analysis.

Temperature	Initial temperature	60°C
	Initial time	0 minutes
	Ramp rate	10°C/minute
	Final temperature	300°C
	Final time	15 minutes
	Injection port temperature	300°C
	Detector temperature	300°C
Detector	Flame ionization	
Gas	Hydrogen gas	
	Air	
	Nitrogen (carrier gas)	
Column flow		1.3ml/minute

3.10.5.3 Calibration and standard stock solutions

Unless noted, all GC standards and stock solutions were prepared in *n*-hexane and were stored at 4°C for no longer than one month.

3.10.5.4 Diesel Standard Stock Solution

A 10% (v/v) diesel standard was prepared by accurately transferring 10ml of fresh diesel sample to a 100ml volumetric flask and diluting to volume with *n*-hexane. Typical working concentration ranged from 0.1% to 5% v/v.

3.10.5.5 Surrogate Standard Stock Solution

Surrogate standards were used to monitor the performance of the analytical system and the effectiveness of the extraction method. The surrogate used through out this study was naphthalene. A 0.5% (w/v) naphthalene stock solution was prepared by accurately weighing out 500mg of naphthalene and diluting to volume with *n*-hexane in a 100ml volumetric flask.

3.10.5.6 Internal Standard Stock Solution

A 0.5% (v/v) stock solution of the first internal standard, 1-phenylhexane, was prepared by accurately transferring 1ml of 1-phenylhexane and diluting to volume with *n*-hexane in a 200ml volumetric flask. A 1% (v/v) stock solution of a second internal standard, 1-phenyltridecane, was prepared by transferring 1ml of 1-phenyltridecane to a 100ml volumetric flask and diluting to the mark with *n*-hexane.

3.10.6 Target compound identification

Straight chain alkanes contained in the complex diesel mixture were identified according to their retention times. A 1% standard of several straight chain alkanes, *n*-C_9 to *n*-C_{25} was prepared in *n*-hexane and injected 5 times in order to determine average retention times. Using the operating conditions listed in Section 3.10.5.2, a 1% diesel standard was injected and by comparing the retention times, straight chain alkanes within the diesel were identified (see Figure 3.3). Diesel standard of 1% was injected 10 times to evaluate the variation in GC output. The variations in peak area ratios relative to the internal standard 1-phenylhexane are shown in Table 3.7.

Table 3.7. Variation in GC output for selected compounds in a 1% diesel standard.

Target compound	**Average peak area ratio to internal standard 1-phenylhexane**	**Standard deviation (10 replicates)**	**Relative standard deviation (%)**
DRO	13.00	1.28	9.91
n-nonane (C9)	0.025	0.0020	8.03
n-decane (C10)	0.0572	0.0034	6.04
n-undecane (C11)	0.0921	0.0013	1.40
n-dodecane (C12)	0.0577	0.0025	4.37
n-tridecane (C13)	0.1722	0.0049	2.87
n-tetradecane (C14)	0.1278	0.0050	3.95
n-pentadecane (C15)	0.1262	0.0093	7.39
n-hexadecane (C16)	0.1874	0.0120	6.42
n-heptadecane (C17)	0.1387	0.0117	8.40
Pristane	0.1030	0.0072	6.98
n-octadecane (C18)	0.1381	0.0103	7.49
Phytane	0.0796	0.0068	8.52
n-nonadecane (C19)	0.1646	0.0144	8.76
n-eicosane (C20)	0.1086	0.0099	9.19
n-heneicocane (C21)	0.0699	0.0042	6.02
n-docosane (C22)	0.0525	0.0053	10.05
n-tricosane (C23)	0.0351	0.0035	9.97
n-tetracosane (C24)	0.0216	0.0017	8.00
n-pentacosane (C25)	0.0103	0.0005	4.95

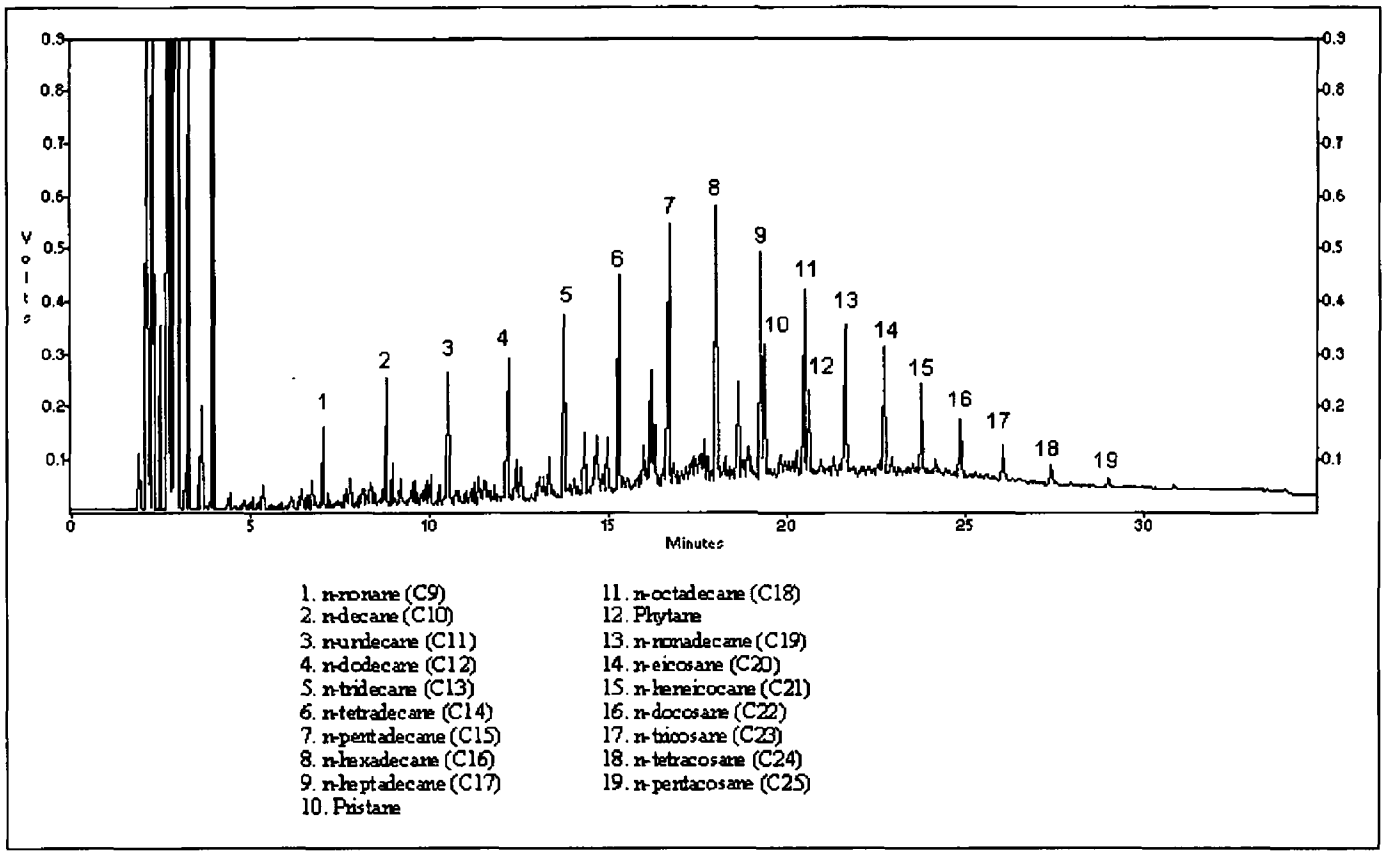


Figure 3.3. Typical gas chromatographic fingerprint of fresh diesel, analysed under conditions listed in Section 3.10.5.2. Peaks labeled 1 to 19, excluding 10 and 12, are the straight chain alkanes. Peak 10 and 12 represent pristane and phytane, respectively.

3.10.7 GC Calibration

3.10.7.1 Initial calibration

Using *n*-hexane, five diesel calibration standards ranging from 0.1% to 5% were made up in 10ml volumetric flasks each containing the surrogate standard (naphthalene 0.05% w/v final concentration) and the two internal standards (1-phenylhexane 0.05% v/v and 1-phenyltridecane 0.1% v/v final concentration). Each standard was injected into the GC under the same conditions, i.e. injection volume and technique, to be used during sample analysis and analysed in triplicate.

The peak area of the target compounds, DRO, total *n*-alkanes (*n*-C_9 to *n*-C_{25}), the isopernoid (pristane) and the UCM, were measured relative to that of the internal standard 1-phenylhexane. Ratios of the peak areas were tabulated and standard curves plotted of peak area ratios against concentrations. Peak area of total n-alkanes and pristane were calculated by valley to valley integration. Horizontal baseline projection from *n*-C_9 to *n*-C_{25} was used to calculate the DRO, while UCM was calculated by subtracting the total resolvable peak area, from *n*-C_9 to *n*-C_{25} using valley to valley integration, from the DRO peak area.

3.10.7.2 Calibration verification

Verification of the working calibration curve was carried out by the analysis of a mid-point calibration standard, usually 1%, at the beginning of each working day and at the end of each batch of 20 samples. If the relative percentage difference was greater than ± 25%, the instrument was recalibration and samples of that batch re-analysed.

3.10.8 Sample analysis

Samples were analysed using the same operating conditions as the standards, and the ratio of peak area of the target compounds and the surrogate standard (naphthalene), to the internal standard (1-phenylhexane) were calculated. The concentrations of the target compounds were extrapolated from the standard curve and final concentrations calculated using the formulae presented in Section 3.10.9.

If the percent recovery of the surrogate standard was less than ± 75% (Formula 3.4), the samples were re-extracted and anlaysed. If the percentage recovery was within the accepted range, then the calculated concentration was corrected to give 100 % recovery.

3.10.9 GC Calculations

Formula 3.1. Diesel range organics in solid matrix.

$$Cs = \left(\frac{Cc}{100}\right) / Ws \; x \; 0.8314$$

where Cs= Concentration of DRO in sample mg/Kg
Cc= Concentration of DRO in extract µL/L
Ws=Weight of sample Kg
0.8314= density of diesel at 18°C.

Formula 3.2. Relative response factor.

$$F = \frac{Peak\ area\ of\ target\ compound}{Peak\ area\ of\ internal\ standard}$$

Formula 3.4. Percent recovery.

$$\%R = \left(\frac{SSr}{Sr}\right) x100$$

Where %R= percent recovery.
SSr = Measured value (peak ratio of surrogate standard to internal standard in sample).
Sr = True Value (average peak ratio of surrogate standard to internal standard in standards).

3.11 Statistical analysis

3.11.1 Paired T-test

The *t*-test is commonly used to evaluate whether the difference in means between two sets of data is greater than what can be attributed to random sampling variation. The t-test is used when variables are normally distributed. In this study when two treatments were being compared for differences the paired t-test was used. The paired t-test examines the changes that occur before and after a single experimental intervention to determine whether or not the treatment had a significant affect. Differences were regarded as being significant if $P < 0.05$.

3.11.2 Analysis of Variance (ANOVA)

For comparison of more than two groups of data ANOVA was used. One way ANOVA was followed by Turkeys comparison of means test to compare treatments. ANOVAs were performed on the data at the 5% ($P=0.05$) significance level using Sigma Stat version 2.03 for windows (SPSS Inc., USA)

3.11.3 Degradation Rates

In this study the degradation rates, both zero and first order degradation rates, were calculated using Sigma Plot version 2001 (SPSS Inc., USA).

4. Results and Discussion

4.1 Biosurfactant production

The first objective of this study was to isolate a microorganism native to the Irish environment that was capable of biosurfactant production, and to produce, recover and characterize the biosurfactant. These results are presented and discussed below.

4.1.1 Isolation of biosurfactant producing microorganism

As it has been well documented that biosurfactants are predominantly synthesized by hydrocarbon degrading microorganisms (Rapp *et al.*, 1979; Kappeli and Finnerty, 1980; Roberts *et al.*, 1989 and Banat *et al.*, 1991; and Banat, 1995), the initial stage in obtaining a biosurfactant producing microorganism involved the isolation of hydrocarbon utilizing microorganisms.

Standard enrichment isolation technique was used for the isolation of diesel degraders. Bushnell-Hass medium supplemented with diesel (5%) as the sole carbon source was the enrichment medium. In all, 22 microorganisms with the ability to utilize diesel as the carbon source were isolated from samples derived from, domestic sewage treatment plants, river and marine water samples, and soil samples, some of which had a history of exposure to petroleum spillage. Characteristics of the different isolates are presented in Table 4.1 and it can be seen that of the 22 purified cultures, 13 were Gram negative (-ve) rods, five were Gram positive (+ve) rods and four were yeasts.

Identification of the purified cultures using the BIOLOG identification system showed the most commonly isolated diesel degraders were members of the genus *Pseudomonas*, followed by *Enterobacter* sp., *Alcaligenes* sp. and *Acinetobacter* sp. (see Table 4.1). All the isolated microorganisms are recognized as hydrocarbon degraders (Watkinson and Morgan, 1990).

Table 4.1. Characteristics of isolated diesel degrading microorganisms.

Strain	Gram stain	Cell shape	Cell hydrophobicity	Growth in Bushnell-Hass + 1% Diesel	Identification	Origin
Isolate A	-	Rods	40.2	+++	*Pseudomonas aeruginosa*	Soil
Isolate B	-	Rods	16.8	+	*Enterobacter sp.*	Soil [a)]
Isolate C	-	Rods	NT	++	*Alcaligenes denitrificans*	Soil [b)]
Isolate D	-	Rods	NT	+++	*Pseudomonas fluorescens*	Soil [b)]
Isolate E	-	Rods	NT	++	*Pseudomonas citronellolis*	Soil [a)]
Isolate F	-	Rods	NT	+	*Acinetobacter johnsonii*	Sea water
Isolate G	-	Rods	NT	+	*Enterobacter asburiae*	Soil [b)]
Isolate GO	-	Rods	<1	++	*Pseudomonas aeruginosa*	River water
Isolate H	yeast		98.89	++	*Yarrowia lipolytica*	Domestic sewage
Isolate I	-	Rods	NT	+++	*Pseudomonas nitroreducens*	Domestic sewage
Isolate J	+	Rods	13.69	+++	No I.D.	Soil [a)]
Isolate K	+	Rods	NT	+++	*Rhodococcus sp.*	Soil [b)]
Isolate L	yeast		90.17	++	*Cryptococcus luteolus*	Domestic sewage
Isolate M	-	Rods	NT	+	*Acinetobacter genospecies 15*	Domestic sewage
Isolate N	-	Rods	NT	+	*Pseudomonas fluorescens*	Domestic sewage
Isolate O	+	Rods	21.39	+++	*Clavibacter sp.*	Soil [a)]
Isolate P	-	Rods	78.79	+	*Alcaligenes xylosoxydans*	Sediment
Isolate Q	+	Rods	39.27	+	*Curtobacterium citreum*	Sediment
Isolate R	yeast		NT	++	*Saccharomycodes ludwigii*	Soil [a)]
Isolate S	-	Rods	9.84	+	No I.D.	Soil [a)]
Isolate Tetra	+	Rods		+++	*Rhodococcus sp.*	Domestic sewage
Isolate U	yeast		99.46	++	*Cryptococcus luteolus*	Domestic sewage

NT = Not tested.
[a)] = Soil with no previous history of hydrocarbon contamination.
[b)] = Soil previously exposed to petroleum contamination.
Visual observations; + = poor growth, ++ = average growth, +++ = Excellent growth.

Having isolated microorganisms with the ability to grow on diesel as a sole carbon source, the isolates were screened for their ability to synthesise biosurfactants. Properties of the isolates were compared to those of a known biosurfactant producer, *Pseudomonas aeruginosa* NCIMB 8626 (Zhang and Miller, 1992). Four different media, which had been reported in the literature to facilitate biosurfactant production by different microorganisms, were used to screen for biosurfactant production. Two of the media used contained water insoluble carbon compounds (i.e. Diesel), while the other two used a water-soluble compound (i.e. Glucose) (Table 4.2). Biosurfactant production was followed by measuring surface tension. The initial surface tension of the different media varied from 61 to 71mN/m, and good biosurfactant producers were considered to be those that decreased the surface tension of the medium to <40 mN/m.

Table 4.2. Media used to screen for biosurfactant producing microorganisms.

Media	**Name of Medium** (refer to Section 3.4.4 for composition)	**Carbon source**	**Surface tension** (mN/m)	**Reference**
1	Bushnell-Hass	Diesel	70	Patel and Desai, 1997.
2	Minimal salts media	Diesel	71	Sim *et al*, 1997; Van Dyke *et al*, 1993.
3	Proteose Peptone Glucose Ammonium salts	Glucose	70	Zhang and Miller, 1992
4	Basal fermentation medium	Glucose	61	Shaikh *et al*, 1997.

After five days of incubation, only three of the twenty three microorganisms (including *Pseudomonas aeruginosa* NCIMB 8626) tested showed satisfactory production of surface active compounds when cultivated on any of the selected media (see Table 4.3). Isolate A, Isolate GO and the known producer *Pseudomonas aeruginosa* NCIMB 8626 lowered the surface tension of PPGAS to 29, 30 and 30mN/m, respectively. Based on this data, these three bacteria were selected for further investigations. Isolate A and GO were identified, using the BIOLOG identification system as strains of *Pseudomonas*

aeruginosa. For the purpose of this study these two cultures are referred to as Isolate A and Isolate GO.

Table 4.3. Cell growth of the isolated microorganisms grown in different culture media, and their effect on culture broth surface tension.

	Cell Growth				Surface tension (mN/m)			
Culture I.D.	**BH**	**MS**	**PPGAS**	**BF**	**BH**	**MS**	**PPGAS**	**BF**
Isolate A	+++	++	+++	+++	56	49	29*	48
Isolate B	+	+	++	++	68	65	58	60
Isolate C	++	++	++	++	59	62	59	49
Isolate D	+++	++	+++	+++	48	56	56	62
Isolate E	++	+	+++	+++	69	68	58	56
Isolate F	+	+	+++	++	56	68	58	60
Isolate G	+	+	++	+++	70	68	60	59
Isolate GO	++	++	+++	+++	48	53	30*	54
Isolate H	++	+	++	++	65	68	60	53
Isolate I	+++	+++	+++	+++	52	53	49	56
Isolate J	+++	++	+++	+++	56	65	49	47
Isolate K	++	+++	+++	++	65	68	58	54
Isolate L	+++	+	++	++	70	70	60	58
Isolate M	+	+	++	+++	71	70	65	64
Isolate N	+	+	++	+++	68	65	60	61
Isolate O	+++	++	++	+++	58	54	60	57
Isolate P	+	+	++	++	65	64	60	58
Isolate Q	+	++	+++	+++	70	68	56	56
Isolate R	++	+	++	+++	64	58	57	54
Isolate S	+	+	++	+++	69	68	57	53
Isolate Tetra	+++	++	+++	+++	58	64	60	60
Isolate U	++	+	++	++	70	65	61	54
Pseudomonas aeruginosa NCIMB 8626	+++	+++	+++	+++	46	70	30*	53

* Microorganisms considered good biosurfactant producers.

BH=Bushnell-Hass medium. MS= Minimal salts medium. PPGAS= Proteose peptone glucose ammonium salys medium. BF= Basal fermentation medium.
Visual observations; + = poor growth, ++ = average growth, +++ = Excellent growth.

4.1.2 Production and recovery of biosurfactants

The three cultures with the most promising potential for production of surface active compounds were identified as being strains of *Pseudomonas aeruginosa.* Most promising production was achieved using PPGAS medium. PPGAS is a phosphate-limited medium and was chosen as numerous reports showed that it facilitated the production of biosurfactants by different strains of *Pseudomonas* (Mulligan and Gibbs, 1989a; Zhang and Miller, 1992; and Wild *et al.*, 1997).

The time course for one of the cultures, Isolate GO, grown in PPGAS with 5 g/L glucose is shown in Figure 4.1. It can be seen that after an initial lag phase of three hours expontional growth commenced, where the optical density increased from 0.002 to 1.546 after 15 hours and reached a stationary phase by 20 hours. Nitrate was utilized rapidly during exponential growth and was fully consumed by the start of the stationary phase. Glucose utilization followed a similar pattern. However it was not fully utilized until after 45 hours of incubation.

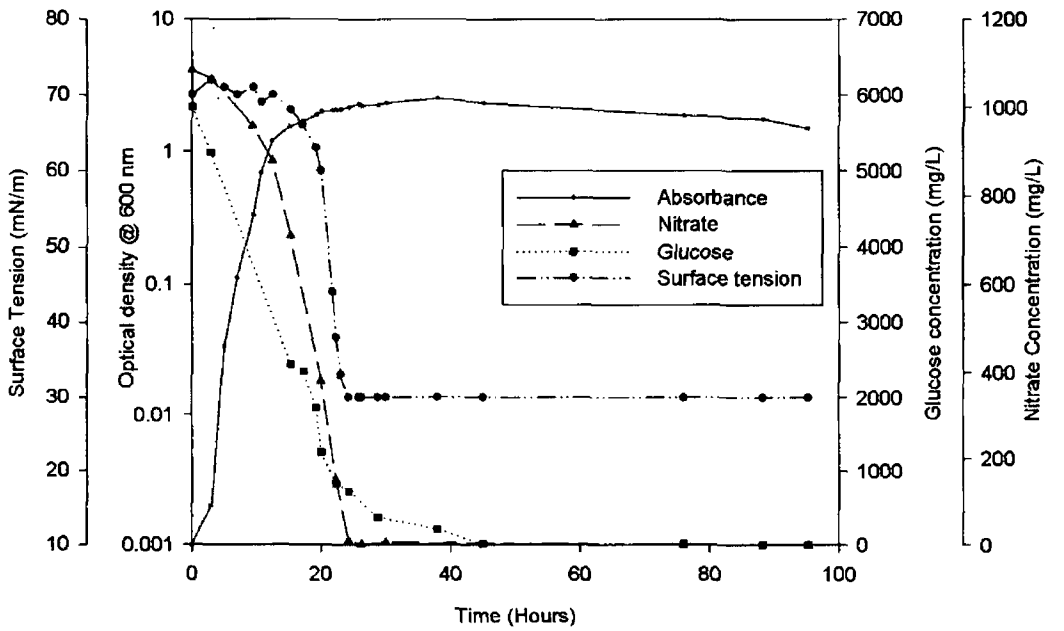


Figure 4.1. Time course of growth, change in surface tension of culture broth and concentration of medium components during the cultivation of Isolate GO, at room temperature in PPGAS medium.

Biosurfactant production was measured indirectly through the measurement of the surface tension of the culture broth. The surface tension of the broth only started to decrease once the culture had reached stationary phase after 20 hours. Surface tension decreased rapidly from 70mN/m to a low of 30mN/m after 24 hours. After this the surface tension of the broth did not decrease any further, indicating that the amount of biosurfactant in the broth had reached its critical micelle concentration (CMC). In order to investigate if there had been further production of biosurfactant during incubation, the surface tension of a 1 in 100 dilution of the broth was carried out. Figure 4.2 shows that the production of biosurfactant continued up until 76 hours as indicated by a reduction of the surface tension of the diluted broth. After 76 hours no further reduction in surface tension was observed, indicating that no more surface active compounds were produced by the culture.

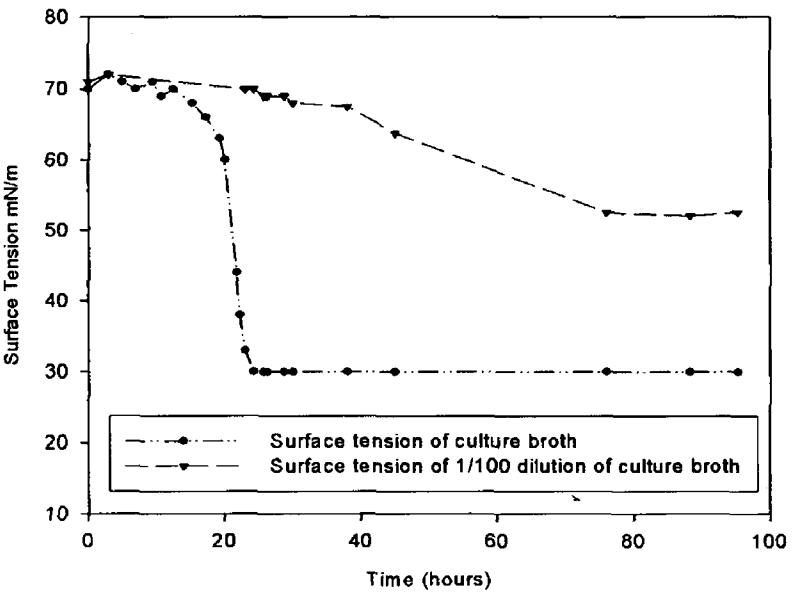


Figure 4.2. Surface activity profiles of undiluted and diluted culture medium during cultivation of Isolate GO, at room temperature, in PPGAS medium.

Biosurfactant production was initiated at the start of the stationary phase, which also coincided with the limitation of nitrate. This implied that biosurfactant production by this culture was triggered by the limitation of nitrate in the growth medium, which concurs with Ramana and Karanth (1989) and Wu and Ju (1998) who reported that biosurfactant production by other strains of *Pseudomonas aeruginosa* was triggered by nitrogen source limitation. As mentioned previously, the biosurfactants produced by *Pseudomonas* sp. are rhamnolipids, which contain rhamnose (sugar) units connected to fatty acid (lipid) residues. Mulligan and Gibbs (1989b) suggested that during biosurfactant biosynthesis it is lipid, not sugar, formation that is the rate limiting factor and that nutrient limitation (via nitrogen) may promote lipid accumulation.

The growth characteristics of Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 followed very similar patterns to that of this culture with maximum surface tension reduction observed after 80 hours of growth (Appendix 2). From these results it was decided to harvest all three biosurfactants after 80 hours of incubation. Weights of recovered crude biosurfactant material were 1.372, 0.794 and 1.156g/L of PPGAS for Isolate A, Isolate GO and *Pseudomonas aeruginosa* NCIMB 8626, respectively. These yields are in close agreement with those of Syldatk *et al.* (1985) who achieved a yield of 1.8g biosurfactant per litre in growing cultures of *Pseudomonas* DSM 2874, while Sim *et al.* (1997) produced 2 g/L crude biosurfactant through the growth of *Pseudomonas aeruginosa* UW-1 in mineral salts medium containing glucose as the carbon source.

4.1.3 Biosurfactant characterisation

Partially purified biosurfactant synthesized by Isolate A, Isolate GO and *Pseudomonas aeruginosa* NCIMB 8626 were assessed for their ability to lower the surface tension of water and the interfacial tension between water and hexane. The results are shown in Table 4.4. The concentration of biosurfactant required to achieve the CMC was approximately 100mg/L for all three biosurfactants (See Figure 4.3a). These results are comparable to those of biosurfactants produced by other *Pseudomonas* species, which

have surface CMC values ranging from 10 to 1000mg/L (Van Dyke *et al.*, 1993). These results show that all three partially purified biosurfactants were capable of lowering surface and interfacial tension of aqueous phases.

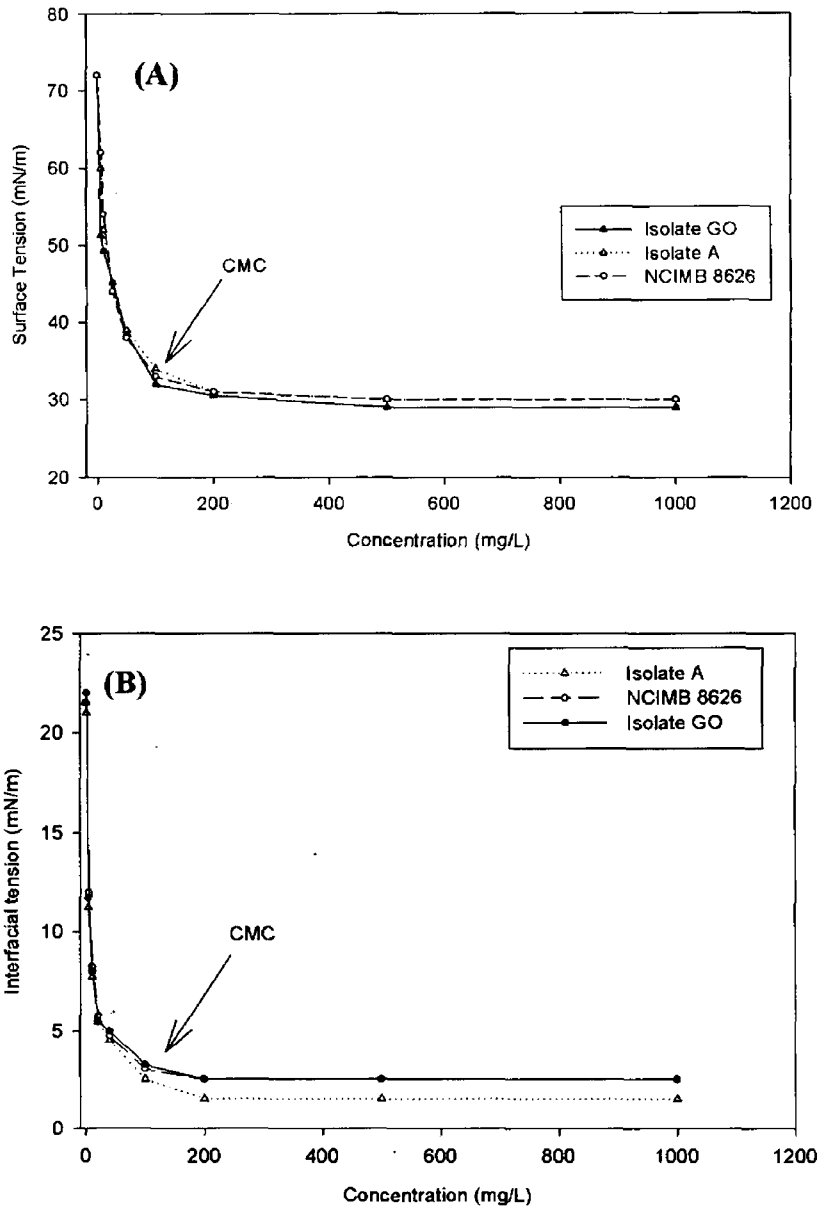


Figure 4.3. Dependence of surface tension (A) and interfacial tension (B) on the concentration of crude biosurfactant produced by Isolate A, Isolate GO and *Pseudomonas aeruginosa* NCIMB 8626. Interfacial tension was measured between hexane and distilled water.

Table 4.4. Minimum surface and interfacial tension achieved using the different biosurfactants based on interpolation of data from Figure 4.3.

Biosurfactant Type	Minimum surface tension achieved (mN/m).	Minimum Interfacial tension achieved (mN/m).
None	72	21.5
Isolate A	29	1.5
Pseudomonas aeruginosa NCIMB 8626.	30	2.5
Isolate GO	30	2.5

In order to determine the ionic nature of the biosurfactant, surface tension of the culture broths was measured under different pH conditions. Culture broth was obtained after 80 hours of growth of the three cultures in PPGAS. They were diluted to their CMC with distilled water. Surface tension of the broth was measured while adjusting the pH with 1N NaOH and 1N HCL. As the solution became more acidic, surface tension was lowered (see Figure 4.4), indicating that the biosurfactants from Isolate A, Isolate GO and *Pseudomonas aeruginosa* NCIMB 8626 were anionic. These results are similar to those produced by Ramana and Karanth, 1989, Roberts *et al.*, 1989, and Cho *et al.*, 1998 which indicated that *Pseudomonas aeruginosa* produces anionic surfactants.

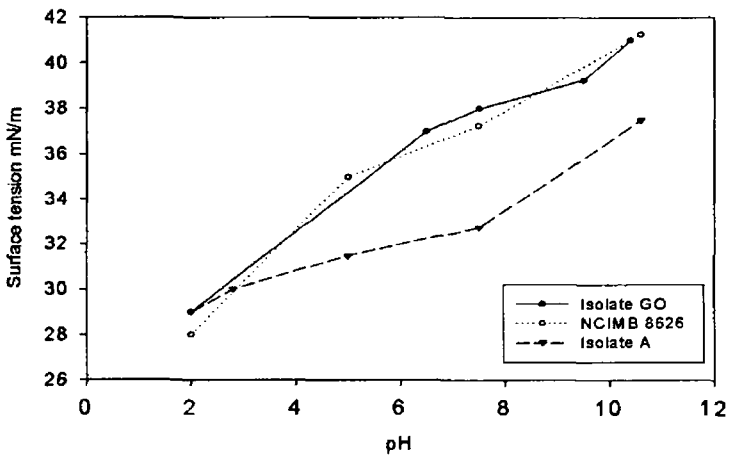


Figure 4.4. Effect of pH on the surface tension of Isolate A, Isolate GO and *Pseudomonas aeruginosa* NCIMB 8626 culture broth. Cultures were grown up in PPGAS for 80 hours. The pH was adjusted with 1N HCL and 1N NaOH.

4.1.4. Qualitative analysis of biosurfactants

It has been reported that biosurfactants produced by *Pseudomonas* species are rhamnose containing glycolipids, referred to as rhamnolipids (Ochsner *et al.*, 1995). L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanote (di-rhamnolipid) and L-Rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanote (mono-rhamnolipid), referred to as rhamnolipid 1 and 2 respectively, are the principal rhamnolipids produced in liquid cultures by *Pseudomonas aeruginosa* (Ochsner *et al.*, 1995). Two other rhamnolipids, 3 and 4, containing only one β-hydroxydecanoyl moiety have also been detected in cultures of resting cells (Syldatk *et al.*1985). However, it is thought that these types may represent degradation products from hydrolysis of rhamnolipids 1 and 2 (Ochsner *et al.*, 1995).

In order to investigate the type of biosurfactants produced in this study, thin layer chromatography of the broth was carried out. Rhamnose containing components were detected using 2% anthrone in concentrated sulphuric solution (Banat *et al.*, 1991).

In this study a rhamnolipid standard solution containing rhamnolipid 1 and 2, received from Francis Environmental BioTek Inc.,Canada, was used as a reference to compare against the biosurfactants produced in the laboratory. These two rhamnolipids were identifiable by the fact that rhamnolipid 2 is more mobile than rhamnolipid 1 (Sim *et al.*, 1997).

The TLC pattern of the biosurfactant standard solution (Figure 4.5. Lane 1) indicates the presence of these components. The least mobile component, rhamnolipid 1, had an R_f value of 0.32, while the other component, rhamnolipid 2, had an R_f value of 0.75. These results are quite similar to those of Cho *et al.* (1998) who reported that rhamnolipids 1 and 2 had R_f values of 0.45 and 0.70 respectively. The slight difference in results may be due to the fact that this author used slightly different ratios of solvent in the developing

solvent, 66:25:4 v/v chloroform methanol:acetic acid compared to 65:15:2 v/v (Banat *et al.*, 1991) in this study.

TLC results for the biosurfactant produced by *Pseudomonas aeruginosa* NCIMB 8626 (Figure 4.5, Lane 2) show that only one spot was detected with an R_f value of 0.76, a value similar to that of mono-rhamnolipid in the standard. This result confirms the work of Wild *et al.* (1997) who reported that this organism, *Pseudomonas aeruginosa* NCIMB 8626 (ATTCC 9027), only accumulates mono-rhamnolipids. It was found that Isolate GO produced a biosurfactant very similar to that of *Pseudomonas aeruginosa* NCIMB 8626 as only one spot was detected (Figure 4.5, Lane 3), which had an R_f value of 0.75, also indicating the presence of mono-rhamnolipid.

The biosurfactants produced by Isolate A were very different. As can be seen in Figure 4.5 Lane 4, TLC analysis of this biosurfactant revealed that it contained four detectable spots. Two of the spots have similar R_f values comparable to that of the standard, indicating the presence of mono- and di-rhamnolipids. Another spot was detected which had an R_f value of 0.53, while a fourth spot which was the least mobile component had an R_f value of 0.23. These results imply that the biosurfactant produced by Isolate A, in PPGAS medium, contained four different types of rhamnolipid, rhamnolipid 1 and 2 and two unidentified rhamnolipids. Without further analysis these unidentified rhamnose containing components could not be identified, but they may be rhamnolipid 3 and 4, biosurfactants which so far have only been produced in resting cultures of *Pseudomonas* sp. DSM 2874 (Syldatk *et al.* 1985), as stated earlier. The amounts of each rhamnolipid produced were not quantified directly but examination of the intensity of the spots on the TLC plates showed that the amount of each rhamnolipid produced was in the order of R1>R2>unidentified compound 1> unidentified compound 2.

