# A Study Into the Potential of Phytoremediation for Diesel Fuel Contaminated Soil

Gillian Adam

Thesis submitted for the degree of Doctor of Philosophy

May 2001

Environmental, Agricultural and Analytical Chemistry

Chemistry Department

University of Glasgow

PAGE
NUMBERING
AS
ORIGINAL

# MISSING

# PRINT

PAGE
NUMBERS
CUT OFF
IN THE
ORIGINAL

TEXT
CUT OFF IN THE
ORIGINAL

## Dedication

*Mum and dad - without your endless encouragement, love and support I would never have achieved half as much. Thank you for all the opportunities you have given me. I love you both.*

## Acknowledgements

Firstly, I would like to thank my supervisor, Dr. Harry Duncan whose knowledge, patience and enthusiasm ' for the science' has carried me through from the start. He encouraged me to explore many areas of research during my PhD, which gave me the opportunity to work with many interesting people, and made my PhD a lot more enjoyable than, I suppose, it should have been. For that I am sincerely grateful. In particular, I would like to acknowledge Margaret Smith of the Marine Technology Centre, University of Glasgow and Professor Keiji Gamoh, Kochi University, Japan who I had the pleasure of working with and Professor Henry Corseuil for extremely helpful discussions.

I would also like to acknowledge the Scottish Crop Research Institute, Dundee for funding this work and especially Professor J. R. Hillman and Dr. Richard Jefferies.

For all the advice and technical help I received during the years, I would like to thank Michael Beglan, Dr. Hugh Flowers, Fareidh Karabasi, Dr. Mazz Marry, Eoin Robertson, Dr. John Cole and Dr. David Morris. I would also like to thank Dr. Pete Ianetta, Scottish Crop Research Institute and Dr. Euan James, Dundee University for pointing me in the right direction to relevant reading material regarding root nodule structure. And not forgetting the number of people in the lab who watered my plants whilst I was on holiday, it was greatly appreciated.

Thanks to my family for always encouraging me to give things a go. And to my sister, Jen, whose thirst for knowledge has always been an inspiration to me. Without their support I would never have begun this PhD. My final thoughts fall to Iain, who has given me so much time and helped me in so many ways since I started my PhD. Thank you for keeping me sane, especially during the last couple of weeks of write up. You make it all worthwhile.

## Declaration

Except where specific reference is made to other sources, the work presented here is the original work of the author. It has not been submitted, in part or in whole, for any other degree. Some of the results have been published elsewhere.

Gillian Adam

## Summary

Diesel fuel is a complex mixture of hydrocarbons with an average carbon number of $C_9$-$C_{23}$. The majority of components consist of alkanes, both straight chained (n-alkanes) and branched and aromatic compounds including mono-, di- and polyaromatic hydrocarbons. Regardless of this complexity, diesel fuel can be readily degraded by a number of soil microorganisms making it a likely candidate for bioremediation. The concept of using plants to enhance bioremediation, termed phytoremediation, is a relatively new area of scientific interest. A branch of phytoremediation particularly suited to the remediation of diesel fuel contaminated soil is rhizodegradation. Rhizodegradation is the breakdown of contaminants in soil by microbial activity that is enhanced by the presence of the root zone (rhizosphere). Rhizodegradation is particularly applicable to diesel fuel contamination as diesel fuel, due to its physical properties, is normally held in the surface soil and does not leach far into the soil profile. Diesel fuel is therefore held within the rooting zone of the plant and is susceptible to rhizodegradation. Plants have been shown to encourage organic contaminant degradation principally by providing an optimal environment for microbial proliferation in the rhizosphere. These degradative processes are influenced not only by the rhizosphere microorganisms but also by the unique properties of the host plant which often leads to enhanced breakdown of organic contaminants in soils that are vegetated, compared to non vegetated soils. In practice however, there are many problems associated with establishing a beneficial plant cover on diesel fuel contaminated soil.

Diesel fuel was seen to be phytotoxic to plants at relatively low concentrations. The type of toxicity induced could be attributed to specific fractions of the total diesel fuel product. Acute toxicity, indicated by inhibition of germination was characteristic of the volatile diesel fuel fraction. Low molecular weight branched cyclohexanes and aromatics, identified by GC-MS to be present in the volatile fraction, were found to cause acute phytotoxic symptoms on selected plant species. A delay in germination was also found to be caused by the physical properties of diesel fuel. Diesel fuel would cause a film of oil to form around the seed which would act as a physical barrier, reducing the transfer of water and oxygen to the germinating seed thus 'suffocating' it. This physical effect was shown to delay seed emergence and in conjunction with the acutely phytotoxic effects of the volatile diesel fuel fraction, was the main cause of the overall inhibitory effect of diesel fuel contaminated soil on germination.

A large variation in the ability of plant species capable of tolerating diesel fuel contamination whilst germinating was observed. This ability was not species specific as members of the same plant family showed differential sensitivity to diesel fuel contamination. Differences were also found within plant subspecies.

Chronic toxicity was initiated by the higher molecular weight diesel fuel components with symptoms developing over time. A decrease in plant height and plant biomass was observed for the majority of plant species investigated during this study which is indicative of chronic toxicity. Developmental effects were also apparent on plants grown in diesel fuel contaminated soil including a delay in maturation of plants indicated by delayed seeding/flowering, a reduction in the number of nodules produced on roots of leguminous plants and differences in their nitrogen-fixing ability and an increase in the number of branched and adventitious roots present on plants grown in diesel fuel contaminated soil.

The physical effects of diesel fuel on soil also had implications for growth of plants on contaminated soils. The influence of diesel fuel on soil water holding capacity differed with the age of the product. Freshly contaminated soil showed a large increase in the volume of water held by the soil whereas 'aged' contaminated soil exhibited the tendency to repel water. This effect must be considered as an additional cause of poor plant performance in diesel fuel contaminated soils as it clearly has an effect on the soils ability to hold water and maintain appropriate moisture conditions for optimal plant growth.

Despite its adverse effect on plants, diesel fuel did not have an obvious detrimental effect on the soil microbial community. Assessment of soil health and nutrient cycling using a series of enzymological methods showed diesel fuel was used as a substrate for microbial proliferation and the cycling of nutrients through the soil system was continuing. Plants grown on this contaminated soil enhanced microbial activity even further by developing a rhizosphere community. The highest enzymic activities were always found in planted soils compared to unplanted soils. The influence of the plant was integral to biodegradation of diesel fuel in soil as was demonstrated by the highest rates of diesel fuel biodegradation being found in planted soils as opposed to unplanted soils. Differences in the amounts of diesel fuel remaining in soil were also found under different plant species. Legumes and their associated rhizosphere community seemed to provide the optimal environment for diesel fuel biodegradation in the rhizosphere.

If the introduction of ethanol to diesel fuel was adopted in the UK, the proposed remedial technique may be ineffective. Diesel fuel with ethanol added would move further in the soil profile, possibly taking the contaminant out of reach of the rooting zone. This would increase the threat of groundwater contamination and would require another method of remediation to be found.

## Contents

# TABLE OF CONTENTS

## CHAPTER ONE

INTRODUCTION



## CHAPTER TWO

MATERIALS AND METHODS

## CHAPTER THREE

METHOD DEVELOPMENT



## CHAPTER FOUR

ANALYSIS OF DIESEL FUEL

## CHAPTER FIVE

BEHAVIOUR OF DIESEL FUEL IN THE SOIL



## CHAPTER SIX

EFFECT OF DIESEL FUEL ON PLANT GROWTH AND DEVELOPMENT :
**EFFECT ON THE SEED**



## CHAPTER SEVEN

EFFECT OF DIESEL FUEL ON PLANT GROWTH AND DEVELOPMENT :
**EFFECT ON THE ROOT**



## CHAPTER EIGHT

EFFECT OF DIESEL FUEL ON PLANT GROWTH AND DEVELOPMENT :
**EFFECT ON THE PLANT**

## CHAPTER NINE

PHYTOREMEDIATION OF DIESEL FUEL CONTAMINATED SOIL



## CHAPTER TEN

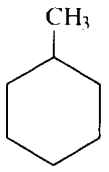
GENERAL DISCUSSION AND CONCLUSIONS

## Common and scientific names of referenced plants

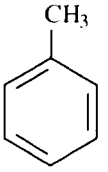
| Common Name | Scientific name |
|---|---|
| Cocksfoot | *Dactylis glomerata L.* |
| Creeping bent | *Agrostis stolonifera L.* |
| Highland bent | *Agrostis castellana L.* |
| Meadow bent | *Agrostis pratensis L.* |
| Common bent | *Agrostis capillaris L.* |
| Black grass | *Alopecurus myosuroides Huds.* |
| Couch grass | *Agropyron repens L.* |
| Sweet vernal grass | *Anthoxanthum odoratum L.* |
| Rough meadow grass | *Poa trivialis L.* |
| Westerwold's ryegrass | *Lolium multiflorum L.* |
| Sheep's fescue | *Festuca ovina L.* |
| Strong creeping red fescue | *Festuca rubra ssp. Rubra L.* |
| Chewing's fescue | *Festuca rubra ssp. Commutata L.* |
| Annual canary grass | *Phalaris canariensis L.* |
| Black medick | *Medicago lupulina L.* |
| Fodder burnet | *Sanguisorba minor ssp. Muricata L.* |
| Common vetch | *Vicia sativa L.* |
| Red clover | *Trifolium pratense L.* |
| White clover | *Trifolium album L.* |
| Little or lesser yellow trefoil | *Trifolium dubium L.* |
| Lucerne | *Medicago sativa L.* |
| Oil seed rape (Winter oil rape) cultivars Martina and Rocket | *Brassica napus var. olifera L.* |
| Flax cultivars Viking And Elise | *Linum usitatissumum L.* |
| Willow variety Rosewarne white | *Salix aurita x cinerea x viminalis* |
| Willow varieties Jorr, Jorrun and Ulv | Swedish varieties of *Salix viminalis* |

Plant species designated using a system of common names and scientific (binomial) names including authority (Emorsgate Seed Catalogue, 1997, Gill and Vear, 1969).

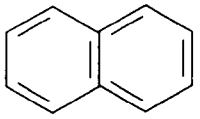
## List and structures of petroleum hydrocarbons used in this study



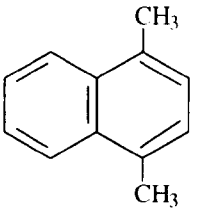

methyl cyclohexane



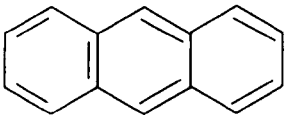

toluene



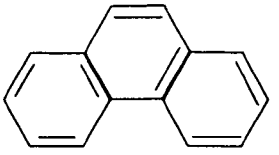
naphthalene



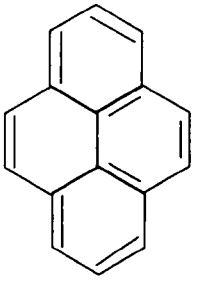

1, 4 dimethyl naphthalene



anthracene



phenanthrene



pyrene

Examples of compounds from each petroleum hydrocarbon group used in this study:

Branched cyclohexanes i.e. methyl cyclohexane

Monoaromatic hydrocarbons i.e. toluene

Diaromatic hydrocarbons i.e. naphthalene and 1,4 dimethyl naphthalene (1,4 DMN)

Polyaromatic hydrocarbons i.e. anthracene, phenanthrene and pyrene.

# CHAPTER ONE

## INTRODUCTION

### 1.1 Environmental Contamination with Petroleum Hydrocarbons

Since its first commercial oil well in 1859, more than 100 billion barrels of oil have been produced in the United States of America. In 1994, world petroleum consumption was at a rate of about 65 million barrels per day (Manahan, 1994). Crude oil is extracted from the earth as a raw material for petroleum refining which provides so much of the worlds energy. The production, transportation and refining of petroleum is, by a conservative estimate, introducing 6 million metric tonnes of oil annually into the oceans (Atlas and Bartha, 1981) and inevitably involves the risk of accidental spills. The wreck of the tanker *Torrey Canyon* in 1969 and the grounding of the *Arrow* tanker the following year (Wang *et al.*, 1994) focused environmental concern on the fate of hydrocarbon pollutants in the oceans (Atlas and Bartha, 1992). In recent years, the number of tanker collisions, releasing crude oil into the aquatic environment seem more frequent, starting with the spill from the *Exxon Valdez* in Prince William Sound, Alaska in 1989, causing widespread pollution and environmental damage. In addition, pollution of the marine environment of the Arabian Gulf, as well as on land, with crude oil following the 1991 Gulf war (Fayad and Overton, 1995) focused attention on pollution of the aquatic environment. These highly topical routes of oil contamination have received much attention and a wealth of literature exists

within the scientific community on oil contamination in the aquatic environment (Atlas and Bartha, 1981 and 1992, Prince, 1994, Fayad and Overton, 1995, MacNaughton *et al.*, 1999). What is less well documented is contamination of the environment by petroleum products and in particular contamination of the terrestrial environment.

Existing literature on petroleum contamination falls into one of three categories: bioremediation of hydrocarbons in soil by landfarming techniques or where fertiliser, aeration or inoculants have been added to artificially stimulate the degradation rate (Dibble and Bartha, 1979, Oudot *et al.*, 1989, Song *et al.*, 1990, Widrig and Manning, 1995, Margensin and Schinner, 1997a and 1997b, Brown *et al.*, 1998, Urarahy *et al.*, 1999, Jørgensen *et al.*, 2000, Roy and Greer, 2000); characterisation of and effect on microorganisms capable of growing on petroleum hydrocarbons (Song and Bartha, 1990, Nichols *et al.*, 1997, Nicolotti and Egli, 1998, Yuste *et al.*, 2000) and plant growth in oil contaminated soil (Dibble and Bartha, 1979, Bossert and Bartha, 1984, Xu and Johnson, 1995, Chaîneau *et al.*, 1997, Xu and Johnson, 1997, Carman *et al.*, 1998a and 1998b). Little information exists specifically on the bioremediation of diesel fuel contaminated soil or the effect of diesel fuel contamination on plant growth and development. The work that has been published on the effect of petroleum products, including diesel fuel, on plants has concentrated on the direct toxic effects of oil on the upper parts of the plant (Baker, 1970). Diesel fuel contamination studies have focused on bioremediation experiments with cold-adapted microorganisms (Margensin and Schinner, 1997a and 1997b), engineered biodegradation techniques in the surface soil or aqueous media (Widrig and Manning, 1995, Šepič *et al.*, 1996, Jørgensen *et al*) or bioremediation with fertiliser addition (Song *et al*, 1990, Roy and Greer, 2000). To my knowledge, only one study has investigated the process of natural attenuation of diesel fuel in soil (Berry and Burton, 1997) and phytoremediation of diesel fuel contaminated sites using Willow trees (Carman *et al.*, 1998a and 1998b).

## 1.2 Petroleum Hydrocarbon Contamination – Past and Present

There are more than 30,000 tonnes of waste oil unaccounted for in the UK each year. Oil accounts for one quarter of all pollution events dealt with by the Environmental Protection agencies. Oil pollution can occur from many sources, both accidental and intentional. Oil may be spilt during delivery or when storage tanks are filled, storage tanks may leak

because they are not maintained or because they are not protected from vandalism, used oil is poured down drains or allowed to seep into the ground. The increase in serious pollution incidents from oil prompted the Environment Agency to examine the effectiveness of current oil storage regulations. This led to the launching of the Oil Care Campaign in January 1995 which brought together the oil industry, local authorities, retailers and regulators in an effort to raise awareness of the impact of oil pollution. Despite the fall in the total number of incidents, the number of major oil pollution incidents rose in 1997. In 1997, in England and Wales, there were 5,542 substantiated fuel and oil pollution incidents, with diesel fuel responsible for 35% of the incidents (Environment Times, 1998). The Scottish Environmental Protection Agency (SEPA) and the three water authorities in Scotland deal with hundreds of oil pollution incidents each year. The most commonly encountered types of oil are diesel, central heating oil, waste oil and petrol. In 1996/1997, the total number of oil pollution incidents, from industrial, agricultural and other sources, was 426 (SEPA, 1997). This figure rose to 480 in 1997/1998 (SEPA, 1998). These incidents are recorded as they generally end up polluting a water body, which the authorities must deal with. Soil sites contaminated with oil will largely go unrecorded.

Petroleum refining unavoidably generates considerable volumes of oil sludges. Common sources of these sludges are storage tank bottoms, oil-water separators, cleaning of processing equipment and soil from minor spills on refinery grounds (Dibble and Bartha, 1979b). The disposal of these sludges on land is common practice (Dibble and Bartha, 1979a and 1979b, Bossert and Bartha, 1985, Ururahy *et al*, 1999, Brown *et al.*, 1998, Jørgensen *et al.*, 2000). Land farming or biopiling, as it is now known (Jørgensen *et al.*, 2000) is another route of petroleum hydrocarbon input into the soil environment.

Land may also be contaminated with petroleum hydrocarbons from railway yards and transportation refuelling areas where continual small spills have lead to chronic fuel pollution. A lot of agricultural farms, factories and army installations have their own fuel storage tanks for refuelling vehicles which is a possible source of fuel contamination. In addition, the large number of petrol stations around the country may add to contamination of the environment through refuelling mishaps and storage problems. Contamination also happens on a large scale when there are major transportation accidents, pipeline bursts or mechanical failure of pumps transporting petroleum products (Dibble and Bartha, 1979a, Widrig and Manning, 1995, Carman *et al.*, 1998a and 1998b).

Historic contamination by petroleum hydrocarbons is also likely to be found in many of the designated contaminated land and Brownfield sites found around the country where the origin and extent of contamination is unknown. In the U.K. alone, there may be up to 250, 000 hectares of contaminated land carrying a potential cleanup cost of almost £20 billion (Whittaker *et al.*, 1995).

From all these sources, the amount of land contaminated with petroleum hydrocarbons such as diesel fuel, in theory, is extensive and a means of remediating this land must be found.

## 1.3 Petroleum Hydrocarbons

Crude oil consists primarily of carbon and hydrogen with a wide variety of elements combined in various forms. Petroleum hydrocarbons are a mixture of hydrocarbons produced from the distillation of crude oil. During the refining process, petroleum products are strongly enriched with hydrocarbons leaving the majority of inorganic materials and other sulphur, nitrogen and oxygen containing organic compounds in the residual material.

### 1.3.1 General Petroleum Chemistry

Petroleum hydrocarbons are divided into two broad families: aliphatic and aromatic hydrocarbons. Aliphatic hydrocarbons are further divided into three main classes: alkanes, alkenes and cycloalkanes.

#### 1.3.1.1 Aliphatic Hydrocarbons

Alkanes contain only single carbon–carbon bonds. Straight chain alkanes or n-alkanes, as they are more commonly known, consist of carbons attached to no more than two other carbons in a linear fashion with two methyl ($CH_3$) terminations. Branched alkanes are structural isomers of n-alkanes which have alkane groups (alkyl) substituted onto hydrocarbon structures. For example, n-pentane, 2 methyl butane or isopentane and 2, 2

dimethyl propane or neopentane are all described by $C_5H_{12}$ but their structures are very different.

Alkenes are hydrocarbons that contain less hydrogen, carbon for carbon, than the corresponding alkane due to the occurrence of one or more double bonds in the alkenes structure. The simplest member of the series is ethene.

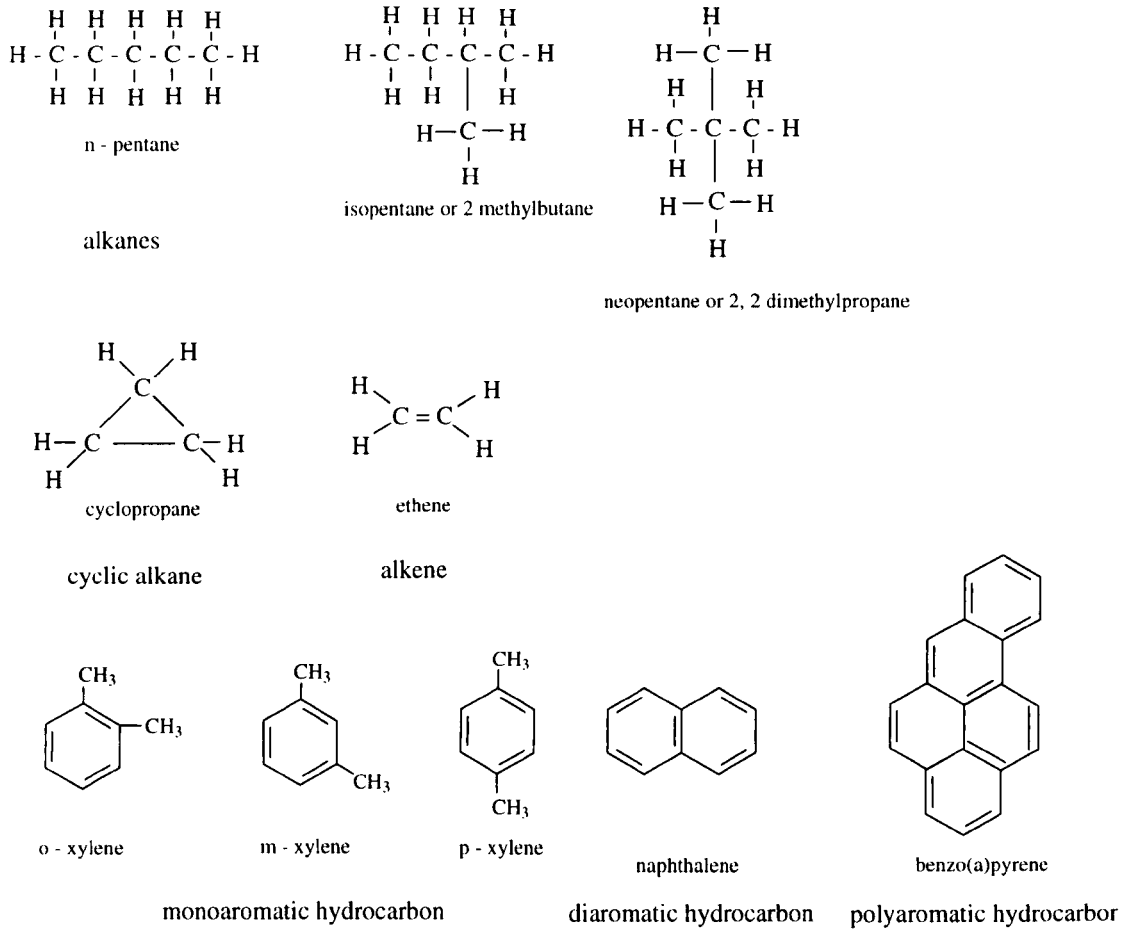
Cycloalkanes are alkanes where carbon atoms form cyclic structures, with the smallest member of the series being cyclopropane. Examples of these aliphatic hydrocarbons are given in Figure 1.3.1.1.

### 1.3.1.2 Aromatic Hydrocarbons

Aromatic hydrocarbons have one or more benzene rings as structural components. A monoaromatic compound has one benzene ring with either six hydrogen groups or a combination of alkyl and hydrogen groups attached. Xylene is a derivative of benzene, which is commonly found in lighter petroleum products and has three isomers.

A diaromatic compound has two fused benzene rings with either eight hydrogen or alkyl groups attached. Naphthalene is a diaromatic hydrocarbon found in the middle range petroleum distillates.

Polyaromatic hydrocarbons (PAHs) have more than two fused benzene rings and they can have various alkyl and hydrogen groups attached. Benzo(a)pyrene is an example of a carcinogenic 5 ringed aromatic which is found in heavier petroleum products and is persistent in the environment. In general, alkyl substituted PAHs predominate in petroleum (SCLF, 1998). Examples of these aromatic hydrocarbons are given in Figure 1.3.1.1.




From Holtzclaw *et al*, (1991) and Gibson and Subramanian, (1984).

Figure 1.3.1.1. Structural representation of selected aliphatic and aromatic hydrocarbons.

### 1.3.1.3 Pattern of Bonding in Hydrocarbons

Aliphatic and aromatic compounds differ by the patterns of bonding between adjacent carbon atoms. Aromatic molecules have ring structures and are basically symmetric with clouds of electrons above and below the plane of the molecule. Aromatic carbon–carbon bonds are termed resonance bonds as electrons are shared between multiple carbon atoms. This 'sharing' of electrons imparts chemical stability to the structure. The bonding pattern of aromatic structures also contributes to their moderate polarity. The electron clouds surrounding the molecules can be deformed by the charge on adjacent molecules resulting in the development of partial positive and negative charges on the molecule.

Aliphatic structures have highly directional bonds where carbon atoms share electrons only with adjacent carbon atoms. This allows free rotation of the structure around these bonds which allows aliphatic structures to assume many different conformations. The bonding pattern of aliphatic structures does not permit the non uniform distribution of charge found with aromatic structures to the same degree, thus aliphatics are non polar or only slightly polar.

The polarity of hydrocarbons governs the degree to which they will interact with themselves and with water and as a general rule, as polarity increases, so does water solubility and boiling point. Therefore aromatics are more water soluble and less volatile than alkanes with the corresponding number of carbon atoms.

Within each hydrocarbon structural family and sub family, there are homologous series. Each member of the series differs from adjacent members in the series by a repeating unit and the physical properties of compounds change with the number of carbon atoms. For example, there is an increase in the boiling point of approximately 20°C for each carbon atom added to an n-alkane chain.

Another feature of petroleum hydrocarbons is that they have large numbers of isomers. Isomers are compounds that have the same elemental formula but have different structural configurations. In general, as the carbon number increases, the number of possible isomers increases rapidly (Holtzclaw *et al.*, 1991). The large number of isomeric compounds accounts for petroleum products high degree of complexity. Petroleum products with high boiling point constituents have high average carbon numbers therefore, they have a large number of isomers and greater chemical complexity than petroleum products with low boiling point constituents.

### 1.3.1.4 Other Components of Petroleum Mixtures

Organic compounds containing sulphur, nitrogen and oxygen may be encountered at significant concentrations in crude oil and in some heavier fuels such as No. 6 fuel oil. Sulphur containing aromatic compounds are the major constituents.
Metals are also encountered in petroleum fuel mixtures in the form of salts of carboxylic acids or as porphyrin chelates.

## 1.3.2 Petroleum Products

Petroleum fuel mixtures are produced from crude oil through a variety of refining and blending processes. After treatment to remove dissolved gas, dirt and water, crude oil is distilled and a variety of petroleum product fractions result. Some of the more important hydrocarbon products derived from the refining of petroleum hydrocarbons are given in Table 1.3.2.1.

### 1.3.2.1 Diesel #2 Fuel

Transportation diesels are manufactured primarily from distilled fractions of crude oil with some blending with cracked gas oils. The major components of diesels are similar to those present in crude oil but with a larger percentage of aromatics (up to 30-40%). There are five different grades of diesel fuel for uses that range from cars, commercial trucks and buses to marine and railroad engines. Additives are used in diesel fuels to protect the fuel system against deposits, rust and corrosion and to keep the fuel system components clean. Table 1.3.2.1.1 shows the typical classes of diesel fuel additives.

| Product | Main carbon no. range | Boiling point range °C | Additional information |
|---|---|---|---|
| Petrol (gasoline) | C4 to C12 | 25 to 215 | Contains light aliphatics that evaporate easily, aromatics primarily BTEX, low amounts of PAHs. |
| Kerosene and jet fuel | C11 to C13 | 150 to 250 | Contains aliphatic and aromatic hydrocarbons, aromatics make up about 10%. |
| Diesel and light fuel oils | C10 to C20 | 160 to 400 | mid range distillates that are less volatile and less water soluble, aromatic content around 25-35%, BTEX levels are low. |
| Heavy fuel oils | C19 to C25 | 315 to 540 | More viscous than water, contain around 15-40% aromatics. |
| Motor and lubricating oils | C20 to C45 | 425 to 540 | Viscous and insoluble in water, may contain 10-30% aromatics including PAHs. |
| Waxes and bitumen | >C30 | >350 | |

Adapted from S.C.L.F., 1998.

Table 1.3.2.1. General characteristics of petroleum products.

| Additive | Composition |
|---|---|
| Ignition improvers | Alkyl nitrates and nitrites, nitro- and nitroso-compounds, peroxides |
| Combustion catalysts / deposit modifiers | Organometallics of Ba, Ca, Mn, Fe<br>Mn, MnO, Mg, MgO, $MgO_2$, $AlO_3$ |
| Anti-oxidants | N-N dialkylphenylenediamines, 2,6 dialkylphenols, chlorinated hydrocarbons |
| Detergents/dispersants | Alcohols, amines, alkylphenols, carboxylic acids, sulfonates and succinamides |

Table 1.3.2.1.1. Typical classes of diesel fuel additives.

The type of diesel fuel investigated during this study was normal, car diesel fuel or diesel #2 fuel. Diesel #2 fuel is classed as a middle petroleum distillate and has a typical carbon range of $C_8$ to $C_{26}$ with the majority of components in the $C_{10}$-$C_{20}$ range. Diesel fuel is a complex mixture of hydrocarbons. The majority of components consist of alkanes, both straight chained, branched and cyclic, and aromatic compounds including mono-, di- and polyaromatic hydrocarbons (PAHs). Of the medium distillate fuels used in terrestrial situations, diesel has the highest content of PAHs and total aromatics (Wang *et al.*, 1990) which make it increasingly difficult to remediate. Regardless of this complexity, diesel fuel can be readily degraded by a number of soil microorganisms making it a likely candidate for bioremediation.

## 1.4 Biodegradation of Petroleum Hydrocarbons in Soil

It has been widely demonstrated that nearly all soils and sediments have populations of microorganisms that are capable of degrading petroleum hydrocarbons. It has been noted that about 20% of all microbial species examined have some capacity to degrade hydrocarbons (Higgins and Burns, 1975). Table 1.4.1 lists examples of the genera of hydrocarbon-degrading microorganisms isolated from soil.

| Bacteria | Fungi |
|---|---|
| *Achromobacter* † | *Aspergillus* ‡ |
| *Acinetobacter* | *Botrytis* |
| *Agrobacterium* | *Candida* |
| *Alcaligenes* † | *Chryososporium* |
| *Azotobacter* | *Fusarium* |
| *Bacillus* | *Gliocladium* |
| *Chromobacterium* | *Mortierella* ‡ |
| *Flavobacterium* † | *Paecilomyces* |
| *Methanobacterium* | *Penicillum* ‡ |
| *Micrococcus*† | *Rhodotorula* |
| *Mycobacterium*† | *Saccharomyces* |
| *Nitrosomonas* | *Spicaria* |
| *Nocardia* † | *Tolypocladium* |
| *Pseudomonas* † | *Trichoderma* ‡ |
| *Sarcina* | *Verticillium* |
| *Vibrio* | |
| *Xanthomonas* | |

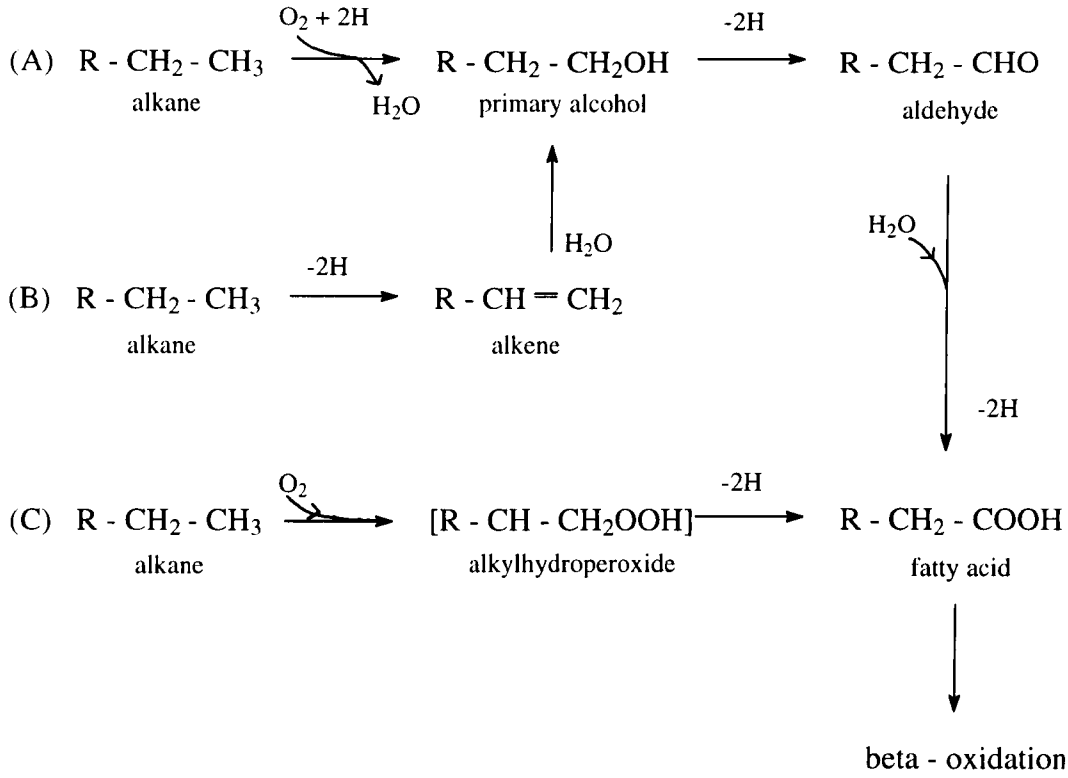
† *most consistently isolated hydrocarbon-degrading bacteria from soil*
‡ *most consistently isolated hydrocarbon-degrading fungi from soil*

compiled from Bossert and Bartha, 1984 and Walton *et al.*, 1993.

Table 1.4.1. Genera of hydrocarbon-degrading microorganisms isolated from soil.

### 1.4.1 Biodegradation of Individual Petroleum Hydrocarbons

Susceptibility to biodegradation varies with the structure and size of the hydrocarbon molecule (Alexander, 1994). The n-alkanes are the most readily degraded by the widest range of microorganisms, with alkanes of intermediate chain length ($C_{10}$-$C_{24}$) degraded most rapidly. Figure 1.4.1.1 shows the initial attack on alkanes which can occur according to three mechanisms. It is generally accepted that both short chain and long chain alkanes are oxidised monoterminally to the corresponding alcohol, aldehyde and fatty acid. Short chain alkanes are toxic to many microorganisms and very long chain alkanes become increasingly resistant to biodegradation (Atlas and Bartha, 1981). Branched n-alkanes are less easily degraded, with rate of degradation being inversely proportional to the degree of branching (Higgins and Burns, 1975). Branching reduces the rate of biodegradation by interfering with the degradation mechanisms or blocking degradation altogether.




(A) oxidation to alcohol, aldehyde and fatty acid, (B) dehydrogenation to alkene followed by conversion to alcohol, aldehyde and fatty acid and (C) formation of fatty acid through labile hydroperoxide intermediate. Pathway B was demonstrated *in vitro* but does not appear to occur on a significant scale in the environment. Further degradation of the fatty acids occurs by beta-oxidation (from Atlas and Bartha, 1981).

Figure 1.4.1.1. Oxidation of terminal methyl groups by alkane degrading microorganisms.

Cyclic alkanes are the least susceptible to microbial attack (Higgins and Burns, 1975) as they are frequently unable to serve as sole carbon sources for microbial growth unless they have a sufficiently long aliphatic side chain. They can however be degraded via co-metabolism by two or more co-operating microbial strains with complementary metabolic capabilities (Atlas and Bartha, 1981). Cyclic hydrocarbons having no terminal methyl groups can be degraded by a few microorganisms by the mechanism shown in Figure 1.4.1.2 but more commonly, co-metabolism is the primary mechanism in the biodegradation of cyclic hydrocarbons.

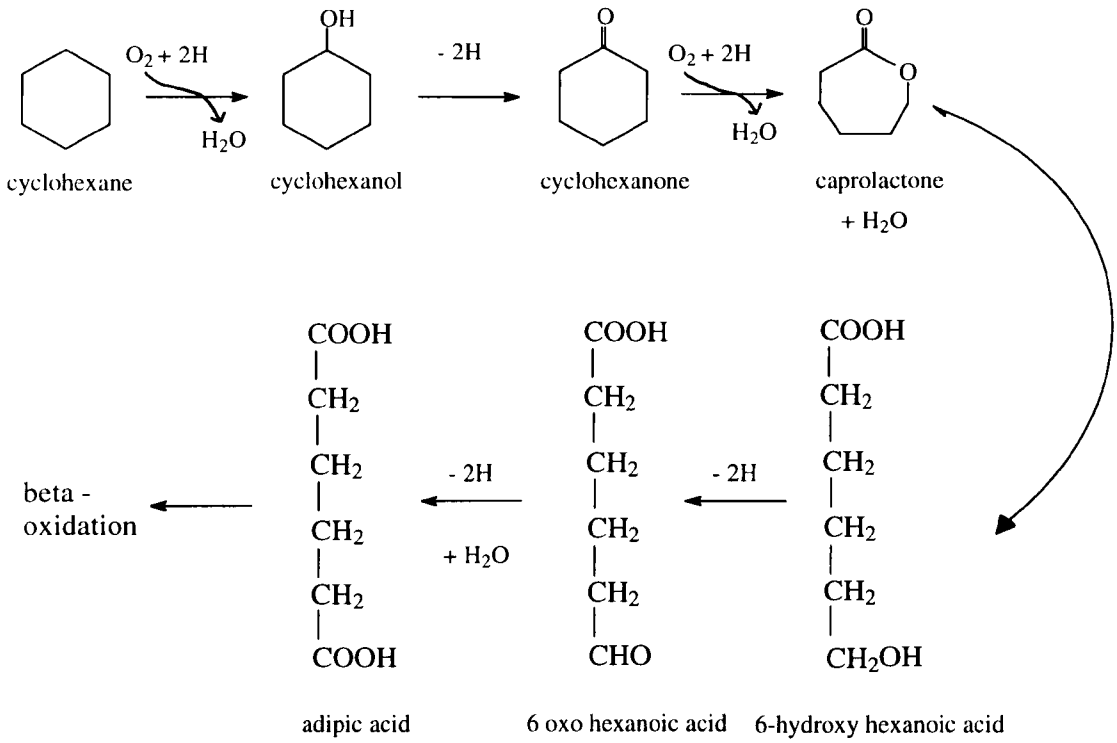



Figure 1.4.1.2. Microbial oxidation of cyclohexane as an example for metabolism of cyclic hydrocarbons (from Atlas and Bartha, 1981).

Aromatic hydrocarbons, especially polyaromatic hydrocarbons, are degraded more slowly than alkanes. Aromatic hydrocarbons are oxidised in one or more steps to catechol then further degraded by opening of the aromatic ring by oxidative *ortho* or *meta* cleavage. Figure 1.4.1.3 illustrates the possible mechanisms of aromatic degradation. Many condensed polyaromatic hydrocarbons are degraded with difficulty or not at all (Atlas and Bartha, 1981).

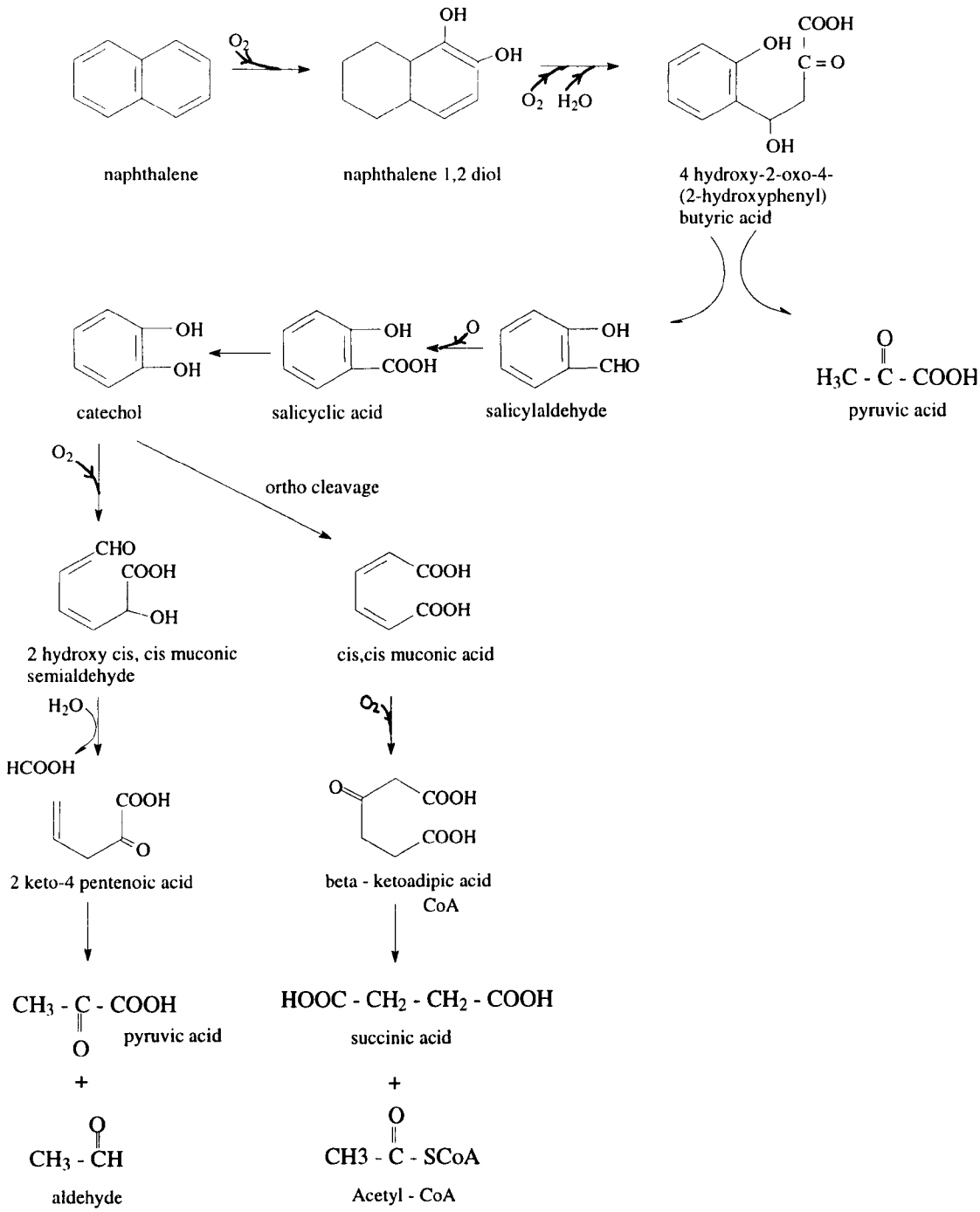



Figure 1.4.1.3. Microbial metabolism of a condensed aromatic ring structure as shown for naphthalene. The resulting catechol is further metabolised by ortho- or meta- cleavage (from Atlas and Bartha, 1981).

### 1.4.2 Biodegradation of Petroleum Products

Biodegradation of petroleum products has been demonstrated in a number of studies. In 1979, Dibble and Bartha demonstrated biodegradation of kerosene petroleum hydrocarbons in agricultural soil following a pipeline break. The disappearance rate of the higher molecular weight compounds (n-$C_{13}$ to n-$C_{17}$) was slightly faster than the disappearance of the n-$C_{10}$ to n-$C_{13}$ compounds. This pattern points to biodegradation as the principle disappearance mechanism, since the compounds disappeared in order of their preferential microbial utilisation rather than in order of their volatility (Dibble and Bartha, 1979). Song *et al* (1990) illustrated that the environmental persistence of medium distillate fuels increased in the order: jet fuel > heating oil > diesel fuel. Bioremediation treatment substantially reduced the persistence of all three of these fuels but a residual fuel content remained. In studies concerning diesel fuel, rapid biodegradation of the n-alkanes ($C_{14}$-$C_{18}$) was observed in aqueous solutions, with aromatic compounds proving to be more resistant to biodegradation (Šepić *et al.*, 1996). Even with biopiling studies, a residual hydrocarbon content is left with total degradation of lubricating and diesel fuel after 5 months being 70% and 71% of the original amount added (Jørgensen *et al.*, 2000).

It is apparent that microorganisms capable of degrading hydrocarbons are widespread and present in most soil environments but biodegradation of heavier petroleum products is never complete. Diesel fuel is a middle distillate fuel composed mainly of n-alkanes, which should, in theory, degrade rapidly. Diesel fuel had a high % of aromatics including polyaromatic hydrocarbons (PAHs) as well as cyclic and branched alkanes, which will prove more difficult to degrade. By encouraging a larger and more varied microbial population to develop in soil contaminated with petroleum hydrocarbons, increased and enhanced biodegradation may be observed.

## 1.5 The Rhizosphere

The microbial population of soil is altered both quantitatively and qualitatively by the presence of plant roots (Rovira and McDougall, 1967). The zone of soil in which this root influence is extended is called the 'rhizosphere' and was first described by Hiltner in 1904 (Curl and Truelove, 1986). This specialised region is characterised by enhanced microbial biomass and activity. The rhizosphere community consists of microorganisms, mainly bacteria, fungi and algae and micro and mesofauna, such as protozoa, nematodes, mites

and insects (Walton *et al.*, 1994a). The abundance of microorganisms in the rhizosphere is commonly 5 to 20 times higher than that of non vegetated soil (Atlas and Bartha, 1993).

Roots provide plants with anchorage and the means to acquire water, nutrients and other growth substances from soil. In addition, roots provide rhizosphere microorganisms with highly favourable conditions for growth. Roots provide structure for microbial colonisation and support microbial communities through inputs of photosynthate to the rhizosphere. Organic compounds released from roots, root exudates and sloughed, dead cells are the cause of the increased microbial biomass in the rhizosphere compared to non rhizosphere soil. This release of photosynthate and the rapid decay of fine roots also increases soil organic matter, which may in turn alter contaminant adsorption, bioavailability and leachability (Schnoor *et al.*, 1995).

### 1.5.1 Plant- Microbial Interactions

The species of plant growing in the soil is a critical factor in the development of a rhizosphere community. Perhaps the best characterised bacterial associations with plant roots are those of the nitrogen fixing bacteria (rhizobia) and leguminous plants. These symbiotic associations are unique for both their nutrient contribution to soils and the physiological relationship between bacteria and plant host. The bacteria invade the root tissues, which respond by forming nodules that become the site of bacterial nitrogen fixation. The interaction of leguminous plants with nitrogen-fixing bacteria results in increased microbial biomass, plant growth and root exudation perhaps because of the increased availability of soil nitrogen in the presence of nitrogen-fixing bacteria. This in turn may lead to enhanced microbial degradation of organic compounds in the rhizosphere by these bacteria (Anderson *et al.*, 1993).

The type, quantity and effectiveness of exudates and enzymes produced by plant roots vary between plant species and even within subspecies. Plants that produce exudates that have been shown to stimulate growth of degrading microorganisms or stimulate co-metabolism would be more beneficial than plants without such directly useful exudates. Red mulberry, crab apple and osage trees produce exudates containing relatively high levels of phenolic compounds at concentrations capable of stimulating growth of PCB-degrading bacteria (Fletcher and Hedge, 1995). Research into this area of rhizodegradation

is still at an early stage and no specific plant species has been found that produce exudates capable of stimulating hydrocarbon-degrading bacteria capable of degrading diesel fuel.

Numerous studies have shown increased biodegradation of organic contaminants in vegetated as opposed to unvegetated soils (see Section 1.6.2.). However, the interactions between plants and bacteria that increase contaminant degradation are not fully understood.

### 1.5.2 Plant-Microbe-Toxicant Interaction in the Rhizosphere

What is the specificity of the interaction between plants and bacteria that leads to contaminant degradation? The answer to this question will determine the strategy used to develop and evaluate phytoremediation methods. One such answer to this question was proposed by Walton *et al* (1994), that plants produce specific signals in response to a contaminant which increases microbial activity causing increased biodegradation of the contaminant of concern. For example, when a chemical stress (contaminant) is present in the soil, a plant may respond by increasing or changing root exudation (carbon allocation) to the rhizosphere. As a result, the microbial community, in turn, increases the detoxication rate of the contaminant. This microbial response could be an increase in microbial numbers, an increase in synthesis of detoxication enzymes or a change in the relative abundance of the microbial strains in the rhizosphere that can degrade the contaminant. Figure 1.5.2.1 illustrates this plant-microbe-toxicant interaction which gives protection to the plant by inducing the metabolic capabilities of the rhizosphere microbial community.

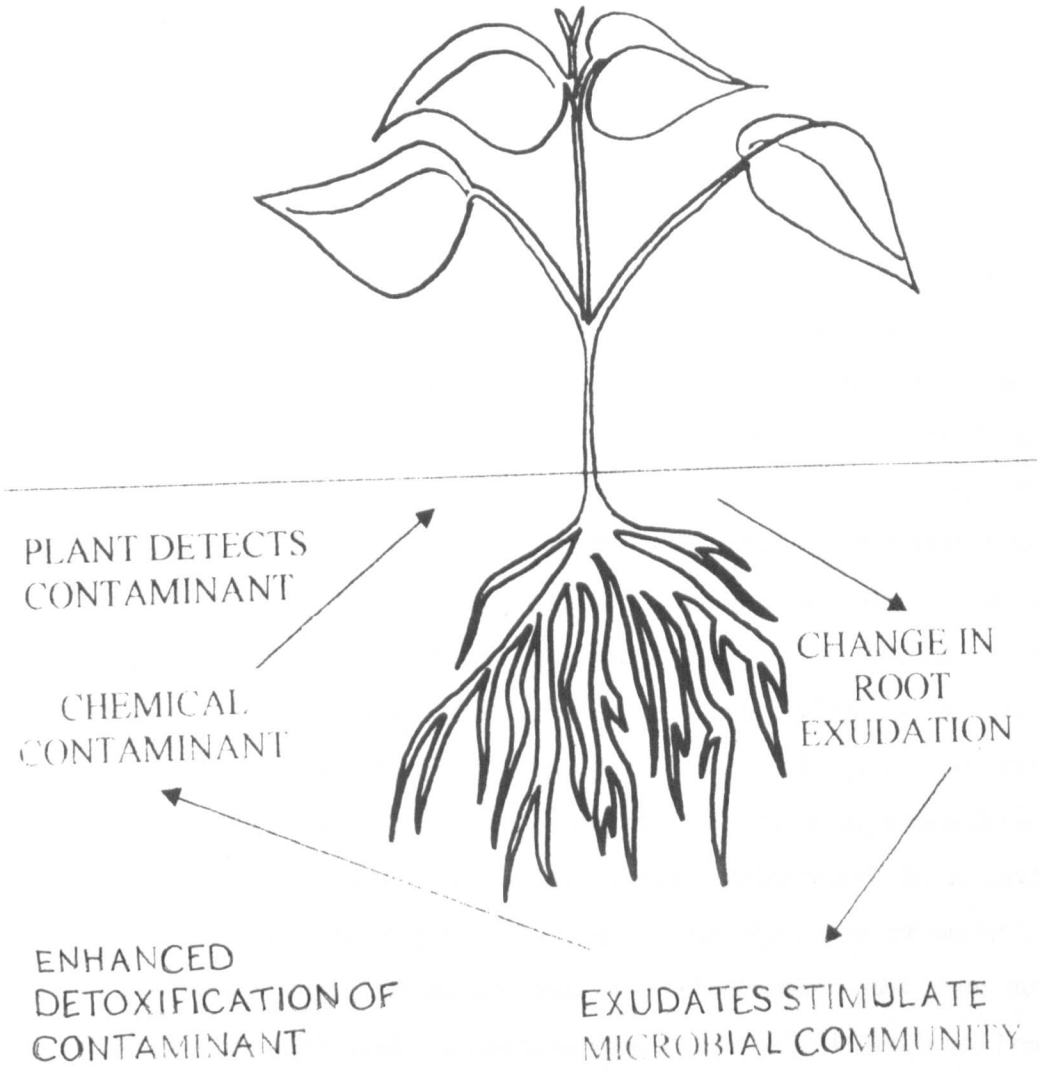



Figure 1.5.2.1. Hypothetical mechanism by which the rhizosphere microbial community may be influenced by the host plant to promote detoxication of a contaminant in soil (positive feedback mechanism).

By encouraging plants to grow on contaminated soil you are enhancing the microbial population that will degrade the contaminant for you. This type of remediation is called phytoremediation and has recently become an area of intense study.

## 1.6 What is Phytoremediation?

The term 'phytoremediation' is formed from the Greek prefix *phyto* (meaning plant) attached to the Latin suffix *remedium* (meaning to cure or restore) (Cunningham *et al.*, 1997). In its simplest form, phytoremediation is the use of green plants to remediate contaminated soil, sediment and water (Cunningham *et al*, 1997, Salt *et al.*, 1998). The use of plant based systems for cleaning up wastes is not new. In fact, the use of plants to cleanse waters contaminated with organic and inorganic pollutants dates back hundreds of years (Cunningham *et al*, 1996) and forms the basis of the reed bed, constructed wetland and municipal waste water treatment technology that we use today. The concept of using plants to remediate contaminated soils is a more recent development and has become an area of intense scientific study. Soil based phytoremediation technologies are in various stages of development, with laboratory research and field trials being conducted to determine the biological, chemical and physical processes that are involved. Within the research community concerned with phytoremediation of contaminated soils, research on the use of plants to remediate inorganic contaminants is extensive and progressing at a more rapid pace than research in organic contaminants. Inorganic contaminants are relatively immutable and therefore their fate in the environment is easier to trace. Organic contaminants, on the other hand, undergo physical, biological and chemical changes, making analysis inherently difficult. Nonetheless, several phytoremediation technologies for the treatment of shallow groundwater organic contaminants are in advanced stages of field trials. Trees with deep roots and high transpiration rates are being tested to address the problems of landfill leachates, pesticide contamination and plant nutrients such as nitrate and phosphate, under commercial names such as Ecolotree®cap, Treemediation and Rhizofiltration (Cunningham *et al.*, 1996). All these processes have targeted relatively water soluble contaminants in surface or shallow subsurface waters. Surface soil decontamination of relatively insoluble/insoluble contaminants *in situ*, is less well documented, both in the laboratory and in the field.

### 1.6.1 The Phytoremediation Industry

The US phytoremediation industry consists of dedicated phytoremediation companies whose sole or primary remediation technology is phytoremediation, other speciality companies diversifying into hazardous waste phytoremediation from areas such as

constructed wetlands, consulting/engineering firms that have developed an expertise in phytoremediation and a large number of academic, government and other non profit research groups conducting research and developing new technologies (D. Glass Associates, 1998). In 1998, D. Glass Associates carried out a survey of phytoremediation markets in the United States of America and found phytoremediation to be applicable to a number of hazardous waste scenarios. The largest phytoremediation market in the U. S. was for the treatment of organic contaminants in groundwater which was estimated at $5-10 million. Other large markets included control of landfill leachate, estimated at $3-5 million and remediation of metals in soils, also about $3-5 million. A few, smaller markets existed including remediation of organics in soils and organics in wastewater, each estimated at $2-3 million and the remediation of radionuclides in soil and groundwater, estimated at $0.5-1 million. Phytoremediation offered an alternative treatment strategy to the remediation of soil and water with sizeable potential markets (D. Glass Associates, 1998). D. Glass Associates predicted the markets involving organic contaminants should see a strong, steady growth in coming years with the markets involving metals and radionuclides capable of dramatic growth as the technology becomes better established.

Britain is at the very early stages of adopting phytoremediation as a viable cleanup strategy for contaminated land, therefore the majority of the research is carried out by academic research groups. A few of the larger companies conduct internal research or tender research to universities on phytoremediation projects particularly relevant to their company. On such example is BP Amoco who has carried out research on methyl tertiary butyl ether (MTBE) remediation in groundwater using willow trees (personal communication). To my knowledge, there has been no similar survey to the one carried out by D. Glass Associates on the potential phytoremediation market in the UK. However, many of the contamination problems encountered in the U.S. are mirrored in the U.K. therefore parallels can be drawn to the potential phytoremediation markets.

### 1.6.2 Contaminants Targeted for Phytoremediation

Phytoremediation is potentially applicable to a diverse range of substances, including many of the major environmental contaminants.

Early work involving metal hyperaccumulators focused on heavy metals (Cd, Cr, Pb, Co, Cu, Ni, Se, Zn) and radionuclides (Cs, Sr, Ur) (Cunningham *et al.*, 1996). However, phytoremediation has been demonstrated successfully against other inorganic

contaminants including arsenic and various salts and nutrients (nitrate, ammonium, phosphate) (Schnoor *et al.*, 1995).

The ability of plants to take up and metabolise a range of environmentally problematic organic contaminants has also been established. Examples include, chlorinated solvents (TCE, PCE) (Walton and Anderson, 1990), ammunition wastes (TNT, DNT, TNB, RDX, HMX), polychlorinated biphenyls (PCBs) and pesticides (Burken and Schnoor, 1997). In addition to the direct uptake and metabolism of organics, plants release exudates from their roots that enhance microbial bioremediation in the root zone (rhizosphere). Examples of contaminants susceptible to rhizosphere degradation are petroleum hydrocarbons (BTEX, PAHs) (GWRTAC, 1996, Ferro *et al.*, 1998, Wetzel *et al.*, 1998, Qui *et al.*, 1998, Binet *et al.*, 2000).

### 1.6.3 Contaminant Distribution and Site Considerations

The distribution of a contaminant in the soil is important to both the feasibility and design of a phytoremediation system. Contaminant distribution will depend on the age of the contaminant, source of the contaminant and nature of the spill as well as the soil type, rainfall and temperature. Contaminant availability is probably the most important parameter in biological remediation strategies as contaminants can be sequestered in a soil in such a way that they cannot interact with soil microorganisms and cannot be metabolised and degraded. Phytoremediation is most effective if soil contamination is limited to 1-2 metres depth from the surface (rooting zone) or if groundwater is within approximately 5 metres of the surface (hydraulic capture zone of plant roots). Sites with low to moderate soil contamination spread over large areas and sites with large volumes of groundwater with low level contamination are particularly suited to phytoremediation.

### 1.6.4 Types of Phytoremediation Processes

Phytoremediation either removes the contaminant from the matrix (decontamination) or sequesters it into the matrix (stabilisation). Several different types of phytoremediation exist based on the natural processes carried out by plants.

Phytodecontamination processes

1. Phytoextraction : extraction and accumulation of contaminant into the plant tissue with subsequent harvesting of plant tissue for destruction e.g. heavy metals, radionuclides.
2. Phytovolatilisation : plants and their associated microbial activity help to increase the rate of volatilisation of a contaminant e.g. certain organics
3. Phytodegradation : plants take up the contaminant and metabolises it to an non harmful by product e.g. chlorinated solvents, pesticides
4. Rhizodegradation : plant roots, their associated microbiota and/or exudates destroy the contaminant in the root zone e.g. petroleum hydrocarbons.

Phytostabilisation processes

1. Humification : incorporation of contaminants into soil humus resulting in lower bioavailability
2. Lignification : toxic components become irreversibly trapped in plant cell wall constituents
3. Irreversible binding : compounds become increasing unavailable due to binding into soil (aging).

### 1.6.5 Plant Species used in Phytoremediation

A variety of naturally occurring and specially selected plant species are used in phytoremediation. Hyperaccumulators, plants that can translocate and tolerate concentrations of heavy metals approximately 100 or more times the usual concentrations (Negri, 1996) seemed ideal candidates for phytoextraction. However, these species appear to hyperaccumulate only one metal in abundance, are slow growing and produce low biomass (Cunningham *et al.*, 1997). No matter how fascinating biologically, hyperaccumulators are poor candidates for phytoremediation systems and researchers are turning to other species as more promising commercial candidates. Deep rooting trees of the *Salicaceae* family (Willow and Poplar) are most commonly used for applications requiring withdrawal of large amounts of water and as barriers to subsurface flow of contaminated groundwater (Stomp *et al.*, 1993) due to their flood tolerance, high transpiration rates and fast growth (Schnoor *et al.*, 1995). Grasses such as ryegrass and fescue are used in the remediation of PAH contaminated soils (Aprill and Sims, 1990, Ferro *et al*, 1998, Binet *et al*, 2000) due to their fibrous root systems with extensive surface

area for microbial colonisation. Grasses appear to be ideal for both phytoremediation and erosion control because their fibrous root systems form a continuous dense rhizosphere (Ferro *et al*, 1998). Nitrogen fixing plants such as legumes (e.g. alfalfa) have been used in some trials because nitrogen is a critical component in the mineralisation of organics in soil.

It is apparent that more plants that grow well in contaminated soils need to be identified and screened for use in phytoremediation technologies.

### 1.6.6 Advantages of Phytoremediation

There are numerous reasons for the increasing level of research on the beneficial effects of vegetation on contaminated soil. Primarily, plants provide a cost effective method of remediating contaminated soil as plants use solar energy to grow, which is inexpensive and widely available. Plants transpire considerable amounts of water which can help bring mobile contaminants to the root zone (rhizosphere) where they are taken up by the plant and accumulated or metabolised. This can slow or even prevent the downward migration of the contaminant. The rhizosphere, due to the increased amount of organic carbon added to the soil, also provides an excellent environment for the adsorption and microbial degradation of organic contaminants. Plants can enhance oxygen transfer to microbial communities by transporting oxygen within the plant and by lowering the water table so that gas phase diffusion can occur in the soil. Vegetation is aesthetically pleasing and can stabilise soils against erosion by wind and water (Ferro *et al.*, 1998). When wind blown dust is controlled, it reduces an important pathway for human exposure via inhalation of soil and ingestion of contaminated food (Schnoor *et al.*, 1995). Vegetation can also be managed inexpensively and can produce biomass for chemical or energy applications (Erickson *et al.*, 1998).

### 1.6.7 Disadvantages of Phytoremediation

There are significant depth limitations to phytoremediation. Phytoremediation is most effective at sites with shallow contaminated soils, where contaminants can be treated in the rhizosphere and by root uptake (Schnoor *et al.*, 1995). Relatively mobile contaminants

could be drawn to plants by a combination of root presence and the hydraulic gradient induced by transpiration from a depth of approximately 10 metres. With relatively immobile contaminants, most researchers consider remediation possible only within the top 0.5 to 2 metres. Although the maximum depths to which roots can be found are often considerably deeper, the root density decreases dramatically with increasing depth (Cunningham *et al.*, 1996). Soluble exudates are produced by plants, which may bind contaminants and enhance their migration off site. The time taken for phytoremediation technologies to show beneficial effects are slow compared to traditional *ex situ* engineering methods. It may take several growing seasons to reach the desired contaminant concentration level. If contaminants are mobilised and accumulated in the plant there is the potential for contamination of the food chain as well as release of contaminants back to the environment if, for example, contaminants collect in leaves and are released during litter fall.

## 1.7 Why Diesel Fuel is a Suitable Candidate for Phytoremediation

Diesel fuel, on entering the terrestrial environment, will spread and seep into the soil. However, the downward migration of diesel fuel through the soil profile is limited due to the physical properties of the fuel. Under normal conditions, diesel fuel will be adsorbed in the organic matter rich surface soil, impeding downward migration. Accidental oil contamination has been shown to accumulate in the surface soil, with the highest percentage being in the top 0-20cm (Kiss et al., 1998). This makes diesel fuel contaminated soil appropriate for phytoremediation techniques as the contaminant is held in the surface soil and within the rooting zone of most plant species. The type of phytoremediation process that is particularly suited to diesel fuel contaminated soil is rhizodegradation. Rhizodegradation is the breakdown of contaminants in soil by microbial activity that is enhanced by the presence of the root zone. As discussed previously, exudates, produced and released from plant roots can increase the microbial biomass and activity in the rhizosphere. These exudates include sugars, amino acids, organic acids, fatty acids, sterols, growth hormones, enzymes and other compounds. Degradation of root exudates can lead to co-metabolism of contaminants in the rhizosphere. The use of exudates as co-metabolites to contaminants in the rhizosphere is particularly pertinent to diesel fuel biodegradation as many of the cyclic alkanes and aromatic compounds can be

successfully degraded by co-metabolism but cannot be used as the sole carbon substrate by microorganisms. Plant roots substantially increase the surface area where active microbial degradation can be stimulated and can affect soil conditions by increasing aeration and moderating soil moisture content, thereby creating more favourable conditions for biodegradation by indigenous microorganisms. Again, the increase in aeration afforded by plant roots is beneficial to diesel fuel degradation as it is an almost strictly aerobic process.

Another aspect of diesel fuel on entering a soil system is evaporation to the atmosphere. A sizeable proportion of diesel fuel is made up of volatile components that will evaporate after a spill. These lighter, more volatile components will disappear rapidly from the soil surface but diesel fuel which has seeped into the surface soil will release these volatile components more slowly. This creates a problem, in a phytoremediation sense, as short chain hydrocarbons and light aromatics are known to be toxic. Few authors have investigated this area of phytotoxicity of volatile diesel fuel components so little is known about the possible effects on plants of these volatile components. What is known, dates back to research into fuels, such as diesel, as herbicides. Previous to 1930, no special weed oils were made and unaltered diesel and smudge oil were used (Overbeek and Blondeau, 1954). The principal reason for the use of oils as herbicides was that they were especially suited to the elimination of weedy grasses and when properly applied, did not harm the crop plants and did not leave toxic residues. Diesel fuel was noted as a valuable general contact herbicide because of its low interfacial tension on plants and because its content of chronic toxicants were effective against grasses but it was too heavy and caused chronic toxicity to which the crop plants were not tolerant (Crafts and Reiber, 1948). This suggests that diesel fuel phytotoxicity comes entirely from the heavier fuel components but a later report by Currier (1951) illustrated the phytotoxicity of benzene derivatives. Currier found toxicity to increase in the order: benzene, toluene, xylene, trimethylbenzenes in both vapour and spray treatments (Currier, 1951). All these compounds are likely to be found in the volatile fraction of diesel fuel and may affect plant growth in diesel fuel contaminated soil.

## 1.8 Soil Enzymology

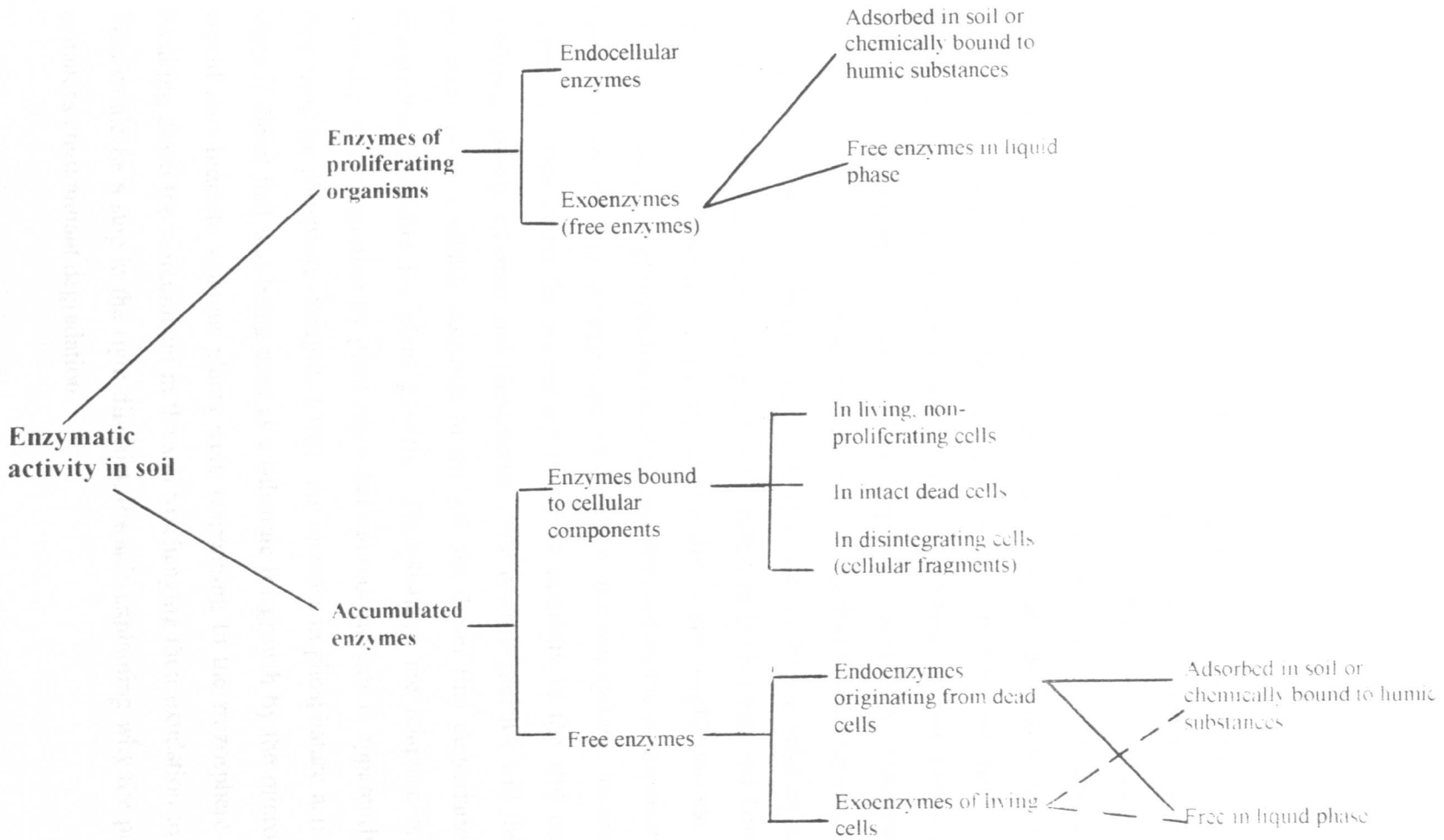
Although enzymes catalyse many degradative processes, soil enzymology has been neglected for decades (Gramss *et al.*, 1999). In recent years, the study of enzyme activity in soil has expanded significantly and as a result, more than 50 enzymes have been examined, many of which are crucial to the cycling of nutrients in soil. Soil enzymes

originate from plants, animals, fungi and bacteria although it is generally agreed that the microbial component is the main source of these enzymes in soil. Because many enzymes are substrate specific and can be chosen from different functional groups (e.g. oxidoreductases, hydrolases etc.) there is an opportunity to determine the potential of a soil to carry out a wide range of reactions that may be critical in the functioning of the ecosystem (Dick, 1997). Alternatively, enzyme activities hold the potential to determine whether contaminated soil is 'impaired' to carry out these biochemical processes. By measuring the changing pattern of specific enzymes during bioremediation of diesel fuel in planted and unplanted soils, the influence that plants have on the level of enzymes, from microorganisms as well as from other sources, could be illustrated. This would provide valuable information as to why enhanced degradation is observed in the rhizosphere of selected plant species compared to unplanted soils.

The enzymes chosen for investigation were essential in cycling matter through the soil system and could therefore be used to assess the breakdown of diesel fuel in soil and the effect of diesel fuel contamination on soil health. These enzymes can be found as an integral part of intact microbial cells or as free enzymes outside microbial cells (exoenzymes). The source of these enzymes may be purely microbial or both plant and microbially produced. Figure 1.8.1 illustrates the various components making up enzyme activities in soils.

### 1.8.1. Dehydrogenase Enzymes

Dehydrogenase enzymes come from the oxidoreductase group of soil enzymes. Biological oxidation of organic compounds is generally a dehydrogenation process, with dehydrogenase enzymes systems fulfilling a significant role in the oxidation of organic matter (Tabatabai, 1982). The dehydrogenase enzymes responsible for dehydrogenase activity in soil are found as an integral part of intact microbial cells and therefore, the results of an assay would indicate the average metabolic activity of the active microbial population. Indeed, several authors have found dehydrogenase activity to closely correlate with respiratory activity in soils (Steubing, 1967, Camiña *et al.*, 1998). Measurement of dehydrogenase activity in this study would illustrate if total microbial activity was enhanced in planted soils as opposed to unplanted soils and if certain plant species increased microbial activity in the rhizosphere more than other plant species. It would also




(Taken from Skujins, 1976).

Figure 1.8.1. Components making up enzymatic activity in soil.

show if diesel fuel was being utilised as a substrate for growth by microorganisms in the soil and at what level diesel fuel became inhibitory to activity of the microbial population.

### 1.8.2 Phosphatase Enzymes

The general name phosphatases is a collective term used to describe a broad group of enzymes that catalyse the hydrolysis of organic phosphorus in soil. They belong to the hydrolase group of soil enzymes. These enzymes have been classified into five major groups but it is the phosphomonoesterases, acid phosphatase and alkaline phosphatase, that hold particular interest because of their importance in soil organic phosphorus mineralization and plant nutrition (Tabatabai, 1982). Since plants utilise only inorganic phosphorus, organic phosphorus compounds must first be hydrolysed by phosphatases which mostly originate from plant roots, fungi and soil microorganisms. Plant roots are major producers of acid phosphatase but do not produce alkaline phosphatase. Alkaline phosphatase originates from soil bacteria, fungi and fauna. Microorganisms also produce acid phosphatases. Phosphatases measured in soils reflect the activity of enzymes bound to soil colloids and humic substances, free phosphatases in the soil solution and phosphatases associated with living and dead plant or microbial cells (Kramer and Green, 2000). The measurement of phosphatase activity during this study would provide information on whether a change in phosphorus cycling was observed in the contaminated soil. Diesel fuel contamination adds a large input of carbon to the soil system. In order to break this usable substrate down the increasing microbial numbers in the soil require additional nutrients, namely nitrogen and phosphorus. Soil microorganisms will therefore be using the majority of available nutrients in the soil for diesel fuel degradation, leaving little phosphorus available for plant growth. Phosphatases are adaptive enzymes and the intensity of the excretion by plant roots and microorganisms is apparently determined by their need for phosphate (Skujinš, 1976). An increase in phosphatase activity would again show if diesel fuel was being used as a substrate for growth by the microorganisms but it would also indicate whether plants were responding to the rhizosphere microorganisms breaking down the contaminant in the soil by changing their exudation to the rhizosphere. This would be a step in the right direction towards explaining why the presence of plants enhances contaminant degradation.

### 1.8.3. Non-Specific Proteases, Lipases and Esterases.

The activity of these enzymes in a soil system is assessed by the measurement of fluorescein diacetate (FDA) hydrolysis in a broad spectrum enzyme assay. The variety of enzymes responsible for FDA activity in soils are found within cells and also as free enzymes (exoenzymes). Non-specific proteases, lipases and esterases are involved in the decomposition of many types of tissue, thus the ability to hydrolyse FDA seems widespread in the soil environment, especially among the major decomposers – bacteria and fungi (Schnürer and Rosswall, 1982). Generally more than 90% of the energy flow in a soil system passes through microbial decomposers therefore an assay which measures microbial decomposer activity will provide a good estimate of total microbial activity. Measurement of FDA activity during this study is similar to the measurement of dehydrogenase activity in the sense that it estimates total metabolic activity of the soil system. How it differs from the dehydrogenase activity assessment is that FDA hydrolysing enzymes are found as free enzymes as well as in live microbial cells. This will provide an estimate of the total microbial activity of the soil as a whole not just of the microbial component and illustrate the influence of plant derived enzymes in the soil system.

## 1.9 Current Research Needs

Crude oil contamination, particularly in the aquatic environment, is well documented. Contamination by petroleum products such as diesel fuel and contamination of the soil environment and those species that interact with the soil environment (microorganisms and plants) is less well documented. When reviewing the literature for this work, the relative scarcity of reports on petroleum biodegradation in soils as compared with those in aquatic systems was notable.

The most common method of dealing with soil contaminated with petroleum hydrocarbons is to remove the contaminated soil to landfill or for incineration. This approach is destructive and costly. Alternative methods for the remediation of contaminated land have been developed including physical, chemical and biological methods. In the last 20 years more selective, sophisticated methods have been developed including soil vapour extraction, extraction followed by wet chemical analysis and

different thermal treatments (Šepič *et al.*, 1996). Chemical techniques include solidification with quicklime and application of surface active additives. The main problem with these methods are they are site specific, dependent on the characteristics of the contaminant and they can be costly to implement. There is a need for inexpensive, *in situ* treatments for reclaiming soil contaminated with petroleum hydrocarbons. Biological treatments for the clean-up of contaminated sites are becoming favourable and an alternative possibility. Phytoremediation is one such technique which may be especially applicable to diesel fuel contaminated land.

## 1.10 Aims and Objectives

The aim of this study was to investigate biodegradation of diesel fuel in the soil environment and determine whether biodegradation could be enhanced by the presence of plants. This investigation encompassed the behaviour of diesel fuel on entering the soil environment, how diesel fuel affected the soil microbial component and the effect of diesel fuel on selected plant species' growth and development. The interaction between plants and their associated microorganisms and the processes involved in the phytoremediation of diesel fuel contaminated soil were also investigated.

A main objective during this study was to develop suitable methods for the characterisation of diesel fuel. As little information was available on diesel fuel composition, thorough analysis of the diesel fuel product was required.

The outcome of this research would hopefully conclude whether phytoremediation is a viable option for diesel fuel contaminated soil.

# CHAPTER TWO

## MATERIALS AND METHODS

All glassware/plasticware was physically washed then soaked in 2–5 % Decon (Phosphate-free surface active cleaning agent) overnight. The glassware/plasticware was rinsed under running water then rinsed 5 times in deionised water. Glassware/plasticware used for nutrient determination was given a final rinse with Purite deionised water. The glassware/plasticware was dried at approximately 40°C in a drying oven. All chemicals, unless otherwise stated, were Analar grade.

## 2.1 Soils Used in the Study

### 2.1.1 Soil Sampling, Storage and Preparation

The soils used in this study were collected fresh from each site. The soil profile was sampled to a depth of approximately 30cm and the removed soil placed in a heavy duty polythene sample bag.

The soils were initially sorted to remove large stones, twigs/plant material, macroinvertebrates etc., and then sieved to 4mm. The soils were stored in tied polythene sample bags at 4°C until used.

## 2.1.2 Description of Soils

The soils used in this study were classified to a Soil Series using the Soil Memoirs and Soil Maps for each area (Grant *et al.* 1962 and Ragg *et al.*, 1976). Only three soils used in this study were not from classified Soil Series. These were two manufactured composts (John Innes seed compost and compost No.2) and the Garscube soil. Information on their site history, preparation and textural class has been given. A brief description of each soil used is given below.

Arkleston soil

The site is located at Arkleston Farm, Paisley, Scotland and is used for intensive cultivation of potatoes. It belongs to the Dreghorn Association, which is formed from raised beach deposits. The series is Dreghorn, which has been classed as a freely drained brown forest soil. Ordnance survey grid reference NS 508655.

Barassie soil

The site is located at Troon, Ayrshire, Scotland and is from a garden. It belongs to the Dreghorn Association, which is developed from raised beach deposits. The soil comes under the Dreghorn Series, which has been classed as a freely drained forest soil. Ordnance survey grid reference NS 328329.

Bargour soil

The site is located at Auchincruive, Ayr, Scotland and is under permanent grass. It belongs to the Bargour Association which is developed from till derived from carboniferous sandstone. The series is Bargour, which has been classed as an imperfectly drained brown forest soil. Ordnance survey grid reference NS 379234.

Caprington soil

The site is also located at Auchincruive, Ayr, Scotland and is under permanent grass and managed for grazing dairy cows. The soil belongs to the Rowanhill Association, which is developed from glacial till derived from sandstone and shale of the productive coal measures. The soil comes under the Caprington Series, which is classed as an imperfectly drained brown forest soil. Ordnance survey grid reference NS 376232.

Darvel soil

The site is located at Lennoxtown, Scotland and is from a garden. It belongs to the Darvel Association which is formed from fluvioglacial sands and gravels derived from

carboniferous igneous and sedimentary rocks. The soil comes under the Darvel Series, which has been classed as a freely drained brown forest soil of low base status. Ordnance survey grid reference NS 635773.

Garscube soil

The site is located at Garscube, Glasgow, Scotland and has had manure applied for an unknown number of years. The soil, analysed for textural properties, has been classed as a sandy loam. Ordnance survey grid reference NS 540702.

John Innes Seed Compost and Compost No. 2

These are manufactured soils formulated by John Innes Horticultural Insitute, UK, which have been classed as sandy by textural analysis (RHS, 1996).

Seed Compost is prepared by mixing 2 parts sterilised loam, 1 part peat and 1 part sand by volume. The loam is prepared by stacking turf for 6 months or more then sterilising it to remove any pathogens present in the soil. Nutrients are added to this mix in the form of 1.2 kg superphosphate of lime and 0.594 kg ground limestone per cubic metre.

Compost No. 2 is prepared by mixing 7 parts loam, 3 parts peat and 1 part sand by volume. To this mixture, 1.2 kg ground limestone, 2.4 kg superphosphate of lime and 1.2 kg potassium sulphate per cubic metre is added.

## 2.2 Characterisation of Soil Types by Physical and Chemical Properties

### 2.2.1 Physical Properties

#### 2.2.1.1 Particle Size Fractionation by Mechanical Analysis

Particle size analysis for the determination of textural class was carried out using a modified method based on ADAS method 57 (ADAS, 1986).

Soil texture describes the size distribution of particles in a soil. The following method provides a quantitative assessment of particle size distribution that allows for an

accurate description of soil texture. The method involves breaking down the soil to its primary particles of sand, silt and clay by destruction of soil organic matter followed by dispersion of the remaining mineral soil. This allows the soil particles to be fractionated according to size by either sieving (sand fractions) or by the sedimentation rate of the particles (silt and clay fractions). The textural class was then determined from the texture chart prepared by the Soil Survey of England and Wales (Hodgson, 1976).

**Destruction of Soil Organic Matter and Soil Dispersion**

The following procedure was repeated for each soil investigated.

Six, labelled porcelain evaporating basins were placed in a 105°C oven and dried for 1 hour. One of the six basins was labelled 'coarse and medium sand', one 'fine sand', two were labelled 'silt plus clay' and the remaining two labelled 'clay'. The basins were removed from the oven and allowed to cool in a desiccator. The basins were then weighed (to four figures) and the oven dry weight recorded.

10g of air dry (sieved < 2mm) soil was weighed accurately (to two figures) into a 400ml glass beaker. 50ml of 6% (w/v) $H_2O_2$ solution was carefully added to the beaker along with two drops of a silicon antifoaming agent. Any initial reaction was allowed to subside then the beaker was heated gently on a steam bath and the mixture stirred occasionally with a glass rod. The mixture was heated until the frothing, caused by the reaction of $H_2O_2$ on organic matter in the soil, had ceased. The beaker was then removed from the steam bath and allowed to cool slightly. A further 50ml 6% $H_2O_2$ solution was added to the mixture and the sides of the beaker washed down with deionised water. The beaker was placed back on the steam bath and heating continued until the reaction was complete. Once the reaction had stopped, the beaker was heated for a further 15 minutes to ensure complete oxidation of organic matter had occurred. The beaker was removed from the steam bath and allowed to cool. Once cool, the sides of the beaker were washed down with deionised water to give approximately a 2cm depth of soil suspension in the beaker. 10ml of 5.7% Calgon (dispersing agent) was added then the suspension dispersed for 5 minutes in an ultrasonic bath.

**Sand Fractionation**

A 1000ml graduated cylinder was set up with a large filter funnel in the neck. 180μm and 53μm sieves were banked together (with the 180μm sieve on top) and placed in the filter funnel. The sieve sizes approximated to the medium sand–fine sand and fine

sand–coarse silt boundaries and allowed separation of the coarse and medium sand fraction and the fine sand fraction.

The beaker containing the dispersed soil suspension was emptied into the 180μm sieve and the beaker washed thoroughly with deionised water to ensure all the soil was added to the sieve. The soil was then washed through the sieves with deionised water until the water passing through the 180μm sieve ran clear. The 180μm sieve containing the coarse plus medium sand fraction was set aside. The fine sand fraction in the 53μm sieve was washed in the same way. The cylinder was then made up to 1000ml with deionised water and stoppered.

The sand fractions were recovered by washing the sand in each sieve with a jet of deionised water into the appropriate labelled porcelain basin. The excess water in the basins was evaporated off in a steam bath then the sand fractions placed in a 105°C oven to dry overnight. The basins were removed from the oven and allowed to cool in a desiccator. The basins were weighed (to four figures) and the % coarse plus medium sand and % fine sand (on an oven dry basis) was calculated as shown below.

$$\% \text{ sand fraction} = \frac{\text{weight of sand fraction}}{\text{weight of oven dry mineral soil}} * 100$$

The weight of oven dry mineral soil can be calculated from :

$$\text{Weight of oven dry mineral soil} = \text{wt. of soil} * \text{c.f.} * \frac{(100 - \% \text{ LOI})}{100}$$

see section 2.2.3 for calculation of % LOI and section 2.2.4 for conversion factor.

**Silt plus clay fraction**

Once the sand fractions were removed, the remaining soil fraction contained in the 1000ml graduated cylinder was used to determine the silt and clay fractions.

As the rate of sedimentation is temperature dependent, the temperature of the suspension was measured and an appropriate sedimentation time was selected (see appendix) for silt plus clay at a depth of 20cm at this temperature (Hodgson, 1976). The 1000ml cylinder was shaken for approximately 60 seconds. The cylinder was then placed on a pipetting stand and timing started on a stopwatch. With the tap closed, a

50ml pipette was lowered into the 1000ml cylinder until the tip of the pipette just failed to touch the surface of the suspension. The height on the pipetting stand scale was noted then 20 seconds before the required sampling time, the pipette was gently lowered exactly 20cm into the suspension. At the appropriate time, the tap was opened and slightly more than 50ml of suspension was withdrawn. The tap was closed and the pipette removed from the 1000ml cylinder. The volume of the pipette was adjusted by running the excess suspension into a waste beaker. The 50ml of suspension was then emptied into the appropriate, labelled porcelain basin. The cylinder was shaken again and the above procedure repeated to obtain a duplicate sample.

The porcelain basins containing silt plus clay fractions were evaporated to dryness on a steam bath. The basins were then placed in a 105°C oven and left to dry overnight. The basins were removed from the oven and allowed to cool in a desiccator. The basins were weighed (to four figures) to obtain % silt plus clay fraction present in the soil.

$$\text{Silt plus clay \%} = \frac{\text{wt. of fraction} - \text{wt. of dispersant}}{\text{wt. of oven dry mineral soil}} * \frac{\text{vol. of cylinder}}{\text{vol. of pipette}} * 100$$

The weight of dispersant can be calculated from :

$$\text{Wt. of dispersant (g)} = \frac{0.57 * \text{vol. of pipette}}{1000}$$

**Clay fraction**

The 1000ml graduated cylinder was shaken as before then placed in an incubator at 25°C. Just before sampling time (see appendix), the cylinder was removed from the incubator and set up on the pipetting stand as before. At the appropriate time, 25ml of suspension was removed from a depth of 10cm and emptied into the labelled porcelain basin. Again, a duplicate sample was taken but the cylinder was not shaken between samples.

The basins containing clay fractions were evaporated to dryness on a steam bath then placed in a 105°C oven overnight. The basins were removed from the oven, cooled in a desiccator then reweighed to obtain the % clay present in the soil.

$$\text{Clay \%} = \frac{\text{wt. of fraction} - \text{wt. of dispersant}}{\text{wt. of oven dry mineral soil}} * \frac{\text{vol. of cylinder}}{\text{vol. of pipette}} * 100$$

**Silt fraction**

The % silt was obtained by subtracting the % clay from the % silt plus clay.

## 2.2.2 Chemical Properties

### 2.2.2.1 Determination of soil pH

This method was carried out according to ADAS method 32 (ADAS, 1986).

**Procedure**

10g fresh soil was weighed, in triplicate, into 4oz glass jars. 25ml of deionised water was added to each jar and the contents shaken, intermittently, for 15 minutes. After 15 minutes the soil suspension was shaken and the pH electrode (calibrated to pH 4.0 and pH 7.0) lowered immediately into the soil suspension. After 30 seconds the pH of the soil suspension was recorded.

### 2.2.2.2 Moisture Content

All air dried soils contain a certain amount of water which is strongly held by the soil particles. The weight of this water, known as hygroscopic water, depends partly on the relative humidity and the temperature of the atmosphere. It is necessary to know precisely what the moisture content of the soil is, as although soil analyses are generally carried out on fresh or air dried soils, the results are reported on an oven dry basis. By working out this conversion to oven dry weight, it is possible to compare results from different laboratories obtained at different times.

**Procedure**

Porcelain basins were placed in a 105°C oven. After 1 hour, the basins were removed and allowed to cool in a desiccator. The basins were then weighed on a 4 figure balance and the weights recorded. 20g fresh soil was then weighed, in triplicate, into each basin. The basins were placed back into the 105°C oven overnight. The basins were

again removed from the oven and cooled in a desiccator and the weight recorded. The % moisture loss from soil, on a fresh weight basis, can be calculated as shown below.

$$\% \text{ moisture loss (fresh soil basis)} = \frac{\text{wt. fresh soil} - \text{wt. oven dry soil}}{\text{wt. fresh soil}} * 100$$

### 2.2.2.3 Organic Matter Content by Loss on Ignition (% LOI)

Ignition of soil at a high temperature (e.g. 700°C) will result in a loss of weight due to loss of organic matter and loss of combined water. In addition, calcareous soil will also lose carbonate. Ignition at a lower temperature (e.g. 400°C) however, will reduce the error but will require longer for complete combustion of the organic matter. The method described below combines these aspects and is used to measure the loss of soil organic matter by loss on ignition.

**Procedure**

5g of soil was weighed, in triplicate, into silica basins and dried overnight at 105°C. The soils were weighed to obtain the oven dry soil weight. The soils were then placed in a muffle furnace and ignited at 500°C for 6 hours. The samples were then reweighed and the weight of ignited soils calculated.

The % organic matter by loss on ignition was calculated by:

$$\% \text{ organic matter (LOI)} = \frac{\text{wt. oven dry soil} - \text{wt. ignited soil}}{\text{wt. oven dry soil}} * 100$$

### 2.2.2.4 Conversion Factors (cf) for Fresh and Air Dried Soil Samples

The conversion factors for relating fresh and air dried soil sample weights to oven dried weights was carried out by the procedure described below. The conversion was necessary to allow results to be compared with results obtained at different times or in different laboratories. This is discussed in Section 2.2.2.2.

**Procedure**

Porcelain basins were placed in a 105°C oven for 1 hour, removed and allowed to cool in a desiccator. 5g of either fresh or air dried soil was weighed, in triplicate, into each porcelain basin and the basins replaced in the oven and dried overnight. Once cooled in a desiccator, the basins were weighed and the oven dry weights recorded. The conversion factor (cf) for air dry or fresh samples weights to an oven dry basis can be calculated as follows:

$$cf = \frac{\text{total oven dry weight}}{\text{total fresh or air dry weight}}$$

The fresh or air dry sample weights can then be multiplied by the cf to give sample weight on an oven dry basis.

### 2.2.2.5 Kjeldahl Nitrogen

Kjeldahl nitrogen content of the soils was determined using the digestion method of Bremner and Mulvaney (1982) with ammonium being determined in the digests by an automated colorimetric method on the Technicon Autoanalyser system. This method does not include oxidised forms of nitrogen such as nitrate.

Samples were digested in concentrated sulphuric acid using potassium sulphate to raise the boiling point and a copper selenium catalyst mixture to accelerate the digestion process. Ammonium in the digests was determined by a modified indophenol green method using a complexing reagent to prevent interferences due to the precipitation of hydroxides in the reagent system.

**Soil Digestion**

**Procedure**

Soil samples were air dried and finely ground using a mortar and pestle. Approximately 0.5g of soil was weighed accurately, using a four figure balance, into a foil weighing boat. The soil was then transferred carefully, to a dry, digestion tube, ensuring all the soil reached the bottom of the tube. The foil weighing boat was reweighed and the

weight recorded. This allowed accurate calculation of the total weight of soil added. 5 replicates for each sample were prepared and the remainder of the rack set up with blanks. Blanks were prepared as above but without the addition of soil.

The rest of the method was carried out in a fume cupboard.

Half a catalyst tablet was added to each digestion tube then, using a dispenser, 5ml of concentrated sulphuric acid was added. The rack was placed on the digestion block and the fume extraction system set up. The block was heated gently until the initial frothing subsided, then the temperature was increased to 375°C and the tubes heated until the digests cleared. A baffle was placed on the front of the rack to promote refluxing higher up the tubes, thereby washing material down into the tubes. Once digestion was complete the block was heated for a further 3 hours.

The tubes were removed from the block and allowed to cool until just warm to the touch. If left too long the digests can solidify. Pointing the tubes to the back of the fume cupboard, 10ml of deionised water was carefully added. The tubes were shaken vigorously to mix the contents then a further 30ml of deionised water was added and the contents mixed.

The contents of each digestion tube were then filtered (Whatman, No2) into 100ml volumetric flasks. The tubes were washed with deionised water and the washings transferred to the filter paper. The filter paper was also washed with deionised water then the volumetric flask was made up to the mark with deionised water.

## Determination of Ammonium in Kjeldahl Digests

### Procedure

The manifold shown in Fig. 2.2.2.5.1 was used for the determination of ammonium-N in the soil digests. The flow rates used at each step in the automated determination are shown in Table 2.2.2.5.2. The samples were analysed using the manifold shown along with standards, blanks and zeros. The samples were run at the rate of 40 per hour with a dilution/neutralisation step before the main manifold (see Table 2.2.2.5.2). The colour was developed in a water bath at 37°C and the colour intensity measured at 650nm. The air was cleaned from atmospheric ammonium by bubbling through 5% sulphuric acid solution. The calibration graph for ammonium is linear from 0-100mg $NH_4$-N $l^{-1}$. For calibration purposes, standards in the range 0-25mg $NH_4$-N $l^{-1}$ were used as my samples were never above this concentration.

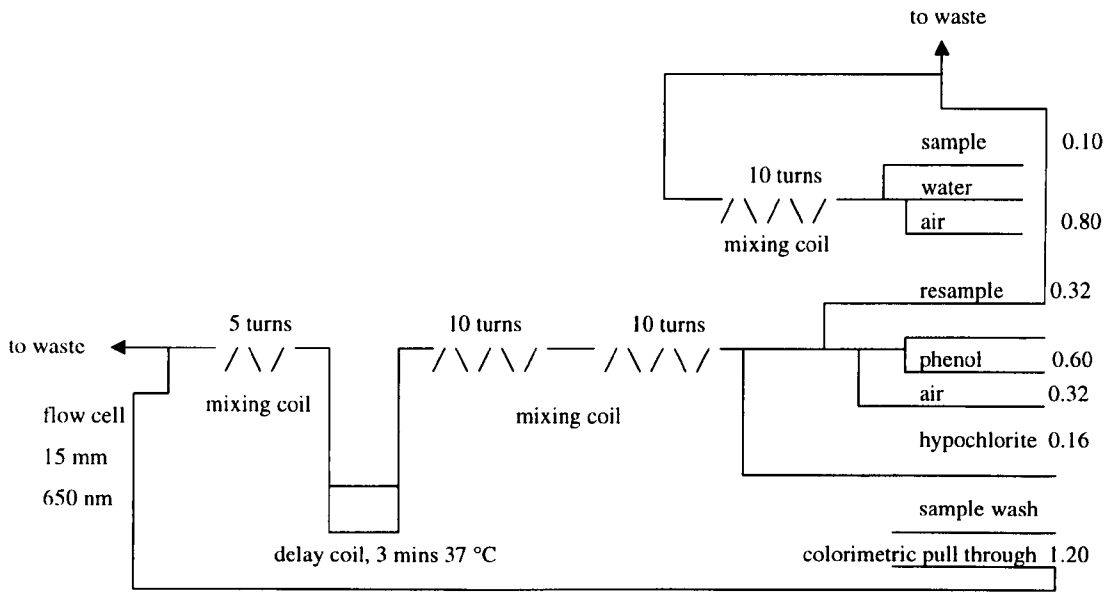



Figure 2.2.2.5.1 Technicon autoanalyser II manifold for the determination of $NH_4$-N.

| Step in procedure | Flow rate ml $min^{-1}$ |
|---|---|
| Dilution/neutralisation | |
| Sample wash solution : 5 % v/v $H_2SO_4$ | |
| Neutralising solution | 2.00 |
| Air | 0.80 |
| Sample | 0.10 |
| Main manifold | |
| Complexing reagent | 0.60 |
| Alkaline phenol | 0.60 |
| Air | 0.32 |
| Resample | 0.32 |
| Hypochlorite | 0.16 |
| 20 turn mixing coil | |
| 3 min delay coil | |
| pull through | 1.20 |

Table 2.2.2.5.2 Flow rates used in the automated determination of $NH_4$-N in soil digests.

## 2.2.2.6 Extractable Phosphorus

Extractable inorganic phosphorus in soil was determined from sodium bicarbonate extracts using ADAS method No. 59 (ADAS, 1986) coupled with an automated determination step. A Technicon Autoanalyzer II was used for the determination of soil inorganic phosphorus. The method is based on the formation of a phosphomolybdate complex using antimony to accelerate the formation of the faintly yellow coloured product. The coloured product is then reduced using ascorbic acid to give a more intense blue colour, which may be measured at 880nm. The method is applicable to a wide range of samples including water samples, soil extracts and digests of plant material.

**Extraction procedure**

2.5g air dried soil (sieved < 2mm) was weighed into a 4oz glass jar. Four replicate samples and two blanks without the addition of soil were prepared. 50ml of 0.5M $NaHCO_3$ was added to each jar and the jars were shaken on an end-over-end shaker for 30 minutes. The samples were filtered (Whatman, No.2) and extracts collected in 100ml plastic bottles.

**Determination of Inorganic Phosphorus in Soil Extracts**

Soil inorganic phosphorus in the soil extracts was determined using the Technicon Autoanalyzer II system. The solutions were analysed along with standards, blanks and zeros. Inorganic phosphate has a linear calibration graph in the range 0–5mg $PO_4$-P $l^{-1}$ and as all my samples were below the top standard concentration there was no need for dilution. The colour was developed in a water bath at 37°C and the colour intensity measured at 880nm. This work was carried out by Fariedh Karabassi.

## 2.2.2.7 Extractable Potassium

Extractable potassium was determined by flame photometry according to a modified version of ADAS Method 63 (ADAS, 1986). The flame photometer (Corning model 410) was calibrated using 0 to 50mg K $l^{-1}$ standards prepared by relevant dilution of the stock solution in 1M ammonium nitrate solution. The resultant calibration graph is curved.

**Extraction procedure**

5g of air dried soil (sieved < 2mm) was weighed, in triplicate, into 4oz glass screw top jars. A blank was included without the addition of soil. 50ml of 1M ammonium nitrate solution was added to each jar and the jars shaken for 30 minutes on an end-over-end shaker. The soil extracts were then filtered (Whatman, No.1) and the filtrate collected in 100ml plastic bottles.

Samples which gave readings above the highest standard concentration were diluted 1:5 in 1M ammonium nitrate solution

## 2.3 Analysis of Diesel Fuel Oil

### 2.3.1 Extraction of Diesel Fuel from Soil

#### 2.3.1.1 Cold Solvent Extraction Step (Short Residence)

Due to the short residence time of diesel fuel in some samples, a method of extraction was developed that allowed the volatile, lighter fuel components to be removed effectively followed by extraction of the heavier fuel components.

A cold shaking extraction method was developed from the mechanical shaking method of Schwab *et al* (1999). 40g of fresh soil (sieved < 2mm) was extracted for 30 minutes in 100ml 1:1 acetone: dichloromethane in an orbital incubator (15°C, 200 rev $min^{-1}$). The extract was filtered (Whatman, No. 2) into 100ml volumetric flask and the volume made up with 1:1 acetone: dichloromethane. This sample was then prepared for Soxhlet extraction as described in Section 2.3.4 to remove the heavier components.

This extract was analysed by GC-FID using the conditions described in Section 2.3.4 and the total petroleum hydrocarbon (TPH) value calculated. The TPH values obtained for each step of the extraction were summed to provide a total TPH value for the sample. Dilution of the original diesel fuel in $CH_2Cl_2$ served as a quantitative analytical standard.

### 2.3.1.2 Soxhlet Extraction Step

40g of soil sample was left to air dry overnight. The sample was ground in a mortar and pestle then transferred to a cellulose thimble for Soxhlet extraction. The Soxhlet method was modified from the US EPA method 3540C for non volatile and semi volatile organic compounds (US EPA, 1986) and the method of Song *et al* (1990).

5g of anhydrous sodium sulphate was added to the bottom of a cellulose extraction thimble then the air dried soil sample added. The thimble was plugged with glass wool then placed into the Soxhlet apparatus. 100ml of either dichloromethane ($CH_2Cl_2$) or 1:1 acetone: dichloromethane was added and the sample extracted for 6 hours. Once cool the extract was transferred, with washings, to a 100ml volumetric flask and the volume made up to 100ml with the appropriate solvent. The extract was analysed by GC-FID as described in Section 2.3.4 and the residual TPH value calculated. Dilution of the original diesel fuel in $CH_2Cl_2$ served as a quantitative analytical standard.

## 2.3.2 Gas Chromatography – Flame Ionisation Detection (GC-FID)

The method for diesel analysis by capillary GC-FID was modified from the US EPA method 8100 for the analysis of polyaromatic hydrocarbons (PAHs) (US EPA, 1986). The development of the method is discussed in Chapter 3, Section 3.1.2. The chromatographic conditions were as follows. Analyses were carried out with a Hewlett-Packard 5890A gas chromatograph and Flame Ionisation detector (FID). The GC was interfaced with a Hewlett-Packard Chemstation data system. Helium carrier gas was adjusted to the recommended linear flow velocity of 20cm $sec^{-1}$ using the non-retained compound butane. Separations were performed on a SGE BPX 5 polysilphenylene-siloxane capillary column (25m x 0.32mm I.D. x 0.5µm). 0.5µl of sample was injected at 35°C with a temperature hold of 3 minutes. The temperature increased 5°C $min^{-1}$ up to 250°C with a 10 minute hold at the end of the run. The injector temperature was 260°C and the detector temperature 270°C.

### 2.3.3 Gas Chromatography- Mass Spectrometry (GC-MS)

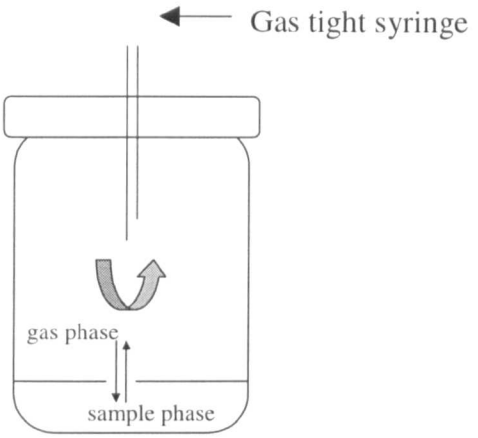
Capillary GC-MS was carried out using a Hewlett-Packard 5971 mass selective detector interfaced to a 5890 series II gas chromatograph and computer (Vectra QS/16S). Mass spectra were recorded at 70 eV on continuous scanning mode. Mass spectra were run through the computer's NBS library for identification. Separations were performed on a HP1 fused silica capillary column (12.5m x 0.2mm I.D. x 0.33μm). The carrier gas used was helium with a flow rate of 1ml $min^{-1}$.

Chromatographic conditions for the analysis of diesel samples were as follows. For pure diesel sample analysis, 0.2μl undiluted diesel was injected at 40°C and this temperature was held for 2 minutes. After this initial time, a temperature programme of 3°C $minute^{-1}$ began up to 275°C and this temperature was held for 1 minute. The sample was split at a ratio of 50:1.

The Total Ion Current (TIC) pattern produced by GC-MS was similar to the pattern produced by capillary GC.

### 2.3.4 Headspace Analysis of Diesel Fuel (GC-FID and GC-MS)

Headspace sampling of volatiles involves placing a sample in a sealed vial and heating it to a predetermined temperature for a period of time. Minimising temperature change and sample volume change is extremely important for headspace sensitivity (Kolb, 1997). The temperature of the sample vial is held for sufficiently long to bring the gas-phase and sample-phase solute concentrations into equilibrium. The volatiles in the gas-phase are then removed and injected directly onto the GC column. This method of equilibrium sampling (Figure 2.3.4.1) was used for the qualitative analysis of the volatile diesel fuel fraction.




Under a constant set of conditions, equilibrium solute concentrations in the headspace are directly proportional to the concentrations in the sample phase.

Figure 2.3.4.1 Basic headspace sampling technique : Equilibrium sampling

As qualitative analysis of diesel was required it was not necessary to determine when the sample reached equilibrium, only when a sufficient gas-phase concentration of the volatile diesel components was reached.

**Procedure**

1g of diesel fuel was weighed into a Chrompack™ headspace vial (4cm x 2cm). The vial was then sealed with a Teflon septum insert and metal collar. This trapped any volatile components within the headspace of the vial and allowed a headspace sample to be withdrawn through the septum. The vial was incubated at 22°C ± 2°C for 48 hours to allow the diesel volatile and non volatile components to equilibrate. A 0.5ml headspace sample was withdrawn from the vial using a 1ml gas syringe (JW Chromatography) and injected directly onto the GC column. The sample was analysed using the programmes described below. Further information on columns used and GC conditions are described in Sections 2.3.2 for GC-FID and 2.3.3 for GC-MS.

**GC-FID Method**

0.5ml of diesel headspace was injected at 35°C with a temperature hold of 3 minutes. The temperature rose steadily at 5°C $min^{-1}$ to 250°C. The temperature was held at 250°C for 10 minutes.

**GC-MS Method**

0.5ml diesel headspace was injected at 35°C with a temperature hold of 3 minutes. The temperature then rose to 250°C on a steady 5°C $min^{-1}$ programme with no temperature hold at the end of the programme. The sample was split at a ratio of approximately 5:1.

## 2.3.5 Separation of Diesel Fuel on Silica Gel

Separation of diesel fuel on silica gel columns was carried out by a modified method based on the method of Wang *et al.*, (1990). The method involved subdividing a diesel fuel sample into saturated, aromatic and polar fractions for total hydrocarbon analysis.

### 2.3.5.1 Preparation of Solvents

All solvents used in the experiment were degassed prior to use.
200ml of Analar hexane, dichloromethane and chloroform:methanol (1:1 v/v) were poured into separate 500ml Buchner flasks. The flasks were placed on magnetic stirrers, at low speed, and a vacuum applied. The solvents were stirred under vacuum for approximately 1 hour until all the gas was removed. Once removed from the vacuum apparatus, the solvents were dried over 4A molecular sieve, which had been activated at 105°C for 24 hours, and the flasks sealed with Nesco™ film to prevent air re-entering the solvent.

### 2.3.5.2 Preparation of Silica Column

Approximately 30g silica gel (Kieselgel Grade 12, 28-200 mesh, Sigma-Aldrich Co.) was weighed into a dry 100ml beaker and activated at 105°C for 12 hours. Once cool, hexane was added to produce a slurry of silica in hexane. A glass column (24cm x 2.3cm ID) with Teflon™ guard was packed with the silica-hexane slurry until approximately 20cm was achieved. The column was allowed to settle for 48 hours

under gravity. The silica was then further packed by running the column at a rate of approximately 3ml $min^{-1}$ for 2 hours. This procedure was repeated after 24 hours. Hexane was replenished from a reservoir connected to the top of the column, which allowed continual replacement of the solvent without letting the column dry out. The column was then ready for use.

### 2.3.5.3 Hydrocarbon Fractionation on Silica Column

0.5g of diesel fuel was added to 3g of silica gel (Kieselgel Grade 12, 28-200 mesh, Sigma-Aldrich Co.) and applied to the top of the column. A 3g layer of anhydrous sodium sulphate was added to the top of the column to absorb any water present and prevent the disturbance of the sample. Hexane was added to wet the top of the column and sample and the column opened to allow a slow flow rate through. The hexane flow brought the sample onto the top of the column and flow rate was set at 30 drops $min^{-1}$ which was equivalent to 1ml $min^{-1}$. Hexane was run through the column until 120ml was collected which contained the saturated fraction. The column was closed and the reservoir changed to dichloromethane. Dichloromethane was run through the column at the same rate until 120ml was collected. This fraction contained the aromatic diesel fuel compounds. Finally, chloroform:methanol 1:1 was added to the reservoir and run through the column as before. This removed the polar fraction.

Each fraction was collected then transferred to a 150ml round bottom flask, with washings. The fractions were concentrated on a rotary evaporator until almost dry then transferred and made up to 1ml in volumetric flasks. The concentrated fractions were then analysed by GC-FID as described in Section 2.3.2.

# 2.4 Behaviour of Diesel Fuel in the Soil

## 2.4.1 Movement of Diesel Through Soil Column

### 2.4.1.1 Leaching Experiment

A column was constructed which allowed the movement of diesel fuel through soil, at depth, to be followed.

**Leaching Column Set Up**

Polyethylene drain pipe was cut into sections (L 10cm x ID 4cm). The sections were sealed together using Parafilm and waterproof tape to provide an airtight seal at the joins. Ten sections were fitted together to create a column 1 metre in length. As the sections were fitted together, the column was filled with John Innes compost No.2 by tapping the soil into each section to create an evenly packed column. John Innes Compost No.2 was chosen as a substitute soil as it is prepared from sterilised loam which provided a low microbial activity soil (Adam and Duncan, 2001). The column was built up, section by section, in this way. The bottom section of the column had a fine Nylon mesh covering the lower end to prevent the soil from escaping but allowing the leachate to freely drain away. An extra section was placed on the top of the column to provide a collar for the water reservoir. The column was run at a temperature of approximately 15°C to reduce microbial activity in the column. The complete set up of the column is illustrated in Figure 2.4.1.1.1.

**Procedure**

10ml of diesel fuel oil was added, by syringe, to the top of the column. The diesel fuel was allowed to penetrate into the soil for approximately 30 minutes. After this time, 50ml of water was poured in to wet the column, then 2 litres of water was added by inverting a 2.5 litre plastic bottle into the top of the column. This acted as a reservoir allowing a constant supply of water to leach through the column. The flow rate depended entirely on gravity flow and the density of the soil packed column. It took approximately 24 hours for 2 litres of water to leach through the column, therefore a

fresh 2 litre reservoir was set up each day. This process was continued for 5 days providing a total of 10 litres of water leached through the column.

The column was dismantled one section at a time and a 40g subsample taken from each section. The samples were then extracted to determine the amount of diesel fuel present in each section as described in Section 2.3.1.

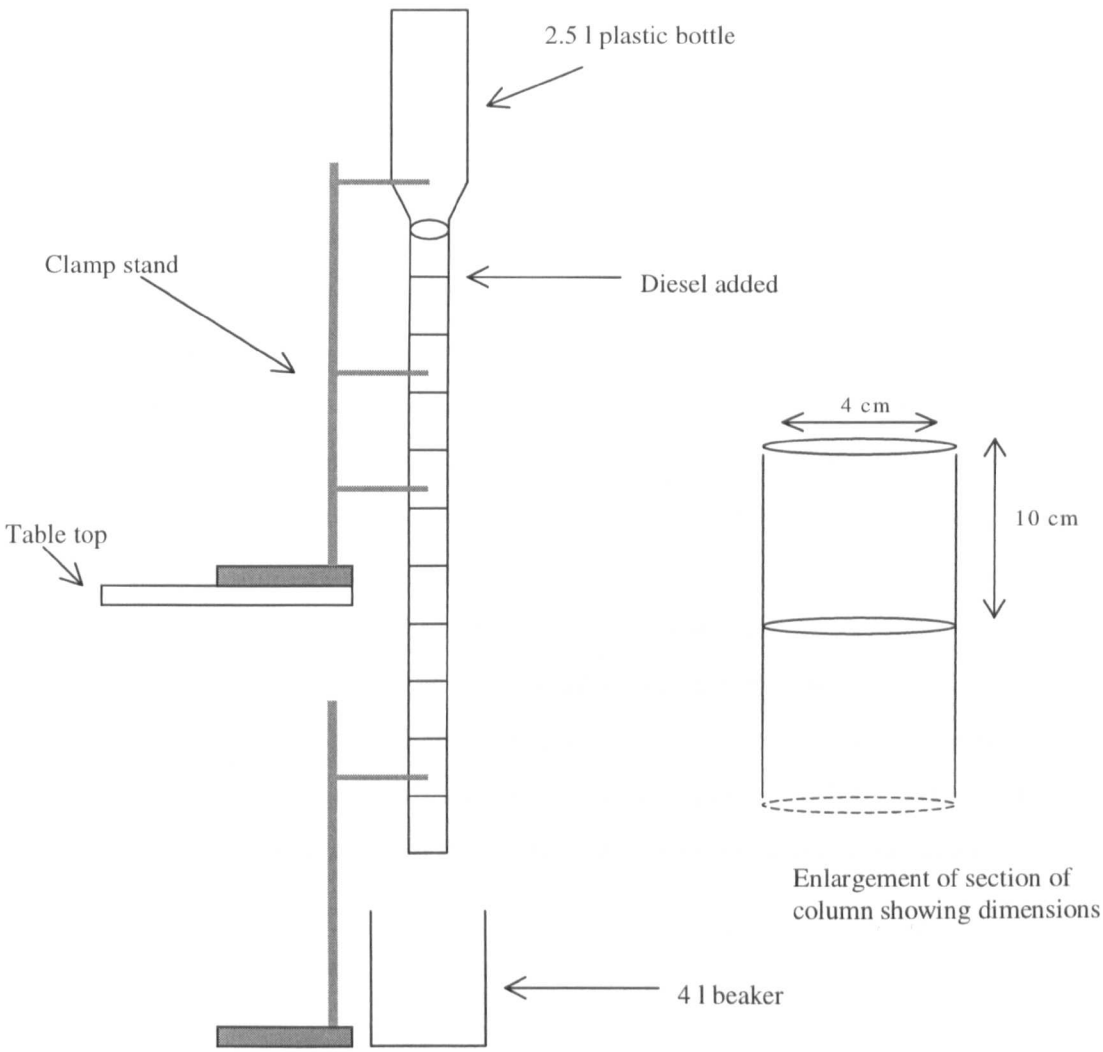



Figure 2.4.1.1.1. Diagrammatic representation of the leaching column set up. The column consisted of 11 sections (one extra section on top of the column to act as a collar for the reservoir) supported by clamp stands. The reservoir was a 2.5 litre plastic bottle containing 2 litres of water. A 4 litre beaker collected the leachate beneath the column.

## 2.4.1.2 Adsorption of Petroleum Hydrocarbons to Soil Components

A laboratory study was undertaken to investigate the movement of various aromatic compounds commonly found in diesel fuel (toluene, naphthalene, 1-ethyl naphthalene, 2,5 dimethylnaphthalene, phenanthrene, anthracene, pyrene and chrysene) through a soil column and if the movement of these compounds was enhanced by ethanol. The effect of soil components on contaminant movement was also investigated. This work was carried out in collaboration with Keiji Gamoh (Kochi University, Japan) whilst working on a joint collaboration on ethanol-additive fuels within the department.

**Preparation of column packing material**

The packing materials used in the study were prepared by sieving the initial air dried samples to < 150μm. This provided material, with an acceptable particle size range and narrower particle size distribution, for packing into a HPLC column that would give constant back pressure values and good chromatographic conditions.

Column G-01 packing material was prepared entirely from sieved Barassie soil. Column G-05 material is the sieved subsoil from the Barassie series. This provides the same soil matrix with lower organic matter, silt and clay content. Column G-01F material is Barassie soil that has been placed in a furnace at 600°C for 6 hours to burn off all the organic matter. For control purposes a column packed with acid washed sand (G-06) and silica gel columns (G-07 and G-08) were included.

Particle size analysis for the determination of size distribution was carried out by mechanical analysis as described in Section 2.2.1.1. Organic matter content was assessed by loss on ignition (LOI) as described in Section 2.2.2.3. Table 2.4.1.2.1 shows the physical characteristics of the six packing materials used in the study.

| | | | Particle size distribution | | | |
|---|---|---|---|---|---|---|
| Column | Description | Organic matter LOI % | Coarse + medium sand % | Fine sand % | Silt % | Clay % |
| G – 01 | Sandy soil | 16.68 | 1.68 | 38.86 | 21.56 | 24.78 |
| G – 05 | Sandy subsoil | 5.01 | 36.00 | 45.33 | 10.17 | 6.97 |
| G – 01F | Sandy soil (OM removed) | 0.00 | 1.95 | 44.75 | 24.80 | 28.50 |
| G – 06 | acid washed sand (Fisher Scientific Chemicals) | 0.00 | 100 | | 0.00 | 0.00 |
| G – 07 | Matrex silica 60 (Fisher Scientific Chemicals) | | | 100 (0.070 - 0.0035 mm) | | |
| G – 08 | Silica Gel 60H (Merck BDH) | | | | 100 (0.0015 mm) | |

% sand values are based on one replicate measurement and % silt and clay are based on duplicate measurements.

Mechanical analysis recoveries for G – 01 and G – 05 were 103.56 % and 103.48 % respectively. Particle size measurements are based on : coarse sand > 0.18 mm, fine sand 0.18 – 0.05 mm, silt 0.05 – 0.002 mm, clay < 0.002 mm.

Table 2.4.1.2.1. Physical characteristics of the packing materials used in this study

## Column Preparation

The prepared material was packed into an empty stainless steel HPLC column (L 100mm x ID 4.6mm) by dry tapping. The packed column was attached to a HPLC pump and 50% aqueous ethanol flowed through (0.1-1.5ml $min^{-1}$) as a packing solvent. After 30 minutes, a small portion of soil saturated in 50% aqueous ethanol was added to the column head to fill up the crack left by the material shifting during packing. Once

the column was successfully packed, 50% aqueous ethanol was run through overnight at 0.1ml $min^{-1}$. Acetone was used to measure the void volume.

**Procedure**

Each petroleum hydrocarbon standard (100mg $l^{-1}$ toluene, naphthalene, 1-ethyl naphthalene, 2,5-dimethyl naphthalene, anthracene, phenanthrene, pyrene and chrysene, prepared in acetone) was added to the selected column individually and varying aqueous ethanol concentrations used as the mobile phase with isocratic elution. The hydrocarbon standards were injected into the column at 1.6ml $min^{-1}$ and detected by UV at 254, 285 and 335nm. Flow rate was set for each column at a rate which provided a constant back pressure. The chromatographic conditions used for each column are outlined in table 2.4.1.2.2. The trend in retention of the hydrocarbons on each column showed good linearity with carbon number, suggesting the column was performing successfully. A good recovery of the petroleum hydrocarbon analytes, from each column, under these conditions was achieved when the absorbance of the eluent from each column was measured by UV spectrophotometry and compared to the corresponding standard solution. The recoveries for mono-and di-aromatic analytes were >99% and >94% for polyaromatic hydrocarbon analytes.

| | Mobile phase | | | | | |
|---|---|---|---|---|---|---|
| | 25 % aqueous ethanol | | | | water | |
| Column | G-01 | G-05 | G-01F | G-06 | Silica A | Silica B |
| Packing weight $g^{-1}$ | 1.6 | 2.6 | 2.4 | 2.2 | 1.2 | 0.85 |
| Flow ml $min^{-1}$ | 1.6 | 1.6 | 1.6 | 1.6 | 0.8 | 1.2 |
| Pressure psi | 90 | 600 | 110 | 20 | 100 | 2000 |

Pressure measured in $lb/in^2$ (psi). Metric conversion – 1 psi ~ 7 kPa.

Table 2.4.1.2.2. Chromatographic conditions used for individual packed columns.

## 2.4.2 Water Holding Capacity of Soil

The water holding capacity of soil is determined by allowing a measured amount of oven dry soil to soak up water. After the soil is saturated and the excess water allowed to drain, the weight increase of the soil is recorded. This value gives 100% of the water holding capacity of that soil.

**Procedure**

Two labelled, porcelain evaporating basins were dried for 1 hour in a 105°C oven. After 1 hour the basins were removed and allowed to cool in a desiccator. The oven dry weight of the basins was recorded.

Two glass leaching columns (L 18cm x ID 2.5cm) were packed with approximately 25g oven dry (105°C) soil (sieved < 2mm). A cotton wool plug was placed at the bottom of the column to prevent soil from escaping. The columns were then completely saturated with water and the excess water allowed to drain. When the columns had stopped dripping, the soil was recovered from each column and emptied into the appropriate, labelled porcelain basin. The basin plus wet soil was weighed and the weight gain recorded.

Weight of water gained (g) = weight of wet soil (g) - weight of oven dry soil (g)

This allows the % water holding capacity to be calculated from :

$$\%\ \text{water holding capacity} = \frac{\text{weight of water gained (g)}}{\text{weight of oven dry soil (g)}} * 100$$

This method was validated by oven drying the porcelain basins containing the wet soil at 105°C overnight. The basins were removed from the oven and allowed to cool in a desiccator. Once cool, the oven dry weights were recorded and the % moisture determined as described in section 2.2.2.2. The % moisture content agreed with the % water holding capacity therefore, the gravimetric method described above was found to be accurate and used for the other samples.

## 2.4.3 Hydrophobicity of soil (Repellency Test)

The molarity of ethanol droplet (MED) test, first proposed by Watson and Letey (1970) and later developed by King (1981), was used to measure soil water repellency.

The water droplet (WD) test was used initially to assess the extent of soil repellency. In the WD test, the time taken for water droplets (approximately 40μL) to penetrate the soil was measured for entry times of 4 minutes or less. If entry took more than 4 minutes, the MED test was used.

The MED test measures the molarity of ethanol in a droplet (approximately 40μL) required for soil infiltration in less than 10 seconds. Soil water repellence was assessed using ethanol concentrations of 0.2M intervals in the range 0.0-5.0M. The repellence rating, stated as the MED index, was interpreted from the guidelines provided by King (1981).

**Procedure**

A Perspex cell with a mesh bottom (L 63mm x W 45mm x D 9mm) was packed with the oven dried (105°C) soil sample (sieved < 2mm) (Fig. 2.4.4.1). The soil was packed evenly by tapping and lightly pressing the soil into the Perspex cell. The excess soil was removed using a spatula to create an even surface.

The WD test involved applying a droplet of water, by Pasteur pipette, at a 90° contact angle (upright position) onto the packed cell's soil surface. The time taken for the droplet to penetrate fully into the soil surface was recorded. If the time taken was over 240 seconds then the sample was retained for the MED test.

The MED test involved applying a droplet of ethanol, by Pasteur pipette, at a 90° contact angle onto the soil surface. A number of ethanol concentrations at 0.2 M interval were used, in the range 0.0-5.0M. For each ethanol concentration, 3 replicate measurements were made in a diagonal across the packed cell. The test was stopped once the ethanol concentration was found which allowed full penetration of the droplet within 10 seconds.

This was carried out for all the soil samples tested and the repellency rating calculated from the interpretation guidelines shown in Table 2.4.4.2.

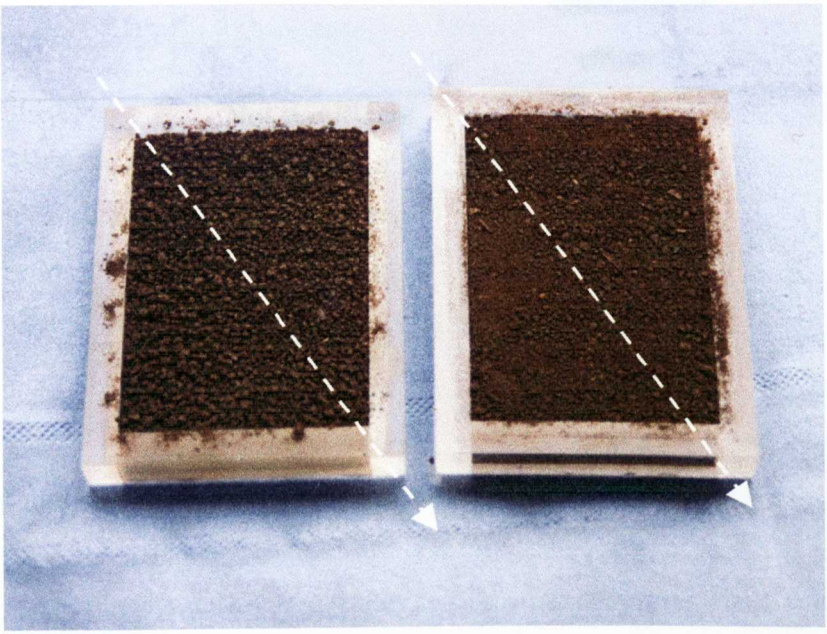


Fig 2.4.4.1 Photograph of the soil packed Perspex cells used for the repellecy tests (WD and MED). Diagonals represent the direction of replicate measurements on each cell.

| | | Tests | |
|---|---|---|---|
| Rating | Severity of Repellence | MED (molarity of ethanol) | WD (s) |
| 1 | Not significant | --- | < 1 |
| 2 | Very low | --- | 1 |
| | | --- | 4 |
| | | --- | 7 |
| 3 | Low | 0.0 | 8 |
| | | 0.2 | 11 |
| 4 | | 0.4 | 16 |
| | | 0.6 | 22 |
| 5 | | 0.8 | 33 |
| | | 1.0 | 53 |
| 6 | Moderate | 1.2 | 85 |
| | | 1.4 | 142 |
| 7 | | 1.6 | 260 |
| | | 1.8 | --- |
| 8 | | 2.0 | --- |
| | | 2.2 | --- |
| 9 | Severe | 2.4 | --- |
| | | 2.6 | --- |
| 10 | | 2.8 | --- |
| | | 3.0 | --- |
| 11 | Very severe | 3.2 | --- |
| | | 3.4 | --- |
| 12 | | 3.6 | --- |
| | | 3.8 | --- |
| | | > 3.8 | --- |

Table 2.4.4.2. Interpretation guidelines for water repellence rating of soils. Modified from King (1981).

# 2.5 Enzyme assays

## 2.5.1 Acid and Alkaline Phosphatase Activity

The measurement of soil phosphatase activity was modified as described below from the method of Tabatabai and Bremner (1982).

The method involves the colorimetric estimation of *p*-nitrophenol released by phosphatase activity when soil is incubated with buffered sodium *p*-nitrophenl solution.

**Procedure**

1.0g of fresh soil (sieved < 2mm) was weighed into 25ml Quickfit conical flasks. 4ml of MUB (pH 6.5 for acid phosphatase assay or pH 11 for alkaline phosphatase assay) was added to each flask and 1ml of 0.0025M *p*-nitrophenyl phosphate solution prepared in the same buffer was added to start the reaction. The flasks were stoppered then shaken to mix the contents of the flasks. The flasks were then incubated at 37°C for 1 hour.

Once removed from the incubator, 1ml of 0.5M $CaCl_2$ was added to each flask and the contents shaken thoroughly. 4ml of 0.5M NaOH was then added and the contents shaken thoroughly. The soil suspension was filtered (Whatman, No.2) and the filtrate measured at 400nm on a spectrophotometer (Hitachi U–1100 spectrophotometer). The *p*-nitrophenol content of each filtrate was calculated by reference to a calibration graph prepared from 0-50µg *p*-nitrophenol standards.

A suitable number of replicates were prepared. Controls were prepared by the procedure described above except the addition of *p*-nitrophenyl phosphate solution was made after the $CaCl_2$ and NaOH had been added.

**Standard preparation**

1ml of standard *p*-nitrophenol solution was diluted to 100ml with deionised water. 0, 1, 2, 3, 4 and 5ml of this diluted standard solution was pipetted into 5ml volumetric flasks. The volume of each flask was then adjusted to 5ml with deionised water. The contents of each flask were then transferred into individual 14ml capped vials. 1ml of 0.5M $CaCl_2$ and 4ml of 0.5M NaOH was then added to each vial and the contents filtered as described above.

If the colour intensity of the filtrates exceeded the 50µg *p*-nitrophenol standard, an aliquot of the filtrate was diluted with deionised water until the spectrophotometer

readings fell within the range of the calibration graph. A 1:5 dilution was most commonly used.

## 2.5.2 Dehydrogenase Activity

The measurement of soil dehydrogenase activity was carried out by the method of Casida *et al* (1964).

The method described below involves the colorimetric determination of 2,3,5-triphenyl formazan (TPF) produced by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by soil microorganisms.

**Procedure**

35g of soil (air dry, sieved <2mm) was weighed into a 4oz screw top glass jar. 0.35g of $Ca_2\ CO_3$ was then added and the contents of the jar shaken to allow the $Ca_2CO_3$ to be thoroughly incorporated throughout the soil. 6g of this soil mixture was accurately weighed into a weighing basin and transferred to a quick fit glass test tube. A suitable number of replicates (5) were prepared.

1ml of triphenyltetrazolium chloride (TTC) aqueous solution (3%) and 2.5ml of deionised water were then added to each test tube and the contents mixed thoroughly with a glass rod. The test tubes were then placed in an incubator at 37°C for 24 hours.

Once removed from the incubator, 10ml of methanol was added to each test tube and the contents shaken by hand for approximately 1 minute. The contents of each test tube were then filtered through a glass filter funnel plugged with non absorbent cotton wool into a 100ml volumetric flask. This step was repeated, adding 10ml aliquots of methanol to the test tubes and quantitatively transferring all the soil to the filter funnel. The soil was then washed with methanol to removed all the triphenyl formazan (TPF) produced and the remaining volume in the volumetric flask made up to the mark with methanol.

The extracts were measured at 485nm on a spectrophotometer and the concentration of TPF produced during the 24 hour incubation calculated by reference to the standard calibration graph. The graph was prepared using 500, 1000, 1500 and 2000 $\mu g\ ml^{-1}$ standards where methanol was used as the zero standard. The resulting graph was linear.

## 2.5.3 Fluorescein Diacetate Hydrolysis

The method for measuring fluorescein diacetate (FDA) activity in soil was carried out by the modified method of Adam and Duncan ( 2001 ) from the original method by Schnürer and Rosswall (1982).

FDA hydrolysis is widely accepted as an accurate method for measuring total microbial activity in a range of environmental samples. The method involves the hydrolysis of colourless fluorescein diacetate by both free and membrane bound enzymes to the coloured end product, fluorescein, which can be measured by spectrophotometry. The development of this method is described fully in Chapter 3, Section 3.2.

**Reagents**

Potassium phosphate buffer pH 7.6, 60mM:

8.7g $K_2HPO_4$ (Riedel-de Haën, Sigma-Aldrich Co. Ltd., Analar) and 1.3g $KH_2PO_4$ (Merck, BDH Analar) were dissolved in approximately 800ml deionised water. The contents were made up to 1 litre with deionised water.

2:1 chloroform/methanol

666ml chloroform (Fisher Scientific UK Limited, analytical grade) was added to a 1 litre volumetric flask. The flask was made up to 1 litre with methanol (Fisher Scientific UK Limited, analytical grade) and the contents mixed thoroughly.

1000µg FDA $ml^{-1}$ stock solution

0.1g fluorescein diacetate (3′ 6′-diacetyl-fluorescein., Sigma-Aldrich Co. Ltd.) was dissolved in approximately 80ml of acetone (Fisher Scientific UK Limited, analytical grade) and the contents of the flask made up to 100ml with acetone. The solution was stored at - 20°C.

2000µg fluorescein $ml^{-1}$ stock solution

0.2265g fluorescein sodium salt (Merck, BDH Analar) was dissolved in approximately 80ml of 60mM potassium phosphate buffer pH 7.6 and the contents made up to 100ml with buffer. Fluorescein sodium salt contains 88.3% fluorescein therefore 2265µg fluorescein sodium salt is required to prepare a 2000µg fluorescein standard solution.

20µg fluorescein ml $^{-1}$ standard solution

1ml of 2000µg fluorescein ml $^{-1}$ stock solution was added to a 100ml volumetric flask and the contents made up to the mark with 60mM potassium phosphate buffer pH 7.6. 1-5 µg ml $^{-1}$ standards were prepared from this standard solution by appropriate dilution in 60mM potassium phosphate buffer pH 7.6.

**Procedure**

2g soil (fresh weight, sieved < 2mm) was placed in a 50ml conical flask and 15ml of 60mM potassium phosphate buffer pH 7.6 added. 0.2ml 1000µg FDA ml$^{-1}$ stock solution was added to start the reaction. Blanks were prepared without the addition of the FDA substrate along with a suitable number of sample replicates. The flasks were stoppered and the contents shaken by hand. The flasks were then placed in an orbital incubator (Gallenkamp Orbital Incubator, 100 rev min$^{-1}$) at 30°C for 20 minutes.