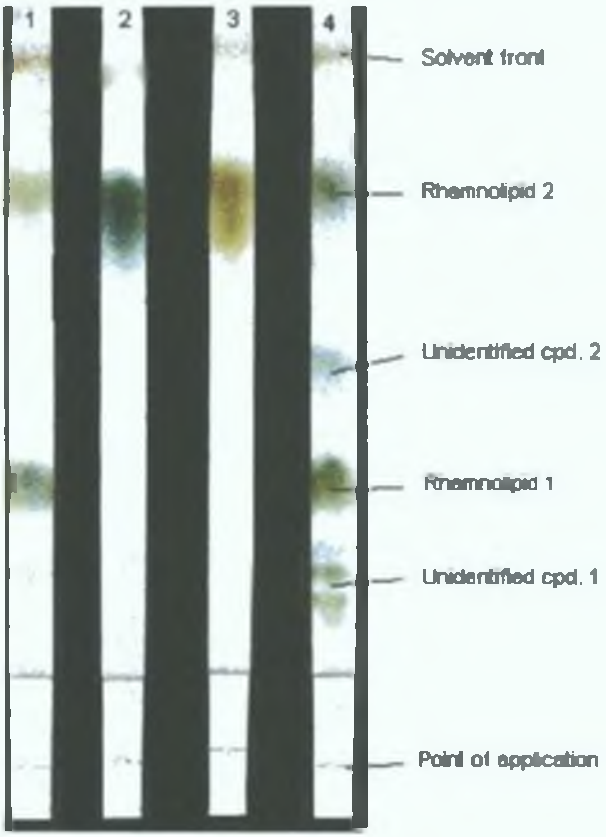


Figure 4.5. Thin layer chromatographs showing the separation of rhamnolipid components of crude biosurfactants.
Lanes correspond to; 1: rhamnolipid standard solution
2: *Pseudomonas aeruginosa* NCIMB 8626 biosurfactant
3: Isolate GO biosurfactant
4: Isolate A biosurfactant

The effect different concentrations of glucose had on biosurfactant production was investigated and it was found that the amount and type of each rhamnolipid produced varied depending on the concentration of glucose present in the medium. When the glucose concentration in PPGAS was doubled from 5g/L to 10 g/L the production of the two unidentified rhamnose containing compounds by Isolate A ceased, indicated by the disappearance of the spots which had R_f values of 0.53 and 0.23 (see Figure 4.6, Lane 2). Reducing the concentration of glucose to 2.5g/L also had a similar effect (Figure 4.6, Lane 3) with the two unidentified compounds disappearing. These results tend to

confirm a proposal by Ochsner *et al.* (1995) that the predominant types of rhamnolipid produced appear to be strain specific and seem to depend also on the environmental and cultivation conditions, and especially on the medium composition.

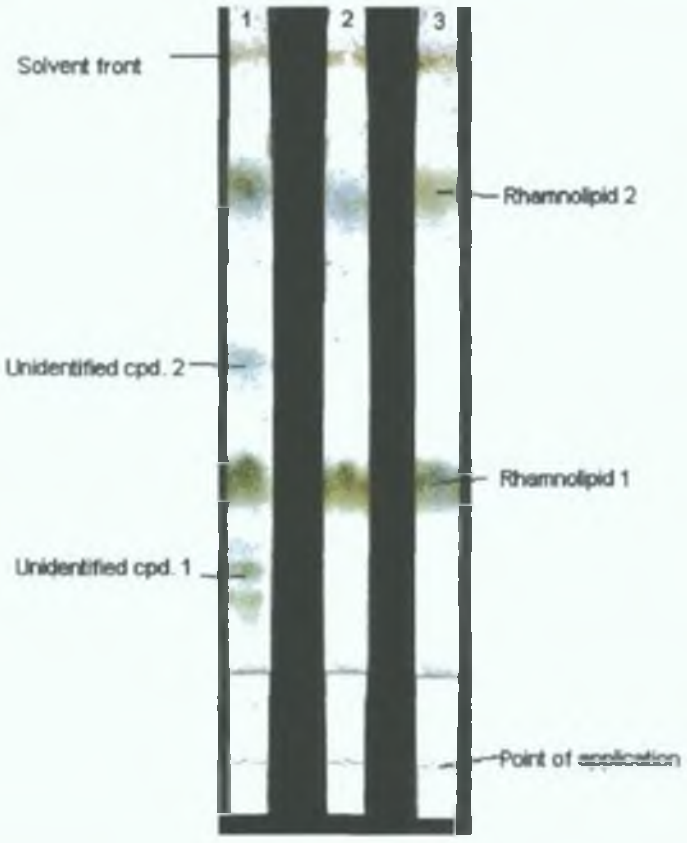


Figure 4.6. The effect of glucose concentrations on the production of biosurfactants by Isolate A. Lanes correspond to: 1: PPGAS + 5g/L glucose
2: PPGAS + 10g/L glucose
3: PPGAS + 2.5g/L glucose

4.1.5 Biosurfactant production using alternative carbon sources

Although interest in biosurfactants is increasing, these surfactants are not economically competitive compared to their synthetic counter-parts (Makkar and Cameotra, 1999). In the biosurfactant production process, the substrate can account for greater than 50% of

the final product cost. Therefore, the use of inexpensive and renewable raw material is important to the overall economics of the biosurfactant production process.

DMSBP is a relatively inexpensive and renewable substrate and it was postulated that due to its high sugar content it may be utilised for the production of biosurfactants. The two cultures chosen to investigate the possibility of using DMSBP for biosurfactant production were *Pseudomonas aeruginosa* NCIMB 8626 and Isolate A. These two cultures were chosen as they both produce biosurfactants with significantly different characteristics. The effect DMSPB had on rhamnolipid production by *Pseudomonas aeruginosa* NCIMB 8626 and Isolate A is illustrated in Table 4.5 and Figure 4.7. The medium used for biosurfactant production was PPGAS in which the glucose was substituted by DMSPB.

The results show that when Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 were grown in PPGAS with DMSBP as the carbon source, the surface tension of both culture broths was reduced significantly. The surface tension of the culture broth was reduced from 70mN/m to 36mN/m in the case of Isolate A, and to 49 mN/m in the case of *Pseudomonas aeruginosa* NCIMB 8626. These results indicate the production of surface active compounds. Analysis of culture broths by TLC revealed that both strains produced only rhamnolipid 1 when grown on DMSBP. Whilst not unusual for Isolate A to produce rhamnolipid 1, it was unusual for *Pseudomonas aeruginosa* NCIMB 8626 to produce this type of rhamnolipid as Wild *et al.* (1997) reported that *Pseudomonas aeruginosa* NCIMB 8626 only accumulated rhamnolipid 2. On the other hand, Ochsner *et al.* (1995) proposed that the predominant types of rhamnolipid produced depended on the medium composition. Overall, these results imply that it may be possible to use DMSBP for the production of biosurfactants, but further work is required in order to optimize the production rate.

Table 4.5. Effect of different carbon sources on the production of biosurfactants by *Pseudomonas aeruginosa* NCIMB 8626 and Isolate A.

Microorganism	Carbon source (5% w/v)	Surface tension mN/m.	Crude extract weight g/L culture
Isolate A	Glucose	29	1.372
Isolate A	DMSBP	36.8	0.414
Pseudomonas aeruginosa NCIMB 8626	Glucose	30	1.146
Pseudomonas aeruginosa NCIMB 8626	DMSBP	49	0.356

All cultures grown for 80 hours under the conditions in Section 3.5.2.

In conclusion, Isolate A and Isolate GO the two cultures isolated from samples obtained in Co. Sligo, had the ability to produce biosurfactants. The biosurfactants produced by Isolate GO when grown in PPGAS were very similar to those produced by a known biosurfactant producer *Pseudomonas aeruginosa* NCIMB 8626 and contained rhamnolipid 2. The biosurfactants produced by Isolate A were significantly different and contained four rhamnolipids when grown in PPGAS. The concentration of glucose in the PPGAS medium played a significant role on the types of rhamnolipids produced. It was also demonstrated that it was possible to use DMSBP as a substrate for biosurfactant production. The biosurfactants produced in this study were stored and used to determine the effect they had on the bioremediation of diesel contaminated soil.

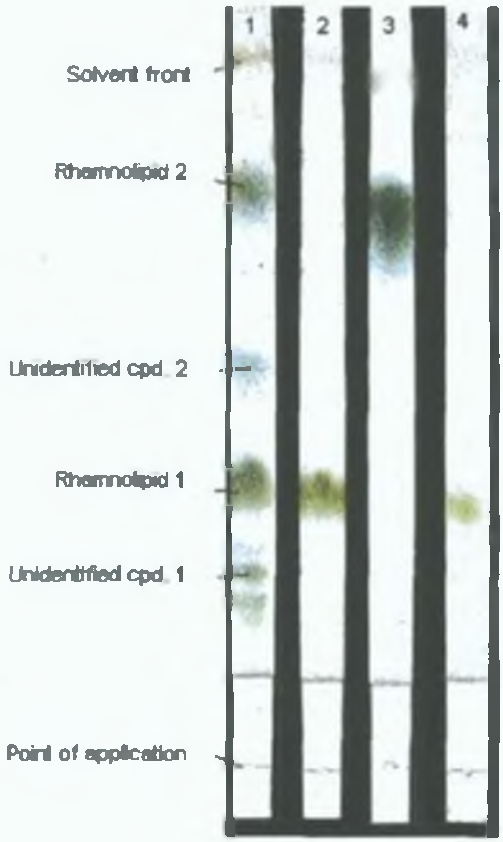


Figure 4.7. TLC plate of biosurfactants produced on different carbon sources by Isolate A and *Pseudomonas aeruginosa* NCIMB 8626.
Lanes correspond to: 1: Isolate A + Glucose
2: Isolate A + DMSBP
3: *Pseudomonas aeruginosa* NCIMB 8626 + Glucose
4: *Pseudomonas aeruginosa* NCIMB 8626 + DMSBP

4.2 Bioremediation of diesel contaminated soil

Before the effect of biosurfactant and organic matter amendments were assessed, studies were conducted to ascertain the amount of diesel lost due to abiotic factors. In addition, the role inorganic nutrients play in bioremediation was studied. These studies were undertaken as certain authors have reported that abiotic losses are insignificant in hydrocarbon removal from soil, while others have shown that such factors can account for a significant fraction of total hydrocarbon removal (Song *et al.*, 1990; and Margesin *et al.* 2000a). Similarly, in relation to nutrient amendments, various authors have reported differing effects of inorganic nutrient addition on the bioremediation of hydrocarbons (Margesin and Schinner, 1997c; and Rhykerd *et al.*, 1999)

4.2.1 Abiotic losses

Abiotic control experiments were carried out to investigate mechanisms, other than biodegradation, contributing to the removal of diesel oil from soil. Abiotic reactions include inorganic, organic, photolytic, surface-catalyzed, sorptive, and transport processes (Sepic *et al.*, 1995a). In the past various authors (Song *et al.*, 1990; Margesin and Schinner, 1997a; 1997b; and Margesin *et al.*, 2000a) have demonstrated that abiotic processes can contribute significantly to the overall removal process of diesel from contaminated soil.

In this study abiotic losses were calculated for the three types of microcosms used throughout the soil experiments. The three types of microcosms, (as described in Section 3.7) included: (1) static, where the soil sample was not mixed once the treatment was added; (2) manually mixed, the soil was manually turned at regular intervals; (3) forced aeration, where a pump was used to deliver air to the soil.

Sterilization of the soils was achieved by poisoning with 0.15% $HgCl_2$ (Walworth and Reynolds, 1995). This treatment rendered the sample microbiologically inactive while

producing minimal changes in the physical and chemical properties of the samples (Hwang, 1999). Throughout the entire experiment no microorganisms were recovered from any of the abiotic systems, using the standard plate count method, which indicated that 0.15% $HgCl_2$ acted as a good biocide.

Trends for the percentage removal of THEM as a function of time are shown in Figure 4.8. The trends shows that, in the static microcosm, abiotic losses resulted in a reduction of 17 % of THEM by day 40, after which no further losses were detected. GC analysis of the THEM on day 112 (Figure 4.8) showed that the lower molecular weight compounds up to *n*-C_9 were completely removed, while only a small portions of *n*-C_{10} to *n*-C_{13} were removed (see Figure 4.10).

Manually mixing the soil resulted in a steady decline in THEM at a rate of 0.16% (per dry weight) per day and after 108 days 24.6 % of the original sample was lost due to abiotic factors. Under these conditions compounds below *n*-C_{12} were totally removed while compounds up to *n*-C_{16} were partially removed (Figure 4.9 and 4.10).

Off the three aeration systems used in this study it was the microcosm which had forced aeration that had the greatest loss due to abiotic factors. In this microcosm the air pump distributed an average of 120L air/kg soil/hour. Over the first 15 days a rapid removal of THEM was observed compared to the other two systems (Figure 4.8). Removal occurred at an average rate of 2.96 % per day for the first 15 days of incubation resulting in approximately 42% removal of THEM. After this initial rapid depletion the rate of removal decreased rapidly with little to no loss observed over the following 100 days. After 112 days of incubation 43 % THEM had been removed in the forced aeration treatment. The chromatograph of the THEM at day 112 (Figure 4.9) shows that, after 112 days, all the peaks before *n*-C_{13} were completely removed while portions of *n*-C_{13} up to *n*-C_{16} were removed (Figure 4.10). This increase in abiotic losses can be attributed to increased volatilization as a result of the increased movement of air through the soil.

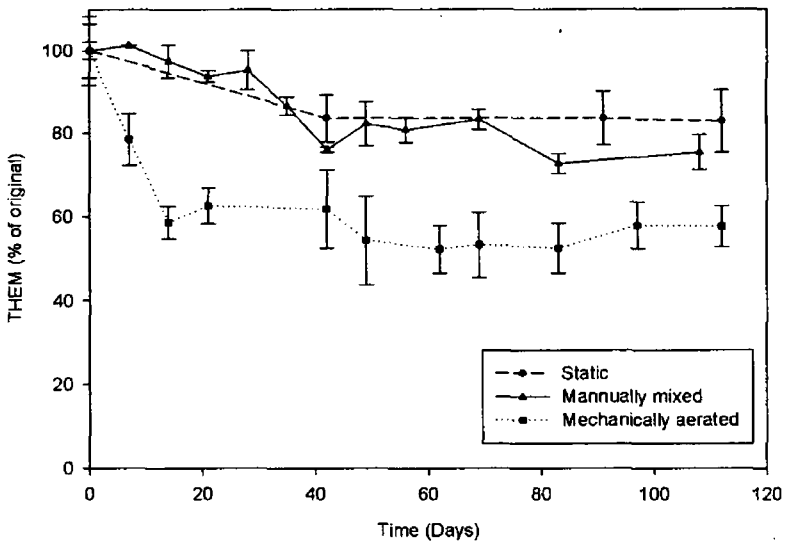


Figure 4.8. The effect of different types of aeration on the abiotic removal of THEM from diesel contaminated soil. Error bars represent ± 1 standard deviation (n = 4).

The results obtained here for the abiotic removal of diesel from a soil system (17 to 42%), compare favourably with those reported by other authors. For example, Margesin *et al.* (2000a) demonstrated that in a manually aerated soil up to 35% of diesel was eliminated due to abiotic losses after an 88 day experimental period, and Song *et al.* (1990) found that after 126 days of incubation approximately 25% removal of diesel was attributed to abiotic factors. These results confirm that abiotic processes can contribute significantly to the overall removal process of diesel from contaminated soil, and would corroborate the proposal of Kroening *et al.* (2001) who postulated that abiotic loss may be a significant method of diesel contamination removal that may have previously been underestimated or overlooked in short term studies. The results found in this study also demonstrated that the type of aeration plays an important role in abiotic losses of diesel from soil.

The problem associated with using abiotic removal as a complete remediation processes for diesel contaminated soils is that while it can account for significant losses in the early stages of a fresh diesel spill, it rates of removal declines significantly once the highly volatile components are removed and the heavier molecular weight, less volatile compounds remain. Alone, abiotic processes could take years to remove these heavier molecular compounds, a time scale not viable for most remediation projects.

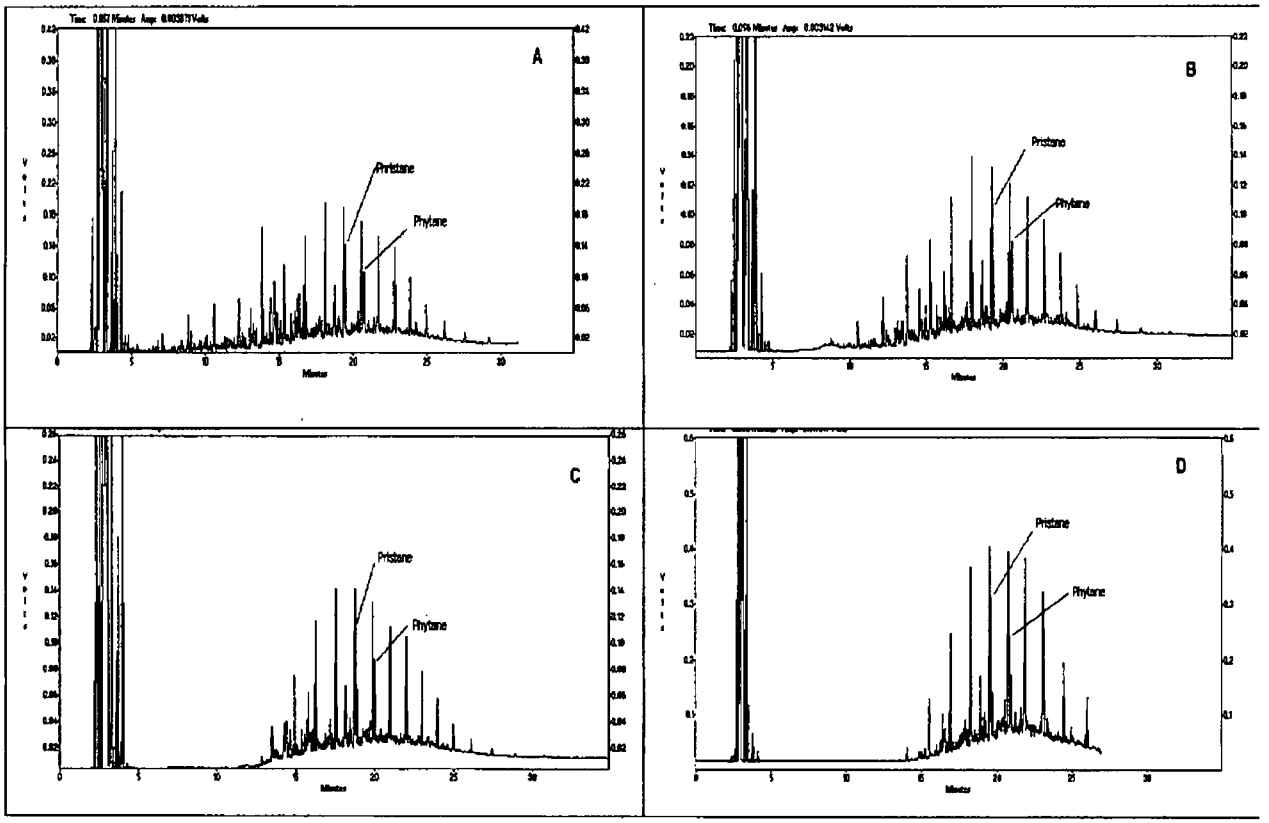


Figure 4.9. Removal of diesel due to abiotic factors. (A) Chromatograph of unweathered diesel; (B) biotic losses in static microcosm after 112 days; (C) abiotic losses in manually mixed microcosm after 108 days; (D) abiotic losses in forced aerated microcosm after 112 days. All microcosms were maintained at room temperature.

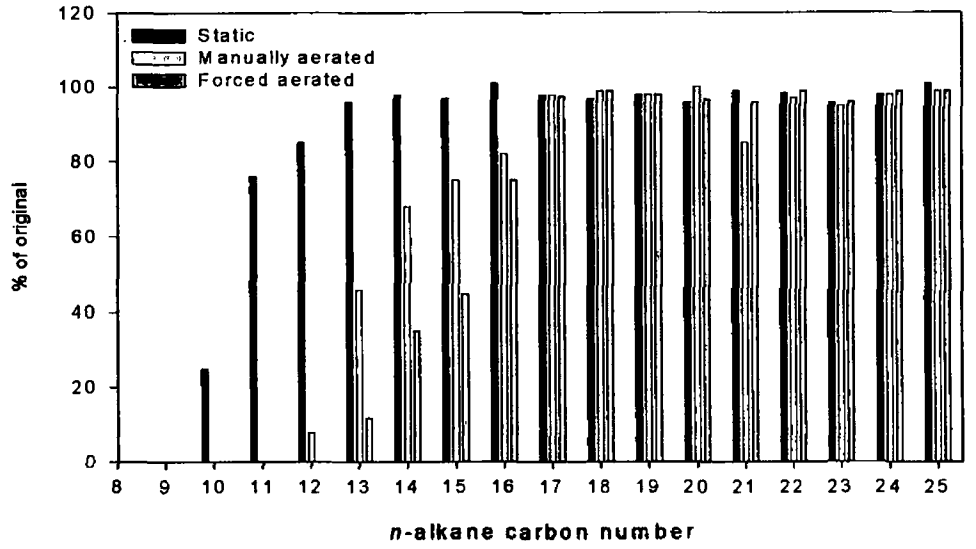


Figure 4.10. The effect of different types of aeration on the abiotic removal of individual *n*-alkanes from diesel contaminated soil after incubation at room temperature for 112 days for the static and forced aerated and 108 days for the manually aerated microcosm.

4.2.2 Effect of inorganic nutrient amendment

It has been well documented in the literature that a major limitation in bioremediation of hydrocarbon-contaminated soil is the bioavailability of nutrients, especially of nitrogen and phosphorus. In general, in contaminated soils, nutrients need to be added in order to enhance bioremediation processes (Margesin and Schinner, 1997a). To date, a large number of both laboratory and field experiments have demonstrated the enhancement and acceleration of hydrocarbon biodegradation in nutrient amended soils (Frankenberger *et al.*, 1989; Morgan and Watkinson, 1989; Rosenberg *et al.*, 1992; Radwan *et al.*, 1997; Demque *et al.*, 1997; Rhykerd *et al.*, 1999; Taylor and Viraraghavan, 1999, Margesin *et al.*, 2000a). The exact amount and type of nutrient required is difficult to predict and depends on the amount of carbon available. C:N:P ratios used by different authors varied from 500:1:0.001 (Huesemann, 1994) to 9:1:0.2 (Morgan and Watkinson, 1989) and the effect on hydrocarbon biodegradation varied considerably. This variation in opinion and resulting outcomes on bioremediation

processes prompted the necessity to establish the effects inorganic nutrient amendment alone had on diesel removal from the contaminated sandy soil used in this study.

Chemical analysis of the sandy soil used throughout this study indicated that the soil was very low in organic carbon and the major nutrients (See Table 3.1). The soil contained 0.41% organic carbon, 150mg/kg total Kjeldahal nitrogen and 12mg/kg available phosphorus. The C:N:P ratio of the soil before it was contaminated with the diesel was approximately 273:10:0.8. In soils contaminated with diesel the amount of carbon in the soil was calculated based on amount of diesel in the soil and 85 % carbon content in the diesel (Rhykerd *et al.*, 1999). Throughout this study, diesel was added to the soil to give a final concentration of approximately 5% which resulted in a C:N:P ratio of 3000:10:0.8 before any amendments were applied.

In the soil bioremediation experiments, unless otherwise stated, nitrogen in the form of NH_4NO_3, and phosphorus and potassium in the form of KH_2PO_4 were added to adjust the C:N:P to approximately 1000:10:1.5 in the contaminated soil. This ratio was chosen as it was in the middle of the extremes reported in the literature. Inorganic nutrient consumption was not monitored during the soil experiments but additional inorganic nutrients were added biweekly to ensure that N, P and K did not become limiting.

An experiment to determine the effectiveness of inorganic nutrients on the bioremediation of diesel contaminated soil was carried out in a manually aerated system, and the results for the trends in biodegradation of DRO, total *n*-alkanes, UCM and pristane in relation to time are presented in Figure 4.11. The correlation coefficients for the kinetic models indicated that the first order model describes the degradation of the different fractions with high correlation ($r > 0.85$). Other authors who have demonstrated that first order kinetics describes the degradation of hydrocarbons with high coefficients, independent of the starting concentration, include Hwang (1999), Jorgesen *et al.* (2000) and Nocentini *et al.* (2000).

In the unamended soil the first order degradation rate constant for DRO was calculated to be 0.0024/day and after 108 days incubation 27% of the DRO had been removed. This percentage removal in the unamended soil was not significantly different ($P<0.05$) to that of the abiotic removal (24.6%) under similar conditions. Examination of the gas chromatographic fingerprint of the residue diesel in the unamended soil after 108 days showed that only the lower molecular weight compounds in the diesel were removed. This chromatographic fingerprint was very similar to that observed in the abiotic microcosm which suggests that very little, if any bioremediation of diesel occurred in the unamended diesel contaminated soil. Another parameter used to quantify hydrocarbon loss to microbial degradation (i.e. ratio of *n*-C_{17}:pristane) indicated that very little bioremediation of the diesel had occurred in the unamended soil. The ratio of *n*-C_{17}:pristane in the residue diesel of the unamended soil at the end of the experiment was 1.5 ± 0.13(n = 4) which was not significantly different to that of fresh diesel 1.6 ± 0.11(n = 4). Ratios of *n*-alkanes to pristane are a traditional weathering indicator which has been used for many years to give estimations of hydrocarbon degradation taking place. Pristane is more resistant to biodegradation than the straight chain *n*-alkanes and therefore a reduction in *n*-C_{17}:pristane ratios indicates bioremediation of the diesel (Kennicutt, 1988; Atlas, 1991; Butler *et al.*, 1991; and Tabak *et al.*, 1991). However, as will be discussed later, there are particular problems associated with using this particular weathering indicator (Section 4.2.4.3).

Amending the soil with inorganic nutrients increased the first order degradation rate constant to 0.0057/day and resulted in a significantly higher removal of DRO compared to the unamended soil. After 108 days of incubation the diesel contaminated soil amended with inorganic nutrients increased the removal of DRO to 51 % compared to 25 % in the unamended soil. The ratio of n-C_{17}:pristane was significantly reduced ($P<0.05$) from 1.6 ± 0.11 on day zero to 0.25 ± 0.01 , which is indicative that bioremediation had occurred. The overall removal of DRO in this study is comparable to the findings of Rhykerd *et al.* (1999) who reported 33% removal of diesel in nutrient amended soil after an 84 day experimental period.

However, the percentage removal of diesel in this study in the nutrient amended soil was relatively low compared to other figures published in the literature. Margesin and Schinner (1997a) reported that 47% of diesel in a nutrient amended arable soil was removed by bioremediation after 30 days at 25°C, while in another report by Margesin *et al.* (2000b) using a higher nutrient application (C:N ratio of 20:1) resulted in the removal of 74% diesel in a 70 day period. Demque *et al.* (1997) established that a similar range of diesel removal (approximately 61-83% in 98 days) from Petawawa sand was achieved through biostimulation with a commercial fertiliser. A possible reason for the difference in rates of diesel removal maybe that the soils originate from entirely different sources and therefore would have totally different microbial populations, which may have a different affinity to diesel and hence its degradation.

The main reason for the increase in DRO removal in the inorganic nutrient amended soil presented here was most probably due to the increased biodegradation of the total *n*-alkanes (Figure 4.11B). The removal rate of the *n*-alkanes, ranging from *n*-C_9 to *n*-C_{25}, was approximately 6.5 times greater in the nutrient amended soil (k =0.013/day) than in the unamended soil (k = 0.002/day). After 108 days 79 % of the total alkanes had been removed leaving behind the more recalcitrant compounds such as branched alkanes (including the isoprenoids pristane and phytane), cyclic alkanes and alkyl aromatics such as naphthalenes and phenanthrenes (Revill *et al.*, 1992) (Figure 4.12). The disappearance of these more dominant resolved components in the gas chromatograph resulted in the detection of a "hump" or unresolved complex mixture (UCM). This UCM is found in most petroleum products but is typically more pronounced in weathered/ biodegraded products (Gough *et al.*, 1992; Revill *et al.*, 1992; and Wraige, 1997). The concentration of the isoprenoid pristane was not significantly reduced (< 10% of original concentration) in either of the treatments (Figure 4.11C), whilst the removal of the UCM fraction of diesel was only slightly enhanced through nutrient addition. At the termination of the experiment, 28.4 % UCM had been removed in the nutrient amended soil, 12% more than in the unamended system (Figure 4.11D).

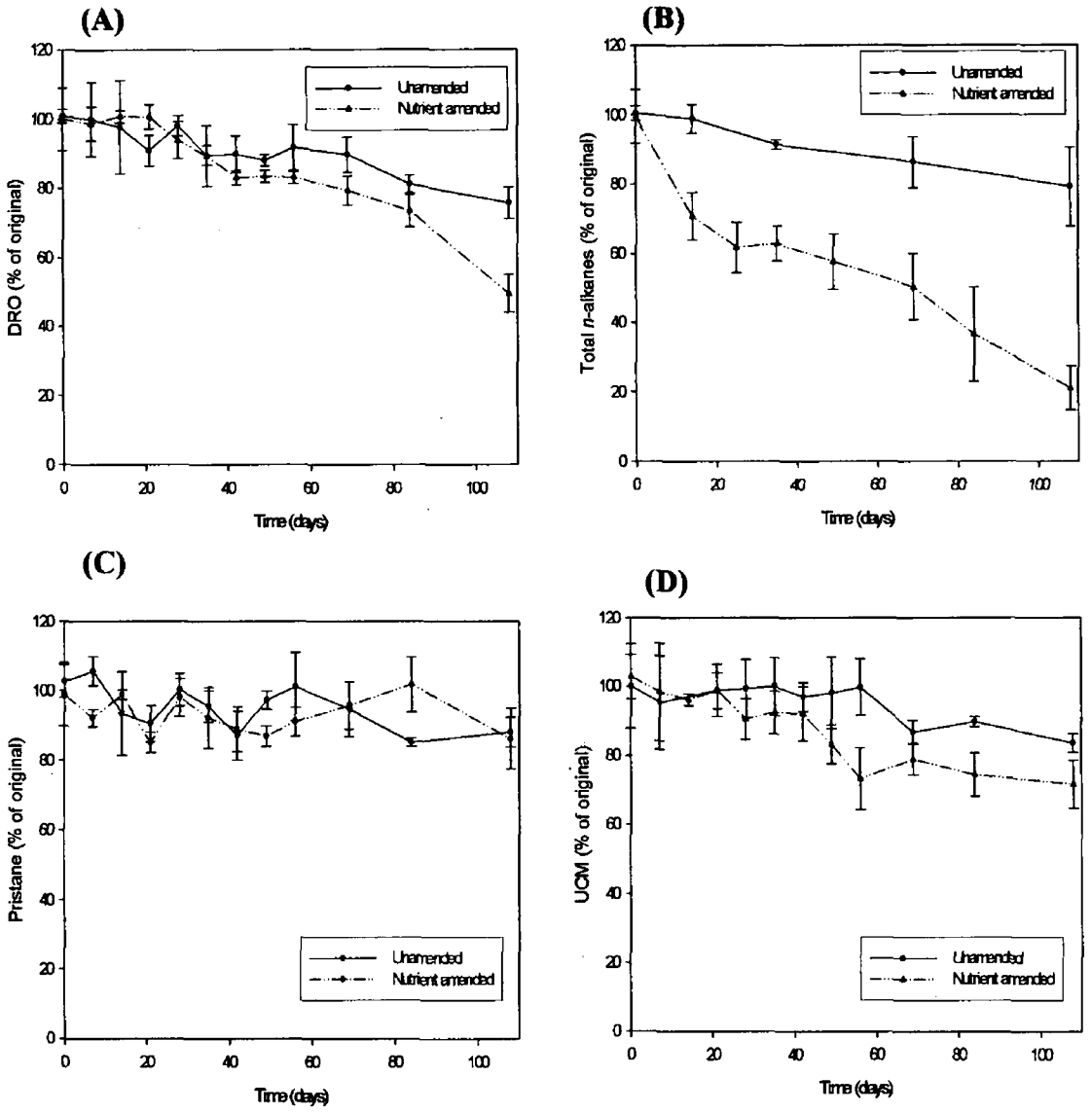


Figure 4.11. Effect of inorganic nutrient amendment on the removal of diesel from a sandy soil. (A) Removal of DRO with time; (B) Removal of total *n*-alkanes with time; (C) Removal of pristane with time; (D) Removal of UCM with time. Conditions are described in Section 3.8.3. . Error bars represent ± 1 standard deviation (n = 4).

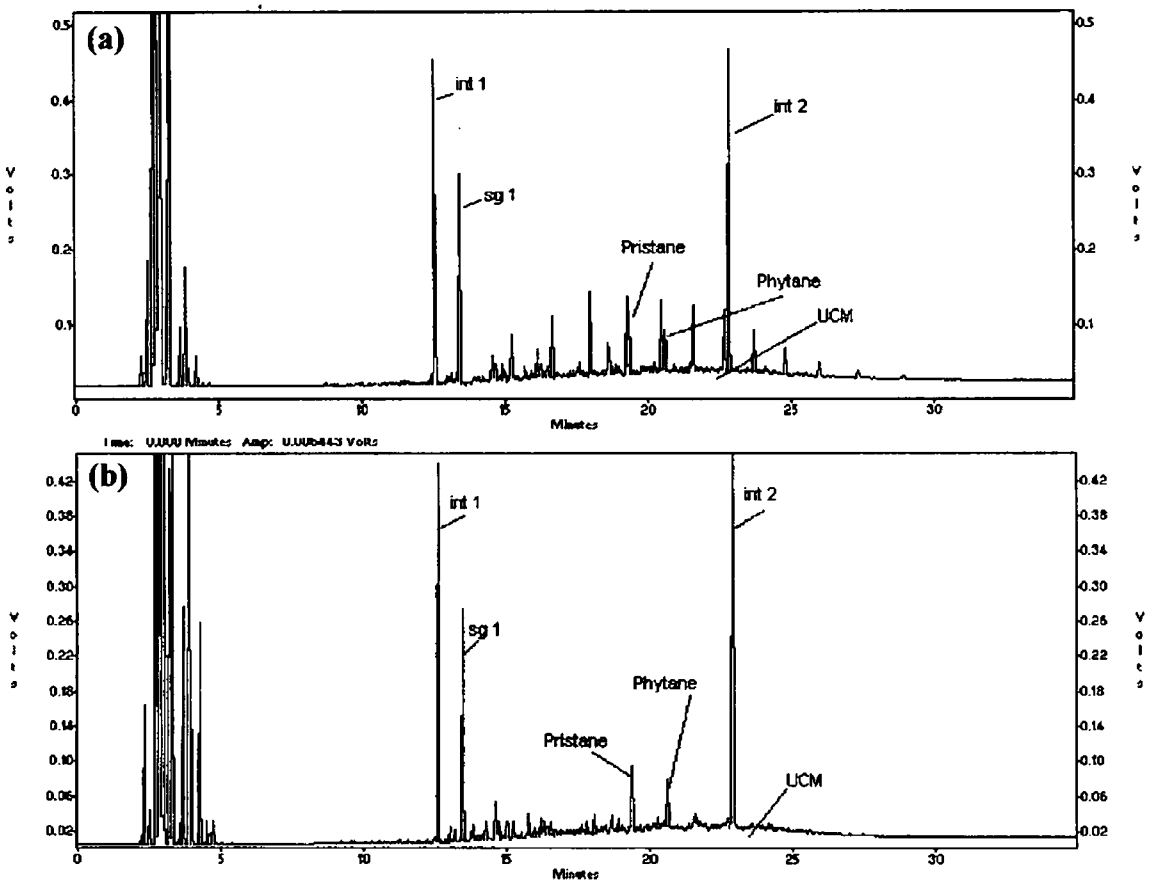


Figure 4.12. Gas chromatograph of the THEM from (a) unamended and (b) inorganic nutrient amended diesel contaminated soil. The soils was incubated at room temperature for 108 days and manually mixed biweekly. (int.1 = internal standard, 1-phenylhexane; int. 2 = internal standard, 1-phenyltridecane; sg1=surrogate standard, naphthalene; and UCM = unresolved complex mixture).