Once removed from the incubator, 15ml of 2:1 chloroform/methanol was added immediately to terminate the reaction. Stoppers were replaced on the flasks and the contents shaken thoroughly by hand. The contents of the conical flasks were then transferred to 50ml centrifuge tubes and centrifuged at 2000 rev min$^{-1}$ for approximately 3 minutes (MSE Scientific Instruments, Coolspin 2 centrifuge). The supernatant from each sample was then filtered (Whatman, No 2) into 50ml conical flasks and the filtrates measured at 490nm on a spectrophotometer (Hitachi U–1100 spectrophotometer).

The concentration of fluorescein released during the assay was calculated using the calibration graph produced from 0-5µg fluorescein ml$^{-1}$ standards which were prepared from a 20µg fluorescein ml$^{-1}$ standard solution. The 0µg ml$^{-1}$ fluorescein standard was used to zero the spectrophotometer before each set of blanks and samples were read.

# 2.6 Plant experiments

## 2.6.1 Germination Experiments

### 2.6.1.1 Plant Screening Experiment

Twenty five plant species including grasses, legumes, herbs and commercial crops were screened for their ability to germinate in diesel fuel contaminated soil.

Plants screened included 14 species of grass: Cocksfoot; Creeping bent; Highland bent; Meadow bent; Common bent; Black grass; Couch grass; Sweet vernal grass; Rough meadow grass; Westerwold's ryegrass; Sheep's fescue; Strong creeping red fescue; Chewing's fescue and Annual canary grass, 2 herbs: Black medick and Fodder burnet, 5 legumes: Common vetch; Red clover; White clover; Little yellow trefoil and Lucerne and 2 cultivar varieties of Oil seed rape :Rocket and Martina and 2 cultivar varieties of Flax: Viking and Elise.

The soil used in this experiment was John Innes seed compost and the diesel fuel was obtained from a local Esso petrol station. To obtain an even distribution of diesel fuel oil in the soil, 5g of diesel was weighed into a beaker containing 5g acetone. This mixture was then added to a beaker containing 100g soil (sieved < 2mm) and the contents mixed thoroughly. The acetone was then allowed to evaporate from the soil leaving a 50g $kg^{-1}$ diesel contaminated soil. This procedure was repeated until enough soil was obtained for the experiment. The same procedure was used to produce 25g $kg^{-1}$ diesel contaminated soil.

10g of uncontaminated, 25g $kg^{-1}$ and 50g $kg^{-1}$ diesel contaminated soil was weighed into petri dishes, in duplicate. Seeds of each species investigated were planted in appropriate petri dishes and the soil moistened. The lids were replaced on the petri dishes and the dishes placed in an incubator at 20°C. The dishes containing contaminated treatments were placed in a desiccator (without silica gel) before being put in the incubator whereas the uncontaminated treatments were placed in directly. The petri dishes were watered when necessary and the rate of germination recorded at one and two weeks.

### 2.6.1.2 Germination of Plant Species at Lower Temperatures

This experiment was designed to test the effect of the volatile diesel fraction on seed germination. Eight plant types were planted in 0, 25 and 50g diesel $kg^{-1}$ soil then incubated at 8°C. Seeds can germinate at 6°C therefore an incubation temperature of 8°C was chosen as it would allow the seeds to germinate but the lower temperature would reduce the concentration of volatile diesel components present in each petri dish.

Fifty seeds of Highland bent, Common bent and Sweet vernal grass and twenty five seeds of Black grass, Rough meadow grass, Fodder burnet, Chewing's fescue and Strong creeping red fescue were planted, in duplicate, in petri dishes containing 0, 25 and 50g diesel $kg^{-1}$ John Innes compost No. 2. The soil was prepared as described in Section 2.6.1.1. The dishes were then moistened with water and placed in a dark incubator at 8°C ± 1°C. Once germination had occurred in the majority of samples, the lights were switched on in the incubator and set to a 16 hour light/ 8 hour dark cycle. The dishes were watered when necessary and the germination rate measured weekly for six weeks.

### 2.6.1.3 Volatility Experiment

A new experimental set up was devised to measure the germination rate of selected plant species where low concentrations of volatile diesel components were present.

Twenty five Westerwold's ryegrass, Sweet vernal grass, Annual canary grass, Lucerne and White clover were planted in 0, 25 and 50g diesel $kg^{-1}$ John Innes compost No.2. Each petri dish was then set up as shown in Figure 2.6.1.3.1 with an acetate collar supporting the petri dish lid. Holes were put in the lid to allow the volatile diesel components to dissipate whilst allowing moisture to be retained. The dishes were covered with black plastic sheeting to encourage germination. Once the seeds had germinated, the sheeting was removed and the dishes watered when necessary. Germination rate was measured at 1 and 2 weeks.

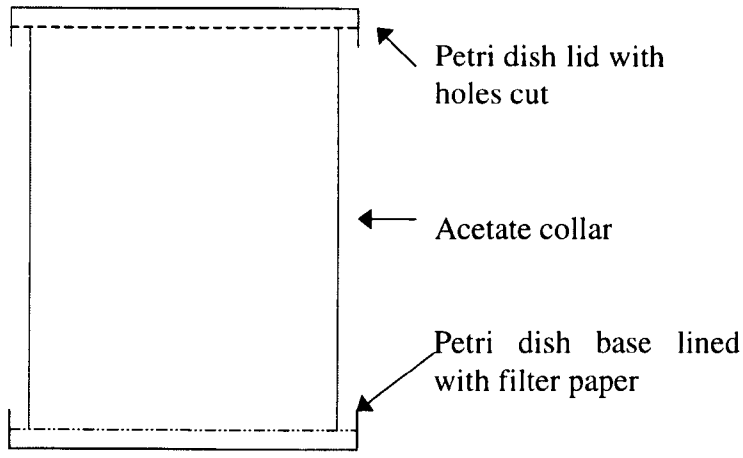



Figure 2.6.1.3.1 Set up for low volatile's germination experiment

## 2.6.1.4 Germination Experiment in 'Aged' Soil

This experiment followed the germination rate of five plant species in 'aged' diesel fuel contaminated soil to illustrate the effect of the volatile fraction on germinating ability of seeds.

Fifty Sweet vernal grass and twenty five Strong creeping red fescue, Sheep's fescue, Chewing's fescue and Black grass seeds were planted in 0, 25 and 50g diesel $kg^{-1}$ John Innes compost No.2 which had been previously contaminated. The soil was contaminated as described in Section 2.6.1.1 and stored in open plastic bags for three weeks. This provided a diesel fuel contaminated soil with the more volatile components at a minimum. The dishes were moistened with water then incubated at 22°C ± 2°C in the dark until the first signs of germination were apparent. The dishes were then positioned in the light and watered when necessary. The germination rate was measured at one and two weeks.

## 2.6.1.5 Seed Viability

This experiment was designed to evaluate a Triphenyl tetrazolium chloride (TTC) method for estimating the germination rates of varying seed species and to determine if diesel fuel had any effect on the viability of seeds.

It is possible to predict the germinability of corn, oats, rye, wheat and barley by observation of the embryo parts that are stained red by the insoluble formazan deposited in viable tissue. It has been reported that new barley, oats and wheat incubated in a 1% TTC solution at 48°C gave a good colour reaction, which appeared to be a quick, reliable index for germination ability (Smith, 1951). The method has been adapted to include other seed species and to evaluate the effectiveness of the method as an indicator of seed germination ability.

**Preliminary investigation**

Seed species chosen for investigation were Flax variety 'Viking', Oil seed rape variety 'Commanche', Canary grass and Red clover. Seeds from each species were randomly sampled from homogeneous populations of each seed type.

Twenty seeds of each type were then incubated at 20°C in a 1.5% TTC solution in petri dishes. Seeds were removed at intervals and dissected using a sharp blade to see the extent of colour development at the embryo. Seeds were cut on the longitudinal axis to expose the embryo clearly. The colour developed was visually rated: red, pink and none which provided an estimate of viability: very viable, may be viable or non viable.

**Effect of diesel fuel on Flax seed viability**

**Procedure**

Flax seed variety 'Viking' was chosen due to the factors stated in the preliminary experiment.

Two hundred and forty randomly sampled flax seeds were split into three groups (80 seeds in each). These seeds were subjected to the following treatments :

1. 80 seeds submerged and soaked in diesel fuel oil for 24 hours,
2. 80 seeds submerged and soaked in diesel fuel oil for 48 hours and
3. 80 seeds submerged and soaked in diesel fuel oil for 168 hours.

Half the seeds from each treatment (40 seeds in total) were removed from the diesel fuel, lightly dried on a paper towels then placed in a 1.5% TTC solution. The seeds were incubated at 20°C for 24 hours to determine the extent of diesel oil penetration and damage to the viability of the seed. The remaining 40 seeds from each treatment were split into 4 groups of 10 seeds and planted in petri dishes containing

John Innes compost No.2. The dishes were moistened and placed in the dark at 20°C until the first signs of germination, when they were removed and allowed to grow in light conditions. The germination rate of the pre soaked seeds was calculated. A control was set up for each test with uncontaminated seeds.

### 2.6.1.6 Phytotoxicity of Individual Petroleum Hydrocarbons

**Branched Cyclohexanes**

The experiment was designed to test the phytotoxicity of individual hydrocarbons commonly found in the lighter fraction of diesel fuel as it has been observed that the volatile fraction of diesel fuel has an effect on seed germinating ability.

**Procedure**

1ml of each cyclohexane solution (methyl, ethyl, propyl and butyl cyclohexane) at each concentration level (0.1, 1 and 5mg $l^{-1}$) was added, in triplicate, to the appropriate, labelled petri dish. Each petri dish was lined with a filter paper (Whatman No.1, 9cm diameter) which absorbed the added contaminants. The acetone was allowed to evaporate for approximately 3 minutes then the filter paper was dampened with water and 15 Westerwold's ryegrass seeds were placed on the filter paper. The petri dished were incubated at 22°C ± 2°C in the dark until the first signs of germination, when they were removed and placed in light conditions. The filter papers were kept moist and the germination rate measured.

**Aromatic Hydrocarbons**

The effect of various aromatic hydrocarbons, commonly found in diesel fuel, on seed germination and development was investigated.

**Procedure**

15g of John Innes compost No.2 was weighed into a petri dish. 1.5ml of 10mg $l^{-1}$ naphthalene solution was added to the soil. The lid was replaced on the petri dish and the contents shaken to mix the contaminant into the soil. This was repeated using the 10mg $l^{-1}$ anthracene solution. The above procedure was repeated for the 50mg $l^{-1}$ 1,4 DMN and 2,3,5 TMN solutions except 0.3ml of each contaminant was added to 15g

soil. This produced soil contaminated with 1mg $l^{-1}$ of each aromatic hydrocarbon. A total of 4 replicate dishes for each contaminant were prepared in this way.

Fifteen Canary grass seeds were planted in each replicate dish, the soil moistened with water and the dishes incubated at 22°C ± 2°C in the dark. Once germination was apparent, the dishes were placed in the light and watered when necessary.

### 2.6.2 Simulated Diesel Fuel Spill

The effect of an aboveground diesel fuel spill on vegetation was investigated by simulating a diesel spill on soil planted with four different plant species.

**Procedure**

Four 35.5cm x 21.5cm seed trays were filled with John Innes compost No.2. Each tray was then seeded with EM3 meadow mix, Westerwold's ryegrass, Red clover or Chewing's fescue. The trays were watered and covered with black plastic sheeting to encourage germination. Once shoots were apparent in the trays, the sheeting was removed and the plants grown in the greenhouse with a 16 hour light/8 hour dark cycle and a constant temperature of 25°C ± 3°C. The plants were grown for two months and the trays were watered when necessary.

After two months, 100ml of diesel fuel was spilled in the centre of each planted tray. Two weeks after diesel addition, the plants were cut back to allow re-growth of new plant material.

The trays were left to grow for a further two months before being destructively sampled. Soil samples were taken from each corner of the tray and one sample from the middle of the tray using a corer (40mm x ID 38mm). The two corner samples from the same side were combined to produce larger samples (see Figure 2.6.2.1). This was repeated for each tray. The collected samples were sieved (< 2 mm) then oven dried (105°C) overnight in porcelain basins. Each soil sample's repellency was evaluated using the MED test as described in Section 2.4.4.

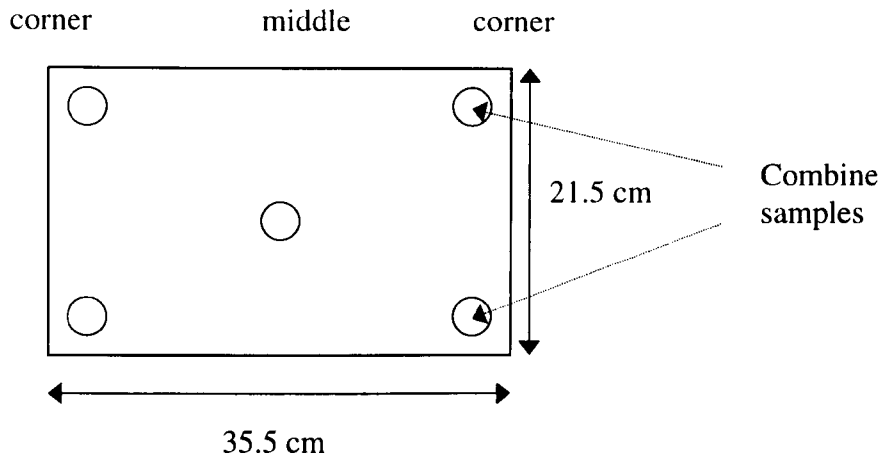



Figure 2.6.2.1. Diagram of sampling pattern of planted trays.

## 2.6.3 Greenhouse Experiments

The most successful plant species observed in the initial germination experiments (Section 2.6.1) were used in a series of greenhouse experiments to evaluate the growth and development of selected species in diesel fuel contaminated soil.

**Preparation of diesel fuel contaminated soil**

Diesel fuel contaminated soil was prepared at different treatment levels by the same procedure for the following pot experiments.

Soil was weighed accurately on a top pan balance (2 figure) and spread out onto heavy duty plastic sheeting. The appropriate amount of diesel fuel, which was obtained from a local Esso petrol station, was weighed into a 4oz glass jar then poured over the soil. The diesel fuel was then mixed in thoroughly through the soil using a hand trowel. The contaminated soil was then weighed into a labelled plastic pot and was ready for planting.

### 2.6.3.1 Initial Mixed Plant

Ten plant species were selected for an initial pot trial. This experiment was split into two sections for ease, with 5 species being investigated in the Set 1 and 5 species in the Set 2.

**Set 1**

Four grass species: Black grass, Cocksfoot, Common bent and Sweet vernal grass and one Oil rape seed cultivar Martina were chosen for investigation.

Ten 4 ″ plastic pots were filled with uncontaminated John Innes Compost No.2, ten pots with 25g diesel $kg^{-1}$ and ten pots with 50g diesel $kg^{-1}$. The diesel fuel contaminated soil was prepared as described in Section 2.6.3. A hundred seeds from each plant species were then sown, in duplicate, into 0g diesel $kg^{-1}$ soil treatment. This was repeated for the 25g diesel $kg^{-1}$ treatment level and the 50g diesel $kg^{-1}$ treatment level. The pots were clearly labelled with an identification number, plant species, treatment level and replicate number and then arranged randomly. The pots were watered and covered with black plastic sheeting to encourage germination. When signs of plant growth were apparent, the sheeting was removed and a set of greenhouse strip lights (3 strip lights in total) placed over the pots. The lights were set on a 16 hour light/8 hour dark cycle and the plants watered when necessary.

**Set 2**

Four grass species were again chosen: Sheep's fescue; Strong creeping red fescue; Chewing's fescue and Westerwold's ryegrass and 1 herb species: Black medick. The pots were set up as before in 0, 25 and 50g diesel $kg^{-1}$ soil except 50 seeds were planted in each pot.

**Greenhouse observations**

The following measurements were made after 3 weeks, 6 weeks then 7 weeks. Tallest plant height (cm), majority plant height (cm), leaf burn (visual rating 1 – 5), stem colour and in addition for the Oil seed rape, maturity rating (ADAS) was measured. Total shoot yield per pot and total root weight per pot were measured at the end of the 7 weeks as described in Section 2.7.1.1 and 2.7.1.2.

## 2.6.3.2 Grasses vs Legumes

Two plant species from the families *Gramineae* and *Leguminosae* were investigated for their potential to grow in soil contaminated with diesel fuel. The experiment was designed to illustrate if the *Leguminosae* grew better in diesel fuel contaminated soil, due to their close association with nitrogen fixing microorganisms, than members of the

*Gramineae* family. The C : N ratio in the soil will be increased by the addition of diesel fuel as diesel fuel is comprised mainly of carbon and hydrogen. This may result in immobilisation of soil nitrogen, leaving none available for plant growth. The *Leguminosae* fix atmospheric nitrogen to produce their own nitrate for growth therefore they may prove more successful in diesel fuel contaminated soil.

**Procedure**

The plant species chosen for this study were Westerwold's ryegrass, Strong creeping red fescue, Common vetch and Red clover.

4.6kg of Darvel soil (sieved < 4 mm) was contaminated with 0, 5, 10 and 20g diesel $kg^{-1}$ soil as described in Section 2.6.3. 350g of soil was weighed into 4″ plastic pots to provide a total of 13 pots contaminated with 0, 5, 10 and 20g diesel $kg^{-1}$. Fifteen seeds from each plant type were planted, in triplicate, into the pots at each treatment. One pot from each treatment was left unplanted. The pots were then laid out in the greenhouse in a randomised design, watered and covered with black plastic sheeting to encourage germination. Once shoot emergence was apparent, the sheeting was removed and the plants were watered when necessary.

**Greenhouse observations**

Longest shoot height and majority shoot height were measured every three weeks until harvest (15 weeks). Germination rate was measured at 3 and 15 weeks. Observations on plant health and development were also made during the experiment. At 15 weeks, the pots were destructively sampled and shoot and root biomass collected for determination as described in Section 2.7.1.1 and 2.7.1.2. Investigation of the Common vetch root nodules was carried out as described in Section 2.7.1.3. In addition, a few nodules from control and contaminated vetch plants were sectioned by hand, placed on a glass slide and viewed by light microscopy (x10 magnification). The light microscope set up is described in Section 2.7.1.4

### 2.6.3.3 Ryegrass vs oil seed rape

A larger greenhouse experiment was set up which compared the growth of Westerwold's ryegrass and Oil seed rape as these seed species consistently grew well in diesel fuel contaminated soil. The experimental design incorporated two plant species

in four diesel fuel treatment levels (0g, 5g, 7.5g and 10g diesel fuel $kg^{-1}$ soil) with two harvest dates (2 and 4 months).

**Procedure**

Arkleston soil was used for this experiment and was contaminated 5kg at a time. Fifty six 9" plastic pots were washed prior to use and labelled with the plant species, contamination level, replicate number and identification number.

In addition to the contaminated soil prepared for the pots, 1kg of soil was also contaminated at each diesel fuel level and stored at 4°C for the duration of the experiment to provide a baseline for diesel lost through abiotic processes.

Twenty one sets of each seed type were counted out and stored in clip top vials until planting. Sixty Westerwold's ryegrass and 40 Oil seed rape cultivar Commanche seeds were in each set. The seeds were planted in the appropriate pot then the pots were laid out in a randomised block design in the greenhouse. The pots were then thoroughly watered and covered with black plastic sheeting to encourage germination. After 4 days the sheeting was removed and the pots were watered when required.

**Greenhouse measurements and soil sample analyses**

Germination rate was measured after 1 week then at the 2 month and 4 month sampling dates. After 2 months the first set of pots were destructively sampled. Plant productivity was assessed by measuring oven dry biomass as described in Section 2.7.1.1.

Enzyme analyses including acid and alkaline phosphatase activity (Section 2.5.1), dehydrogenase activity (Section 2.5.2) and FDA activity (Section 2.5.3) were carried out on soil subsamples from each pot. Finally, diesel analysis was carried out on soil subsamples from each pot, the soils kept at 4°C for the duration of the experiment and freshly contaminated soils at each diesel fuel level.

## 2.6.3.4 Legume vs Non-Legume

This pot trial involved three plant species plus a control with no plants at four different diesel fuel treatment levels (0, 5, 10 and 15g diesel $kg^{-1}$ soil). The trial incorporated two sampling dates at 2 months and 4 months growth

**Procedure**

Eighty 7″ plastic pots and eighty 9″ plastic saucers were washed prior to use. Each pot was labelled with an identification number, plant type, treatment level and replicate number. Twenty four sets of each seed type were counted out and stored in clip top vials. Twenty seeds per set was decided on for both Westerwold's ryegrass and Common vetch as this would provide adequate plant cover but with enough room for growth over the 4 month experiment. Because the EM3 special general purpose meadow mix was a mixture of seed species, counting out individual seeds was unrealistic. Instead, 0.09g ± 0.005 of EM3 was weighed into each set as suggested by the supplier's guidelines for sow rate (4g per $m^2$).

Each pot holds 2kg soil therefore 40kg of uncontaminated, 5g diesel $kg^{-1}$, 10g diesel $kg^{-1}$ and 15 g diesel $kg^{-1}$ John Innes compost No.2 was required. The diesel fuel treated soil was contaminated 2kg at a time as described in Section 2.6.3. The pots were then laid out in the greenhouse. The seeds were planted into each pot and the pots thoroughly watered. The pots were then covered with black plastic sheeting to aid germination and prevent the surface soil from drying out. After 4 days, when most of the seeds were showing signs of germinating, the covering was removed. Light was provided by a central lamp (Phillips 400 watt HP1 plus) set on a 16 hour light/8 hour dark cycle. The pots were watered initially every day from above, to provide the germinating seeds with water but once the plants were reasonably well grown, the saucers were filled and the plants obtained water from below.

## 2.6.3.5 Willow Clone Trial

This experiment was designed to determine if variability exists among willow clones for performance in diesel fuel contaminated soil.

**Plant and soil selection**

For this study, four willow clones were chosen. One traditional British variety of basket willow, Rosewarne white (*Salix aurita x cinerea x viminalis*) and three recently bred Swedish varieties of *Salix viminalis* : Jorr, Jorrun and Ulv were selected for comparison.

2kg of John Innes seed compost was contaminated at a time using the procedure described in Section 2.6.1 to obtain 0, 25 and 50g diesel $kg^{-1}$ contaminated soils. This procedure was repeated until 20kg of each contaminated soil was produced. The

contaminated soils were then transferred to 25kg troughs. A trough was also set up with 20kg of uncontaminated soil as a control.

**Experimental design**

The stools of each willow clone were labelled with a number from 1–12. Four stools from each willow clone were planted into 0, 25 or 50g $kg^{-1}$ contaminated soil. The design was a randomised complete block with four replications.

**Greenhouse observations**

The following agronomic traits were measured every two months: shoot length, number of shoots and diameter of longest shoot at half height. Total yield, number of leaves per shoot and root mass was measured at the end of the experiment.

**Harvesting of willow**

After 10 months growth, the final measurements were taken and the willow trees were harvested. The trees were harvested by cutting the shoots level with the top of the original stool. The shoot and leaf material were put in plastic sample bags until oven dry biomass was assessed.

The stools and roots were removed from the troughs and any soil adhering to the roots shaken back into the trough. The root material and stools were then placed in plastic sample bags. It was extremely difficult to separate the roots of individual trees so the overall root biomass for each trough was measured.

The shoot material was prepared as described in section 2.7.1. To obtain the total oven dry shoot biomass per plant.

The soil from each trough was emptied onto heavy duty plastic sheeting and allowed to air dry for 48 hours. The soil was then sieved (< 2mm) to remove the rest of the root material. This root material was added to the material already collected for each trough. The soil was then subsampled and stored in a tied plastic sample bag at 4°C until diesel analysis was carried out. The root material was prepared and dried as described in Section 2.7.2.

**Diesel analysis**

The diesel fuel remaining in the soil after 10 months was extracted from a subsample of each trough using the Soxhlet method (section 2.3.1) followed by GC-FID analysis (Section 2.3.2).

### 2.6.3.6 Detoxification Experiment

The experiment was designed to investigate the effect of the more volatile fraction of diesel fuel on plants at different stages of growth. Both seeds and transplanted seedlings were grown in freshly contaminated and 'aged' diesel fuel contaminated soils.

**Procedure**

Three hundred and fifty Westerwold's ryegrass seeds were planted in 35.5cm x 21.5cm seed trays, approximately 2 weeks before the experiment start date. John Innes compost No.2 was contaminated at 25g and 50g diesel $kg^{-1}$ soil treatment levels as described in section 2.6.3. Two kg of soil at each contamination level was prepared 4 weeks in advance of the experiment start date. Four 4″ pots were filled with this soil and left to 'age' in the greenhouse. This procedure was repeated 1 week before the experiment start date, with four 4" pot again being filled with contaminated soil at each level. The day before the experiment was due to commence, 2kg of soil was freshly contaminated at each level and transferred to 4″ pots. Twenty five seeds or twenty five 2 week old seedlings were planted, in duplicate, at each level of diesel fuel contamination. The pots were thoroughly watered and laid out in the greenhouse. The pots were watered when necessary.

**Greenhouse observations and harvesting**

The general condition of the plants was monitored along with germination rate for the seeded treatments and survival rate for the transplanted seedlings. The longest shoot was also recorded. Once the experiment was complete, the shoot biomass was collected and oven dried as described in Section 2.7.1.

## 2.7 Harvesting of Greenhouse Trials

### 2.7.1 Preparation of Shoot Material

The shoot material was cut at the root-shoot interface, one plant at a time. This allowed the number of plants per pot to be calculated accurately at the time of harvest. The shoot material from each plant was bulked together, cut down to manageable lengths (if required) and then placed in pre-weighed foil envelopes. The envelopes containing

shoot material were oven dried for 72 hours at 75°C. After this time, envelopes containing shoot material were removed from the oven and cooled in a desiccator. Once cool, the envelopes containing shoot material were weighed (to four figures) to obtain the oven dry weight of shoot material.

Willow shoot material was cut level with the top of the original stool. The shoots were then cut using secateurs to approximately 5cm lengths to allow easier drying. The cut shoot material was placed in preweighed foil basins then oven dried at 75°C for 96 hours. The foil basins were then removed from the oven and allowed to cool in a desiccator. The foil basins containing oven dried shoot material were weighed on a two figure balance and the weight recorded.

Determination of dry weight should be carried out at 60-75°C as destruction of plant material is prevented at these lower temperatures (Böhm, 1979).

### 2.7.2 Preparation of Root Material

When harvesting, the pot was upturned onto heavy plastic sheeting and the plants plus soil tipped out. The shoot (top growth) material was then cut at the root-shoot interface and retained for further preparation. The root and soil mixture was shaken to dislodge the majority of soil. The larger roots were hand picked from the soil and set aside. The soil was then sieved (< 2mm) to retain the remaining root material and this was added to the rest of the root material. The soil (sieved < 2mm) was sub-sampled for enzyme and diesel analyses if required.

The collected root material was air dried then sorted by hand to remove most of the soil debris. The roots were then washed by hand through a 1mm sieve in continuos running water as a final cleaning step.

The washed roots were air dried to near dryness then placed in a pre-weighed foil envelope and dried at 75°C for 72 hours. After this time, the envelopes containing root material were removed and allowed to cool in a desiccator. Once cool, the envelopes containing root material were weighed (to four figures) and the weight of oven dry root material calculated.

Samples prepared in this way may lose fine roots and older root segments. Due to this, a 10% total root weight error was placed on the oven dry root weights (Böhm, 1979).

### 2.7.3 Determination of Root Nodules

Leguminous plants were also prepared for root nodule investigation. The plants were shaken free from soil then washed thoroughly under running water. After the root sample was washed, the number of nodules was determined on a per plant basis. The roots nodules were then prepared for microscopy (Section 2.7.1.4) or dry root weight determination (Section 2.7.1.2).

### 2.7.4 Nodule Embedding and Microscopy

The preparation of root nodules for microscopic examination was carried out by Mr Eoin Robertson of the IBLS Electron Microscopy Unit.

Freshly washed root nodules were fixed in gluteraldehyde in 0.2M cacodylate buffer for 6 hours then dehydrated through an ethanol series. The dehydrated nodules were then infiltrated in LR white resin for 48 hours and polymerised at 60°C overnight. Sections (2 microns) were cut using a glass knife on a LKB ultratone III and dried onto glass slides over a hot plate at 60°C. Half the sections were stained at 60°C for 10 seconds with 1% toluidine blue in 1% sodium tetraborate (Borox). The sections were visualised at 10x and 40x magnification on a Leica ATC ™ 2000 compound microscope with camera attachment.

## 2.8 Spatial Distribution of Roots

An experimental system was set up which enabled the pattern of root development of selected plant species to be followed in a model soil system contaminated with diesel fuel.

**Experimental set up**

Two glass boxes were constructed (40cm x 100cm) from heavy glass plates and set up as shown in Figure 2.8.1. Different patterns of diesel oil contamination were investigated to follow how root development in selected plant species altered when diesel fuel was encountered. The different set ups are shown below.

In each set up, John Innes seed compost (sieved < 2mm) was used and the different concentrations of diesel oil contaminated soil prepared as described in section

2.6.3. The glass boxes were set up in the greenhouse where light was provided on a 16 hour light/8 hour dark cycle from a central lamp (Phillips 400 watt HP1 Plus) and the temperature was maintained at 25°C ± 3°C. The glass boxes were covered with black plastic sheeting to create a realistic soil environment and to allow the seeds to germinate. The plants in the glass boxes were watered when necessary.

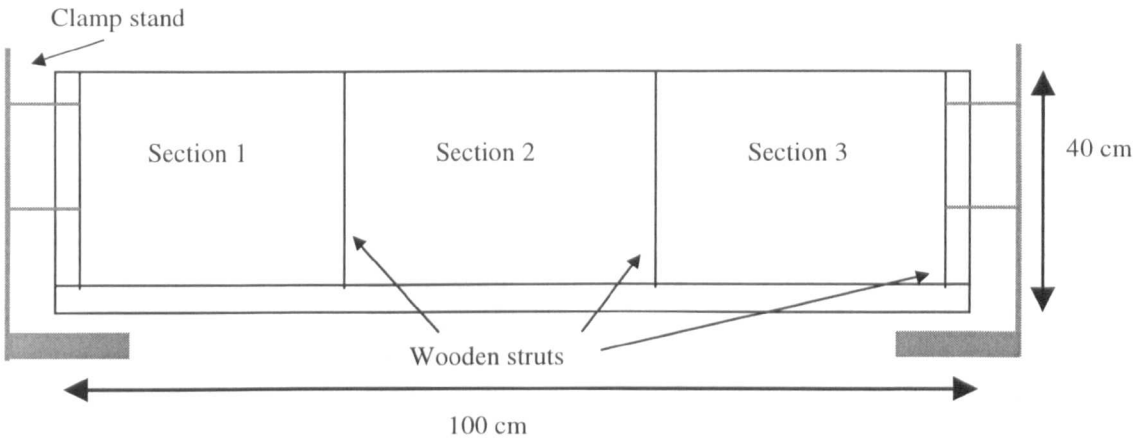



Figure 2.8.1 Basic set up of glass box for following the pattern of root development *in situ*

**Set up 1:** One glass box was left as a control and filled with uncontaminated soil. The second box was contaminated with a layer of diesel fuel (10ml for each section) at a depth of 10cm and uncontaminated soil was placed on top of the contaminated layer. Each box was split into three sections to allow comparison of three different plant species (see Figure 2.8.2). The plant species chosen were two grasses, Common bent and Sweet vernal grass and an Oil seed rape cultivar variety Martina. One hundred seeds from each species were sown into the relevant section of each box and the boxes watered.

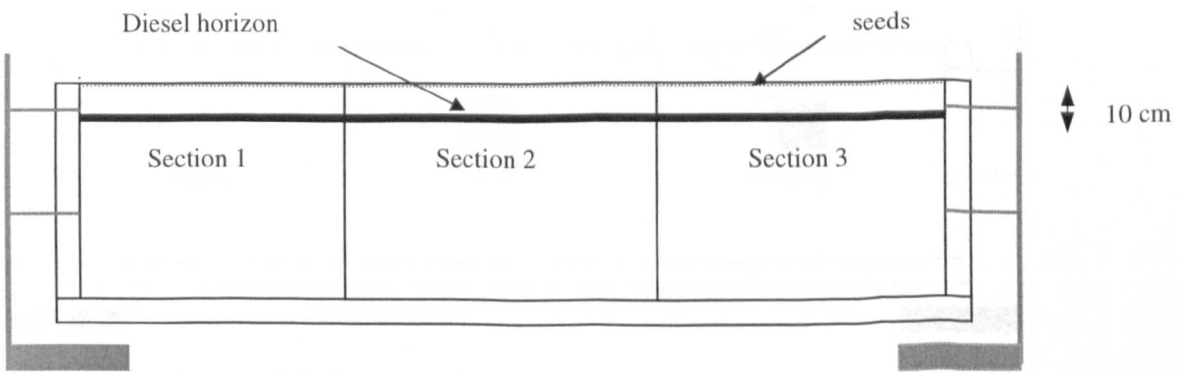



Figure 2.8.2. Impact of a diesel horizon on plant root growth.

**Set up 2:** Glass boxes were set up in three sections as in set up 1. Two of the three sections were further split into two halves to provide one half with contaminated soil and one half with uncontaminated soil (see Figure 2.8.3.). 25g $kg^{-1}$ and 50g $kg^{-1}$ contaminated soil was prepared and packed into the allocated area. A 10cm depth of uncontaminated soil was included on top of all three sections.

50 Cocksfoot seeds and 25 Fodder burnet seeds were planted in each section of the appropriate box. The boxes were watered and covered with plastic sheeting as before.

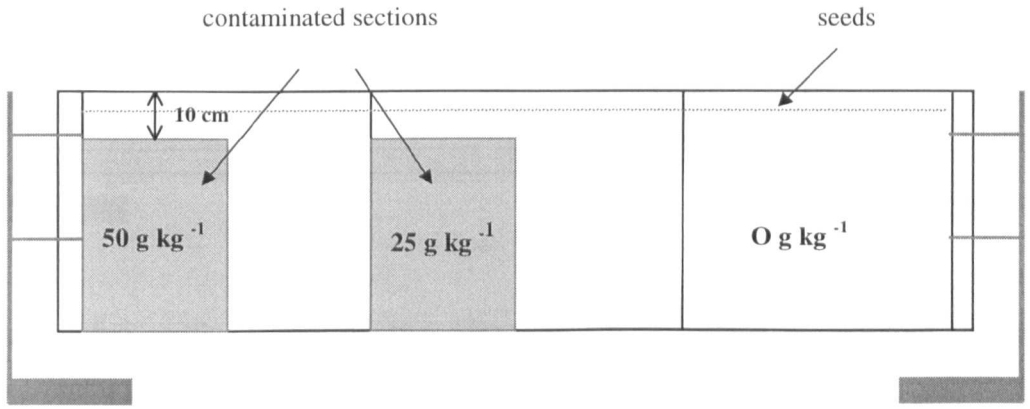



Figure 2.8.3. Development of roots when in contact with uncontaminated and contaminated soil

**Set up 3:** Glass boxes were set up as before except each section had a contaminated patch of diesel fuel oil. 25g $kg^{-1}$ and 50g $kg^{-1}$ contaminated soil was prepared as before and then used to pack a 6cm x 6cm square of the glass box section. A control section was included with no contaminated soil. One glass box was planted with Oil seed rape variety Commanche, 20 seeds per section and the second box planted with Flax variety Viking, 30 seeds per section. The boxes were covered with plastic sheeting and watered when necessary.

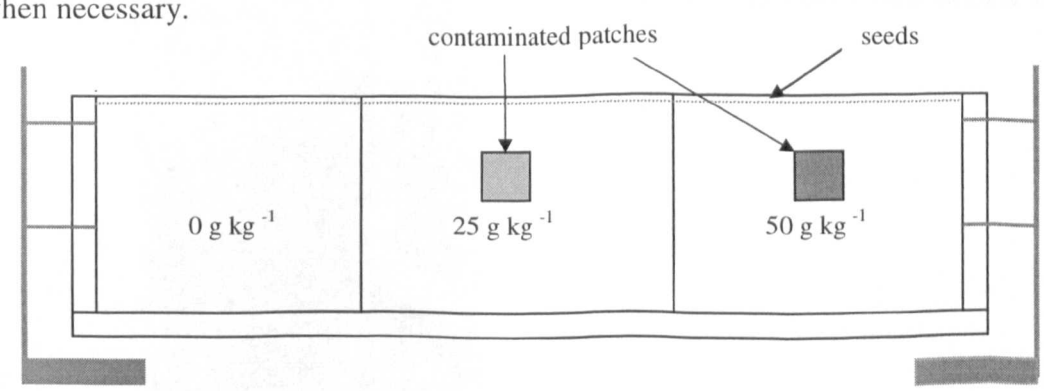



Figure 2.8.4. Pattern of root growth where diesel fuel contaminated is present in distinct patches.

**Set up 4:** Glass boxes were constructed as before but each section contained two contaminated squares per section (4cm x 2.5cm). The levels of diesel fuel oil contamination were 0, 5, 10g $kg^{-1}$ in box 1 and 15, 25 and 50g $kg^{-1}$ in box 2. Twenty Oil seed rape seeds variety Commanche were planted in each section and the glass boxes watered.

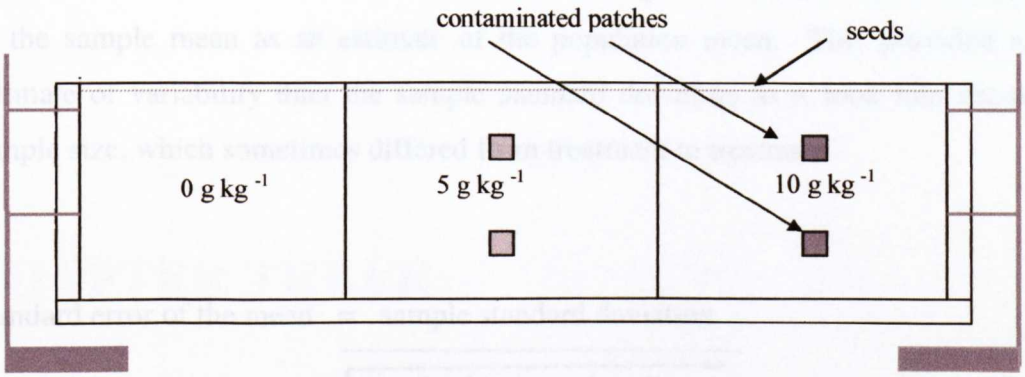



Figure 2.8.5. Illustration of box showing smaller areas of discrete diesel fuel contamination.

**Set up 5:** One glass box was constructed as shown in Figure 2.8.6. with the glass positioned lengthwise. This gave a 1 metre deep soil profile which was used to measure the rooting depth of Westerwold's ryegrass. The box was packed entirely with uncontaminated soil and 3 seeds planted. The box was watered and covered with plastic sheeting and the root depth measured on a weekly basis.

Figure 2.8.6. Photograph of vertical glass box set up for monitoring rooting

## 2.9 Statistical Analysis

Sample standard deviations, calculated using the Microsoft Excel statistical function, were used to work out the standard error of the mean and the coefficient of variation.

The standard error of the mean was used to provide an estimate of the precision of the sample mean as an estimate of the population mean. This provided a better estimate of variability than the sample standard deviation as it took into account the sample size, which sometimes differed from treatment to treatment.

$$\text{Standard error of the mean} = \frac{\text{sample standard deviation}}{\sqrt{\text{sample size (no. of replicates)}}}$$

The coefficient of variation was used to examine the degree of variability between two or more sample means where the mean values were on incomparable scales. This was calculated from :

$$\text{Coefficient of variation} = \frac{\text{sample standard deviation}}{\text{sample mean}} * 100$$

Analysis of variance was carried out using the Minitab package and Tukeys One Way Anova function which sets a 95% confidence interval. The Anova values indicated whether or not there was a significant difference between treatment means.

# CHAPTER THREE

## METHOD DEVELOPMENT

The methods for diesel fuel extraction and analysis and Fluorescein diacetate (FDA) hydrolytic activity were integral parts of this study and were chosen for further development.

## 3.1 Analysis of Diesel Fuel

### 3.1.1 Extraction of Diesel Fuel from Soil

Due to the different concentrations of diesel fuel present in the soil samples analysed (ranging from 5g–50g diesel $kg^{-1}$ soil) during this study and the different residence times of diesel fuel in these soil samples (ranging from 1 week to 10 months), different extraction techniques had to be developed to effectively remove the remaining diesel fuel.

Soil samples containing diesel fuel with a short residence time, such as the soil samples taken after 1 week from the leaching experiment described in Section 2.4.1, contained both volatile diesel fuel components and heavier diesel fuel components. The extraction method for these samples required a two-step extraction procedure whereby

the more volatile components were initially extracted followed by removal of the heavier components.

Samples containing diesel fuel with longer residence times (>1 month) did not require this initial extraction step as the majority of volatile components were already lost by this time. Instead, a hot solvent extraction could be performed directly on these samples. Depending on the initial concentration of diesel fuel in soil, different sample preparations were performed and different solvents used for the extraction.

#### 3.1.1.1 Long Residence Time Samples (>1 month)

It has been observed during the course of this study that the majority of the volatile diesel fuel components are lost from soil within 2–3 weeks. Therefore, a hot solvent extraction technique was used directly on long residence time soil samples, as no initial extraction was required for removal of the lighter, more volatile components. The hot solvent, soxhlet extraction technique was carried out using the method of Song *et al* (1990). The type of solvent used during the extraction was dependent on the initial diesel fuel concentration of the soil samples.

**High Level Diesel Fuel Samples (25–50g diesel $kg^{-1}$ soil range)**

The higher diesel fuel concentrations were used in the Willow clone trial described in Section 2.6.3.5. Because the initial concentrations were high, the overall recovery of diesel fuel from soil was achieved relatively easily using the soxhlet extraction method. 100ml of dichloromethane was used to extract a 40g air dried (<2mm) diesel fuel contaminated soil sample. The recovery value of the method was tested by adding a specified concentration of diesel fuel to soil, then allowing the diesel fuel to interact with the sample for 24 hours. This contaminated soil sample was air dried and sieved < 2mm, then extracted with hot dichloromethane for 6 hours. Quantitative recovery of this 0 time sample was determined on total petroleum hydrocarbon (TPH) recovery as well as on recovery of individual hydrocarbons. Dilution of the original diesel fuel in dichloromethane served as a quantitative analytical standard. The results for TPH recovery and individual hydrocarbons are given in Table 3.1.1.1.1 below.

| | Peak area | | | | |
|---|---|---|---|---|---|
| | Standard original diesel fuel | | 0 time freshly extracted 25g $kg^{-1}$ | | Recovery % |
| TPH | 159514 | | 119198 | | 74.7 |
| Individual petroleum hydrocarbons | Ret time | Peak area | Ret time | Peak area | |
| C10 | 16.847 | 2823 | 16.947 | 1074 | 38.0 |
| C11 | 20.392 | 4820 | 20.496 | 3078 | 63.9 |
| C12 | 23.710 | 5336 | 23.819 | 4234 | 79.3 |
| C13 | 26.818 | 5417 | 26.932 | 4753 | 87.7 |
| C14 | 29.735 | 5529 | 29.852 | 4966 | 89.8 |
| C15 | 32.483 | 5467 | 32.603 | 4993 | 91.3 |
| C16 | 35.076 | 4995 | 35.200 | 4636 | 92.8 |
| C17 | 37.531 | 7101 | 37.659 | 6322 | 89.0 |
| C18 | 39.871 | 5484 | 40.003 | 5280 | 96.3 |
| C19 | 42.083 | 3094 | 42.218 | 3104 | 100.3 |
| C20 | 44.196 | 2479 | 44.334 | 2447 | 98.7 |

Diesel fuel standard is prepared from a 1:100 diltuion of original diesel fuel in dichloromethane. 0 time standard is a 40g sample of 25g diesel $kg^{-1}$ soil extracted with 100ml dichloromethane which is equal to a 1:100 dilution.

Total petroleum hydrocarbon value (TPH) is the sum of all the peak areas measured.

Individual petroleum hydrocarbons are n-alkanes with carbon numbers ranging from 10 – 20. Retention time is the peak retention time in minutes.

Table 3.1.1.1.1. TPH recovery and individual petroleum hydrocarbon recovery values used to assess efficiency of the dichloromethane soxhlet extraction procedure, n = 3 where % difference between triplicate injections <5%.

Due to the acceptable recovery achieved by this method (approximately 75% TPH), the method was not modified for high level diesel fuel samples. The majority of the 25% diesel fuel components that were not recovered were probably volatile diesel fuel components that would be quickly lost from the soil. The extremely high recovery of the heavier diesel fuel components (approximately 80–100%) indicated the method was working successfully. However, when using this method for lower level diesel fuel samples, the recovery rate was much lower than achieved before (approximately 50%

recovery, data not shown). The method was therefore modified to allow quantitative extraction of diesel fuel from low level samples (5-20 g diesel $kg^{-1}$ soil ) with better recovery rates.

**Low Level Diesel Fuel Samples (5–20g diesel $kg^{-1}$ soil range)**

To enhance the extraction of diesel fuel components from soil, 5g diesel $kg^{-1}$ soil samples were ground prior to extraction and the recovery rates compared to samples that were sieved <2 mm prior to extraction. Grinding of soil samples prior to extraction increased the recovery of diesel fuel components only slightly (1.5%). The most noticeable increase in recovery was observed when different solvents were used during the extraction. The US EPA method 3540C for non volatile and semi volatile organic compounds suggests using a mixture of dichloromethane and acetone (1:1) for extraction of petroleum hydrocarbons. This solvent mixture was assessed and the recovery rates obtained compared to a dichloromethane extraction on its own. The results showed a large increase in recovery rate when the dichloromethane : acetone (1:1) mix was used. Ground 5g diesel $kg^{-1}$ soil samples extracted with dichloromethane recovered only 48% of the total diesel fuel added. This value increased to 60% recovered when dichloromethane : acetone (1:1) was used. Again, the recovery rates were calculated using dilution of the original diesel fuel in dichloromethane. The low level diesel fuel samples were used in long term growing experiments (> 1 month) so although this value for recovery appears to be quite low, when you consider the contribution the volatile diesel fuel components would make to this value, the recovery is acceptable. An estimate of the total peak area lost from volatile diesel fuel components was determined by calculating the sum of the peak areas of every peak measured in the original diesel fuel standard below 21 minutes retention time. From headspace analyses carried out in Section 4.4 it was observed that diesel fuel components of retention times of 21 minutes or less could volatilise freely at room temperature. It was therefore decided that components below 21 minutes retention time coming off the GC column would be lost naturally through volatilisation and would therefore not be present in diesel fuel contaminated soil samples of 1 month or over. The recovery of diesel fuel components from this low level sample (5g diesel $kg^{-1}$ soil) using dichloromethane : acetone (1:1) increased to approximately 76% recovery when the estimate of volatiles diesel fuel components is included. This recovery rate was deemed acceptable for the lowest level sample used during the course of this study (5g diesel $kg^{-1}$ soil) and theoretically, the recovery would increase with the other higher concentrations of the low level samples (10–20 g diesel $kg^{-1}$ soil).

### 3.1.1.2 Short Residence Time Samples (1 week)

Soil samples containing diesel fuel with a short residence time still contain the majority of volatile diesel fuel components found in fresh diesel fuel. Because of this, an initial extraction must be performed to ensure quantitative removal of the volatile diesel fuel fraction. As these diesel fuel components are low molecular weight, a cold solvent extraction is necessary to prevent volatilisation of the components of interest. A cold shaking extraction was developed from the method of Schwab *et al* (1999) using the solvent dichloromethane : acetone (1:1) mixture which performed extremely well in previous extractions.

A 0 time, 5g diesel $kg^{-1}$ soil sample was prepared as a standard to test the recovery of this method. Again, suitable dilution of the original diesel fuel (0.2g) in dichloromethane (100ml) served as a quantitative analytical standard. The fresh sample was extracted by mechanical shaking using 100ml dichloromethane : acetone (1:1). After 30 minutes extracting, the sample was filtered into 100ml volumetric flask and made up to the mark with dichloromethane : acetone (1:1). This sample was then analysed by GC-FID to obtain the TPH value as described in Section 2.3.4. The recovery of diesel fuel components using the modified mechanical shaking method was excellent. 82.6% total petroleum hydrocarbons (TPH) was recovered using the initial cold extraction. Individual petroleum hydrocarbon recoveries were also assessed, with recoveries ranging from approximately 79–87 % for n-alkanes (C12- C21). The recovery of the more volatile diesel fuel components was greatly improved compared to the hot solvent soxhlet extraction. The recovery values for TPH as well as individual petroleum hydrocarbons are given in Table 3.1.1.2.1.

To increase the recovery further, samples that had previously been cold solvent extracted were kept for additional extraction by the hot solvent soxhlet method. This would hopefully improve the recovery of the heavier diesel fuel components whose recoveries were not as high using the cold solvent method as previously observed using the hot solvent soxhlet extraction. The samples were prepared and extracted using dichloromethane : acetone (1:1) as described in Section 3.1.1.1 and the extracts analysed by GC-FID to obtain TPH values and individual petroleum hydrocarbon values. The recovery of diesel fuel components by this extra step was increased and the results are given in Table 3.1.1.2.1.

Table 3.1.1.2.1 (shown on next page). TPH recovery and individual petroleum hydrocarbon recovery values used to assess efficiency of the mechanical shaking and soxhlet extraction procedure, n = 3 where % difference between triplicate injections <5%.

Diesel fuel standard is prepared from a 0.2:100 diltuion of original diesel fuel in dichloromethane. 0 time standards are 40g samples of 5g diesel $kg^{-1}$ soil extracted with 100ml dichloromethane : acetone which is equal to a 0.2:100 dilution. Total petroleum hydrocarbon value (TPH) is the sum of all the peak areas measured. Individual petroleum hydrocarbons are n-alkanes with carbon numbers ranging from 12–21. Retention time is the peak retention time in minutes. Increased recovery % is the total peak area measured by adding the shaking and soxhlet extracted peak area values together and displaying as a percentage of the original diesel fuel standard.

| | Peak area | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Standard original diesel fuel | | 0 time shaking freshly extracted 5g diesel $kg^{-1}$ soil | | Recovery % | 0 time soxhlet freshly extracted 5g diesel $kg^{-1}$ soil | | Increased recovery % |
| TPH | 56019 | | 46266 | | 82.6 | 4322 | | 90.3 |
| Individual hydrocarbons | Ret time | Peak area | Ret time | Peak area | | Ret time | Peak area | |
| C12 | 23.015 | 1953 | 23.003 | 1607 | 82.2 | 23.016 | 31.2 | 83.9 |
| C13 | 26.106 | 1887 | 26.094 | 1567 | 83.1 | 26.106 | 82.5 | 87.4 |
| C14 | 29.008 | 1971 | 28.996 | 1648 | 83.6 | 29.008 | 139.8 | 90.7 |
| C15 | 31.744 | 1858 | 31.733 | 1587 | 85.4 | 31.744 | 187.3 | 95.5 |
| C16 | 34.327 | 1787 | 34.316 | 1533 | 85.8 | 34.326 | 193.8 | 96.6 |
| C17 | 36.771 | 2518 | 36.759 | 2185 | 86.8 | 36.770 | 332.7 | 99.9 |
| C18 | 39.102 | 1959 | 39.091 | 1663 | 84.9 | 39.102 | 292.1 | 99.8 |
| C19 | 41.309 | 1239 | 41.297 | 1047 | 84.5 | 41.309 | 178.6 | 98.9 |
| C20 | 43.415 | 1128 | 43.404 | 899 | 79.7 | 43.418 | 220.4 | 99.2 |
| C21 | 45.426 | 760 | 45.415 | 621 | 81.8 | 45.429 | 127.0 | 98.4 |

Table 3.1.1.2.1 TPH recovery and individual petroleum hydrocarbon recovery values used to assess efficiency of the mechanical shaking and soxhlet extraction procedure, n = 3 where % difference between triplicate injections <5%.

The development of the methods described in the previous section allowed quantitative extraction of diesel fuel from a range of diesel fuel contaminated soil samples with acceptable recovery rates. As diesel fuel contaminated soil ages, volatile diesel fuel components are lost and the remaining diesel fuel components become more difficult to extract. A hot solvent soxhlet extraction was therefore required to remove the 'aged' diesel fuel successfully from the long residence time samples. Recovery of 'aged' diesel fuel was acceptable in the high level diesel fuel samples using dichloromethane but not in the low level diesel fuel samples. A mixture of acetone : dichloromethane (1:1) was used instead, with great success. The advantage of acetone : dichloromethane (1:1) against dichloromethane on its own may be due to the increased polarity of the extracting solvent causing a 'wetting' effect on the soil components thereby enhancing the extraction of diesel fuel.

The recoveries obtained for each extraction method were acceptable and the loss in recovery observed could be easily explained by loss of the more volatile diesel fuel components. Therefore, the methods described here were used as standard methods for the extraction of diesel fuel from soil samples throughout the rest of this study. The final extraction procedures are described in full in Section 2.3.1.

## 3.1.2 Capillary GC-FID Method for Diesel Fuel Analysis

The method for diesel analysis by GC-FID was modified from the US EPA method 8100 for the analysis of polyaromatic hydrocarbons (PAHs) (US EPA, 1986). Method 8100 describes the use of both packed and capillary columns however, the capillary column method was chosen as a starting point as better resolution of individual hydrocarbon components would be achieved.

### 3.1.2.1 Summary of Method

The method provides gas chromatographic conditions for the detection of parts per billion (ppb) levels of certain PAHs. The samples must first be extracted, then both neat and diluted samples may be analysed by direct injection. A 2 to 5µl aliquot of the sample is injected into the GC using the solvent flush technique and the compounds in the effluent detected by flame ionisation detection (FID).

### 3.1.2.2 Choice of Solvent

The suggested solvents for use in this method were analytical grade isooctane and hexane. Isooctane was found to contain impurities, most of which were in the time range of the diesel fuel component peaks which provided an unsatisfactory baseline. In order to minimise the levels of background noise created by the solvent, hexane was tested. The resultant trace showed considerably lower levels of impurities, which provided a steady baseline with low background noise. Hexane proved a satisfactory solvent to use for the analysis.

### 3.1.2.3 Gas Chromatograph (GC)

Analyses were carried out on a Hewlett-Packard 5890A gas chromatograph. The GC was interfaced with a Hewlett-Packard Chemstation data system which comprised of a HP 9000 300 series computer, HP 9153C disc drive and Thinkjet printer.

Hydrogen and air were used as detector support gases with nitrogen as the make up gas. Helium was used as the carrier gas as it is the preferred gas for temperature programmed analyses. The column head pressure was set at 30kPa. The carrier gas linear flow velocity was checked using the non-retained compound, butane. The flow was adjusted as near to the recommended 20cm $s^{-1}$ as possible (actual linear velocity was 19.3cm $s^{-1}$).

The injection mode was purged splitless injection with a purge delay of 36 seconds. This delay allowed volatile peaks to be obtained from the column with the least amount of interference from the solvent tail.

### 3.1.2.4 Choice of Column

Method 8100 suggests using a 30m x 0.25mm I.D. or 30m x 0.32mm I.D. fused silica capillary column. In this case a 25m x 0.32mm I.D. SGE BPX 5 (5% phenyl (equiv.) polysilphenylene siloxane) capillary column (SGE Chromatography Products) was used for the analysis of diesel fuel oil. Diesel fuel is a complex mixture of volatile, semi-volatile and non-volatile components. The BPX 5 capillary column provided a versatile column that has been successfully used to analyse non-volatile PAHs as well as semi-volatile hydrocarbons. In addition, the BPX 5 capillary column proved capable of resolving volatile hydrocarbons.

The BPX phase type has a greater stability through substitution of a benzene ring for some of the oxygen atoms in the siloxane backbone. This substitution dramatically improves both the chemical and thermal stability of the stationary phase. This allows column bleed to be kept to a minimum, high operating temperatures to be achieved and an extended column life (SGE Chromatography Products Catalogue, 1998). Low column bleed is particularly important, as most of the diesel fuel analyses will be in the trace range where low column bleed enhances the detection and quantitation of analytes. Reduced column bleed also makes the column more detector compatible, minimising rising baselines and detector contamination. This aspect is most important for the analysis of biodegraded and weathered fuel products, as baseline rise is unavoidable in these samples.

### 3.1.2.5 Detector

Flame Ionisation Detection (FID) was chosen as it exhibits a nearly universal response to all organic compounds, it is sensitive, stable and has a large linear range.

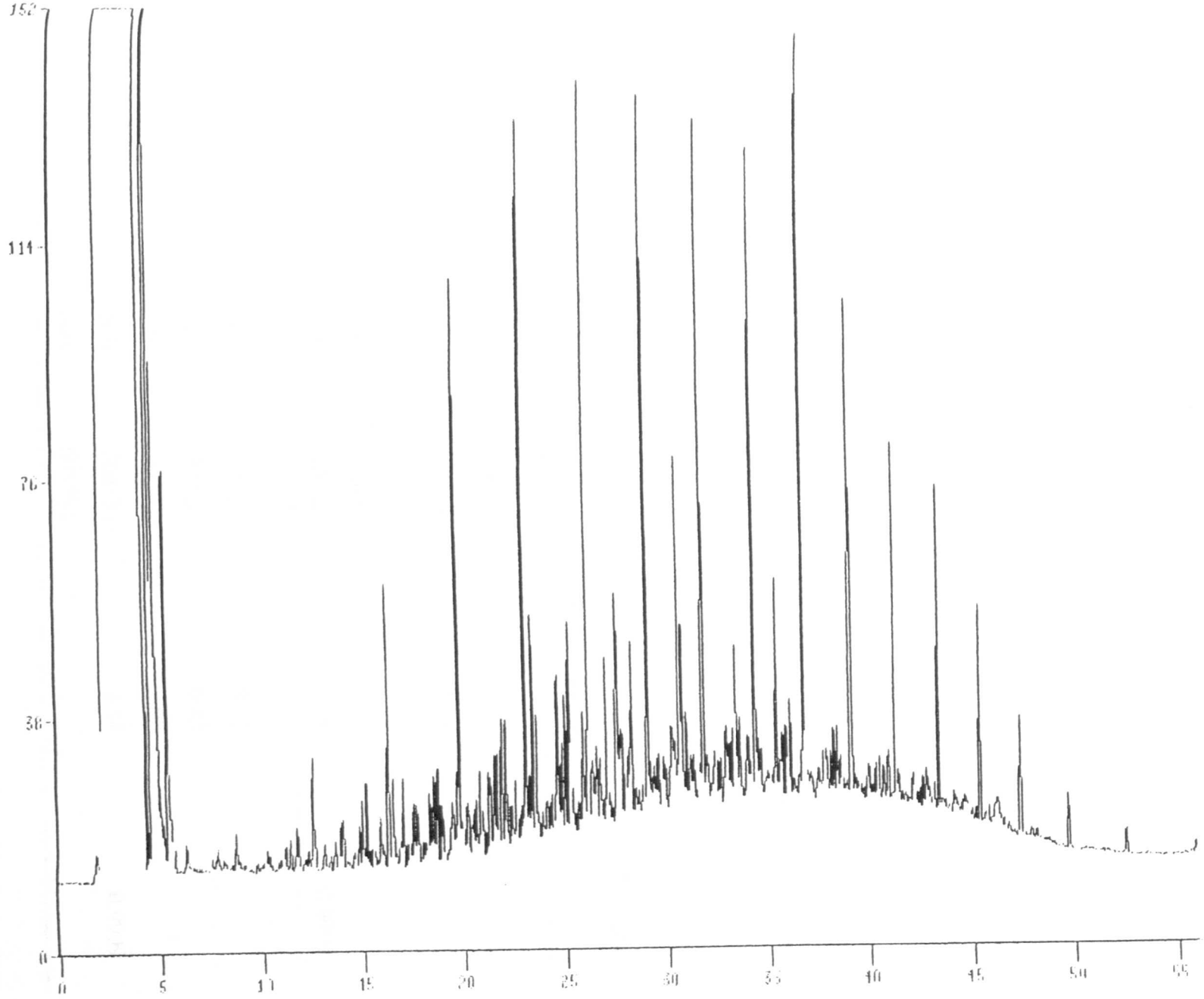
### 3.1.2.6 Temperature Programme

The recommended temperature programme was: set the column temperature at 35°C for 2 minutes, then programme at 10°C $min^{-1}$ to 265°C and hold for 3 minutes.

Initially the programme was not altered except for the final operating temperature. The HP 5890A GC has a maximum operating temperature of 250°C. This temperature is sufficiently high to allow elution of all the diesel fuel components from the column.

Using the described conditions, a 0.5µl sample of diesel fuel diluted 1/1000 in hexane (analytical grade) was injected onto the column. The resultant trace was poorly resolved therefore the temperature programme was modified. In order to resolve the large number of diesel fuel peaks successfully, the temperature gradient was reduced to 5°C $min^{-1}$. This produced a satisfactory resolution of all the major peaks present in diesel fuel (see Figure 3.1.2.6.1).

Figure 3.1.2.6.1. (on next page) GC trace of diluted diesel fuel using the modified temperature conditions.


152
111
76
38
0
0
5
10
15
20
25
30
35
40
45
50
55

### 3.1.2.7 Linear Response, Detection Level and Quantifiable Level

The linear response was calculated using n-alkane and polyaromatic hydrocarbon standards.

**Preparation of n-decane and anthracene standards, 0-1000 mg $l^{-1}$**

0.1g of n-decane (Sigma-Aldrich, 99% pure) was weighed into a 100ml volumetric flask and the flask made up to volume with hexane (analytical grade). This provided a 1000mg $l^{-1}$ decane standard solution. This standard solution was used to prepare 0.5, 5, 50 and 500mg $l^{-1}$ decane standard solutions by appropriate dilution in hexane. The procedure was repeated to prepare a range of anthracene standards except acetone (analytical grade) was used as the solvent.

The response was found to be linear up to 1000mg $l^{-1}$ which is within the range of the analyses (see Table 3.1.2.7.1).

| Standard | concentration mg $l^{-1}$ | av. peak area | % difference between replicates area and av. area | $R^2$ value |
|---|---|---|---|---|
| n-decane | 0.5 | 34.83 | 0.89 | |
| | 5 | 353.6 | 0.20 | |
| | 50 | 3207.5 | 0.33 | |
| | 500 | 32289.0 | 1.60 | |
| | 1000 | 65812.0 | 1.18 | 0.9999 |
| anthracene | 0.5 | 25.5 | 4.33 | |
| | 5 | 342.25 | 0.82 | |
| | 50 | 3479.0 | 5.00 | |
| | 500 | 30413.0 | 0.64 | 0.9999 |
| | 1000 | 60826.0* | | |

* indicates theoretical value. $R^2$ value indicates the deviation from a linear trend with values of 1 (or close to) showing a linear relationship.

Table 3.1.2.7.1 Linear detection range values for n-decane and anthracene.

The limit of detection was 0.05mg $l^{-1}$ and the lowest quantifiable level was 0.5mg $l^{-1}$. Where very low concentrations of diesel required quantification, integrator events were set no lower than the events acceptable for the 0.5mg $l^{-1}$ standard i.e., threshold 0 with a peak area reject of 20.

### 3.1.2.8 Petroleum Hydrocarbon Standards

Standards were injected to confirm some of the major peaks in the diesel fuel chromatograph.

**Preparation of n-Alkane and Aromatic Hydrocarbon Standards**

The appropriate amount of each standard was weighed accurately into a 100ml volumetric flask then the contents of the flask made up to volume with the relevant solvent. n-alkane, cyclohexane and naphthalene standards were prepared in hexane (analytical grade) with the more polar PAHs being prepared in acetone (analytical grade).

Both qualitative and quantitative standards were prepared and injected into the GC as individual compounds and as mixtures of compounds. 0.5µl of each individual standard solution or mixed standard solution was injected directly onto the column and analysed using the GC conditions described in Section 2.3.2. Triplicate injections of each standard were obtained for reproducibility and the retention time and peak area calculated for quantitative identification of diesel fuel components (Table 3.1.2.8.1).

**Calculation of Standard Reproducibility**

Peak areas of replicate injections were added together then divided by the replicate number to give the average peak area. The average peak area was then divided by 100 to give 1% of the average peak area. The average peak area was subtracted from the replicate peak area (or vice versa depending on which value was larger), and then the difference between the two is divided by the 1% average peak area value. This gives the % difference between the average peak area and the replicate peak area (see example below).

**Example for 998mg $l^{-1}$ 1,8 Dimethylnaphthalene**

Replicate 1: peak area 45935
Replicate 2: 46253
Replicate 3: 46057

Average peak area 46081.7

$$1\ \%\ \text{average peak area} = \frac{46081.7}{100} = 460.8$$

% difference between average peak area and replicate peak area:

$$\frac{\text{av. peak area} - \text{peak area rep. 1}}{1\ \%\ \text{av. peak area}} = \frac{46081.7 - 45935}{460.8} = 0.32\ \%\ \text{difference}$$

$$\frac{\text{peak area rep. 2} - \text{av. peak area}}{1\ \%\ \text{av. peak area}} = \frac{46253 - 46081.7}{460.8} = 0.37\ \%\ \text{difference}$$

$$\frac{\text{av. peak area} - \text{peak area rep. 3}}{1\ \%\ \text{av. peak area}} = \frac{46081.7 - 46057}{460.8} = 0.05\ \%\ \text{difference}$$

Average % difference = 0.25 %

Reproducibility of the standards was satisfactory with peaks areas of triplicate injections lying within 5% of each other and retention times varying no more than ± 0.01 minute.