The enhanced removal of diesel components in the inorganic nutrient amended soil corresponded with stimulation in microbiological parameters. The total number of mesophilic microorganisms increased in both inorganic nutrient amended and unamended soils over the first few days (Figure 4.13). From day 0 to day 4 there was an initial increase in CFUs from $2.2x10^5$ to 1.6×10^7 CFU/g dry soil in the unamended soil and to $4.3x10^7$ CFU/g dry soil in the inorganic nutrient amended soil. After day 4 the numbers in the unamended sample did not increase any further, averaging between 10^6 and 10^7 for the duration of the experiment. In the amended soil the numbers of CFU increased further to $5.7x10^8$ by day seven, after which the numbers stayed significantly

higher throughout the experiment, ranging from 10^8 to 10^9. These results indicated that the increased removal of diesel through the addition of inorganic nutrients was as a result of stimulation of the indigenous microbial population.

In this study, it was observed that the numbers of bacteria increased following biostimulation through the addition of inorganic nutrients, but also a significant increase was observed in the soil that did not receive nutrients but had diesel present. This increase was attributed to the stimulatory effects of moisture content adjustment and the addition of the diesel, a phenomenon previously encountered by Harrison *et al.* (2000).

Data was obtained on changes in microbial activity upon inorganic nutrient addition by measurement of soil respiration using a BI-2000 electrolytic respirometer (Section 3.8.6). Respirometry is a method of determining metabolic activity as measured by changes in concentrations of carbon dioxide and oxygen and has been used repeatedly during bioremediation projects (Van der Waarde *et al.*, 1995; Margesin and Schinner, 1997a; Hwang 1999; Margesin *et al.*, 2000a and Namkoong *et al.*, 2002). Soil respirometery was carried out, as quantification of viable soil microorganisms alone gives no information about the efficiency or microbial activity of the populations. It is also well known that only a small portion of soil microorganisms can be isolated and cultivated on laboratory media (Margesin *et al.*, 2000a).

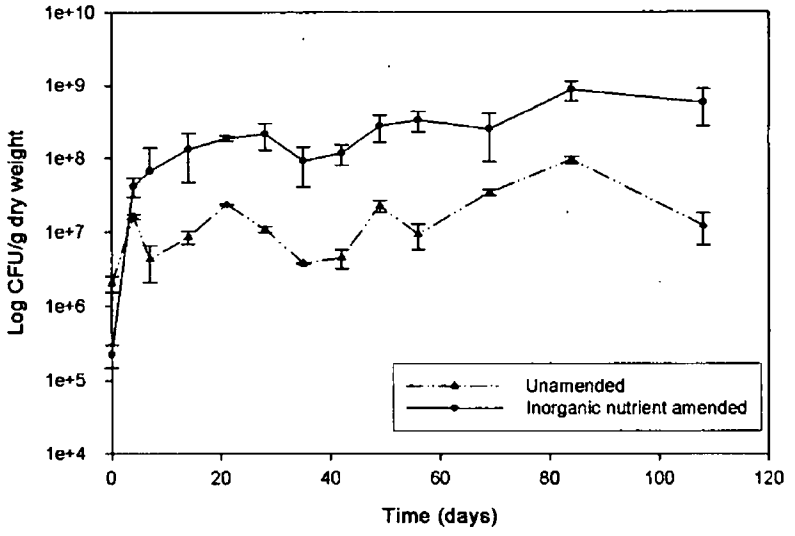


Figure 4.13. Trends in total mesophilic bacteria numbers recovered from diesel contaminated soil, amended and unamended with inorganic nutrients. Error bars represent ± 1 standard deviation (n = 4).

Trends in soil respiration as a function of time are shown in Figure 4.14. After a lag phase of four days the amount of O_2 consumed in the nutrient amended sample started to increase at a steady rate of 202 mg O_2/kg soil/day and after 20 days of incubation, at room temperature, 4,111mg O_2/kg soil was consumed in the inorganic nutrient treated soil. This consumption after 20 days in the inorganic nutrient amended soil was almost four times greater than in the unamended soil which had consumed 1,439 mg O_2/kg in the same time period. The lag phase can be attributed to the fact that the indigenous microorganisms which, have had no previous exposure to diesel, take time acclimatising to the new carbon source. Siddiqui and Adams (2002) found a similar pattern in O_2 consumption in an inorganic nutrient amended diesel contaminated soil. They reported that when diesel plus inorganic nutrients were added to soil with no previous history of hydrocarbon contamination, soil respiration response showed a lag phase of six days. The same authors also showed that the lag phase was reduced to only two days when diesel plus inorganic nutrients were added to a soil with a previous history of hydrocarbon contamination. The increase in soil respiration after nutrient application, as compared to the sample without nutrients, has repeatedly been described (Margesin and Schinner 1997a; Hwang, 1999; and Margesin *et al.*, 2000a) and is interpreted as an indication of enhanced hydrocarbon minerilization.

The combined results for, THEM removal, total mesophilic bacterial numbers and soil respiration, demonstrate that application of inorganic nutrients at the rate used in this study increased the microbial activity significantly and enhanced the degradation rate of diesel in contaminated soil compared to an unamended control.

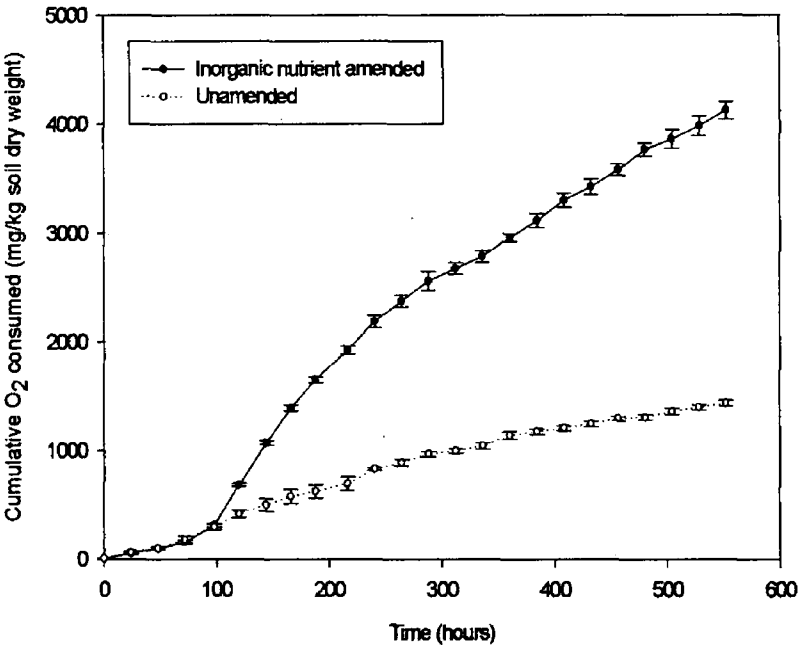


Figure 4.14. Trends in O_2 consumption in diesel contaminated soil with and without inorganic nutrient amendment. Error bars represent ± 1 standard deviation (n = 3).

4.2.3 Effects of biosurfactant amendment on diesel bioremediation

The bioremediation of organic contaminants, such as diesel, suffers from several bottlenecks, one of which is the low bioavailability of hydrophobic organic contaminants. This poor bioavailability is caused by low mass transfer rates of the contaminants to the degrading microorganisms from sites where they are inaccessible (Noordman, 1999). In these situations the use of biosurfactants to increase the

bioavailability of the contaminants has been suggested as a way of enhancing the bioremediation process. To-date limited research has been carried out with regard to the use of surfactants or biosurfactants to enhance the bioremediation of diesel contaminated soils. In this section, the biosurfactants produced by Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 were investigated for their possible use in enhancing the remediation of diesel contaminated soil.

Solubilising agents such as biosurfactants may enhance bioremediation of soils contaminated with hydrophobic organic contaminants by diminishing sorption of the contaminants or increasing desorption rates. The effectiveness of the two biosurfactants to enhance the removal of sorbed diesel from soil was determined using a washing procedure and compared with the use of a synthetic surfactant. Diesel contaminated soil (approximately 5% diesel v/w) was washed with solutions of the different surfactants at various concentrations, and the DRO in the aqueous wash solution were calculated.

The results for DRO removal by the different surfactant solutions are presented in Figure 4.15. It can be seen that the amount of DRO removed was different for each surfactant and was dependant on surfactant concentration. For all surfactants tested the removal of DRO increased with increase in surfactant concentration. At the lowest concentration of 0.1 times their CMC the surfactants did not significantly ($P<0.05$) enhance DRO removal compared to water alone. Increasing the concentration to 1, 2 and 5 times their CMC resulted in significant ($P<0.05$) enhancement in DRO removal compared to washing with water alone. The most efficient of the surfactants tested was the biosurfactant produced by Isolate A which removed 54.4 % of the added DROs when applied at five times its CMC. Triton X-100 (synthetic surfactant) and the biosurfactant produced by *Pseudomonas aeruginosa* NCIMB 8626 removed 41 and 21 % of the DRO, respectively, when applied at a similar concentration of five times their CMC. These results indicate that increasing the concentration of each surfactant tested to above their CMC significantly enhanced the removal of diesel. The results obtained in this study compare favorably to findings by Scheibenbogen *et al.* (1994) and Bai *et al.* (1997), both of whom reported that biosurfactants, produced by *Pseudomonas aeruginosa,* were most

effective at removing residual hydrocarbon from sandy soil at concentrations above their CMC. Ang and Abdul (1991) postulated three mechanisms for removal of hydrocarbon from soil columns; displacement (e.g. as by water); dispersion (surfactants lower surface tension, leading to oil displacement); and solubilisation (dissolution) of oil by surfactant micelles. It is likely that all three mechanisms were involved in the removal of hydrocarbons by the surfactants tested in this study. However, as there was a large increase in removal at concentrations above the CMC value it was postulated that solubilisation was the most important mechanism for removal of diesel from soil.

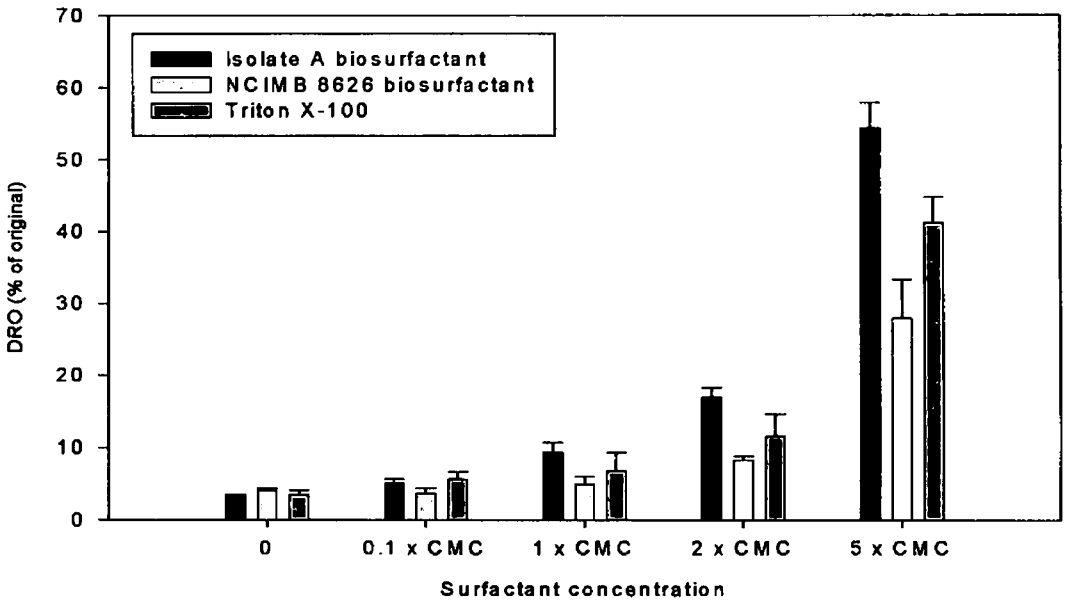


Figure 4.15. Removal of diesel from soil by washing with the biosurfactants produced by Isolate A and *Pseudomonas aeruginosa* NCIMB 8626, and the synthetic surfactant Triton X-100. Error bars represent ± 1 standard deviation (n = 4).

Comparison of the results for the three surfactants tested show that the biosurfactant produced by Isolate A was the most effective at removing DRO from the sandy soil followed by the synthetic surfactant Triton X-100, while the biosurfactant produced by *Pseudomonas aeruginosa* NCIMB 8626 was the least effective at removing DRO from the sandy soil.

Examination of the removal of individual *n*-alkanes and the isoprenoids pristane and phytane after washing of the diesel contaminated soil with the biosurfactant solutions produced by Isolate A demonstrated that there was no preferential removal of the lower molecular weight compounds compared to the higher molecular weight compounds (Figure 4.16). This was illustrated by the fact that the concentrations of the selected compounds increased by similar proportions as the concentration of the biosurfactant increased. i.e. the concentration of all the selected *n*-alkanes and the two isoprenoids removed increased by a factor of 1.7 ± 0.14 when washed with biosurfactant at two times its CMC, compared to washing with water. Increasing the concentration of the biosurfactant to five times its CMC resulted in a further increase in the percentage removal, by a factor of 3.5 ± 0.14.

All the above results demonstrate that the surfactants tested enhanced the removal of a major portion (up to 54%) of sorbed diesel from soil relative to treatment without the surfactants (4%). However, enhanced removal of sorbed contaminants does not necessarily result in an enhancement in the bioremediation of the contaminant and therefore further studies were carried out to investigate if the biosurfactants would enhance the bioremediation of diesel contamination.

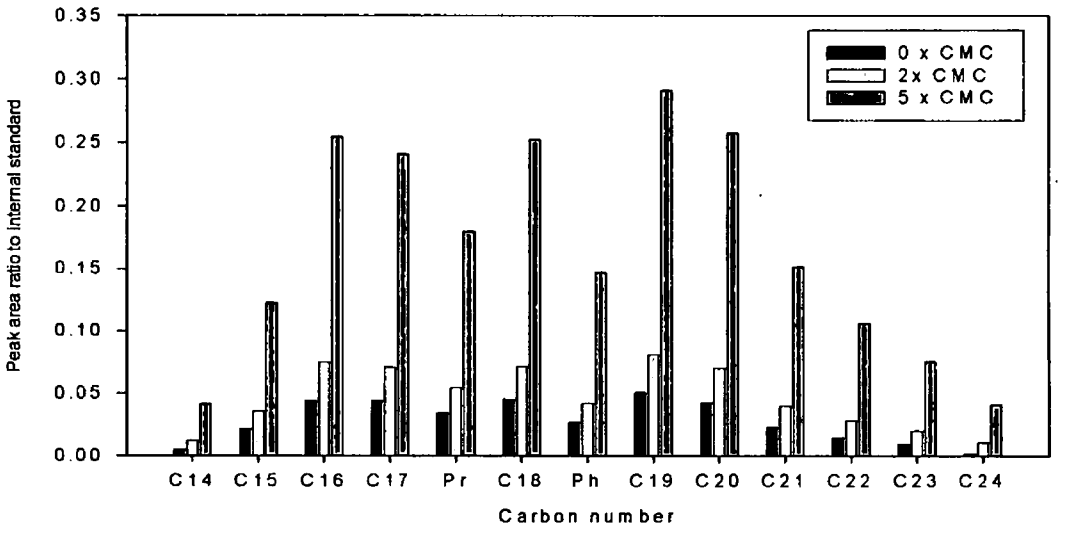


Figure 4.16. Recovery of hydrocarbon fractions from diesel contaminated soil by washing with different concentrations of the biosurfactant produced by Isolate A. C14 to C24 represent straight chain alkanes, Pr and Ph refer to pristane and phytane.

Before surfactants were used in bioremediation studies it was important to establish that they were not toxic to the degrading microbial populations as this would result in inhibition of the bioremediation process. It is well known that surfactants can be toxic to bacteria (Noordman, 1999). In this present study the toxicity of biosurfactants produced by Isolate A, Isolate GO and *Pseudomonas aeruginosa* NCIMB 8626, towards the indigenous soil microbial population was investigated. Each surfactant was tested at a concentration equivalent to five times their CMC, which was higher than that used in the bioremediation experiments. The growth patterns of the soil consortium, grown in nutrient broth in the presence of the different surfactants, as measured through optical density determination at 600nm, are shown in Figure 4.17. The results indicate that there was no significant difference in the growth pattern of the soil consortium in the presence of the biosurfactants, compared to the control. This would suggest that the surfactants were not toxic to the soil consortium. However, due to the complexity of the indigenous soil microbial populations it is impossible to say that all of the individual microorganisms, important in the overall degradation of diesel, are unaffected by the presence of these biosurfactants.

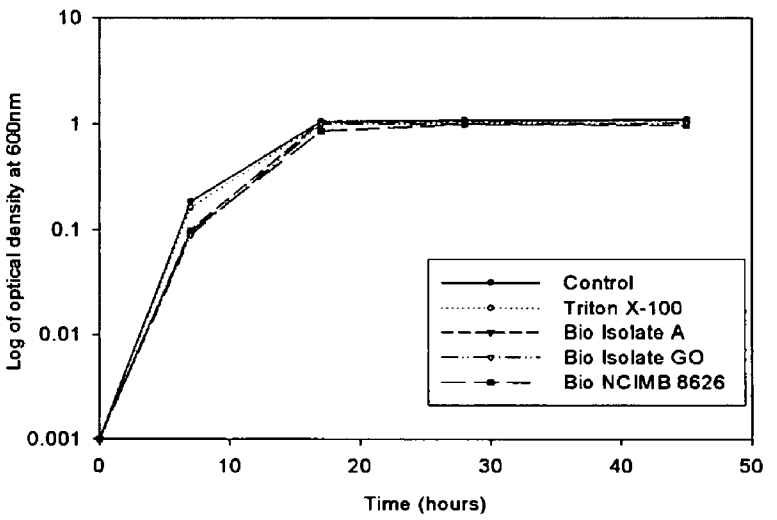


Figure 4.17. The effects of the different surfactants on the growth of soil microorganisms in nutrient broth grown in flask cultures. All surfactants applied at 5 x CMC.

Even though the importance of inorganic nutrients in the biodegradation of diesel in soil has been discussed, a study was conducted to investigate whether a single application of biosurfactants to diesel contaminated soil, without nutrient amendment, would have an effect on the rate of diesel biodegradation. Biosurfactants produced by Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 were applied at concentrations of 0.1, 1.0 and 3 times their CMC (see Table 3.5) and the microcosms (Figure 3.1A) were left static throughout the experiment. At the end of the experimental period of 90 days, no significant difference in the THEM removal was observed between the soils with or without biosurfactant amendment (see Appendix 3). GC analysis of the THEM showed that at the end of the experiment only the *n*-alkanes with retention times less than *n*-C_{13} were removed in any of the treatments and none of the chromatographic fingerprints differed significantly from that of the abiotic sample. Calculations of the ratios of *n*-C_{17}:pristane in the residue diesels on day 90 (Figure 4.18) indicated that no reduction in the ratios had occurred and thus confirmed that no biodegradation had occurred.

Analysis of the soil for biosurfactant activity at the end of the 90 day experimental period showed that neither of the biosurfactants were detectable in the soil, independent of the starting concentration. These results suggested that the two biosurfactants were biodegraded by the indigenous soil microorganisms and prompted the necessity for an investigation into the fate of the biosurfactants in the sandy soil in order to determine how quickly they are degraded.

Therefore it may be concluded that a one off addition of these biosurfactants on their own to a diesel contaminated soil would have no impact on bioremediation.

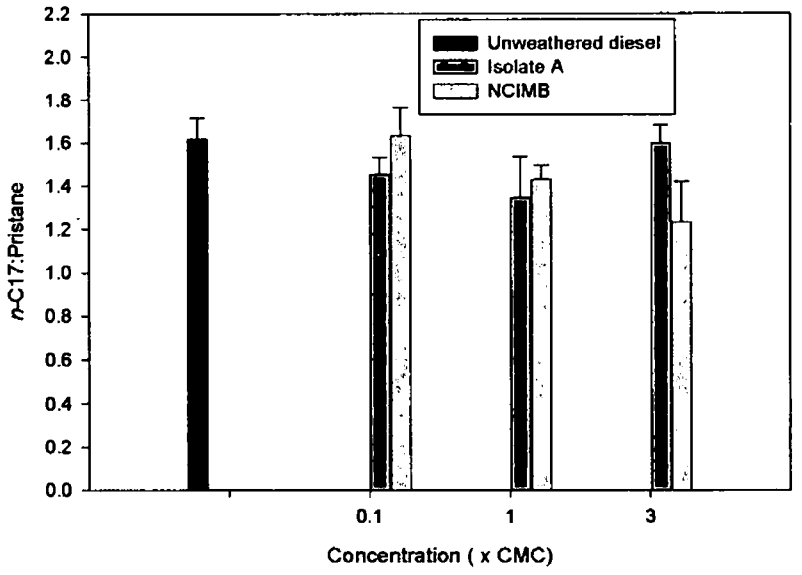


Figure 4.18. Effect of biosurfactant addition on n-C_{17}:pristane ratio in soils contaminated with diesel in the absence of inorganic nutrients. Biosurfactants were applied in a single application to the diesel contaminated soil and above represents results for the ratio of n-C_{17}:pristane on day 90 of the experiment. Values are expressed as the mean of 3 replicates. Error bars represent ± 1 standard deviation.

As no surface activity was found to be present at the end of the period of analysis, it was decided to investigate the fate of biosurfactants in the soil. The fate of the biosurfactants in soil was investigated by amending the soil with the biosurfactants and measuring the water extractable surfactant over time (Section 3.6.6). The results (Figure 4.19) established that both biosurfactants disappeared rapidly from diesel contaminated sandy soil, i.e. within a matter of days, as measured by the disappearance of the surface activity. When applied at a concentration of approximately equivalent to their CMC, the two biosurfactants were removed from the soil system within six days. To confirm that this removal was due to biodegradation and not abiotic losses such as volatilization or their sorption to soil particles, an abiotic control was run in which Isolate A biosurfactant was added into sterile soil (autoclaved). Over a 20-day period no removal of the biosurfactant was observed in the sterile soil as measured by retained surface activity (Figure 4.19), confirming biodegradation as the mechanism for biosurfactant removal from the soil. These results compare favourably with those of Bregnard *et al.* (1998) who reported that rhamnolipid biosurfactants, similar to those used in this study,

were biodegraded by indigenous sandy soil microorganisms. These findings further suggest that biosurfactants would have to be added on a regular basis in order to be of any value in promoting bioremediation.

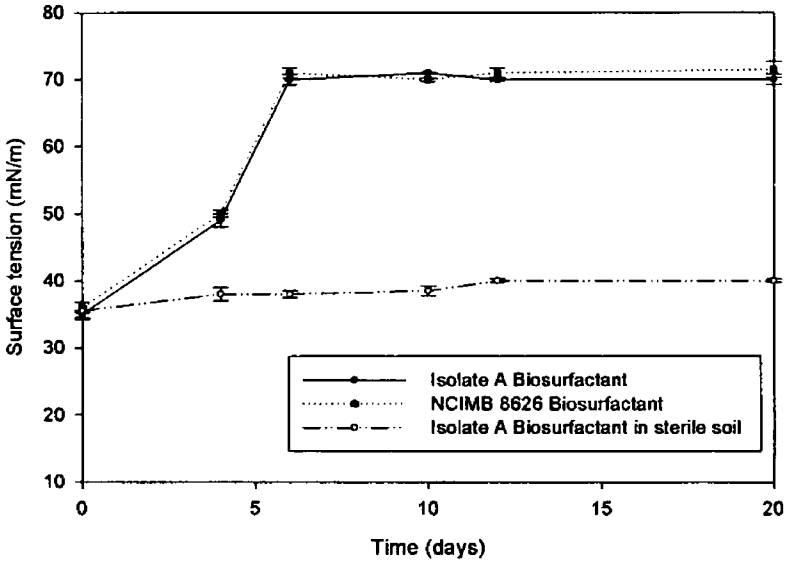


Figure 4.19. Fate of the biosurfactants in the diesel contaminated soil. Soil samples were washed with distilled water and the surface tension of centrifuged aqueous phase was determined. The background surface tension of the soil is not presented, but averaged 71.25 mNm^{-1} throughout the experiment. Error bars represent ± 1 standard deviation (n = 3).

As the effect of a single application of biosurfactants with no additional nutrients had no impact on bioremediation of diesel contaminated soil, it was decided to investigate the effect multiple additions of biosurfactant had on the bioremediation process. Biosurfactants from Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 were added every week (on the basis of previous findings regarding their biodegradation) to the diesel contaminated sandy soil (5%), to maintain a concentration equivalent to three times their CMC. On this occasion the soil contained inorganic nutrients, NH_4NO_3 and KH_2PO_4 as per Section 3.8.3, in order to achieve a more realistic situation for bioremediation to take place. The microcosms (Figure 3.2) were force aerated over a period of 98 days and the degradation patterns of the THEM found in the different treatments are presented in Figure 4.20. It can be seen that over the first 15 days there

was no significant difference in the percentage removal of THEM in all the treatments compared to the control which had no biosurfactants (33-45% removal), with the degradation rates varying from 2.1 to 2.9 % THEM per dry weight soil per day. After day 15 the degradation rates slowed down in the nutrient amended system to a rate of 0.23% per day. In the biosurfactant amended soils the rates also slowed down but continued at a slightly higher rate than the control, at 0.41 and 0.38% per day for *Psuedomonas aeruginosa* NCIMB 8626 and Isolate A biosurfactants, respectively.

By the end of the experimental period there was no statistically significant ($P<0.5$) difference between the two biosurfactant amended microcosms compared to the inorganic nutrient only amended system. After 98, days Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 biosurfactants had removed 74 and 72 % THEM, respectively compared to 65 % in the inorganic nutrient amended system. These results suggested that the addition of biosurfactants from Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 had no positive effect on the removal of THEM from a diesel contaminated soil compared to amendment with inorganic nutrients only.

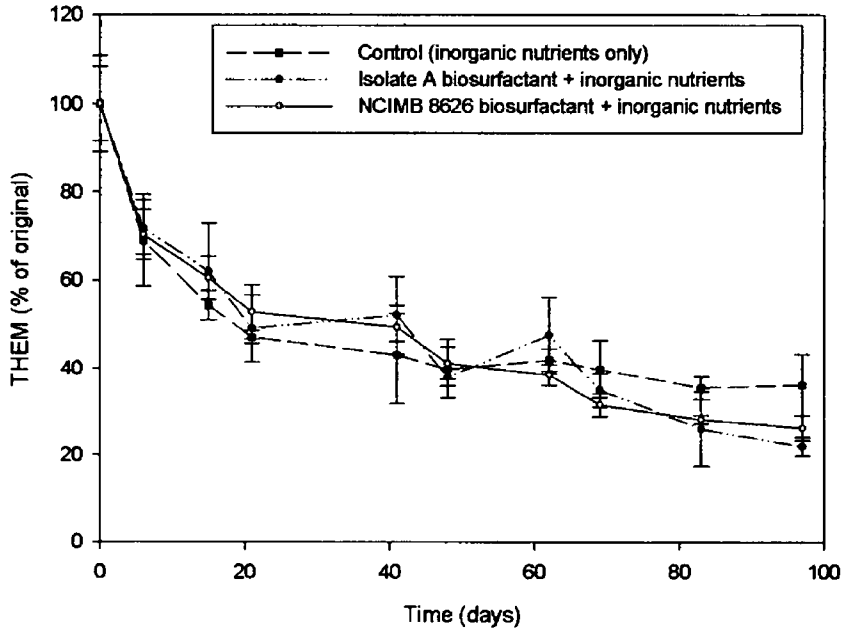


Figure 4.20. Effect of biosurfactants plus inorganic nutrients on removal of THEM from a sandy soil freshly contaminated with diesel. Error bars represent ± 1 standard deviation (n = 4).

In order to determine whether specific components of the diesel had preferentially been removed under these conditions, qualitative analysis of the THEM was performed by GC. The results indicated that by the end of the experimental period all the large resovable peaks (i.e. straight chain *n*-alkanes) had been removed under all conditions and to the same extent, whereas isoprenoid alkanes, such as pristane and phytane were still detectable. The UCM had become more prominent by the day 98 as a consequence of *n*-alkane removal (See appendix 4).

Semi qualitative analysis of *n*-alkanes was carried out by calculating the ratio of *n*-alkanes to pristane. The trends for a selection of *n*-alkanes to pristane ratios are shown in Figure 4.21 where it can be seen that there were no significant difference between the rates of removal in biosurfactant treated and untreated systems, with all the *n*-alkanes being degraded within 60 days. Also Figure 4.21 shows that the ratio of phytane to pristane did not change through out the experiment indicating that phytane was not degraded under these conditions. In this experiment only *n*-alkanes and phytane were semi quantified as no internal standard was used. Visual comparison of the gas chromatographs on day 98 showed there were no significant differences in the gas chromatographic fingerprints from any of the treatments (See appendix 4). Each sample, and the control, still contained pristane and phytane as well as a prominent UCM. Figure 4.22 represents a typical chromatographic fingerprint of the extracted material on day 98. It can be concluded that the addition of biosurfactant to the diesel contaminated soil did not significantly enhance the removal of any of the selected individual components of diesel and over the experimental period tested did not enhance the overall THEM removal compared to amendment with inorganic nutrient only.

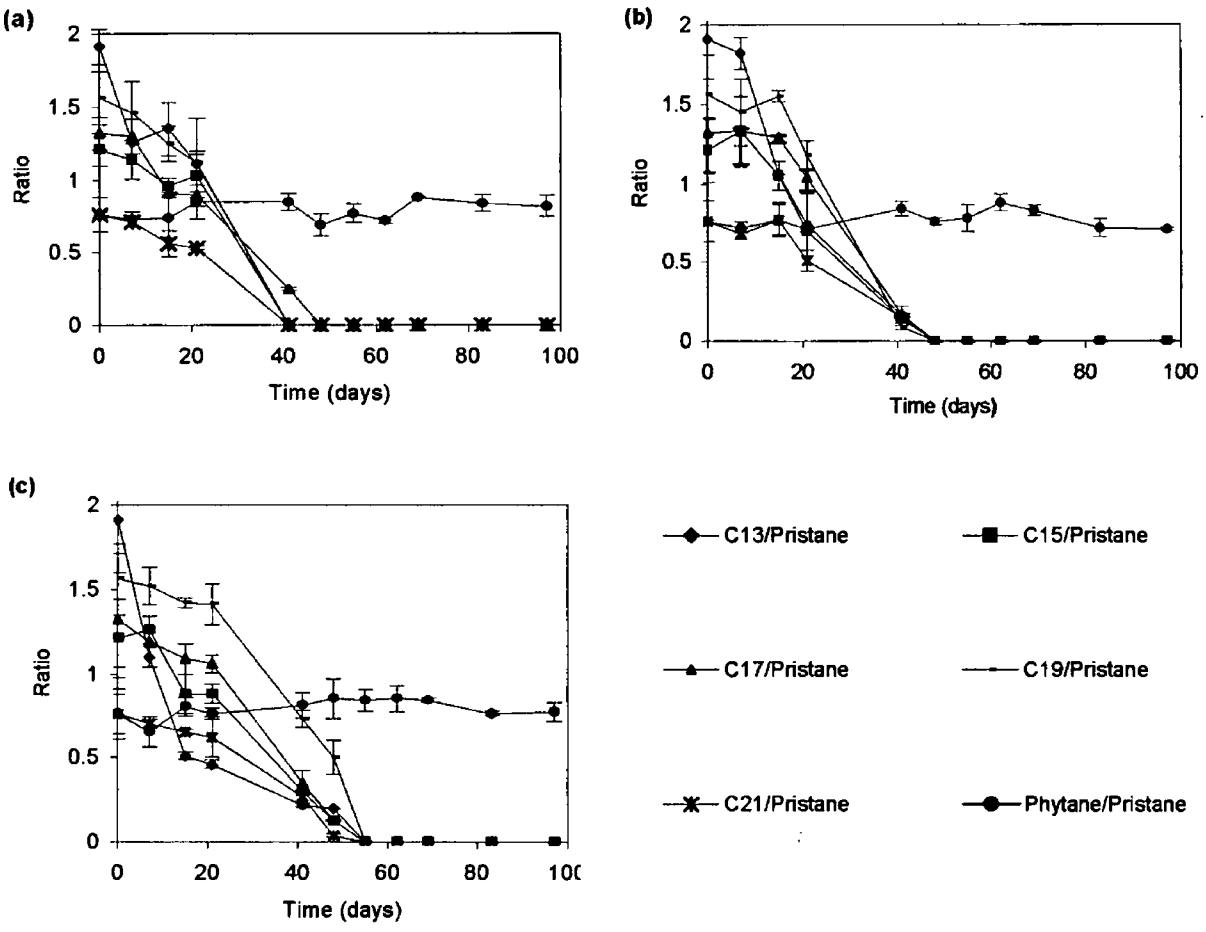


Figure 4.21. Ratios of selected *n*-alkanes to pristane in (a) inorganic nutrient amended, (b) inorganic nutrient amendment + Isolate A biosurfactant, (c) inorganic nutrient amendment + *Pseudomonas aeruginosa* NCIMB 8626 biosurfactant. Error bars represent ± 1 standard deviation (n = 4).

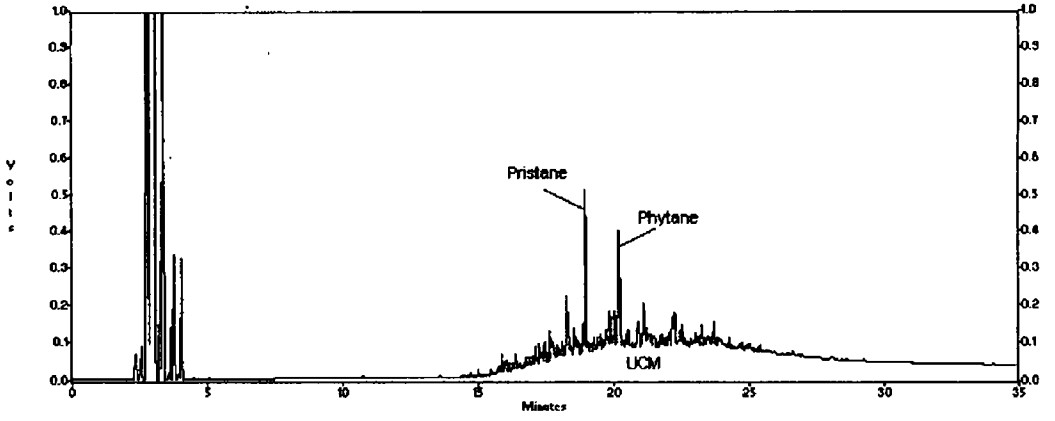


Figure 4.22. Chromatographic fingerprint of residual diesel extracted from soil after 98 days treatment with biosurfactants in the presence of nutrients. This chromatograph represents a typical chromatographic fingerprint of residual diesel after treatment with either, inorganic nutrients only, inorganic nutrients + Isolate A biosurfactant, or inorganic nutrient + *Pseudomonas aeruginosa* NCIMB 8626 biosurfactant (for other chromatographic fingerprints see Appendix 4)

At the same time THEM analysis was being carried out changes in microbial populations were monitored by recovering total mesophilic bacteria (TMB) at 22°C. The patterns of TMB through out the experimental period are presented in Figure 4.23. In the initial stages i.e. within 7 days of the start, a rapid increase in TMB was observed in both the control and in the biosurfactant amended soils. The CFU/g soil (dry weight) increased from an average of $1.7x10^5$ to $4.6x10^8$. After this initial rapid increase, the numbers remained elevated up until day 41. However, after day 41 the numbers started to decline and continued to decline until the end of the experiment at day 98. Matching the pattern of microbial populations with the presence of *n*-alkanes, indicated that this decline in microorganism numbers coincided with the complete disappearance of *n*-alkanes. This suggested that once the *n*-alkanes were removed the majority of the indigenous microbial population did not have the ability to metabolize the remaining more recalcitrant compounds, such as the isoprenoids and the compounds present in the UCM and, consequently they died off, lowering the total microbial population.

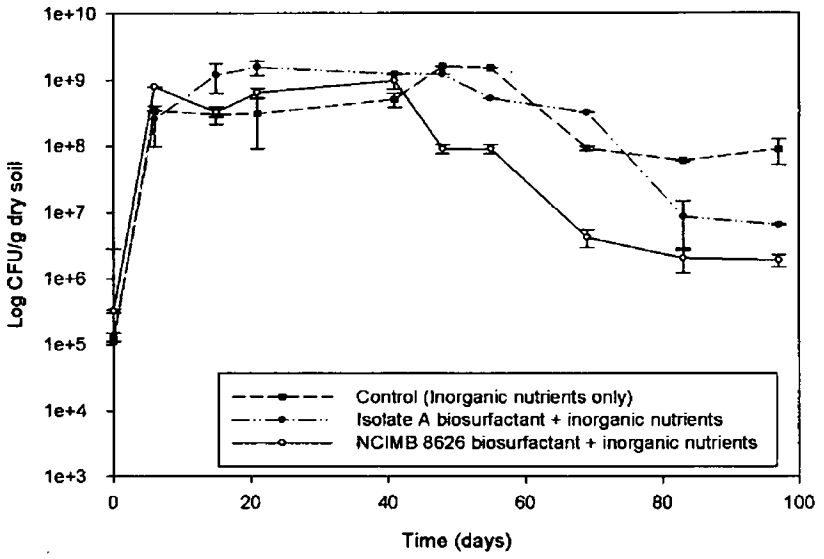


Figure 4.23. Trends in total mesophilic bacteria in freshly contaminated diesel soil amended with biosurfactant plus inorganic nutrients. Error bars represent ± 1 standard deviation (n = 4).

It was postulated that once the readily biodegradable compounds had disappeared that the presence of biosurfactants would increase the bioavailability of the remaining compounds and in-turn enhance their biodegradation, as it had been suggested by Bregnard *et al.* (1996 and 1998) that the initial enhanced degradation rates of hydrocarbons often decline despite a sufficient supply of oxidants and nutrients due to reduced bioavailability of the remaining fraction. However, these results indicated that the application of biosurfactants produced by Isolate A and *Pseudomonas aeruginosa* NCIMB 8626 had only a slight enhanced effect on the removal of THEM, with their presence having no significant effect on the removal of individual components within the diesel sample.

To date limited work has being carried out on the amendment of diesel contaminated soil with biosurfactants, with only some data available on the effects of synthetic surfactant amendment to diesel contaminated soil. Bregnard *et al.* (1998) demonstrated that biodegradation of weathered diesel fuel under denitrifying conditions was not stimulated by the addition of a rhamnolipid biosurfactant and Triton X-100. They found

that after 138 days of incubation at 25°C between 80 and 95% of the original diesel was still recovered from aquifer material. Margesin and Schinner, (1999) found that, in freshly contaminated diesel soil, biodegradation by the indigenous microorganisms was inhibited at all concentrations of sodium dodecyl sulfate concentrations tested (50 – 1,000mg/L). Some work has been carried out on the effects of surfactants on soils contaminated with other hydrocarbons i.e. Providenti *et al.* (1995) reported that a biosurfactant produced by *Psuedomonas aeruginosa* UG2 inhibited the degradation of phenanthrene in sandy loam and silt loam soil slurries. Holden *et al.* (2002) found that in sand the *in-situ* production of surface-active compounds by *Pseudomonas aeruginosa* did not enhance the biodegradation of hexadecane. In relation to polycyclic aromatic hydrocarbons (PAH) contaminants, Deschenes *et al.* (1996) studied the effect of biosurfactant produced by *Pseudomonas aeruginosa* UG2 at three concentrations and observed no enhanced removal of 13 EPA priority PAHs in a creosote-contaminated soil.

In this study it was found that biosurfactants added to the soil were degraded quite rapidly which lead to the conclusion that the lack of a significant enhancement of diesel bioremediation may have been due to the effectiveness of the biosurfactant being lost as a result of their biodegradability. This phenomenon has also been observed by several other authors (Deschenes *et al.*, 1996; Bregnard *et al.*, 1998; Riser-Roberts, 1998; Margesin and Schinner, 1999; and Vipulananda and Ren, 2000). Bregnard *et al.* (1998) even found that the preferential degradation of biosurfactants actually inhibited diesel biodegradation, an observation not noted in this study.

One of the major problems associated with the use of surfactants in bioremediation processes is that, on the one hand, surfactants need to be biodegradable in order that they do not persist in the environment after the target contaminant is removed, whilst on the other hand, like in this study, if the surfactants are too biodegradable their effectiveness can be lost or they may be utilized preferentially to the target contaminant thereby inhibiting the biodegradation process.

Results of this study and other studies indicate that the potential of using biosurfactants to effect enhanced bioremediation may have limited application. However they may find applications in areas such as soil washing and enhanced petroleum recovery, for example..

4.2.4 Effect of organic matter amendment on diesel bioremediation

As mentioned previously, various organic materials including, chopped bermudagrass hay, sawdust (Rhykerd *et al.*, 1999), bark chips (Jorgensen *et al.*, 2000), poultry litter (Williams *et al.*, 1999, and Rahman *et al.*, 2002), coir pith, (Rahman *et al.*, 2002), sewage sludge, compost (Hupe *et al.*, 1996, and Namkoong *et al.*, 2002), wheat bran (Vasudevan and Rajaram, 2001), corn-slash (Piehler *et al.*, 1999) and protein hydrolysate (Harrison *et al.*, 2000) have been investigated as possible amendments used in the bioremediation of hydrocarbon contaminated soils. Some of these organics have been added as bulking agents to condition soils, whilst others have been added as co-substrates and sources of nutrients, organic carbon and microorganisms. The degree of success of these amendments has varied considerably and greatly depends on the type and concentration of amendment and also on the type of hydrocarbon to be degraded.

In this study two organic materials, which to the best of the authors knowledge have not been used previously as amendments for hydrocarbon contaminated soils, were investigated for their ability to enhance the bioremediation of diesel contaminated sandy soil. The two organics used were, dried molassed sugar beet pulp (DMSBP) and spent brewery grain (SBG).

SBG is a by-product of beer brewing. Each year large quantities of the material are produced world wide, and in Guinness Ireland approximately 80,000 tonnes of the material is produced annually. Currently the material is used mainly as a low grade animal feed ingredient, however, in more recent years research has being carried out on its possible use as a substrate from mushroom cultivation (Sánchez and Royse, 2001;

and Wang *et al.*, 2001). The physical properties of SBG, such as particle size, volume weight, specific density, porosity, and water-holding capacity, are conducive to its use as a bulking agent. In addition this material is rich in lignocelluloses, protein, fat, fibre and other essential elements such as nitrogen (See Table 3.3 and Appendix 5) which are essential for optimum microbial activity. Another advantage of SBG is that it is available at low or no cost throughout the year not only from large factories but also from a number of local breweries (Wang *et al.*, 2001).

DMSPB, a by-product from the Irish sugar industry and like SBG, this material is coarse in nature and available in large quantities which make it ideal as a bulking agent. DMSBP is also high in sugars, which are thought may act as a carbon co-substrate and enhance diesel bioremediation. The material is also rich in protein, fibre, and the 22 to 30 % molasses (Ryan, 2002) in the material is also high in organic acids, vitamins and minerals (See Table 3.2 and Appendix 6), all of which promote microbial growth.

Throughout this study both organic materials were added to the diesel contaminated soil at an application rate of 5% w/w on a dry weight basis, unless otherwise stated. The organically amended soil was supplemented with the inorganic nutrients, NH_4NO_3 and KH_2PO_4, and maintained throughout the experimental period as described in Section 3.8.3. A control containing only inorganic nutrients was included in order to compare the effectiveness of the organic material addition. The microcosm (Figure 3.2) was maintained at room temperature throughput the experiment and was manually aerated on a weekly basis.

4.2.4.1 Bioremediation of DRO in organically amended soils

As can be seen from Figure 4.24, the two amendments significantly ($P<0.05$) enhanced the removal of DRO in comparison to the control. Degradation of DRO was affected by the type of organic material used. However, the general trend of DRO removal in both organically amended microcosms followed a similar pattern. A rapid decrease in DRO

was observed in the early stages of the experiment, i.e. within 40 days. Following this decrease, the residual DRO was degraded at a much slower rate. This pattern of high degradation initially followed by a much slower rate is typical of first order degradation, and is the characteristic pattern found in the literature in relation to petroleum bioremediation studies (Rosenberg *et al.*, 1992; Hupe *et al.*, 1996; Venosa *et al.*, 1996; Demque *et al.*, 1997; Harrison *et al.*, 2000 and Jorgensen *et al.*, 2000).

A comparison of the soils amended with the two organic test compounds, showed that by the fourteenth day of the experiment a statistically significant difference ($P<0.05$) in the degradation of DRO was observed in the presence of SBG, with a 20% enhancement in DRO removal compared to DMSBP. From day fourteen to the end of the experimental period, day 108, SBG continued to have a significantly enhanced ($P<0.05$) effect on the biodegradation of DRO compared to DMSBP. On the final day SBG had enhanced the removal of DRO by 17% in comparison to DMSBP.

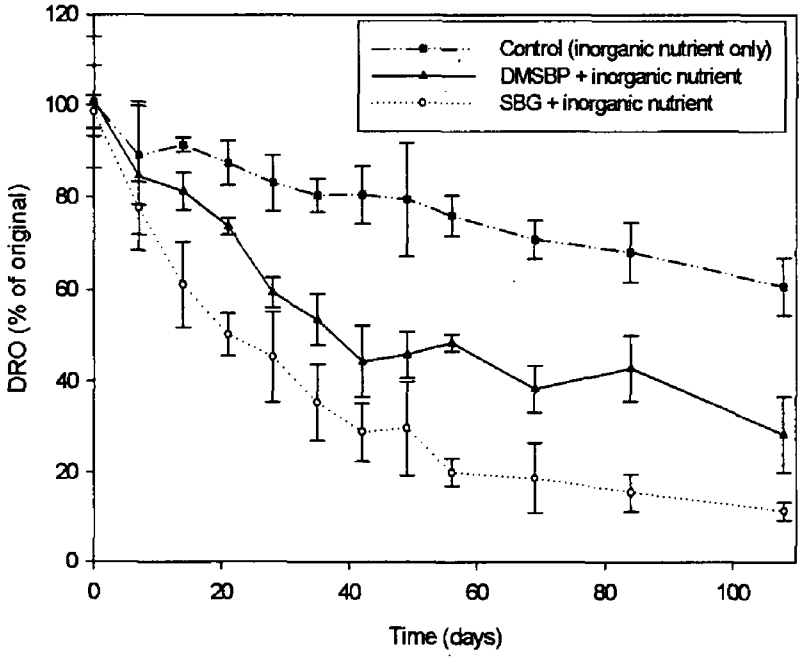


Figure 4.24. Degradation patterns of DRO in diesel contaminated soil amended with DMSBP and SBG compared to a control. All soils were also amended with inorganic nutrients. Error bars represent ± 1 standard deviation (n = 4).

The initial concentration of DROs in the freshly contaminated soils was 45,345, 43,286, 48,756 mg/kg (dry weight basis) for the control, DMSBP and SBG amended soils, respectively. After 108 days of incubation, DRO concentrations were reduced to 26,754 mg/kg, 12,360 mg/kg and 5,363 mg/kg for the control, DMSBP and SBG amended soils, respectively. These represented a 41, 72 and 89% degradation of DRO over the test period, respectively. At the end of the 108 day test period DMSBP and SBG had enhanced the overall removal of diesel by 31 and 47 % compared to the control which had received inorganic nutrients only. This indicated that the addition of either DMSBP or SBG significantly enhanced the removal of diesel from the sandy soil.

As shown previously, abiotic processes can contribute to the overall removal of diesel from soil. Abiotic processes result in the lower molecular weight, highly volatile compounds being removed first followed by the less volatile compounds. It is possible that such processes may have contributed to some of the DRO removal in this experiment. However, examination of the chromatographic fingerprints (Figure 4.26-4.28) illustrated that in the early stages of the experiment not only the low molecular weight, highly volatile compounds were removed but also the higher molecular weight, less volatile compound were removed. This demonstrated that bioremediation of the diesel is the more dominant of the processes and it was therefore concluded that the contribution of abiotic process towards the removal of diesel under these conditions was insignificant.

The two standard kinetic models, zero and first order, were considered for the purpose of evaluating the removal of the different fractions of the diesel. Graphical evaluation using both models is presented in Figure 4.25. The UCM from SBG amended soil was used to represent degradation patterns from experiments because similar patterns were observed in all experiments for the different diesel fractions. As can be seen in Figure 4.25 the regression analysis of the data shows that the first order model describes the data best.

The correlation coefficients of both models are presented in Table 4.6. Both models had high correlation (Clark, 1994) with first order degradation having higher correlation coefficients than the zero order model. Therefore, the first order kinetic model was used to discuss the degradation of the different fractions of diesel.

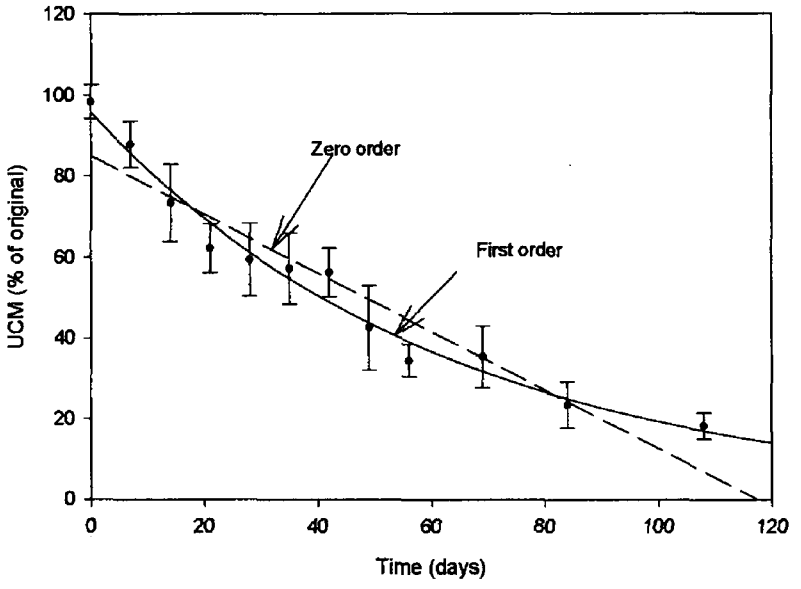


Figure 4.25. Curve fits based on zero and first order models for the UCM in SBG amended diesel contaminated soil. Error bars represent ± 1 standard deviation (n = 4).

The results obtained, applying first order degradation rate constants, showed that DMSBP (k= 0.011/day) was almost three times more effective at removing DRO than the control (k= 0.004/day), while SBG (k= 0.018/day) was approximately 4.5 times more efficient. These increases in degradation rates reduced the half life of DRO from 173 days in the control to 63 and 38.5 days for DMSBP and SBG, respectively.

Table 4.6. Estimation of removal and degradation kinetic parameters based on the zero order and first order kinetic models, in diesel contaminated soil amended and unamended with organic SBG and DMSBP.

Amendments	*Diesel fraction*	*Zero order*	*First order*		
		r^c	k^d	r^c	½ life
Nutrient only	DRO[a]	0.94	0.004	0.96	173
	n-alkanes[b]	0.91	0.011	0.91	40
	UCM	0.93	0.004	0.94	173
	Pristane	0.94	0.001	0.95	533
Nutrient + DMSBP	DRO	0.81	0.011	0.94	63
	n-alkanes	0.99	0.067	0.93	8.5
	UCM	0.89	0.008	0.95	86.6
	Pristane	0.88	0.015	0.95	49.5
Nutrient + SBG	DRO	0.78	0.018	0.95	38.5
	n-alkanes	0.98	0.120	0.90	5.13
	UCM	0.90	0.016	0.98	43.3
	Pristane	0.78	0.035	0.96	19.8

a) Total area resolved from *n*-C9 to *n*-C25.
b) Sum of individual n-alkane ranging from *n*-C9 to *n*-C25.
c) r = correlation coefficient.
d) k = the first order kinetic constant.

These results for the degradation of DRO are comparable to, if not better, than those reported in the literature. In a study by Jorgensen *et al.* (2000), the total amount of diesel in bark chip amended soils decreased by 71% from 700 to 200 mg(kg dry w)$^{-1}$ over a 150 day period. Namkoong *et al.* (2002) reported that the addition of two organic materials, sewage sludge and compost, enhanced the removal of diesel from a sandy loam soil almost three times that of the unamended soil, with TPH first order degradation rate constants increasing from 0.037/day in unamended soil to 0.124/day after organic amendment. They found that the effectiveness of the amendments was dependant on the ratio of organic material to soil and that increasing the organic

concentration did not necessarily increase the rate of hydrocarbon degradation. The first order degradation rates in the report by Namkoong *et al.* (2002) were significantly higher than those reported here, but upon examination of the experimental procedures, the types of soils used were different to those in this study, and the ratio of amendment was much higher (10 to 50% amendment as wet weight basis compared to the 5% dry used in this study). Also, Namkoong *et al.* (2002) started with an initial diesel concentration of 10,000mg/kg compared to an initial diesel concentration of approximately 50,000mg/kg used in this study. So, proportionally the amount of diesel removal was significantly higher in this study due to the elevated initial concentration. Furthermore, the application rate of the organic amendments was significantly lower in this study which would be advantageous in cutting overall cost.

Other authors to report enhanced degradation of hydrocarbons in the presence of organic amendments include, Hupe *et al.* (1996) who reported that the amendment of a sandy loam with organic kitchen and garden waste increased the removal of lubricating oil by 40% over a 162 day test period. Harrison *et al.* (2000) reported that the amount of diesel removed from a sandy gley soil was 21% greater with the addition of protein hydrolysate, when compared to an inorganic nutrient amended control. Whilst Rhykerd *et al.* (1999) reported that over a 210 day experiment the addition of two organics, hay and sawdust, enhanced bioremediation of an oil contaminated sandy soil from 77 % in inorganic nutrient amended to 90% in the organically amended soil.

The results of this investigation into the effect of different organic material amendments on remediation of diesel contaminated soil showed that they acted to enhance degradation of diesel components compared to controls. This suggests that the organic material tested did not act as a competing energy source for the degrading microorganisms. This is in contrast to other authors who reported that certain organic materials were preferentially degraded over the target compounds (Thomas *et al.* 1992, LaGrega *et al.*, 1994, and Cookson, 1995).

Visual examination of the gas chromatographic fingerprints of the DRO at different time periods, clearly demonstrated that the two organic materials had a significant affect on the degradation of diesel compared to the control (See Figures 4.26 to 4.28). It can be seen that the pattern of eluted peaks changed significantly with time, with the disappearance of the large resolvable peaks, leaving behind the unresolvable fraction referred to as the "hump" or UCM. Quantitative analysis of different fractions within the diesel samples was carried out and their trends in the different treatments are discussed below.

4.2.4.2 Bioremediation of n-alkane in organically amended soils

The removal of *n*-alkanes in the diesel contaminated soils amended with the DMSBP and SBG are presented in Figure 4.29. The results show that in the two organic amended soils, *n*-alkanes were degraded more rapidly than DRO (Figure 4.24). The *n*-alkanes in the DMSBP amended soil were totally degraded after 70 days of the experiment, while the presence of SBG significantly ($P<0.05$) decreased this time, with the *n*-alkanes being completely degraded in less than 40 days. The first order degradation rate constant for total *n*-alkanes was significantly greater than those for DRO regardless of the type of amendment applied (see Table 4.6). This signifies that *n*-alkanes are preferentially degraded compared to other compounds in DRO, a phenomenon reported by numerous authors (Frankenberger *et al.*, 1991; Thomas *et al.*, 1992; Huesemann 1994; Marquez-Rocha *et al.*, 2001 and Namkoong *et al.*, 2002).

A plot of the first order degradation rate constants of *n*-alkanes against the first order degradation rate constants of DRO is shown in Figure 4.30. Linear regression analysis indicated that there was a significant correlation between the two ($r = 0.9997$), with the degradation rate of *n*-alkanes being approximately six times greater than that of DRO. The only other quantitative data, available in the literature, on the preferential degradation of *n*-alkanes was in a report by Namkoong *et al.* (2002). They found that in

a diesel contaminated sandy loam, the degradation rate of *n*-alkanes, ranging from *n*-C_{10}-*n*-C_{20}, was about twice that of TPH. These results suggest that the indigenous microbial population in the sandy soil used in this study had a greater preference towards *n*-alkanes than the indigenous population in the sandy loam soil used by Namkoong *et al.* (2002).

The two organic amendments used in this study significantly decreased the half life of *n*-alkanes compared to the control. In the soil amended with DMSBP the half life of *n*-alkanes was reduced from 40 days in the control to 8.5 days in the amended soil. The amendment of the diesel contaminated soil with SBG reduced this time significantly more to a value of 5.13 days (Table 4.6). These results indicate that addition of the two organic materials significantly enhanced the removal of *n*-alkanes with SBG being considerably better than DMSBP.

This preferential degradation of the *n*-alkanes is the main reason for the DRO degradation pattern of rapid decrease in the initial phases followed by a slower degradation rate. The initial swift degradation of DRO is due essentially to the rapid degradation of these readily biodegradable *n*-alkanes, and once these are removed the degradation rate decreases as the remaining compounds are more recalcitrant to microbial degradation.

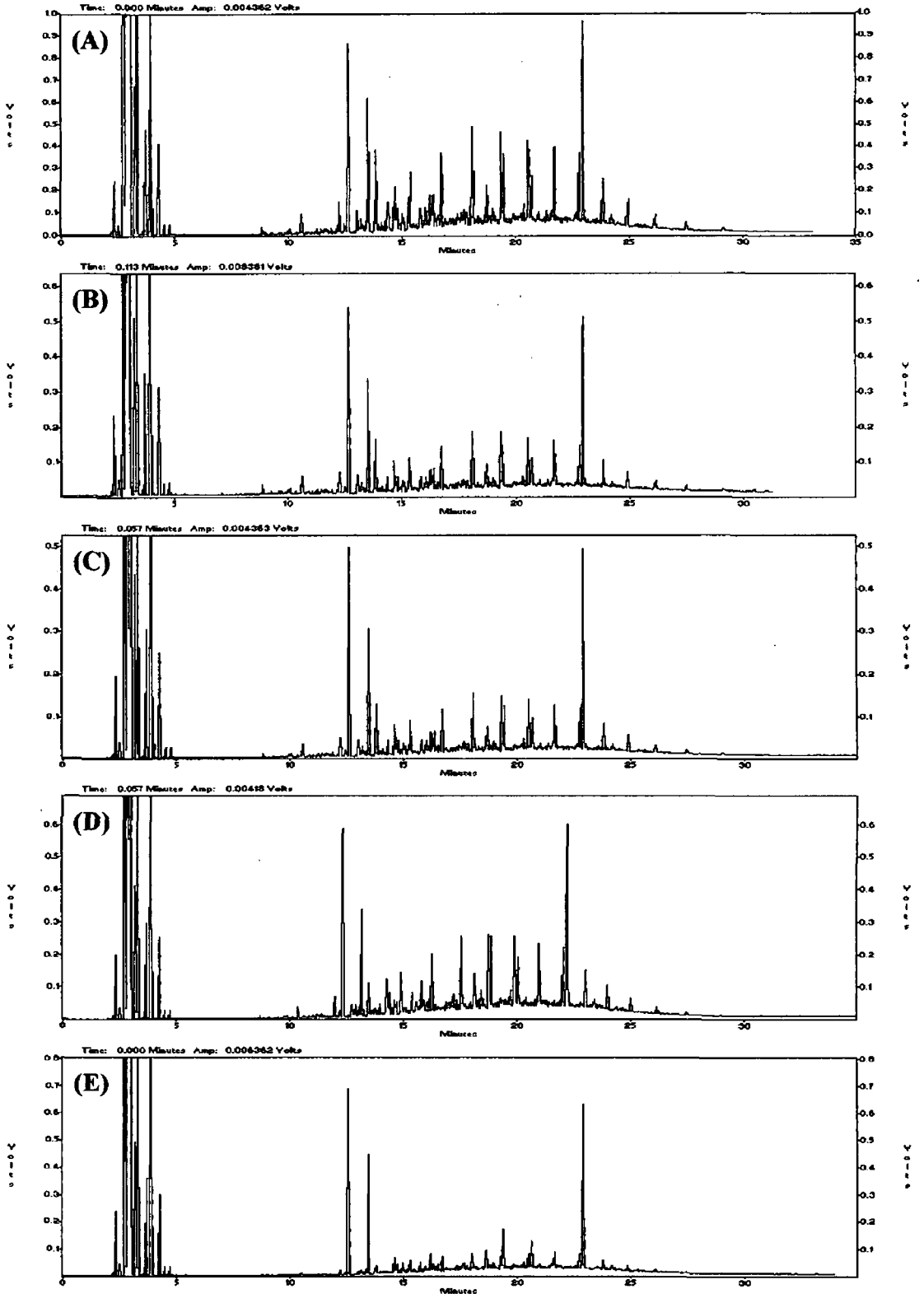


Figure 4.26. Chromatographic fingerprints of THEM from diesel contaminated soil amended with inorganic nutrients. (A) Day 0; (B) Day 11; (C) Day 25; (D) Day 53; (E) Day 105.

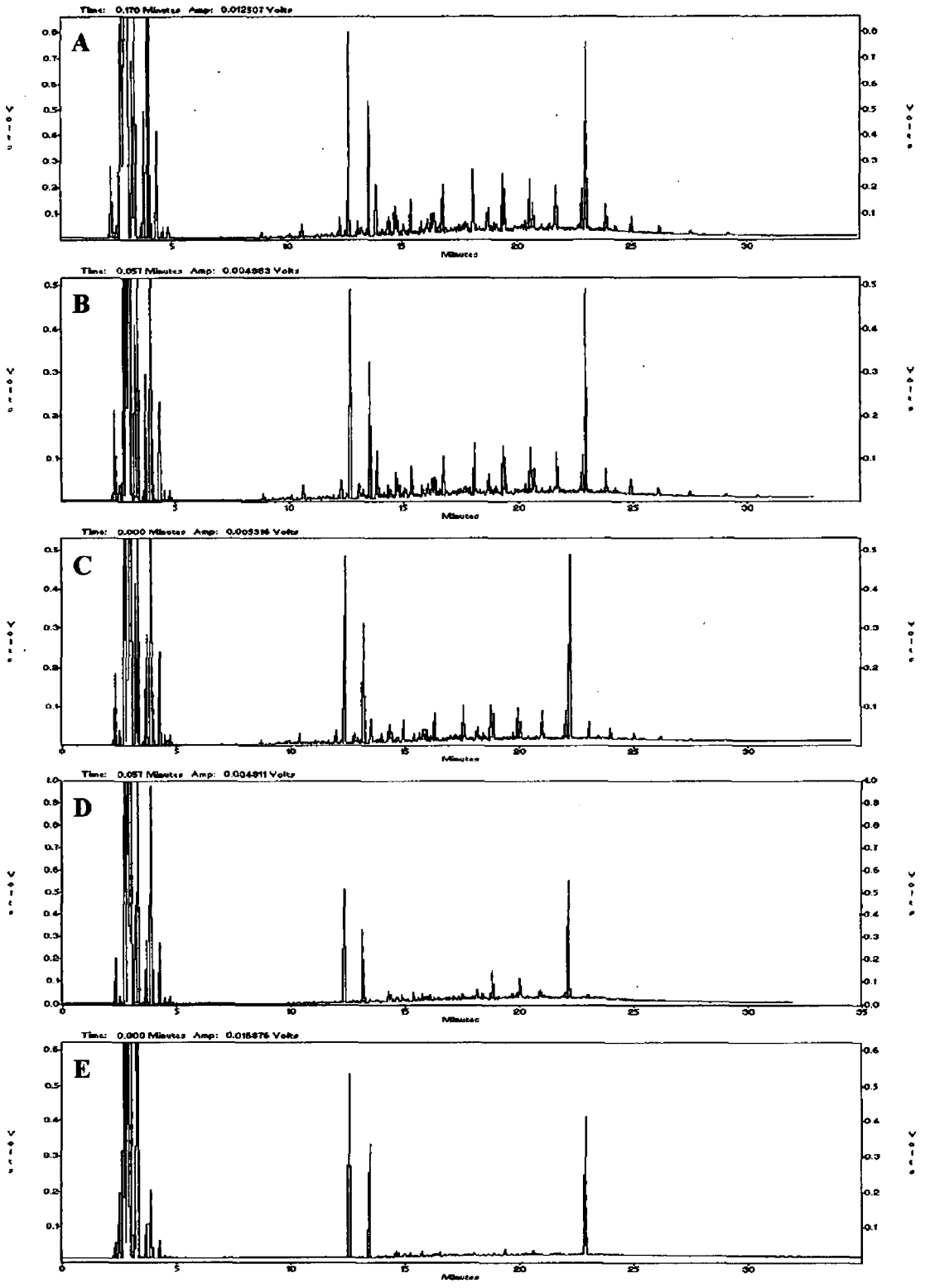


Figure 4.27. Chromatographic fingerprints of THEM from diesel contaminated soil amended with dried molassed sugar beet pulp and inorganic nutrients. (A) Day 0; (B) Day 11; (C) Day 25; (D) Day 53; (E) Day 105.

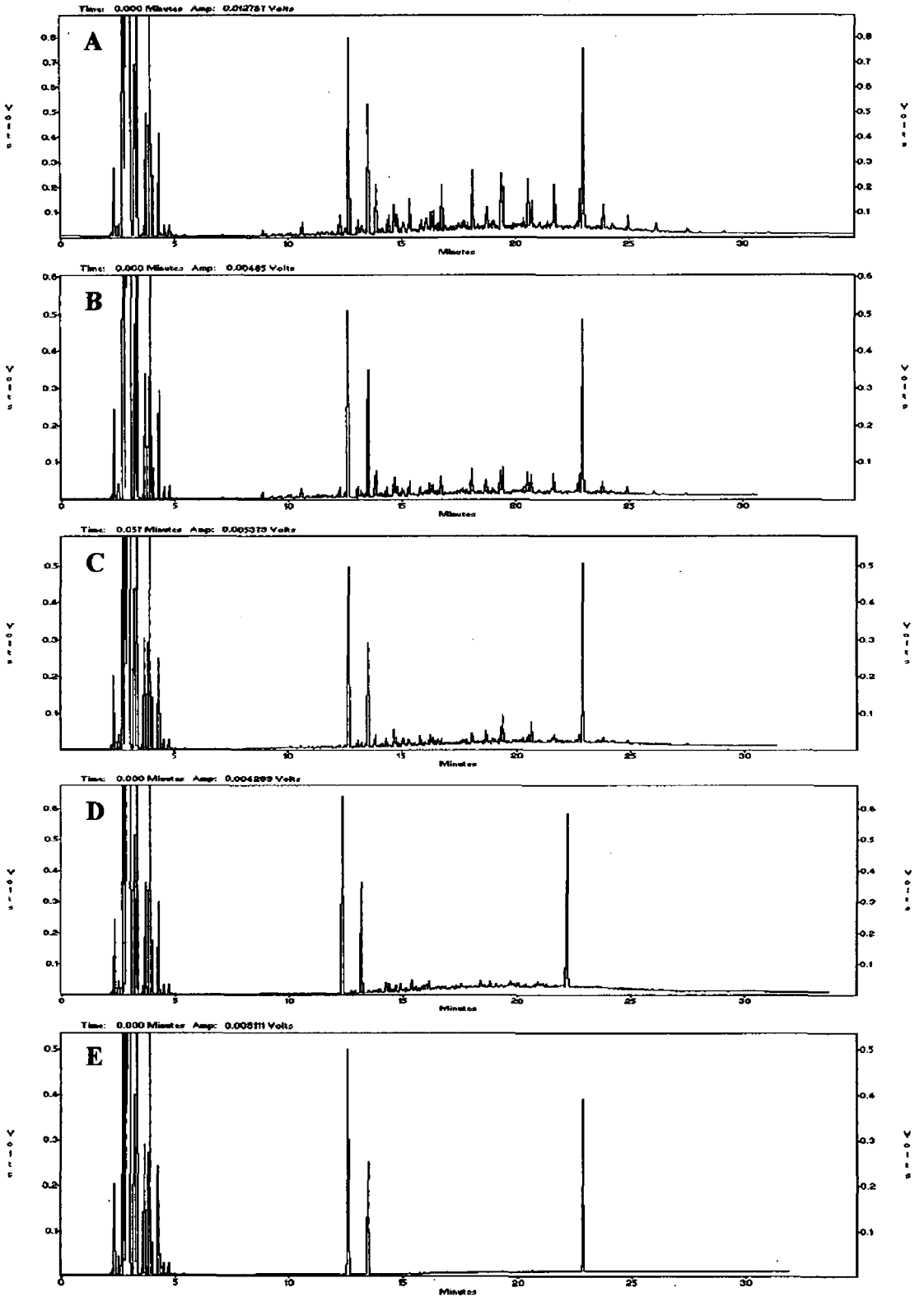


Figure 4.28. Chromatographic fingerprints of THEM from diesel contaminated soil amended with spent brewery grain and inorganic nutrients. (A) Day 0; (B) Day 11; (C) Day 25; (D) Day 53; (E) Day 105.

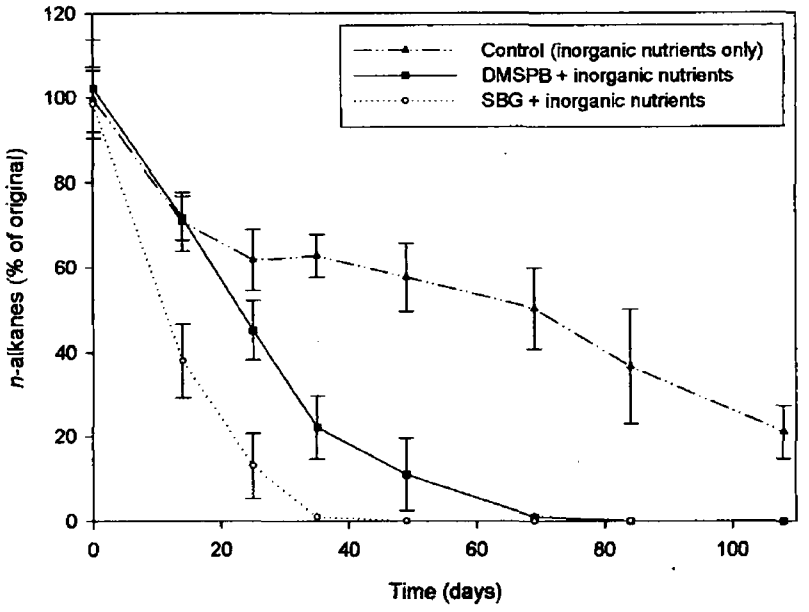


Figure 4.29. Degradation patterns of total *n*-alkanes in diesel contaminated soil amended with DMSBP and SBG compared to a control. All soils were also amended with inorganic nutrients. Error bars represent ± 1 standard deviation (n = 4).

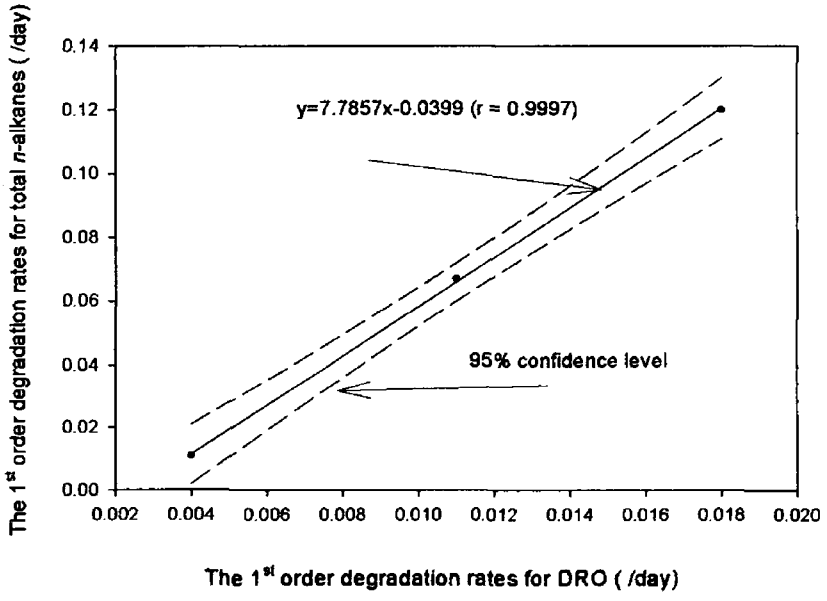


Figure 4.30. Relationship between the first order degradation rate constants of DRO and *n*-alkanes in diesel contaminated soil amended with DMSPB, SBG and inorganic nutrients.