Table 3.1.2.8.1 (on next page) Example of some petroleum hydrocarbon standards used for quantitative identification of diesel fuel components.

| standard | Concentration mg $l^{-1}$ | Av. Retention time ± SE | Av. PA | % difference of Replicate PA and av. PA | Theoretical 1000 mg $l^{-1}$ PA |
|---|---|---|---|---|---|
| **Branched cyclohexanes** | | | | | |
| Methyl cyclohexane | 583 | 6.29 ± 0.004 | 32922 | 4.26 | 56470 |
| Ethyl cyclohexane | 507 | 10.21 ± 0.001 | 30558 | 3.19 | 60272 |
| Propyl cyclohexane | 507 | 13.89 ± 0.003 | 32630 | 3.59 | 64358 |
| Butyl cyclohexane | 499 | 17.66 ± 0.004 | 33233 | 3.40 | 66599 |
| Hexyl cyclohexane | 497 | 24.61 ± 0.004 | 34054 | 3.07 | 68520 |
| **n-alkanes** | | | | | |
| nonane | 562 | 12.50 ± 0.002 | 37442 | 1.75 | 66623 |
| decane | 502 | 16.23 ± 0.004 | 33038 | 1.65 | 65812 |
| undecane | 488 | 19.76 ± 0.005 | 32672 | 1.63 | 66950 |
| dodecane | 493 | 23.06 ± 0.005 | 33924 | 1.43 | 68811 |
| tridecane | 558 | 26.16 ± 0.005 | 38179 | 1.27 | 68422 |
| tetradecane | 502 | 29.06 ± 0.006 | 34079 | 1.29 | 67886 |
| hexadecane | 567 | 34.38 ± 0.006 | 37610 | 1.55 | 66331 |
| heptadecane | 583 | 36.83 ± 0.005 | 38802 | 2.22 | 66555 |
| **Aromatics** | | | | | |
| Naphthalene | 111 | 23.53 ± 0.002 | 6707 | 5.46 | 60426 |
| 2,6 diisopropyl naphthalene | 286 | 29.95 ± 0.001 | 17600 | 3.66 | 61325 |
| hexamethyl benzene | 100 | 31.19 ± 0.002 | 6007 | 3.89 | 60070 |
| 1,5 dimethyl naphthalene | 109 | 31.31 ± 0.002 | 6328 | 4.25 | 58058 |
| 1,8 dimethyl naphthalene | 104 | 32.29 ± 0.002 | 5786 | 5.60 | 55641 |
| 4,4, dichloro biphenyl | 107 | 40.22 ± 0.002 | 2302 | 5.08 | 21520 |

Assessment of petroleum hydrocarbon contaminated land is hampered by the lack of readily available information describing the composition of common petroleum products. Petroleum products are highly complex and variable mixtures that require specific chemical analysis that is time consuming and challenging. Once a petroleum hydrocarbon mixtures is released into the environment, its composition changes due to processes such as biodegradation, dissolution into water and volatilisation. These processes affect each hydrocarbon group differently, resulting in a pattern of hydrocarbon distribution that may be very different from the original petroleum product.

In order to assess the behaviour of diesel fuel in the soil and monitor any changes that may occur to the diesel fuel composition, a GC-FID method was developed. The method gave excellent separation of the main diesel fuel components in an acceptable run time that allowed changes in diesel fuel composition to be observed and quantitated.

## 3.2 Fluorescein Diacetate (FDA) Hydrolysis

The method for measuring total microbial activity by FDA hydrolysis was modified from the original method of Schnürer and Rosswall (1982). A quick and easy method to measure the overall activity of the soils microbial population was essential for investigating the impact of diesel fuel on soil health. The most widely used method for soil studies, after thorough searching of the literature, was developed by Schnürer and Rosswall in 1982. The method showed great potential but was very limited in the range of soil types it could successfully be applied to. The method was critically assessed to provide a more sensitive and accurate method for the determination of FDA hydrolysis in a wide range of soils. The development of the method is discussed below.

### 3.2.1 Background

The use of fluorescein esters as a measure of enzyme activity was first noted by Kramer and Guilbault (1963) where a simple procedure was described for the assay of lipase activity in the presence of other esterases. It was not until 1980 that the use of fluorescein esters as a measure of microbial activity was applied to environmental samples. Swisher and Carroll (1980) demonstrated that the amount of fluorescein

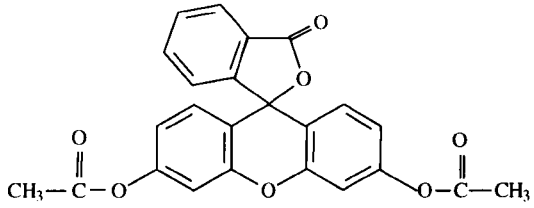
produced by the hydrolysis of fluorescein diacetate (FDA) was directly proportional to the microbial population growing on Douglas Fir foliage and a standardised method was developed. This method was later evaluated by Schnürer and Rosswall (1982) who used FDA hydrolysis to determine total microbial activity in soil and straw litter as well as cell density in pure microbial cultures.

Fluorescein diacetate (3′ 6′ -diacetyl-fluorescein) is a fluorescein conjugated to two acetate radicals. This colourless compound is hydrolysed by both free (exoenzymes) and membrane bound enzymes (Stubberfield and Shaw, 1990), releasing a coloured end product, fluorescein. Figure 3.2.1.1 illustrates the enzymic conversion of FDA to fluorescein which appears to be primarily a hydrolysis followed by a dehydration reaction. This end product absorbs strongly in the visible (490nm) and can be measured by spectrophotometry. The enzymes responsible for FDA hydrolysis are plentiful in the soil environment. Non-specific esterases, proteases and lipases, which have been shown to hydrolyse FDA, are involved in the decomposition of many types of tissue. The ability to hydrolyse FDA thus seems widespread, especially among the major decomposers, bacteria and fungi (Schnürer and Rosswall, 1982). Generally more than 90% of the energy flow in a soil system passes through microbial decomposers therefore an assay which measures microbial decomposer activity will provide a good estimate of total microbial activity.

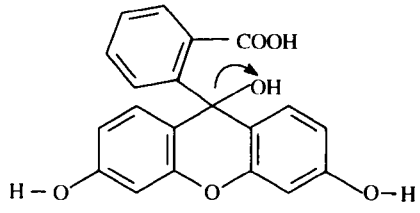
The FDA method was also shown to correlate well with some of the most accurate measures of microbial biomass such as ATP content and cell density studies (Stubberfield and Shaw, 1990) and radio-labelled thymidine incorporation into microbial DNA (Federle *et al.*, 1990). Whereas these methods are time consuming and difficult to perform, enzyme assays are generally rapid and simple.

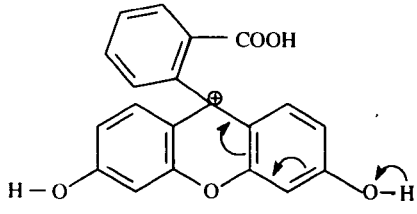
Since 1982, FDA hydrolysis has been used to measure total microbial activity in a range of samples from mould growth on wood and other building materials (Bjurman, 1993), to plant residues (Zablotowicz *et al.*, 1998), to stream sediment biofilms (Battin, 1997), activated sludge (Fontvieille, 1992) and deep sea clay and sand sediment profiles (Gumprecht *et al.*, 1995).

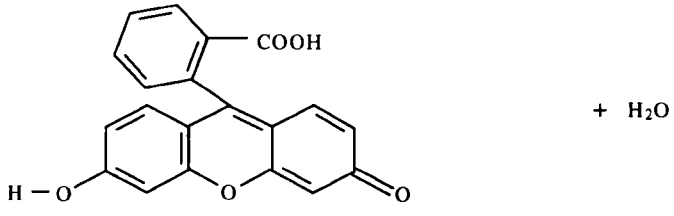
The advantage of this method being simple, rapid and sensitive, coupled with the widespread acceptance of FDA hydrolysis as a measure of total microbial activity, suggested that this would be an extremely useful method to optimise to include a wide range of soils.




Fluorescein diacetate (FDA)
colourless




Fluorescein coloured acid yellow
visible 490 nm

The enzymic conversion of fluorescein diacetate (FDA) to fluorescein appears to be primarily a hydrolysis reaction followed by a dehydration reaction. The two acetate groups are hydrolysed at their ester linkage and the lactone part of the structure is cleaved at its internal ester link (step 1). The resultant OH group leaves, creating a positively charged bond (step 2). This charge must be satisfied so the above intermediary step occurs starting from a loss of H at the terminal position (step 3). This results in an overall loss of water.

Figure 3.2.1.1 Enzymic conversion of fluorescein diacetate (FDA) to fluorescein.

### 3.2.2 Limitations of the Original Method

Schnürer and Rosswall found that FDA activity was very low in sand and clay samples. The low activity observed for these soil types was made more difficult to measure by the authors' choice of solvent for terminating the hydrolysis reaction. Because of the rapidity of FDA hydrolysis, it is necessary when working with many samples to find a way of terminating hydrolysis at a specific time. Schnürer and Rosswall found acetone (50% v/v) to be most efficient, totally stopping hydrolysis in a soil sample for 2 hours. However, a substantial decrease in the absorbance of fluorescein produced by the soil samples was observed when acetone was added. This dramatic colour loss is independent of initial fluorescein concentration but makes the measurement of FDA hydrolytic activity very difficult in soils with low microbial activity i.e. sandy and/or clayey soils.

Also, the original method for preparing standards involved preparing hydrolysed fluorescein by boiling FDA solutions of known concentrations in a water bath. This method proved extremely inaccurate and was not reproducible hence was deemed an unsuitable method for preparing standards.

Despite these limitations, the method had many features that conveyed its potential as a rapid and accurate measurement of total microbial activity in soil, which many other methods lacked. It was therefore decided that the method should be developed.

The individual parameters of the FDA reaction were studied to optimise the assay for the measurement of soil samples. These factors included effect of pH, amount of soil, amount of substrate, time of incubation, optimum temperature of incubation, choice of solvent for terminating the hydrolysis reaction and preparation of suitable standards. The results from each parameter studied were culminated to produce the final assay procedure (Section 2.5.3). Each parameter studied is discussed below.

## 3.2.3 Method Development

### 3.2.3.1 Materials

**Soils Used in the Study**

A total of five surface soils and one manufactured soil were selected to obtain a range of textural properties within the sandy and /or clayey textural class and cover a range of enzymic activities. Particle size analysis for the determination of textural class was carried out as described in Section 2.2.1.1 and dehydrogenase activity assessed as described in Section 2.5.2. The total nitrogen content of the soils, soil pH and organic matter content was assessed as outlined in Sections 2.2.2.5, 2.2.2.1 and 2.2.2.3. Table 3.2.3.1.1 shows the textural, chemical and biological properties of the six soils chosen.

| Soils | % coarse sand | % fine sand | % silt | % clay | Textural class |
|---|---|---|---|---|---|
| Barassie† | 7.7 | 14.2 | 4.1 | 4.4 | Sand |
| Bargour‡ | 39.4 | 27.5 | 11.3 | 21.8 | Sandy loam |
| Caprington‡ | 29.2 | 22.0 | 25.7 | 23.1 | Sandy clay loam |
| Dreghorn† | 32.7 | 35.3 | 16.3 | 15.7 | Sandy loam |
| Garscube | 51.7 | 20.8 | 12.8 | 15.2 | Loamy sand |
| John Innes compost | 72.2 | 17.6 | 4.9 | 8.4 | Sand |

| Soils | pH (water) | LOI (%) | Total N (%) | Dehydrogenase activity (μg TPF $g^{-1}$ oven dry soil 24 $h^{-1}$) |
|---|---|---|---|---|
| Barassie† | 7.20 | 5.7 | 0.20 | 173.59 |
| Bargour‡ | 5.46 | 6.9 | 0.18 | 145.79 |
| Caprington‡ | 6.46 | 10.5 | 0.28 | 151.16 |
| Dreghorn† | 6.86 | 6.5 | 0.17 | 67.80 |
| Garscube | 7.23 | 9.6 | 0.35 | 224.70 |
| John Innes compost | 7.06 | 10.2 | 0.19 | 76.88 |

Coarse sand > 0.18 mm, fine sand 0.18 – 0.05 mm, silt 0.05 – 0.002, clay < 0.002 mm.

† Textural properties and Total N (%) taken from Metwaly (Ph.D. thesis, University of Glasgow, 1999).

‡ Textural properties and Total N (%) taken from Khan (Ph.D. thesis, University of Glasgow, 1987).

Table 3.2.3.1.1. Textural, chemical and biological properties of the six soils chosen.

**Reagents**

60mM potassium phosphate buffer pH 7.6

8.7g $K_2HPO_4$ (Riedel-de Haën, Sigma-Aldrich Co. Ltd., Analar) and 1.3g $KH_2PO_4$ (Merck, BDH Analar) were dissolved in approximately 800ml deionised water. The contents were made up to 1 litre with deionised water. The buffer was stored in the fridge (4°C) and pH checked on day of use.

2:1 chloroform/methanol

666ml chloroform (Fisher Scientific UK Limited, analytical grade) was added to a 1 litre volumetric flask. The flask was made up to 1 litre with methanol (Fisher Scientific UK Limited, analytical grade) and the contents mixed thoroughly.

1000µg FDA $ml^{-1}$ stock solution

0.1g fluorescein diacetate (3′ 6′-diacetyl-fluorescein, Sigma-Aldrich Co. Ltd.) was dissolved in approximately 80ml of acetone (Fisher Scientific UK Limited, analytical grade) and the contents of the flask made up to 100ml with acetone. The solution was stored at -20°C.

2000µg fluorescein $ml^{-1}$ stock solution

0.2265g fluorescein sodium salt (Merck, BDH Analar) was dissolved in approximately 80ml of 60mM potassium phosphate buffer pH 7.6 and the contents made up to 100ml with buffer

20µg fluorescein $ml^{-1}$ standard solution

1ml of 2000µg fluorescein $ml^{-1}$ stock solution was added to a 100ml volumetric flask and the contents made up to the mark with 60mM potassium phosphate buffer pH 7.6.

1-5 µg $ml^{-1}$ standards were prepared from this standard solution by appropriate dilution in 60mM potassium phosphate buffer pH 7.6.

## 3.2.3.2 Investigation of Method Parameters

### Effect of pH

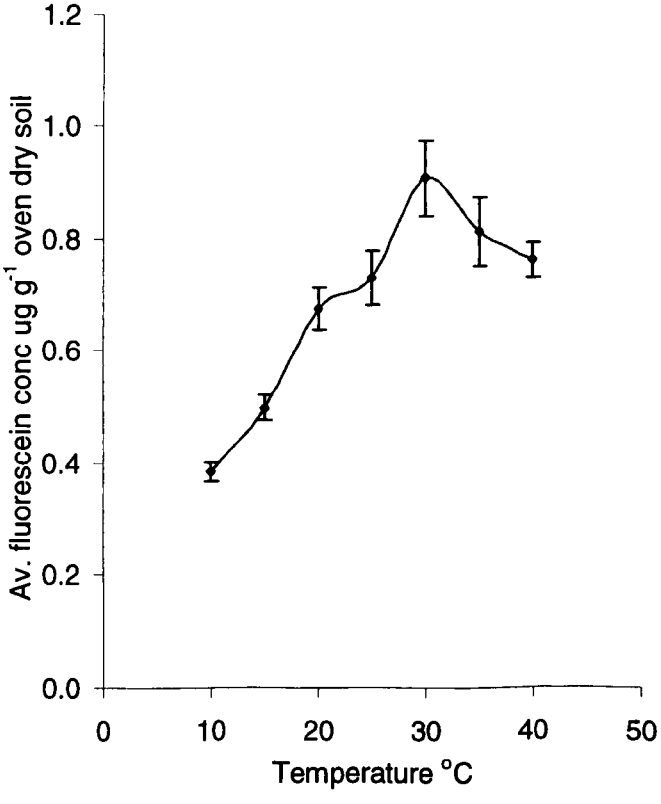
The rate of hydrolysis of fluorescein compounds reaches a maximum between pH 7.0 and 8.0 (Guilbault and Kramer, 1964). Fluorescein diacetate was found to exhibit a maximum rate of hydrolysis at pH 7.6 (Swisher and Carroll, 1980). Carrying out the enzymic reaction at this pH was advantageous for many reasons. At high and low pHs, solubilisation of organic matter in the soil samples caused interference problems with the measurement of fluorescein released, by creating blanks with very high background absorbances. Carrying out the reaction at pH 7.6 removed this interference problem. Spontaneous hydrolysis of fluorescein esters is known to occur at high pHs (Guilbault and Kramer, 1964). At pH 7.6 no spontaneous hydrolysis of fluorescein diacetate was observed. Finally, the product of FDA hydrolysis, fluorescein, exhibits a maximum fluorescence at about pH 8.0 (Guilbault and Kramer, 1964). This was verified when standards were prepared in buffers at different pHs. The absorbance values measured for the same concentration of fluorescein standards were more than double in the pH 7.6 potassium phosphate buffer compared with the pH 5.6 buffer (Table 3.2.3.2.1). This illustrates that fluorescein is near its maximum absorbance at pH 7.6.

| pH 5.6 | | pH 7.6 | |
|---|---|---|---|
| Fluorescein conc (µg/ml) | abs 400nm | Fluorescein conc (µg/ml) | abs 400nm |
| 0 | 0 | 0 | 0 |
| 2 | 0.175 | 1 | 0.248 |
| 4 | 0.344 | 2 | 0.403 |
| 6 | 0.511 | 3 | 0.636 |
| 8 | 0.682 | 4 | 0.830 |
| 10 | 0.844 | 5 | 1.056 |
| 12 | 1.009 | | |

Table 3.2.3.2.1 Absorbance of fluorescein standards in buffers of varying pH.

### Effect of Temperature

The rate of hydrolysis of a substrate by an enzyme depends on the temperature of incubation. A study of FDA activity in soil as a function of temperature showed maximum activity occurred at 30°C. This is in agreement with findings by Breeuwer *et al* (1995) who observed maximum FDA activity by yeast esterases at this temperature. The activity rapidly decreased just above 30°C suggesting inactivation of the enzymes involved at this elevated temperature. Fig 3.2.3.2.2 shows the effect of temperature on FDA hydrolysis in a sandy soil. At high temperatures considerable spontaneous hydrolysis of fluorescein esters can occur (Guilbault and Kramer, 1964), adversely affecting the accuracy and reproducibility of the method. No spontaneous hydrolysis of FDA was found to occur between 20-40°C, which covers the range around the temperature chosen for this assay.

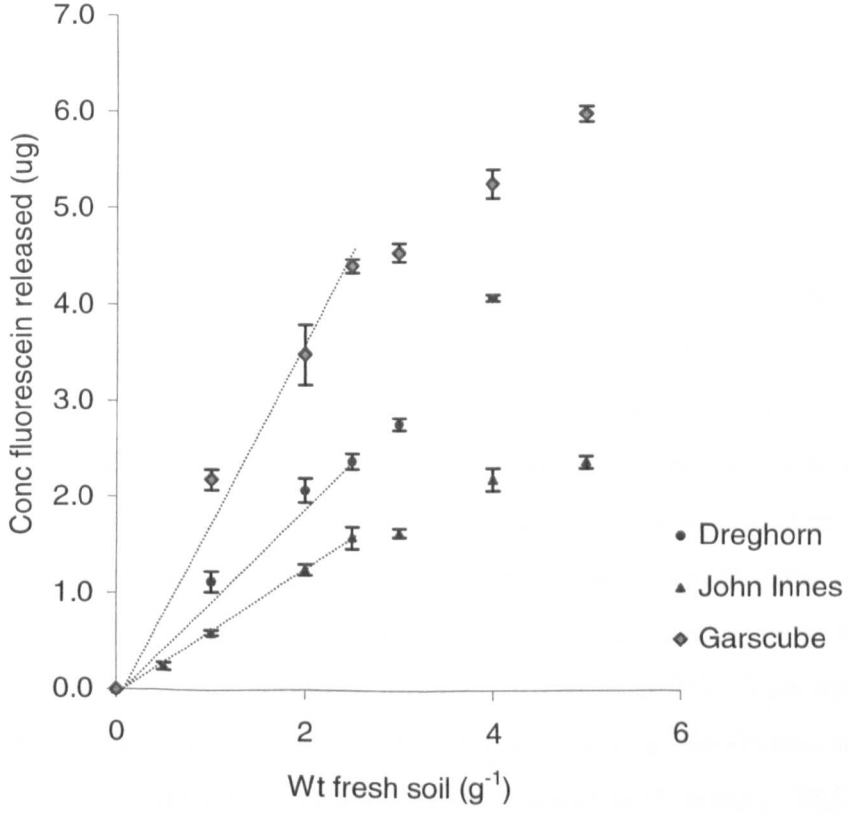



Given are mean values with standard error bars, n = 4.

Fig 3.2.3.2.2 Effect of temperature on FDA hydrolysis by enzymes present in a sandy soil (Barassie).

### Amount of Soil

The initial reaction rates of soil enzymes are usually proportional to the amounts of soil added to the assay (Frankenberger and Johanson, 1983). Using the conditions described for the final assay procedure, soil weights (fresh weight, sieved < 2mm) ranging from 0.5-5g were incubated to discover the optimal amount of soil required for the reaction to proceed at a steady rate without substrate becoming limiting. A linear relationship was observed between soil weight and fluorescein released up to 2.5g. Fig. 3.2.3.2.3 shows the effect of the amount of soil on the hydrolysis rate of FDA. The deviation from linearity when soil weights greater than 2.5g were used indicates substrate concentration was probably becoming a limiting factor




Given are mean values with standard error bars, n = 3.

Fig. 3.2.3.2.3 3 Relationship between soil weight and FDA hydrolysis.

A soil weight of 2g was chosen for the final assay procedure. This allowed the reaction to proceed at a steady rate, for all the soils tested, without substrate becoming limiting. It also ensured that the amount of fluorescein hydrolysed during the assay fell within the sensitivity range of the spectrophotometer (0.1-1.0). The three soils chosen to illustrate this in Fig. 3.2.3.2.3.3 were: (a) a manufactured compost (John Innes Compost No 2) which represents a sandy soil with low microbial activity; (b) Dreghorn which represents a sandy loam with an intermediate microbial activity and (c) Garscube soil which is a loamy sand with high microbial activity. The low activity soil released enough fluorescein during the assay to be measured accurately by spectrophotometry (absorbance values greater than 0.1) and the high activity soil released enough fluorescein to lie within the range of the spectrophotometer without dilution (absorbance values below 1.0). These three soil types hopefully represent the range of microbial activities encountered in most soils.

**Adsorption of Fluorescein onto Soil**

The amount of fluorescein adsorbed onto soil was considered before carrying out the enzyme assay. When a new soil is investigated, the amount adsorbed onto soil should be calculated so the values obtained for the assay can be corrected for the loss. Table 3.2.3.2.3.4 shows the results from a simple experiment showing the adsorption of fluorescein to soils with differing textural properties. Soil samples were incubated using the conditions described for the final assay procedure in 15ml fluorescein standard at each concentration (0-5μg $ml^{-1}$). Blanks were prepared without the addition of soil. Samples and blanks were centrifuged and filtered as described in the final assay procedure. The amount of fluorescein adsorbed at each concentration is shown as % fluorescein adsorbed and an average total value is given for each textural class. Generally the amount of fluorescein adsorbed is less than 5%. This observation was noted by the original authors who found the adsorption of fluorescein to soil did not exceed 7% and was mostly lower than 5% (Schnürer and Rosswall, 1982). A soil with a high silt–clay ratio and high organic matter content, such as Caprington can however adsorb up to 13.7% fluorescein which is a large proportion of the total released.

| | % fluorescein μg $ml^{-1}$ adsorbed by soil | | |
|---|---|---|---|
| Fluorescein conc μg $ml^{-1}$ in blank | Barassie sand | Dreghorn sandy loam | Caprington sandy clay loam |
| 0 | 0 | 0 | 0 |
| 1 | 5.7 ± 0.83 | 3.3 ± 0.69 | 20.2 ± 0.47 |
| 2 | 4.3 ± 0.60 | 7.1 ± 2.57 | 14.6 ± 0.39 |
| 3 | 3.2 ± 0.15 | 5.8 ± 0.27 | 11.1 ± 0.93 |
| 4 | 4.1 ± 0.29 | 3.3 ± 0.74 | 10.5 ± 0.26 |
| 5 | 2.7 ± 0.24 | 2.4 ± 1.07 | 11.7 ± 1.53 |
| Average total adsorbed % | 4.0 | 3.6 | 13.7 |

Given are means ± S.E., n = 3.

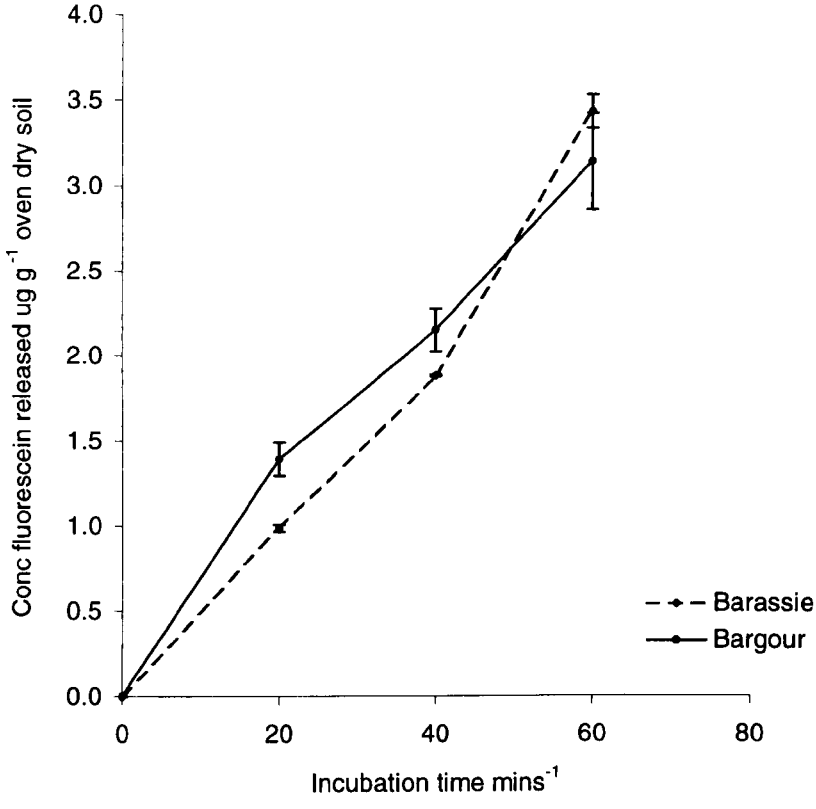
Table 3.2.3.2.3.4 Adsorption of fluorescein onto soils with differing textural properties.

**Time of incubation**

It has been suggested that an assay for soil enzymes should not require a long incubation time because the risk of error through microbial proliferation increases with increasing incubation time. Part of this error can be minimised by the addition of toluene as a bacteriostat to enzyme assays although many authors do not favour the use of toluene for this purpose. Toluene has been shown to inhibit some enzymes as well as having an activating effect on others due to increased permeability of the cell membrane in the presence of toluene, allowing entry of the substrate. (Skujins, 1967). In this study the use of toluene as a bacteriostat in the assay of FDA hydrolysing enzymes was dismissed as it was found to inhibit FDA hydrolysis in the soil samples investigated by approximately 35%. It was therefore decided to keep the incubation time as short as possible.

The hydrolysis reaction was found to be linear with time up to 40 minutes for the soils investigated using the conditions described for the final assay procedure. Figure 3.2.3.2.3.5 indicates the linear increase in FDA hydrolysis up to 40 minutes in

two of the soil types investigated. The assay was not limited by substrate concentration over this time period.




Given are mean values with standard error bars, n = 4.

Figure 3.2.3.2.3.5 Determination of optimum incubation time.

An incubation time of 20 minutes was chosen for the final assay procedure because this time allowed the concentration of substrate hydrolysed to lie within the range of the spectrophotometer for all the soil types investigated without the need for dilution. Removing a possible dilution step makes the method quicker and easier to perform as well as removing an extra step where error could occur.

**Choice of Solvent for Terminating Hydrolysis**

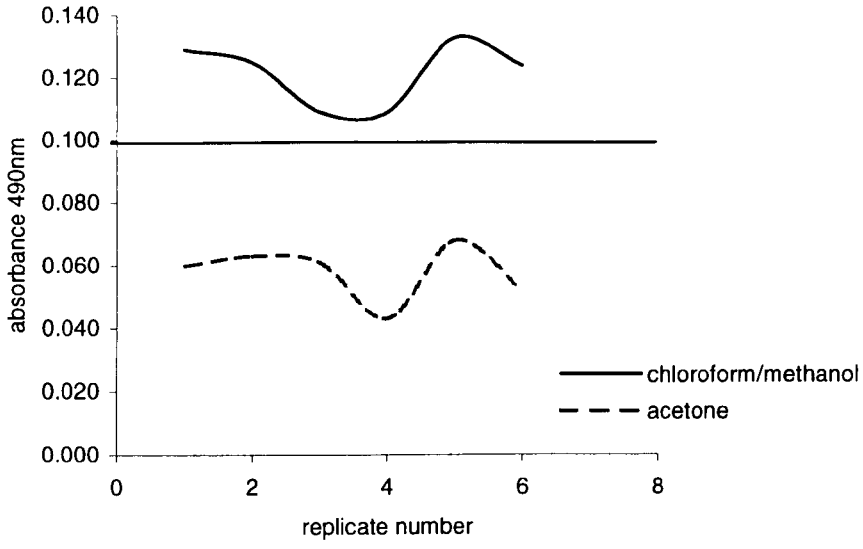
It is necessary when carrying out enzyme assays to be able to stop the enzymic reaction at a given point. As mentioned before, Schnürer and Rosswall found acetone (50% v/v) to be most efficient, totally stopping hydrolysis in a soil sample for 2 hours. The addition of acetone to the soil samples did terminate hydrolysis but it also caused a decrease in the amount of fluorescein measurable by spectrophotometry. Although the

drop in colour was by the same ratio each time (ranged from 39.8-41.0% in 1-5μg $ml^{-1}$ fluorescein standards where acetone was added), samples with low microbial activity which only release a small amount of fluorescein were made increasingly more difficult to measure. This decrease in colour, which was more than a dilution effect, was shown on average to be 37% of the colour developed in a sandy soil. Table 3.2.3.2.3.6 illustrates the decrease in fluorescein concentration of samples where acetone has been used to stop the hydrolysis reaction compared to samples where chloroform/methanol (2:1 v/v) has been used. The samples sometimes dropped below the range of the spectrophotometer when acetone was added. The absorbance values for the samples where chloroform/methanol (2:1 v/v) has been used to terminate hydrolysis lie just above 0.1, which is the minimum absorbance that can be measured accurately by spectrophotometry. The sample values where acetone has been used to stop the reaction have fallen below the range that can be measured accurately (see Figure 3.2.3.2.3.7). The relative precision of the method, defined by the coefficient of variation of replicate measurements, is also increased when chloroform/methanol (2:1 v/v) is used instead of acetone. Therefore the use of acetone was deemed unsuitable for this purpose and chloroform/methanol (2:1 v/v) was proposed as the new solvent for terminating FDA hydrolysis in soil samples.

| | chloroform/methanol (2:1 v/v) | | Acetone (50% v/v) | | |
|---|---|---|---|---|---|
| replicate | abs 490 nm | conc (μg $g^{-1}$) soil | abs 490 nm | conc (μg $g^{-1}$) soil | Corrected conc (μg $g^{-1}$) soil |
| 1 | 0.129 | 0.543 | 0.060 | 0.249 | 0.332 |
| 2 | 0.125 | 0.5.33 | 0.063 | 0.268 | 0.356 |
| 3 | 0.109 | 0.467 | 0.061 | 0.255 | 0.340 |
| 4 | 0.109 | 0.455 | 0.043 | 0.180 | 0.239 |
| 5 | 0.133 | 0.568 | 0.068 | 0.288 | 0.383 |
| 6 | 0.124 | 0.518 | 0.052 | 0.221 | 0.294 |
| Final vol. of filtrate (ml) | | 20 | | 30 | 20 |
| av. conc (μg $g^{-1}$) soil | | 0.514 | | 0.244 | 0.324 |
| CV | | 8.61 | | | 15.74 |

All replicate absorbance values and concentration values in μg fluorescein $g^{-1}$ oven dry soil are given. The final volumes of filtrate collected were 20 ml in the chloroform/methanol (2:1 v/v) samples and 30 ml in the acetone samples. Due to the differences in the final volumes a conversion factor of 0.33 was used on the acetone values to counteract this dilution effect. The corrected concentration values for the acetone samples give fluorescein concentrations (μg $g^{-1}$ oven dry soil) in a 20 ml final volume. These values can be compared directly to the values obtained for the chloroform/methanol (2:1 v/v) samples. CV = coefficient of variation.

Table 3.2.3.2.3.6 Decrease in fluorescein concentration (μg $ml^{-1}$) of Barassie soil in acetone terminated samples compared with chloroform/methanol (2:1 v/v) terminated samples.

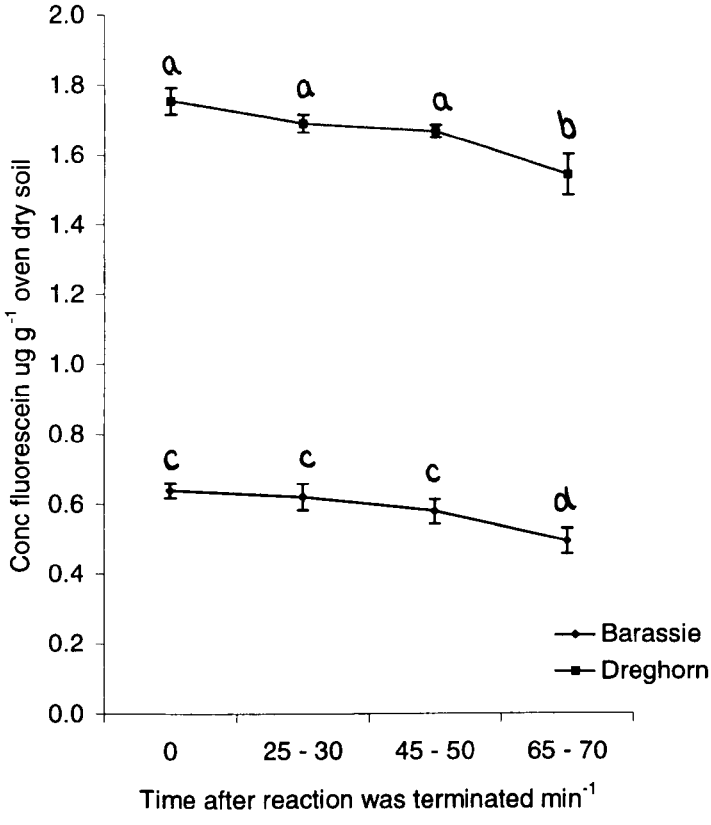



All replicate absorbance values are plotted.

Figure 3.2.3.2.3.7 Decrease in absorbance by addition of acetone compared to chloroform/methanol (2:1) in Barassie soil.

**Change in hydrolysis over time**

Changing from acetone to another means of terminating the hydrolysis reaction involved finding a substitute that would stop hydrolysis successfully without causing the same loss of colour observed with acetone. A 2:1 ratio of chloroform/methanol (v/v) was found to be most efficient, stopping hydrolysis from continuing for up to 50 minutes after its addition (Figure 3.2.3.2.3.8). The length of time the reaction was terminated for was sufficient to allow the measurement of a large number of samples without changes occurring in the samples.




Given are mean values and standard error bars, n = 5.

Means followed by the same letter are not significantly different at $P < 0.05$.

Figure 3.2.3.2.3.8 Change in fluorescein concentration over time after termination of the hydrolysis reaction.

In addition, chloroform will help solubilise cell membranes, as acetone did, facilitating the extraction of fluorescein. FDA, being non-polar, readily penetrates into the cell and is hydrolysed to fluorescein. The polarity of fluorescein impedes its transport back through the cell membrane causing intracellular accumulation. Fluorescein is liberated into the environment only after the storage capacity of the cell has been exceeded and the excess is excreted (Rotman and Papermaster, 1966). Chloroform will help solubilise cell membranes aiding the extraction of fluorescein. The presence of methanol will help the chloroform interact with the moist soil hence increasing its ability to terminate the reaction effectively. Fluorescein released during the incubation also moves preferentially into the more polar potassium phosphate buffer/methanol phase, which increases the efficiency of the extraction procedure. Acetone also removed a lot of dissolved organic matter from the samples producing

blanks with very high background absorbances whereas the chloroform/methanol (2:1 v/v) does not. All these advantages make chloroform/methanol a more beneficial solvent to use in terminating the hydrolysis reaction.

**Substrate Concentration**

2000μg $ml^{-1}$ fluorescein diacetate (FDA) solution was used by most authors as the substrate for the reaction. By adding 0.2ml of 2000μg $ml^{-1}$ FDA, 400μg FDA was achieved in each replicate. This concentration was found to be unnecessarily high for the conditions chosen for the final procedure. High concentrations of FDA should be avoided as FDA is poorly soluble in water and other polar solutions (Breeuwer *et al.*, 1995). Even in acetone, high concentrations of FDA produce slightly cloudy solutions suggesting not all the FDA added is in solution, hence available to the microorganisms. Instead a 1000μg $ml^{-1}$ FDA solution was chosen to start the reaction. This supplied 200μg FDA to each replicate, which can in turn, release a maximum of 160μg of fluorescein. This 160μg of fluorescein is diluted in 15ml 60mM potassium phosphate buffer pH 7.6 then a further 5ml of methanol (from 15ml chloroform/methanol (2:1 v/v) only the methanol is added to the filtrate). This gives a final possible fluorescein concentration of 8μg $ml^{-1}$. The maximum concentration of fluorescein is never released by the conditions described for the final procedure therefore standards are prepared covering a range of 0-5μg fluorescein $ml^{-1}$. All the soils investigated, using the conditions set for the assay, were within the range described by the standards.

**Preparation of standards**

The original method stated that standards should be prepared using hydrolysed fluorescein diacetate (FDA). This was achieved by boiling FDA solutions of known concentrations in a water bath for 30 minutes (Schnürer and Rosswall, 1982). Other authors increased the boiling time to 60 minutes (Chen *et al.*, 1988). This method for obtaining reproducible standards proved too variable. Figure 3.2.3.2.3.9A shows the results of 0-200μg FDA standards prepared in this way. 0-200μg concentrations of FDA were added to 5ml of 60mM potassium phosphate buffer pH 7.6 in screw top vials. The lids were replaced and the standards placed in a boiling water bath for a set time (30 or 60 minutes). Once cool a further 10ml of 60mM potassium phosphate buffer pH 7.6 was added to keep all volumes the same as the final procedure. 15ml of chloroform/methanol (2:1 v/v) was added and the standards were centrifuged and filtered as described by the final procedure. The standards were measured at 490nm and

the results plotted to produce a standard calibration graph. The results differed for both hydrolysis times. Fluorescein diacetate can in fact be hydrolysed for up to 6 hours in a water bath, although a slight plateau is reached after 4 hours. Figure 3.2.3.2.3.9B illustrates the continued hydrolysis of a 100µg FDA standard. Replicate 100µg FDA standards in 5ml 60mM potassium phosphate buffer pH 7.6 were placed in a boiling water bath. A duplicate set of replicates were removed every hour and the fluorescein released measured as described above. Whether FDA is continuing to be hydrolysed or whether it is being degraded in the prolonged heating is unclear but the method for preparing standards is clearly unacceptable.

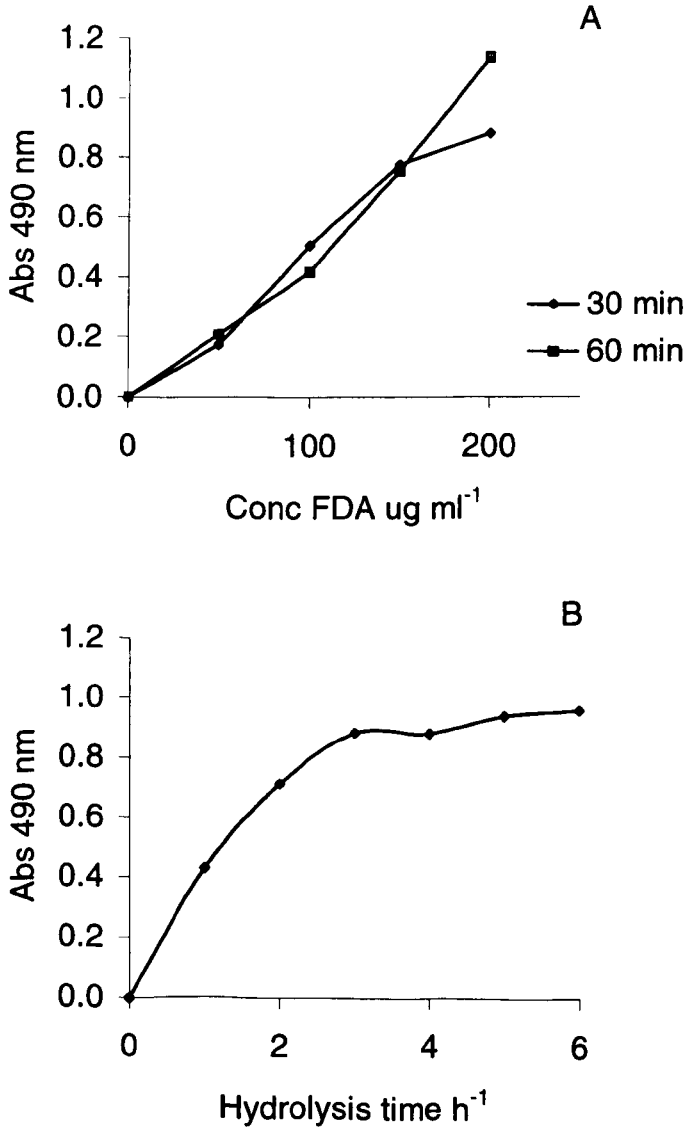



Figure 3.2.3.2.3.9A Fluorescein diacetate (FDA) hydrolysed in boiling water bath (diluted 1 : 1 in 60mM potassium phosphate buffer pH 7.6) and B continued hydrolysis of 100 µg FDA standard in boiling water bath.

Sodium fluorescein salt was chosen instead to prepare the standards. Sodium fluorescein salt released the same acid yellow coloured fluorescein as FDA released allowing direct measurement of fluorescein released from FDA in soil by a standard calibration graph prepared from sodium fluorescein salt. Certain pure fluorescein preparations should be avoided as this compound is generally solvent yellow coloured and adds an error to the absorbance measurements. The fluorescein salt can be weighed accurately and known concentrations of fluorescein obtained so standard results rarely differ. Standards prepared by this method did not alter significantly over three separate months.

By using the boiling water bath method for preparing standards the amount of fluorescein hydrolysed by the samples and the amount of FDA hydrolysed during standard preparation was continually underestimated and was not always consistent. Preparing standards from sodium fluorescein salt is a much more accurate method.

### 3.2.3.3 Conclusions

The potential of fluorescein diacetate (FDA) hydrolysis as a measure of total microbial activity has been recognised by many authors and used on a wide range of samples. The most frequently used method for measuring FDA activity in soil was found to be limited in the range of soil types it could measure successfully. The method described in this study critically assessed each individual parameter of the FDA hydrolysis assay and optimised each one for the measurement of a wide range of soils.

The pH the assay was carried out at was unchanged from the original method as FDA had a maximum rate of hydrolysis at this pH. pH 7.6 was also beneficial as no spontaneous hydrolysis of FDA occurred at this pH, no solubilisation of organic matter was observed and the product of the hydrolysis reaction, fluorescein, was near its maximum absorbance. The optimum temperature of incubation was found to be 30°C and a 20 minute incubation time was chosen for the final assay procedure. FDA hydrolysis was linear with time up to 40 minutes but 20 minutes was sufficient for obtaining easily measurable fluorescein concentrations within the range of the spectrophotometer. A linear relationship was found between soil weight and FDA hydrolysis up to 2.5g soil. Two g was chosen as the final soil weight as it allowed a steady reaction rate without substrate becoming limited for all the soils investigated and

it also ensured that the fluorescein concentrations released lay within the range of the spectrophototmeter. It was also noted that fluorescein can be adsorbed onto soil causing error in the amount of fluorescein measurable after hydrolysis. For most soil types this value was below 5% but could be as high as 13% in clayey soils. It was therefore important to work out adsorption loss when a new soil type was being investigated.

The most important parameter assessed during this study was the choice of solvent for terminating the reaction. By changing the solvent from acetone (50% v/v) to chloroform/methanol (2:1 v/v) low activity soils, such as sandy and clayey soils, could be measured successfully. This increased sensitivity was achieved as no loss of colour was observed when chloroform/methanol (2:1 v/v) was used. Chloroform/methanol (2:1 v/v) also aids extraction of fluorescein from microbial cells by altering or destroying the membrane properties allowing fluorescein to be released. There is no need to place samples in an ice bath, as the original method states, to prevent volatilisation when using chloroform/methanol (2:1 v/v) and the presence of chloroform increases the extraction efficiency of fluorescein by pushing fluorescein into the more polar methanol/phosphate buffer layer which is then filtered and measured. This increased extraction efficiency was verified by the decrease in replicate variability when chloroform/methanol (2:1 v/v) was used. The variability was measured by the coefficient of variation which was 8.6% in the chloroform/methanol (2:1 v/v) samples as opposed to 15.7% in the acetone samples. For this soil type 4% of the variability can be attributed to adsorption of fluorescein onto soil. Chloroform/methanol (2:1 v/v) stopped hydrolysis for up to 50 minutes in the samples investigated which was sufficient to allow measurement of a large number of samples. The chloroform/methanol (2:1 v/v) does not remove dissolved organic matter, as acetone did, hence produce clear blanks with low background absorbance. The substrate concentration used provided enough substrate for the assay to proceed without limitation and was below the saturation level of FDA in polar solutions. Finally, the altered method for preparing standards provides reproducible standards with consistent fluorescein concentrations. By modifying the individual parameters and optimising the assay conditions, the new method proposed allows for a faster determination of FDA activity in a wide range of soils with increased precision and sensitivity.

Fluorescein diacetate (FDA) hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in a range of environmental samples, including soils. The current method for measuring FDA hydrolysis in soils was limited in its application. As the measurement of soil microbial activity is extremely important in assessing the impact of contamination on soil systems, the original method was optimised for the measurement of a wide range of soil types. The new, more sensitive method was applied to the range of soils used in this study, both diesel fuel contaminated and uncontaminated. The final procedure is described in Section 2.5.3.

# CHAPTER FOUR

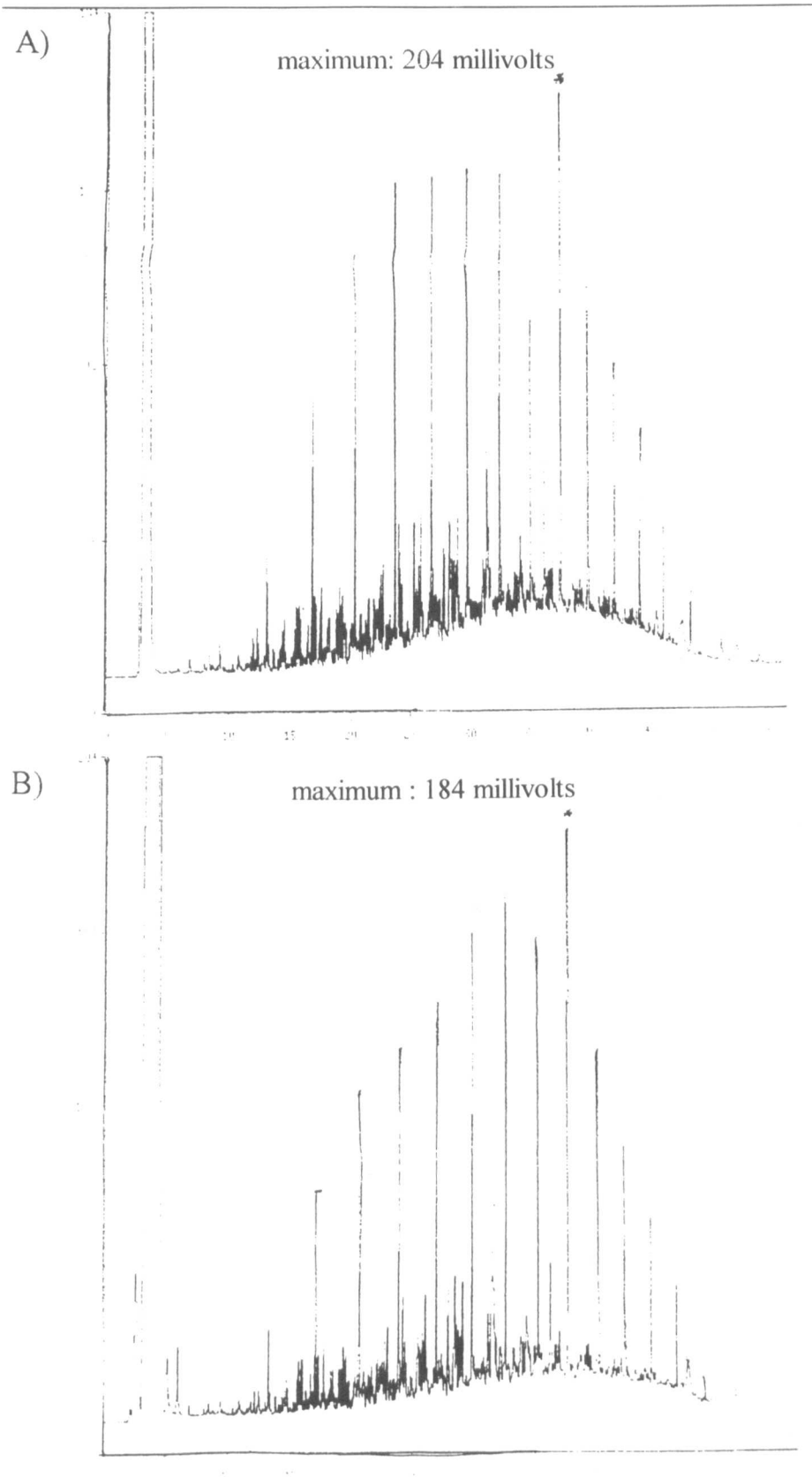
## ANALYSIS OF DIESEL FUEL

A large part of this study involved following the changes in diesel fuel composition after it had been released into the soil environment. Changes occur due to volatilisation, leaching, adsorption and biodegradation of diesel fuel, which affects each hydrocarbon family differently. Petroleum characterisation (fingerprinting) is a technique that uses gas chromatography to identify petroleum hydrocarbons definitive characteristics which allows the type of petroleum product to be defined. This method could be used to assess the diesel fuel composition and therefore identify any changes to that composition. As petroleum fingerprinting relies on the expertise of the analyst, it was extremely important to obtain detailed compositional and structural information which would allow me to become familiar with the chromatographic profile and help define whether losses observed were due to abiotic (non-biological) or biotic (biological) processes. In depth analysis of diesel fuel was carried out by GC-FID as described in Section 2.3.2 and GC-MS as described in Section 2.3.3 on pure diesel products, the volatile diesel fraction (Section 2.3.4) and by petroleum hydrocarbon class separation on silica gel (Section 2.3.5).

## 4.1 Diesel Fuel Fingerprinting

Hydrocarbon products such as gasoline, diesel fuel and asphalts are all produced from crude oil by a variety of refining and distillation processes. Each product is created by the combination of multiple individual hydrocarbon compounds, all of which have slightly different properties such as boiling and vaporisation temperatures. The middle range distillates are used in different proportions to create products such as kerosene, diesel and heating oil (Wigger *et al.*, 1998).

Diesel fuels are complex mixtures of hydrocarbons with an average carbon number of C8-C26. The majority of components consist of alkanes, both straight chained (n-alkanes) and branched and aromatic compounds including mono-,. di- and polyaromatic hydrocarbons (PAHs). There are five different grades of diesel fuels for uses that range from cars, commercial trucks and buses to marine and railway engines. The type of diesel fuel used throughout this study was for use in cars, sometimes called diesel #2. Transportation diesels are manufactured primarily from distilled fractions of crude oil with some blending with cracked gas oils. The majority of components are similar to those present in the crude oil, but include a higher fraction of aromatics. Differences in the composition of diesel fuel depend on the source of the crude oil and on refinery processes. Fortunately, the majority of diesel fuel produced in Scotland comes from BP-Amoco's Grangemouth refinery. This minimises the different compositions of diesel fuel likely to be found in Scotland. The only retailer that is not supplied by diesel refined from Grangemouth is Esso (personal communication – BP Amoco). Figure 4.1.1 shows GC-FID chromatograms of diesel fuel from BP and Esso service stations. The chromatograms are similar but the pattern of the tall, evenly spaced n-alkanes differ. The majority of hydrocarbons present in each diesel fuel are the same but the concentration of each hydrocarbon is different which produces a slightly different hydrocarbon fingerprint. Because of this difference in composition, Esso diesel fuel was used throughout the duration of this study to avoid any errors incurred by using fuels produced by different refineries.

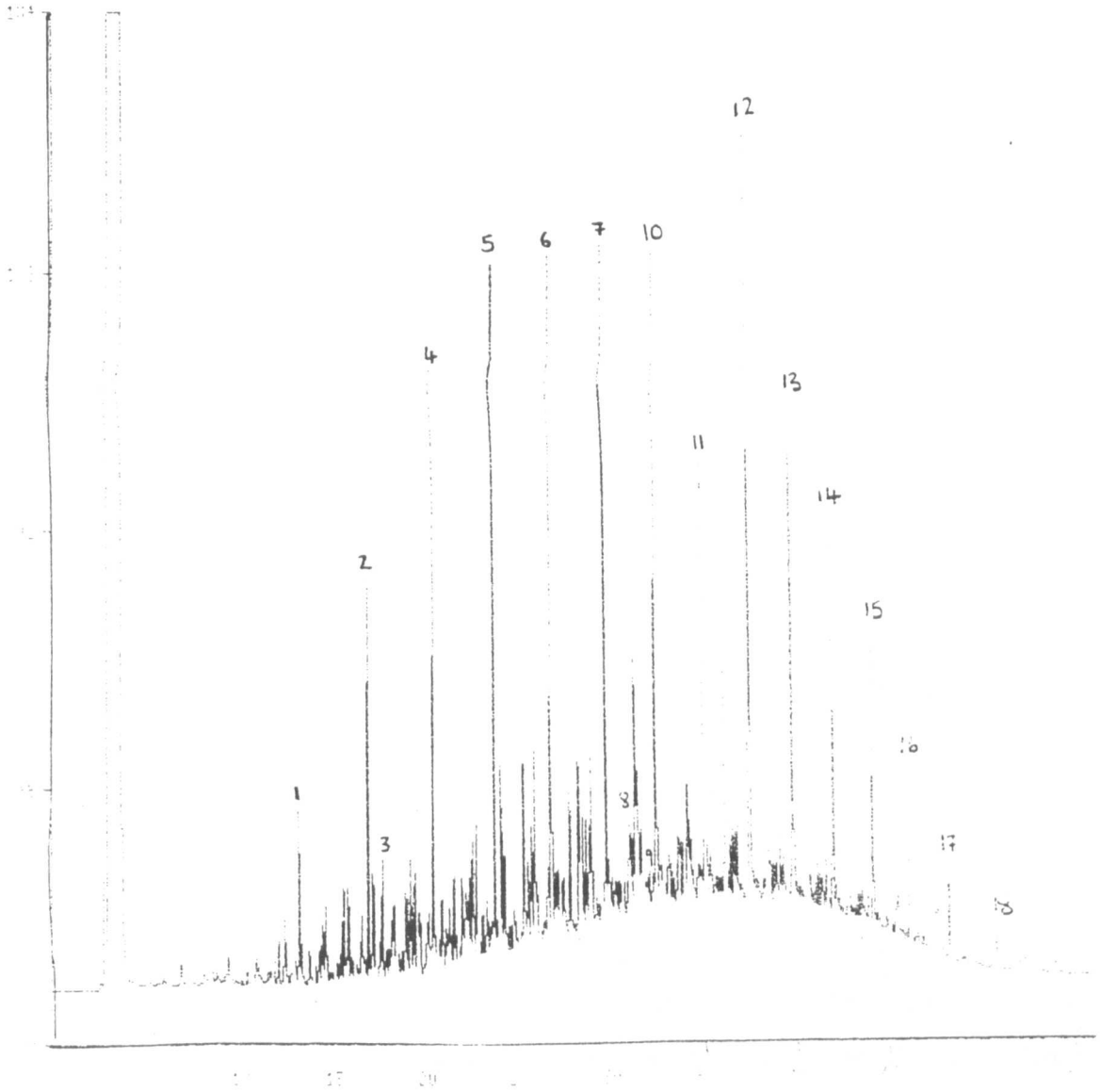



The n-alkane containing 17 carbons, n- heptadecane (peak designated by *) is in the highest concentration in both chromatograms but the concentrations of C13, C14, C15 and C16 are very different.

Figure 4.1.1. GC-FID chromatograms of diesel fuel from A) Esso and B) BP service stations. Both fuel samples were analysed using the same chromatographic conditions as described in Section 2.3.2

## 4.2 GC-FID Analysis of Diluted Diesel Fuel

The analysis of diesel fuel began by separating a diluted diesel fuel sample by capillary gas chromatography using the optimum chromatographic conditions for the separation determined in Chapter 3, Section 3.1.2. The developed method provided good resolution of all the major peaks in the diesel fuel chromatogram within a reasonable run time (56 minutes). Identification of individual peaks present in the diesel fuel sample was carried out by comparison with external standards injected separately. Alkane, cycloalkane, mono-, di- and polyaromatic hydrocarbon standards were prepared (as described in Section 3.1.2.8) and used to identify the corresponding peaks in the diesel fuel sample. Figure 4.2.1 shows the GC-FID chromatogram of diesel fuel diluted 1:100 with hexane. Each identified peak has been assigned a number which relates to the numbers given below the chromatogram.




1- nonane, 2 - decane, 3 - butyl cyclohexane, 4 - undecane, 5 - dodecane, 6 - tridecane, 7 - tetradecane, 8 - hexamethyl benzene, 9 - 1,8 dimethyl naphthalene, 10 - pentadecane, 11- hexadecane, 12 - heptadecane, 13 - octadecane, 14 - nonadecane, 15 - eicosane, 16 -heneicosane, 17 - docosane, 18 - tricosane.

Figure 4.2.1 GC-FID chromatogram of diesel fuel diluted 1:100 with hexane.

## 4.3 GC-MS Analysis of Pure Diesel Fuel

To provide positive identification of the lesser hydrocarbon peaks found in the diesel fuel chromatogram, pure diesel fuel was separated by capillary gas chromatography then individual peaks analysed by mass spectrometry. GC-MS is an extremely useful method for identifying unknown compounds within hydrocarbon samples but the complexity of the diesel fuel sample sometimes made the interpretation of the resulting mass spectra difficult. For example, less defined, broader peaks would sometimes contain mixtures of two or three compounds due to poor resolution of all the compounds present. If you took a MS measurement from the front of the peak you could get a completely different mass spectrum than if you took a measurement from the back of the peak. Therefore, measurements were taken from areas of the peak thought to contain only one compound then repositioned on another such area within the peak. This was very time consuming, as two or three mass spectra were taken from each peak. Overall, the data produced from this thorough examination of one pure diesel fuel chromatogram led to a detailed description of the different hydrocarbons present in diesel fuel.

Figure 4.3.1 illustrates the Total Ion Concentration (TIC) chromatogram produced by GC-MS analysis of a pure diesel fuel sample. As the sample was injected into the GC in its undiluted form, no solvent peak was present to mask the more volatile diesel compounds that come off the column during the first few minutes of the analysis. This allowed a complete diesel fuel fingerprint to be obtained.

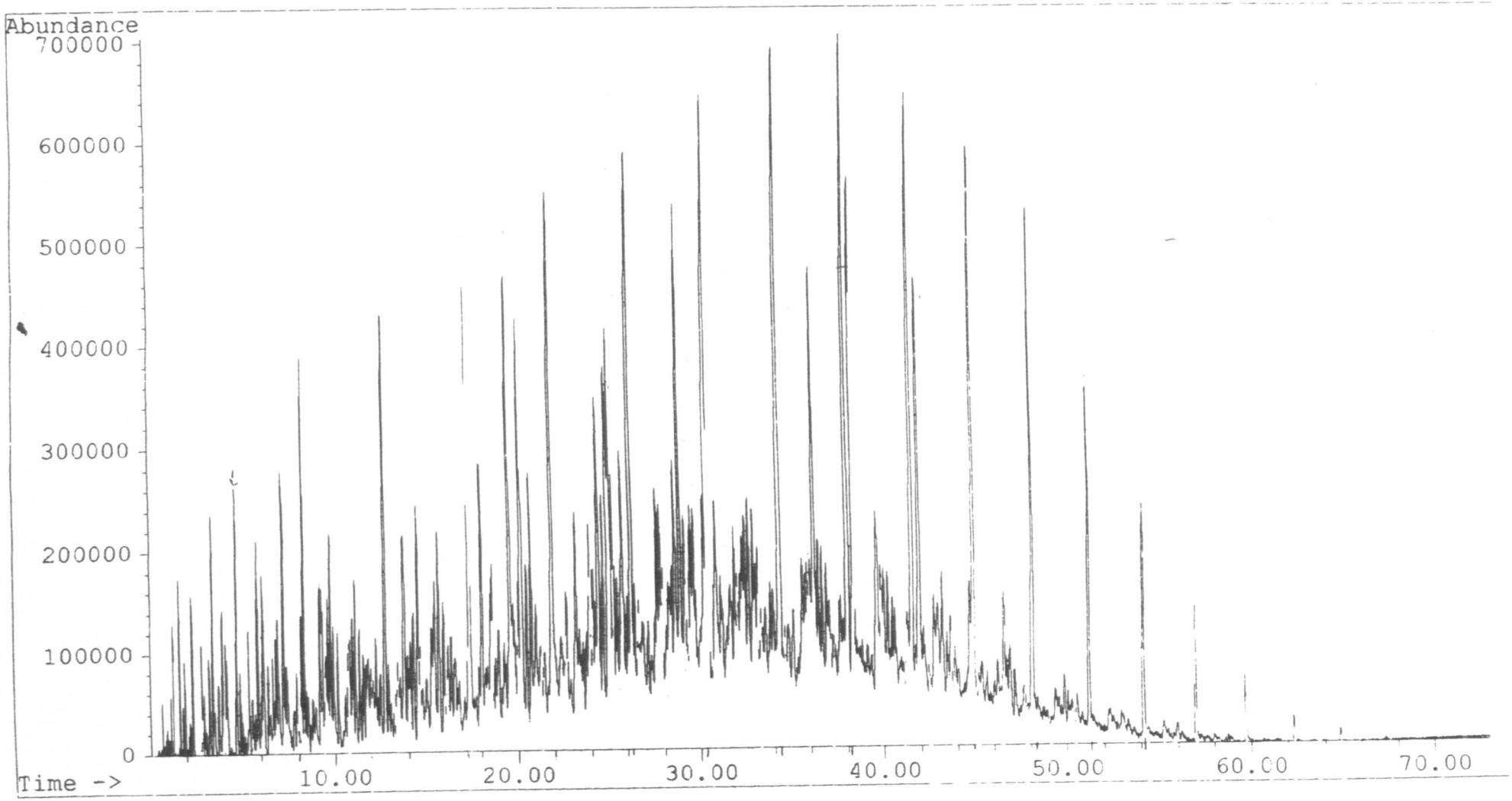
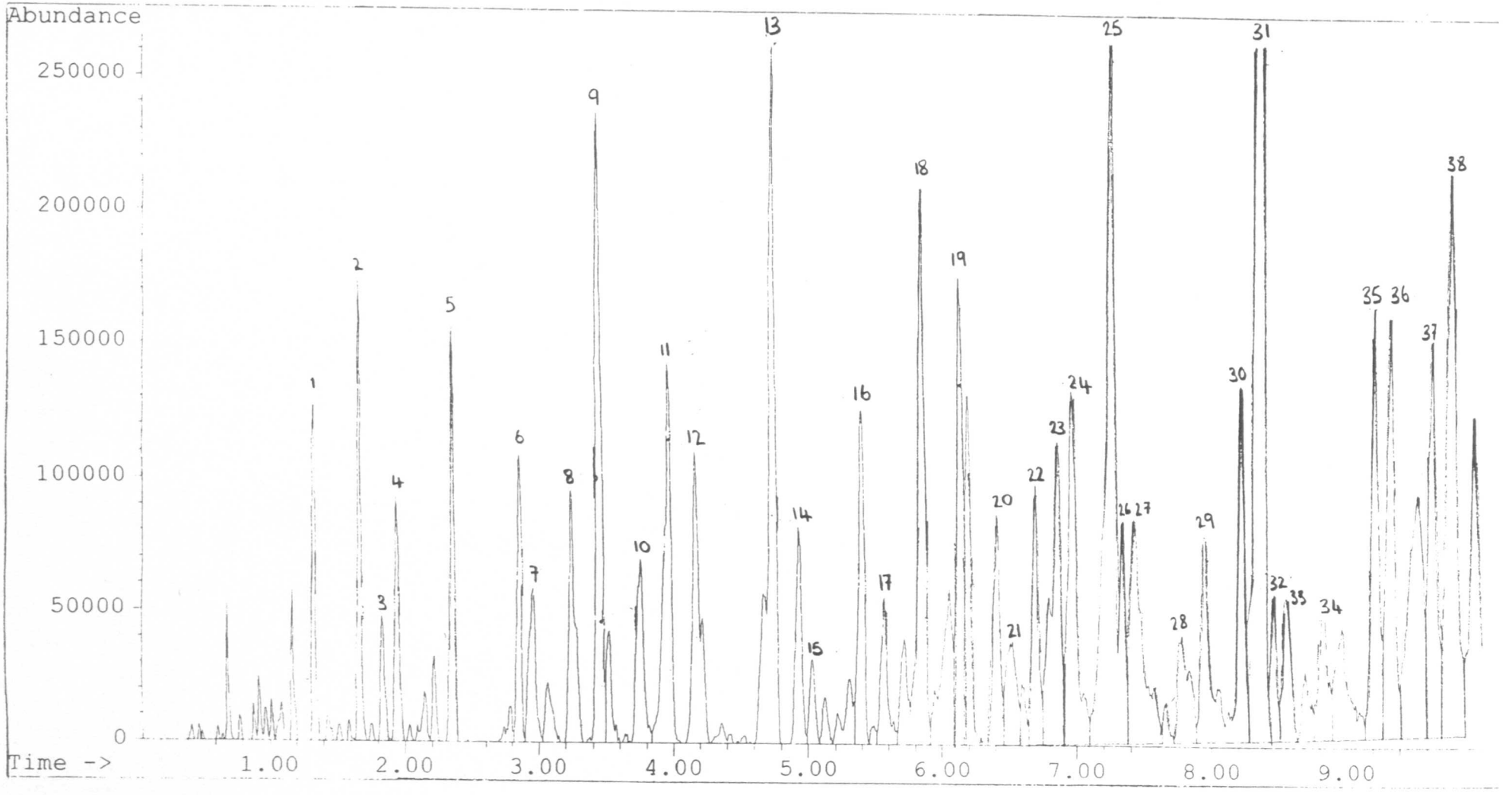

Abundance
700000
600000
500000
400000
300000
200000
100000
0
Time ->
10.00
20.00
30.00
40.00
50.00
60.00
70.00

Figure 4.3.2 illustrates the same TIC diesel fuel chromatogram as in Figure 4.3.1. The chromatograms (shown on the next few pages) were obtained by displaying the original chromatogram stored in the memory of the data system in 10 minute segments and selecting the optimum attenuation of the particular segment before printing. Each peak on the chromatogram analysed by mass spectrometry has been designated a number and these numbers correspond to the peak numbers listed in Table 4.3.3. Very small or very 'messy' peaks were not analysed by mass spectrometry. Table 4.3.3 (shown as the mirror image of each chromatographic section) gives full information on each peak identified.