4.2.4.3 Bioremediation of pristane in organically amended soil

Two compounds which are commonly identified in partially biologically weathered petroleum products are pristane and phytane. These two compounds are isoprenoid alkanes which are probably derived from the thermal degradation of tocopherols and/or from the catagenic decomposition of methlytrimethyltridecylchromans (Bregnard *et al.*, 1997). The biodegradation of pristane is of particular interest as up until quite recently this compound was thought to be nonbiodegradable and was used as an inert biomarker in oil degradation studies (Pritchard and Costa, 1991; and Atlas and Bartha 1992). Ratios of n-C_{18}/phytane and n-C_{17}/pristane have traditionally been used, and in some cases are still being used, as weathering indicators of biodegradation (Butler *et al.*, 1991; and Santas *et al.*, 1999).

The removal rate of pristane in the organically amended diesel contaminated soil is presented in Figure 4.31. The graph illustrates that in the control there was no significant removal of pristane over the sampling period of 108 days. In comparison, it can be seen that in the soil which received organic treatment, pristane was degraded quite rapidly by the indigenous population.

In soils amended with SBG amendment, a lag phase of 7 days was observed before pristane degradation began, after which it was degraded quite rapidly. By day 69 of the experiment more than 90% of the pristane was removed and by the end of the experiment, day 108, 98% of the pristane was removed in the SBG amended system.

DMSBP amended soils did remove pristane but was not as effective as SBG. A longer lag phase of 28 days was observed and significantly less pristane ($P<0.05$) was removed by the end of the experimental period, 64% removal compared the 98% in the SBG amended soil. Comparison of first order degradation rate constants (see Table 4.6) showed that the application of DMSBP and SBG caused a 15 and 36 fold increase, respectively, in the rate of pristane degradation compared to the control. The half life of

pristane was reduced from 533 days in the control to 49.5 and 19.8 for DMSBP and SBG amended soils.

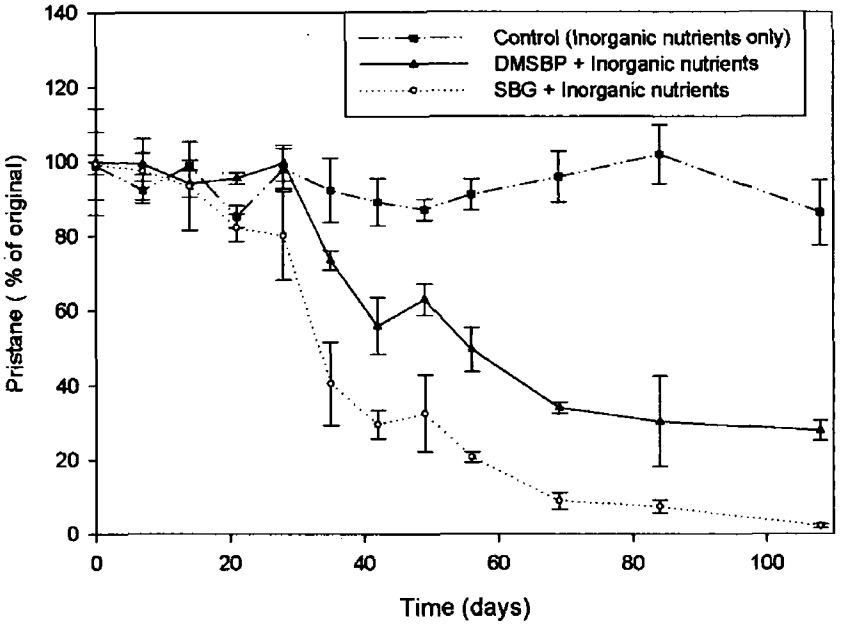


Figure 4.31. Degradation patterns of pristane in diesel contaminated soil amended with DMSPB, SBG and inorganic nutrients. Error bars represent ± 1 standard deviation (n = 4).

These results for pristane degradation rates are considerably higher than previously reported in the literature. Bregnard *et al.* (1996) carried out a study on partially weathered diesel contaminated soil in which all the *n*-alkanes had been removed and consisted of isoprenoid alkanes and a UCM. They found that even after addition of nutrients and incubation for 440 days, traces of pristane and phytane still remained. Brown *et al.* (1998) discovered that after 110 days of degradation of weathered petroleum compounds amended with organic material (wood products) the isoprenoids pristane and phytane had nearly disappeared but were still detectable.

Venosa *et al.* (1996) reported that the first order degradation rates for pristane in an unamended diesel contaminated sandy soil was approximately 0.01/day and was increased to 0.02/day through the addition of nutrients. The first order degradation rate constant of pristane in the SBG experiment (k=0.036/day) was significantly higher compared to the results of Venosa *et al.* (1996), which means that the addition of SBG was very effective at enhancing the bioremediation of pristane in a diesel contaminated soil.

As mentioned earlier, up until recently and even today the ratios of pristane to *n*-alkanes is used as an indicator of biodegradation effectiveness (Seklemova *et al.*, 2001). However, more recently, Atlas and Cernigila (1995) reported during the Exxon Valdez oil spill, pristane and phytane were found to be degraded quite rapidly and could only be used as an internal marker for a few weeks to months. This study demonstrated that pristane can no longer be regarded as a recalcitrant biomarker and that its ratio to *n*-alkanes can only be used as a biodegradation weathering indicator in the very early stages of a process, as later readings may substantially underestimate the extent of biodegradation. Other authors to confirm that pristane and phytane are inadequate biomarkers when conditions favour bioremediation are Ko and Lebeault (1999) who reported that a pure bacterial strain, *Rhodococcus equi* P 1, was capable of degrading 10 g/L pristane within 24 hours incubation in liquid culture at 35°C. Bej *et al.* (2000) also found that a *Rhodococcus* species isolated from the Antarctica was able to utilize pristane quite readily.

Even in anaerobic systems, care should be taken in using *n*-alkane:pristane ratios as Bregnard *et al.* (1997) demonstrated that, in a liquid system, within 102 days, more than 90% of added pristane was degraded by enrichment cultures under anaerobic conditions.

4.2.4.4 Bioremediation of UCM in organically amended soil

Most research on the degradation of diesel and other petroleum products has concentrated on those compounds that are resolvable by high performance liquid chromatography and gas chromatography, even though the unresolved complex mixture (UCM) clearly dominates gas chromatographs of weathered petroleum (Gough and Rowland, 1990; Killops and Al-Juboori, 1990; and Rowland *et al.*, 2001). In fact, as a group, UCM are more abundant than the resolved hydrocarbons, even in unweathered oils, though this is seldom realized due to normalization of most chromatographs on the resolved peaks (Revill *et al.*, 1992).

Should clean up goals be defined in terms of concentration or even percentage removal of contamination, removal of the UCM may be critical to achieving desired clean up objectives (McGowan, 1999). Also the removal of UCM from soil or water systems is important, as a number of components of UCMs have been reported to have toxic effects. Rowland *et al.* (2001) demonstrated that the monoaromatic components of a UCM of hydrocarbons from a crude oil elicit a sub-lethal toxic response in a typical marine pollution indicator organism (the mussel, *Mytilus edulis)*. Ehrhardt and Petrick (1984) and Ehrhardt and Burns (1990) reported that the oxidation of the aromatic components of UCM increased their toxicity with the formation of carboxylic acids, aromatic ketones and alcohols. The aliphatic UCM hydrocarbons had no toxic effect on the feeding rates of mussels, whilst oxidation resulted in an increase in toxicity (Thomas *et al.*, 1995).

In this study the UCM made up 65% of the fresh diesel sample. During the bioremediation experiments the concentration of UCM decreased with time. The trend of UCM removal in relation to time is presented in Figure 4.32. The results show that the two organic amendments significantly increased the removal of UCM compared to the control. By day 14 both the organically treated soils had statistically significantly enhanced the removal of UCM ($P<0.05$). This enhancement continued until the end of

the experimental period by which time DMSBP and SBG had enhanced UCM removal from 29% in the control to 72% and 82%, respectively. This represented an increase in UCM removal of 43 and 53% for DMSBP and SBG amended soils, respectively, compared to the control.

First order kinetics modeled the degradation pattern of UCM with high correlation, i.e. r = 0.95 to 0.98 (See Table 4.6). Calculation of first order degradation rate constant, indicated that DMSBP doubled (k= 0.008/day) the rate of UCM removal compared to the control (k = 0.004/day), while SBG (k= 0.016/day) increased the rate of removal by a factor of four. The half life of UCM was decreased dramatically from 173 days in the control to 86.6 and 43.3 days in DMSBP and SBG amended soil, respectively. These results indicate that the amendment of diesel contaminated soil with either DMSPB or SBG significantly enhanced the bioremediation of the UCM of diesel. Of the two organics, SBG significantly ($P<0.05$) enhanced the removal of the UCM compared to DMSBP.

The proportion of DRO occupied by the UCM changed extensively throughout the experimental period. As the treatments progressed the UCM became more pronounced. On day zero the UCM occupied 65% of the DROs, but by the end of the experiment, day 108, the proportion occupied by UCM had increased to 99 % in the SBG amended system, and to 88% in the DMSBP system. These results show that at the end of the experiment the UCM made up the majority of the DRO in the organically treated soils, indicating that in a diesel sample the most recalcitrant fraction are the components within the UCM. This is in agreement with the general consensus which is that the UCM is a mixture of many structurally complex isomers and homologues of branched and cyclic hydrocarbons (Eglinton *et al.*, 1975; Sanders and Tibbetts, 1987; and Thomas *et al.*, 1995) which are relatively inert to microbial degradation (Gough *et al.*, 1992).

In relation to the degradation of the UCM, very little work has been carried out previously. One study by Gough *et al.* (1992) reported that in a liquid culture 17 % of the UCM from commercially available lubricating oil was degraded by *Pseudomonas*

fluorescens after 25 days. McGowan (1999) reported that in a marine spill, the first order degradation rate of UCM in a weathered crude oil increased by 1.33 fold from 0.0021 to 0.0028/day when supplemented with nutrients. Comparison of these degradation rates to those found in this study suggests that addition of the organic materials DMSPB and SBG, would be very effective for the degradation of UCM.

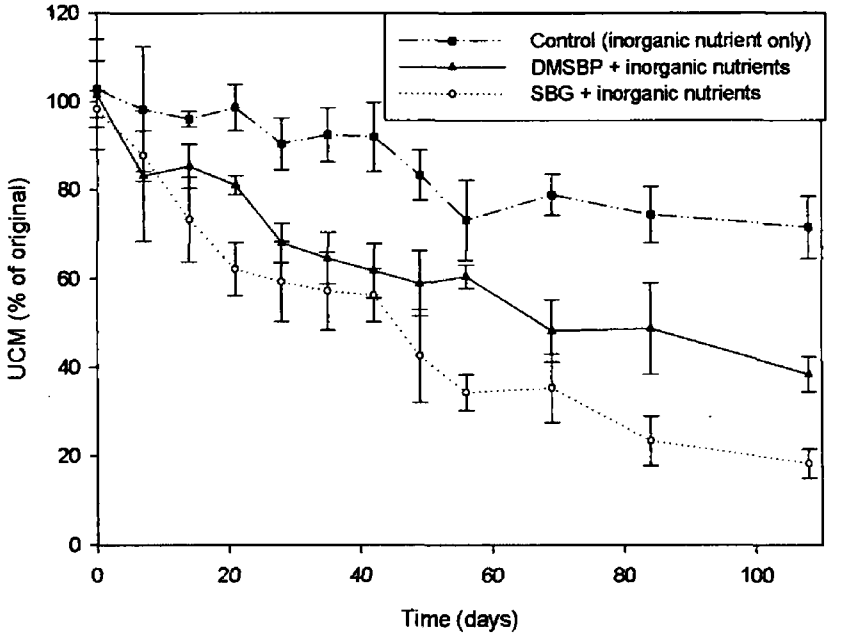


Figure 4.32. Degradation pattern of the UCM in diesel contaminated soil amended with DMSBP, SBG and inorganic nutrients. Error bars represent ± 1 standard deviation (n = 4).

4.2.4.5 Microbiological patterns in organically amended soils

The enhanced removal of the diesel in the different treatments corresponded with increases in all the microbiological parameters measured, and the increase in numbers appeared to mirror closely the removal of diesel from the systems, with a rapid increase in the numbers during the first 7 days of the experiment. Figure 4.33 illustrates the effect

the two organic materials had on the total number of microorganisms present in the soil. The addition of DMSPB increased the total numbers of microorganisms from $1.6x10^5$ CFU/g soil (dry weight basis) to $3.5x10^9$ after 7 days, after which the total number of microorganisms remained fairly constant for the duration of the experiment ranging from $7.6x10^8$ to $4.2x10^9$ CFU/g soil (dry weight basis). A similar pattern was observed in the soil which had been amended with the SBG, a rapid increase occurred over the first 7 days from $1.3x10^5$ to $4.0x10^9$ CFU/g soil (dry weight basis), and again after this initial increase the numbers remained constant through out the experiment. As can be seen from the results, the numbers of total bacteria in the control test were statistically significantly lower ($P<0.05$) than in the organically amended soils. At each sampling point except for the final day the total mesophilic bacteria in the control system were approximately 100 fold less than those in the organically treated soil. On day 108 the total number of bacteria in the DMSBP had dropped to below the control. This result demonstrates that addition of either DMSBP or SBG significantly enhanced ($P<0.05$) the numbers of microorganisms in the diesel contaminated soil. It is hypothesised that this increase in microbial numbers is the main reason for the enhanced bioremediation of the diesel.

This increase in numbers of microorganisms following the application of the two organics was not as a result of the introduction of microorganisms present in the organic material itself, but was due to enhancement of the indigenous soil population. This was confirmed through microbial analysis of the two organic substrates prior to addition. Analysis of the two organic materials prior to their use as amendments showed that they contained very low numbers of microorganisms. The DMSPB contained an average of 21 CFU/g (dry weight basis), while SBG contained an average of 11 CFU/ g (dry weight basis). The reason for such low numbers in both organic materials is that prior to application both materials had been oven dried at 100°C for 24 hours which would have killed off most microorganisms.

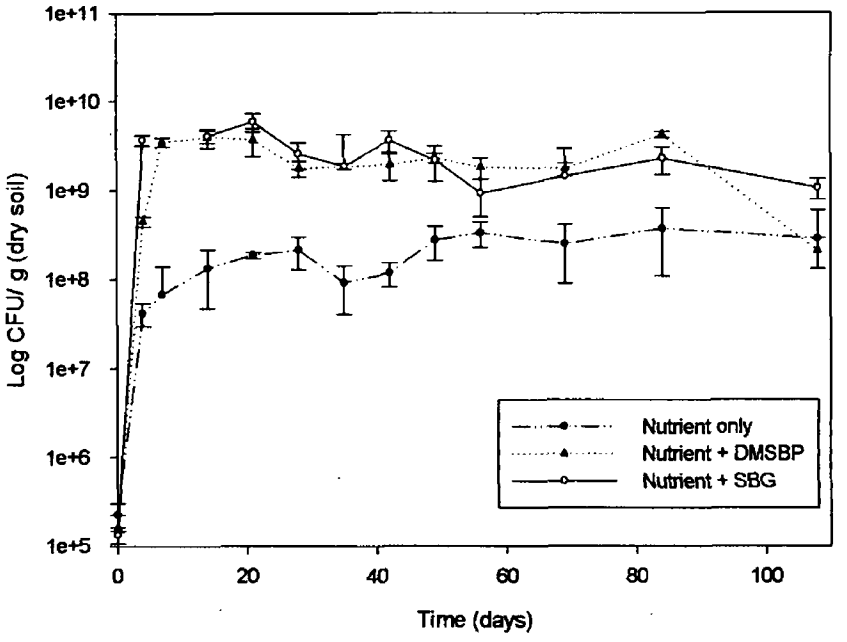


Figure 4.33. Pattern of total mesophilic bacteria in diesel contaminated soil amended with DMSPB, SBG and inorganic nutrients compared to the control. Error bars represent ± 1 standard deviation (n = 4).

Even though the proportion of hydrocarbon degrading microorganisms present was not known, the results showed that by stimulating the growth of the overall microbial population, the numbers of hydrocarbon degrading microorganisms increased concomitantly. Hydrocarbon degrading microbes are capable of using a wide range of substrates, and are not reliant on hydrocarbons as a sole source of carbon and energy. It is believed that the organic amendments acted as an easily metabolisable alternative carbon and energy source for the hydrocarbon degrading microorganisms to use whilst degrading the diesel components by co-metabolism. This phenomenon has also been demonstrated by other authors using different organic materials. Harrison *et al.* (2000) found that in a diesel contaminated soil the application of protein hydrolysate, an easily metabolisable carbon source, increased the number of total heterotrophic microorganisms, which in turn significantly enhanced diesel removal. Guzev *et al.*

(1997) established that the biodegradation of diesel in soil can be controlled by easily metabolisable carbohydrates and that the introduction of glucose in an amount of up to 1mg/kg augmented the population of hydrocarbon-utilizing microorganisms about ten-fold and enhanced the rate of diesel degradation threefold. The same authors reported that the amount of these easily metabolisable carbons added to contaminated soil must be carefully monitored as large amounts may lead to the so-called "substrate-accelerated" death. Also, as mentioned earlier the carbon source in the amendment materials must not present a preferential carbon source that pre-empts degradation of the target contaminant (LaGrega *et al.*, 1994).

Linked to the information about changes in microbial numbers, data was obtained about changes in microbial activity as measured by O_2 consumption. The addition of both organic substrates significantly increased the amount of oxygen consumed by the indigenous microbial populations. Respirometery was used as a means of monitoring O_2 consumption by microbial populations in soils undergoing various treatments. The oxygen uptake by the indigenous microorganisms in the presence of DMSBP + nutrients, DMSBP + nutrients + diesel, and the control which contained diesel + nutrients gave cumulative values of 29,100 ± 2,530, 36,100 ± 3,252 and 4,111 ± 354 mg O_2 consumed per kg of soil, respectively, after 23 days incubation at room temperature (see Figure 4.34). A significant difference ($P<0.05$) between DMSBP amended and unamended soils was first detectable after 12 hours, thereafter, the oxygen consumption curves diverted further until the end of the experiment. After 23 days the amount of oxygen consumed in the soils amended with DMSBP was approximately 9 times higher than in the control with no DMSBP. These results confirm that the addition of DMSBP to the diesel contaminated soil significantly enhanced the numbers and activity of the indigenous microbial population. The only other reports on the use of molasses (a component of DMSBP) in hydrocarbon bioremediation processes are those by Al-Hadhrami *et al.* (1996 and 1997). These authors investigated the effect of cane molasses, which is very similar in composition to beet molasses, addition on the biodegradation of *n*-alkanes by *Pseudomonas aeruginosa*. In a liquid medium they found that molasses

addition stimulated rapid respiration, which was accompanied by substantial *n*-alkane breakdown. After 16 hours they reported that molasses addition had increased the oxidation rates of *Pseudomonas aeruginosa* by a factor of seven in comparison to the control.

In relation to the soil respiration in the SBG amended soils, the results were very similar to those obtained for the DMSBP amended soil. High cumulative oxygen uptake occurred in the SBG amended soil when compared to the soil deficient of SBG. At the end of the 23 day experimental period, SBG caused an approximate eight fold increase in O_2 consumption (see Figure 4.35), resulting in the uptake of 31,660 ± 1,005 mg O_2/kg soil compared to 4,111 ± 354 mg O_2/kg soil in the control. These results imply addition of SBG not only enhanced the number of bacteria in a diesel contaminated soil but also stimulated microbial activity. As similar studies were not found in the literature in relation to SBG amendments of hydrocarbon contaminated soils, no comparisons could be made.

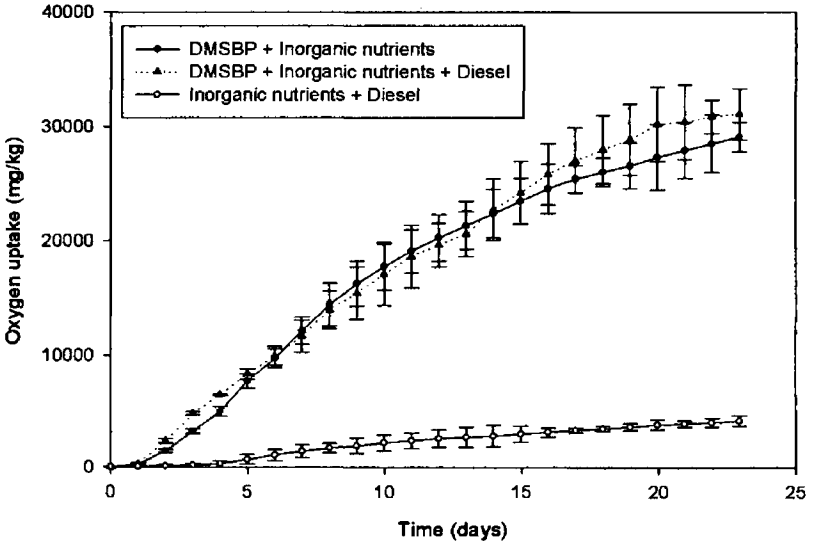


Figure 4.34. Cumulative oxygen uptake in diesel contaminated soil amended with DMSBP, compared to a control. Error bars represent ± 1 standard deviation (n = 3)

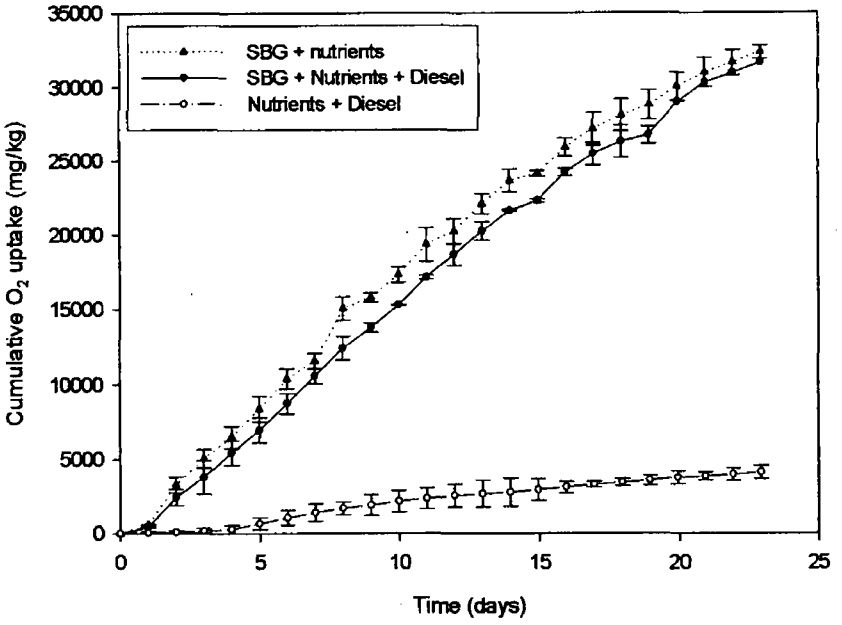


Figure 4.35. Cumulative oxygen uptake in diesel contaminated soil amended with SBG compared to a control. Error bars represent ± 1 standard deviation (n = 3).

It is well known that oil contamination causes significant changes in microbial populations and activity. Many authors have observed an increase in the total numbers of soil microorganisms with time, during biological decontamination, correlating with biodegradation and biological activities (Song *et al.*, 1990; and Margesin *et al.*, 2000b). In this study it was observed that the number of microorganisms increased to similar levels in both the DMSBP and SBG amended systems. The O_2 consumption was also very similar in both types of treatment, but in relation to diesel removal there was significantly more diesel biodegradation in the SBG amended system, suggesting that the changes in microbial numbers and respirometery activity are indicative of a stimulated biodegradation process, but do not represent an accurate measurement of the actual biodegradation. Similar observations were made by Van Der Waarde *et al.* (1995) and Margesin *et al.* (2000a), who found that changes in bacterial numbers are indicative of a stimulated bioremediation process, but do not represent an accurate measurement of the actual biodegradation.

The activity of viable soil microorganisms alone gives no information about the efficacy of the populations, and it is well known that only a small proportion of soil microorganisms can be isolated and cultivated on laboratory media. While the quantification of soil biological activities, e.g. soil respiration, are often used to interpret the intensity of microbial metabolism in soil (Schinner *et al.*, 1996; and Margesin *et al.*, 2000a), care should also be taken in interpretating results, because as was shown here, increased O_2 consumption does not necessarily mean increased degradation of the target compound. In conclusion, soil biological methods should not be used on their own, but should be used to complement chemical methods when evaluating and assessing the success of decontamination methods.

At the end of the experimental period the DROs in the two organically amended soil systems were still quite high, 12,360 mg/kg and 5,363 mg/kg in DMSBP and SBG amended soils, respectively, and therefore would not meet the legislative cleanup requirements of many countries (Section 2.5). However, these DRO residual levels may be acceptable in Ireland as Irish legislation has set no numerical cleanup requirement for petroleum contaminated sites and state that the cleanup level required is land use specific. However, before this soil could be released to the environment, toxicity testing would be necessary in order to establish if the residual hydrocarbons are toxic.

There are a number of reasons why the DRO levels remained high at the end of this experiment. Firstly, the initial starting concentration of 50,000mg/L is extremely high and would rarely be experienced in real samples. A review of the literature showed that the majority of real samples have hydrocarbon concentrations ranging from 500 to 18,000 mg/kg THP (Troy *et al.*, 1992; Demque *et al.*, 1997; Berry and Burton, 1997; and Zytner *et al.*, 2001). Also, the time scale of this experiment, 108 days, was quite short and due to the fact that the DRO was still being degraded at the end of the experiment, extending the incubation period may lower the hydrocarbon concentration further.

4.2.5 Effect of forced aeration on diesel bioremediation in SBG amended soil

A study was conducted to determine if changing the method of aeration from manual aeration to forced aeration would further stimulate the biodegradation process. As low O_2 content has been shown to limit the bioremediation of soils contaminated with petroleum hydrocarbons (von Wedel *et al.*, 1988) it was postulated that forced aeration would keep a more constant and higher concentration of O_2 in the contaminated soil compared to the manual aeration. Forced aeration has also been shown to decrease the amount of space required for treatment of contaminated soil compared to windrows which are manually aerated (Tiquia and Tam, 2002). The forced aeration experiment focused exclusively on the use of SBG as the organic supplement as previous studies indicated SBG significantly enhanced diesel removal compared to other treatments.

DRO removal from soil is shown in Figure 4.36 and it can be seen that the method of aeration influences the rate of DRO removal. At all times DRO removal was higher in the force aerated system by comparison to the manually aerated system. By the end of the experimental period of 65 days, the DRO removal in the forced aeration system was approximately 92%. This represents a 15% greater removal than in the manually aerated system. This increase in DRO removal is more than likely due a higher supply of oxygen in the forced aerated system. In the forced aerated system an air pump constantly supplies oxygen to the soil every 15 minutes, while in the manually aerated system oxygen is only replenished every week, when the sample is manually turned.

The rate of total *n*-alkane degradation was also enhanced by the forced aeration (see Figure 4.37). By the fourteenth day a statistically significant difference ($P<0.05$) appeared between the two aeration systems. After 20 days, all the n-alkanes had disappeared in the force aerated system, compared to requiring 30 days in the manually aerated system. Pristane degradation was also significantly enhanced (Figure 4.38). By day 45 almost all the pristane was removed (>98%) in the system undergoing forced aeration. It took 108 days in the manually aerated system to reach the same degree of pristane removal.

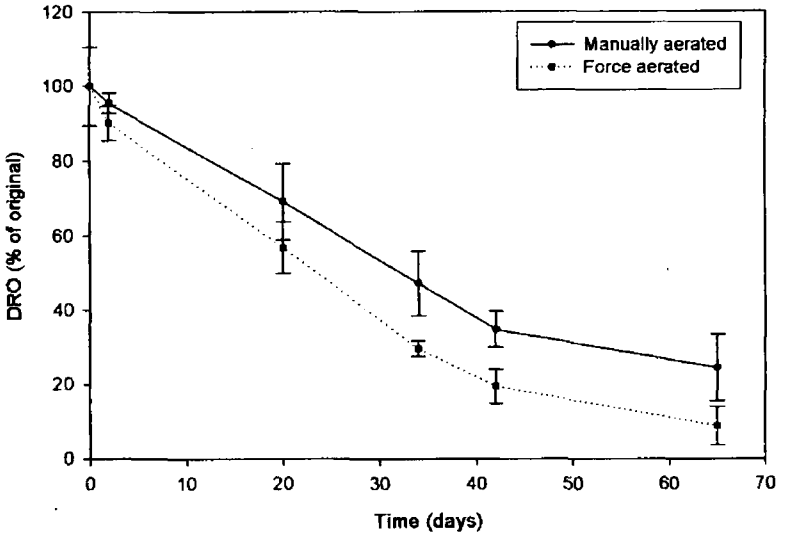


Figure 4.36. Comparison of the effects of forced and manual aeration on DRO removal in SBG amended soil. Error bars represent ± 1 standard deviation (n = 4).

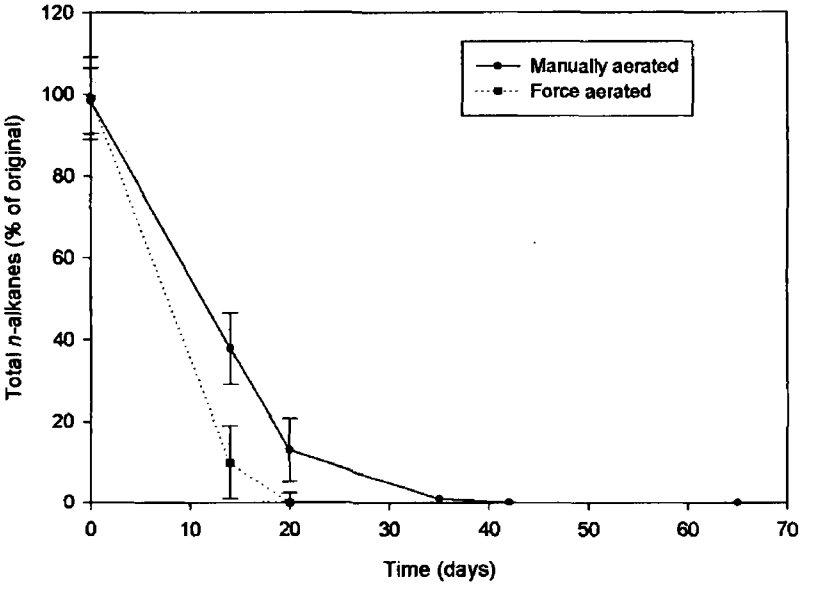


Figure 4.37. Comparison of the effects of forced and manual aeration on the degradation of total *n*-alkanes from a diesel contaminated soil amended with SBG. Error bars represent ± 1 standard deviation (n = 4).

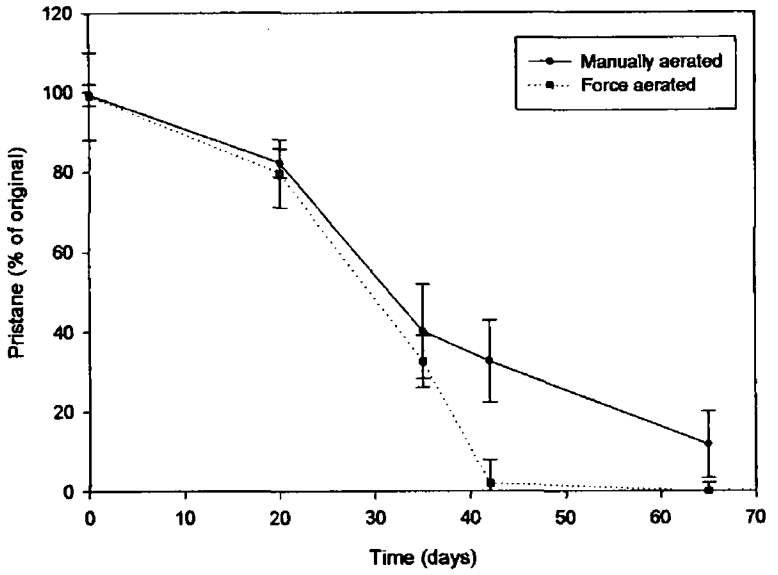


Figure 4.38. Comparison of the effects of forced and manual aeration on the degradation of pristane from a diesel contaminated soil amended with SBG. Error bars represent ± 1 standard deviation (n = 4).

The rate of the UCM fraction removal was again found to be significantly ($p<0.05$) higher in the forced aeration system compared to the manually aerated system (Figure 4.39). At the end of the experimental period, the forced aerated system had achieved 23% more UCM removal that the manually aerated system. On day 65 the soil receiving the forced aeration had only 22% of the original concentration of UCM remaining compared to 45% remaining in the manually aerated sample.

A look at the gas chromatographic fingerprints (Figure 4.40) from the final sampling day showed that in the force aerated system the UCM is much smaller and confined to the narrower region of the chromatograph (between 17 and 27.5 minutes), while in the manually aerated system the UCM is larger and occupied a greater extent of the chromatograph (12.5 to 27.5 minutes). These results would suggest that under optimum bioremediation conditions the lower molecular weight compounds within the UCM of diesel are more degradable that the higher molecular weight compounds.

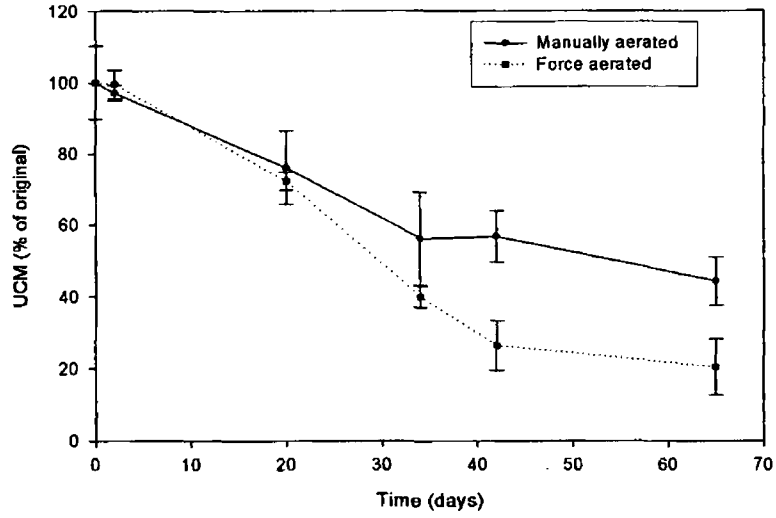


Figure 4.39. Comparison of the effects of forced and manual aeration on the degradation of the UCM fraction from a diesel contaminated soil amended with SBG. Error bars represent ± 1 standard deviation (n = 4).

Other authors who have investigated the effects of forced aeration on the bioremediation processes have reported similar findings. Battaglia and Morgan (1994) found that the biodegradation of hydrocarbon contaminated material by indigenous microorganisms was stimulated through forced aeration. It is not only the bioremediation of hydrocarbon contaminated soil that is enhanced through forced aeration. Boopathy (2000) enhanced the bioremediation of explosive contaminated soils containing, 2,4,6-trinitrotoluene (TNT); trinitrobenzene (TNB); 2,4-dinitrotoluene (2,4-DNT); hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX); and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX), through forced aeration.

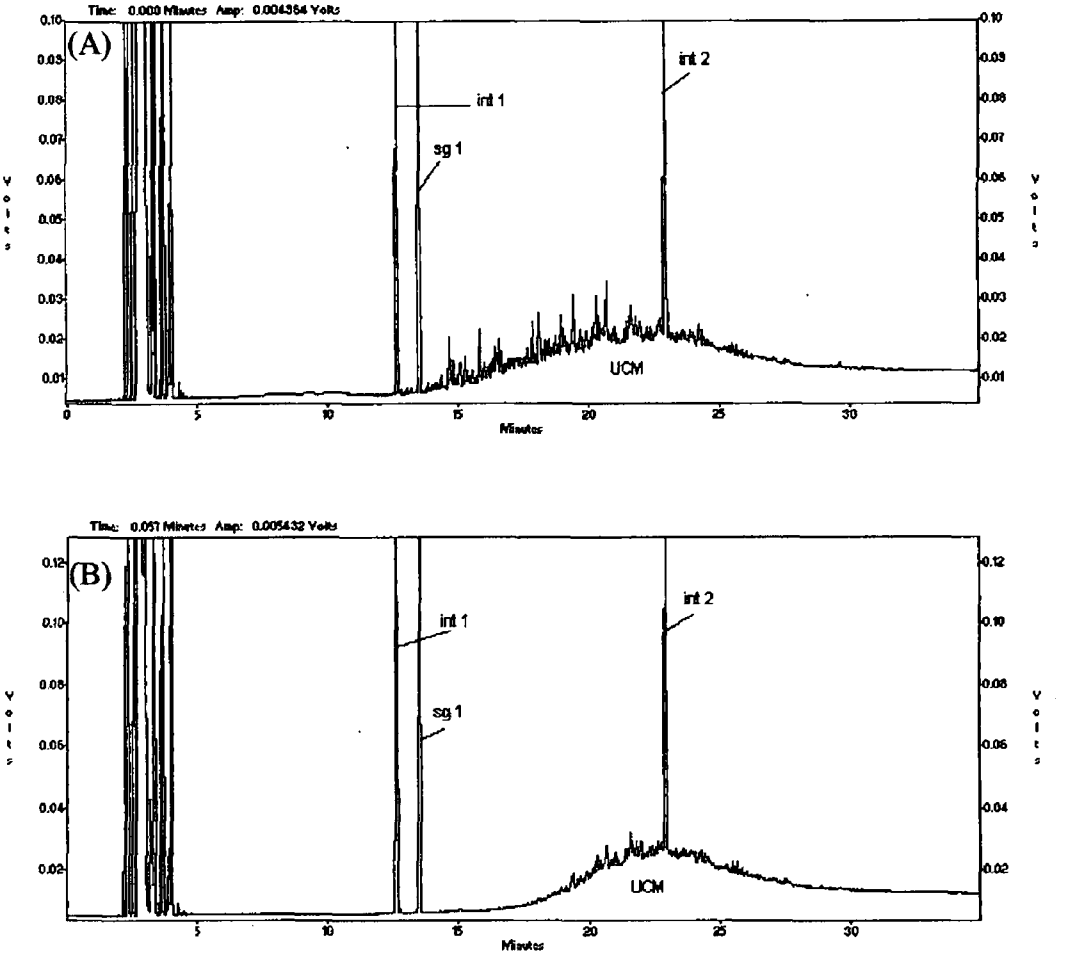


Figure 4.40. Chromatographic fingerprints of residue diesel from SBG amended soil which had received manual and forced aeration. Chromatographs represent residual diesel in SBG amended soil after 65 days treatment in (A) manually aerated, (B) forced aeration systems. Int 1 = internal standard 1-phenylhexane. Int 2 = internal standard 1-phenyltridecane. Sg 1 = surrogate standard naphthalene. UCM = unresolved complex mixture.

4.2.6 Possible reasons for enhanced bioremediation through organic amendment

All the results obtained in this section, in relation to amending diesel contaminated soil with DMSBP and SBG, suggest that both are very effective at stimulating the growth of indigenous microbial populations and increasing the rate and extent of diesel biodegradation.

In the literature numerous explanations have been put forward as to why the addition of inexpensive organic amendments, such as the two used in this study, to hydrocarbon contaminated soils may have beneficial effects on the bioremediation process. A number of authors have shown that organic amendments can be used for soil moisture control (Atlas, 1977; Hornick, 1983; and Huesemann, 1994). In soils with poor moisture retention capacities, organic matter can increase water holding capacity by swelling when wet to absorb two to three times its weight in water, and in saturated soils organic material can provide for better drainage. The two organics used in this study increased the water holding capacity of the sandy soil. As the concentration of both organics in the soil was increased the WHC of the soil increased (Figure 4.41). At a concentration of 20 % w/w (dry weight basis) DMSBP increased the WHC of the sandy soil from 36.6% to 54 %, while SBG increased it further to 71%. At the application rate used through out this study (i.e. 5% w/w dry weight basis) SBG and DMSBP increased the WHC from 36.50% to 41.27 and 39.95%, respectively.

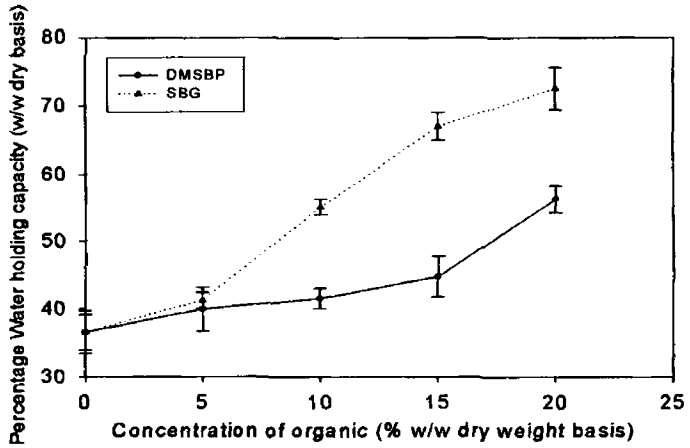


Figure 4.41. Effect of organic amendment on the water holding capacity of the soil. Error bars represent ± 1 std (n = 4).

However, this does not seem to be the main reason for the enhanced diesel removal observed, as an experiment carried out on the water extractable material from SBG showed that the extracted material from SBG alone stimulated the rate of diesel biodegradation to the same extent as the SBG. The SBG was soaked in boiling water for 6 hours, in order to extract compounds such as proteins, minerals and vitamins, and then the extracted material was added to the diesel contaminated soil at a rate equivalent to 5% SBG. The results presented in Figure 4.42 show that the extract had a similar effect on the removal of DRO and total n-alkanes, when compared to the original SBG. These results suggest that it is not the physical presence of SBG that stimulates diesel degradation, but the high protein, fat, mineral and carbon content that are responsible for the enhanced effect on diesel degradation. A similar experiment was not carried out using DMSBP extract, but other authors who studied the effect of molasses on hydrocarbon degradation reported that the high mineral and vitamin content of molasses was partially responsible for the enhanced effect on n-alkane degradation (Al-Hadhrami *et al.*, 1996 and 1997).

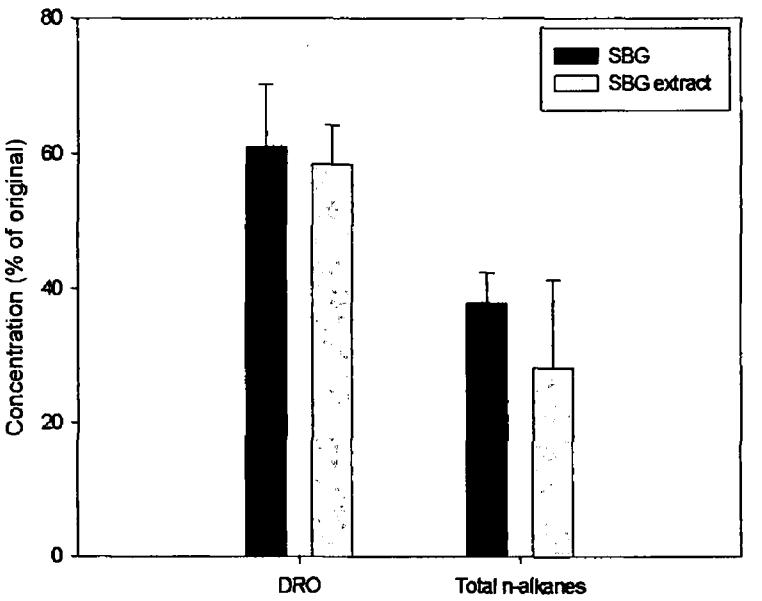


Figure 4.42. Comparison of diesel removal from soil amended with SBG and the water extractable material from SBG. Error bars represent ± 1 standard deviation (n = 4).

These additional carbon and energy sources may have stimulated the metabolism of diesel through cometabolism (Alexander, 1981). Alternatively they may have simply induced and supported the growth of a higher population of diverse microbes which, on exhaustion of the preferred added substrate, may have mineralized the diesel as a secondary carbon/energy source, a mechanism referred to as analog enrichment or cross-acclimation (Colleran, 1997).

A further possible reason for the improved diesel transformation may be due to the organic material enhancing the growth of associated microorganisms involved in the overall indigenous microbial population, as synergistic microbial interactions have been reported to be of particular importance in aerobic and anaerobic environments (Colleran, 1997)

4.2.7 Anaerobic bioremediation of diesel

Aerobic conditions are considered necessary for optimal rates of bioremediation of soils contaminated with petroleum hydrocarbons (Rhykerd *et al.*, 1999). However, Bregnard *et al.* (1996) suggested that diesel degradation may occur under anaerobic conditions. This prompted an experiment to investigate the anaerobic degradation of diesel in this sandy soil and to establish if SBG amendment would stimulate the anaerobic degradation process.

Anaerobic biodegradation of diesel contaminated sandy soil, with and without SBG treatment, was examined by placing the soil microcosms in anaerobic jars and comparing the results to that of a similar microcosm left exposed to the atmosphere, i.e. the aerobic system was left static and received no aeration. All microcosms were amended with nutrients (KH_2PO_4 and NH_4NO_3) and incubation was carried out at room temperature.

After 140 days of incubation there was little removal of DRO in either the SBG amended or unamended anaerobic systems. In the anaerobic microcosm that had received nutrients only, an average of 12% of DRO was removed, consisting of 14% removal of the total *n*-alkanes and 3% removal of the UCM fraction (Figure 4.43b). In the anaerobic system that had been amended with SBG, 9% DRO, 12% of the *n*-alkanes and 4 % of the UCM had been removed in a similar time scale, indicating that the SBG amendment did not stimulate the anaerobic degradation process. In comparison, in the aerobic microcosm 39% removal of the DRO, including 47% of the *n*-alkanes and 37% of the UCM had been removed (Figure 4.43C).

Gas chromatographs of the final day samples (see Figure 4.44) showed that neither of the anaerobic systems had any significant changes in chromatographic fingerprints when compared to fresh diesel sample used to contaminate the soil on day zero. Straight chain alkanes as low as *n*-C_{10}, were still detectable in both anaerobic systems after 140 days of incubation. In comparison the chromatographic fingerprint for static aerobic system showed that a large portion of the larger peaks (*n*-alkanes) had been removed.

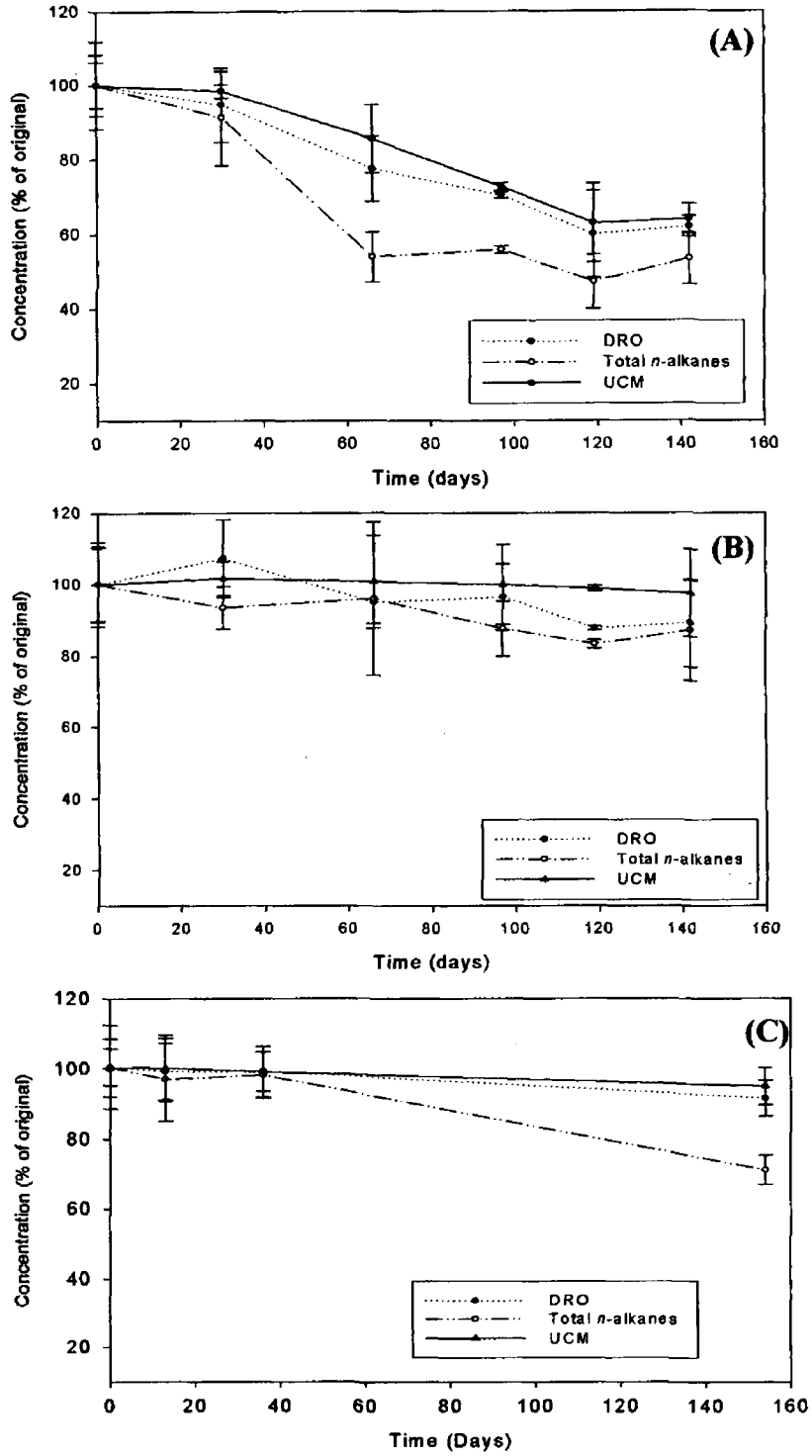


Figure 4.43. Trends in removal of different fractions of diesel in anaerobic microcosms. (A) Represent data for nutrient amended aerobic soil microcosm; (B) Anaerobic microcosm with inorganic nutrient amendment, (C) Anaerobic microcosm amended with nutrients and SBG. Error bars represent ± 1 std (n = 4).

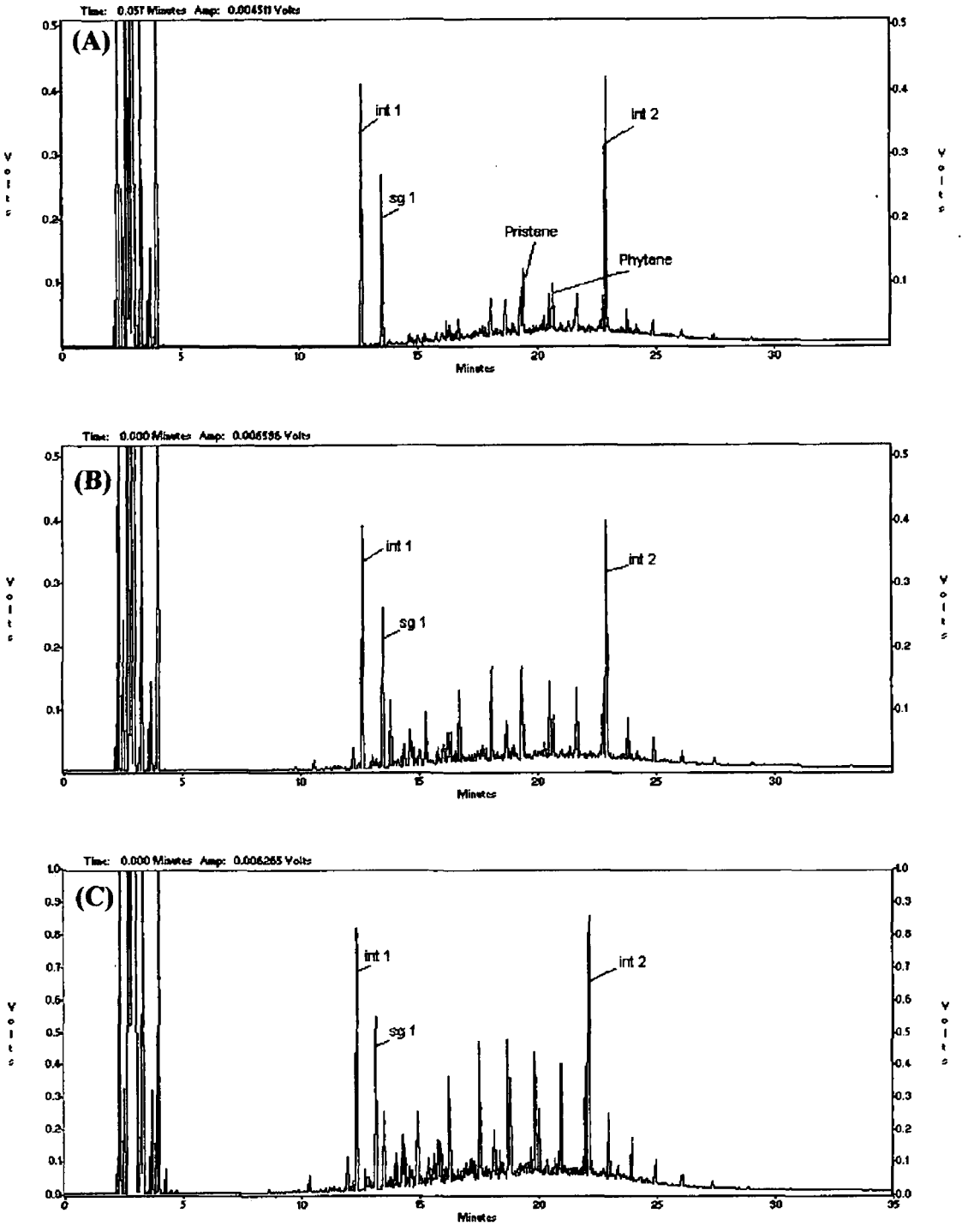


Figure 4.44. Chromatographic fingerprints of residual diesel in aerobic and anaerobic systems. Residual diesel from, (A) static aerobic microcosm amended with inorganic nutrients, (B) Anaerobic microcosm amended with inorganic nutrients, (C) Anaerobic microcosm treated with inorganic nutrients and SBG. Int 1 = internal standard 1-phenylhexane. Int 2 = internal standard 1-phenyltridecane. Sg 1 = surrogate standard naphthalene.

Microbial analysis was also carried out on the three systems and the results (Figure 4.45) show that the total numbers of mesophilic microorganism in the anaerobic system which received inorganic nutrients increased slightly over the duration of the experiment. The numbers increased from $1.23x10^5$ CFU/g soil (dry weight basis) to $1.0x10^7$ by day 140 in the nutrient amended anaerobic system. These numbers were significantly ($P<0.05$) lower than the numbers in the inorganic nutrient amended aerobic system. Amendment of the anaerobic microcosm with SBG significantly increased the total number of mesophilic microorganisms. Numbers of microorganisms in the SBG amended soil reached levels similar to those in the aerobic system which had received inorganic nutrients. However this increase in the microbial population due to SBG did not result in an enhancement in diesel removal. The above results would suggest that microorganisms were present in the anaerobic systems but the correct environmental conditions were not available for them to utilize the diesel.

Possible reasons why the biodegradation of diesel under anaerobic conditions was so limited may be that alternative electron acceptors i.e. nitrate, sulfate, iron and carbon dioxide, were not present in large enough quantities. Bausum and Taylor (1986) reported that anaerobic metabolism of hydrocarbons must begin with the oxidation step, and therefore in the absence of O_2 an alternative electron acceptor must be present. When nitrate is the electron acceptor, a solution of water containing 50mg nitrate/L would require 90,000kg water to degrade 1kg hydrocarbon (Hinchee and Miller, 1990). In this study only a small amount of NO_3 (62mg/kg soil) was available as an alternative electron acceptor and it is highly probable that this deficiency of alternative electron acceptors was the explanation for the lack of biodegradation. Another possible explanation is that the acclimation period required for anaerobic degradation can be immediate, or it can take up to 18 months (Alexander, 1994) and therefore a longer period may have been required for the indigenous microbial population to become acclimatized.

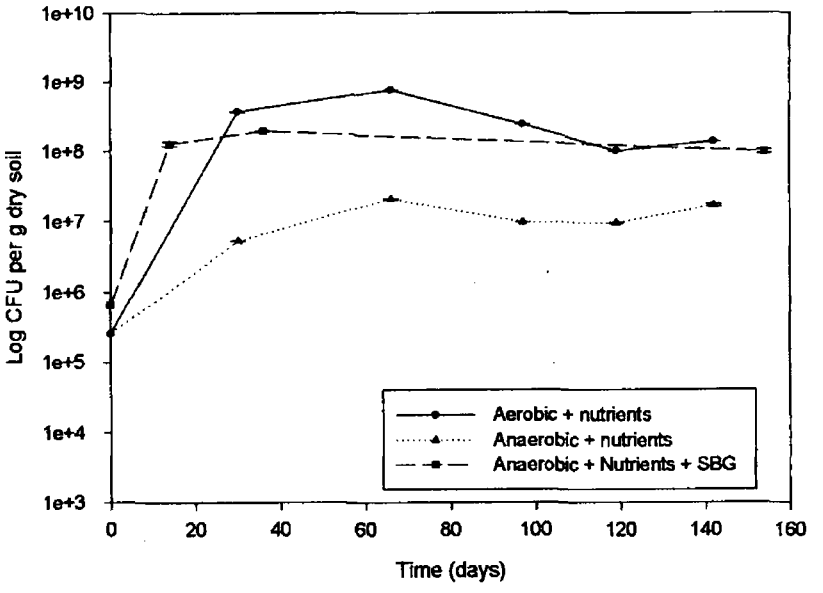


Figure 4.45. Comparison of total mesophilic bacteria in aerobic and anaerobic diesel contaminated soil. Error bars represent ± 1 std (n= 4).

The results of this experiment demonstrated that anaerobic biodegradation of diesel contaminated sandy soil occurred at a very slow rate, if at all. In the literature no studies on the anaerobic degradation of diesel contamination were found for the direct comparison of results. However, in a study by Grishchenkov *et al.* (2000) on the anaerobic degradation of crude oil, he reported that after 50 days of incubation 15-18% of total extractable material, 20-25% of some alkanes and 15-18% of selected PAHs were degraded under anaerobic conditions. Bregnard *et al.* (1996) carried out an investigation on the anaerobic degradation of a weathered diesel sample and found that the isoprenoids and UCM were only reduced to about 50% of the initial concentration after 470 days of incubation, in comparison to 91% in similar aerobic systems.

5.0 Conclusions

From the experimental results of this research, the following conclusions could be drawn.

- The biosurfactants produced by Isolate A, a strain of *Pseudomonas aeruginosa*, contained four different rhamnolipids unlike the majority of *Pseudomonads* which produce biosurfactants with only two types of rhamnolipids.

- The biosurfactants investigated in this research had a neutral effect on the biodegradation of diesel contaminated sandy soil. This was hypothesized as being due to the fact that the biosurfactants themselves were readily biodegradable.

- Whilst not advantageous in the bioremediation of diesel contaminated soil, the biosurfactants did significantly enhance the physical removal of diesel from the sandy soil compared to washing with water alone. Of the surfactants tested the biosurfactants from Isolate A was found to be the most effective.

- In bioremediation processes the type of aeration used had a significant effect on the abiotic removal of diesel. Forced aeration resulted in the greatest abiotic removal while there was no significant difference between manually aerated and static systems.

- Amendment of the diesel contaminated sandy soil with inorganic nutrients significantly enhanced the removal of diesel and in particular the removal of the *n*-alkanes.

- Further amendment of the diesel contaminated soil with organic materials significantly enhanced the biodegradation of all the diesel fractions monitored in

this study, compared to inorganic nutrients only. Of the two organic materials SBG significantly enhanced removal in comparison to DMSBP.

- Pristane, an isoprenoid which up until recently was believed to be relatively resistant to biodegradation, was readily degraded in both organically amended soils, but especially in the SBG amended soil. This compound should no longer be used as a recalcitrant biomarker in soil bioremediation processes, and *n*-C_{17}:pristane ratios should only be used as weathering indicators in the very early stages of bioremediation, hours to days rather than weeks to months.

- The addition of the different organic materials significantly enhanced the overall indigenous microbial population in soil. It was concluded from the results that by increasing the overall microbial populations the hydrocarbon degrading microorganisms are concomitantly increased.

- The forced aeration system significantly enhanced the biodegradation of all diesel fractions in the SBG amended soil, compared to the manual aeration system.

- Anaerobic degradation of diesel, under the conditions used in this study, was minimal and the addition of SBG did not enhance this process.

- The main reason why SBG enhanced the biodegradation of diesel appears to be due to certain chemical components within the material and not due to physical conditioning of the contaminated soil by the SBG.

References.

Abdul, A.S., and Gibson, T.L. (1990). Selection of surfactants for the removal of petroleum products from shallow sandy aquifers. *Groundwater*. 28, 920-926.

Abdul, A.S., and Gibson, T.L. (1991). Laboratory studies of surfactant-enhanced washing of polychlorinated biphenyl from sandy material. *Environ. Sci. Technol.* 25, 665-671.

Abdul, A.S., Gibson, T.L., Ang, C.C., Smith, J.C., and Sobczynski, R.E. (1992). *In-situ* surfactant washing of polychlorinated biphenyls and oils from a contaminated site. *Groundwater*. 30(2), 210-231.

Abu-Ruwaida, A.S., Banat, I.M., Haditirto, S., and Khamis, A. (1991). Nutrient requirements and growth characterization of a biosurfactant-producing *Rhodococcus* bacterium. *World J. Microbiol. Biotechnol.* 7, 53-61.

Admassu, W. and Korus, R.A. (1996). Engineering of bioremediation processes: needs and limitations. In: *Bioremediation: Principles and Applications*. Crawford, R.L. and Crawford, D.L. (Eds.). Cambridge University Press, U.K.

Agency for Toxic Substances and Disease Registry (2002). *Toxicity profiles for fuel oils. 1995*. (Online), Available: http://www.atsdr.cdc.gov (2001, June 1).

Ahearn, D.G., Meyers, S.P., and Standard, P.G. (1971). The role of yeasts in the decomposition of oils in marine environments. *Dev. Ind. Microbiol.* 12, 126-134.

Alexander, M. (1981). Biodegradation of chemicals of environmental concern. *Science*. 211, 132-138.

Alexander, M. (1994). *Biodegradation and bioremediation*. Alexander, M. (ed). Academic Press, San Diego, CA.

Al-Hadhrami, M.N., Lappin-Scott, H.M., and Fisher, P.J. (1996). Effects of the addition of organic carbon sources on bacterial respiration and *n*-alkane biodegradation of Omani crude oil. *Mar. Pollut. Bull.* 32(4), 351-357.

Al-Hadhrami, M.N., Lappin-Scott, H.M., and Fisher, P.J. (1997). Studies on the biodegradation of three groups of pure *n*-alkanes in the presence of molasses and mineral fertilizer by *Pseudomonas aeruginosa*. *Mar. Pollut. Bull.* 34(11), 969-974.

American Petroleum Institute. (1978). Teratology study in rats: Unleaded gasoline. EBI. Project no. 20698-6. American Petroleum Institute. Washington, DC.

Amdurer, M., Fellman, R., and Abdelhamid, S. (1985). *In situ* treatment technologies and superfund. In *Proc., International Conference for New Frontiers for Hazardous Waste Management*. EPA-600/9-85/025.

Ang, C.C., and Abdul, A.S. (1991). Aqueous surfactant washing of residual oil contamination from sandy soil. *Ground Water Monit. Rev.* 11, 121-127.

April, T.M., Abbott, S.P., Foght, J.M., and Currah, R.S. (1998). Degradation of hydrocarbons in crude oil by the ascomycete *Pseudallescheria boydii* (Microascaceae). *Can. J. Microbiol.* 44, 270-278.

Aronstein, B.N., and Alexander, M. (1992). Surfactants at low concentrations stimulate biodegradation of sorbed hydrocarbons in samples of aquifer sands and soil slurries. *Environ. Toxicol. Chem.* 11, 1227-1233.

Aronstein, B.N., Calvillo, Y.M., and Alexander, M. (1991). Effects of surfactants at low concentration on the desorption and biodegradation of sorbed aromatic compounds in soil. *Environ. Sci. Technol.* 25, 1728-1731.

Arora, H.S., Cantor, R.R. and Nemth, J.C. (1982). Land treatment: a viable and successful method of treating petroleum industry waste. *Environ. Int.* 7, 285-295.

Arthur, P., Stiver, W.H., and Zytne, R.G. (1995). Passive volatilization of gasoline from soil. *J. Soil Contam.* 4(2), 1-13.

Atlas, R.M. (1977). Stimulated petroleum biodegradation. *Crit. Rev. Microbiol.* 5, 371-386.

Atlas, R.M. (1982a). Microbial Degradation of Petroleum Hydrocarbons: an Environmental Perspective. *Microbiol. Rev.* 45(1), 180-209.

Atlas, R.M. (1982b). Molecular methods for environmental monitoring and containment of genetically engineered microorganisms. *Biodegradation.* 3, 137-146.

Atlas, R.M. (1991). Microbial hydrocarbon degradation:- Bioremediation of oil spills. *J. Chem. Technol. Biotechnol.* 52, 149-156.

Atlas, R.M. (1992). Petroleum microbiology. In: *Encyclopedia of Microbiology.* Vol. 3. Academic Press, Inc., New York.

Atlas, R.M. (1994). Microbial hydrocarbon degradation-bioremediation of oil spills. *J. Chem. Tech. Biotechnol.* 52, 149-156.

Atlas, R.M., and Bartha, R. (1987). *Microbial Ecology- Fundamentals and Applications*. 2nd ed. Benjamin Cummings, Melon Park, CA.

Atlas, R.M., and Bartha, R. (1992). Hydrocarbon biodegradation and oil spill bioremediation. In: *Advances in Microbial Ecology, Vol. 12.* Marshall K.C. (Ed.). Plenum Press, New York, NY

Atlas, R.M., and Bartha, R. (1993). Ecological aspects of bioremediation control: soil, waste, and water management. In: *Microbial Ecology.* Atlas, R.M., and Bartha, R. (Eds.). Benjamin/Cummings, Redwood City, California.

Atlas, R.M. and Cerniglia, C.E. (1995). Bioremediation of petroleum pollutants: Diversity and environmental aspects of hydrocarbon biodegradation. *BioScience*. 45(5), 332-338

Bai, G.Y., Brusseau, M.L., and Miller, R.M. (1997). Biosurfactant enhanced removal of residual hydrocarbons from soil. *J. Cont. Hydrol.* 25, 157-170.

Baldi, F., Ivosevic, N., Minacci, A., Pepi, M., Fani, R., Svetlicic, V., and Zutic, V. (1999). Adhesion of *Acinetobacter venetianus* to diesel fuel droplets studied with *in situ* electrochemical and moleculer probes. *Appl. Environ. Microbiol.* 65(5), 2041-2048.

Banat, I.M. (1995). Biosurfactant production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review. *Bioresources Technol.* 51, 1-12.

Banat, I.M. (1993). The isolation of a thermophilic biosurfactant producing *Bacillus sp. Biotech. Letters.* 15, 591-594.

Banat, I.M., Samarah, N., Murad, M., Horne, R., and Banerjee, S. (1991). Biosurfactant production and use in oil tank clean-up. *World J. Microbiol. Biotechnol.* 7, 80-88.

Banat, I.M., Makkar, R.S., and Cameotra, S.S. (2000). Potential commercial applications of microbial surfactants. *Appl. Microbial. Biotechnol.* 53, 495-508.

Barathi, S., and Vasudevan, N. (2001). Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. *Environ. Inter.* 26, 413-416.

Bardi, L., Mattei, A., Steffan, S., and Marzona, M. (2000). Hydrocarbon degradation by a soil microbial population with β-cyclodextrin as surfactant to enhance bioavailability. *Enzyme and Microbiol. Technol.* 27(9) 709-713.

Bardos, R.P., Nathanail, C.P., and Weenk, A. (1999). *Review of Added Environmental Value: A Tool to Help Understand the Effects of Remediation within the Context of Sustainable Development.* R&D Technical Report P5-022. Environ. Agency, Britsol, U.K.

Barkay, T., Navon-Venezia, S., Ron, E.Z. and Rosenberg, E. (1999). Enhancement of solubilization of polyaromatic hydrocarbons by the bioemulsifier Alasan. *Appl. Environ. Microbiol.* 65, 2697-2702.

Baryshnikova, L.M., Chemeris, N.A., and Golovlev, E.L. (1994). Glucose effect in the transport of glucose by *Rhodococcus minimus. Microbiol.* 63(3), 405-410.

Battaglia, A., and Morgan, D.J. (1994). *Ex-situ* forced aeration of soil piles: a physical model. *Environ. Prog.* 13(3), 178-187.

Bausum, H.T., and Taylor, G.W. (1986). *Literature survey and data base assessment: Microbial fate of diesel fuels and fog oils.* Technical report 8408. ADA167799. Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD.

Beeba, J.L., and Umbreit, W.W. (1971). Extracellular lipid of *Thiobacillus thiooxidans. J. Bacteriol.* 108, 612-615.

Bej, A.K., Saul, D., and Aislabie, J. (2000). Cold tolerance of alkane degrading *Rhodococcus* sp. from Antarctica. *Polar Biology.* 23, 100-105.

Bell, C., Kostecki, P.T., and Calabrese, E.J. (1989). State research and regulatory approaches of state agencies for the cleanup of petroleum contaminated soils. In *Petroleum Contaminated soils.* Calabrese, E.J., and Kostecki, P.T.(Eds.). Lewis Publishers, Chelsea, MI.

Bento, F.M., and Gaylarde, C.C. (1996). Biodeterioration of stored diesel oil: studies in Brazil. *Int. Biodeter. Biodeg.* 47(2), 107-112.

Berg, M.S., Loehr, R.C., and Webster, M.T. (1998). Release of petroleum hydrocarbons from bioremediated Soils. *J. Soil Contam.* 7(6), 675-695

Berry, K.A., and Burton, D.L. (1997). Natural attenuation of diesel fuel in heavy clay soil. *Can. J. Soil Sci.* 469-477.

Black, C.A. *Methods of Soil Analysis.* Part 2. Black, C.A. (ed.). American Society of Agronomy. Madison, Wisconsin.

Boopathy, R. (2000). Bioremediation of explosives contaminated soil. *Internat. Biodeter. Biodegrad.* 46(1), 29-36.

Bossert, I. and Bartha, R. (1984). The fate of petroleum in soil ecosystems. In: *Petroleum Microbiology.* Atlas, R.M. (Ed). Macmillan, New York.

Bouchez, M., Blanchet, D., and Vandecastelle, P.J. (1995). Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. *Appl. Microbiol. Biotechnol.* 43, 156-164.

Bregnard, T,P,-A., Hohener, P., Haner, P., and Zeyer, J. (1996). Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms. *Environ. Toxicol. Chem.* 15(3), 299-307.

Bregnard, T,P,-A., Hohener, P., Haner, P., and Zeyer, J. (1997). Anaerobic degradation of pristane in nitrate-reducing microcosms and enrichment cultures. *Appl. Environ. Microbiol.* 63(5), 2077-2081.

Bregnard, T,P,-A., Hohener, P., and Zeyer, J. (1998). Bioavailability and biodegradation of weathered diesel fuel in aquifer material under denitrifying conditions. *Environ. Toxicol. Chem.* 17(7), 1222-1229.

Britvic, S., Lucic, D., and Kurelec, B. (1993). Bile fluorescence and some early biological effects in fish as indicators of pollution by xenobiotics. *Environ. Toxicol. Chem.* 12, 765-773.

Brown, E. J., Resnick, S.M., Rebstock, C., Luong, H.V., and Lindstrom, J. (1991). UAF Radiorespirometric Protocol for Assessing Hydrocarbon Mineralization Potential in Environmental Samples. *EPA/600/J-92/389. Biodegradation.* 2:121-127.