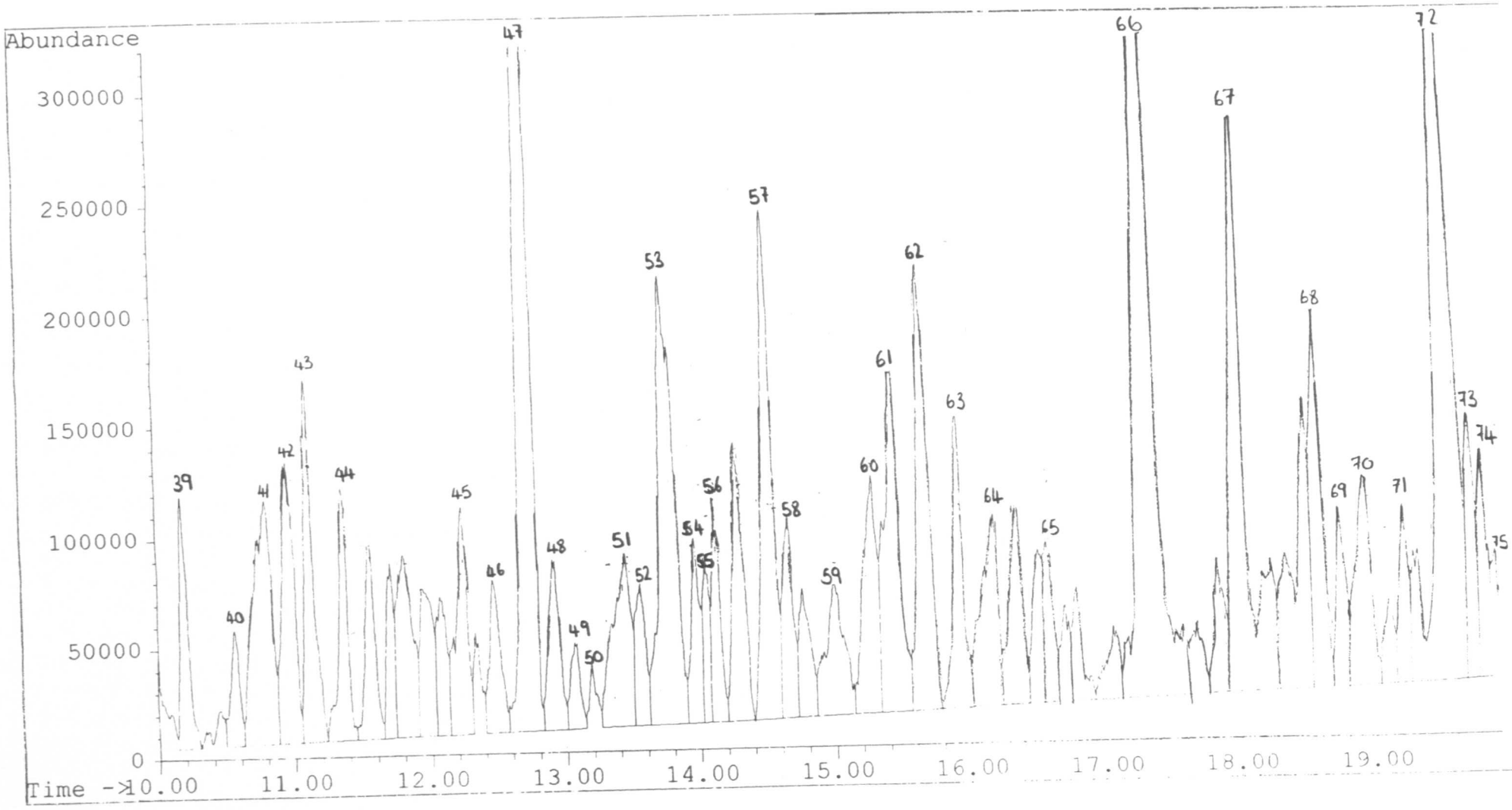
| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 1 | 1.33 | 0.099 | methyl cyclohexane |
| 2 | 1.66 | 0.131 | benzene |
| 3 | 1.83 | 0.049 | dihydro 4,4 dimethyl 2 (3H) furanone |
| 4 | 1.95 | 0.099 | 1,3 dimethyl cyclohexane |
| 5 | 2.35 | 0.150 | 3 ethyl hexane |
| 6 | 2.84 | 0.116 | ethyl cyclohexane |
| 7 | 2.94 | 0.094 | 1,1,3 trimethyl cyclohexane |
| 8 | 3.21 | 0.147 | ethylbenzene |
| 9 | 3.34 | - | unknown |
| 10 | 3.74 | 0.123 | 2,3 dimethyl heptane |
| 11 | 3.98 | 0.267 | a benzene |
| 12 | 4.14 | 0.194 | 3 ethyl 4,4 dimethyl 2 pentene |
| 13 | 4.75 | 0.466 | n - nonane |
| 14 | 4.90 | 0.114 | 1 methylethyl benzene |
| 15 | 5.03 | 0.043 | 1 methylethyl cyclohexane |
| 16 | 5.40 | 0.177 | propyl cyclohexane |
| 17 | 5.56 | 0.095 | butyl cyclopentane |
| 18 | 5.87 | 0.347 | a benzene |
| 19 | 6.12 | 0.258 | a naphthalene |
| 20 | 6.40 | 0.138 | a naphthalene |
| 21 | 6.54 | 0.104 | 1 ethyl 2,3 dimethyl cyclohexane |
| 22 | 6.68 | 0.152 | a naphthalene |
| 23 | 6.88 | 0.273 | 2, 3 dimethyl octane |
| 24 | 6.98 | 0.286 | an alkane |
| 25 | 7.26 | 0.584 | a naphthalene |
| 26 | 7.32 | 0.124 | a benzene |
| 27 | 7.42 | 0.261 | a benzene |
| 28 | 7.77 | - | 4 propyl 3 heptene |
| 29 | 7.93 | 0.157 | (1 methylpropyl) benzene |
| 30 | 8.21 | 0.237 | a benzene |
| 31 | 8.35 | 0.840 | n - decane |
| 32 | 8.45 | 0.820 | 1 methyl 3-(1 methylethyl) benzene |
| 33 | 8.54 | 0.117 | a benzene |
| 34 | 8.83 | 0.120 | a benzene |
| 35 | 9.20 | 0.297 | butyl cyclohexane |
| 36 | 9.35 | 0.317 | 2,6 dimethyl nonane |
| 37 | 9.63 | 0.283 | 1 methyl 3 propyl benzene |
| 38 | 9.80 | 0.547 | bicyclo nona-dien-3-one |

Table 4.3.3. Full peak information relating to the chromatogram of undiluted diesel fuel in Figure 4.3.2. Peak numbers 1 – 38 relating to 0 – 10.00 minutes on the chromatogram.


Abundance
250000
200000
150000
100000
50000
0
Time ->
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38

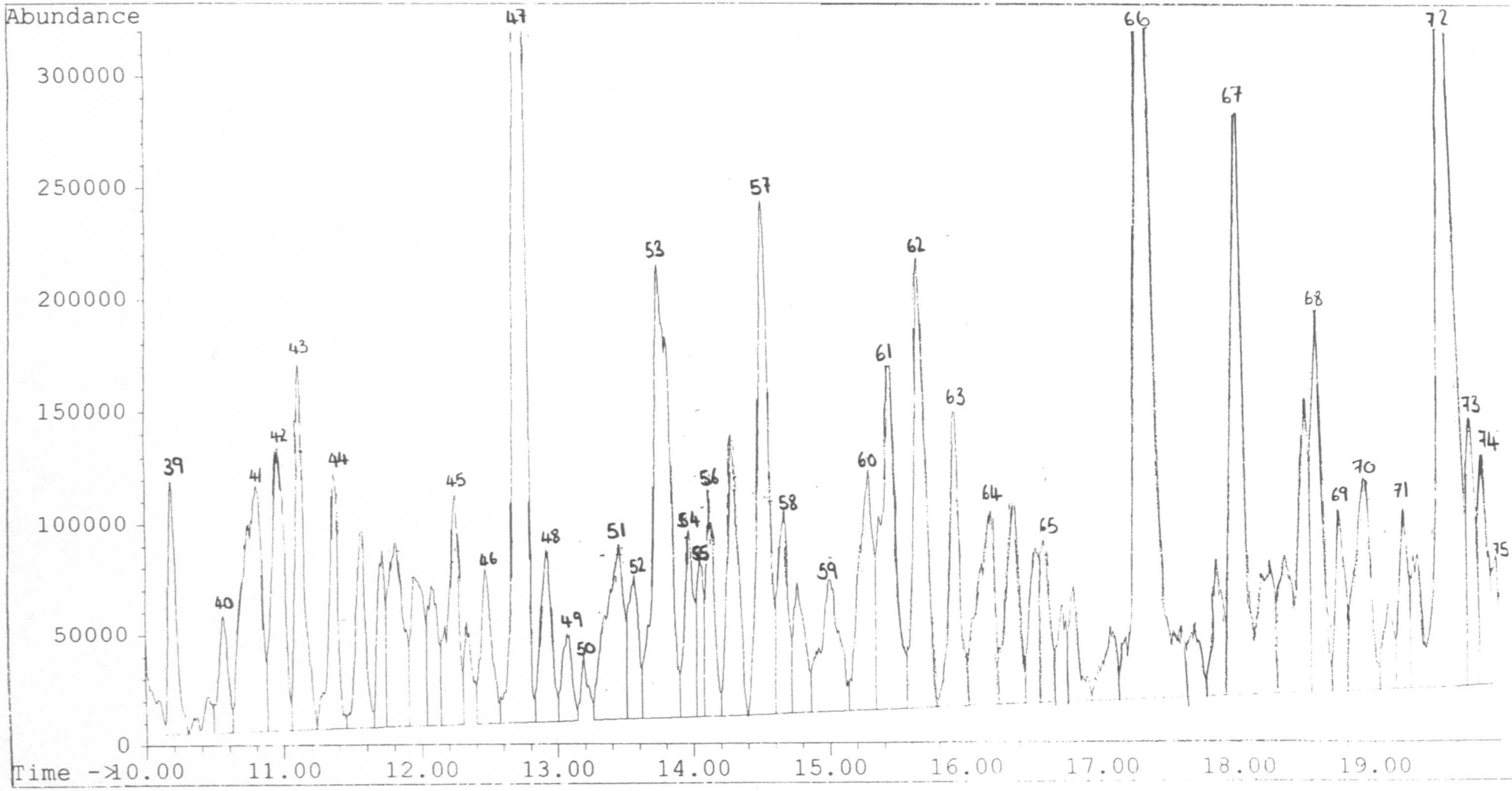
| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 39 | 10.20 | 0.211 | 1 methyl 4 propyl benzene |
| 40 | 10.57 | 0.112 | aromatic |
| 41 | 10.83 | 0.504 | 5 methyl decane |
| 42 | 10.99 | 0.380 | aromatic |
| 43 | 11.10 | 0.355 | 4,5 dimethyl nonane |
| 44 | 11.36 | 0.250 | 3 methyl decane |
| 45 | 12.24 | 0.281 | 4,7,7 trimethyl bicyclo heptan-3-one |
| 46 | 12.47 | 0.186 | aromatic |
| 47 | 12.74 | 1.245 | n - undecane |
| 48 | 12.92 | 0.215 | decahydro-2 methyl naphthalene |
| 49 | 13.06 | 0.215 | aromatic |
| 50 | 13.19 | 0.095 | 2,3 dihydro 1,6 dimethyl indene |
| 51 | 13.46 | 0.345 | 2,3 dihydro 5 methyl indene |
| 52 | 13.57 | 0.160 | 5 methyl undecane |
| 53 | 13.73 | 0.795 | pentyl cyclohexane |
| 54 | 13.96 | 0.184 | 4,5 dimethyl nonane |
| 55 | 14.04 | - | unknown |
| 56 | 14.12 | 0.194 | 1 methyl 4(2 methylpropyl) benzene |
| 57 | 14.53 | 0.611 | 1 methylene indene |
| 58 | 14.65 | 0.159 | 1 methyl 4,2 methylpropyl benzene |
| 59 | 15.00 | 0.275 | aromatic |
| 60 | 15.27 | 0.360 | alkane |
| 61 | 15.45 | 0.555 | 4 methyl undecane |
| 62 | 15.61 | 0.593 | alkane |
| 63 | 15.95 | 0.327 | alkane |
| 64 | 16.19 | 0.342 | branched cyclohexane |
| 65 | 16.60 | 0.182 | 1 methyl 3 pentyl cyclohexane |
| 66 | 17.33 | 1.931 | n - dodecane |
| 67 | 18.00 | 0.759 | alkane |
| 68 | 18.49 | - | hexyl cyclohexane |
| 69 | 18.75 | 0.170 | unknown |
| 70 | 18.97 | 0.365 | 4 butyl benzaldehyde |
| 71 | 19.22 | 0.187 | 1,5 dimethyl bicyclo[3.2.2.]nona-6,8-dien-3-one |
| 72 | 19.53 | 1.499 | 1 methyl naphthalene |
| 73 | 19.72 | 0.254 | alkane |
| 74 | 19.80 | 0.224 | 5 methyl dodecane |
| 75 | 20.00 | 0.312 | 4 methyl dodecane |

Table 4.3.3 continued. Peak numbers 39 – 75 relating to 10.00 – 20.00 minutes on the chromatogram.


Abundance
300000
250000
200000
150000
100000
50000
0
Time ->
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75

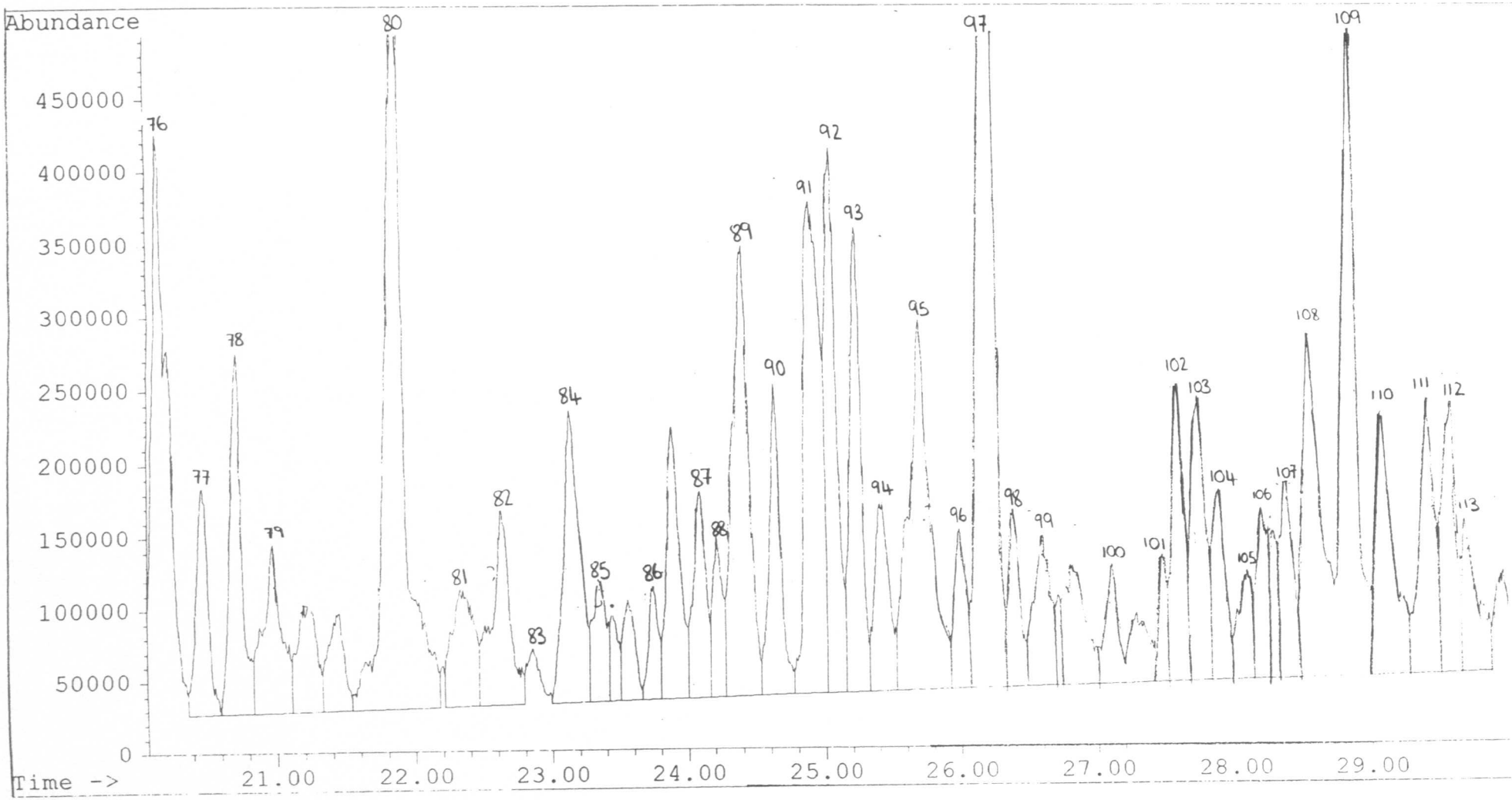
| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 39 | 10.20 | 0.211 | 1 methyl 4 propyl benzene |
| 40 | 10.57 | 0.112 | aromatic |
| 41 | 10.83 | 0.504 | 5 methyl decane |
| 42 | 10.99 | 0.380 | aromatic |
| 43 | 11.10 | 0.355 | 4,5 dimethyl nonane |
| 44 | 11.36 | 0.250 | 3 methyl decane |
| 45 | 12.24 | 0.281 | 4,7,7 trimethyl bicyclo heptan-3-one |
| 46 | 12.47 | 0.186 | aromatic |
| 47 | 12.74 | 1.245 | n - undecane |
| 48 | 12.92 | 0.215 | decahydro-2 methyl naphthalene |
| 49 | 13.06 | 0.215 | aromatic |
| 50 | 13.19 | 0.095 | 2,3 dihydro 1,6 dimethyl indene |
| 51 | 13.46 | 0.345 | 2,3 dihydro 5 methyl indene |
| 52 | 13.57 | 0.160 | 5 methyl undecane |
| 53 | 13.73 | 0.795 | pentyl cyclohexane |
| 54 | 13.96 | 0.184 | 4,5 dimethyl nonane |
| 55 | 14.04 | - | unknown |
| 56 | 14.12 | 0.194 | 1 methyl 4(2 methylpropyl) benzene |
| 57 | 14.53 | 0.611 | 1 methylene indene |
| 58 | 14.65 | 0.159 | 1 methyl 4,2 methylpropyl benzene |
| 59 | 15.00 | 0.275 | aromatic |
| 60 | 15.27 | 0.360 | alkane |
| 61 | 15.45 | 0.555 | 4 methyl undecane |
| 62 | 15.61 | 0.593 | alkane |
| 63 | 15.95 | 0.327 | alkane |
| 64 | 16.19 | 0.342 | branched cyclohexane |
| 65 | 16.60 | 0.182 | 1 methyl 3 pentyl cyclohexane |
| 66 | 17.33 | 1.931 | n - dodecane |
| 67 | 18.00 | 0.759 | alkane |
| 68 | 18.49 | - | hexyl cyclohexane |
| 69 | 18.75 | 0.170 | unknown |
| 70 | 18.97 | 0.365 | 4 butyl benzaldehyde |
| 71 | 19.22 | 0.187 | 1,5 dimethyl bicyclo[3.2.2.]nona-6,8-dien-3-one |
| 72 | 19.53 | 1.499 | 1 methyl naphthalene |
| 73 | 19.72 | 0.254 | alkane |
| 74 | 19.80 | 0.224 | 5 methyl dodecane |
| 75 | 20.00 | 0.312 | 4 methyl dodecane |

Table 4.3.3 continued. Peak numbers 39 – 75 relating to 10.00 – 20.00 minutes on the chromatogram.


Abundance
300000
250000
200000
150000
100000
50000
0
Time ->
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75

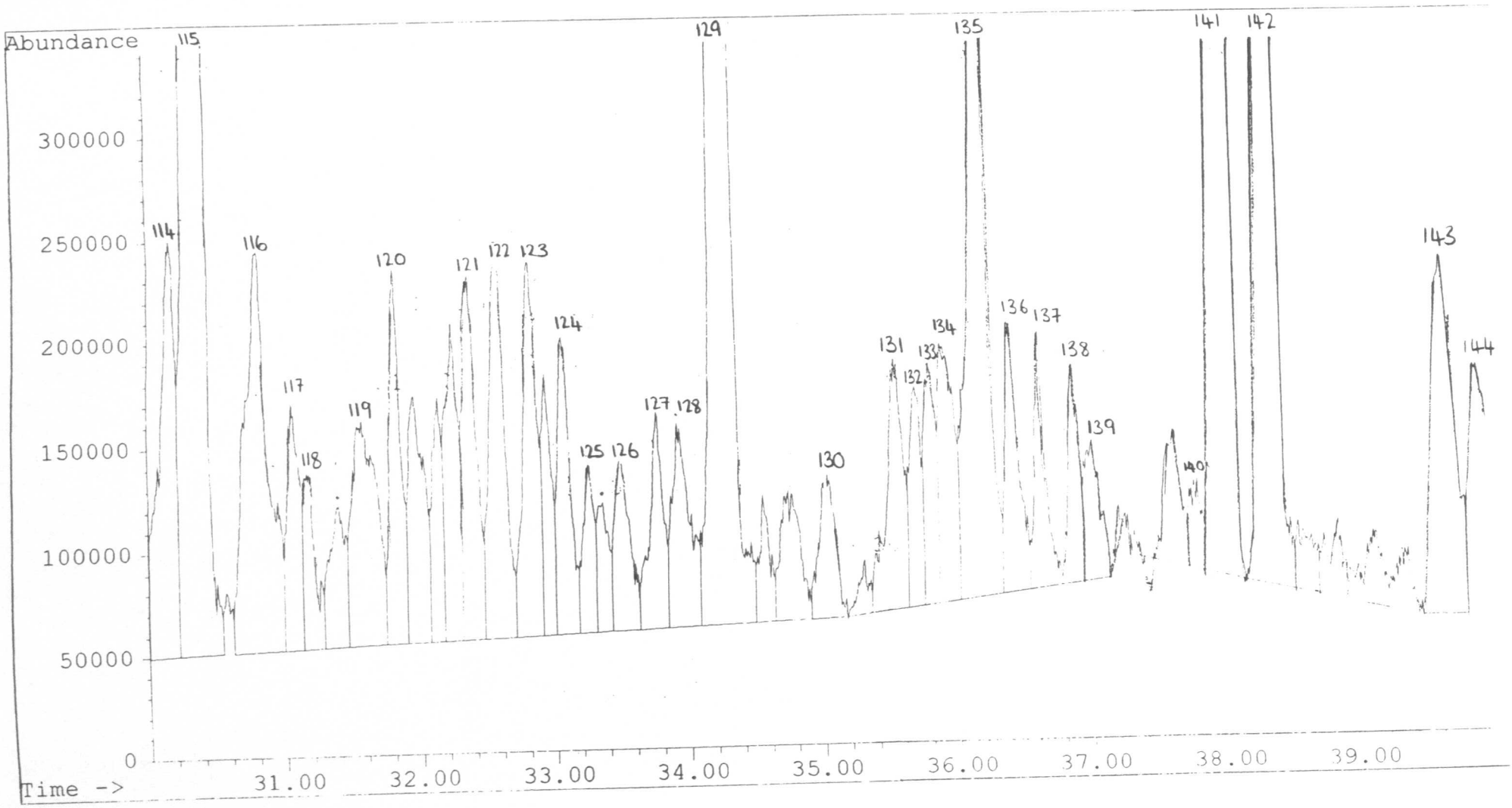
| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 76 | 20.13 | 1.524 | aromatic |
| 77 | 20.50 | 0.448 | alkane |
| 78 | 20.71 | 0.662 | 7 methyl dodecane |
| 79 | 20.97 | 0.511 | cyclohexyl benzene |
| 80 | 21.86 | 2.359 | n - tridecane |
| 81 | 22.27 | - | alkane |
| 82 | 22.65 | 0.59 | alkane |
| 83 | 22.85 | - | alkane |
| 84 | 23.14 | 0.785 | heptyl cyclohexane |
| 85 | 23.23 | - | 2 ethenyl naphthalene |
| 86 | 23.73 | 0.194 | 1 methyl 2 hexylbenzene |
| 87 | 24.09 | 0.428 | alkane |
| 88 | 24.26 | 0.268 | 5 methyl tridecane |
| 89 | 24.41 | 1.251 | 2,6 dimethyl naphthalene |
| 90 | 24.64 | 0.581 | 2 methyl tridecane |
| 91 | 24.91 | 1.367 | a naphthalene |
| 92 | 25.08 | 0.976 | a naphthalene |
| 93 | 25.26 | 0.871 | 2,6,10 trimethyldodecane |
| 94 | 25.41 | 0.471 | aromatic |
| 95 | 25.72 | 1.339 | a naphthalene |
| 96 | 25.99 | 0.321 | aromatic |
| 97 | 26.22 | 2.393 | n - tetradecane |
| 98 | 26.40 | 0.321 | alkane |
| 99 | 26.60 | 0.431 | aromatic |
| 100 | 27.11 | 0.262 | alkane |
| 101 | 27.49 | 0.194 | aromatic |
| 102 | 27.60 | 0.573 | octyl cyclohexane |
| 103 | 27.74 | 0.684 | 4 methyl 1,1 biphenyl |
| 104 | 27.93 | 0.381 | octyl benzene |
| 105 | 28.08 | 0.256 | (1methylheptyl) benzene |
| 106 | 28.26 | 0.206 | alkane |
| 107 | 28.42 | 0.406 | alkane |
| 108 | 28.51 | 1.027 | 2(-1 methylethyl) naphthalene |
| 109 | 28.87 | 1.622 | alkane |
| 110 | 29.06 | 0.746 | alkane |
| 111 | 29.41 | 0.698 | aromatic |
| 112 | 29.60 | 0.688 | a naphthalene |
| 113 | 29.71 | 0.356 | 1-(1-5-dimethylhexyl)-4-methyl cyclohexane |

Table 4.3.3 continued. Peak numbers 76 – 113 relating to 20.00 – 30.00 minutes on the chromatogram.


Abundance
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Time ->
21.00
22.00
23.00
24.00
25.00
26.00
27.00
28.00
29.00
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113

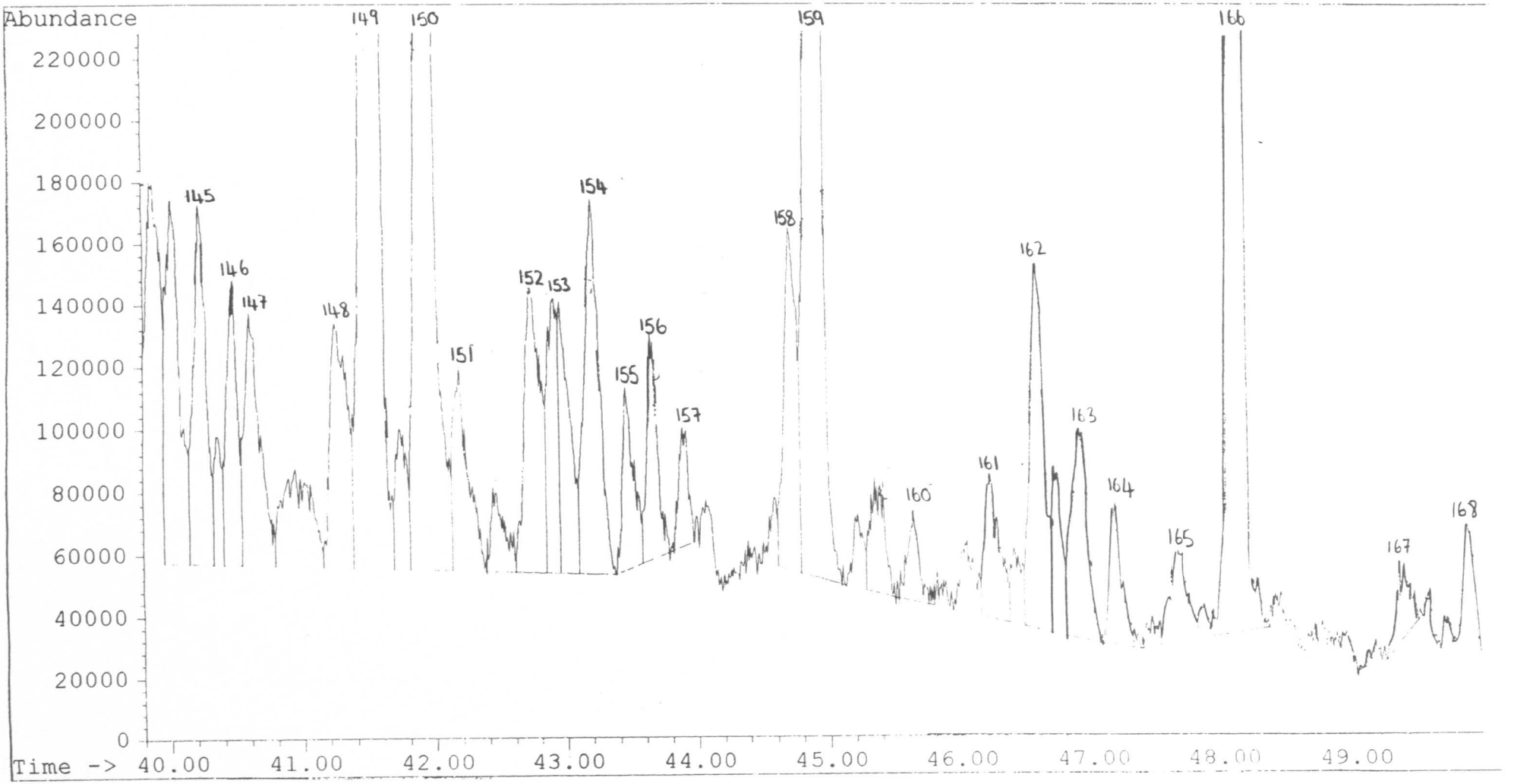
| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 114 | 30.17 | 0.759 | a naphthalene |
| 115 | 30.38 | 2.814 | n - pentadecane |
| 116 | 30.70 | 1.086 | a naphthalene |
| 117 | 31.07 | 0.338 | 9H fluorene |
| 118 | 31.16 | 0.248 | 1 methyl 7,1 methylethyl naphthalene |
| 119 | 31.58 | 0.650 | a naphthalene |
| 120 | 31.80 | 0.490 | nonyl cyclohexane |
| 121 | 32.38 | 0.569 | 2 methyl 1 propyl naphthalene |
| 122 | 32.58 | 0.722 | alkane |
| 123 | 32.78 | 0.616 | 9 silafluorene |
| 124 | 33.07 | 0.430 | 3 methyl pentadecane |
| 125 | 33.24 | 0.215 | 4 methyl benzofuran |
| 126 | 33.49 | 0.310 | dibenzofuran |
| 127 | 33.76 | 0.351 | aromatic |
| 128 | 33.88 | 0.419 | 1 methyl-7-(1 methylethyl) naphthalene |
| 129 | 34.30 | 2.834 | n - hexadecane |
| 130 | 35.03 | 0.288 | aromatic |
| 131 | 35.54 | 0.496 | 4 methyl 9H fluorene |
| 132 | 35.70 | 0.301 | aromatic |
| 133 | 35.80 | 0.317 | decyl cyclohexane |
| 134 | 35.98 | 0.471 | alkane |
| 135 | 36.22 | 1.676 | alkane |
| 136 | 36.42 | 0.425 | alkane |
| 137 | 36.62 | 0.316 | alkane |
| 138 | 36.87 | 0.270 | alkane |
| 139 | 37.00 | 0.258 | 2 methoxy 1,1 biphenyl |
| 140 | 37.89 | 0.121 | anthracene |
| 141 | 38.06 | 2.613 | n - heptadecane |
| 142 | 38.38 | 1.911 | 2,6,10,14 tetramethyl pentadecane |
| 143 | 39.62 | 0.909 | undecyl cyclohexane |
| 144 | 39.86 | 0.479 | alkane |

Table 4.3.3 continued. Peak numbers 114 – 144 relating to 30.00 – 40.00 minutes on the chromatogram.


Abundance
300000
250000
200000
150000
100000
50000
0
Time ->
31.00
32.00
33.00
34.00
35.00
36.00
37.00
38.00
39.00
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144

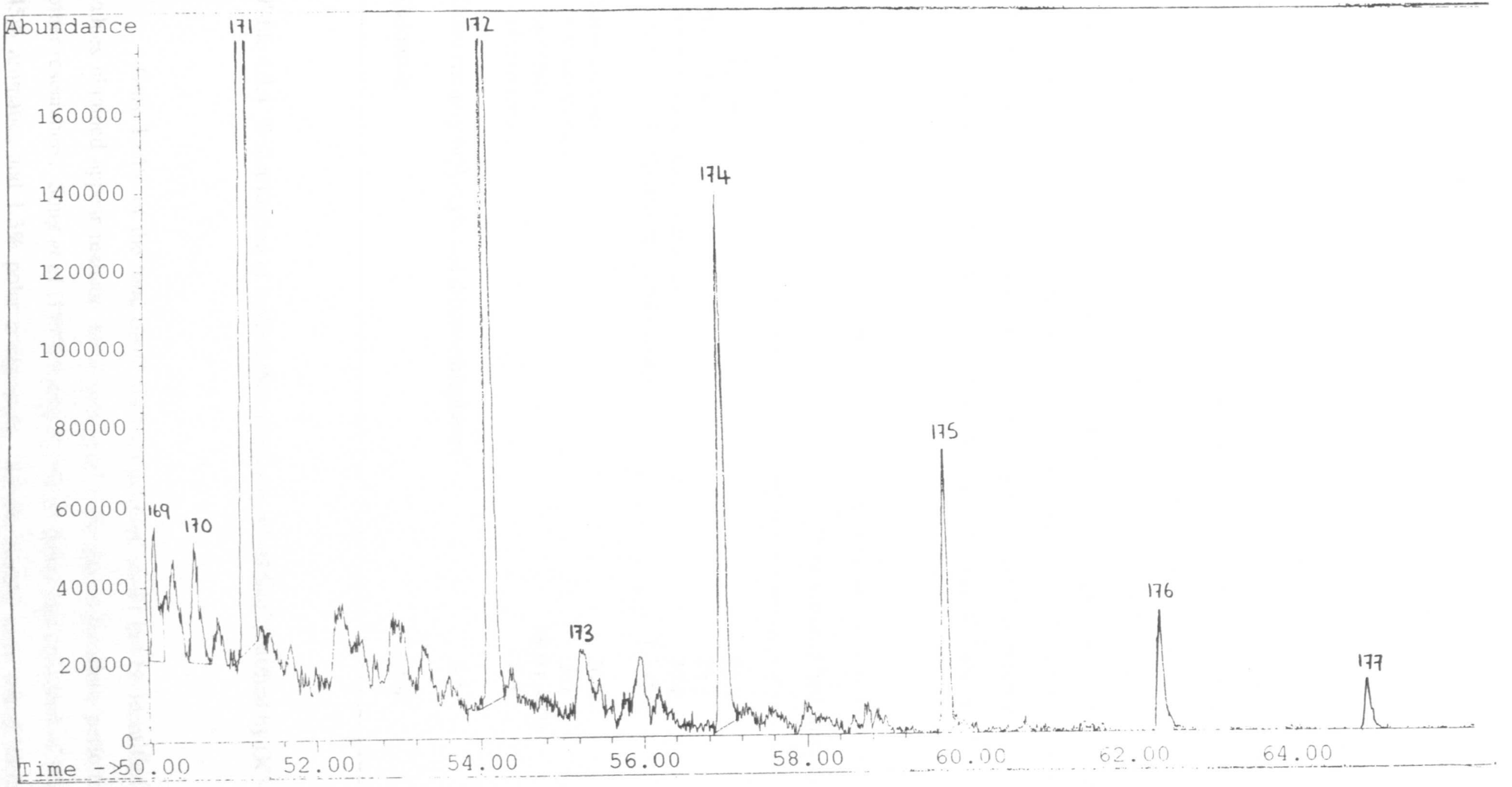
| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 145 | 40.23 | 0.354 | alkane |
| 146 | 40.47 | 0.239 | alkane |
| 147 | 40.62 | 0.318 | methyl dibenzothiophene |
| 148 | 41.26 | 0.332 | methyl dibenzothiophene |
| 149 | 41.57 | 2.218 | n - octadecane |
| 150 | 41.94 | 1.618 | 2,6,10,14 tetramethyl hexadecane |
| 151 | 42.19 | 0.242 | 2 methyl phenanthrene |
| 152 | 42.74 | 0.368 | unknown |
| 153 | 42.95 | 0.246 | alkane |
| 154 | 43.22 | 0.448 | dodecyl cyclohexane |
| 155 | 43.46 | 0.161 | alkane |
| 156 | 43.66 | 0.189 | 3 methyl heptadecane |
| 157 | 43.88 | 0.088 | 3 methyl octadecane |
| 158 | 44.72 | 0.348 | branched alkane |
| 159 | 44.93 | 1.809 | n - nonadecane |
| 160 | 45.65 | 0.085 | unknown |
| 161 | 46.23 | 0.171 | unknown |
| 162 | 46.61 | 0.410 | tridecyl cyclohexane |
| 163 | 46.93 | 0.269 | 2 methyl nonadecane |
| 164 | 47.18 | 0.139 | 3 methyl nonadecane |
| 165 | 47.65 | 0.106 | alkane |
| 166 | 48.14 | 1.506 | n - eicosane |
| 167 | 49.38 | 0.083 | unknown |
| 168 | 49.85 | 0.115 | tetradecyl cyclohexane |

Table 4.3.3 continued. Peak numbers 145 – 168 relating to 40.00 – 50.00 minutes on the chromatogram.


Abundance
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Time -> 40.00
41.00
42.00
43.00
44.00
45.00
46.00
47.00
48.00
49.00
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168

| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 169 | 50.06 | 0.090 | Alkane |
| 170 | 50.56 | 0.093 | Alkane |
| 171 | 51.21 | 0.882 | n - heneicosane |
| 172 | 54.14 | 0.584 | n - docosane |
| 173 | 55.95 | - | Branched cyclohexane |
| 174 | 56.96 | 0.325 | n - tricosane |
| 175 | 59.73 | 0.173 | n - tetracosane |
| 176 | 62.34 | 0.086 | n - pentacosane |
| 177 | 64.42 | - | n- hexacosane |

Table 4.3.3 continued. Peak numbers 169 – 177 relating to 50 – 65.00 minutes on the chromatogram.


Abundance
171
172
174
175
176
177
169
170
173
160000
140000
120000
100000
80000
60000
40000
20000
0
Time ->50.00
52.00
54.00
56.00
58.00
60.00
62.00
64.00

In the chromatogram shown (Figure 4.3.2), a total of 177 peaks were indicated and from these 106 were identified. It should however, be emphasized that out of the 61 unidentified peaks only 7 are unknown. The other 54 peaks mass spectra allowed these peaks to be classed as an alkane, cycloalkane, benzene, naphthalene or other aromatic. Although the identity of these peaks is not known, the hydrocarbon class of each peak was identified. This allowed the percentage distribution of hydrocarbon classes present in this diesel fuel sample to be determined. Table 4.3.4 shows the percentage distribution of hydrocarbon classes in diesel fuel identified by GC-MS. Each peak was identified to its hydrocarbon class then the percentage peak areas from each peak were added together to give a total for that particular class. The small or 'messy' peaks not analysed by mass spectrometry were included in this calculation and are represented by the unknown class of compounds at the end of Table 4.3.4.

| | % total diesel fuel composition |
|---|---|
| Total alkanes | 56.5 |
| n-alkanes | 25.1 |
| branched alkanes and alkenes | 24.2 |
| cyclic alkanes (branched cyclohexane) | 7.2 (5.8) |
| Total aromatics | 26.6 |
| monoaromatics | 5.0 |
| diaromatics | 19.0 (14.3) |
| polyaromatics | 2.9 |
| Other (mainly biphenyls and dibenzothiophenes) | 3.5 |
| Unknown | 13.4 |

Table 4.3.4 % distribution of hydrocarbon classes in diesel fuel identified by GC-MS.

Only 13.4% of the total diesel fuel composition could not be identified. The values obtained appear realistic when compared with class separations performed by other researchers. Song *et al* (1990) found 53.7% of diesel fuel consisted of saturates, 45% aromatics and 1.3% polar compounds. Similar results were found during this investigation, with diesel fuel containing 56.5% saturates. A lower value for total

aromatics and polar compounds was observed however. The values obtained for total aromatics were also found by Bundt *et al* (1991). The authors observed total aromatics to equal 28.3% of diesel fuel with mono-, di- and polyaromatic hydrocarbons (PAHs plus biphenyls) making up 16.4%, 8.0% and 3.9% of the total respectively. I found the diesel fuel analysed contained 26.5% total aromatics with 5.0%, 19.0% and 2.9% consisting of mono-, di- and PAHs. 3.5% of this diesel was also found to contain more polar compounds such as biphenyls and dibenzothiophenes.

The differences in percentage distribution can be attributed to diesel fuel composition differing due to the source of crude oil used and refining processes. The values represented here do appear to be a realistic measure of the percentage distribution of hydrocarbon classes present in a typical diesel fuel.

Figure 4.3.5 shows the percentage distribution of hydrocarbon classes in diesel fuel from Table 4.3.4 represented as chromatogram-style graphs. Each graph shows the full diesel composition with a specific class highlighted. For example, Figure 4.3.5.A illustrates the n-alkane and branched alkanes present in diesel fuel. This class of compounds is spread throughout the whole chromatogram. Figure 4.3.5 E however shows the diaromatic compounds which can only be found in the mid-range of the chromatogram. This figure clearly illustrates the elution pattern of each hydrocarbon class and gives an indication of the percentage of the total diesel fuel made up by each hydrocarbon class.

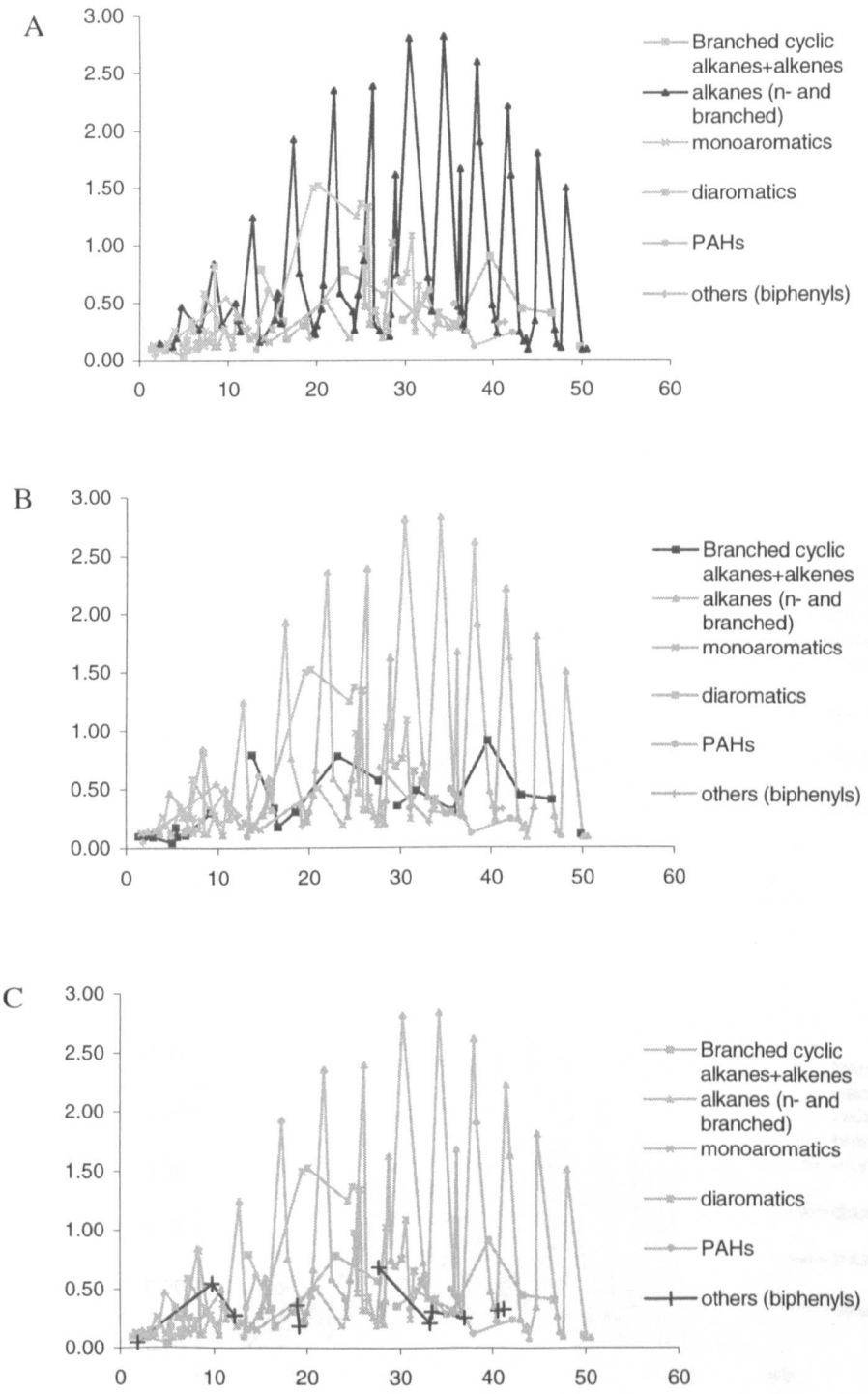



Figure 4.3.5. Graphical representation of the percentage distribution of hydrocarbon classes in diesel fuel.

A) n-alkanes, branched alkanes and alkenes, B) cyclic alkanes and C) other compounds (mainly biphenyl and dibenzothiophenes).

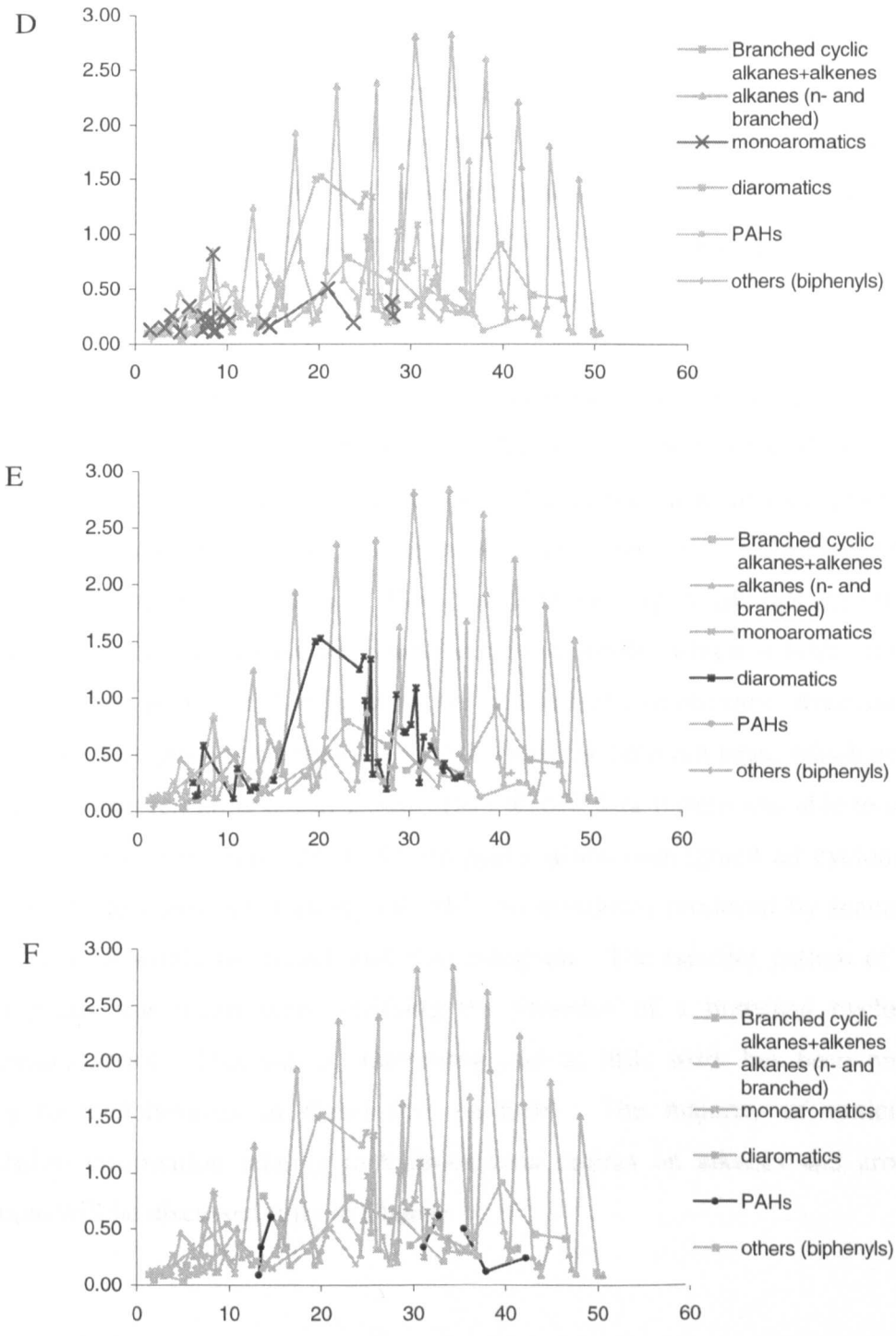
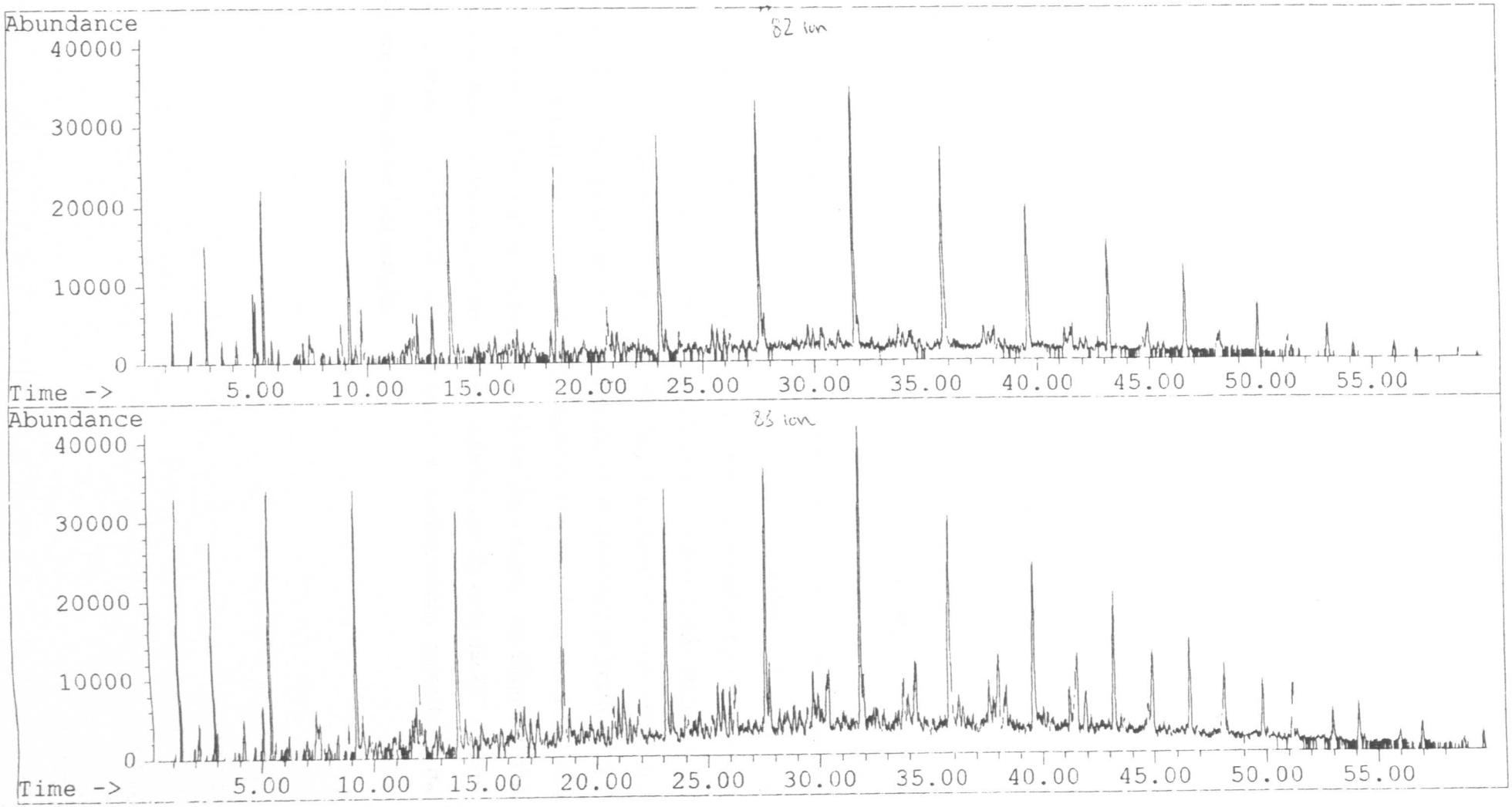



Figure 4.3.5 continued. Graphical representation of the percentage distribution of hydrocarbon classes in diesel fuel.

D) monoaromatics, E) diaromatics and F) polyaromatics (PAHs).

The distinctive pattern of petroleum hydrocarbon chromatograms is due to the presence of homologous series of hydrocarbons that differ from adjacent members in the series by a repeating unit. The most apparent homologous series in diesel fuel is the n-alkane family. The group starts with n-octane, a compound containing 8 carbons, and increases by addition of $CH_2$ through the series until n-hexacosane, a C26 hydrocarbon. These n-alkanes provide the 'backbone' to the diesel fuel chromatogram (as illustrated on Figure 4.3.1).

A less obvious homologous series was identified during GC-MS investigation of the diesel fuel chromatogram. A branched cyclohexane series was found ranging from methyl cyclohexane to hexadecyl cyclohexane. This series was difficult to see in the original diesel fuel chromatogram as the cyclohexane peaks were hidden in the dense area of peaks beneath the n-alkanes. However, with the help of GC-MS the series was identified. Cyclohexanes have an identifiable mass spectra which is built around the large 82/83 ion peak which represents the individual cyclohexane structure. The remaining peaks represent the branching units from this main ion peak, which generally increase by 15 representing a $CH_2$ group. The GC-MS data system was able to scan the diesel fuel chromatogram for all 82/83 ion peaks which highlighted all cyclohexanes. Figure 4.3.4 illustrates the resulting GC-MS chromatogram produced by scanning for ions 82 and 83 within the diesel fuel chromatogram. The familiar pattern of evenly spaced peaks was again seen, verifying the presence of a branched cyclohexane homologous series. This was an interesting find as little work has been presented relating to cyclohexanes in diesel fuel products. The majority of toxicity and degradation information relating to hydrocarbons centres on alkanes and aromatics. This topic will be discussed fully in Chapter 6.

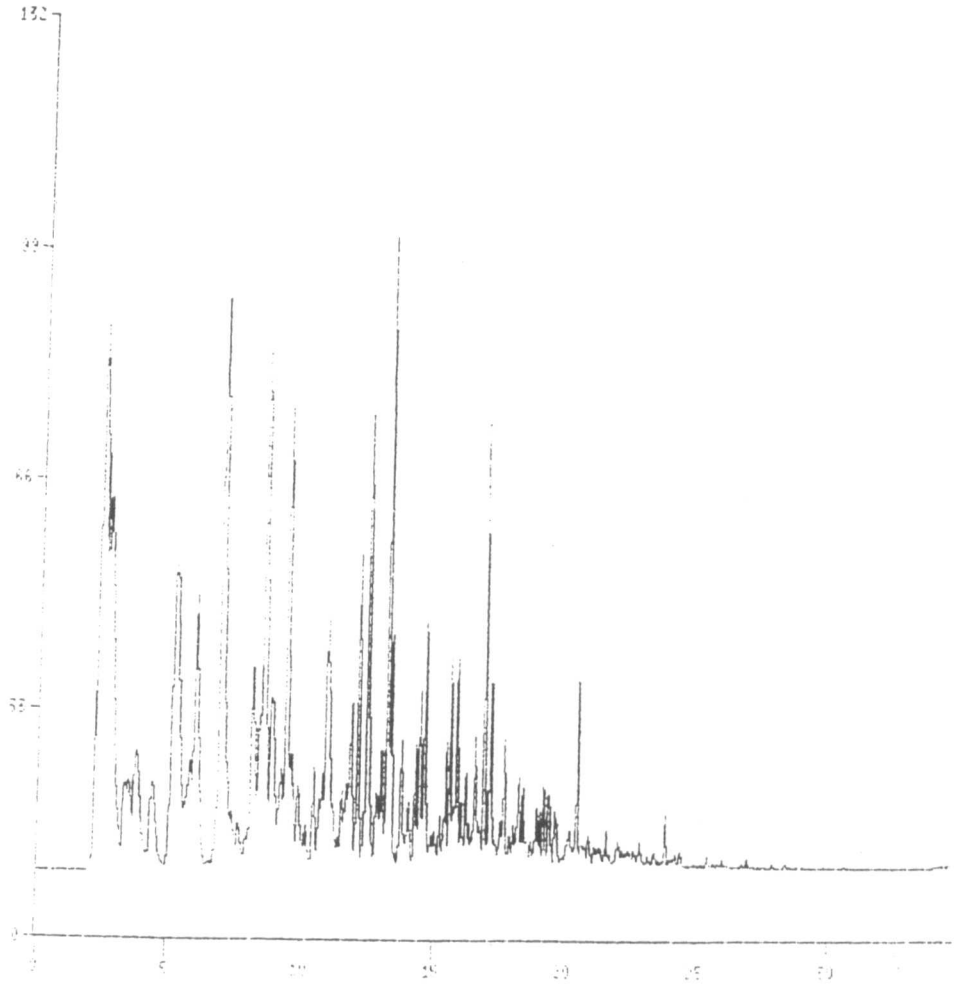

Abundance
82 ion
40000
30000
20000
10000
0
Time ->
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
Abundance
83 ion
40000
30000
20000
10000
0
Time ->
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00

## 4.4 GC-FID and GC-MS Analysis of Volatile Diesel Fuel

As mentioned at the start of the chapter, diesel fuel composition alters when released into the soil environment due to weathering by both biological and non-biological processes. Biological weathering caused by microbial degradation or plant uptake will be discussed in later chapters.

There are two main processes which affect the non-biological weathering of diesel fuel, these being leaching and volatilisation. Although both these factors will be discussed in later chapters it was necessary to quantify the loss of diesel fuel by volatilisation, as volatilisation is the most important non-biological weathering process affecting diesel fuel composition.

Diesel fuel is a complex mixture of both volatile and non-volatile compounds. It was important to determine which compounds would volatilise, under normal conditions, from diesel fuel and how this changed the diesel fuel fingerprint. Theoretically, the compounds identified during the GC-MS analysis of diesel fuel could be assessed individually on their volatility by reference to values for each pure compound. However, the volatility of these compounds is retarded by the fact that diesel fuel is a complex mixture, therefore volatility values for pure compounds have no use in this situation. Instead, a sample of diesel fuel was placed in a vial, sealed and then stored at 20°C for equilibrium headspace sampling as described in Section 2.3.4. The compounds, which were volatile at 20°C, began to evaporate from the diesel fuel until they reached equilibrium in the headspace above the sample. An aliquot of this headspace was then withdrawn from the vial and injected directly onto the GC column. Figure 4.4.1 shows the GC-FID chromatogram of hydrocarbons present in the headspace above the diesel fuel sample.



Attenuation maximum : 132 millivolts.

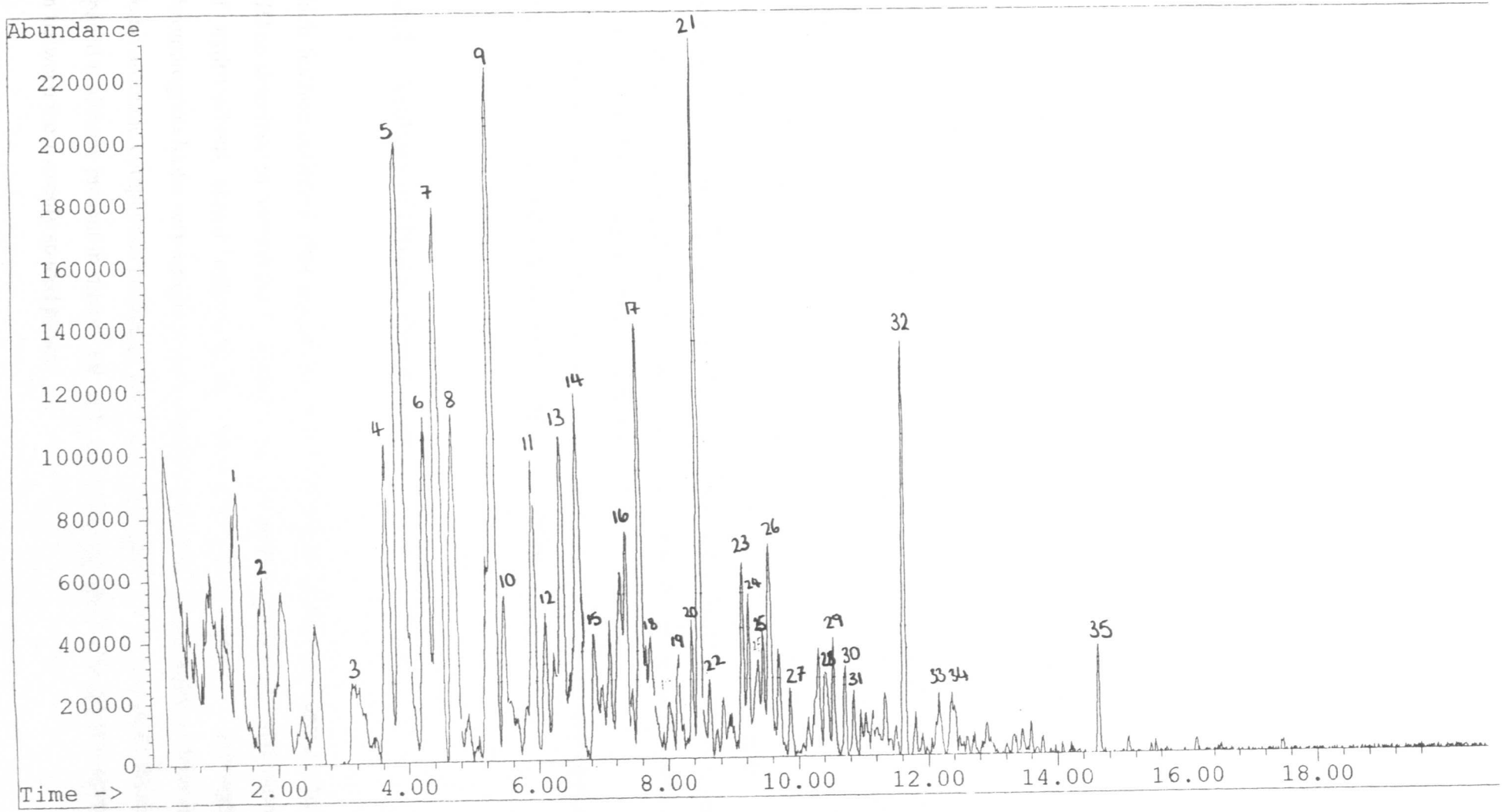
Figure 4.4.1. GC-FID chromatogram of volatile diesel fuel fraction at 20°C.

No peaks were detected after 25 minutes on the chromatogram so the GC run was stopped at 35 minutes.