Brown, J.L., Syslo, J.L., Lin, Y.-H.L., Getty, S.L., Vemuri, R.L., and Nadeau R.L. (1998). On-Site Treatment of Contaminated Soils: An Approach to Bioremediation of Weathered Petroleum Compounds. *J. Soil Contam.* 7(6), 773-800.

Brueil, C., and Kushner, D.J. (1980). Effects of lipids, fatty acids an other detergents on bacterial utilization of hexadecane. *Can. J. Microbiol.* 26, 223-231.

Bruheim, P., Bredholt, H., and Eimhjellen, K. (1997). Bacterial degradation of emulsified crude oil and the effect of various surfactants. *Can. J. Microbiol.* 43(1), 17-22.

Butler, E.L., Douglas, G.S., Steinhauer, W.G., Prince, R.C.,Aczel, T., Hsu, C.S., Bronson, M.T., Clark, J.R., and Lindstrom, J.E. (1991). Hopane, a new chemical tool for measuring oil biodegradation. In: *On-site bioreclamination: Processes for Xenobiotic and Hydrocarbon Treatment.* Hinchee, R.E., and Olfenbuttel, R.F.(Eds.) Butterworth-Heinemann, Stoneham, MA.

Cappuccino, J.G., and Sherman, N. (1998). Microbiology: Laboratory manual. Cappuccino, J.G., and Sherman, N. (Eds.). Benjamin-Cummings Publishing Company, San Francisco.

Carriere, P.P.E., and Mesania, F.A. (1995). Enhanced biodegradation of creosote-contaminated soil. *Waste Management.* 15(18), 579-583.

Chandler, P.T. (1993). Feeding grewers grain. (online) Available. http://www.inform.umd.edu/EdRes/Topic/AgrEnv/ndd/feeding/FEEDING_BREWERS_GRAIN.html (1 may 2002).

Chang, B.V., Wei, S.H., and Yuan, S.Y. (2001). Biodegradation of phenanthrene in soil. *J. Environ. Sci. Health.* 36(2), 177-187.

Chang, Z.Z., and Weaver, R.W. (1998). Organic bulking agents for enhancing oil bioremediation in soil. *J. Soil Contam.* 1,173-180.

Cho, J.-H., Jeong, Y.-L., Park, O-J., Yoon, B.-D., and Yang, J.-W. (1998). Characterization of glycolipid biosurfactant from an isolated strain of *Pseudomonas aeruginosa* YPJ80. *J. Microbiol. Biotechnol.* 8(6), 645-649.

Churchill, P.F., and Churchill, S.A. (1997). Surfactant-enhanced biodegradation of solid alkanes. *J. Environ. Sci. Health.* 32, 293-306.

Cirigliano, M.C., and Carman, G.M. (1984). Isolation of a biosurfactant from *Candida lipolytica. Appl. Environ. Microbiol.* 48(4), 747-750.

Clark, G.M. (1994). *Statistics and experimental design-an introduction for biologists and biochemists.* Clark, G.M.(Rd.). Arnold, London.

Colleran, E. (1997). Uses of bacteria in bioremediation. In: *Bioremediation protocols.* Sheehan, D. (Ed.). Human Press, Totowa, New Jersey.

Conaway CC, Schreiner CA, Cragg ST. 1984. Mutagenicity evaluation of petroleum hydrocarbons. In: *Advances in modern environmental toxicology. Vol. 6: Applied toxicology of petroleum hydrocarbons.* MacFarland, H.N., Holdsworth C.E., MacGregor, J.A., Call, R.W., and Lane, M.L. (Eds.). Princeton Scientific Publishers Inc., Princeton, NJ.

Cookson, J.T. (1995). *Bioremediation Engineering Design and Application.* Cookson, J.T. (Eds.). McGraw-Hill, Inc., Columbus, OH.

Cooper, D.G., and Goldenberg, B.G. (1987). Surface-active agents from two *Bacillus* species. *Appl. Environ. Microbiol.* 53(2), 224-229.

Cooper, D.G., and Paddock, D.A. (1983). *Torulopsis petrophilum* and surface activity. *Appl. Environ. Microbiol.* 46(6), 1426-1429.

Cooper, D.G., MacDonald, C.R., Duff. S.J.B., and Kosaric, N. (1981). Enhanced production of surfactin from *Bacillus subtilis* by continous product removal and metal cation additions. *Appl. Environ. Microbiol.* 42(3), 408-412.

Cooper, D.G., Zajic, J.E., and Gerson, D.F. (1979). Production of surface active lipids by *Corynebacterium lepus*. *Appl. Environ. Microbiol.* 37, 4-10.

Creedy, J. (1977). A Laboratory Mannual for Schools and Colleges. Creedy, J. (ed). Athenaeum Press Ltd., Newcastle upon Tyne, U.K.

Curry, S., Ciuffetti, L., and Hyman, M. (1996). Inhibition of growth of a *Graphium* sp. on gaseous *n*alkanes by gaseous *n*-alkynes and *n*-alkenes. *Appl. Environ. Microbiol.* 62, 2198-2200.

Custance, S.R., McCaw, P.A., Kopf, A.C., and Sullivan, M.J. (1992). Fate of the Chemical Mixtures: Crude Oil, JP-5, Mineral Spirits, and Diesel Fuel. In: *Hydrocarbons Contaminated Soils and Groundwater Vol. 3.* Calabrese E.T., and Kostecki P.T. (Eds.). Lewis Publishers, Bactoaton, Florida.

Dagher, F., Déziel, E., Lirette, P., Paquette, G., Bisaillon, J.G., and Villemur, R. (1997). Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils. *Can. J. Microbiol.* 43(4), 368-377.

Davies, J.S., and Westlake, D.W. (1979). Crude oil utilization by fungi. *Can. J. Microbiol.* 25(2), 146-156.

Day, P.R., (1965). Particle fractionation and particle-size analysis. In: *Methods of Soil Analysis*. Part I. Black, C.A. (ed.). American Society of Agronomy. Madison, Wisconsin.

Delille, D., Basseres, A., and Dessommes, A. (1997). Seasonal variation in bacteria in sea ice contaminated by diesel fuel and dispersed crude oil. *Microbiol. Ecol.* 33, 97-105.

Demque, D.E., Biggar, K.W., and Heroux, J.A. (1997). Land treatment of diesel contaminatd sand. *Can. Geotech.* 34, 421-431.

Desai, J.D., and Banat, I.M. (1997). Microbial production of surfactants and their commercial potential. *Microbiol. Molecular Biol. Rev.* 63(1), 47-64.

Desai, J.D. and Desai, A.J. (1993). Production of biosurfactants. In: *Biosurfactants: Production, Properties and Application.* Kosaric, N. (ed) Marcel Dekker, Inc. New York, New York.

Deschenes, L., Lafrance, P., Villeneuve, J.P., and Samson, R. (1996). Adding sodium dodecyl sulfate and *Pseudomonas aeruginosa* UG2 biosurfactants inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote-contaminated soil. *Appl. Microbiol. Biotechnol.* 46. 638-646.

Devinny, J.S., Medine, V.F., and Hodge, D.S. (1994). Biofiltration for treatment of gasoline vapors. In: *Hydrocarbon bioremediation.* Hinchee, R.E., Alleman, B.C., Hoeppel, R.E., and Miller, R.N. (Eds). CRC Press, Boca Raton, FL.

Dibble J.T., and Bartha, R. (1979). Effects of environmental parameters on the biodegradation of oil sludge. *Appl. Environ. Microbiol.* 37, 720-749.

Dragun, J. (1998). The soil chemistry of hazardous material. Dragun, J. (Ed), Amerst Scientific Publishers, Amerst, Massachusetts.

Dupont, R.R. (1993). Fundamentals of bioventing applied to fuel contaminated sites. *Environ. Prog.* 12(1), 45-53

Duvnjak, Z., Cooper, D.G., and Kosaric, N. (1982). Production of surfactant by *Arthrobacter paraffineus* ATCC 19558. *Biotech. Bioeng.* 24: 165-175.

Eglinton, G., Maxwell, J.R., and Pjilip, R.P. (1975). Organic geochemistry of sediments from contemporary aquatic environments. In: *Advances in Organic Chemistry.* Tissot, B., and Bienner, F. (Eds.). Editions Technip, Paris.

Ehrhardt, M.G., and Burns, K.A. (1990). Petroleum derived dissolved organic compounds concentrated from inshore waters in Bermuda. *J. Exp. Mar. Biol. Ecol.* 138, 9-34.

Ehrhardt, M.G., and Petrick, G. (1984). On the sensitized photo-oxidation of alkylbenzenes in sea water. *Mar. Chem.* 15, 47-58.

Ekundaya, E.O., and Obuekwe, C.O. (2000). Hydrocarbon utilization in yeast isolates found to grow in association with petroleum in a polluted ultisol of Midwestern Nigeria. *Enviorn. Monitoring Assess.* 63(2), 381-387.

Energy Information Administration. (2002). *U.S. Heating Oil, Diesel Fuel, and Distillate,* (Online), Available: http://www.eia.doe.gov (2001, June 1).

Ermolenko, Z.M., Kholodenko, V.P., Chugunov, V.A., Zhirkova, N.A., and Rasulova, G.E. (1997). A mycobacterial train isolated from the oil of the Ukinskoe oil field- identification and degradative properties. *Microbiol.* 66(5), 542-545.

Etkin, D.S. (2001). Analysis of oil spill trends in the US and worldwide. In: *Preceedings of the 2001 International Oil Spill Conference*. 1,291-1300.

Ferguson, C., and Kasamas, H. (1999). *Risk assessment for contaminated sites in Europe. Vol. 2 : Policy frameworks*. Ferguson, C., and Kasamas, H. (Eds.). LQMS Press, UK.

Fiechter, A. (1992). Biosurfactants: moving towards industrial application. *Tibtech*. 10: 208-217.

Fingas, M.F. (1995). A Literature Review of the Physics and Predictive Modeling of Oil Spill Evaporation. J. *Hazardous Material*. 42, 57-175.

Fingas, M.F. (1998). Studies on the Evaporation of Crude Oil and Petroleum Products: II.Boundary Layer Regulation. *J. Haz. Materials*. 57, 41-58.

Foght, J.M., Gutnick, D.L., and Westlake, D.W. (1989). Effect of emulsan on biodegradation of crude oil by pure and mixed bacterial cultures. *Appl. Environ. Microbiol.* 55, 36-42.

Frankenberger, W.T., Emerson, K.D., and Turner, D.W. (1989). *In-situ* bioremediation of an underground diesel fuel spill: A case history. *Environ. Management*. 13(3), 325-332.

Frankenberger, W.T. (1991). The need for laboratory feasibility study in bioremediation of petroleum hydrocarbons. In: *Hydrocarbon Contaminated Soils and Groundwater*. Calabrese E.T., and Kostecki, P.T.(Eds.). Lewis Publishers, Baco Raton, Florida.

Fu, M.H., and Alexander, M. (1995). Use of surfactants and slurrying to enhance the biodegradation in soil of compounds initially dissolved in nonaqueous-phase liquids. *Appl. Microbiol. Biotechnol.* 43, 551-558.

Gao, B., Yang, L.Y., Wang, X.R., Zhao, J.C., and Sheng, G.Y. (2000). Influence of modified soils on the removal of diesel fuel oil from water and the growth of oil degradation microorganism. *Chemosphere*. 41(3), 419-426.

Gibson, D.T., and Subramanian, V. (1984). Microbial degradation of aromatic hydrocarbons. In: *Microbial degradation of organic compounds*. Gibson, D.T. (Ed.). Marcel Dekkar, New York.

Gilbert, C.E., and Calabrese, E.J. (1992). Developing a standard for methyl butyl ether in drinking water. In: *Regulating Drinking Water Quality*. Gilbert, C.E., Calabrese, E.J (Eds.). Lewis Publishers, Chelsea, Michigan.

Gobdout, J.G., Comeau, Y., and Greer, C.W. (1995). Soil characteristics effects on introduced bacterial survival and activity. In: *Bioaugmentation for site remediation.* Hinchee, R.E., Fredrickson, J., and Alleman, B.C. (Eds.). Battelle Press, Columbus, OH.

Gold-Bouchot, G., Zavala-Coral, M., Zapata-Pérez, O., and Ceja-Moreno, V. (1997). Hydrocarbon concentrations in oysters (*Crassostrea virginica*) and recent sediments from three coastal lagoons in Tabasco, Mexico. *Bulletin Environ. Contam. Toxicol.* 59(3), 430-437.

Goldstein, R.M., Mallory, L.M., and Alexander, M. (1985). Reasons for possible failure of inoculation to enhance biodegradation. *Appl. Environ. Microbiol.* 45, 1122-1125.

Gough, M.A., and Rowland, S.J. (1990). Characterization of Unresolved complex mixtures of hydrocarbons in petroleum. *Nature*. 344, 648-650.

Gough, M.A., Rhead, M.M., and Rowland, S.J. (1992). Biodegradation studies of unresolved complex mixture hydrocarbons: model UCM hydrocarbons and the aliphatic UCM. *Org. Geochem.* 18(1), 17-22.

Greene, A.E., Kay, J.G., Stehmeier, L.G. and Voordouw, G. (2002). Microbial community composition at an ethane pyrolysis plant site at different hydrocarbon inputs. *FEMS Microbiol. Ecol.* 40(3), 233-241.

Grishchenkov, V.G., Townsend, R.T., McDonald, T.J., Autenrieth, R.L., Bonner, J.S., and Boronin, A.M. (2000). Degradation of petroleum hydrocarbons by facultative anaerobic bacteria under aerobic and anaerobic conditions. *Process Biochem.* 35, 889-896.

Gurjar, m., Khire, J.M., and Khan, M.I. (1995). Bioemulsifier production by *Bacillus stearothermophilus* VR-8 isolate. *Lett. Appl. Microbiol.* 21, 83-86.

Gutnick, D.L., and Rosemberg, E. (1977). Oil tankers and pollution: a microbiological approach. *Annual Rev. Microbiol.* 31, 379-396.

Guzev, V.S., Khalimov, E.M., Volde, M.I., and Kulichevskaya, I.S. (1997). Regulatory effect of glucose on hydrocarbon-oxidising microorganisms in soil. *Microbiol.* 66(2), 154-159.

Haba, E. Espuny, M.J., Busquets, M., and Manresa, A. (2000). Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 20044 from waste frying oils. *J. Appl. Microbiol.* 88, 379-387.

Harrison, A.B., Ripley, M.B., Dart, R.K., Betts, W.B., and Wilson, A.J. (2000). Effects of protein hydrolysate on the degradation of diesel fuel in soil. *Enzyme Microbiol. Technol.* 26(5-6), 388-393.

Heitkamp, M.A., Franklin, W., and Cernigila, C.E. (1988). Microbial metabolism of polycyclic aromayic hydrocarbons: Isolation and characterization of a pyrene-degrading bacterium. *Appl. Environ. Microbiol.* 54, 2549-2555.

Henderson, T.R., Li, A.P., Royer, R.E., and Clark, C,R. (1981). Increased cytotoxicity and mutagenicity of diesel fuel after reaction with NO_2. *Environl. Mutagenesis.* 3(3), 211-220.

Herman, D.C., Zhang, Y., and Miller, R.M. (1997). Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. *Appl. Environ. Microbiol.* 63, 3622-3627.

Hinchee, R.E., and Miller, R.N. (1990). Bioreclamination of hydrocarbons in the unsaturated zone. In: *Hazardous waste management of contaminated sites and industrial risk assessment*. Pillman, W., and Zirm, K. (Eds.).

Hisatsuka, K., Nakahara, T., Sano, N., and Yamada, K. (1971). Formation of rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. *Agric. Biol. Chem.* 35, 686-692.

Holden, P.A., LaMontagne, M.G., Bruce, A,K., Miller, W.G., and Lindow, S.E. (2002). Assessing the Role of *Pseudomonas aeruginosa* Surface-Active Gene Expression in Hexadecane Biodegradation in Sand. *Appl. Environ. Microbiol.* 6(5), 2509-2518.

Hornick, S.B. (1983). The interaction of soils with waste constituents. In: *Land Treatment of Hazardous Wastes.* Parr, J.F., Marsh, P.B., and Kla, J.M. (Eds.). Noyes Data Corp., Park Ridge, NJ.

Hubert, C., Shen, Y., and Voordouw, G. (1999). Composition of toluene-degrading microbial communities from soil at different concentrations of toluene. *Appl. Environ. Microbiol.* 65(7), 3064-3070.

Huesemann, M.H. (1994). Guidelines for land-treating petroleum hydrocarbon-contaminated soils. *J. Soil Contam.* 3(3), 1-20.

Hunt, P.G., Rickard, W.E., Deneke, F.J., Koutz,, F.R., and Murman, R.P (1973). Terrestrial oil spills in Alaska: Environmental effects and recovery. In: *Prevention and Control of Oil Spills.* APE/EPA-USCG. American Petroleum Institute: Washington, D.C.

Hupe, K., Luth, J,-C., Heerenklage, J., and Stegmann, R. (1996). Enhancement of the biological degradation of soils contaminated with oil by the addition of compost. *Acta Biotechnol.* 16(1), 19-30.

Hwang, E.Y. (1999). Bioremediation of diesel-contaminated soil using stimulated composting process. Ph.D. Konkuk University, Seoul, South Korea.

Irkhin, A.I., Kondrashenko, T.N., and Puchkov, E.O. (1989). Sensitivity of *Escherichia coli* cell membranes to different classes of detergents. *Microbiol.* 58, 217-221.

Jahan, K., Ahmed, T., and Maier, W.J. (1997). Factors affecting the nonionic surfactant enhanced biodegradation of phenanthrene in the soil. *Water Environ. Res.* 69(3), 317-325.

Jahan, K., Ahmed, T., and Maier, W.J. (1999). Modeling the influence of nonionic surfactants on biodegradation of phenanthrene. *Water Environ. Res.* 33(9), 2181-2193.

Jain, D.J., Lee, H., and Trevors. (1992). Effects of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactant on the biodegradation of selected hydrocarbons in soil. *J. Ind. Microbiol.* 10, 87-93.

Jenny, K, Käppeli, O; Fiechter, A. (1991). Biosurfactants from Bacillus licheniformis: structural analysis and characterization. *Appl. Microbiol. Biotechnol.* 36(1), 5-13.

Johnson, E.A., and Schroeder, W.A. (1995). Astaxanthin from the yeast *Phaffia rhodozyma*. *Studies in Mycology* 38, 81-90.

Joong-Hoon, Cho., Jeong, Y,L. Park, O.J. Yoon, B.D., and Yang, J.W. (1998). Characterization of glycolipid biosurfactant from an isolated strain *Pseudomonas aeruginosa* YPJ80. *J. Microbiol. Biotechnol.* 8(6), 645-649.

Jorgensen, K.S., Puustinen, J., and Suortti, A.M. (2000). Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. *Environ. Pollution.* 107(2), 245-254.

Kaestner, M., and Mahro, B. (1996). Microbial degradation of polycyclic aromatic hydrocarbons in soils affected by the organic matrix of the compost. *Appl. Microbiol. Biotechnol.* 44(5), 668-675.

Kappeli, O., and Finnerty, W.R. (1980). Characterization of hexadecane partition by the growth medium of *Acinetobacter* sp. *Biotechnol. Bioeng.* 22, 495-503.

Kennicutt, M.C. (1988). The effect of biodegradation of crude oil bulk and molecular composition. *Oil Chem. Pollut.* 4, 89-112.

Killops, S.D., and Al-Juboori, M.A.H.A. (1990). Characterization of the unresolved complex mixture (UCM) in the gas chromatograms of biodegraded petroleum. *Org. Geochem.* 15, 147-160.

King, R. B., Long, G.M., and Sheldon, J.K. (1997). *Practical Environmental Pollution.* King, R. B., Long, G.M., and Sheldon, J.K. (Eds.). Lewis Publishers, Boca Raton, Florida.

Ko, S.H., and Lebeault, J.M. (1999). Effect of a mixed culture on co-oxidation during degradation of saturated hydrocarbon mixture. *J. Appl. Microbiol.* 87, 72-79.

Kom S.H., and Lebeault, J.M. (1999). Effect of mixed culture on the co-oxidation during the degradation of saturated hydrocarbon mixture. *J. Appl. Microbiol.* 87, 72-79.

Komagata, K., Nakasa, T., and Katsu, N. (1964). Assimilation of hydrocarbons by yeasts. I. Preliminary screening. *J. Gen. Appl. Microbiol.* 10, 313-321.

Kosaric, N., Cairns, W.L., Gray, N.C.C., Stechey, D. and Wood, J. (1984). The role of nitrogen in multiorganism strategies for biosurfactant production. *JAOCS.* 61(11), 1735-1743.

Kostecki, P.T. Calabrese, E.J., and Garnick, E. (1988). Regulatory policies for petroleum contaminated soils: How states have traditionally dealt with the problem. In: *Soils Contaminated by Petroleum.* Kostecki, P.T., and Calabrese, E.J. (Eds.). John Wiley Interscience, New York.

Kostecki, P.T. Calabrese, E.J., and Simmons, K. (2001). Survey of states 2000 soils cleanup standards for petroleum contamination. *Soil and Sediment Contam.* 10(2), 117-196.

Kretschmer, A., Bock, H., and Wagner. (1982). Chemical and physical characterization of interfacial-avtive lipids from *Rhodococcus erythropplis* grown on *n*-alkane. *Appl. Environ. Microbiol.* 44(4), 864-870.

Kroening, S.J., Leung, D.W., Greenfield, L.G., and Galilee, C. (2001). Losses of diesel oil by volatilisation and effects of diesel oil on seed germination and seedling growth. *Environ. Technol.* 22(9), 1113-117.

LaGrega, M.D., Buckingham, P.L., and Evans, J.C. (1994). Hazardous waste management. LaGrega, M.D., Buckingham, P.L., and Evans, J.C. (Eds). McGraw-Hill Book Co., New York.

Laha, S., and Luthy, R.G. (1991). Inhibition of phenanthrene minerilization by nonionic surfactants in soil-water systems. *Environ. Sci. Technol.* 25, 1920-1930.

Laha, S., and Luthy, R.G. (1992). Effects of nonionic surfactants on the solubilization and minerilization of phenanthrene in soil-water systems. *Biotechnol. Bioeng.* 40, 1367-1380.

Lang, S. and Wullbrandt, D. (1999). Rhamnose lipids- biosynthesis, microbial production and application potential. *Appl. Microbiol. Biotechnol.* 51, 22-32.

Leahy, J.G., and Colwell, R.R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbial. Rev.* 54, 305-313.

Lee, K (2001). *In situ* bioremediation of oiled shoreline environments. In: *Opportunities for Environmental Applications of Marine Biotechnology: Proceedings of the October 5-6, 1999, Workshop*. National Academy Press, Washington DC.

Lee, K., and Levy, E.M. (1987). Enhanced biodegradation of light crude oil in sandy beaches. In: *Proceedings, 1987 oil spill conference.* American Petroleum Inst., Washington, DC.

Lee, K., and Levy, E.M. (1989). Biodegradation of petroleum in the marine environment and its enhancement. In: *Aquatic Toxicology and Water Quality Management.* New York.

Lee, M.D. and Ward, C.H. (1985). Environmental and biological methods for the restoration of contaminated aquifers. *Environ. Toxicol. Chem.* 4, 557-565.

Lin, S.C., Minton, M.A., Sharma, M.M., and Georgiou, G. (1994). Structural and Immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. *Appl. Environ. Micribiol.* 60(1),31-38.

Little, C.D., Palumbo, A.V., Herbes, S.E., Lidstrom, M.E., Tyndall, R.L., and Gilmer, P.J. (1988). Trichloroethylene biodegradation by a methane-oxidising bacterium. *Appl. Environ. Microbiol.* 54, 951-956.

Lowery, O.H., Rosenbourg, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Lupton, F.S., and Marshall, K.C. (1979). Effectiveness of surfactants in the microbial degradation of oil. *Geomicrobiol. J.* 1(3), 235-247.

Lytle, D. A., and Peckarsky, B.L. (2001). Spatial and temporal impacts of a diesel spill on stream invertebrates. *Freshwater Biology.* 46, 693-704.

MacDonald, C.R., Cooper, D.G., and Zajic, J.E. (1981). Surface active lipids from *Nocardia* grown on hydrocarbons. *Appl. Environ. Microbiol.* 41, 117-123.

Madsen, E.L. (1991). Determining *in situ* biodegradation. *Environ. Sci. Technol.* 25, 1663-1673.

Makkar, R.S., and Cameotra, S.S. (1999). Bisurfactant production by microorganisms on unconventional carbon sources. *J. Surfactants and Detergents.* 2(2), 237-241.

Marahiel, M., Denders, W. Krause, M., and Kleinkauf, H. (1977). Biological role of gramicidin S in spore functions. Studies on gramicidin S negative mutants of *Bacillus brevis* 9999. *Eur. J. Biochem.* 99: 49-52.

Margesin, R., and Schinner, F. (1997a). Efficiency of indigenous and inoculated cold adapted soil microorganisms for biodegradation of diesel oil in Alpine soils. *Appl. Environ. Microbiol.*. 63(7), 2660-2664.

Margesin, R., and Schinner, F. (1997b). Bioremediation of diesel-oil-contaminated alpine soils at low temperatures. *Appl. Microbiol. Biotechnol.* 47, 462-468.

Margesin, R., and Schinner, F. (1997c). Laboratory bioremediation experiments with soil from a diesel-oil contaminated site-significant role of cold-adapted microorganisms and fertilizers. *J. Chem. Technol. Biotechnol.* 70, 92-98.

Margesin, R., Walder, G., and Schinner, F. (1999). Biological decontamination of oil spills in cold environments. *J. Chem. Technol. Biotechnol.* 74, 381-389.

Margesin, R., Walder, G., and Schinner, F. (2000a). The impact of hydrocarbon remediation (Diesel oil and polycyclic aromatic hydrocarbons) on enzyme activities and microbial properties of soil. *Acta Biotechnol.* 20(2-3), 313-333.

Margesin, R., Zimmerbauer, A., and Schinner, F. (2000b). Monitoring of bioremediation by soil biological activities. *Chemosphere.* 40, 339-346.

Marin, M., Pedregosa, A., Rios, S., Ortiz, M.L., and Laborda, F. (1995). Biodegradation of diesel and heating oil by *Acinetobacter calcoaceticus* MM5: its possible application on biodegradation. *Intern. Biodeter. Biodeg.* 296-285.

Marquez-Rocha, F. J., Hernandez-Rodriguez, V., and Lamela, M.A.T. (2001). Biodegradation of Diesel Oil in Soil by a Microbial Consortium. *Water, Air, and Soil Poll.* 128 (3-4), 313-320.

McGowan, E. (1999). Bioremediation of unresolved complex mixtures in marine oil spills. Final report: Irish Marine Institute Fisheries research Centre. EU Project B4-3040/95/928/jnb/C4.

Mercade, M.E., Manresa, A., Robert, M., Espuny, M.J., de Andres, C., and Guinea, J. (1993). Olive oil mill effluent (OOME). New substrate for biosurfactant production. *Biores. Technol.* 43, 1-6.

Mihelcic, J.R., Lueking, D.R., Mitzell, R.J., and Stapleton, J.M. (1993). Bioavailability of sorbed- and separate-phase chemicals. *Biodegradation.* 4, 141-153.

Millner, G.C., Nye, A.C., and James, R.C. (1992). Human health based soil cleanup guidelines for diesel fuel. In: *Contaminated soils, diesel fuel contamination*. Kostecki, P.T. and Calabrese, E.J. (Eds). Lewis Publishers, Chelsea, Michigan.

Molitor. G.T.T. (2002). From my perspective; Five economic activities likely to dominate the new millennium V. New atomic age. *Technological Forecasting and Social Change* In Press. Available: http//www.sciencedirect.com (22 June 2002).

Morgan, P., Lee, S.A., Lewis, S.T., Sheppard, A.N., and Watkinson, R.J. (1993). Growth and biodegradation of white-rot fungi in soil. *Soil Biol, Biochem.* 40, 466-470.

Morgan, P., and Watkinson, R.J. (1989). Hydrocarbon degradation in soils and methods for soil biotreatment. *Crit. Rev. Biotechnol.* 8, 505-333.

Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y., and Imanaka. (1993). A new lipopeptide biosurfactant produced by *Arthrobacter sp.* Strain MIS38. *J. Bacteriol.* 174(20), 6459-6466.

Morikawa, M., Ito, M., and Imanaka, T. (1992). Isolation of a new surfactin producing *Bacillus pumilus* A-1 and cloning and nucleotide sequence of the regulatory gene, psf-1. *J. Ferment. Bioeng.* 74, 255-261.

Morkes, J. (1993). Oil-spills whose technology will clean up. *Res. Devel. Magazine.* 35, 54-56.

Mulligan, C.N., and Gibbs, B.F. (1989a). Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 55(11), 3016-3019.

Mulligan, C.N., Mahmourides, G., and Gibbs, B.F. (1989b). The influence of phosphate metabolism on biosurfactant production by *Pseudomonas aeruginosa*. *J. Biotechnol.* 12, 199-210.

Mulligan, C.N., Yong, R.N., and Gibbs, B.F. (2001). Heavy metal removal from sediments by biosurfactants. *J. Haz. Mat.* 85(1-2), 111-125.

Namkoong, W., Hwang, E.Y., Park, J.S., and Choi, J.Y. (2002). Bioremediation of diesel-contaminated soil with composting. *Environ. Pollution.* 119, 23-31.

Neff, J.M., Ostazeski, S., Gardiner, W., and Stejskal, I. (2000). Effects of weathering on the toxicity of three offshore Australian crude oils and a diesel fuel to marine animals. *Environ. Toxicol. Chem.* 19(7), 1809-1821.

Neu, T.R., Hertner, T., and Poralla, K. (1990). Surface active properties of viscosin a peptidolipid antibiotic. *Appl. Microbiol. Biotechnol.* 32, 521-525.

Nocentini, N., Pinelli, D., and Fava, F. (2000). Bioremediation of soil contaminated by hydrocarbon mixtures: the residual concentration problem. *Chemosphere.* 41, 1115-1123.

Noordman, W.H. (1999). Biosurfactant-enhanced soil remediation: effect of rhamnolipid on the bioavailability and microbial uptake of hydrophobic compounds.(Online).Available:http://www.ub.rug.nl/eldoc/dis/science/w.h.noordman/ (May 2002).

Oberbremer, A., Muller-Hurtig, R., and Wagner, F. (1990). Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. *Appl. Biotechnol.* 32, 485-489.

Ochsner, U.A., Hembach, T. and Fiechter, A. (1995). Production of rhamnolipid biosurfactants. *Adv. Biochem. Eng. Biotech.* 53, 89-118.

Ochsner, U.A., Koch, A.K., Fiechter, A., and Reiser, J. (1994). Isolation and characterization of a regulatory gene affecting rhamnolipid synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 176, 2044-2054.

Ogram , A., Sun, W., Brockman, F.J., and Fredrickson, J.K. (1985). Isolation and characterization of RNA from low-biomass deep subsurface sediments. *Appl. Environ. Microbiol.* 61, 582-587.

Oh, Y,S., Sim, D.S., and Kim, S.J. (2001). Effects of nutrients on crude oil biodegradation in the upper intertidal zone, *Marine Pollution Bulletin.* 42(12),1367-1372.

Olivera, F.L., Loehr, R.C., Coplin, B.C., Eby, H.C., and Webster, M.T. (1998). Prepared bed land treatment of soils containing diesel and crude oil Hydrocarbons. *J. Soil Contam.* 7(6), 657-674.

Page, C.A., Bonner, J.S., Kanga, S.A., Mills, M.A., and Autenrieth, R.L. (1999). Biosurfactant solubilization of PAHS. *Environ. Eng. Sci.* 16(6), 465-476.

Parr, J.F., Sikora, L.J., and Burge, W.D. (1983). Factors affecting the degradation and inactivation of waste constituents in soils. In: *Land Treatment of Hazardous Wastes.* Parr, J.F., Marsh, P.B. and Kla, J.M. (Eds). Noyes Data Corp., Park Ridge, NJ.

Patel, R.M., and Desai, A.J. (1997). Surface-active properties of rhamnolipids from *Pseudomonas aeruginosa* GS3. *J. Basic Microbiol.* 4, 281-286.

Piehler, M.F., Swistak, J.G., Pinckney, J.L., and Paerl, H.W. (1999). Stimulation of diesel fuel biodegradation by indigenous nitrogen fixing bacterial consortia. *Microbiol. Ecol.* 38, 69-78.

Potter, T.L., and Simmons, K.E. (1998). Composition of petroleum mixtures. In: *Total petroleum hydrocarbon wirking criteria working group.* Vol. 2. Amerst Scientific Publishers, Amerst, Massachusetts.

Pramer, D., and Bartha, R. (1972). Preparation and processing of soil samples for biodegradation studies. *Environ. Letters.* 2, 217-224.

Pritchard, P.H., and Costa, C.F. (1991). EPA's Alaska oil spill bioremediation project. *Environ. Sci. Technol.* 25, 372-379.

Providenti, M.A., Flemming, C.A., Lee. H., and Trevors, J.T. (1995). Effect of addition of rhamnolipid biosurfactant or rhamnolipid-producing *Pseudomonas aeruginosa* on phenanthrene minerilization in soil slurries. *FEMS Microbiol. Ecol.* 17, 15-26.

Radwan, S.S., Al-Mailem, D, El-Nemr, I.M., and Salamah, S. (2000). Enhanced remediation of hydrocarbon contaminated desert soil fertilized with organic carbons. *Inter. Biodeter. Biodegrad.* 46, 129-132.

Radwan, S.S., Sorkhoh, N.A., El-nemr, I.M., and El-Desouky, A.F. (1997). A feasibility study on seeding as a bioremediation practice for the oily Kuwaiti desert. *J. Appl. Microbiol.* 83, 353-358.

Radwan, S.S., Sorkhoh, N.A., Fardoun, F., and Al-Hasan, R.H. (1995). Soil management enhancing hydrocarbon biodegradation in the polluted Kuwaiti desert. *Appl. Microbiol. Biotechnol.* 44, 265-270.

Rahman, K.S.M., Banat, I.M., Thahira, J., Thayumanavam, T., and Lakshmanaperumalsamy, P. (2002). Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. *Bioresource Technol.* 81, 25-32.

Ram, N.M., Bass, D.H., Falotico, R., and Leahy, M. (1993). A decision framework for selecting remediation technologies at hydrocarbon-contaminated sites. *J. Soil Contam.* 2(2), 1-23.

Ramana, K.V., and N.G. Karanth. (1989). Factors affecting biosurfactant production using *Pseudomonas aeruginosa* CFTR-6 under submerged conditions. *J. Chem. Tech. Biotech.* 45, 249-257

Rapp, P., Bock, H., Wray, V., and Wager, F. (1979). Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on n-alkane. *J. Gen. Microbiol.* 11, 491-503.

Reham, H.J., and Reiff, I. (1981). Mechanisms and occurrence of microbial oxidation of long-chain alkanes. *Adv. Biochem. Eng.* 19, 175-215.

Revill, A.T., Carr, M.R., and Rowland, S.J. (1992). Use of oxidation degradation followed by capillary gas chromatography-mass spectrometry and multi-dimensional scaling analysis to fingerprint unresolved complex mixtures of hydrocarbons. *J. Chromatography.* 589, 281-286.

Rhykerd, R.L., Crews, B., McInnes, K.J., and Weaver, R.W. (1999). Impact of bulking agents, forced aeration, and tillage on remediation of oil-contaminted soil. *Bioresource Technol.* 67, 279-285.

Richard, J.Y., and Vogal, T.M. (1999). Characterization of a soil bacterial consortium capable of degrading diesel fuel. *Inter. Biodeter. Biodegrad.* 44, 93-100.

Riser-Roberts, E. (1992). *Bioremediation of petroleum contaminated sites.* Riser-roberts (Ed). CRC Press, Boca Raton, Florida.

Riser-Roberts, E. (1998). *Remediation of Petroleum contaminated soils-Biological, physical and chemical processes.* Riser-Roberts, E. (Ed). Lewis Publishers, Washington, D.C.

Rittmann, B. E., E. Seagren, B. A. Wrenn, A. J. Valocchi, C. Ray, and L. Raskin (1994). *In Situ* Bioremediation, 2nd edition. Rittmann, B. E., E. Seagren, B. A. Wrenn, A. J. Valocchi, C. Ray, and L. Raskin. (Eds.). Noyes Publishers, Inc., Park Ridge, NJ.