To identify the peaks present in the diesel fuel headspace, another diesel sample was prepared for equilibrium headspace sampling and analysed by GC-MS. The chromatographic conditions used for the GC-MS analysis were the same as for GC-FID analysis except the type of column used (see Section 2.3.4). However, very similar chromatographic traces were obtained using both methods. Figure 4.4.2 illustrates the GC-MS chromatogram of the headspace diesel fuel fraction. Peaks analysed by mass spectrometry were assigned a peak number and this number relates to the peak numbers listed in Table 4.4.3.

| Peak No. | Retention Time | Peak Area (%) | Compound |
|---|---|---|---|
| 1 | 1.417 | 3.217 | methyl cyclohexane |
| 2 | 1.788 | 2.041 | toluene |
| 3 | 3.154 | 1.650 | Ethyl cyclohexane |
| 4 | 3.672 | 3.417 | o-xylene |
| 5 | 3.903 | 9.120 | m-xylene |
| 6 | 4.270 | 3.981 | branched alkane |
| 7 | 4.470 | 5.968 | p-xylene |
| 8 | 4.702 | 3.937 | iso-cyclohexane |
| 9 | 5.313 | 7.681 | n-nonane |
| 10 | 5.474 | 1.487 | alkyl benzene |
| 11 | 5.921 | 2.466 | propyl cyclohexane |
| 12 | 6.112 | 1.192 | nonene |
| 13 | 6.364 | 3.154 | alkyl benzene |
| 14 | 6.623 | 4.040 | alkyl benzene |
| 15 | 6.852 | 1.036 | alkyl benzene |
| 16 | 7.359 | 1.506 | decene |
| 17 | 7.577 | 3.005 | ethyl benzene |
| 18 | 7.728 | 1.102 | (ethylpropyl) cyclopentane |
| 19 | 8.157 | 0.426 | alkyl benzene |
| 20 | 8.372 | 0.620 | (methylethyl) benzene |
| 21 | 8.484 | 3.895 | decane |
| 22 | 8.643 | 0.373 | alkyl benzene |
| 23 | 9.151 | 1.107 | butyl cyclohexane |
| 24 | 9.245 | 0.861 | ethyl heptane |
| 25 | 9.468 | 0.600 | alkyl benzene |
| 26 | 9.566 | 1.603 | alkyl benzene |
| 27 | 9.883 | 0.349 | alkyl benzene |
| 28 | 10.422 | 0.681 | alkyl benzene |
| 29 | 10.543 | 0.550 | methyl octane |
| 30 | 10.720 | 0.391 | branched alkane |
| 31 | 10.861 | 0.305 | branched cyclohexane |
| 32 | 11.640 | 1.913 | n-undecane |
| 33 | 12.178 | 0.531 | branched alkane |
| 34 | 12.378 | 0.381 | (methylpropyl) cyclohexane |
| 35 | 14.639 | 0.464 | n-dodecane |

Table 4.4.3. Full peak information relating to the chromatogram of diesel fuel headspace in Figure 7.4.2. Peak numbers 1 – 35 relating to 0 – 15.00 minutes on the chromatogram.


Abundance
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Time ->
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

35 peaks were analysed and 20 peaks were identified. The other 15 peaks were grouped into their respective hydrocarbon family or sub-family by analysis of the mass spectra. The dominant peaks were the low molecular weight alkanes (C9–C12), BTEX compounds (benzene, toluene, ethyl benzene and the xylenes, m-, o- and p-xylene) and branched cyclohexanes (methyl–butyl).

The volatile fraction in diesel fuel at 20°C made up between 5 and 10% of the total diesel fuel.

## 4.5 Hydrocarbon Group-Type Analysis by Fractionation

As demonstrated in the previous sections of this chapter, the major problem in the analysis of diesel fuel is in its complexity. The pre-separation of diesel fuel according to classes of similar structure prior to their identification would make the process of identifying individual compounds much easier, especially for the determination of minor compounds. Numerous separation procedures for crude oil, shale oil and coal derived materials exist from liquid-liquid extractions to semi-preparative HPLC using backflush techniques (Akhlaq, 1993). In 1961, Snyder established the classical method of column chromatography on silica gel and alumina to separate petroleum hydrocarbons. Since then a variety of adsorbents have been tested.

Diesel fuel fractionation into alkane, aromatic and polar groups was carried out by the modified method of Wang *et al* (1990) as described in Section 2.3.5.

### 4.5.1 Analysis of Hydrocarbon Classes

Each fraction collected after separation on the silica gel column was analysed by GC-FID as described in Section 2.3.2. Figure 4.5.1.1 illustrates the GC-FID chromatogram of hydrocarbons eluted with n-hexane from the silica column. Although the chromatogram looks very similar to the original diesel fuel chromatogram, there should be no aromatic compounds present in this fraction. Figure 4.5.1.1 shows all the evenly spaced n-alkanes present in diesel fuel, with the branched and cyclic alkanes appearing in between these evenly spaced peaks.

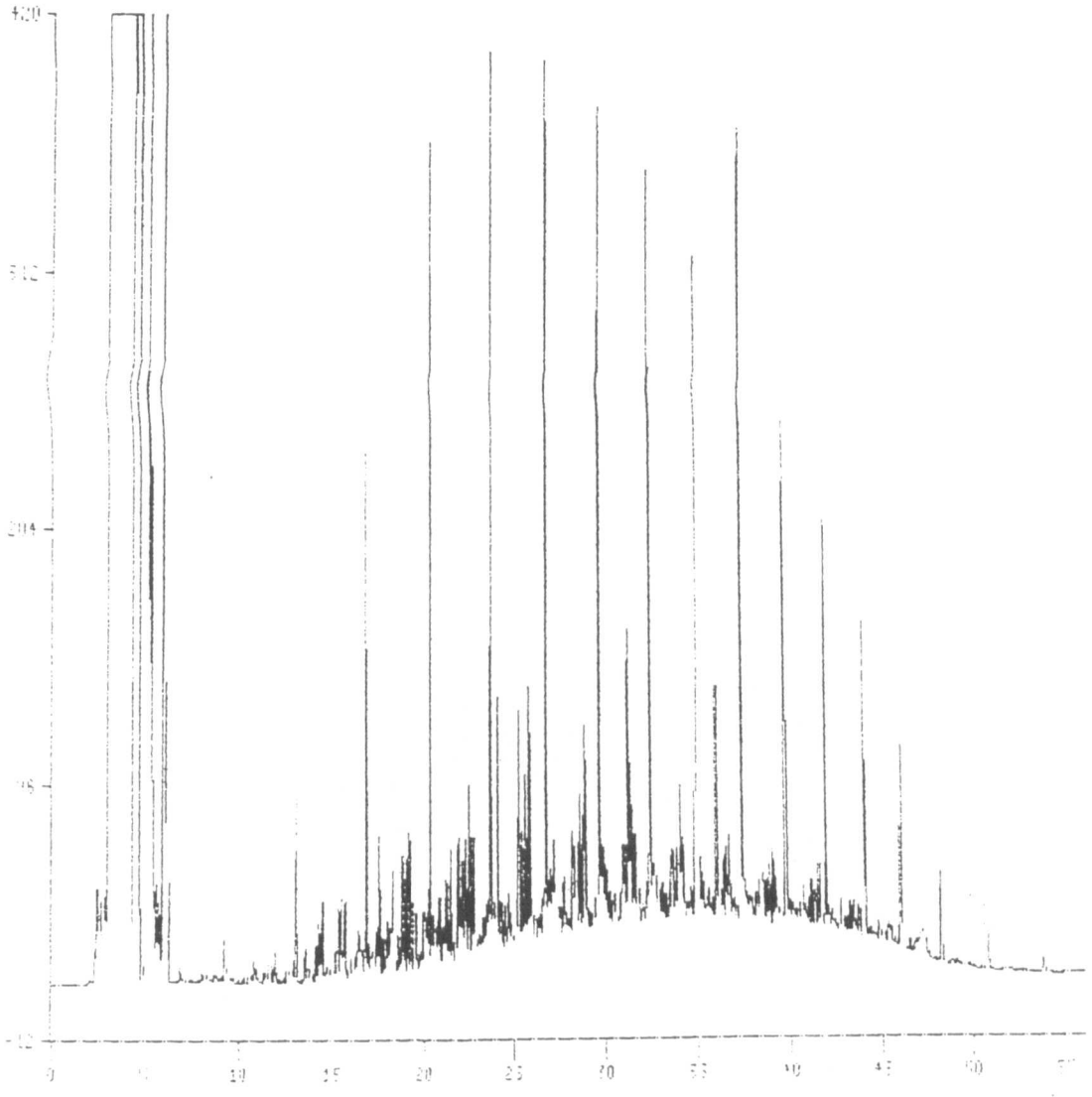


Figure 4.5.1.1. GC-FID chromatogram of alkane fraction of diesel fuel.

Figure 4.5.1.2 illustrates the GC-FID chromatogram of hydrocarbons eluted with dichloromethane from the silica column. This fraction contains aromatic compounds such as mono-, di- and polyaromatic hydrocarbons (PAHs). The peaks appear near the start of the chromatogram and lessen as the time increases. This result was expected, as the majority of aromatic compounds found in diesel fuel are mono- and diaromatic hydrocarbons, which elute at the early to middle stages of the GC run time (see Figure 4.3.5). The fingerprint I obtained for the aromatic fraction was similar to the results published by Šepič *et al* (1996) for diesel fuel.

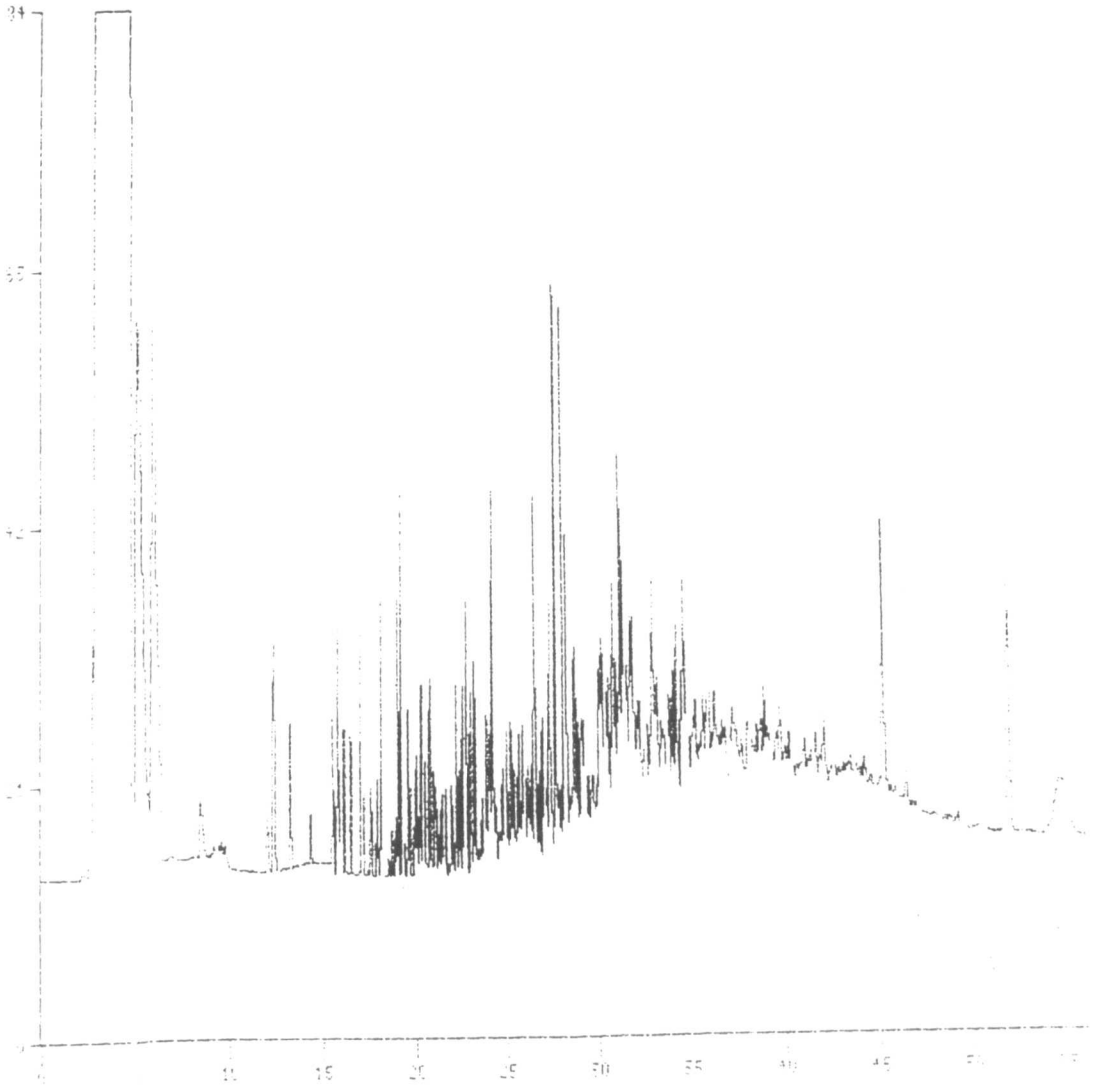


Figure 4.5.1.2 GC - FID chromatogram of aromatic fraction of diesel fuel.

Unfortunately, the column packing partially dried out before I could run chloroform:methanol (1/1 v/v) to collect the polar fraction.

Although the silica column method does separate diesel fuel into its relevant fractions successfully, the method itself is extremely time consuming and difficult to perform. The column method was not suited to routine analysis of numerous samples but it did provide information on the distribution of compounds in both the alkane and aromatic fractions.

GC-FID and GC-MS analysis provided a huge amount of information on the composition of diesel fuel which was an integral part of this study. The majority of hydrocarbons present were identified which allowed the distribution of hydrocarbon classes in diesel fuel to be calculated. The information gained on diesel fuel composition allowed diesel fuel biodegradation in the soil to be followed and provided information on the type of diesel fuel components that were being degraded. In addition, in depth analysis of the pure diesel fuel product and the volatile diesel fuel fraction by GC-FID and GC-MS, added greatly to the lack of readily available information on the composition of petroleum products.

# CHAPTER FIVE

## BEHAVIOUR OF DIESEL FUEL IN THE SOIL

When considering the overall effect of contamination on a soil system, change to the soil physical as well as biological characteristics must be investigated. The behaviour of diesel fuel on entering a soil system was investigated and the effect diesel fuel had on soil physical properties ascertained.

## 5.1 Influence on Soil Water Holding Capacity

Soil water holding capacity is the total amount of water a soil can absorb without draining. The amount of water a soil can hold is dependent on soil physical properties that influence soil structure. The most important factors influencing soil water holding capacity are the amount of organic matter present and the silt and clay content. It is widely known that the higher the organic matter and fine particle content of the soil, the larger the volume of water that soil can hold. This statement was verified by comparing the soil water holding capacities of two soils with differing textural properties (Section 2.2.1.1). Barassie soil, which has been classed as a sand, has an organic matter content (LOI % value) of 5.7% and a water holding capacity of 41.6%. Darvel soil, which is in the sandy clay loam class, contains 9.1% organic matter (LOI % value) and has a water holding capacity of 54.3%. These values clearly show the influence that the physical

characteristics of soil haveon the soil water holding capacity of soil. It was therefore concluded that addition of organic material, in the form of diesel fuel hydrocarbons, may influence the water holding capacity of the soil. This theory was investigated by contaminating soil with diesel fuel to produce 100g of 50g diesel $kg^{-1}$ soil and the water holding capacity of the contaminated soil compared to the uncontaminated soil. The results showed an increase of 13.7% in soil water holding capacity of soil contaminated with diesel fuel compared to uncontaminated soil.

Few authors have mentioned an increase in water holding capacity when investigating soils contaminated with petroleum hydrocarbons. In fact, Li *et al* (1997) noted that contamination did not change the saturation percentage of soil. These observations were made on soils containing 'aged' hydrocarbon residues however. By using freshly contaminated soil, an unrealistic situation for comparison has been set up. It was therefore decided that the soil samples, planted with Meadow mix, collected from the final greenhouse trial (Section 2.6.3.4) after 4 months would be tested to provide a more realistic estimate of the influence of residual diesel fuel contamination on soil water holding capacity.

The soil water holding capacity of Meadow mix planted and unplanted, uncontaminated soil and planted and unplanted diesel fuel contaminated soil at all levels (5g, 10g and 15g diesel $kg^{-1}$ soil) was tested. Table 5.1.1 shows the percentage soil water holding capacity of the Meadow mix soil samples.

| Treatment | % water holding capacity |
|---|---|
| Uncontaminated, no plants | 36.7 |
| Uncontaminated, plants | 36.7 |
| 5g diesel $kg^{-1}$, no plants | 39.4 |
| 5g diesel $kg^{-1}$, plants | 41.1 |
| 10g diesel $kg^{-1}$, no plants | 42.6 |
| 10g diesel $kg^{-1}$, plants | 42.3 |
| 15g diesel $kg^{-1}$, no plants | 40.9 |
| 15g diesel $kg^{-1}$, no plants | 42.6 |

Diesel residues ranged from <1%, 1.89% and 3.39% in 5g, 10g and 15g diesel kg-1 soil under planting.

Table 5.1.1. % soil water holding capacity of aged diesel fuel contaminated soil planted with Meadow mix, n = 1.

Little difference was found between the uncontaminated soil and the uncontaminated soil planted with Meadow mix. This was first thought of as unusual as plants produce exudates which increase the organic carbon content of the soil. You would therefore expect an increase in soil water holding capacity of planted soils compared to unplanted soils. However, the plants had only been growing for 4 months, which may not have been enough time to influence the organic carbon content of the surrounding soil. Also, the sample was taken from the bulk soil and not the rhizosphere soil so any organic matter added to the soil surrounding the roots would be diluted in the bulk soil.

An increase was found between the contaminated and uncontaminated soil samples. Water holding capacity was increased by an average 4.8% (range 2.7–5.9%), in the diesel fuel contaminated soils tested. This small increase is due to the residual diesel fuel in the soil after 4 months treatment and the influence of microorganisms and plant growth on soil organic carbon content.

The influence of diesel fuel on soil water holding capacity obviously differed with the age of the product. Freshly contaminated soil showed a large increase in the volume of water held by the soil. This may be due to the entire diesel fuel fraction being present which means the more hydrophilic aromatic compounds were still present which would attract water and increase the water holding capacity of the soil. When the contaminated soil ages however, these hydrophilic compounds are degraded readily, leaving behind more hydrophobic residues. This more realistic scenario showed as fuel ages and degrades in the soil, less of an impact was observed on soil water holding capacity but an increase in water repellence was observed.

## 5.2 Occurrence of Repellent Soil

Hydrophobic mineral soils have been observed in various environments over the years, including burned forest soils, citrus groves, sandy soils of Australia and New Zealand and cultivated soils (King, 1981, Roy and McGill, 1997). Soil water repellency is attributed to the presence of hydrophobic organic substances forming a coating over the surface of normally hydrophilic soil particles. It was always thought that soil water repellency was attributed solely to the presence of organic material of unusual composition however, a theory that is gaining wider acceptance is that the molecular

orientation of organic matter coatings on soil particles may be determining whether soils repel or adsorb water (Ma'Shum and Farmer, 1995). According to this theory, amphiphilic or surface active molecules such as humic, fulvic and fatty acids, can impart water repellant character to a soil when their hydrophilic ends are oriented towards soil particle surfaces and their hydrophobic ends extend toward the open pore space (Anderson *et al.*, 1995). The reverse arrangement is thought to prevail in soils that adsorb water normally where the hydrophilic functional groups interact mostly with the soil solution whereas the hydrophobic functional groups interact mostly with themselves and with the surface of organic matter coatings. Changes in the interfacial conformation of amphiphilic organic molecules on the surface of soil particles has been postulated to occur during soil drying and following treatment with certain non polar organic solvents (Ma'Shum and Farmer, 1995).

Soils contaminated with hydrophobic materials, such as hydrocarbon residues have the potential to develop water repellency (Li *et al.*, 1997) which may result in impaired plant-soil water relations (Brown *et al.*, 1982). The formation of repellant soil characteristics due to diesel fuel contamination was investigated using the method of King (1981) as described in Section 2.4.3.

Two sets of samples were tested for development of water repellency, one with a low diesel fuel content (ranging from <1–3.45% diesel fuel residue) and one with a high diesel fuel content (approximately 7.5%). Firstly, one set of samples collected from the pot experiment described in Section 2.6.3.4 was investigated. These samples contained both diesel fuel contaminated and uncontaminated soil and both Westerwold's ryegrass planted and unplanted soil. The soils were packed into perspex cells as described in Section 2.4.3 and the water droplet (WD) test performed. The results from this experiment are given in Table 5.2.1 with a repellency rating given based on the interpretation guidelines for water repellency rating of soils by King (1981).

| | Water Droplet (WD) Test | | |
|---|---|---|---|
| Sample | Infiltration rate ($s^{-1}$) | Rating | Explanation |
| Uncontaminated, unplanted | <1, 1, <1 | 1 | Not significant |
| Uncontaminated, planted | 3, 3, 3 | 2 | Very low |
| 5g diesel $kg^{-1}$ soil, planted | 16, 13, 14 | 3 | Low |
| 10g diesel $kg^{-1}$ soil, planted | 62, 62, 54 | 5 | Low |
| 15g diesel $kg^{-1}$ soil, planted | 158, 146, 137, 153 | 6 | Moderate |

5g, 10g and 15g diesel kg-1 soil residual values were <1% (ND), 2.12% and 3.45%.

Table 5.2.1 Repellency rating determined by the Water Droplet Test for contaminated and uncontaminated, planted and unplanted soils.

The results show that uncontaminated soil has a very low rating providing no significant water repellence but by growing plants in uncontaminated soil, the repellency rating goes up one point. This slight increase in repellency was probably caused by plant exudates and increased microbial biomass in planted soil as opposed to unplanted soil. This observation has been noted by several authors, with repellency being formed by decomposing plant material and exudates, under certain stands of plant species such as Eucalyptus, pine and yucca (King, 1981). The addition of diesel fuel decreased the infiltration rate of water into soil significantly. Increase in diesel fuel addition caused a subsequent increase in the repellency of soil. As these soils have been remediated for 4 months, the diesel fuel content is small. However, the diesel fuel that remains in the soil is in the form of more resistant, hydrophobic residues which is reflected by the soil repellency results.

The second set of samples tested for repellency were samples collected from the simulated diesel fuel spill as described in Section 2.6.2. Samples were collected from the corner positions and the middle of each planted tray after the experiment had ended. Each tray had been contaminated with 100 ml of diesel fuel, spilled on the surface of each planted tray to simulate an aboveground diesel fuel spill. Soil samples were prepared and the water droplet (WD) and molarity of ethanol (MED) test performed as described in Section 2.4.3. The results given in Table 5.2.2 for the four plant species

tested using both WD and MED tests have again been given a repellency rating as described by King (1981).

| | | Test | |
|---|---|---|---|
| Plant type | position | WD rating | MED rating |
| Westerwold's ryegrass | Middle * | | 12 |
| | Corner 1 | | 12 |
| | Corner 2 | | 12 |
| Red clover | Middle * | | 12 |
| | Corner 1 | N/A | 10 |
| | Corner 2 | | 11 |
| Chewing's fescue | Middle * | | 12 |
| | Corner 1 | | 12 |
| | Corner 2 | | 12 |
| Meadow mix | Middle * | | 12 |
| | Corner 1 | | 12 |
| | Corner 2 | | 12 |

N/A not applicable. MED ratings > 9 – 11 indicate severe conditions and >11 indicate very severe repellent conditions (from the Interpretation guidelines for water repellence rating of soils, modified by King, 1981).

Approximately 7.5% diesel fuel added per tray – higher levels of diesel fuel at middle positions than corner positions. * point source of diesel fuel contamination.

Table 5.2.2 Repellency rating of soils under four different planting treatments determined by the Water Droplet (WD) Test and Molarity of Ethanol (MED) Test.

The water droplet test was initially performed on the first few samples but the infiltration rate of the water droplet was greater than 4 minutes therefore the test was abandoned. In fact, the soils used in the simulated diesel fuel spill experiment were so repellant, the water droplet placed on the soil surface stayed, perfectly formed on the soil surface for >1 hour. No infiltration of water occurred at all. This led to the molarity of ethanol (MED) test being performed on these samples. The molarity of

ethanol required to break the surface tension of the repellent soil surface in these samples was very high (>3.8 M ethanol). This gave a repellency rating of 11 or 12 for the majority of samples tested which is very severe. The extreme repellency of the soil was one of the reasons for the lack of plant regrowth in the simulated diesel oil spill experiment (Section 2.6.2). The soil could not retain enough water and the plants could not obtain adequate water to begin regrowing. An example of the effect of the simulated diesel fuel spill on plant growth is illustrated in Figure 5.2.3. Red clover plants before the spill, immediately after and 2 months after being cut back and allowed to regrow show how badly the plants were affected by the fuel spill and that regrowth was seen only around the edges of the spill.

Figure 5.2.3. Simulated diesel fuel spill on Red clover planted soil. Example of Red clover plants before the spill (top), immediately after the spill (middle) and 2 months after being cut back (bottom).

The introduction of diesel fuel into the soil clearly has an effect on the soil's ability to hold water and maintain appropriate moisture conditions for optimal plant growth. This effect must be considered as an additional cause of poor plant performance in diesel fuel contaminated soils. Although the residual diesel fuel content of the soil may be low enough not to cause any phytotoxic effects on plant growth, the influence these residues have on the development of repellant soil may cause reduced plant yield and unhealthy plants.

## 5.3 Downward Migration of Diesel Fuel in Soil

Another important factor on diesel fuel entering a soil system is its subsequent lateral and vertical movement in the soil profile. Rainfall can encourage contaminant leaching through the soil profile which can lead to surface water and groundwater contamination. Diesel fuel, due to its hydrophobic character, should not move far in the soil profile. However, this statement is highly dependent on the characteristics of the soil the diesel fuel is contaminating and whether diesel fuel is contaminating from an aboveground or underground source. The difference in the surface and subsurface soil characteristics allow diesel fuel, on entering these systems, to behave very differently.

The presence of other contaminants commonly found with diesel fuel may also influence its migratory behaviour. The results discussed below include work carried out by Professor Keiji Gamoh and myself on ethanol additive fuels. Although this work is not directly related to the thesis subject matter, it is extremely pertinent to this chapter on the behaviour of diesel fuel in the soil. Diesel fuel consists of many additives which improve the performance of the fuel. Addition of oxygenates, such as ethanol and methyl-tertiary-butyl ether (MtBE) to fuels to reduce vehicular emissions is common practice. At present, only MtBE and similar ethers are used in the U.K. (Environment Agency, 1999). These additives, although beneficial in reducing atmospheric pollution, may in fact increase groundwater contamination due to cosolvency of petroleum hydrocarbons and by provision of a preferential substrate for microbial utilisation (personal communication). Because these additives are present in diesel fuel, their influence on contaminant movement should not be discounted. The rest of this chapter therefore discusses diesel fuel movement in the soil profile and how the inclusion of additives, such as ethanol, influence this movement.

### 5.3.1 Vertical Movement of Diesel Fuel Through a Soil Column

Vertical movement of diesel fuel from an above ground spillage was investigated using a 1 m soil packed column which was leached with water as described in Section 2.4.3. The distribution of diesel fuel down the soil profile was determined by GC-FID analysis of extracted diesel fuel from 10 cm sections of the column.

The results from the 1 m soil columns suggested that the downward migration of diesel fuel in the soil profile was enhanced by ethanol addition. Figure 5.3.1 shows the percentage distribution of diesel fuel in 1 m soil columns leached with 10 l of water, where only diesel fuel has been added and where diesel fuel with 5 % ethanol has been added. Little movement of diesel fuel was observed in the diesel fuel only column with diesel fuel distribution decreasing evenly from the top of the column (Section 1) to a depth of 30 cm (Section 3). Negligible amounts of diesel fuel were found below this depth.

Diesel fuel with 5 % ethanol, on the other hand, was seen to migrate to a depth of 40 cm and the pattern of diesel fuel distribution in the soil profile was very different from that seen in the diesel fuel only column. Again, the largest percentage of diesel fuel was found in the top 10 cm (Section 1). The percentage of diesel fuel in Section 2 and 3 decreased, as before, to 22.5 % and 13.8 %. However, the percentage of diesel fuel in Section 4 rose to 24.1 % of the total diesel fuel added. No diesel fuel was found in sections below this depth.

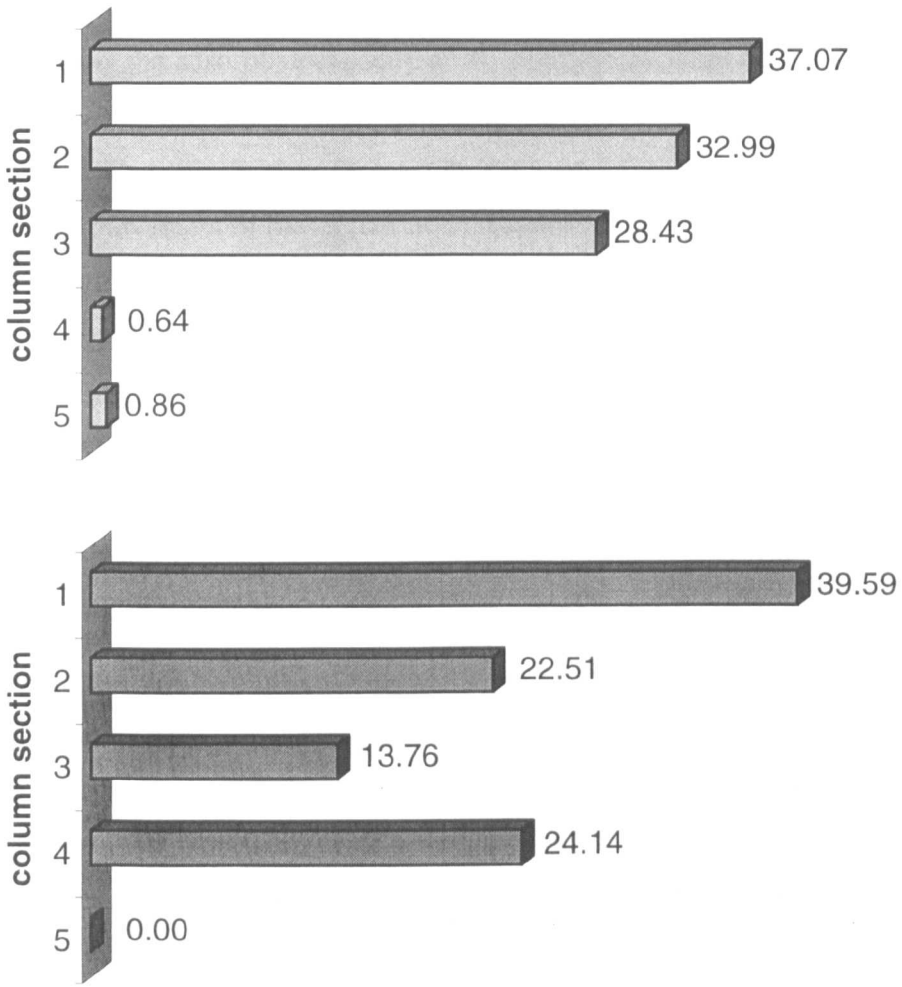



Figure 5.3.1.1. % distribution of diesel in 1m soil columns for diesel only (top) and diesel plus 5% ethanol (bottom). Only sections 1-5 are shown as no diesel was found in the lower sections of the column (sections 6-10).

The pattern of diesel fuel distribution in the soil profile clearly showed the enhancement of diesel fuel movement through the soil column due to ethanol addition. Gas chromatographic (GC) analysis of diesel fuel extracted from each soil section resulted in GC traces whose pattern of hydrocarbon distribution were very similar. There was no indication that specific components or fractions of the diesel fuel were being mobilised and moving further down the soil profile than other components or fractions of the diesel fuel. This implies that the effect ethanol has on enhancing diesel fuel movement in the soil may be due to the 'wetting' effect of ethanol on the soil components allowing mass movement of diesel fuel. As previously discussed, ethanol

can break the surface tension of repellent soils allowing infiltration. Ethanol present within diesel fuel would therefore enhance infiltration of diesel fuel into the soil profile by lessening the adsorptive capacity of the hydrophobic sites of soil components such as organic matter and allow diesel fuel to move further down the soil profile. It can be concluded that by further increasing the concentration of ethanol in diesel fuel, increased movement of diesel fuel into the subsurface would be observed.

To quantitate what sort of concentrations of ethanol were required to enhance movement of diesel fuel into the subsurface a more thorough investigation was undertaken.

### 5.3.2 Movement of Petroleum Hydrocarbons Through A Soil Column

The effect of soil components on contaminant movement was investigated by a novel method using HPLC packed columns carried out by Professor Keiji Gamoh and as described in Section 2.4.4. This allowed the retentive behaviour of different soil components on petroleum hydrocarbons to be studied. Conclusions could then be drawn from these results on the behaviour of petroleum hydrocarbon movement in surface soils and subsurface soils.

Initially, a soil (Barassie series : 40.5% sand, 21.5% silt and 25.0% clay) with an average organic matter content (16.7%) was assessed to determine if aromatic hydrocarbons, commonly found in diesel fuel, could move through a soil column and what levels of ethanol addition enhanced this movement. Aqueous ethanol concentrations ranging from 0 to 50% ethanol were used as the mobile phase in this column (G-01). An aqueous ethanol concentration of above 10% was required for any movement of hydrocarbons in column G-01 to occur. At 25% aqueous ethanol as the mobile phase, the lighter, more soluble aromatic hydrocarbons eluted slowly from the column whereas the larger aromatics (1,5 dimethyl naphthalene, phenanthrene, anthracene, pyrene and chrysene) were retained on the column. Toluene, naphthalene and 1-ethyl naphthalene had retention times of 2.49, 9.86 and 28.00 minutes respectively. The length of time taken for these hydrocarbons to come off the G-01 column suggests the soil packing has hydrophobic sites capable of retaining aromatic hydrocarbons but the adsorption of the lighter hydrocarbons on these sites can be overcome by 25% aqueous ethanol. When the aqueous ethanol concentration of the mobile phase was further raised to 50% ethanol, all the aromatic hydrocarbons added

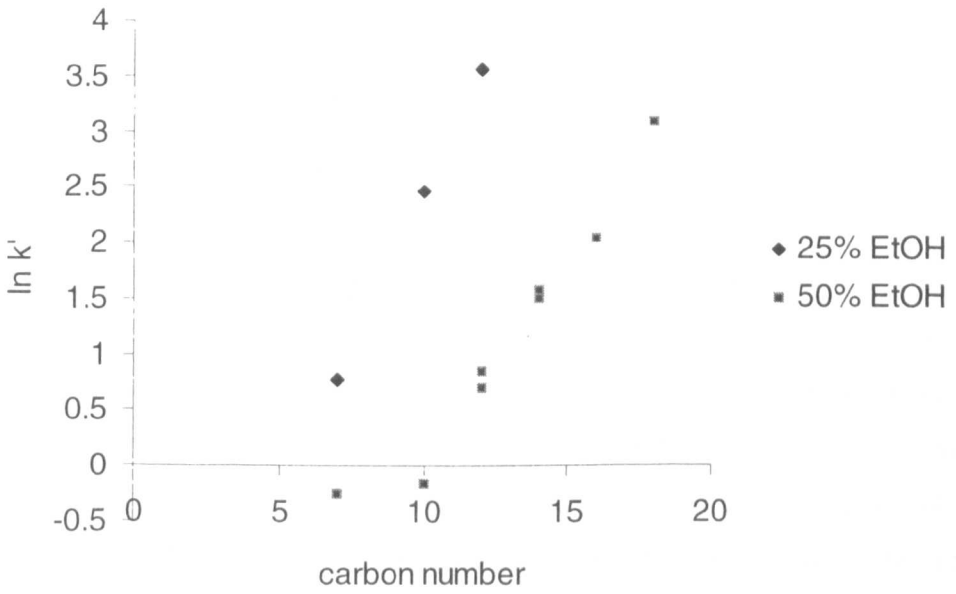
could be eluted from the column and the time taken for elution was much quicker than with 25% aqueous ethanol. This implies the retentive behaviour of the soil column was lessened by increasing concentrations of ethanol. This is supported by the observations made in the previous section and the MED test results whereby increasing ethanol concentration breaks the surface tension of repellent soil allowing penetration. Table 5.3.2.1 shows the retention time ($t_R$) and capacity factor (k') of hydrocarbons on the Barassie soil column (G-01) using different ethanol mobile phase concentrations. Capacity factors are included as although a peak can be identified by its retention time, this varies with column length and mobile phase flow rate (Lindsay, 1992). The same columns lengths are used throughout this experiment but the mobile phase flow rates differ. By using capacity factors instead of retention times, a direct comparison can be drawn between different column results.

| | Mobile phase | | | |
|---|---|---|---|---|
| | 25% EtOH | | 50% EtOH | |
| Hydrocarbon | $t_R$ | k' | $t_R$ | k' |
| Acetone, | $t_o = 0.78$ | | $t_o = 0.78$ | |
| toluene | 2.49 | 2.19 | 1.40 | 0.79 |
| Naphthalene | 9.86 | 11.64 | 1.45 | 0.86 |
| 1 ethyl naphthalene | 28.00 | 34.89 | 2.35 | 2.01 |
| 1, 5 dimethyl naphthalene | - | - | 2.61 | 2.35 |
| phenanthrene | - | - | 4.28 | 4.49 |
| anthracene | - | - | 4.55 | 4.83 |
| pyrene | - | - | 6.69 | 7.58 |
| chrysene | | | 18.00 | 22.08 |

Capacity factor $k' = \frac{t_R - t_o}{t_o}$ where $t_R$ is the analyte peak retention time and $t_o$ is the peak of the unretained solvent front

Table 5.3.2.1 Retention time ($t_R$) and capacity factor (k') of hydrocarbons on the Barassie soil column (G-01) using different ethanol mobile phase concentrations.

Figure 5.3.2.2 illustrates the information given in Table 5.3.2.1 on the retentive behaviour of the Barassie soil column (G-01). The capacity factors are quite large so the natural log (ln) of each capacity factor (k') has been taken to bring the ln k' values to between 1 and 10, which is the normal range for k' values (Lindsay, 1992). Using 25% aqueous ethanol as the mobile phase eluted only the smaller aromatic hydrocarbons (toluene, naphthalene and 1 ethyl naphthelene), the larger aromatics (1,5 dimethyl naphthalene, phenanthrene, anthracene, pyrene and chrysene) were retained on the column. By increasing the aqueous ethanol concentration to 50%, all the aromatic hydrocarbons added to the column were eluted and more quickly than when 25% aqueous ethanol was used as the mobile phase.




Carbon number relates to : C7 – toluene, C10 – naphthalene, C12 – 1 ethyl naphthalene and 1, 5 dimethyl naphthalene, C14 – phenanthrene and anthracene, C16 – pyrene and C18 – chrysene.

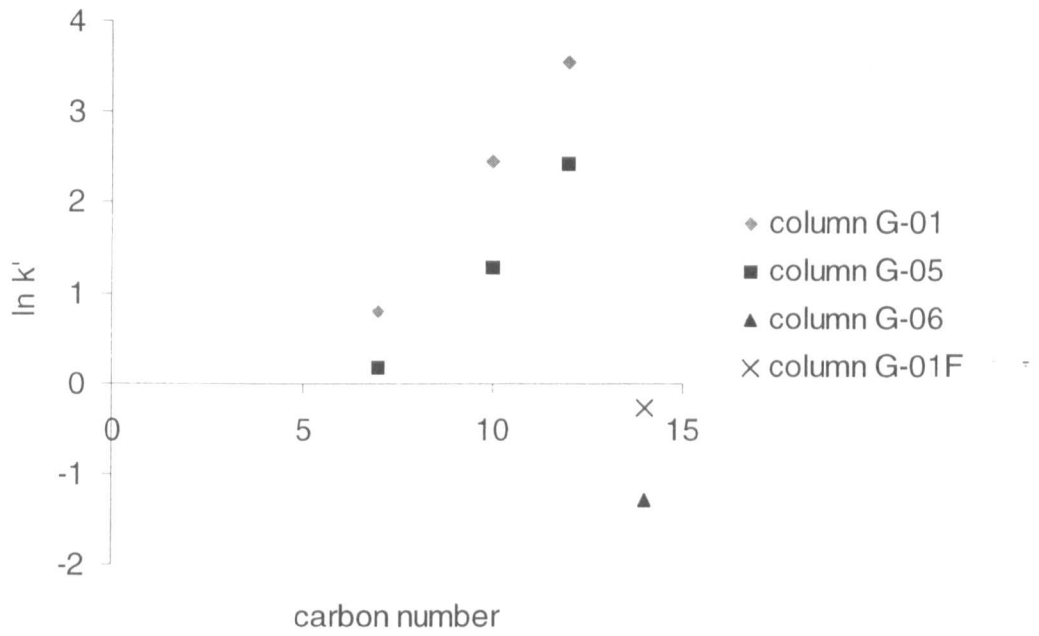
Figure 5.3.2.2 Comparison of retentive behaviour of G-01 soil column using different mobile phase concentrations of ethanol.

Increasing ln k' values indicate increased retention of hydrocarbons due to increased adsorption to soil sites. The 25% aqueous ethanol results lie above the 50% aqueous ethanol results as the hydrocarbons were retained longer on the column using 25% aqueous ethanol as the mobile phase than they were using 50% aqueous ethanol mobile phase.

It can be clearly seen that ethanol enhances hydrocarbon mobility through soil columns and increasing the ethanol concentration, in turn, increases the mobility of the hydrocarbons. The aromatic hydrocarbons were strongly held in the soil by adsorption to soil components and could not be mobilised by water or up to 10% ethanol as the mobile phase. At 25% aqueous ethanol, only the lighter aromatic compounds (toluene, naphthalene and 1 ethyl naphthalene) were mobilised. As the ethanol concentration increased to 50% however, all the aromatic hydrocarbons added to the column were mobilised and eluted from the column.

To determine what effect various soil components had on the adsorption of aromatic hydrocarbons a series of soil columns were prepared with varying ratios of organic matter, sand, silt and clay.

Column G-05 was prepared from the subsurface soil of the Barassie series used in column G-01. This subsurface soil consisted of a very large proportion of sand (approximately 81%) with low organic matter (approximately 5%), silt (approximately 10%) and clay (approximately 7%). Column G-01F was packed from the Barassie soil used in column G-01 that had been ignited for 6 hours in a 500 °C furnace to remove all the organic matter. Column G-06 is a manufactured sand sample (Fisher Scientific Chemicals, 40-100 mesh) which provided a measure for the mineral fraction of soil and finally, columns G-07 and G-08 which are packed from silica of varying particle size. Column G-07 contains particles in the fine sand to silt range and column G-08 contains only silt-sized particles. These columns were included to investigate the influence of particle size distribution on hydrocarbon adsorption. Figure 5.3.2.3 shows the trend in retention of hydrocarbons on various soil columns. 25% aqueous ethanol was used as the mobile phase as it was found to enhance hydrocarbon mobility in the original soil column (G-01) and is an important environmental value as fuel in Brazil and many states in the U. S. A. contain 24% ethanol (Massad *et al.*, 1993).




Carbon number relates to : C7 – toluene, C10 – naphthalene, C12 – 1 ethyl naphthalene and C14 – phenanthrene.

Figure 5.3.2.3 shows the trend in retention of hydrocarbons on various soil columns.

The original soil column (G-01) which contained average organic matter, sand, silt and clay values had the highest adsorptive capacity for aromatic hydrocarbons which is shown in Figure 5.3.2.3 by the trend in retention lying above all the other column values. The sandy subsoil, which contained high levels of sand but with low organic matter, silt and clay content had the next highest adsorptive capacity. The presence of organic matter was the most important factor in the adsorption of petroleum hydrocarbons because when all the organic matter was removed, as in column G-01F, the retentive behaviour of the soil column was drastically reduced. Column G-01F was basically the same as column G-01 except no organic matter was present. This allowed the retentive behaviour of the column to be attributed, almost entirely, to the presence of organic matter. However, some retention of the larger aromatic hydrocarbons, such as phenanthrene, was observed which suggests other factors are involved in retaining hydrocarbons on soil. Column G-06, which is a manufactured sand of narrow particle size range, also showed signs of retaining the larger aromatic hydrocarbons suggesting sand particles themselves have some retentive behaviour.

Two silica columns were used to distinguish between the influence of particle size on hydrocarbon retention. The results are given in Table 5.3.2.4. The silica used as packing material in these columns (G-07 and G-08) had no surface coatings hence they had little adsorptive capacity. Because of this water was used as the mobile phase.

| | Mobile phase : water | | | |
|---|---|---|---|---|
| | Column G-07 | | Column G-08 | |
| Hydrocarbon | $t_R$ | k' | $t_R$ | k' |
| Acetone, | $t_0$ = 1.47 | | $t_0$ = 2.86 | |
| toluene | 2.10 | 0.42 | 4.81 | 0.68 |
| Naphthalene | 3.81 | 1.19 | 7.83 | 1.73 |
| 1 ethyl naphthalene | 5.97 | 2.43 | 15.72 | 4.49 |
| phenanthrene | 6.17 | 2.54 | 18.95 | 5.62 |

Table 5.3.2.4 Retention times and capacity factors for petroleum hydrocarbons on silica columns with varying particle size ranges.

Column G-07 had a larger particle size distribution (diameters ranging from 0.0035 mm to 0.70 mm particles) than column G-08 (0.0015 mm particle size diameter). This was reflected by the capacity factors of the aromatic hydrocarbons on each column. The time taken for elution of each hydrocarbon was almost double on column G-08 compared with column G-07. These results show that mineral particles such as sand, may influence the adsorption of petroleum hydrocarbons even when they are not coated with organic matter or other active functional groupings. Generally, fine particles and organic matter are responsible for the adsorption of pollutants to the soil matrix. However, the results obtained during this investigation using various sandy soils and surrogate sand columns indicate other factors may be involved in the adsorption of petroleum hydrocarbons. Other authors have found that sandy soil can bind hydrocarbons adsorptively although neither silty material nor significant amounts of organic matter was present. Loser *et al* (1999) proposed that soil particles are covered with micropores, which enlarge the soil surface in comparison with the macroscopic surface area. This microporosity is the reason for hydrocarbons being

more strongly adsorbed to sandy soil than expected (Loser *et al.*, 1999). This theory seems a likely explanation for the slightly retentive behaviour of both the silica columns tested (columns G-07 and G-08) and the sandy soil column with no organic matter present (column G-01F).

These results suggest a greater possibility of groundwater contamination by aromatic hydrocarbons in ethanol additive diesel fuel spills occurring from both underground storage tanks and above ground spills. Surface soil components such as soil organic matter, as well as silt and clay, play an extremely important role in retaining petroleum hydrocarbons near the soil surface. However, ethanol was shown to enhance movement of both individual petroleum hydrocarbons and diesel fuel by lessening the adsorptive capacity of the surface soil components. If these petroleum hydrocarbons leak at the subsurface level, the low organic matter content and the lower silt and clay contents allow hydrocarbon migration to occur more freely. In addition, an underground petroleum hydrocarbon spill, which would normally migrate and contaminate groundwater quite readily, would be further enhanced by the addition of ethanol.

Having demonstrated the behaviour of diesel fuel on entering a soil system and the influence it has on the physical properties of soil I turn to the main focus of this study which considers whether phytoremediation was a viable option for the clean up of diesel fuel contaminated land and whether growing plants enhanced the breakdown of diesel fuel in contaminated soil. The remaining results chapters therefore investigate the effect of diesel fuel on plants at various stages of growth and development in an attempt to determine which plant species are most successful in diesel fuel contaminated soil. The most successful plant species from the initial screening studies were then used in large scale greenhouse trials to: examine the effect of diesel fuel on plant growth and development; investigate the influence of diesel fuel and plant growth on soil enzyme activity and to quantify the degradation of diesel fuel in planted and unplanted soil.

The data collected from these plant trials lead to a better understanding of the changes occurring in soil due to the addition of diesel fuel and growing plants and gave a clearer picture of the effect of phytoremediation on the plant-soil-microorganism-contaminant interactions.

# CHAPTER SIX

## EFFECT OF DIESEL FUEL ON PLANT GROWTH AND DEVELOPMENT : **EFFECT ON THE SEED**

## 6.1 Plant Screening

A selection of plant species were screened for their ability to germinate in soil contaminated with diesel fuel as described in Section 2.6.1.1. The response varied with diesel fuel concentration and plant species. At relatively low levels of diesel fuel contamination, delayed seed emergence and reduced germination were observed for the majority of plant species investigated (Table 6.1.1).

The ability of seeds to germinate in diesel fuel contaminated soil ranged from completely unaffected (e.g. Oil seed rape, Flax cv. Elise) to completely susceptible (e.g. Couch grass, Rough meadow grass). Some plant species were affected initially, resulting in a delayed seed emergence. These species had low percentage germination rates at 7 days but germination increased significantly by 14 days. This effect is clearly illustrated by Common vetch, with percentage germination being 64%, 26% and 12% at 7 days in 0g, 25g and 50g diesel $kg^{-1}$ soil respectively. By day 14, the germination rate had risen to 64%, 60% and 42% in 0g, 25g and 50g diesel $kg^{-1}$ soil. This trend is also seen with Red clover, White clover and Little yellow trefoil.

| Plant species | | Germination rate % Diesel concentration g kg $^{-1}$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | 7 days | | | 14 days | | |
| Common Name | Latin Name | 0 | 25 | 50 | 0 | 25 | 50 |
| Grasses | | | | | | | |
| Cocksfoot | *Dactylis glomerata* | 47 | 17 | 0 | 53 | 20 | 0 |
| Creeping bent [b] | *Agrostis stolonifera* | 20 | 20 | 3 | 30 | 38 | 5 |
| Highland bent [b] | *Agrostis castellana* | 85 | 45 | 45 | 85 | 50 | 46 |
| Common bent [b] | *Agrostis capillaris* | 96 | 32 | 18 | 98 | 34 | 20 |
| Black grass | *Alopecurus myosuroides* | 37 | 23 | 3 | 60 | 30 | 3 |
| Red grass | *Alopecurus pratensis* | 20 | 13 | 0 | 17 | 13 | 0 |
| Sweet vernal grass [b] | *Anthoxanthum odoratum* | 90 | 55 | 10 | 90 | 60 | 15 |
| Rough meadow grass [b] | *Poa trivialis* | 50 | 6 | 0 | 55 | 10 | 0 |
| Westerwold's ryegrass | *Lolium multiflorum* | 84 | 72 | 46 | 78 | 64 | 50 |
| Couch grass | *Agropyron repens* | 17 | 0 | 0 | 20 | 0 | 0 |
| Sheep's fescue | *Festuca ovina* | 64 | 26 | 6 | 58 | 38 | 24 |
| Strong creeping red fescue | *Festuca rubra ssp. rubra* | 88 | 68 | 20 | 82 | 88 | 40 |
| Chewing's fescue | *Festuca rubra ssp. commutata* | 56 | 38 | 18 | 48 | 50 | 20 |
| Annual canary grass | *Phalaris canariensis* | 84 | 70 | 14 | 72 | 60 | 10 |

| Plant species | | Germination rate % | | | | | |
|---|---|---|---|---|---|---|---|
| | | Diesel concentration g kg $^{-1}$ | | | | | |
| | | 7 days | | | 14 days | | |
| Common Name | Latin Name | 0 | 25 | 50 | 0 | 25 | 50 |
| Herbs and legumes | | | | | | | |
| Black medick | *Medicago lupulina* | 28 | 24 | 34 | 20 | 20 | 24 |
| Fodder burnet | *Sanguisorba minor ssp. muricata* | 28 | 8 | 10 | 18 | 16 | 2 |
| Common vetch | *Vicia sativa* | 64 | 26 | 12 | 64 | 60 | 42 |
| Red clover | *Trifolium pratense* | 44 | 44 | 32 | 56 | 56 | 40 |
| White clover | *Trifolium album* | 42 | 10 | 8 | 68 | 36 | 12 |
| Little yellow trefoil | *Trifolium dubium* | 40 | 0 | 4 | 40 | 36 | 18 |
| Lucerne | *Medicago sativa* | 80 | 88 | 78 | 74 | 84 | 66 |
| Commercial crops | | | | | | | |
| Oil seed rape cv. Rocket | *Brassica napus var. olifera* | 90 | 100 | 95 | 100 | 100 | 95 |
| Oil seed rape cv. Martina | *Brassica napus var. olifera* | 96 | 100 | 95 | 100 | 100 | 95 |
| Flax cv. Viking | *Linum usitatissimum* | 72 | 66 | 36 | 74 | 66 | 38 |
| Flax cv. Elise | *Linum usitatissimum* | 94 | 94 | 94 | 94 | 96 | 98 |

Table 6.1.1 Germination rates (%)[a] of plant species exposed to varying concentrations of diesel fuel, measured 7 and 14 days after planting at 20 °C.

[a] 100 % germination rate equals every seed planted germinating and producing a sizeable shoot (> 2 mm), [b] these seed species were planted at a sow rate of 100 per replicate. The remaining seed species were planted 25 seeds per replicate.

The inhibition of germination generally increased with increasing diesel fuel concentration with some species showing an almost linear decrease in germination with increasing diesel fuel concentration (e.g. Black grass, White Clover). The ability to tolerate diesel fuel contamination whilst germinating was not species specific as members of the same plant family showed differential sensitivity to diesel fuel contamination. The most apparent example of this was found in the family *Gramineae* (Grasses) with some species germinating well (e.g. Westerwold's ryegrass) whilst others would not germinate at all (e.g. Couch grass). Differences were also found within subspecies (e.g. Fescues).

The differential sensitivity of plants to hydrocarbon toxicity is well known (Crafts and Reiber, 1948; Currier, 1951; Baker, 1970; Warner *et al.*, 1983; Gauvrit and Cabanne, 1993; Chaînea *et al.*, 1997) and exploited to man's benefit. For example, members of the family *Umbelliferae* (e.g. carrots) are notably tolerant to injury by lighter oils (low molecular weight, BP range 150-275°C) whereas grasses are intolerant. This specificity allowed oils to be successfully used as post emergence herbicides in vegetable crops (Gauvrit and Cabanne, 1993). Although the toxicity of oils described in these articles apply to the oil being sprayed directly onto the plant and not applied to the soil, as in my case, many helpful parallels can be drawn.

Phytotoxicity was seen to increase with gravity through the series gasoline, kerosene, diesel fuel and heavy fuel oil, indicating that the lighter fractions, either because they are more volatile or because the compounds present are less toxic, caused less long term damage to the plants than the heavier fraction (Crafts and Rieber, 1948). In apparent contradiction to that statement, the smaller the hydrocarbon molecule, the more toxic the oil is to plants (van Overbeek and Blondeau, 1954). Highly volatile hydrocarbons, primarily those that are small and lightweight, are able to move through cell membranes easily. Depending on the nature of the chemical, this penetration can cause toxic effects, which are acute but generally short lived. Therefore, these lightweight components of oils are initially extremely toxic to plants but are too volatile to cause lasting damage.

The type of toxicity induced by petroleum hydrocarbons was also related to the molecular weight of their components, with acute toxicity being induced by low molecular weight components and chronic toxicity induced by high molecular weight components (Gauvrit and Cabanne, 1993). Acute toxicity was exhibited by gasoline

and the light ends of oils and also by benzene, toluene, xylene, cyclohexane, cyclohexene and many of their derivatives. Such toxicity is violent but non persistent because the compounds causing the toxicity are relatively volatile and soon leave the plant (Crafts and Reiber, 1948). Acute toxicity caused by the lighter fraction of diesel fuel may explain the delayed seed emergence and reduction in germination displayed by plants in the initial screening experiment. This theory was investigated further in a series of germination experiments designed to determine the effect of diesel fuel's volatile components on seed germination.

Chronic toxicity, which is initiated by high molecular weight components, results from application of diesel fuel and heavy fuel oils (Crafts and Reiber, 1948). Expression of chronic toxicity may vary over time with symptoms developing slowly. As the initial screening experiment was conducted over a short time period, the occurrence of symptoms relating to chronic toxicity was unlikely. This effect was studied in detail in a series of pot experiments that will be discussed in chapter 8.

## 6.2 Phytotoxicity of Volatile Diesel Fraction

The inhibition of seed germination by the volatile fraction of diesel fuel was investigated further in a series of germination experiments. The experiments were designed to minimise the concentration of volatile diesel fuel components in close proximity to the germinating seed.

The first experiment described in Section 2.6.1.2 involved germinating seeds in petri dishes at 8°C instead of 20°C. This would minimise the volatilisation of the low molecular weight components of diesel fuel but still allow germination to proceed. Eight plant species were chosen for comparison which were reasonably cold tolerant. The germination rate of plants at this temperature was dramatically reduced compared to germination rates at 20°C, however a noticeable increase in germination rate was again apparent for all species tested in all treatment levels compared to the germination rates of the same species in freshly contaminated soil. Measurements were made over the course of 6 weeks due to the slowed germination response. Table 6.2.1 shows the percentage germination of the eight species tested. After 6 weeks, the response of both Chewing's fescue and Strong creeping red fescue had greatly improved compared to the original screening experiment results. In freshly contaminated soil, both these plants'

percentage germination fell to 50% or below the control value in 50g diesel $kg^{-1}$ soil as shown in Table 6.1.1. When grown at 8°C, their percentage germination was the same or just slightly below the control at this treatment level. A few of the plant species, namely Highland bent, Common bent and Rough meadow grass, did not grow well at this low temperature. These species were possibly more susceptible to the cold and the germination results obtained will be biased by this factor and are therefore included with caution. Germinating seeds at a lower temperature appeared to slow volatilisation of diesel fuel components present in the petri dishes, which resulted in a less detrimental effect on germination. This observation has been noted by several authors (Bossert and Bartha, 1984; Rogers *et al.*, 1996), who came to the same conclusion.

| | Germination % | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diesel concentration g $kg^{-1}$ | | | | | | | | |
| Plant species | 2 weeks | | | 4 weeks | | | 6 weeks | | |
| | 0 | 25 | 50 | 0 | 25 | 50 | 0 | 25 | 50 |
| Highland bent | 11 | 0 | 0 | 22 | 7 | 6 | 31 | 21 | 22 |
| Common bent | 25 | 0 | 0 | 25 | 2 | 3 | 43 | 6 | 10 |
| Sweet vernal grass | 41 | 31 | 17 | 48 | 32 | 23 | 57 | 46 | 42 |
| Black grass | 40 | 30 | 18 | 40 | 38 | 24 | 48 | 36 | 28 |
| Rough meadow grass | 16 | 0 | 0 | 18 | 0 | 0 | 20 | 8 | 4 |
| Fodder burnet | 32 | 14 | 0 | 60 | 30 | 16 | 72 | 52 | 38 |
| Chewing's fescue | 40 | 18 | 14 | 40 | 26 | 28 | 66 | 62 | 68 |
| Strong creeping red fescue | 82 | 54 | 48 | 82 | 62 | 64 | 88 | 88 | 80 |

Table 6.2.1 Germination at low temperature 8 °C

Another germination experiment was set up to verify the results obtained from the last experiment as the differential response of the species chosen to the lower germination temperature caused an unnecessary bias. Seed species were again germinated in petri dishes, in the same levels of diesel fuel contaminated soil as before (0g, 25g and 50g diesel $kg^{-1}$ soil) except an acetate collar was used to create a volume of air space above the germinating seeds which would allow the diesel fuel volatiles to dissipate (Section 2.6.1.3). Table 6.2.2 shows the results of this experiment where percentage germination has again been measured after 7 and 14 days.

| | Germination % | | | | | |
|---|---|---|---|---|---|---|
| | Diesel concentration g $kg^{-1}$ | | | | | |
| | 7 days | | | 14 days | | |
| Plant species | 0 | 25 | 50 | 0 | 25 | 50 |
| Canary grass | 34 | 28 | 54 | 46 | 44 | 60 |
| Sweet vernal grass | 34 | 26 | 22 | 38 | 34 | 28 |
| White clover | 54 | 40 | 50 | 54 | 48 | 56 |
| Westerwold's ryegrass | 78 | 72 | 62 | 80 | 80 | 80 |
| Lucerne | 88 | 66 | 76 | 88 | 74 | 90 |

Table 6.2.2. Germination of seeds of varying plant species in diesel fuel contaminated soil with low volatile diesel fuel components.

Although the results are variable, the overall germination rate of the seeds in both treatment levels is more similar to the control germination rate. Because the volatile fraction of diesel is less concentrated in this experimental set up, the results suggest the volatile fraction has an influential role in delaying seed emergence and reducing germination.

To test this conclusion, five plant species were germinated in diesel fuel contaminated soil that had been 'aged' for three weeks before the seeds were sown (Section 2.6.1.4). By aging the soil, this ensured the majority of the volatile components of diesel had volatilised. The results for this experiment given in Figure 6.2.3 clearly shows the influence that the volatile fraction of diesel fuel had on seed germination. The values show the ratio of percentage germination of each treatment compared to the control, which will have a value of 1 (equals 100% germination). The results compare the 14 days screening experiment germination values with the 14 days 'aged' soil germination values. All five plant species tested had higher germination rates, at all treatment levels, in the 'aged' soil compared with the freshly contaminated soil, which was used in the screening experiment. The largest difference in germination rates was seen with Black grass which germinated badly in freshly contaminated soil but improved greatly, particularly at the 25g diesel $kg^{-1}$ soil level, in 'aged' soil. At the

lower level, 25g $kg^{-1}$, most plants showed a slight increase in germination in the 'aged' soil but a large improvement was seen at the 50g $kg^{-1}$ level.

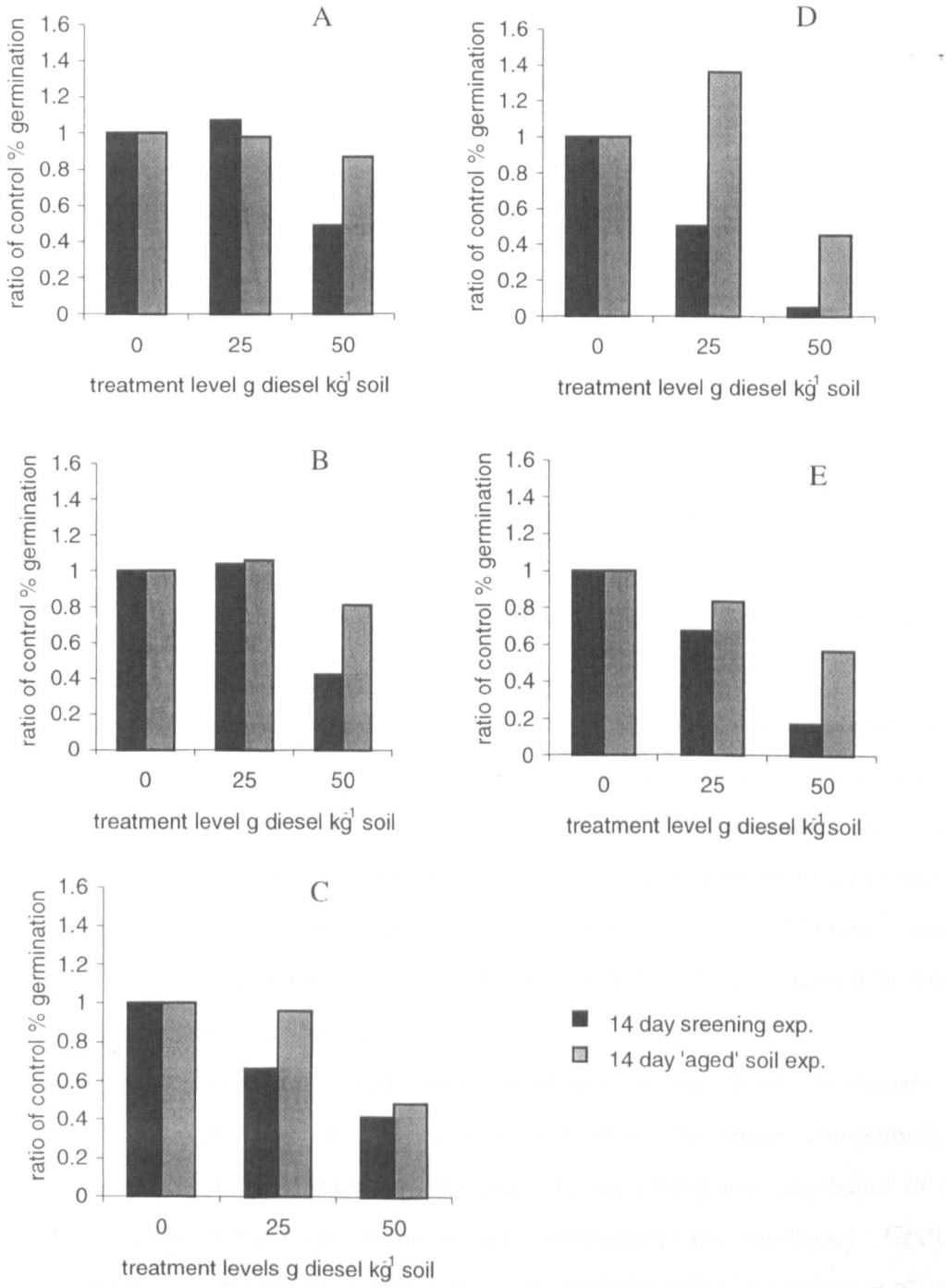



Figure 6.2.3. Germination of varying plant species in 'aged' diesel fuel contaminated soil compared to freshly contaminated soil. A) Strong creeping red fescue, B) Chewing's fescue, C) Sheep's fescue, D) Black grass and E) Sweet vernal grass.

A set of germination experiments were set up to help explain the toxic effect of the volatile fraction of diesel fuel and attribute the effect to a specific group of components of diesel fuel. Seeds were germinated in soils contaminated with a variety of pure petroleum hydrocarbons and the germination rates were measured.

Headspace analysis of diesel fuel was carried out as described in 2.3.4 to determine which hydrocarbons would be present in the volatile fraction of diesel fuel surrounding the germinating seeds. GC-FID analysis concluded that approximately 5-10% of diesel fuel consists of compounds that would volatilise at 20 °C under normal conditions. GC-MS allowed identification of some of the compounds commonly found in the volatile fraction of diesel fuel. The results of diesel fuel headspace analysis are discussed fully in Section 4.4.

## 6.3 Phytotoxicity of Individual Hydrocarbons

The predominant volatile hydrocarbons found in diesel fuel headspace were the isomers of xylene (m-, o- and p-), alkanes (C9–C12) and alkylbenzenes. There were also low levels of toluene, branched cyclohexanes (methyl-, to butylcyclohexane) and alkenes.

A lot of work has previously been done on toxicity of volatile hydrocarbons. Aliphatic hydrocarbons ($C_6$–$C_{12}$) were shown to be non-toxic to plants by spray application (Crafts and Reiber, 1948) and introducing a double bond into n-alkane chains only increased the toxicity slightly. Toxicity in both vapour and spray treatments increased in the order benzene, toluene, and the xylene (Currier, 1951) confirming the conclusions of Crafts and Reiber (1948) who stated that toxicity increased as the side chain was lengthened in this series.