Roberts, M., Mercade, M.E., Bosch, M.P., Parra, J.L., Espuny, M.J., Manresa, M.A., and Guinea, J. (1989). Effects of carbon source on biosurfactant production by *Pseudomonas aeruginosa*. *Botechnol. Lett.* 11, 871-874.

Roch, C., and Infante, C. (1997). Enhanced oily sludge biodegradation by a tension-active agent isolated from *Pseudomonas aeruginosa* USB-CS1. *Appl. Microbiol. Biotechnol.* 47, 615-619.

Roch, F., and Alexander, M. (1995). Biodegradation of hydrophobic compounds in the presence of surfactants. *Environ. Tox. Chem.* 14(7), 1151-1158.

Rosenberg, E. (1993a). Microbial diversity as a source of useful biopolymers. *J. Ind. Microbiol.* 11, 131-137.

Rosenberg, E. (1993b). Microbial surfactants. *Crit. Rev. Biotechnol.* 3, 109-132.

Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., and Ron. (1992). Petroleum bioremediation – a multiphase problem. *Biodegradation.* 3, 337-350.

Rosenberg, E., and Ron, E.Z. (1996). Bioremediation of petroleum contamination. In: *Bioremediation: Principles and Applications.* Crawford, R.L. and Crawford, D.L. (Eds.), Cambridge University Press, U.K.

Rosenberg, M., and Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbial. Lett.* 9, 29-33.

Rowland, S., Donkin, E.S., and Wraiger, E. (2001). Aromatic hydrocarbon "humps" in the marine environment: Unrecognised Toxins? *Evviron. Sci. Technol.* 35, 2640-2644.

Roy, D., Kommalapati, R.R., Mandava, S.S., Valsaraj, K.T., and Constant, W.D. (1997). Soil washing potential of a natural surfactant. *Environ. Sci. Technol.* 31(3), 670-675.

Ryan, M. Technician at Green Vale animal feeds. Conversation with author, 13 March, 2002.

Sánchez, J.E. and Royse, D.J. Adapting substrate formulas used for shiitake for production of brown *Agaricus bisporus*. *Bioresource Technol.* 77(1), 65-69.

Sanders, P.F., and Tibbetts, P.J.C. (1987). Effects of discarded drill muds on microbial populations. *Philos. Trans. R. Soc. London.* 316, 567-585.

Sandvik, S., Lobe, A., and Pederson, T.A. (1986). Biodegradation of oily sludge in Norwegian soils. *Appl. Microbiol. Biotechnol.* 23, 297-301.

Santas, R., Korda, A., Tenente, A., Buchholz, K., and Santas, P.H. (1999). Mesocosm assays of oil spill bioremediation with oleophilic fertilizers: Inipol, F1 or both? *Marine Poll. Bulletin.* 38(1), 44-48.

Scheibenbogen, K., Zytner, R.G., Lee, H., and Trevors, J.T. (1994). Enhanced removal of selected hydrocarbons from soil by *Pseudomonas aeruginosa* UG2 biosurfactants and some chemicall surfactants. *J. Chem. Tech. Biotech.* 59, 53-59.

Schinner, F., Ohlinger, R., Kandeler, E., and Margesin, R. (1996) In: *Methods in Soil Biology*. Schinner, F., Ohlinger, R., Kandeler, E., and Margesin, R. (Eds,) Springer Verlag, Berlin.

Schulz, D., Passeri, A., Schmidt, M., Lang, S., Wagner, F., Wray, V. and Gunkel. (1991). Marine biosurfactants, I. Screening for biosurfactants among crude oil degrading marine microorganisms from the North Sea. Z. *Nature*. 46, 197-203.

Schwendinger, R.B. (1968). Reclamation of soil contaminated with oil. *J. Inst. Petrol.* I54, 182-197.

Seklemova, E., Pavlova, A., and Kovacheva, K. (2001). Biostimulation-based bioremediation of diesel fuel: field demonstration. *Biodegradation.* 12(5), 311-316.

Sepic, E., Leskovsek, H., and Trier, C. (1995a). Aerobic bacterial degradation of selected polyaromatic compounds and *n*-alkanes found in petroleum. *J. Chromatography.* 697(1-2), 515-523.

Sepic, E., Trier, C., and Leskovsek, H. (1995b). Biodegradation study of selected hydrocarbons from diesel oil. *Analyst.* 121(10), 1451-1456.

Setti, L., Lanzarini, G., and Pifferi, P.G. (1995). Dibenzothiophene biodegradation by a *Pseudomonas* sp. in model solutions. *Process Biochemistry.* 30(8), 721-728.

Shaikh, A.U., Hawk, R.N., Sims, R.A., and Scott, H.D. (1997). Redox potential and oxygen diffusion rate as parameters for moniroring biodegradation of some organic wastes in soil. *Nucl. Chem. Waste Manage.* 5, 337-343.

Shin, S.U., Oh, Y.S., and Kim, S.J. (1999). Biodegradation of phenanthrene by *Sphingomonas* sp. strain KH3-2. *J. Microbiol.* 37(4), 185-192.

Shreve, G.S., Inguva, S., and Gunnam, S. (1995). Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *Pseudomonas aeruginosa*. *Molecular Marine Biology and Biotechnol.* 4(4), 331-337.

Siddiqui, S., and Adams, W.A. (2002). The fate of diesel hydrocarbons in soils and their effect on the germination of perennial ryegrass. *Environ. Toxicol.* 17(1), 49-62.

Sikdar, S.K., and Irvine, R.L. (1998). *Bioremediation: Principles and Practice*.Vol. 1. Sikdar, S.K., and Irvine, R.L.(Eds.). Technomic Publishers, Lancaster, PA.

Sim, L., Ward, O.P., and Li, Z.Y. (1997). Production and characterization of a biosurfactant isolated from *Pseudomonas aeruginosa UW*-1. *J. Ind. Microbiol. Biotechnol.* 19, 232-238.

Sims, R., and Bass, J. (1984). Review of in-place treatments techniques for contaminated surface soils. EPA-540/2-84-003a.

Singer, M.E., and Finnerty, W.R. (1984). Microbial metabolism of straight-chain and branched alkanes. In: *Petroleum microbiology*, Altas, R. (ed) Macmillan Publishing Co., New York.

Song, H.G., Wang, X., and Bartha, R. (1990). Bioremediation potential of terrestrial fuel spills. *Appl. Environ. Microbiol.* 56, 652-656.

Stelmack, P.L., Gray, M.R., and Pichard, M.A. (1999). Bacterial adhesion to soil contaminants in the presence of surfactants. *Appl. Environ. Microbiol.* 65(1), 163-168.

Stephenson, G.L., Koper, N., Atkinson, G.F., Solomon, K.R., and Scroggins, S. (2000). Use of linear regression techniques for describing concentration-response relationships for plant species exposed to contaminated site soil. *Environ. Toxicol. Chem.* 19, 292-242.

Storm-Kristiansen, T., Daling, P.S., and Lewis, A. (1993). Weathering properties and chemical dispersability of crude oils transported in US waters. *Technical Report Series 93-032.* Marine Spill Response Corporation, Washington, DC.

Suzuki, T., Tanaka, H., and Itoh, I. (1974). Source lipids of *Arthrobacteria, Corynsbacteria* and *Norcardia* grown on sucrose. *Agric. Biol. Chem.* 33, 190-195.

Syldatk, C., Lang, S., Wagne, F., Wray, V., and Witte, L. (1985). Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas spec.* DSM 2874 grown on *n*-alkanes. *Nature*. 40, 51-60.

Tabak, H.H., Glaser, J.A., Stohofer, S., Kupferle, M.J., Scarpino, P., and Tabor, M.W. (1991). Characterization and optimization of treatment of organic wastes and toxic organic compounds by a lignolytic white rot fungus in bench-scale bioreactors. In: *On-Site Bioreclamination: Processes for Xenobiotic and Hydrocarbon Treatment.* Hinchee, R.E., and Olfenbuttel, R.F. (Eds.). Butterworth-Heinemann, Stoneham, MA.

Taylor, C., and Viraraghavan, T. (1999). A bench-scale investigation of land treatment of soil contaminated with diesel fuel. *Chemosphere.* 39(10), 1583-1593.

Thomas, J.M., Ward, C.H., Raymond, R.L., Wilson, J.T., and Loehr, R.C. (1992). Bioremediation. In: *Encyclopedia of Microbiology. Vol.1.* Academic Press, New York.

Thomas, K.V., Donkin, P., and Rowland, S.J. (1995). Toxicity enhancement of an aliphatic petrogenic unresolved complex mixture (UCM) by chemical oxidation. *Water Res.* 29(1), 379-382.

Thibault, G.T., and Elliott, N.W. (1980). Biological detoxification of hazardous organic chemical spill. In *Proceedings 1980 conference on Hazardous materials and Spills.* May 13-15. Louisvill, KY.

Thibault, S. L., Anderson, M., and Frankenberger, W. T. (1996). Influence of surfactants on pyrene desorption and degradation in soils. *Appl. Environ. Microbiol.* 62, 283-287.

Tiehm, A. (1994). Degradation of polycyclic aromatic hydrocarbons in the preence of synthetic surfactants. *Appl. Environ. Microbiol.* 60, 258-263.

Tiquia, S.M., and Tam, N.F.Y. (2002). Characterization and composting of poultry litter in forced-aeration piles. *Process Biochemistry.* 37(8), 869-880.

Troy, M.A., McGinn, S.R., Greenwald, B.P., Jerger, D.E., and Allen, L.B. (1992). Bioremediation of diesel fuel contaminated soil at a former railroad fueling yard. In *Contaminated Soils Diesel Fuel Contamination.* Kostecki, P.T., and Calabrese, E.J. (Eds.). Lewis Publishers, Inc.,Chelsea, MI.

US Chemical Substance Inventory. (2002). In: *Toxic Substance Control Act.*

USEPA, (1985). Handbook of remedial action at waste disposal sites. EPA/625/6-85/006.

USEPA. (1996a). Composting, Engineering Bulletin, EPA/540/5-96/502.

USEPA. (1996b). Method 3550B. Ultrasonic extraction.

USEPA. (1998b). Technical protocol for evaluating natural attenuation of chlorinated solvents in ground water. EPA/600/R-98/128

USEPA (2002). Office of underground storage tanks (OUST). Available: http://www.epa.gov/swerust1/cat/camarchv.htm. (2002, May 21).

Van Der Waarde, J.J., Dijkhuis, E.J., Henssen, M.J.C., and Keuning, S. (1995). Enzyme Assays as Indicators for Biodegradation. In: *Monitoring and Verification of Bioremediation.* Hinchee, R.E., Douglas, G.S., and Ong, S.K. (Eds.). Battelle Press, Columbus, OH.

Van Dyke, M.I., Gully, S.L., Lee, H., and Trevors, J.T. (1993). Evaluation of microbial surfactants for recovery of hydrophobic polluatants from soil. *J. Ind. Microbiol.* 11, 163-170.

Van Dyke, M.I., Lee, H. and Trevors. (1991). Applications of microbial surfactants. *Biotechnol. Adv.* 9, 241-252.

Van Loosdrecht, M.C.M., Lyklema, J., Norder, W., and Zehnder, A.J.B. (1990). Influence of interfaces on microbial activity. *Microbiol. Rev.* 54, 75-87.

Vasudevan, N., and Rajaram, P. (2001). Bioremediation of oil sludge-contaminated soil. *Environ. Inter.* 26, 409-411.

Velraeds, M.M., Van der Mei, H.C., Reid, G., and Busscher, H.J. (1997). Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* to solid substrata by an adsorbed biosurfactant layer from *Lactobacillus acidophilus*. *Urology.* 49(5), 790-794.

Venosa, A.D., Suidan, M.T., Strohmeier, K.L., Haines, J.R., Eberhart, N.L., King, D., and Holder, E. (1996). Bioremediation of an experimental oil spill of Delaware Bay. *Environ. Sci. Technol.* 30, 1764-1775.

Vik, E.A., Bardos R.P., Brogan, J. Edwards, D. Gondi, F. Henrysson T., Jensen B.K., Jorge C., Mariotti C., Nathanail P., and Papassiopi N. (2001). Towards a Framework for Selecting Remediation Technologies for Contaminated Sites. *Land Contamination and Reclamation.* 9(1), 119-127.

Vipulananda, C., and Ren, X. (2000). Enhanced solubility and biodegradation of naphthalene with biosurfactant. *J. Environ. Eng.* 126(7), 629-634.

Vogel, T.M., Criddle, C.S., and McCarty, P.L. (1987). Transformations of halogenated aliphatic compounds. *Environ. Sci. Technol.* 21, 722-736.

Volkering, F., Breure, A.M., van Andel, J.G., and Rulkens, W.H. (1995). Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. *Appl. Environ. Microbiol.* 61, 1699-1705.

von Wedel, R.J., Mosquera, J.F., Goldsmith, C.D., Hater, G.R., Wong, A., Fox, T.A., Hunt, W.T., Paules, M.S., Quiros, J.M., and Wiegand, J.W. (1988). Bacterial biodegradation of petroleum hydrocarbons in groundwater: *in-situ* augmented bioreclamination with enrichment isolates in California. *Water Sci. Technol.* 20, 501-503.

Walker, J.D., and Colwell, R.R. (1974). Microbial petroleum degradation: Use of mixed hydrocarbon substrate. *Appl. Microbiol.* 27, 1053-1060.

Walworth, J.L., and Reynolds, C.M. (1995) Bioremediation of a Petroleum-Contaminated Cryic Soil: Effects of Phosphorus, Nitrogen, and Temperature. *J. Soil Contam.* 4(3), 299-310.

Wang, D., Sakoda, A., and Suzuki, M. (2001). Biological efficiency and nutritional value of *Pleuroyus ostreatus* cultivated on spent beer grain. *Bioresource Technol.* 78, 293-300.

Wang, X., and Bartha, R. (1994). Effects of Bioremediation on Toxicity, Mutagenesis, and Microbiota in Hydrocarbon-Polluted Soils. In *Remediation of Hazardous Waste Contaminated Soils.* Wise, D.L., and Trantolo, D.J. (Eds.). Marcel Dekker Inc., New York.

Wardley-Smith, J. (1976). *The Control of oil pollution on the sea and inland waters: The effects of oil spills on the marine environment and methods of dealing with them.* Wardley-Smith, J. (Ed.). Graton and Trotman Ltd., UK.

Watkinson,R.J., and Morgan, P. (1990). Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegradation.* 1(2-3), 79-92.

Westlake, J.L., Jobson, A., Phillippe, R., and Cook, D.F. (1974). Biodegradability and crude oil composition. *Can. J. Microbiol.* 20, 915-928.

Whyte, L.G., Hawari J., Zhou, E., Bourbonniere, L., Inniss, W.E., Greer, C.W. (1998). Biodegradation of variable-chain-length alkanes at low temperatures by a phychrotrophic *Rhodococcus* sp. *Appl. Environ. Microbiol.* 67(7), 2578-2584.

Whyte, L.G., Slagman, S.J., Pietrantonio, F., Bourbonniere, L., Koval, S.F., Lawerance, J.R., Inniss, W.E., and Greer, C.W. (1999). Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. *Appl. Environ. Microbiol.* 65(7), 2961-2968.

Wild, M., Cora, A.D., Hernandez, A.L., Miller, R.M. and Soberon-Chavez, G. (1997). Selection and partial characterization of a *Pseudomonas aeruginosa* mono-rhamnolipid deficient mutant. *FEMS Microbiol. Lett.* 153, 279-285.

Williams, C.M., Grimes, J.L., and Mikkelsen, R.L. (1999). The use of poultry litter as co-substrate and source of inorganic nutrients and microorganisms for the *ex-situ* biodegradation of petroleum compounds. *Poult. Sci.* 78(7), 956-964.

Wilson, N.G. and Bradely, G. (1996). The effect of immobilization on rhamnolipid production by *Pseudomonas fluorescens. J. Appl. Bacteriology.* 81(5), 525-530.

Wilson, N.G., and Bradely, G. (1997). A studt if a bacterial mobilization substratum for use in the bioremediation of crude oil in a saline water system. *J. Appl. Microbiol.* 83(4), 524-530.

Wraige, E.J. (1997). Studies of the Synthesis, Environmental Occurrence and Toxicity of Unresolved Complex Mixtures (UCMs) of Hydrocarbons. PhD Thesis. University of Plymouth, UK.

Wu, J., and Ju, L.-K. (1998) Extracellular particles of polymeric material formed in n-hexadecane fermentation by *Pseudomonas aeruginosa. J. Biotechnol.* 59(3), 193-202.

Yagi, O., and Soudo, R. (1980). Degradaion of polychlorinated biphrnyls by microorganisms. *J. Water Pollut. Control Fed.* 52, 1035-1043.

Yakimov, M.M., Timmis, K.N., Wray, V., and Fredrickson, H.L. (1995). Characterization of a new lipopepide surfactant produced by a thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl. Envir. Microbiol.* 61(5), 1706-1713.

Zajic, J.E., Guignard, E., and Gerson, D.F. 1977. Properties and biodegradation of a bioemulsifier from *Corynebacterium hydrocarboclastus. Biotech. Bioeng.* 19, 1303-1320.

Zhang, Y., and Miller, R.M. (1992). Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). *Appl. Environ. Microbiol.* 58(10), 3276-3282.

Zhang, Y., and Miller, R.M. (1994). Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. *Appl. Environ. Microbiol.* 60, 2101-2106.

Zhang, Y., Maier, W.J., and Miller, R.M. (1997). Effects of rhamnolipids on the dissolution, bioavailability, and biodegradation of phenanthrene. *Environ. Sci. Technol.* 31(8), 2211-2217.

Zhou, E., and Crawford, R.L. (1995). Effects of Oxygen, nitrogen and temperature on gasoline biodegradation in soil. *Biodegradation.* 6, 127-140.

Zhou, Q.H., and Kosaric, N. (1995). Utilization of canola oil and laclose to produce biosurfactant with *Candida bombicola. JAOCS.* 72(1), 67-71.

Zitrides, T. (1983). Biodecontamination of spill sites. *Pollut. Eng.* 15, 24-27.

Zytner, R.G., Salb, A., Brook, T.R., Leunissen, M., and Stiver, W.H. (2001). Bioremediation of diesel fuel contaminated soil. *Can. J. Civ. Eng.* 28(1), 131-140.

Appendix 1.

Phosphate buffer, 20mM, (pH 7.0).

The 20mM phosphate buffer (pH 7.0) was made up according to the method by (Creedy, 1977). In brief, 0.02M stock solutions of sodium dihydrogen phosphate (3.12 g of $NaH_2PO_4.2H_2O$ per litre) and disodium hydrogen phosphate (2.839 g of Na_2HPO_4 per litre or 7.17 g of $NaHPO_4.12H_2O$ per litre) were made up in deionised water. To make up the required 20mM buffer (pH 7.0), 19.5mls of the stock solution of NaH_2PO_4 was added to 30.5mls of the Na_2HPO_4 stock solution and made up to 100mls with deionized water. This buffer was stored at 4°C for up to two months.

Appendix 2.

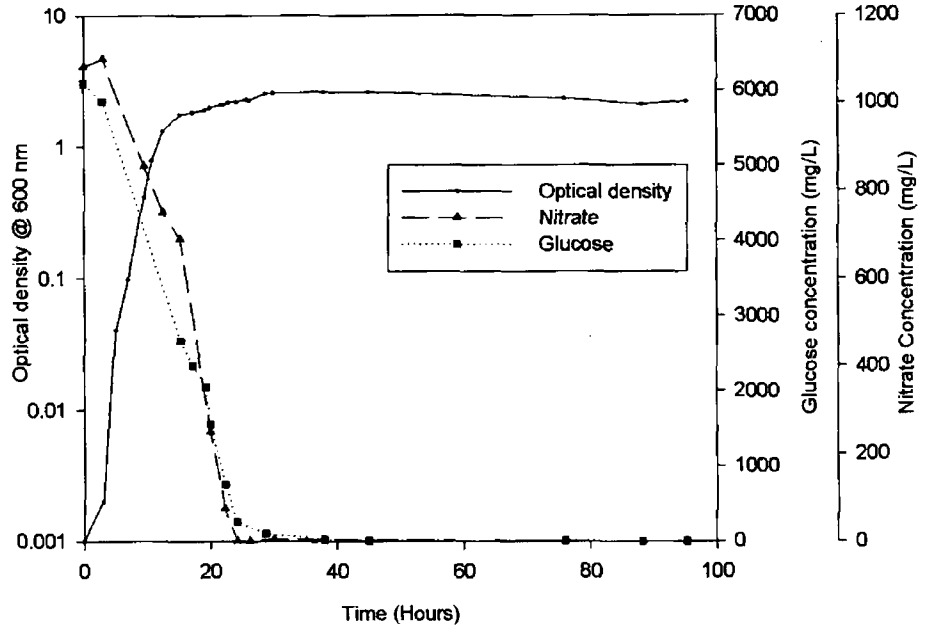


Figure 1. Time course of growth, change in surface tension of culture broth and concentration of medium components during the cultivation of Isolate A, at room temperature in PPGAS medium, in flask culture.

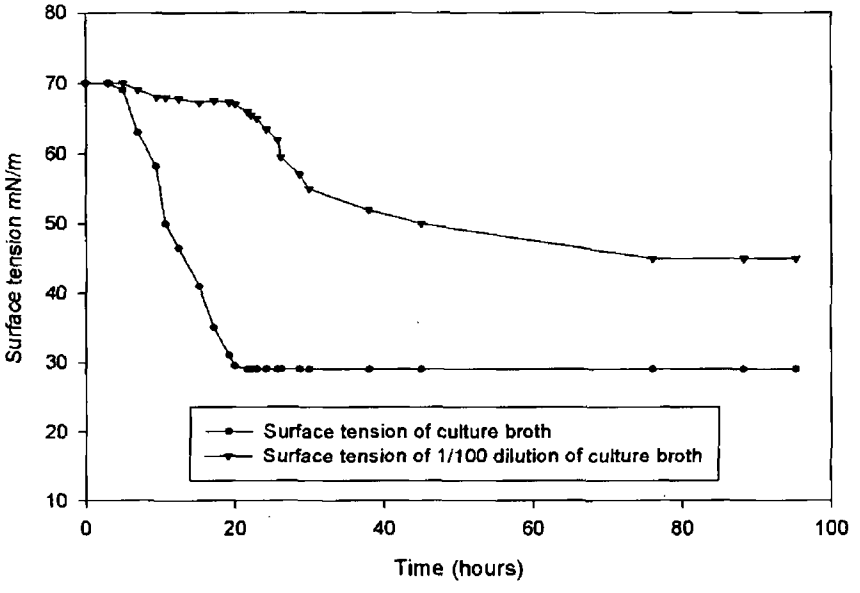


Figure 2. Surface activity profiles of undiluted and diluted culture medium during cultivation of Isolate A, at room temperature, in PPGAS medium, in flask culture.

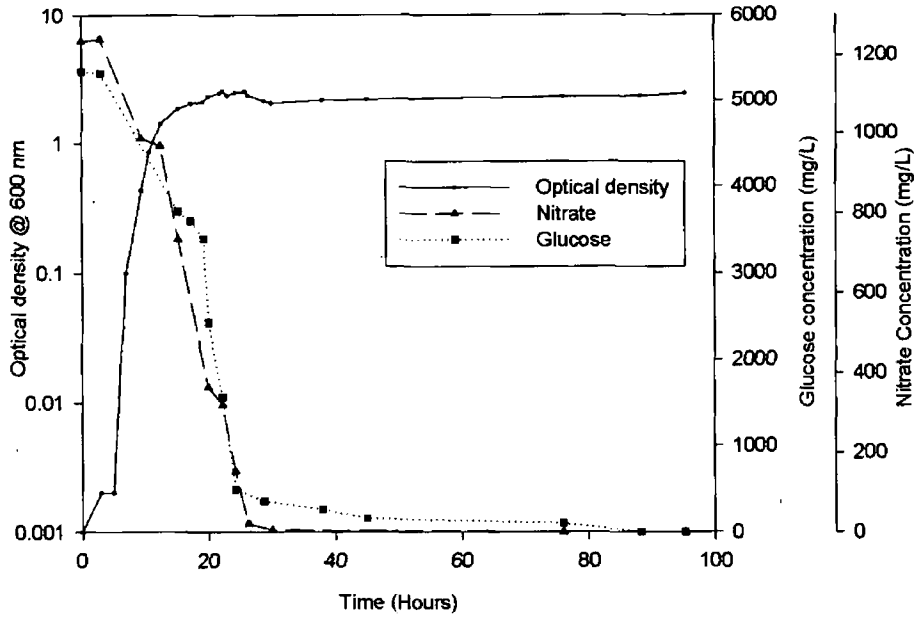


Figure 3. Time course of growth, change in surface tension of culture broth and concentration of medium components during the cultivation of *Pseudomonas aeruginosa* NCIMB 8626, at room temperature in PPGAS medium, in flask culture.

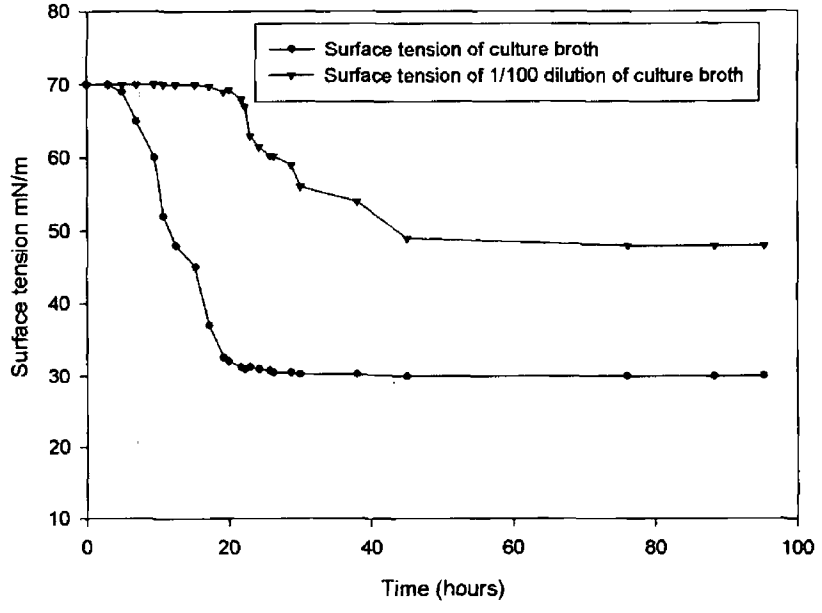


Figure 4. Surface activity profiles of undiluted and diluted culture medium during cultivation of *Pseudomonas aeruginosa* NCIMB 8626, at room temperature, in PPGAS medium, in flask culture.

Appendix 3.

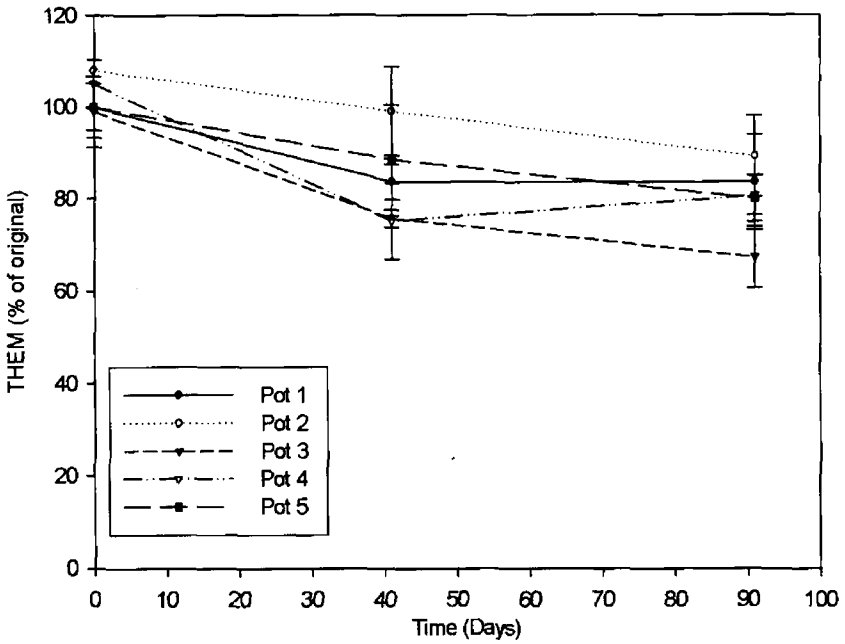


Figure 1. Variation in THEM in a diesel contaminated sandy soil treated with Biosurfactant produced by NCIMB 8626 and no inorganic nutrients. Pot 1 = abiotic control, Pot 2 = natural remediation, Pot 3 = Biosurfactant @ 10 x CMC, Pot 4 = Biosurfactant @ 1 x CMC and Pot 5 = Biosurfactant @ 0.1 x CMC (Error bars represent ± 1 STD, n = 4).

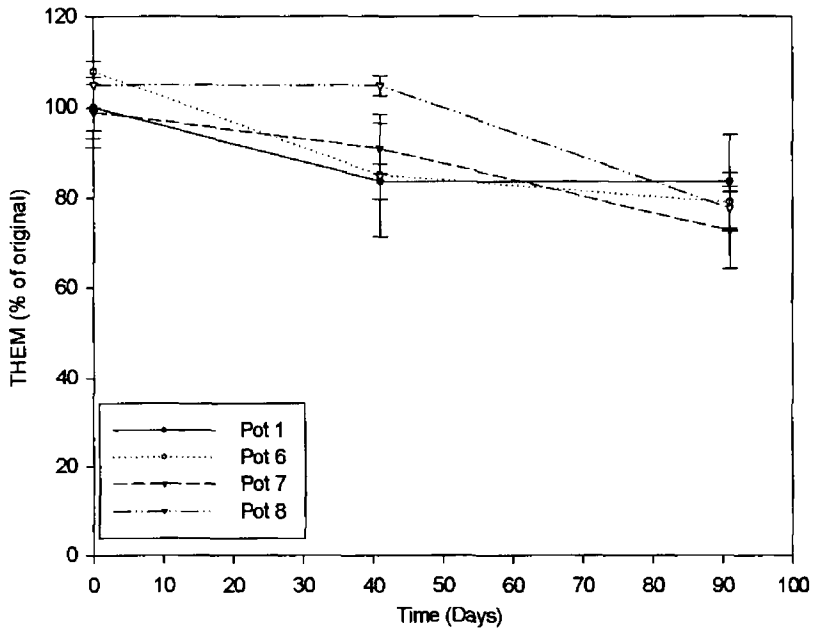


Figure 2. Variation in THEM in a diesel contaminated sandy soil treated with Biosurfactant produced by Isolate A and no inorganic nutrients. Pot 1 = abiotic control, Pot 6 = Biosurfactant @ 3 x CMC, Pot 7 = Biosurfactant @ 1 x CMC and Pot 8 = Biosurfactant @ 0.1 x CMC (Error bars represent ± 1 STD, n = 4).

Appendix 4.

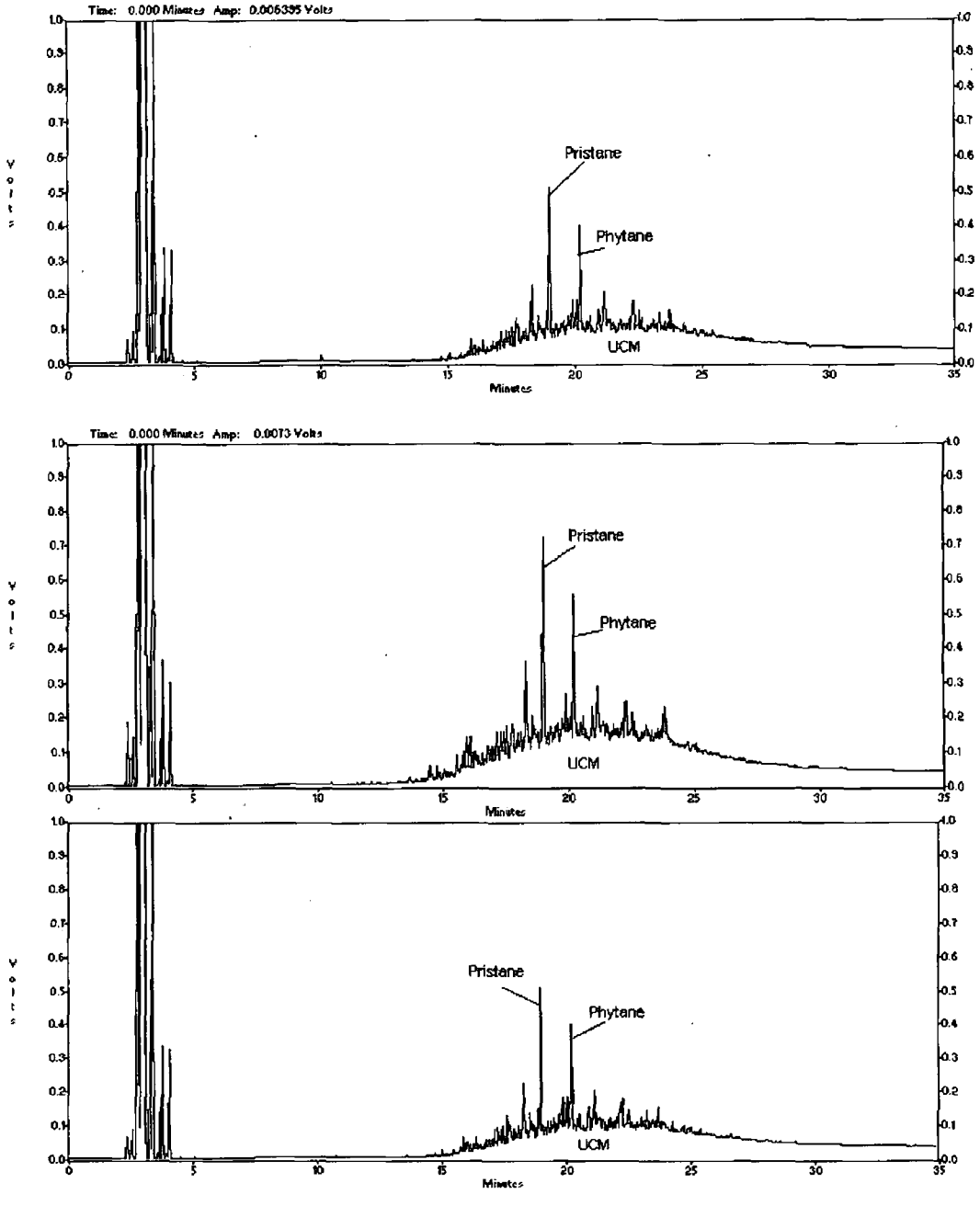


Figure 3. Chromatographic fingerprint of residual diesel extracted from soil after 98 days treatment with biosurfactants in the presence of inorganic nutrients. (a) Diesel contaminated soil amended with organic nutrients (b) inorganic nutrients + isolate A biosurfactant, (c) inorganic nutrient + *Pseudomonas aeruginosa* NCIMB 8626 biosurfactant.

Appendix 5.

Table 1. Chemical composition of SBG (Chandler, P.T., 1993)

		Eight Brewery Locations		
Nutrient	**Unit**	**Mean**	**Std.**	**C.V.**
Ash	%	4.4	0.44	9.92
Calcium	%	0.37	0.05	12.36
Phosphorus	%	0.66	0.06	9.78
Magnesium	%	0.26	0.02	6.26
Sodium	%	0.02	0.005	22.8
Potassium	%	0.09	0.02	18.12
Sulphur	%	0.23	0.04	18.12
Iron	ppm	148.09	21.77	14.70
Copper	ppm	22.28	5.12	22.97
Manganese	ppm	44.97	4.18	9.29
Zinc	ppm	87.84	5.25	5.97

Appendix 6.

Composition of beet molasses which makes up 22 to 30 % of DMSBP (Ryan, 2002).

Table 1. Average values for vitamins (Ryan, 2002).

Vitamin	**Concentration mg/kg**
Biotin	0.04/0.13
Folic Acid	ca 0.2
Inositol	5800/8000
Ca-Oantothenate	50/100
Pyridoxine	ca 5.4
Riboflavin	ca 0.4
Thiamine	ca 1.3
Nicotinic Acid	20/45
Choline	400/600