The most interesting components identified in the diesel headspace were branched cyclohexanes as little work has been done on these compounds. A homologous series of cyclohexane with increasing branching was identified in diesel fuel and the lighter of these cyclohexanes were identified in the headspace. Crafts and Reiber showed cyclohexane was more toxic that methylcyclohexane when applied as a spray diluted in paraffin oil. However, the activity of hydrocarbons is dependant on diluent. On a molar concentration basis, a hydrocarbon diluted with air is roughly 30 times more phytotoxic than a hydrocarbon diluted with water and approximately 3,000 times more phytotoxic than if it were diluted in paraffin oil (Currier, 1951). So

although work has been carried out on the toxicity of these hydrocarbons, little is known about their toxicity to germination whilst in the vapour phase. A germination experiment was designed to test the effect of increasing branching of cyclohexane on Westerwold's ryegrass seeds.

Controls started to germinate after 3 days whereas the contaminated seeds germination was delayed until day 6. By day 9, measurable shoots had been produced in both control and contaminated dishes.

| cyclohexane | Concentration mg $l^{-1}$ | Germination (%) | Shoot length (cm) ± SE |
|---|---|---|---|
| control | 0 | 64 ± 2.22 | 63.5 ± 2.02 |
| methyl | 0.1 | 62 ± 5.88 | 8.50 ±0.91 |
| | 1.0 | 64 ± 2.22 | 8.53 ± 0.73 |
| | 5.0 | 58 ± 2.22 | 7.95 ± 0.23 |
| ethyl | 0.1 | 53 ± 0.00 | 8.73 ± 1.11 |
| | 1.0 | 62 ± 4.44 | 8.80 ± 0.68 |
| | 5.0 | 44 ± 9.69 | 7.50 ± 0.52 |
| propyl | 0.1 | 73 ± 7.70 | 9.53 ± 1.22 |
| | 1.0 | 60 ± 3.85 | 9.10 ± 1.19 |
| | 5.0 | 69 ± 4.44 | 9.73 ± 0.19 |
| butyl | 0.1 | 47 ± 3.85 | 9.20 ± 0.61 |
| | 1.0 | 49 ± 2.22 | 9.60 ± 0.51 |
| | 5.0 | 62 ± 8.01 | 8.17 ± 0.74 |

Average values given ± SE, n = 3.

Table 6.3.1 % germination and shoot length values for Westerwold's ryegrass grown for 9 days in varying concentrations of branched cyclohexanes.

The results were varied but showed branched cyclohexanes delayed seed emergence and caused a reduction in shoot length. The reduction in shoot length cannot be directly attributed to the delayed seed emergence, therefore the cyclohexanes must be having a detrimental effect on growth. No significant difference was found between the germination results however, the trend in results suggests increasing levels of methyl,

ethyl and butyl cyclohexane had a detrimental effect on germination but propyl cyclohexane seemed to enhance germination slightly.

This experiment was repeated using a less tolerant plant species to try and obtain a clearer picture of the effect of branched cyclohexanes on germination. Sheep's fescue seeds were germinated in 0.1–5.0mg $l^{-1}$ of each cyclohexane contaminant as before. Sheep's fescue proved more susceptible to diesel fuel contamination and therefore may provide a better indication of the toxic effect of branched cyclohexanes. The results shown in Table 6.3.2 were much easier to interpret.

| Cyclohexane | Concentration mg $l^{-1}$ | Germination (%) |
|---|---|---|
| control | 0 | 43 ± 1.76 |
| methyl | 0.1 | 57 ± 0.67 |
| | 1.0 | 37 ± 1.20 |
| | 5.0 | 37 ± 0.33 |
| ethyl | 0.1 | 3 ± 0.33 |
| | 1.0 | 0 ± 0.00 |
| | 5.0 | 0 ± 0.00 |
| propyl | 0.1 | 20 ± 1.00 |
| | 1.0 | 53 ± 1.15 |
| | 5.0 | 23 ± 1.53 |
| butyl | 0.1 | 47 ± 1.53 |
| | 1.0 | 0 ± 0.00 |
| | 5.0 | 0 ± 0.00 |

Average values given ± SE, n = 3.

Table 6.3.2 % germination of Sheep's fescue after 17 days growing in varying concentrations of branched cyclohexanes.

The ethyl and butyl branched cyclohexanes had a huge impact on germination rate with practically no seeds germinating in any of the ethyl cyclohexane concentrations and only the lowest level of butyl cyclohexane allowing a normal germination rate. By stark comparison, the methyl and propyl cyclohexanes had an almost normal germination rate. At 0.1mg methyl cyclohexane $l^{-1}$ , germination

appeared to be enhanced by the contaminant and at higher concentration levels the germination rate was just slightly below the control germination rate. Propyl cyclohexane reduced germination rate by approximately 50% of the control at 0.1 and 5.0mg $l^{-1}$ levels but appeared to enhance the germination rate at 1.0mg $l^{-1}$ level. This result was odd but may have been caused by a non-homogeneous selection of seeds being used. When carrying out the plant experiments, a homogeneous selection of seeds were chosen to provide as even a response to germination and growth as possible. However, it was very difficult to obtain homogeneity with small seeds or seeds with a husk such as Sheep's fescue.

An unusual pattern of germination was apparent in this experiment, with length of cyclohexane branching determining toxicity to seeds. The methyl and butyl cyclohexanes appeared to be extremely toxic to seeds whilst the methyl and propyl cyclohexanes had no or slight toxicity. A similar observation has been noted with toxicity of substituted benzenes. Crafts and Reiber (1948) found benzene toxicity increased with increasing number of isopropyl substitutions but not in a simple series. The mono- and tri- substitutions were low in toxicity and the di- and tetra- substitutions were high in toxicity.

These experiments have shown that the lightweight, volatile hydrocarbons present in diesel fuel have a large influence on the germination of seeds of varying plant species. However, germination results of the contaminated treatments with minimal volatile diesel fuel components were never as high as the control germination results. This suggests that the influence of the volatile fraction of diesel fuel is not the only factor inhibiting seed germination. The remaining diesel fuel still has some level of toxicity to the seeds.

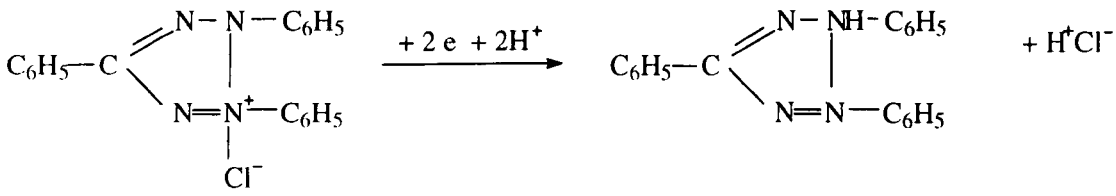
Amakiri and Onofeghara (1984) have shown that crude oil and its distillates are potent contact herbicides. The embryo of a seed could be injured or killed if it comes into contact with crude oil. Oil may enter seeds via the micropylar end of dicotyledonous seeds or the coleorhizal end of monocotyledonous seeds, or simply through a crack or scar in the seed coat (Amakiri and Onofeghara, 1984). Whichever way it enters, penetration of oil would endanger the activity of the embryo, which is vital to germination. Injury to the embryo that was not fatal resulted in delayed seed emergence.

To determine whether diesel fuel had similar contact herbicidal properties as described for crude oil by Amakiri and Onofeghara (1984), a modified germination experiment was conducted on seeds previously soaked in diesel fuel (Section 2.6.1.5).

## 6.4 Effect of Diesel Fuel on Seed Viability

A preliminary experiment was carried out to assess the effectiveness of the Tetrazolium method by Smith (1951) for determining germinating ability of cereals and if this method could be applied to other seed species.

In the presence of viable tissue, colourless solutions of triphenyl tetrazolium chloride (TTC) form insoluble red triphenyl formazan (TPF) by the following reaction (Smith, 1951):




colourless red colour

It is then possible to predict the germinating ability of seeds by observation of the embryo parts that are stained red by the insoluble TPF deposited in viable tissue.

After 24 hours incubation in 1.5% TTC solution at 20°C, the Red clover and Flax seeds were showing signs of colour development but the Canary grass and Oil seed rape seeds were not. These seeds were placed back into the TTC solution and incubated for a further 96 hours.

The Red clover seeds had colour development ratings of 45% red, 20% pink and 0% white. This ties in well with the initial screening experiment germination results (Table 6.1.1) that showed percentage germination of Red clover seeds after 7 days to be 44%. This figure rose to 56% after 14 days. Some of the pink rated seeds by the Tetrazolium test must be viable enough to germinate but a slight lag phase is seen.

The Flax seeds had ratings of 70% red, 30% pink and 0% white, suggesting 70% of the seeds were very viable. Again this is in agreement with the initial screening

experiment results where 72% germination was recorded after 7 days and 74% germination after 14 days.

The Oil seed rape and Canary grass seeds, which were incubated for a total of 120 hours, appeared to have good penetration of the TTC by this time. The Oil seed rape seeds were harder to assess and a colour development rating of 69% red and 31% white was observed. This value underestimates the actual percentage germination as normal germination rates are observed in the high 90% range. The same problem was found when assessing the Canary grass seeds and a ratio of 60% red to 40% white was found. Again this underestimates the true percentage germination, as it is normally approximately 70-80%. The problem may be caused by both Oil seed rape and Canary grass seeds having hard seed coats which may affect the penetrability of the TTC solution.

From this preliminary investigation, Red clover and Flax seeds were found to be the most successful seed species for use in viability studies as they allowed easy penetration of the TTC solution through their seed coats facilitating rapid colour development (within 24 hours), the seeds were easy to dissect and the stained embryo was clearly visible. Flax seeds were chosen for the next part of the investigation (Section 2.6.1.5), as they were larger and more manageable than the small Red clover seeds. The Oil seed rape and Canary grass seeds were discarded as suitable species for investigation as the incubation time in TTC solution was larger than the other species tested and the penetration of TTC solution in this time was inconsistent.

Seeds of the Flax variety 'Viking' pre-soaked in diesel fuel differed from control seeds in their pattern of germination. Oil soaked seeds tended to have a larger lag phase preceding germination and the lag in germination seemed to increase with increasing pre-soaking time (Figure 6.4.1).

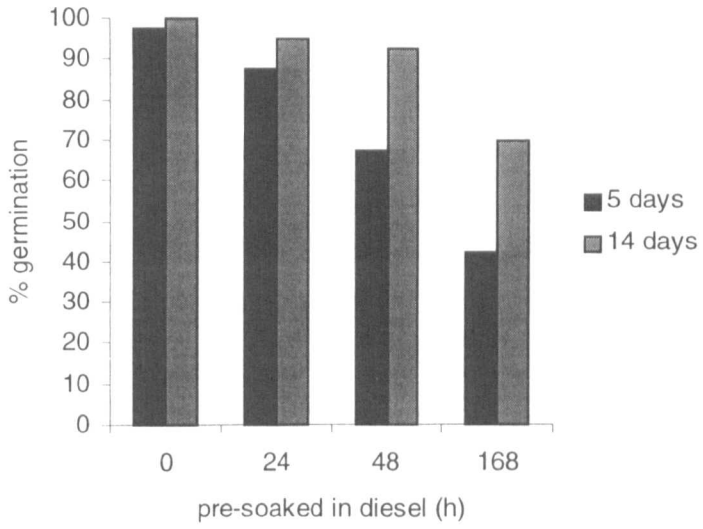



Figure 6.4.2 Germination of flax seeds pre-soaked in diesel fuel.

The percentage germination fell from 97.5% in the control to 87.5% after 24 hours soaking, then to 67.5% and 42.5% after 48 and 168 hours respectively. Despite the longer lag period, the overall percentage germination of diesel fuel soaked seeds after 14 days was not badly affected until the 168 hour pre-soaking treatment, where the percentage germination fell from the 90% range to 70%.

Flax seeds dissected after pre-soaking in diesel fuel showed that the seeds had retained their viability even after soaking for 168 hours (Table 6.4.3). Pre-soaking for 48 and 168 hours showed only 2.5% of the seeds to be completely non-viable. This may suggest that diesel fuel has penetrated into the seed and killed the embryo making it non-viable or it may be just a few of the seeds were non-viable to begin with. The majority of the pre-soaked seeds were showing signs of viability using the Tetrazolium test but the germination rate in the 168 hour pre-soaked treatment was drastically reduced. This inhibitory effect on germination could be attributed more to physical constraints than biological damage on the seeds resulting from the physical and chemical characteristics of the diesel fuel. As mentioned previously, diesel fuel was used in the past as a herbicide (Crafts and Reiber, 1948; van Overbeek and Blondeau, 1953). Although the toxicity of diesel fuel has decreased in recent years due to the removal of a lot of the aromatic compounds present in the diesel fraction, it is still toxic to plants at certain concentrations. The embryo of a seed could easily be injured or killed if it were to come in contact with diesel fuel. There was little indication that the embryos were being killed in this investigation however, as the majority of seeds were showing tetrazolium reducing properties, suggesting respiring, healthy seeds. Amakiri

and Onofeghara (1984) showed that seeds of *Capsicum frutescens* retained almost 100% viability after 32 weeks pre-soaking in crude oil. The lag phase preceding germination was increased threefold.

The seeds have a primary line of defence preventing diesel fuel penetration – their seed coat. The integrity and hardness of the seed coat affect the rate of oil penetration (Amakiri and Onofeghara, 1984). Only 2.5% of the 48 and 168 hour pre-soaked seeds were classed as non-viable. Injury to the embryo may not have been fatal, but reduced the growth activity of the embryo, which resulted in delayed seed emergence. These results indicate that seed coats resistant to oil penetration will be virtually unaffected, and therefore a prerequisite to embryo damage by oil is tissue penetration.

| | Visual appearance of TTC reduction | | |
|---|---|---|---|
| Treatment | Development of stained colour * | | |
| Soaked in diesel (hrs) | Red | Pink | None (white) |
| 0 | 70.0 | 30.0 | 0.0 |
| 24 | 87.5 | 12.5 | 0.0 |
| 48 | 77.5 | 20.0 | 2.5 |
| 168 | 66.7 | 30.8 | 2.5 |

* average of 40 seeds

Table 6.4.3 Development of TPF staining in viable seeds pre-soaked for varying lengths of time in diesel fuel.

A more likely reason for the inhibitory effect of diesel fuel on germination is its physical water repellent property. The film of oil around the seeds may act as a physical barrier, preventing or reducing both water and oxygen from entering thus 'suffocating' the seed. This would explain why the seeds still reduced

TTC to TPF after being submerged in diesel fuel but had an extended lag phase before germinating.

The effects of diesel fuel on the later stages of growth and development was observed by measuring the shoot and root lengths of the pre-soaked diesel fuel seedlings after 14 days growth (Table 6.4.4).

| Treatment soaked in diesel (h) | Germination % ± SE | Shoot height (cm) ± SE | Root length (cm) ± SE |
|---|---|---|---|
| 0 | 100 ± 0.00 | 8.5 ± 0.24 | 11.8 ± 0.12 |
| 24 | 95 ± 0.29 | 8.0 ± 0.15 | 11.2 ± 0.19 |
| 48 | 92.5 ± 0.58 | 6.8 ± 0.33 | 10.8 ± 0.57 |
| 168 | 70 ± 0.87 | 6.7 ± 0.41 | 9.1 ± 1.10 |

Table 6.4.4 Summary of average germination, shoot and root lengths of 14 day old Flax seedlings pre-soaked in diesel fuel.

There was a slight decrease in shoot length observed in the pre-soaked seed treatments compared to the control. The longer the pre-soaking, the greater the reduction in shoot length. The control and 24 hour pre-soaked seed shoot length were not significantly different but both these values were significantly different from the 48 and 168 hour pre-soaked seed shoot lengths. This suggests soaking the seeds in diesel fuel for longer than 24 hours has a significant effect on plant development. This trend was also seen for the root length values although the differences in lengths were not significantly different.

The other noticeable difference on plant development was the production of secondary leaves on the Flax seedlings. The secondary leaves were quite noticeable on the control and 24 hour pre-soaked seedlings but were small or not present on the other treated seedlings. This showed the effect that the lag phase, caused by pre-soaking in diesel fuel, had on plant development.

In summary, germination of seeds in diesel fuel contaminated soil was highly dependent on plant species. Some species were notably tolerant whilst other species were completely intolerant of diesel fuel contamination. The ability to tolerate diesel fuel was not species specific, with members of the same plant family showing differential sensitivity to diesel fuel. Of the more tolerant species, a delay in seed emergence was generally observed. This delay was caused, in part, by the volatile fraction of diesel fuel. Individual volatile hydrocarbons were shown to delay seed emergence and have a detrimental effect on plant development with branched cyclohexanes being particularly phytotoxic to certain plant species. Another cause of this inhibitory effect on germination may be attributed to the physical constraints induced by diesel fuel on the seed. Diesel fuel would cause a film of oil to form around the seed which would act as a physical barrier, preventing or reducing both water and oxygen transfer thus 'suffocating' the seed. The physical effect was also shown to delay seed emergence and therefore could be a factor in the overall inhibitory effect of diesel fuel contamination on germination.

# CHAPTER SEVEN

## EFFECT OF DIESEL FUEL ON PLANT GROWTH AND DEVELOPMENT: **EFFECT ON THE ROOT**

### 7.1 Changes to Root Structure

When conducting the initial screening experiments where seeds were germinated in freshly contaminated soil (Section 2.6.1.1) an unusual developmental effect occurred on some of the seed species being investigated. Branching of roots from seedlings grown in diesel fuel contaminated soil was observed, where none was seen in the uncontaminated control seedlings. This observation kept recurring during the initial screening experiment and was therefore investigated more fully.

Figure 7.1.1 illustrates two week old Annual canary grass and Flax seedlings grown in 50g diesel $kg^{-1}$ contaminated soil and uncontaminated soil. The effect on plant development is clearly noticeable with the contaminated seedlings being extremely 'stunted' compared to the control seedlings. The most interesting effect however, was the appearance of branches on the root structure of Flax seedlings grown in contaminated soil. The roots of contaminated seedlings were highly branched whereas, the seedlings grown in uncontaminated soil had one, long root.

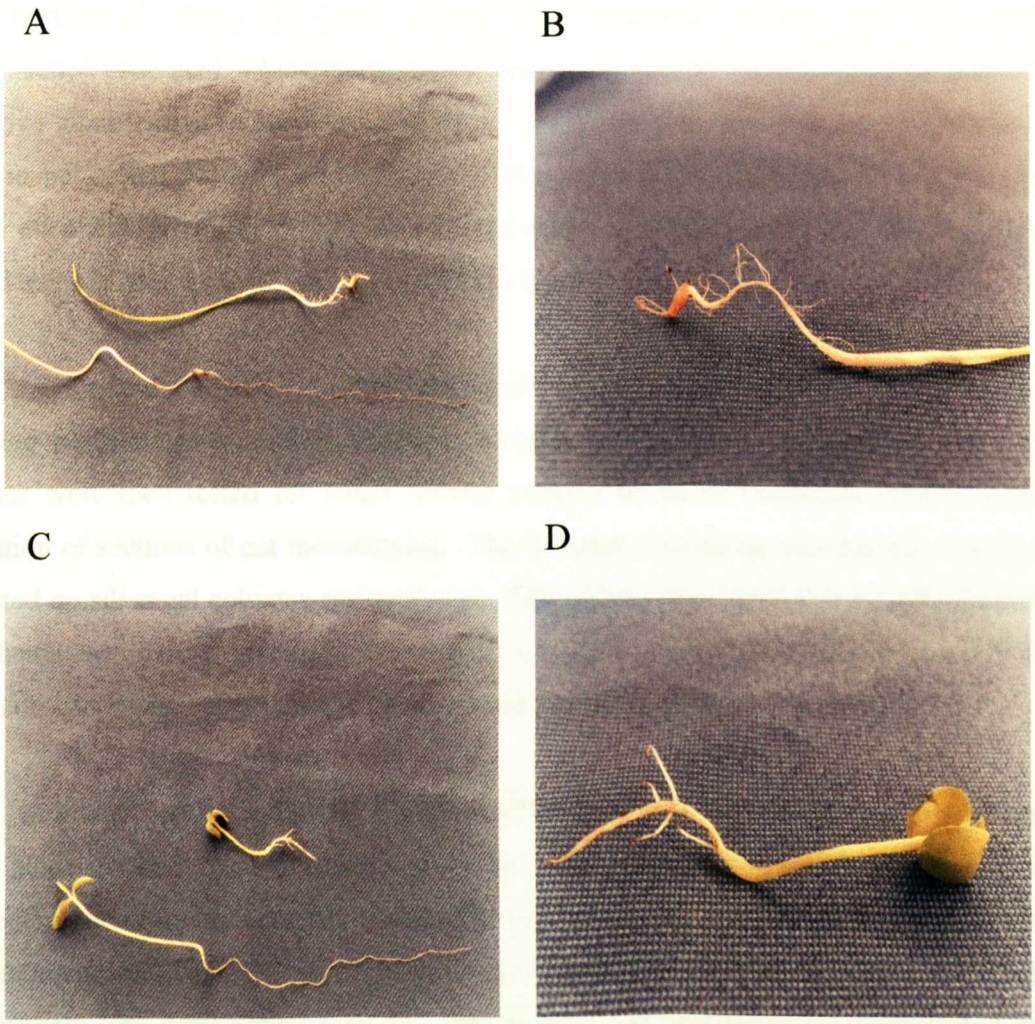



Figure 7.1.1. Effect of diesel fuel on root formation of two week old seedlings.

(A) Annual canary grass – seedling grown in diesel fuel contaminated (top) and uncontaminated (bottom) soil; (B) Annual canary grass – enlargement of seedling grown in contaminated soil; (C) Flax - seedling grown in diesel fuel contaminated (top) and uncontaminated (bottom) soil and (D) Flax - enlargement of seedling grown in contaminated soil.

Can ary grass seedlings grown in contaminated soil showed root structures appearing on the stems of seedlings. This effect was not observed in the control plants grown in uncontaminated soil. Root structures that appear in unusual places on plants are termed adventitious roots. The development of adventitious roots has been shown to be caused by the plant growth hormone, auxin. Salisbury and Ross (1991) noted that added auxin often caused emergence of many adventitious roots in the lower internodal

added auxin often caused emergence of many adventitious roots in the lower internodal stem region and these roots were not restricted to the base of stems, but could form on the lower surface of stems that were placed in a horizontal position and kept moist. Naphthalene acetic acid, which is a synthetic auxin, and several derivatives of benzoic acid also have auxin activity (Salisbury and Ross, 1991). Similar substances to these can be found in petroleum products and it is not unlikely that they would induce hormone type effects on plants grown in petroleum contaminated soils. This effect has been noted by several authors (Bossert and Bartha, 1985; Gudin and Harada, 1974a and b).

Gudin and Harada (1974a) fractionated crude oil as well as other distillates, including kerosene and residual asphaltic fraction, by successive solvent elution. The fractions were then tested for auxin activity using a series of biological tests including elongation of sections of oat mesocotyles. The fractions containing active auxin were then separated on silica gel columns and analysed. The authors concluded that naphthenic acids and phenyl acetic acid identified from crude oil and its distillates were responsible for certain developmental effects found in vegetation grown on petroleum polluted sites.

In a later paper, Gudin and Harada (1974b) also found that the presence of phenyl acetic acid, isolated from petroleum fractions, had a significant effect on geotropism. Geotropism involves the correct orientation of the root and shoot tips emerging from germinating seeds. During a pot trial involving Oil seed rape (described in Section 2.6.3.1), an extremely low germination rate was observed for Oil seed rape in contaminated soil (Section 8.1) although germination was high in the initial screening experiment for the same seed type (Section 6.1). After further investigation of the seeds remaining in the soil after the pot experiment had ended it became apparent what had caused the low germination rate. The majority of seeds had split their seed coats but the root and shoot tips failed to orient properly and were shrivelled before emerging from the soil. The root tips appeared to grow horizontally or curved upwards, while the shoot tips and cotyledons were frequently oriented downwards or sideways. This observation was noted by Bossert and Bartha (1985) when growing Soybeans (*Glycine max*) in oily sludge material. They concluded the disruption of geotropism was likely to be caused by hydrocarbon residues with plant hormone effects.

These observations suggest diesel fuel may contain compounds that resemble the structure of and exhibit plant growth hormone effects similar to those found by Gudin and Harada (1974a and b).

To try and distinguish between the components of diesel fuel that mimic plant growth hormones, a germination experiment was set up to test individual petroleum hydrocarbons (Section 2.6.1.6). No branching was observed when germination experiments using cyclohexanes were conducted so this group was excluded from the trial. For a chemical to induce similar effects as a plant growth hormone their structures must be similar. The effects observed when growing plants in petroleum contaminated soils were mostly attributed to an auxin type chemical. Therefore the most obvious group of chemicals to start with were those which appeared similar to auxin (Figure 7.1.2 A). Gudin and Harada (1974) identified naphthenic acids and phenyl acetic acid (Figure 7.1.2 B and C) as components of crude oil and its distillates which exhibited auxin activity. Commercial powders into which cut ends of stems are dipped to facilitate root production usually contain Naphthalene acetic acid (NAA) (Salisbury and Ross, 1991). It was therefore decided that the diaromatic and polyaromatic hydrocarbons (PAHs) should be tested first.

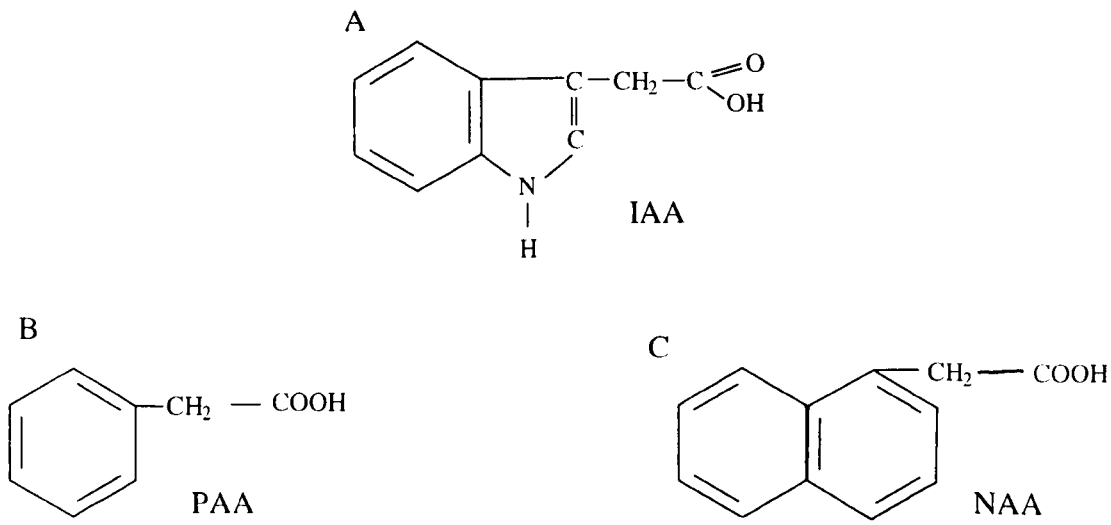



Figure 7.1.2 (A) Indole-3-acetic acid (IAA) – most common auxin plant growth hormone and other types of auxin including (B) Phenylacetic acid (PAA) which is widespread among plants but is less active than IAA and (C) Naphthalene acetic acid (NAA) which is a synthetic auxin and more effective at root initiation than IAA.

To test whether branching of aromatics or size of aromatic compound had an effect on root development a germination experiment was set up. Canary grass seeds, which had shown development of adventitious roots previously, were germinated in 1mg $l^{-1}$ naphthalene, 1,4 dimethylnaphthalene (1,4 DMN), 2,3,5 trimethylnaphthalene (2,3,5 TMN) and anthracene contaminated soil. The results are given in Table 5.1.2 for percentage germination, root length and percentage of branching roots and shoot length. Although there was a large drop in germination rate in 1,4 DMN contaminated soil compared to the control, the germination rate was not significantly different from the others. The length of root appeared to be reduced by growth in naphthalene but again the difference was not significant. However, a significant reduction in shoot length was observed for seedlings grown in 1,4 DMN contaminated soil compared to the control.

| contaminant | % branched roots | % germination ± SE | av. shoot length ± SE | av. root length ± SE |
|---|---|---|---|---|
| Control | 26 | 33.3 ± 2.66 | 25.5 ± 0.56 | 9.85 ± 1.03 |
| Naphthalene | 23 | 28.3 ± 8.02 | 25.48 ± 0.51 | 6.44 ± 1.66 |
| 1,4 DMN | 33 | 19.3 ± 4.96 | 22.49 ± 0.83 | 9.48 ± 0.84 |
| 2,3,5 TMN | 65 | 33.5 ± 6.12 | 26.04 ± 0.80 | 10.18 ± 1.53 |
| Anthracene | 37.5 | 40.0 ± 6.0 | 24.47 ± 0.70 | 7.85 ± 0.10 |

Table 7.1.2 Development of Canary grass seedlings grown in 1mg $l^{-1}$ concentrations of various aromatic compounds.

The number of branching roots was also measured in each replicate to ascertain whether the contaminant being tested was enhancing the formation of adventitious roots. Unexpectedly, the control had some branching roots present on seedlings grown in uncontaminated soil (26%). This may be due to the shallow depth of soil in the petri dishes causing the plant to produce adventitious roots for support. Seedlings grown in naphthalene contaminated soil showed no sign of enhanced root branching with 23% of seedlings having branching roots. There was a slight increase in branching observed with 1,4 DMN and anthracene contaminated soils with the number of seedlings having branched roots rising to 33% and 37.5% respectively. The largest and most significant increase was seen with 2,3,5 TMN which enhanced the number of seedlings exhibiting

signs of root branching to 65%. This value is clearly different from the control value, indicating that branched naphthalenes such as 2,3,5 TMN, have an influence on root development. The trend in results observed during this experiment suggest an increase in the number of branches on naphthalene enhances the occurrence of adventitious root formation on Canary grass seedlings. This may be due to branching causing the chemical to persist longer whereas unbranched naphthalene would be volatile and would disappear quickly from the vicinity of the growing seedling.

## 7.2 Spatial Distribution of Roots

An experimental system was set up which enabled the pattern of root development of selected plant species to be followed in a model soil system contaminated with diesel fuel (Section 2.8).

Set up 1 was designed to examine the reaction of plant roots to a continuous subsurface layer of diesel fuel. The seeds were germinated and allowed to grow initially in uncontaminated soil as a 10 cm layer of uncontaminated soil was placed on top of the layer of diesel fuel. Therefore, there was very little difference between the germination rate in the control and contaminated sections of the glass box (approximately 2%). Although three plant species were chosen for investigation, only the oil seed rape results are discussed in full. The reason for this being, the germination rate of both grass species in the glass box set up were quite low with Sweet vernal grass having a germination rate of approximately 50% and Common bent having a germination rate of approximately 40%. The roots produced by both these grasses were very fine and difficult to visualise in the glass box set up unlike the Oil seed rape roots, which were extremely thick and white in appearance. The roots of both grass species were slow growing whereas the roots of the oil seed rape grew quickly and were altogether easier to assess. It was therefore decided that the glass boxes containing oil seed rape plants should be thoroughly studied to help explain the interaction of roots with areas of diesel fuel oil contamination.

After 2 weeks growth, the oil seed rape plants were growing well. The plants grown in the control glass box with no diesel fuel addition had produced a considerable top growth biomass and had long, vertical roots reaching almost to the bottom of the glass box (approximately 30 cm). The plants grown in the contaminated glass box were

the same size as the plants grown in the uncontaminated box, however the roots appeared to grow to the level of the diesel fuel layer and no further. After 3 weeks growth, the roots of the oil seed rape plants growing in the contaminated box began to grow through the diesel fuel layer into the uncontaminated soil below. The plants began to look less healthy at this stage, with small yellow blotches appearing on the leaves and the stems were beginning to go purple. At 3 weeks, the difference in top growth biomass was apparent between the control and contaminated plants. By 5 weeks, the majority of the roots from the oil seed rape plants growing in the control box had reached the bottom of the glass box (approximately 40 cm) and the plants were healthy and the top growth abundant. The oil seed rape plants grown in the contaminated box were much shorter than the control plants and at least a growth stage behind the control plants according to the ADAS rating for growth stages in oil seed rape. The control plants were showing signs of their third or fourth true leaf whereas the contaminated plants had only their second true leaves. The cotyledons of the contaminated plants were also very yellow and dropping off the plant whereas the cotyledons of control plants were green and perfectly unfolded. The roots of the contaminated plants had grown through the diesel layer but the roots themselves were very fine and had less bulk than the rest of the roots growing in uncontaminated soil. There was a noticeable line of differentiation between these two root areas, which can be clearly seen in Figure 7.2.1. After 8 weeks the top growth biomass was harvested from each glass box and dried as described in Section 2.7.1.1. The biomass collected from the contaminated box was 43% of the total biomass collected from the control glass box. The large reduction in biomass was exaggerated due to the number of plants that died in the contaminated glass box as well as the smaller, less healthy plants.

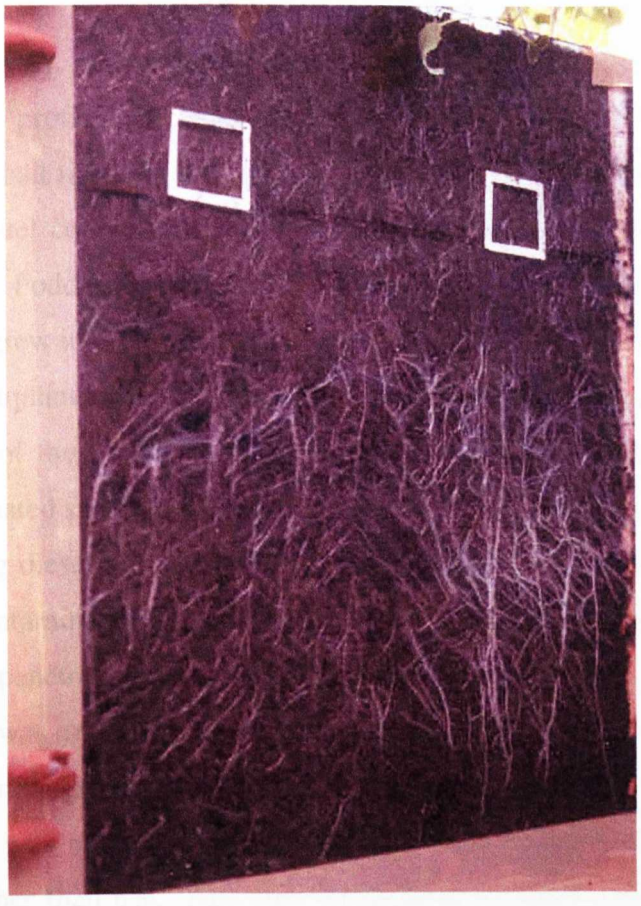


Figure 7.2.1. Oil seed rape plants growing in a glass box set up with a subsurface layer or diesel fuel. The arrow indicates the line of differentiation between the root mass above and below the diesel contaminated horizon.

Set up 2 was designed to evaluate whether roots would grow into diesel fuel contaminated soil when there was also a supply of 'clean', uncontaminated soil present. Each glass box was split into three sections and half of each section filled with either uncontaminated, 25g $kg^{-1}$ or 50g $kg^{-1}$ diesel fuel contaminated soil. Each glass box also had a 10cm layer of uncontaminated soil, as described in set up 1, to allow germination to proceed unhindered. Two deep rooting plant species were chosen for this investigation. Cocksfoot, which is a grass and Fodder burnet, which is a herb. The seeds began to germinate and shoot growth was apparent by week one. However, there was a distinct difference between the germination rates of Fodder burnet seeds in each section. The control, uncontaminated section had a 56% germination rate whereas the 25g $kg^{-1}$ and 50g $kg^{-1}$ diesel fuel contaminated sections had germination rates of 36%

and 20% respectively. The volatile fraction of the diesel fuel may have been diffusing through the soil profile, affecting seed germination. After 4 weeks growth, the germination rate had risen to 72%, 52% and 32% in the uncontaminated, 25g $kg^{-1}$ and 50g $kg^{-1}$ diesel fuel contaminated sections and the plants were growing successfully. The roots of the Fodder burnet plants stopped at the contaminated sections and the remaining roots grew into the uncontaminated half. After 3 months growth, the roots of the Fodder burnet plants were still avoiding the contaminated regions in each section.

The root of the Cocksfoot plants on the other hand, appeared to grow straight into the contaminated sections although the rate of root growth through the sections was slower in the 50g diesel $kg^{-1}$ section than the 25g diesel $kg^{-1}$ section. After 3 months growth, the uncontaminated and the 25g diesel $kg^{-1}$ sections were packed with roots and the roots were penetrating the 50g diesel $kg^{-1}$ section. The germination rate of Cocksfoot seeds was extremely poor in all sections (approximately 6%), which must be due to the different growth conditions in the glass box. The germination rate of Cocksfoot seeds was never high (53% in control soil shown in Table 6.1.1) but was considerably higher than 6%. Although the number of seeds growing in each section was low, root penetration and overall root mass in each section suggests that Cocksfoot plants are capable of growing into diesel contaminated areas quite successfully as shown by Figure 7.2.2.

After three months growth, measurement of total oven dried shoot and root biomass (as described in Sections 2.7.1 And 2.7.2) showed an increase in Cocksfoot plant biomass growing in the 25g $kg^{-1}$ contaminated glass box compared to plants growing in the control glass box. The total shoot biomass increased to 118% of the control biomass and the root biomass increased to 123% of the control root biomass. The increase in root biomass was much larger than shoot biomass in the 25g $kg^{-1}$ contaminated treatment which altered the shoot : root ratio. The shoot : root ratio of control plants was 0.99 which is in the normal range for healthy plants. The shoot biomass is generally larger than the root biomass giving a value of 1 or slightly below (Böhm, 1979). This ratio fell to 0.94 in contaminated plants, as the root biomass was much larger than the shoot biomass. This indicates that plants growing in 25g $kg^{-1}$ contaminated treatment were stressed, as stress, particularly water stress, results in a larger proportion of carbon being allocated to the roots (Li *et al.*, 1997). This stress may have been caused by the physical influence of diesel fuel on the soil reducing its ability to hold water thus reducing the amount available for plant uptake. The effect of diesel fuel on soil physical properties leading to factors such as water stress are discussed fully in Chapter 5.

The 50g diesel $kg^{-1}$ contaminated plants showed a decrease in both shoot and root biomass, with shoot biomass falling to 46% of the control and root biomass falling to 57% of the control root biomass. Figure 7.2.2 illustrates an example of shoot and root biomass collected from Cocksfoot plants grown in each section of the glass box set up.

Figure 7.2.2 (overpage) Example of Cocksfoot plants harvested from A) control, B) 25g diesel $kg^{-1}$ contaminated treatment and C) 50g diesel $kg^{-1}$ contaminated treatment after 3 months growth.

Figure 7.2.2. Examples of Cocksfoot plants grown in 0g, 25g and 50g diesel $kg^{-1}$ soil in the glass box set up.

Set up 3 was designed to test whether small areas of contamination would be tackled by probing roots in the soil or if those roots would preferentially avoid contaminated areas and grow into areas of 'clean' soil. Glass boxes were set up as shown in Section 2.8 with patches (6 cm x 6 cm) of 0, 25g and 50g diesel $kg^{-1}$ contaminated soil below the soil surface. Flax variety ' Viking' and Oil seed rape variety 'Commanche' were chosen for this experiment as both were notably tolerant to diesel fuel and produced sizeable roots which would be easily identifiable in the glass box set up. Seeds were planted and germinated well in the uncontaminated soil above the contaminated patches. Germination rates in each section of the glass box were similar with average percentage germination reaching 98% for Flax plants and 64% for Oil seed rape plants. After 3 weeks, the roots of both plants were growing through the area which would have been contaminated in the control, uncontaminated section of each glass box but not into the contaminated sections. The majority of the roots avoided the contaminated patches entirely, which is illustrated in Figure 7.2.3.

(A) (B)

Figure 7.2.3. Growth of Oil seed rape in glass boxes contaminated with (A) 0g and (B) 25g diesel kg-1 soil patches. Photographs illustrate the pattern of root development after 3 weeks growth.

After 8 weeks growth, the roots of both plant species had filled the areas of uncontaminated soil completely. The Oil seed rape plant roots were beginning to grow into the 25g diesel $kg^{-1}$ soil patch but were less inclined to move into the 50g diesel $kg^{-1}$ soil patch. Figure 7.2.4 shows a close up of both these contaminated areas.

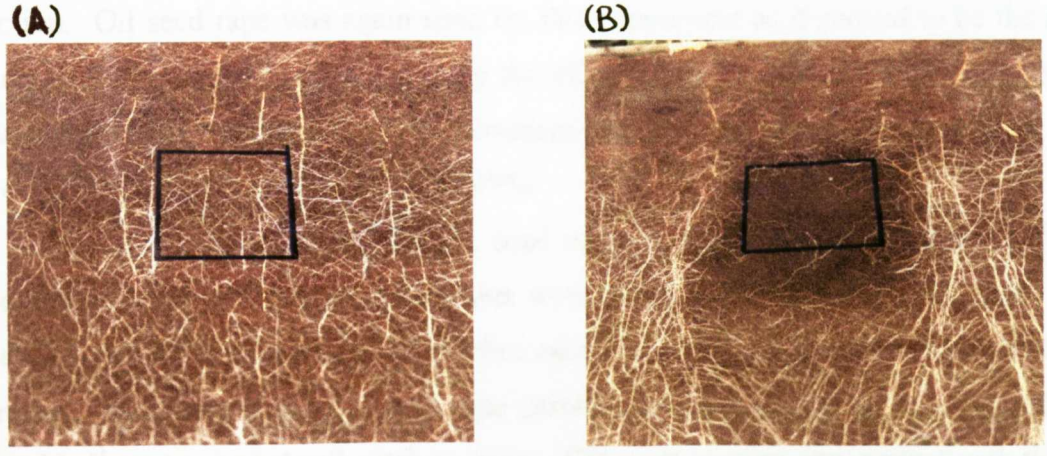



Figure 7.2.4. Close up of Oil seed rape plant roots in (A) 0g and (B) 25g diesel $kg^{-1}$ contaminated soil at 8 weeks.

The Flax plant roots had a similar response but avoided the contaminated areas more readily than the Oil seed rape roots. Figure 7.2.5 again shows a close up of Flax roots in 0g and 50g diesel $kg^{-1}$ contaminated soil.

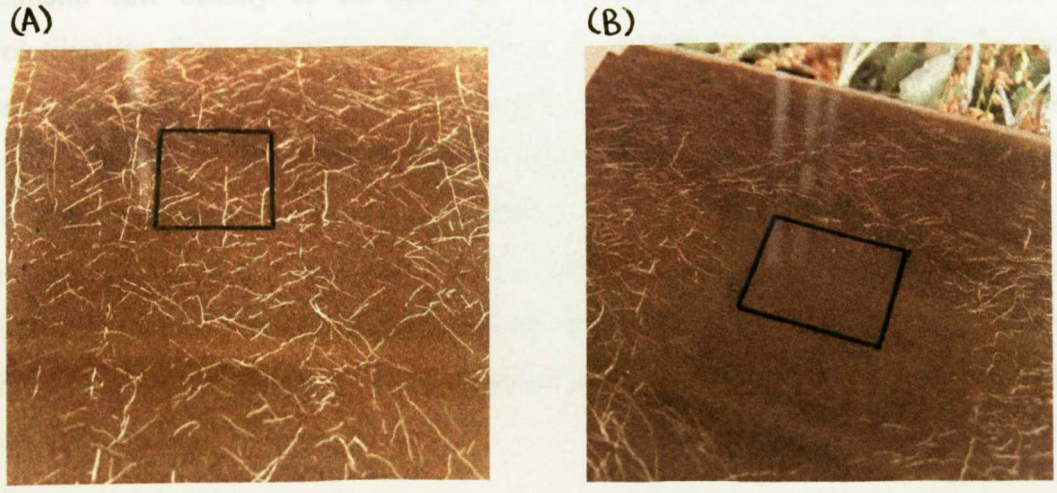
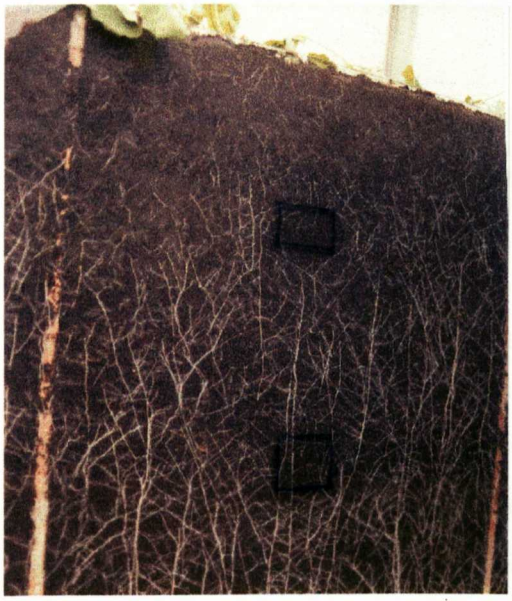



Figure 7.2.5. Close up of Flax plant roots in (A) 0g and (B) 50g diesel $kg^{-1}$ contaminated soil at 8 weeks.

Set up 4 was designed to evaluate the response of plant roots to patches of diesel fuel contamination and if the age of the root affected its ability to grow into contaminated areas. The glass boxes were split into three sections and each section contaminated with small patches (4cm x 2.5cm) of diesel fuel contaminated soil as shown in Figure 2.8.5. Each section contained either 0g, 5g, 10g, 15g, 25g or 50g diesel $kg^{-1}$ contaminated soil patches positioned near the top and bottom of each glass box section. Oil seed rape was again used for this experiment as it proved to be the most successful plant species for illustrating the effect of diesel fuel contamination on the pattern of root development. The germination rate was similar in each section with average percentage germination being 97%.

After 4 weeks growth, the Oil seed rape plants were growing well in all the sections of the glass boxes. The plants were green and healthy and the roots were growing into all the contaminated patches except the 50g diesel $kg^{-1}$ contaminated soil patches. The roots of the Oil seed rape plants grew through the 0g, 5g, 10g and 15g diesel $kg^{-1}$ contaminated soil patches easily. The roots coming into contact with the top 25g diesel $kg^{-1}$ contaminated soil patch did not grow straight through this area but appeared to skirt around the edges. The roots reaching the bottom contaminated patch however, grew straight through suggesting the length of time it took for the roots to reach the bottom patch was sufficient to allow a reduction in the diesel fuel's toxicity either due to dissipation of the volatile fraction or biodegradation, therefore they were not deterred from entering this area. The soil profile was tightly packed to allow a realistic soil density to be achieved that would greatly reduce any diesel fuel volatilisation from occurring. In addition, the length of time it took for the roots to reach the bottom contaminated patch was only 7 days more from the time they reached the first patch. It was unlikely that biodegradation of diesel fuel over this time period could account for the roots readily penetrating the lower contaminated patch. The most likely explanation therefore was that the roots had become acclimatised to the diesel fuel contamination and the plant was more developed and able to tackle the presence of this contaminant. Figure 7.2.6 illustrates root growth in 25g and 50g diesel $kg^{-1}$ contaminated soil sections.



(A)

(B)

Figure 7.2.6. Growth of Oil seed rape plants in glass boxes contaminated with small patches of (A) 25g and (B) 50g diesel $kg^{-1}$ soil.

Finally, a glass box was set up and positioned lengthwise as shown in Figure 2.8.6 to test the depth of rooting of Westerwold's ryegrass. Some authors suggest grass roots only grow about 50cm into the soil profile, which would imply they are of no use for phytoremediation at depths greater than 50cm. Westerwold's ryegrass was chosen as it is not considered a deep rooting grass and was capable of growing in reasonably high levels of diesel fuel contaminated soil, hence a likely plant species for phytoremediation.

After 2 months growth, Westerwold's ryegrass roots had extended down to a depth of 1 metre, filling the entire length of the glass box set up. This proves that grasses may extend their root systems far into the soil profile and should not be discarded as candidates for surface soil phytoremediation practices.

## 7.3 Effect on Nodulation of Leguminous Plants

During the harvesting of pot experiments containing leguminous plants, a recurring difference in the number and formation of the nodules present on control and contaminated Common vetch (*Vicia sativa*) plants was observed. This observation was investigated further during harvesting of the pot experiment described in Section 2.6.3.4 where Vetch plants, along with Westerwold's ryegrass and Meadow mix, were grown in varying levels of diesel fuel contaminated soil and harvested after 2 and 4 months growth. Plants were removed from the soil and washed to remove any adhering soil and the number of nodules per plant counted. Five nodules were then removed from five separate control plants and five separate contaminated plants and sectioned for light microscopy as described in Section 2.7.1.4.

Observation of nodule sections by light microscopy illustrated clear differences between nodules taken from control Vetch plants and plants grown in diesel fuel contaminated soil. The majority of the nodules from control plants were spherical and appeared to be at the initial stages of nodule differentiation. Few bacteroids were present within the central body of the nodule suggesting the nodule was immature. This agrees with the visual observations made during harvesting of this experiment (Section 2.6.3.4) and the pot experiment described in Section 2.6.3.2 where the nodules of control Vetch plants were spherical and greenish/white in appearance when cut open to expose the central nodule structure. These observations indicated that the nodules were at an early stage of development and may not have been effectively fixing atmospheric $N_2$. Figure 7.3.1A shows a section of a Vetch root nodule grown in uncontaminated soil. An enlargement of this nodule is also shown (Figure 7.3.1.B), illustrating clearly the bacteroids and surrounding cortical cells.

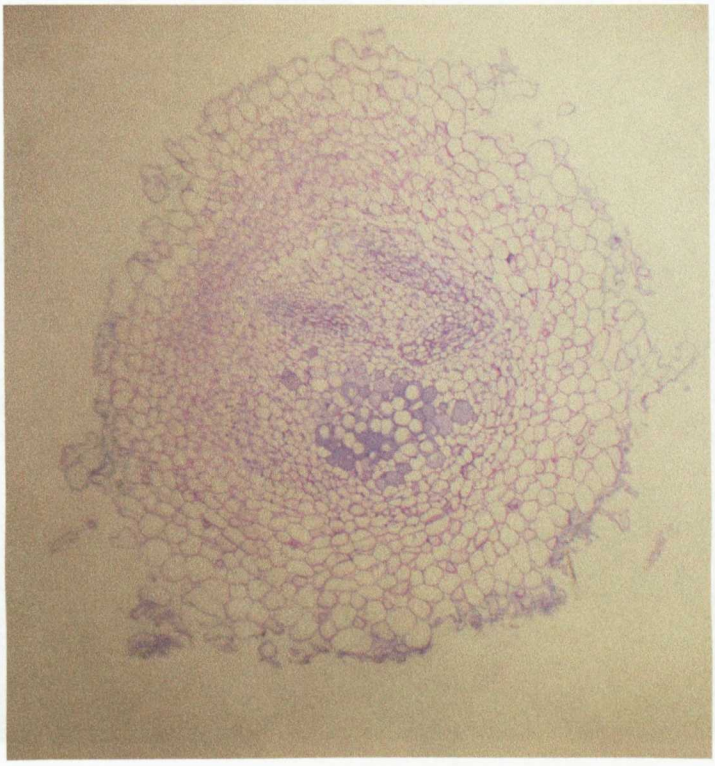
Figures 7.3.1 and 7.3.2 (shown overpage). Sections of Common vetch root nodules grown in uncontaminated and diesel fuel contaminated soil.

In the longitudinal axis of the nodule, the central mass of tissue shows successive stages of host cell invasion and differentiation by rhizobium with the nodules grown in uncontaminated soil having few cells harbouring rhizobia (Figure 7.3.1) and nodules grown in contaminated soil having a large central mass of infected cells and well differentiated cell types (Figure 7.3.2).

While some cells became infected by rhizobia, other cells remained uninfected and developed into a variety of specialised cell types. At an early stage, nodule endodermis (E) developed as a single layer of cells having suberised cell walls, dividing the outer cortex (OC) from the central nodule parenchyma or inner cortex (IC). The inner cortex is distinctive as the cells are tightly packed without intercellular air spaces which constitutes the major barrier for oxygen diffusion.

Within the inner cortex, rhizobia differentiate into intracellular symbionts or bacteroids which are harboured within organelle-like structures called symbiosomes which form bacteroid clusters within cells (BC). Bacteroids induce the nitrogen-fixing (nitrogenase) enzyme system which are protected in the low oxygen microenvironent created by the inner cortex. Vascular tissue (VT) extends into the nodule body allowing exchange of nutrients and water between the nodule and the plant (Brewin, 1991).

(A)



(B)

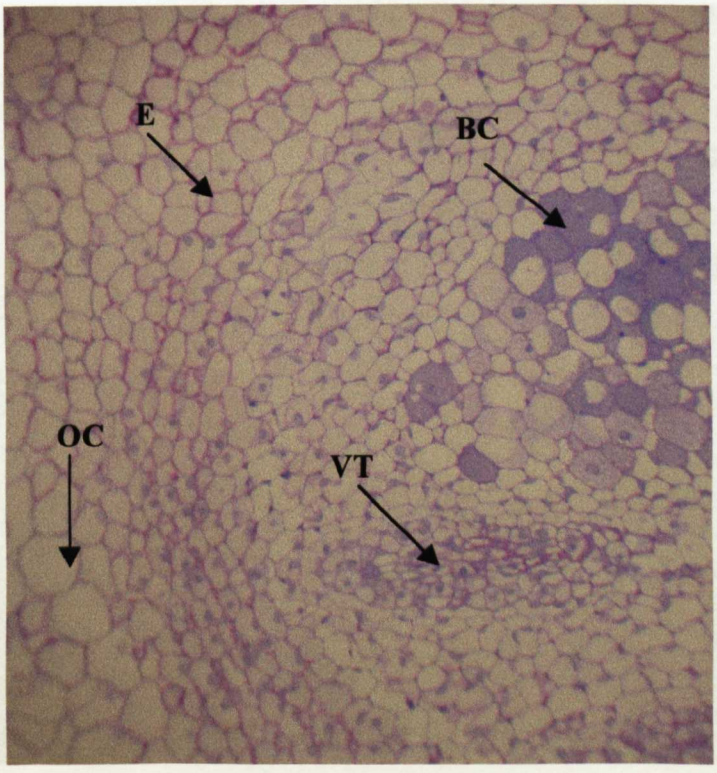
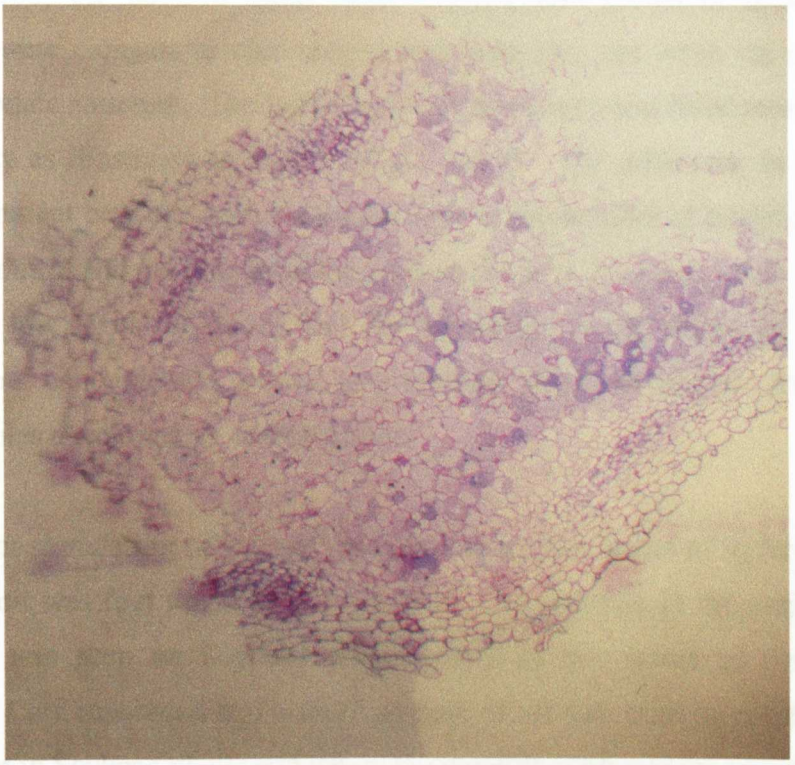



Figure 7.3.1. Light microscopy section (2μ) of a Common vetch nodule (magnification x 40) grown in uncontaminated soil (A) and (B) enlargement of this nodule (magnification x 100).

(A)



(B)

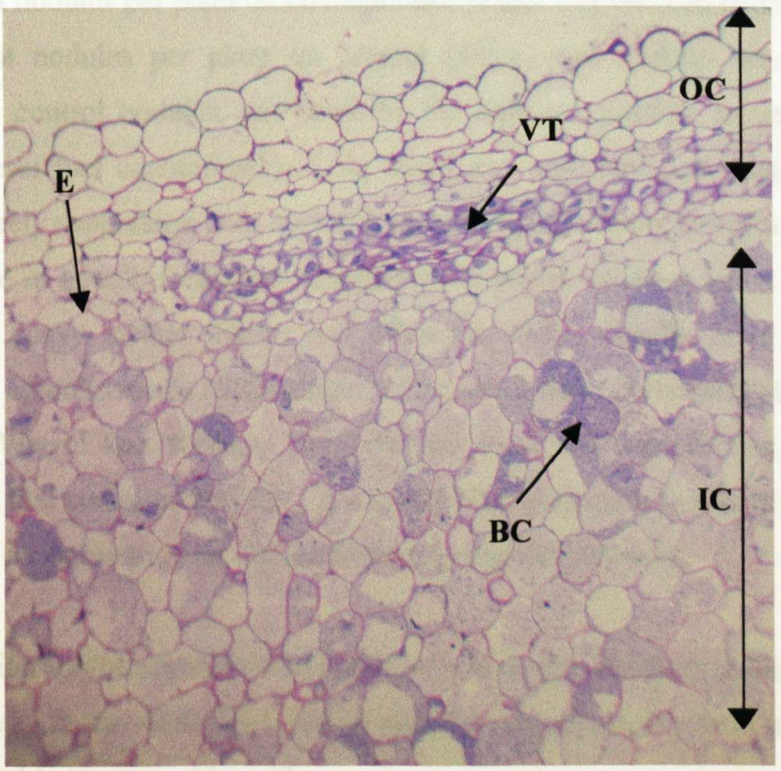



Figure 7.3.2. (A) Light microscopy section (2μ) of a Common vetch nodule (magnification x40) grown in contaminated soil and (B) enlargement of this nodule (magnification x100).

In comparison, the nodules taken from Vetch plants grown in diesel fuel contaminated soil were elongate or club shaped and were pink/red when cut open to expose the inner nodule structure. The bacteroids were numerous and filled most of the central nodule body as illustrated in Figure 7.3.2A and B. The difference in nodule shape is clearly apparent between each section as well as the number of bacteria filled cells between the control and the contaminated nodules (Figures 7.3.1 and 7.3.2). These results suggest, at low levels of diesel fuel contamination (5-10g diesel $kg^{-1}$ soil), nodules formed on contaminated plants are actually more developed than the corresponding nodules developed on control plants.

The apparent stimulation of legume root nodules by low levels of hydrocarbon contamination in soil was first noted by Carr in 1919. An increase in the number of nodules per plant was seen on Soybean plants grown in low levels of crude oil contaminated soil. Carr concluded that a small amount of oil may even be desirable in nodule development in Soybean plants and where the amount of oil was increased to the extent of damaging the plant, there was still some nodule development. Although the total number of root nodules per plant on contaminated plants was reduced compared to the number of root nodules per plant on control plants, root nodules were more developed than the control nodules, as shown by the light microscopy sections. At higher levels of diesel fuel contamination (15g diesel $kg^{-1}$ soil) however, root nodule formation was severely suppressed with only two nodules being present out of a total of 13 plants. Decrease in nodule formation of leguminous plants has been noted in soils contaminated with heavy metals (Porter and Sheridan, 1981, Casella *et al.*, 1988, Mårtensson, 1992), agrochemicals (Mårtensson, 1992), acid rain (Porter and Sheridan, 1981) and PAHs (Wetzel and Werner, 1995) but no work has been carried out on nodulation in diesel fuel contaminated soil. It was therefore important to investigate the effect of diesel fuel contamination on nodule formation and development.

An explanation for the apparent stimulation of nodule development in diesel fuel contaminated soil may be the additional carbon added to the soil in the form of diesel fuel, changed the soil C:N ratio. The addition of a huge carbon source, such as diesel fuel, would widen the C:N ratio which in turn would leave less N available for plant uptake. This has been observed for soils contaminated with petroleum hydrocarbons (Xu and Johnson, 1997) with N becoming immobilised in the microbial biomass hence less N is available for plant uptake. This may cause the Vetch plants growing in contaminated soil to nodulate quicker than Vetch plants grown in uncontaminated soil

which would explain why the contaminated nodules appeared more developed and at a later stage of differentiation than the control nodules even though the seeds were planted at the same time.

In summary, growth of plants in diesel fuel contaminated soil induced the formation of adventitious roots on certain plant species. This unusual developmental effect was likely to have been caused by the presence of petroleum hydrocarbons that had growth hormone-type effects. Branched naphthalenes showed signs of enhancing the development of adventitious roots on Canary grass seedlings. Similar hormone-type substances present in diesel fuel may also have induced other developmental effects, such as negative geotropism.

The pattern of root development was also altered when presented with diesel fuel contaminated soil. Plant roots would avoid diesel fuel contaminated patches if there was 'clean' soil to grow into. Once the 'clean' soil was utilised, only then would plant roots grow into the contaminated patches. As the plants grew, their ability to grow into contaminated soil increased suggesting an acclimation period was present.

Finally, diesel fuel contamination did not affect the strain of microorganisms responsible for root nodule formation in the soil. Root nodules were found on plants grown in contaminated soil and they appeared more developed than the corresponding nodules found on control plants. This unusual observation was attributed to the need for nitrogen fixation by the plants grown in contaminated soil. The soil C:N ratio was off balance, in favour of carbon, due to the huge input of petroleum hydrocarbons therefore the plant-microbial interaction would try to compensate for this.

# CHAPTER EIGHT

## EFFECT OF DIESEL FUEL ON PLANT GROWTH AND DEVELOPMENT: **EFFECT ON THE PLANT**

The previous two chapters have shown some effects of diesel fuel on the viability and germination of selected plant species and its effect on root growth and development.

The plant species that germinated most successfully in diesel fuel contaminated soil in the initial screening experiments (described in Section 6.1) were used in a series of small pot experiments to examine the effect of diesel fuel on the later stages of plant growth and development. A pot trial was also set up using Willow to determine whether variability exists among Willow clones in their ability to grow in diesel fuel contaminated soil.

### 8.1 Initial Mixed Plant Experiment

Ten plant species were chosen from the initial plant screening experiment (see Section 6.1) either for their high germination rate in diesel fuel contaminated soil or, for comparison, their consistently poor germination in differing levels of diesel fuel contaminated soil. These species were used in a small pot experiment, which was split into two sets for ease of sampling. The results could then be used to compare the effect of diesel fuel contamination on susceptible and non-susceptible plant species.

Five species were used in Set up 1 and five species in Set up 2, as described in Section 2.6.3.1. The soil used in the experiment was manufactured John Innes compost which provided a model soil with adequate nutrients for plant growth. The physical and chemical characteristics of this soil type are given in Table 8.1.1.

| Textural properties | | Chemical properties | |
|---|---|---|---|
| % coarse sand | 72.2 | pH | 7.06 |
| % fine sand | 17.6 | LOI % | 10.2 ± 0.20 |
| % silt | 4.9 | Total N % | 0.19 ± 0.01 |
| % clay | 8.4 | Ext. P mg $kg^{-1}$ | 43.6 ± 0.59 |
| Textural class | sand | Ext. K mg $kg^{-1}$ | 292.2 ± 0.80 |

Average values are given. LOI % ± SE, n = 3. Total N % ± SE, n = 5. Extractable P ± SE, n = 4 and Extractable K ± SE, n = 3.

Table 8.1.1 Physical and chemical properties of the John Innes Compost no. 2.

However, the germination rate of both the susceptible and non-susceptible plant species was extremely poor as shown by the results in Table 8.1.2. This was unexpected as many of the seed species were chosen for their high germinating ability in the presence of diesel fuel (e.g., Strong creeping red fescue, Sheep's fescue, Westerwold's ryegrass).

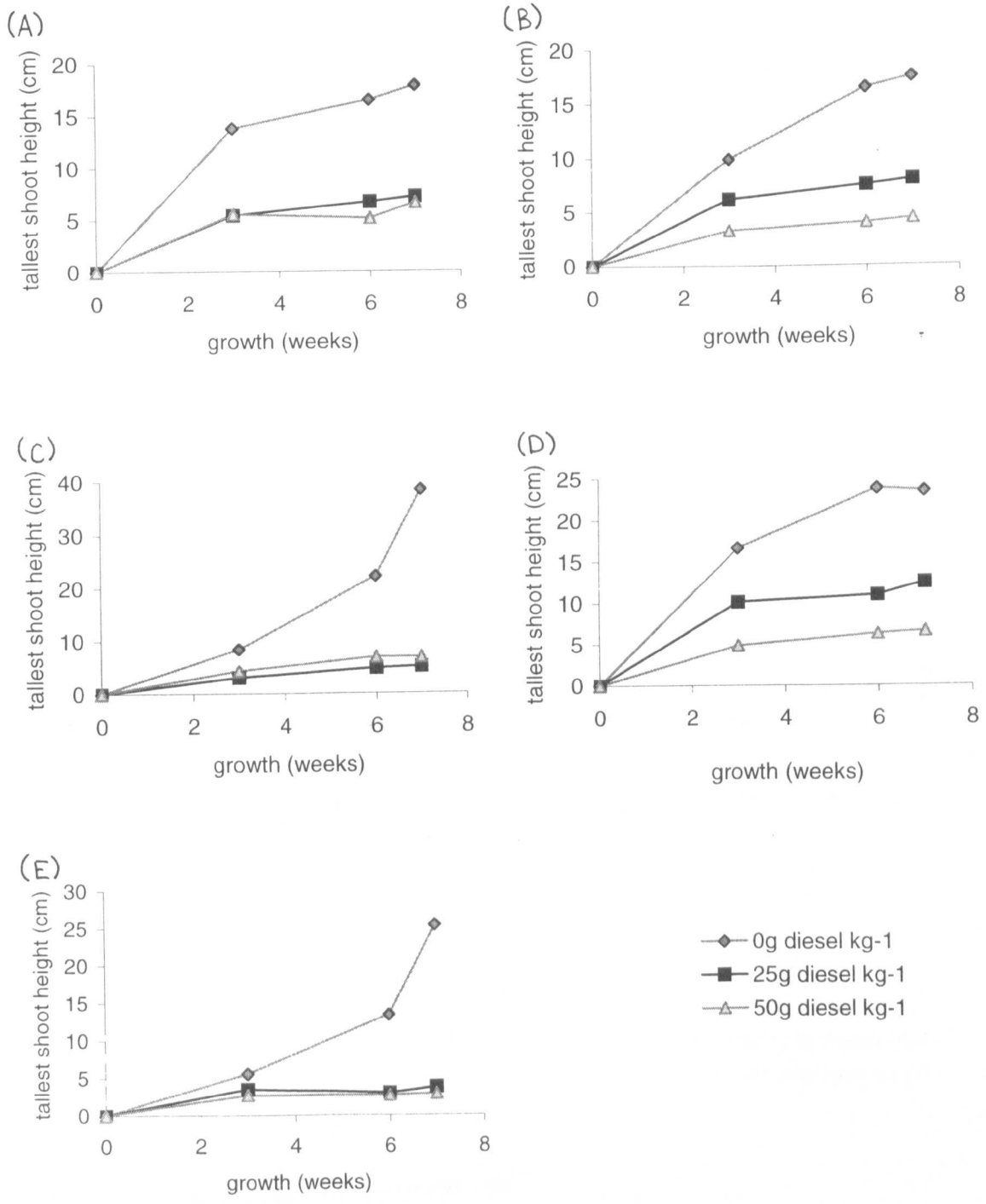
| | % Germination | | |
|---|---|---|---|
| | treatment, g diesel $kg^{-1}$ soil | | |
| Plant species | 0 | 25 | 50 |
| Black grass | - | 24 ± 1.5 | 6 ± 0.5 |
| Cocksfoot | 36 ± 2.0 | 5 ± 1.5 | 2 ± 1.0 |
| Common bent | - | - | - |
| Sweet vernal grass | - | 38 ± 9.5 | 9.5 ± 0.5 |
| Oil seed rape | 76 ± 1.0 | 61 ± 6.0 | 48 ± 1.0 |
| Sheep's fescue | 59 ± 2.5 | 24 ± 3.0 | 7 ± 0.5 |
| Strong creeping red fescue | 64 ± 2.0 | 45 ± 2.5 | 8 ± 0.0 |
| Chewing's fescue | 79 ± 3.5 | 13 ± 4.5 | 6 ± 1.0 |
| Westerwold's ryegrass | 71 ± 0.5 | 28 ± 3.0 | 2 ± 0.0 |
| Black medick | 8 ± 1.0 | 0 ± 0.0 | 2 ± 0.0 |

Average values given ± SE, n = 2. - (blank values) germination rate not recorded

Table 8.1.2 Germination results from initial mixed plant experiment (Set up 1 and 2).

The only seed species that germinated well in both the initial screening experiment and the initial pot trial was Oil seed rape. The germination rate was much lower however, than in the initial screening experiment. The concentrations of diesel fuel chosen for the initial pot experiment were the same as those used in the initial screening experiment (0, 25 and 50g diesel $kg^{-1}$ soil) as although the concentrations were high, the seeds seemed capable of germinating at these concentrations. This appeared not to be the case in the pot experiment. It became apparent that covering the seeds with soil had a greater effect on the germination rate and health of the plant than expected. The seed would be surrounded by diesel fuel contaminated soil and when the shoot and root tips started to emerge from the germinating seed, they would be in close proximity to the diesel fuel. The entire germinating seed would also be enclosed by diesel fuel volatiles, which may also affect germination. These factors were investigated and the results discussed in Sections 6.2 (Phytotoxicity of volatile diesel fuel) and Section 6.4 (Effect of diesel fuel on seed viability). The results from these investigations showed that the volatile diesel fuel fraction had a large influence on germination and seed viability was also affected by diesel fuel contaminated soil in close proximity to the seed.

The small number of plants that did germinate and grow in the 25g and 50g diesel $kg^{-1}$ contaminated soils were very different from the plants of the same species grown in uncontaminated soil. The overall heights of plants grown in diesel fuel contaminated soil were stunted compared to plants grown in uncontaminated soil as shown in Figure 8.1.3. All the plant heights of species grown in diesel fuel contaminated soil were reduced by more than 50% of the control plant height except for Westerwold's ryegrass, whose height improved towards the end of the experiment (Figure 8.1.3 G). Sweet vernal grass (Figure 8.1.3 B) and Meadow foxtail (Figure 8.1.3 D) showed a distinct difference between plant height in the 25g and 50g diesel $kg^{-1}$ soil treatments. The remaining plant species were just as badly affected by 25g diesel $kg^{-1}$ soil treatment as they were by the 50g diesel $kg^{-1}$ soil treatment. As the weeks progressed, plant height increased slightly in most of the plant species investigated, suggesting whatever had suppressed growth initially was less phytotoxic or the plant was adapting to tolerate the conditions.




Average values given, n = 2.

Figure 8.1.3. Tallest shoot height of selected plant species grown in varying levels of diesel fuel contaminated soil after 7 weeks. A) Oil seed rape cv. Martina, B) Sweet vernal grass, C) Cocksfoot, D) Meadow foxtail and E) Common bent.

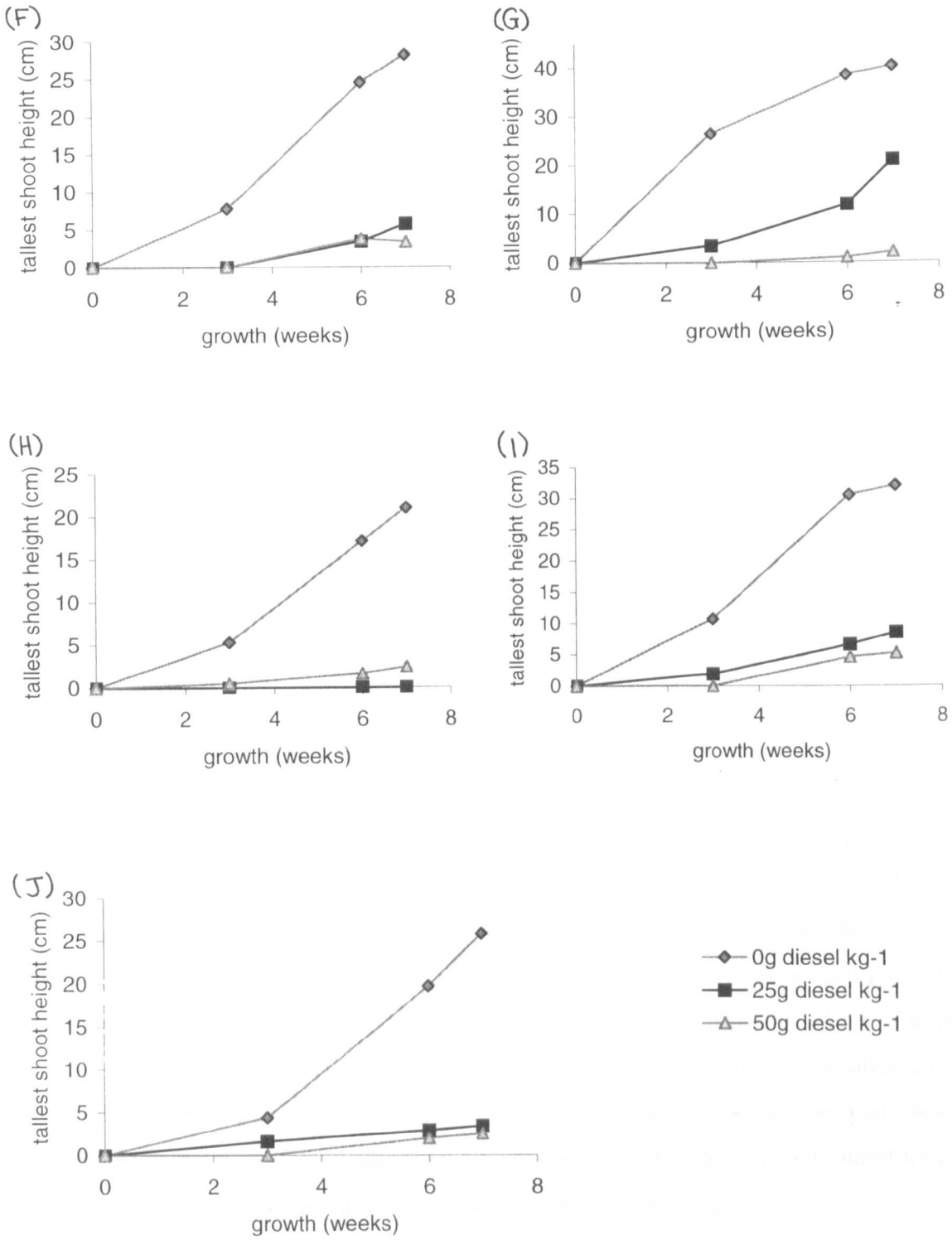



Figure 8.1.3 continued. Tallest shoot heights of selected plant species grown in varying levels of diesel fuel contaminated soil after 7 weeks.

F) Chewing's fescue, G) Westerwold's ryegrass, H) Black medick, I) Strong creeping red fescue and J) Sheep's fescue.

The visual condition of the plants grown in contaminated soil was good, apart from the oil seed rape, whose leaves were badly discoloured. There was no sign of severe leaf burn, which would indicate the plant was transpiring some of the more volatile hydrocarbons. All in all, the general condition of the plants was good but the drastic reduction in plant biomass showed just how badly affected the plants grown in diesel fuel contaminated soil really were. Growth in diesel fuel contaminated soil noticeably reduced the production of top growth of every plant screened. Figure 8.1.4 shows two plant species grown in 0g, 25g and 50g diesel $kg^{-1}$ contaminated soil for six weeks. Oil seed rape was one of the more successful plant species chosen as its germination rate was 76%, 61% and 48% in 0, 25 and 50g diesel $kg^{-1}$ soil respectively. Although the germination rate was only moderately affected, the rate of growth was severely affected, as is illustrated in Figure 8.1.4. The Meadow foxtail's germination rate was badly affected but the overall plant height was only slightly reduced in the 25g diesel $kg^{-1}$ soil treatment compared to the control plants (Figure 8.1.4). Meadow foxtail did not grow well at the higher level of contamination (50g diesel $kg^{-1}$ soil).

The reduction in the average height of contaminated plants grown in diesel fuel contaminated soil is reflected in the overall shoot and root biomass results collected from contaminated plants. At harvest, shoot and root biomass was collected from both control and contaminated treatments then oven dried as described in Section 2.7.1 and 2.7.2 to obtain oven dried biomass weights. The results from Set up 1 and Set up 2 are given separately in Tables 8.1.5 and 8.1.6. The total oven dried shoot weight per pot is given for plants in Set up 1 due to overcrowding of the pots caused by a high planting density. The high density of plants in each pot prevented an accurate germination count from being conducted hence the results could not be given on a weight per plant basis. The density of planting was reduced in Set up 2 to overcome this problem therefore the results from Set up 2 are presented as total oven dry weight per plant.

The results clearly show the effect that growing plants on diesel fuel contaminated soil had on plant biomass. All the plant species investigated had reduced biomass when grown in diesel fuel contaminated soil but the reduction in biomass varied greatly between plant species. For example, Black grass plant biomass was reduced to 44% of the control biomass value in 25g diesel $kg^{-1}$ contaminated soil and to 0.2% in 50g diesel $kg^{-1}$ contaminated soil. Oil seed rape plant biomass on the other hand, was reduced to 14% and 10% of the control biomass values in 25g and 50g diesel $kg^{-1}$ contaminated soil.


Figure 8.1.4. Oil seed rape (top) and Meadow foxtail (bottom) plants grown in 0g, 25g and 50g diesel $kg^{-1}$ soil for 6 weeks.

| | Av. total shoot dry wt. per pot (mg) | | |
|---|---|---|---|
| | Treatment, g diesel $kg^{-1}$ soil | | |
| Plant species | 0 | 25 | 50 |
| Black grass | 1784 ± 356.8 | 781.4 ± 0.15 | 3.9 ± 0.6 |
| Cocksfoot | 926 ± 247.8 | 10.3 * | 2.8 * |
| Common bent | 1075 ± 43.9 | 52.7 ± 20.6 | 0 |
| Sweet vernal grass | 1261 ± 79.2 | 70.5 ± 30.0 | 12.4 ± 1.7 |
| Oil seed rape | 5044 ± 8.0 | 722.9 ± 70.6 | 527.6 ± 00.5 |

Average values are given (in mg) ± standard errors, n = 2 unless superscripted by* where n = 1.

Table 8.1.5. Total shoot weights (oven dry weight) per pot for plant species grown in Set up 1.

| | Av. Shoot wt. per plant (mg) | | | Av. Root wt. per plant (mg) | | |
|---|---|---|---|---|---|---|
| | Treatment, g diesel $kg^{-1}$ soil | | | | | |
| Plant species | 0 | 25 | 50 | 0 | 25 | 50 |
| Sheep's fescue | 128.2 ± 44.0 | 1.1 ± 0.13 | 0.7 ± 0.44 | 37.0 ± 1.2 | 1.2 ± 0.00 | 1.5 ± 0.7 |
| Strong creeping red fescue | 170.0 ± 14.9 | 12.75 ± 10.3 | 1.4 ± 0.00 | 55.5 ± 6.8 | 2.25 ± 0.3 | 0.7 ± 0.0 |
| Chewing's fescue | 8.6 ± 1.2 | 0.9 ± 0.3 | 0.97 ± 0.74 | 65.0 ± 12.0 | 3.1 ± 1.6 | 4.3 ± 2.0 |
| Westerwold's ryegrass | 161.8 ± 4.6 | 10.9 ± 6.7 | 8.1 ± 0.00 | 188.0 ± 7.45 | 6.75 ± 4.1 | 0 |
| Black medick | 1198.4 ± 279.8 | 0 | 5.35 ± 2.65 | 325.5 ± 98 | 0 | 3.3 ± 0.30 |

Average values are given (in mg) ± SE, n = 2

Table 8.1.6. Total shoot and root weights (oven dry weight) per plant for plant species grown in Set up 2.

Drastic reductions in both shoot and root biomass were observed for all the plant species investigated in this experimental set up. Although the seed species chosen for this experiment were capable of germinating in high levels of diesel fuel (e.g. 25g and 50g diesel $kg^{-1}$ soil) as illustrated in Section 6.1, they did extremely poorly in this experimental set up. It was therefore decided that lower diesel fuel contamination levels would be used in subsequent plant experiments to allow a more sizeable plant biomass to develop. This should allow any difference in diesel fuel bioremediation due to the influence of plant growth to be seen.

## 8.2 Grasses versus Legumes

Four plant species were chosen from the initial germination experiment (see Section 6.1) that germinated well in diesel fuel contaminated soil. Two species from the family *Gramineae* and two species from *Leguminosae* were chosen to investigate if the *Leguminosae* grew better in diesel fuel contaminated soil, due to their close association with nitrogen fixing microorganisms than members of the *Gramineae*.

The soil used in this experiment (Darvel soil) was a 'real' soil, sampled fresh from site and prepared for use. A freshly collected soil was chosen to provide realistic soil conditions. The soils physical and chemical charateristics are given in Table 8.2.1 below.

| Textural properties | | Chemical properties | |
|---|---|---|---|
| % coarse sand | 33.5 | pH | 7.30 |
| % fine sand | 20.0 | LOI % | 9.1 ± 0.16 |
| % silt | 22.0 | Total N % | 0.32 ± 0.01 |
| % clay | 24.4 | Ext. P mg $kg^{-1}$ | 49.4 ± 0.72 |
| Textural class | Sandy clay loam | Ext. K mg $kg^{-1}$ | 169.0 ± 0.75 |

Average values are given. LOI % ± SE, n = 3. Total N % ± SE, n = 5. Extractable P ± SE, n = 4 and Extractable K ± SE, n = 3.

Textural characteristics taken from Metwaly, PhD Thesis, University of Glasgow, 1999.

Table 8.2.1. Physical and chemical properties of Darvel soil.

Agronomic performance in diesel fuel contaminated soil was again assessed by measuring germination rate, plant height (tallest shoot height and majority shoot height) and shoot and root biomass. In addition, the ability of Common vetch plants and Red clover to develop root nodules and the onset of maturation indicated by flowering/seeding of each species was investigated.

Germination rate was measured every three weeks from planting until harvest at 15 weeks but only the results from the 3 week and 15 week measurements are given in Table 8.2.2 on the next page.

A reduction in germination rate was observed for all the plant species investigated except for the Common vetch plants grown in 5g diesel $kg^{-1}$ soil initially. At three weeks the % germination rate was increased to 91.1% in the 5g diesel $kg^{-1}$ soil compared to 82.2% in the control soil. By harvest time at 15 weeks however, the number of plants surviving had fallen to 60% in the 5g diesel $kg^{-1}$ soil compared to 84.4% in the control soil. This initially high germination rate in the low level of diesel fuel contaminated soil may indicate the Common vetch seeds were not affected by low levels of diesel fuel until the seed coats had split and germination had begun. Once the primary root and shoot had emerged, the seedlings were more susceptible to diesel fuel toxicity. The Red clover and Westerwold's ryegrass seeds responded much the same to diesel fuel contamination at the start of the experiment as they did at the end whereas the Common vetch and Strong creeping red fescue seeds appeared to improve by the 15 week measurement. Common vetch plants grown in 20g diesel $kg^{-1}$ soil increased from 8.9% germination rate at 3 weeks to 37.8% germination rate at 15 weeks. Strong creeping red fescue increased to 17.8% germination rate in 10g diesel $kg^{-1}$ soil at 15 weeks from 4.4% germination rate at 3 weeks.

| | % germination | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 weeks g diesel $kg^{-1}$ soil | | | | 15 weeks g diesel $kg^{-1}$ soil | | | |
| | 0 | 5 | 10 | 20 | 0 | 5 | 10 | 20 |
| Plant species | | | | | | | | |
| Westerwold's ryegrass | 93.1 ± 0.7 | 17.8 ± 2.2 | 6.7 ± 0.6 | 0 ± 0 | 93.3 ± 0.6 | 20 ± 2.1 | 4.4 ± 0.3 | 0 ± 0 |
| Common Vetch | 82.2 ± 1.3 | 91.1 ± 1.3 | 35.6 ± 1.7 | 8.9 ± 0.9 | 84.4 ± 1.2 | 60 ± 1.5 | 24.4 ± 1.7 | 37.8 ± 1.2 |
| Red clover | 42.2 ± 1.3 | 20 ± 0.6 | 11.1 ± 0.7 | 6.7 ± 1.0 | 37.8 ± 1.2 | 22.2 ± 0.7 | 15.6 ± 1.3 | 6.7 ± 1.0 |
| Strong Creeping Red Fescue | 97.8 ± 0.3 | 46.7 ± 2.5 | 4.4 ± 0.7 | 4.4 ± 0.7 | 97.8 ± 0.3 | 37.8 ± 2.3 | 17.8 ± 1.3 | 0 ± 0 |

Average values are given ± standard errors (SE), n = 3.

Table 8.2.2 Germination rate for species of grass and legumes grown in diesel fuel contaminated soil at 3 and 15 weeks growth.

Tallest plant height and majority plant height were measured throughout the experiment until harvest. The results for tallest plant height are illustrated in Figure 8.2.3. Plants grown in contaminated soil were stunted compared to the same plants grown in control soil. Common vetch plants showed an almost linear decrease in plant height with increasing diesel fuel concentration. Westerwold's ryegrass plant height was very similar at the 5g and 10g diesel $kg^{-1}$ soil level but Westerwold's ryegrass failed to grow at the 20g diesel $kg^{-1}$ soil level. Red clover and Strong creeping red fescue plant heights were reduced compared to the control plants with Red clover performing slightly better at the higher levels of diesel fuel contamination than Strong creeping red fescue.

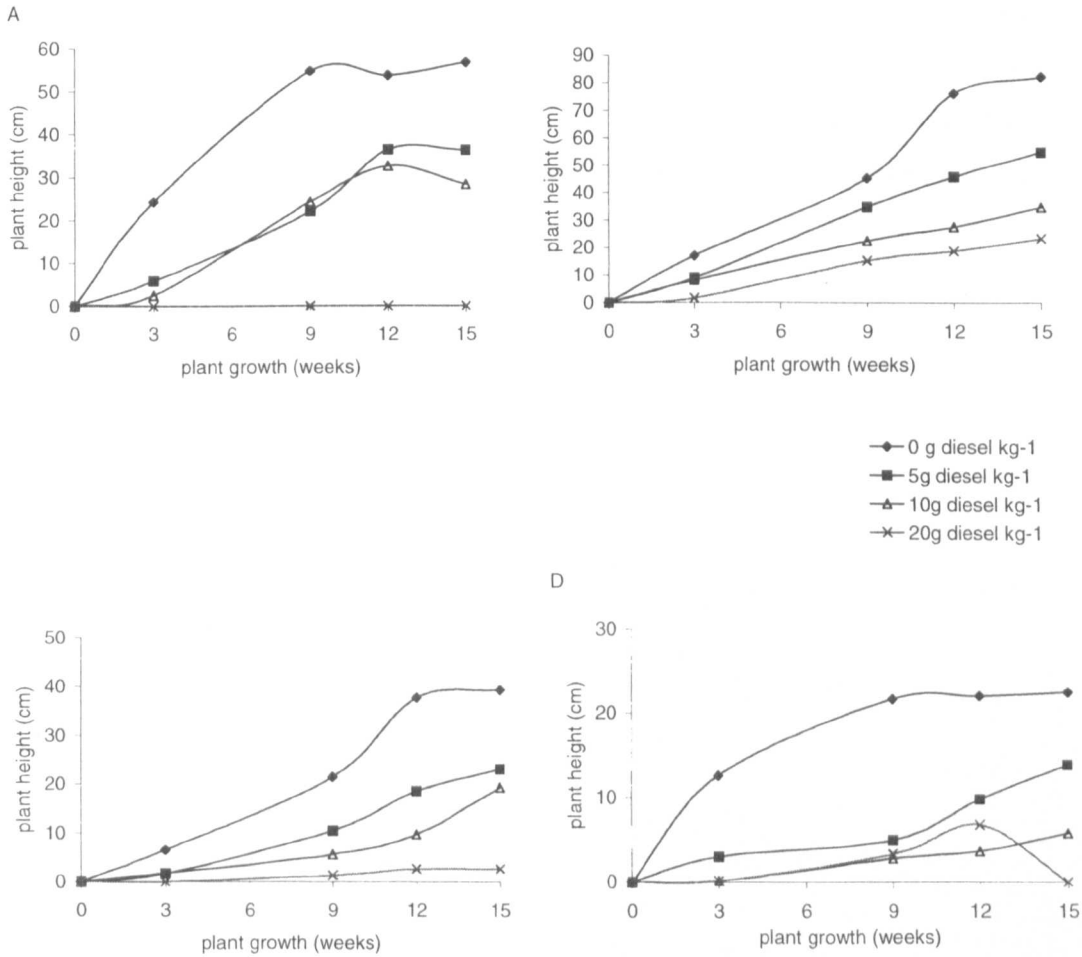



Figure 8.2.3 Tallest plant height measurements of A) Westerwold's ryegrass, B) Common vetch, C) Red clover and D) Strong creeping red fescue over the length of the experiment.

The decrease in plant growth indicated by the reduction in shoot height was reflected by the shoot and root biomass results for the majority of plant species investigated. The only exception to this rule was Westerwold's ryegrass, whose shoot biomass values per plant appeared stimulated by growing in low levels of diesel fuel contaminated soil (5g and 10g diesel $kg^{-1}$ soil). Table 8.2.4 illustrates the average oven dry shoot and root biomass results for the four plant species investigated. Shoot biomass per plant was increased by 32% and 347% compared to the control plants biomass per plant in 5g and 10g diesel $kg^{-1}$ soil respectively. This large increase in shoot biomass for plants grown in the 10g diesel $kg^{-1}$ soil treatment was reflected in the root biomass results by an increase of 158% compared to the control root biomass. This apparent stimulation of growth may be due, in part, to the additional space allowed to plants grown in contaminated soil pots. Germination rates were reduced in contaminated soil hence the planting density was less in contaminated pots compared to control pots. This would allow more room for contaminated plants to grow and produce biomass. The increase in biomass cannot be explained entirely by differences in planting density and it appears that Westerwold's ryegrass growth is stimulated in low levels of diesel fuel contaminated soil.

| | g Shoot biomass oven dry wt per plant | | | | g Root biomass oven dry wt per plant | | | |
|---|---|---|---|---|---|---|---|---|
| | g diesel $kg^{-1}$ soil | | | | g diesel $kg^{-1}$ soil | | | |
| | 0 | 5 | 10 | 20 | 0 | 5 | 10 | 20 |
| Plant species | | * | * | | | * | * | |
| Westerwold's ryegrass | 0.091 ± 0.07 | 0.120 ± 0.13 | 0.407 ± 0.17 | 0 ± 0 | 0.076 ± 0.08 | 0.067 ± 0.07 | 0.196 ± 0.05 | 0 ± 0 |
| Common Vetch | 0.275 ± 0.12 | 0.119 ± 0.26 | 0.072 ± 0.10 | 0.042 ± 0.05 † | 0.117 ± 0.07 | 0.066 ± 0.30 | 0.044± 0.08 | 0.046 ± 0.05 |
| Red clover | 0.792 ± 0.19 | 0.396 ± 0.15 | 0.100 ± 0.11 * | 0.093 ± 0 | 0.529 ± 0.09 | 0.163 ± 0.06 | 0.46 ± 0.05 * | 0.041 ± 0 |
| Strong Creeping Red Fescue | 0.056 ± 0.05 | 0.009 ± 0.02 | 0.003 ± 0.01 | 0 ± 0 | 0.067± 0.07 | 0.013 ± 0.04 | 0.005 ± 0.01 | 0 ± 0 |

Average values are given ± standard errors (SE), n = 3 unless denoted * n = 2 or † n = 1.

Table 8.2.4 Average oven dry shoot and root biomass results for Westerwold's ryegrass, Red clover, Common vetch and Strong creeping red fescue plants grown in varying levels of diesel fuel contaminated soil.

Two other observations were made during the course of this pot experiment regarding the developmental behaviour of plants grown in diesel fuel contaminated soil. The first observation was concerned with the development of root nodules on the two species of legume used in this trial. The possible advantage that legumes would have over other plant species would be in their ability to fix atmospheric nitrogen to produce their own source of nitrogen. This nitrogen fixing ability would allow leguminous plants to grow in soil with low levels of available nitrogen which is frequently the case with hydrocarbon contaminated soils. A huge input of petroleum hydrocarbons in the form of diesel fuel for example, would alter the C:N ratio of the soil in favour of carbon and the little available nitrogen present would become immobilised in microbial biomass. Any plant that produced its own source of available nitrogen may therefore grow more successfully in diesel fuel contaminated soils. Infection of roots of leguminous plants with the appropriate species of *Rhizobium* or *Brady-rhizobium* is required for the formation of root nodules (Brock and Madigan, 1991). Decrease in nodule formation has been noted by other authors in contaminated soils (Porter and Sheridan, 1981., Casella *et al.*, 1988., Mårtensson, 1992., Wetzel and Werner, 1995) but no work has been carried out on nodulation of legumes in diesel fuel contaminated soil. Observations from this pot experiment showed that nodules were present on roots of both control and contaminated plants which suggests that the species of *Rhizobium* responsible for infecting the roots of both Red clover and Common vetch plants were unaffected by diesel fuel contamination. In addition, when root nodules taken from control and contaminated plants were dissected to show their internal structures, a very clear difference between control and contaminated nodules was observed. Nodules taken from control plants of both Red clover and Common vetch grown in uncontaminated soil had small nodules with pale, almost white internal structures. This suggests a lack of leghaemoglobin, the red coloured protein responsible for binding oxygen within the nodule structure, which is always found in healthy nitrogen fixing nodules (Brock and Madigan, 1991). Nodules taken from contaminated Red clover and Common vetch plants had swollen nodules with extremely pink or red internal structures, which suggests the nodules were actively fixing nitrogen. Although this observation was noted on both Red clover and Common vetch plants, the difference was more apparent in Common vetch root nodules. This difference in the development of root nodules was investigated in more detail in a later pot experiment (Section 9.2) and the results from this experiment discussed fully in Section 5.3.

The second observation made during the course of the experiment was the onset of maturation of the plants under study. Plants grown in diesel fuel contaminated soil were found, time and time again, to be stunted compared to plants grown in uncontaminated soil. The reduction in plant height suggested that diesel fuel was retarding growth of the plant but it was unclear whether developmental changes in the plant, such as the onset of flowering, were being retarded also. The control Red clover plants began to flower after 10 weeks growth where no flowering was apparent on the contaminated plants even at harvesting (15 weeks). The Common vetch plants began to flower and produce seed pods after 12 weeks growth where no seed pods were present on any of the contaminated plants. At harvesting, the Common vetch plants grown in 5g diesel $kg^{-1}$ soil were flowering and had seed pods present but none of the plants grown in higher diesel fuel contamination levels showed signs of doing so. Figure 8.2.5 illustrates the production of seed pods on Common vetch plants where none are present on the corresponding contaminated plants. The Westerwold's ryegrass plants growing in both control and contaminated soil began to seed around 14 weeks growth. The Strong creeping red fescue had not seeded by the harvest date. The lag phase observed in flowering/seeding of the plant species investigated suggest diesel fuel is affecting plant physiology and slowing the plants usual flowering cycle. The fact that Common vetch plants did eventually flower and produce seed pods suggests that diesel fuel contamination is delaying the developmental process rather than preventing it from happening.

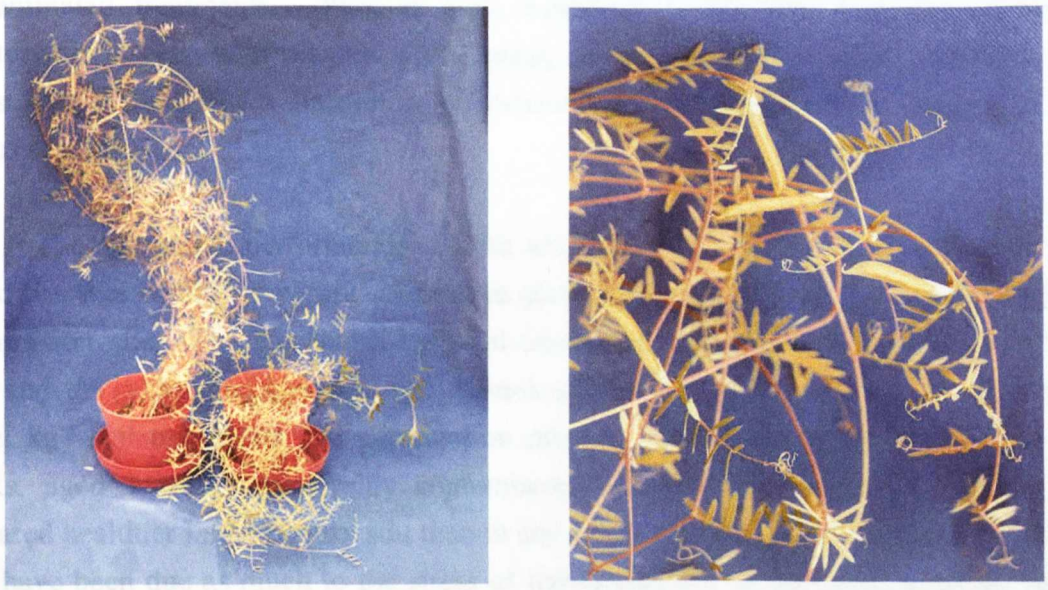


Figure 8.2.5 Production of seed pods on Common vetch plants where none are present on the corresponding contaminated plants. A) Control plant on left and contaminated plant on right. B) Enlargement of control plant showing seed pods.

## 8.3 Toxicity of 'Aged' Soil

A recurring problem observed during the small pot experiments was the low germination rate of seeds in diesel fuel contaminated soil that had previously been found to germinate well. Seeds in the initial screening experiment (Section 6.1) germinated and grew successfully but when the same seeds were planted in contaminated soil in the pot experiments, their germination rate decreased. The effect that covering the seeds with contaminated soil had on germination and growth was investigated in Section 6.2 and Section 6.4. It was found that volatile diesel fuel components had a large influence on seed germination and that diesel fuel affected the viability of seeds possibly by physically impeding the germinating seed.

An experiment was designed, similar to the experiment described in Section 2.6.1.4, to further test the influence of volatile and low molecular weight diesel fuel components on a larger scale. 0g, 25g and 50g diesel $kg^{-1}$ soil treatments were set up at progressively later stages of aging of contaminated soil as described in Section 2.6.3.6. John Innes compost No. 2 was contaminated with diesel fuel at different times to

prepare treatments that had aged 4 weeks, treatments that had aged 1 week and freshly contaminated treatments. Pots at each treatment level were then seeded with Westerwold's ryegrass or planted with 2 week old Westerwold's ryegrass seedlings to investigate if there was a discernible difference between different plant stages – seed versus seedling.

The agronomic performance of both seed and seedlings was assessed. After 1 week, the Westewold's ryegrass seeds were germinating in the control soil with a 82% germination rate. The 25g diesel $kg^{-1}$ soil treatments had germination rates of 48%, 66% and 4% in the aged 4 weeks, aged 1 week and freshly contaminated soils. The 50g diesel $kg^{-1}$ soil treatments had germination rates of 10%, 4% and 0% in the aged 4 weeks, aged 1 week and freshly contaminated soils. The transplanted seedlings appeared healthier in the control soil than in any of the contaminated treatments but this may have been due as much to the stress of transplantation as the effect of diesel fuel contamination. As the experiment progressed, the seedlings grew more happily in all the treatments.

After 3 weeks, the seeded pots were improving, with germination rates increasing in all pots except the freshly contaminated 50g diesel $kg^{-1}$ soil treatment which failed to show any signs of growth. Table 8.3.1 shows the germination results of the seeded treatments.

| Treatment g diesel $kg^{-1}$ soil | Germination rate % |
|---|---|
| 0g | 84 ± 0.5 |
| 25g fresh | 8 ± 0.0 |
| 25g 1 week | 72 ± 5.5 |
| 25g 4 weeks | 76 ± 2.0 |
| 50g fresh | 0 ± 0.0 |
| 50g 1 week | 16 ± 0.0 |
| 50g 4 weeks | 40 ± 2.5 |

Average values are given ± SE, n = 2.

Table 8.3.1 Germination results of the Westerwold's ryegrass seeded treatments.

After 6 and 9 weeks growth the germination rate and tallest shoot height were measured for the seeded pots and survival rate of seedling and tallest shoot height measured for the pots transplanted with seedlings. The results are given in Table 8.3.2.

The germination rate of the seeds had not increased since the 3 week measurement. In fact, the number of plants decreased in the 4 week aged 50g diesel $kg^{-1}$ soil from 40% germination rate at 3 weeks to 30% germination rate at 9 weeks. The number of seedlings surviving transplantation into contaminated soil was high. Only at the freshly contaminated 50g diesel $kg^{-1}$ soil level was the survival rate drastically reduced as illustrated in Table 8.3.2. Plant heights were tallest in control soil for both the seeded pots and pots containing seedlings. Plants heights were lower in the 50g diesel $kg^{-1}$ soil contaminated treatments than in the 25g diesel $kg^{-1}$ soil. It was apparent that diesel fuel contamination was still affecting plant growth after 4 weeks aging as plant height and germination rate were lower than the control values.

| | 6 weeks | | 9 weeks | |
|---|---|---|---|---|
| Treatments | % germination | Tallest shoot height cm | % germination | Tallest shoot height cm |
| Seeds | | | | |
| 0g | 84 ± 0.5 | 39.5 ± 1.5 | 84 ± 0.5 | 45.0 ± 1.0 |
| 25g fresh | 8 ± 0.0 | 20.8 ± 0.8 | 8 ± 0.0 | 24.3 ± 0.8 |
| 25g 1 week | 72 ± 4.0 | 24.5 ± 1.5 | 72 ± 4.0 | 26.8 ± 1.8 |
| 25g 4 weeks | 74 ± 0.5 | 27.8 ± 0.3 | 74 ± 0.5 | 27.5 ± 0.3 |
| 50g fresh | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 |
| 50g 1 week | 16 ± 0.0 | 8.5 ± 0.0 | 14 ± 0.5 | 12.0 ± 1.0 |
| 50g 4 weeks | 32 ± 4.0 | 10.5 ± 0.5 | 30 ± 3.5 | 15.0 ± 7.5 |
| | % survival rate | Tallest shoot height cm | % survival rate | Tallest shoot height cm |
| Seedlings | | | | |
| 0g | 96 ± 1.0 | 38 ± 4.0 | 96 ± 1.0 | 38.5 ± 3.5 |
| 25g fresh | 88 | 25.5 | 88 | 24.0 |
| 25g 1 week | 88 | 23.0 | 88 | 26.0 |
| 25g 4 weeks | 66 ± 0.5 | 25.3 ± 2.8 | 68 ± 1.0 | 32.0 ± 4.0 |
| 50g fresh | 28 ± 0.0 | 12.3 ± 0.3 | 28 ± 1.0 | 15.0 ± 0.5 |
| 50g 1 week | 68 | 23.0 | 84 | 28.0 |
| 50g 4 weeks | 68 | 18.5 | 60 | 22.0 |

Average values are given ± SE, n = 2 unless no SE given where n = 1.

Table 8.3.2 Germination rate and tallest shoot height of the seeded pots and survival rate and tallest shoot height for pots transplanted with seedlings.

The seedlings appeared to perform slightly better in the contaminated soils than the seeded treatments as can be seen in Figures 8.3.3 and 8.3.4. Figure 8.3.3 shows the seeded treatments at all levels of diesel fuel contamination after 4 and 9 weeks growth. The seeds germinated and grew more successfully at the lower contamination level (25g diesel $kg^{-1}$ soil) and in the 4 week aged soil. Figure 8.3.4 shows the treatments transplanted with seedlings after 4 and 9 weeks growth. After 9 weeks, there was a difference between the 25g and the 50g seedling treatments but it was not as apparent as with the seeded treatments. Again the seedlings appeared to grow more successfully in the aged soils but the biomass values do not support this statement. Table 8.3.5 shows the oven dry shoot and root biomass results for Westerwold's ryegrass plants in all the treatment levels.

Both shoot and root biomass per plant was drastically reduced in all levels of diesel fuel treated soil for the seeded treatments. As expected, the reduction was more drastic in the 50g diesel $kg^{-1}$ soil level than in the 25g diesel $kg^{-1}$ soil level. The reduction in shoot biomass was actually less in the freshly contaminated 25g diesel $kg^{-1}$ soil treatment than in the aged treatments with biomass being 19.3%, 9.2% and 12.3% of the control biomass in the freshly contaminated, aged 1 week and aged 4 weeks treatments. The root biomass results followed this trend. No seeds germinated in the freshly contaminated 50g diesel $kg^{-1}$ soil treatment and shoot biomass was reduced to 3.4% of the control biomass in both the aged 1 week and aged 4 weeks treatments. The seedlings appeared to grow more successfully in the contaminated soils in Figure 8.3.4 but the biomass results again show a drastic reduction in biomass. Shoot biomass was reduced to 19.5%, 20% and 24.5% of the controls shoot biomass in the freshly contaminated 25g diesel $kg^{-1}$ soil, aged 1 week and aged 4 weeks seedling treatments. Root biomass was reduced even further with the 25g contaminated treatment averaging a root biomass of 5.4% of the controls root biomass. Seedlings grown in the 50g diesel $kg^{-1}$ soil treatments were, on average, reduced to 10.8% of the controls shoot biomass and 6.7% of the controls root biomass.

Figure 8.3.3. Seeded treatment at all levels of diesel fuel contamination after 4 (left) and 9 (right) weeks growth.

Figure 8.3.4. Seedling treatment at all levels of diesel fuel contamination after 4 (left) and 9 (right) weeks growth.

| | Shoot biomass | Root biomass |
|---|---|---|
| Treatment | Dry weight mg per plant | Dry weight mg per plant |
| Seeds | | |
| 0g | 128.3 ± 1.3 | 266.4 ± 47.6 |
| 25g fresh | 24.8 ± 0.1 | 22.8 ± 2.9 |
| 25g 1 week | 11.8 ± 0.7 | 18.0 ± 1.3 |
| 25g 4 weeks | 15.8 ± 1.0 | 16.1 ± 5.7 |
| 50g fresh | 0 ± 0.0 | 0 ± 0.0 |
| 50g 1 week | 4.3 ± 0.4 | 3.6 ± 0.2 |
| 50g 4 weeks | 4.2 ± 3.5 | 4.5 ± 3.0 |
| Seedlings | | |
| 0g | 116.1 ± 3.2 | 453.0 ± 60.0 |
| 25g fresh | 22.6 | 28.1 |
| 25g 1 week | 23.2 | 21.9 |
| 25g 4 weeks | 28.5 ± 6.2 | 23.9 ± 9.1 |
| 50g fresh | 8.8 ± 3.1 | 9.0 ± 0.1 |
| 50g 1 week | 15.2 | 13.9 |
| 50g 4 weeks | 13.7 | 7.4 |

Average values are given ± SE, n = 2 unless no SE given where n = 1.

Table 8.3.5. Shoot and root biomass values for Westerwold's ryegrass seeded and seedling transplanted treatments after 9 weeks growth.

A clear difference was seen between the freshly contaminated and aged soil treatment levels in the seeded treatments but not in the seedling treatments. The seedlings seemed to survive and grow more successfully than the newly germinated seeds in the contaminated treatments and the total biomass collected at harvest was greater for the seedlings in all treatments than the seeds. This may be due to the 'head start' the seedlings have over the seeds on producing biomass. It appeared that plants at a later stage of growth could survive and grow more successfully in diesel fuel contaminated soil than seeds. However, transplanting seedlings was extremely hard to do and the plants were probably unnecessarily stressed and may have been damaged due to this transplantation process, adding an error to the results. In addition, the 4 week aged diesel fuel contaminated soil had considerable water repellence making wetting of the soil very difficult. This could also have biased the results. Therefore the statement that plants at later stages of growth are more capable of growing in diesel fuel contaminated soil than seeds should be viewed tentatively, as the results were variable.

## 8.4 Growth of Willow on Diesel Fuel Contaminated Soil

Willow has been used extensively in the remediation of metal contaminated sites due to its abundant growth on poor soils, uptake and accumulation mechanisms for certain metals and its ability to transpire large volumes of water reducing metal leaching into the subsurface. Little work has been carried out on the growth of willow on petroleum hydrocarbon contaminated sites however. Carman *et al* (1998) chose a hybrid willow, species Prairie cascade, to remediate a diesel fuel contaminated site due to its superior agronomic performance in fuel contaminated soil. The experiment described in Section 2.6.3.5 was designed to evaluate if variability exists among willow clones in their ability to grow in diesel fuel contaminated soil.

Four willow clones, one British variety and three Swedish varieties, were planted in varying levels of diesel fuel contaminated soil. The growth of willow in contaminated soil was assessed and the quantity of diesel fuel remaining in the soil after 10 months measured to determine the potential of willow as candidates for phytoremediation of diesel fuel contaminated sites.

## 8.4.1 Rooting of Willow Cuttings (stools)

Each willow stool was planted directly into the soil at all treatment levels without prior rooting. This allowed the rooting ability of the stool in diesel fuel contaminated soil to be evaluated. Willow is generally planted after initial rooting has occurred. Carman *et al* (1998) transplanted cuttings into sand to allow rooting then further transplanted the rooted cuttings into 'clean' soil to allow leaf and root development to begin before planting in contaminated soil. This method of planting provides a bias in the assessment of willow clones for their ability to grow in contaminated soil as the contaminated soil may slow down or even prevent rooting from occurring and the willow cutting is much older and possibly less susceptible to the contaminants when it is transplanted. By allowing the cuttings to produce root and shoots, you are also making the job of planting much more difficult as you have to be careful not to damage the delicate roots and new shoots.

After two weeks growth in 0g, 25g and 50g diesel $kg^{-1}$ contaminated soil, all the stools in the control soil had developing roots and shoots whereas one Jorr stool in the 25g diesel $kg^{-1}$ treatment and one Jorr and one Jorrun stool in the 50g diesel $kg^{-1}$ treatment showed no signs of growth. This suggests the diesel fuel contaminated soil may be suppressing the initial stages of root and shoot development. Figure 8.4.1.1 illustrates the initial stages of root and shoot growth (2 weeks) in 0g, 25g and 50g diesel $kg^{-1}$ contaminated soil treatments.

## 8.4.2 Agronomic Assessment

### 8.4.2.1 Shoot Height

Differences in shoot length and plant health were apparent for all willow clones grown in contaminated soil compared to the control soil (0g diesel $kg^{-1}$). Initially, the mean value for average shoot length of each replicate was reduced more in the Ulv and Rosewarne White clones then in the Jorr and Jorrun clones. Average shoot length in the Ulv clone was reduced by 37% and 45% of the control average shoot length in the 25g and 50g diesel $kg^{-1}$ contaminated soil respectively. A similar response was shown by the Rosewarne White clone with average shoot length being reduced by 37% and 43%. The Jorrun and Jorr clones appeared to grow more happily in diesel fuel contaminated soil with reduction in shoot lengths being 7.4% and 33% and 10% and 48% respectively


Figure 8.4.1.1. Initial stages of shoot and root growth (2 weeks) on willow clones grown in 0g, 25g and 50g diesel $kg^{-1}$ soil.

at the two treatment levels. After six months the trend in pattern of growth shown by the clones initially altered. The overall growth of the Ulv clone improved at the 25g diesel $kg^{-1}$ level with average shoot length being 74% of the control average shoot length. A slight reduction was seen at the 50g diesel $kg^{-1}$ level. The Jorrun clone deteriorated badly in both treatments over the six month period with average shoot lengths falling to 63% and 47% of the control. The Rosewarne White clone, which was badly affected to begin with, worsened over the six months. Average shoot length dropped to 53% of the control in both treatments. There was no distinction between the higher and lower levels of diesel fuel contamination. The Jorr clone, on the other hand, continued to grow well at the 25g diesel $kg^{-1}$ level and improved significantly at the 50g diesel $kg^{-1}$ level. The average shoot lengths of the replicates grown in control (0g diesel $kg^{-1}$) and 25g diesel $kg^{-1}$ soil treatments were almost identical. This pattern of growth continued until harvesting of the willow at ten months. The Rosewarne White and Jorrun clones did not improve further whereas the Ulv and Jorr clones stayed at the same level of growth. Figure 8.4.2.1.1 illustrates the pattern of shoot length over the 10 month growing period for each clone investigated. A slight decrease in shoot height is noted at the 10 month measurements for all clones in all treatment levels as growth begins to slow at the end of the growing season.

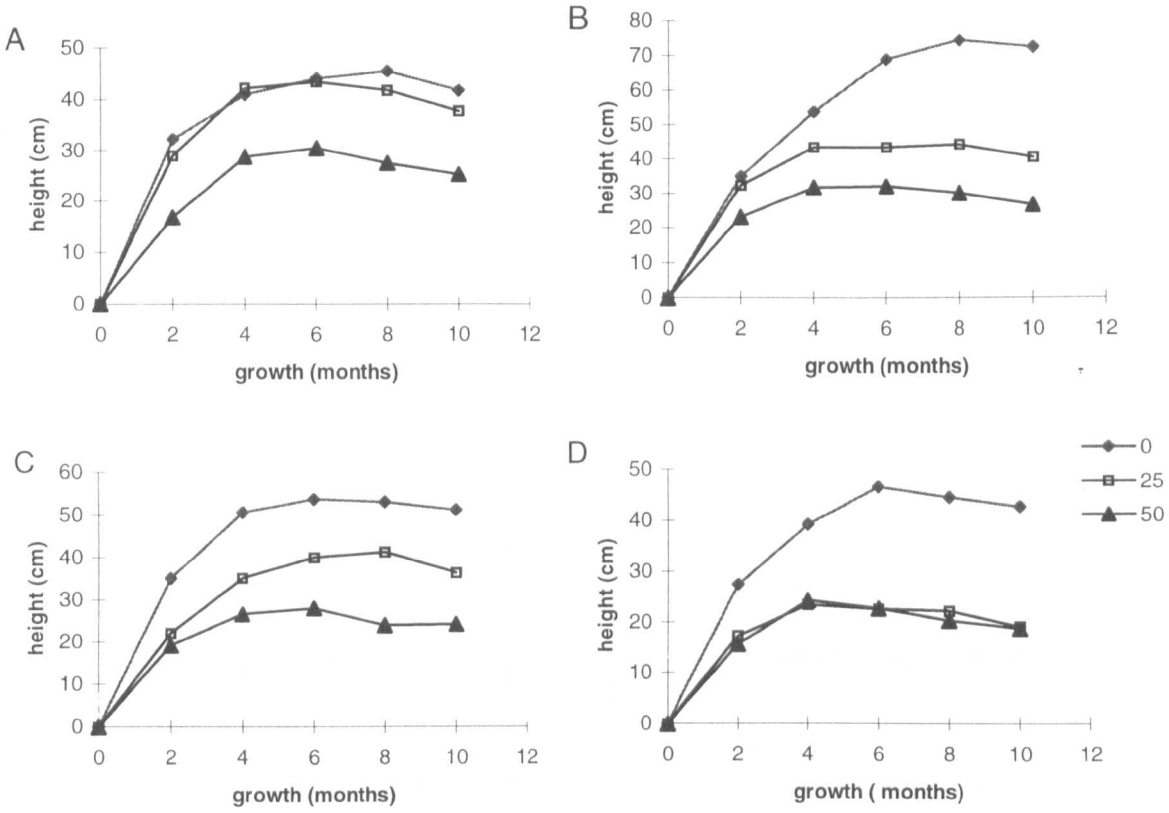



Figure 8.4.2.1.1. Average shoot heights of willow clones grown in 0g, 25g and 50g diesel $kg^{-1}$ contaminated soil over 10 month growth period. A) Jorr, B) Jorrun, C) Ulv and D) Rosewarne white.

## 8.4.2.2 Shoot Diameter

The diameter of the longest shoot was also measured at half its height. Figure 8.4.2.2.1 shows the shoot diameter measurements for each clone over the 10 month growing period. It is generally seen that the length of shoot relates to the diameter of shoot. This statement appears to be true for the willow clones grown in control soil (0g diesel $kg^{-1}$). As illustrated in Figure 8.4.2.2.1, shoot length increases and the diameter of the shoot also increases slightly. This pattern is also followed at the 25g and 50g diesel $kg^{-1}$ treatment levels for the Rosewarne White, Ulv and Jorrun clones but not for the Jorr clone. Jorr replicates grown in 25g diesel $kg^{-1}$ soil showed little difference in shoot height (Figure 8.4.2.1.1) but the measured shoot diameter is very different between the control and contaminated treatments (Figure 8.4.2.2.1). The reduction is shoot diameter may indicate a symptom of diesel fuel toxicity that the measurement of only shoot height would not have uncovered.

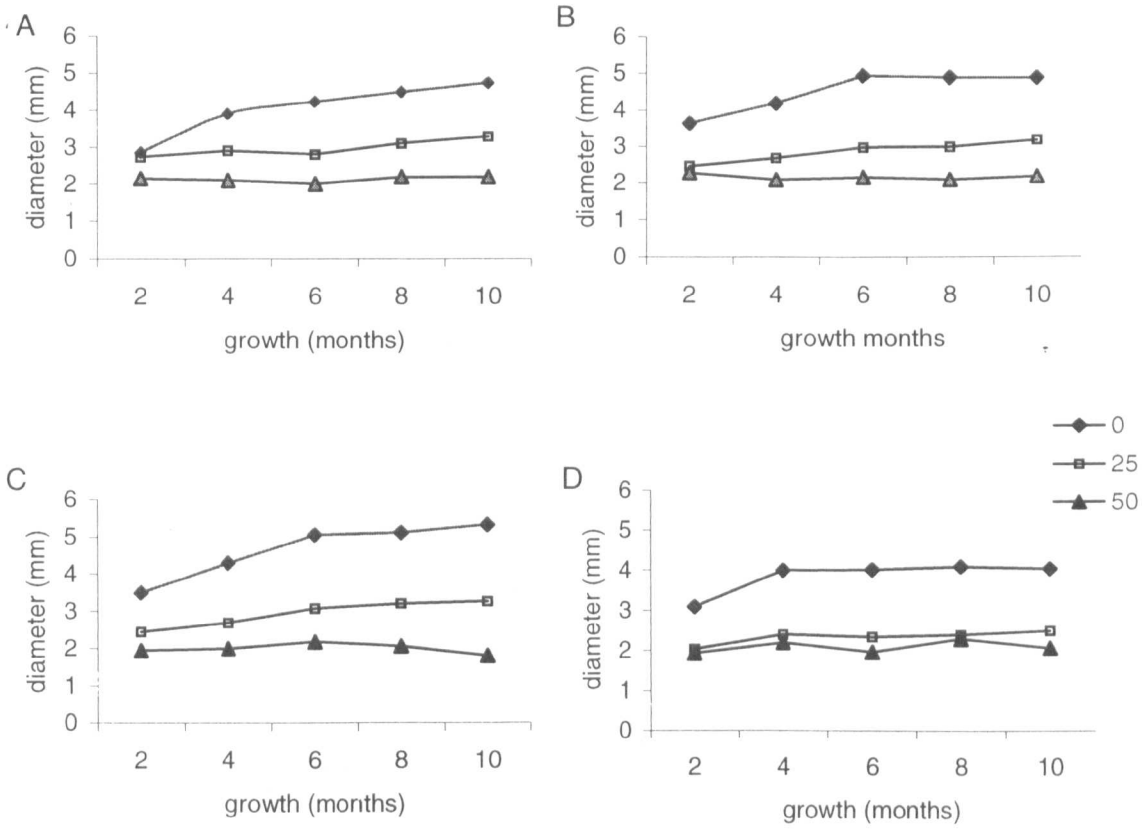



Figure 8.4.2.2.1. Average shoot diameters (at half shoot height) of willow clones grown in 0g, 25g and 50g diesel kg-1 contaminated soil over 10 months. A) Jorr, B) Jorrun, C) Ulv and D) Rosewarne white.

### 8.4.2.3 Shoot Biomass

After 10 months the willows were harvested. The leaves on each willow were removed and counted then the shoot biomass collected and dried as described in Section 2.7.1. The number of leaves per willow and the reduction in shoot biomass clearly reflect the retardation of growth and affect on plant health that diesel fuel contamination had on willow. The number of leaves was reduced on contaminated willow compared to control willow. Even the Jorr clones grown in 25g diesel $kg^{-1}$, whose shoot height was very similar to clones grown on 0g diesel $kg^{-1}$ soil, had fewer leaves, on average. The number of leaves per willow fell to 59.2% and 38.8% of the control in the 25g and 50g diesel $kg^{-1}$ soil respectively.

Shoot biomass was badly affected in all the clones investigated and tied in well with the measurements of shoot height and shoot diameter. The Jorr clone biomass

dropped to 47% in 25g diesel $kg^{-1}$ soil compared to the control. Although the shoot height between the control and the 25g diesel $kg^{-1}$ treatment was not greatly different, the overall biomass was greatly reduced. This was expected as the shoot diameter was greatly reduced in the 25g diesel $kg^{-1}$ treatment compared to the control. The Jorrun and Ulv clones had similar reductions in shoot biomass with percentages falling to 32.9 and 6.8 and 29.4 and 4.8 in the 25g diesel $kg^{-1}$ soil and 50g diesel $kg^{-1}$ soil treatments respectively. The Rosewarne white shoot biomass was as badly affected at the 25g diesel $kg^{-1}$ treatment level as it was at the 50g diesel $kg^{-1}$ treatment level. Shoot biomass fell to 15.3 % and 14.4 % of the control biomass in the two treatments. Table 8.4.2.3.1 summarises the agronomic performance of the four willow clones grown in 0g, 25g and 50g diesel $kg^{-1}$ soil.

### 8.4.2.4 Root Biomass

Although root mass was not measured individually for each willow clone in this experiment, the overall root mass was collected from each treatment. It proved very difficult to separate the roots from individual willow cuttings in each treatment level due to the roots becoming entangled with each other. Instead, the roots were collected from each treatment and bulked together to provide measurements for total root mass in each diesel fuel treatment level. The 25g diesel $kg^{-1}$ soil treatment had a reduced root mass of 22.8 % of the control and the 50g diesel $kg^{-1}$ soil treatment dropped to 10.2 % of the control.

| Clone | Treatment | Av. shoot length (cm) | Av. diameter (mm) | Av. biomass (dry weight) per clone (g) | Av. no. leaves per clone |
|---|---|---|---|---|---|
| Jorr | 0 | 41.8 ± 3.2 | 4.8 ± 0.3 | 5.73 ± 0.9 | 28 ± 4.6 |
| | 25 * | 37.8 ± 1.6 | 3.3 ± 0.2 | 2.15 ± 0.3 | 19 ± 1.3 |
| | 50 | 25.3 ± 2.3 | 2.2 ± 0.1 | 0.55 ± 0.1 | 10 ± 1.4 |
| Jorrun | 0 | 72.5 ± 11.1 | 4.9 ± 0.3 | 9.94 ± 1.3 | 49 ± 6.3 |
| | 25 | 40.6 ± 8.4 | 3.2 ± 0.3 | 1.87 ± 0.7 | 29 ± 8.0 |
| | 50 * | 26.8 ± 3.1 | 2.2 ± 0.2 | 0.65 ± 0.1 | 19 ± 5.8 |
| Ulv | 0 | 51.1 ± 3.6 | 5.3 ± 0.3 | 8.09 ± 0.7 | 41 ± 7.2 |
| | 25 | 36.3 ± 2.7 | 3.3 ± 0.2 | 2.09 ± 0.2 | 18 ± 1.8 |
| | 50 | 24.2 ± 1.9 | 1.8 ± 0.2 | 0.49 ± 0.1 | 8 ± 2.1 |
| Rosewarne White | 0 | 42.6 ± 13.3 | 4.1 ± 0.4 | 4.71 ± 2.2 | 30 ± 10.3 |
| | 25 | 19.2 ± 0.7 | 2.5 ± 0.1 | 0.64 ± 0.0 | 11 ± 1.1 |
| | 50 * | 18.80 ± 1.7 | 2.1 ± 0.4 | 0.30 ± 0.1 | 8 ± 3.5 |

Average values are given ± standard error (SE), n = 4 unless denoted by * n = 3.
Average diameter values are calculated from diameters measured at half shoot height.

Table 8.4.2..3.1. Agronomic performance of Willow clones in 0g, 25g and 50g diesel $kg^{-1}$ soil after 10 months growth.

## 8.4.3 Analysis of Residual Diesel Fuel

The amount of diesel fuel remaining in the soil after 10 months was determined by extracting the diesel fuel residue from subsamples taken at each treatment level. The diesel fuel was extracted using the Soxhlet method described in Section 2.3.2 and analysed by GC-FID to obtain total petroleum hydrocarbon (TPH) values (as described in Section 2.3.4). The recovery of TPH from soil using this procedure for extraction and analysis was approximately 75 % (see Section 3.1.1.1) with extremely high recovery of >C13 hydrocarbons (> 87 % recovery).

After 10 months growth, the level of diesel fuel had decreased by approximately 50% in both the 25g and 50g diesel $kg^{-1}$ soil treatments, as shown in Table 8.4.3.1. This level of breakdown is not as high as expected, but the concentration of diesel fuel added was high.

| treatment | TPH peak area | % difference between replicates | Av. TPH peak area | % diesel remaining |
|---|---|---|---|---|
| Original diesel | 159515 | 4.9 | | |
| Fresh 25g (time zero) | 119198 | 1.0 | | |
| 25g willow 1 | 57324 | 1.0 | | |
| 25g willow 2 | 60663 | 2.4 | | |
| 25g willow 3 | 58875 | 0.6 | 58954 | 49.5 |
| Fresh 50g (time zero estimate) | 238396 | | | |
| 50g willow 1 | 120109 | 4.7 | | |
| 50g willow 2 | 93527 | 1.3 | 107564 | 45.0 |
| 50g willow 3 | 109055 | 4.1 | | |

TPH peak areas calculated from replicates, n = 3. Fresh 50g (zero time) TPH peak area is an estimate calculated by doubling the TPH peak area extracted from the Fresh 25g (zero time) sample.

Table 8.4.3.1. Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 10 months for Willow planted troughs.

The troughs in which the Willow trees were grown in had no drainage, which meant that waterlogged conditions were frequently encountered in these troughs. The Willow trees themselves are capable of surviving under wet conditions but waterlogging of the soil would cause anaerobic conditions to arise which is not conducive to rapid breakdown of petroleum hydrocarbons. Hydrocarbon breakdown is a predominantly aerobic process. If the conditions the Willow were grown in maintained an aerobic environment at all times, an increase in degradation may have occurred. Nevertheless, Willow are capable of growing on high levels of diesel fuel contaminated soil and encourage breakdown of the diesel fuel contaminant.

Figure 8.4.3.2 illustrates the pattern of breakdown of diesel fuel remaining in the 25g diesel $kg^{-1}$ soil trough after 10 months compared to the original extracted diesel fuel (25g diesel $kg^{-1}$ soil at time zero). Hydrocarbons from the front of the diesel fuel chromatogram were preferentially degraded under these conditions. n-alkanes ranging from C9-C14 were greatly reduced, along with the lower molecular weight branched alkanes and aromatics. Some of these compounds could be lost by volatilisation but the majority of the compounds lost (anything over 24 minutes on the chromatogram) were not volatile, therefore they must have been biologically degraded.

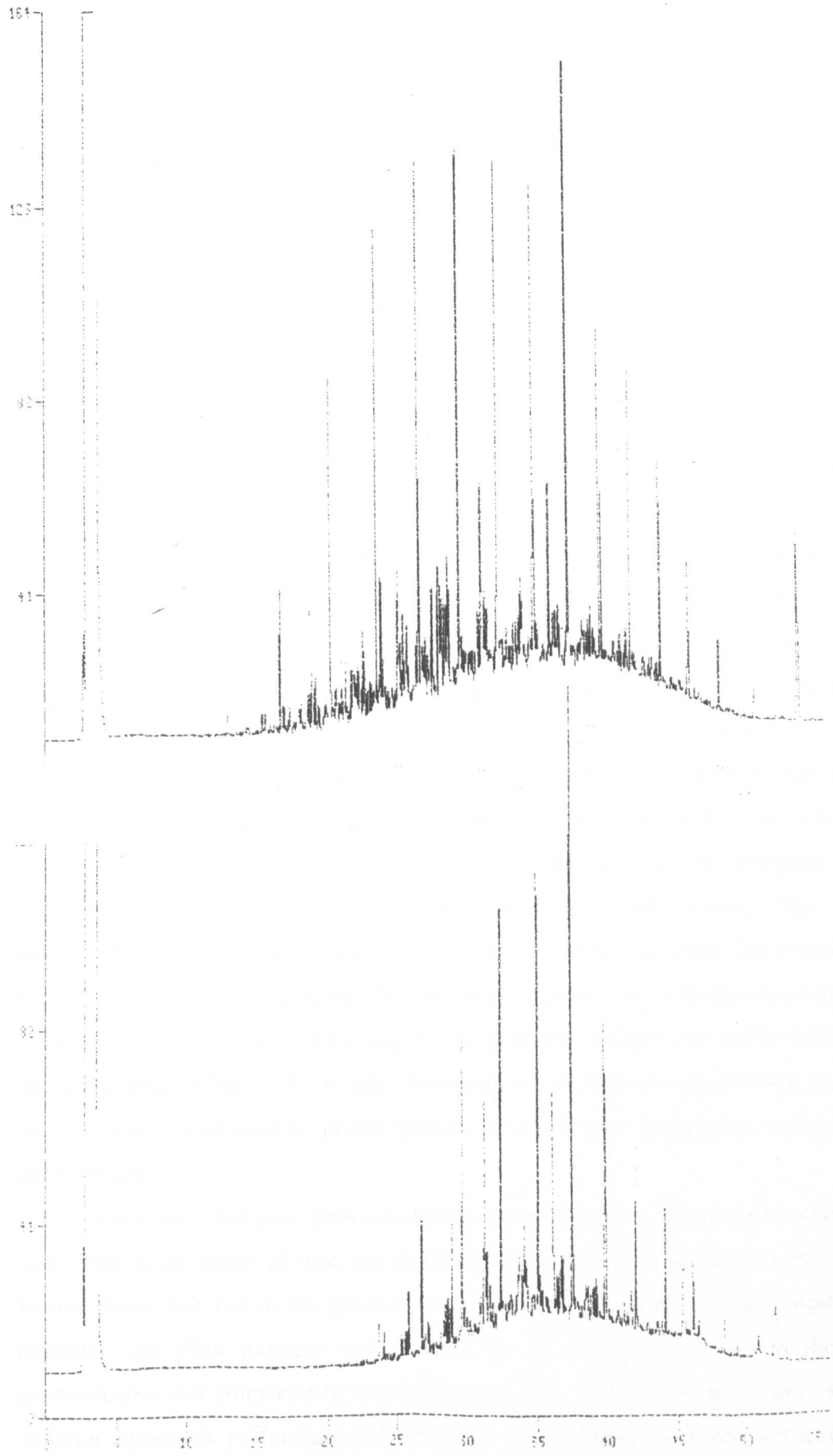


Figure 8.4.3.2. Pattern of diesel fuel remaining in 25g diesel $kg^{-1}$ soil after 10 months (bottom) compared to 25g diesel $kg^{-1}$ soil at time zero (top).

In summary, the series of small pot experiments have shown that seed germination was severely reduced by covering the entire seed with contaminated soil. This finding was extremely important as an integral part of this study involved finding suitable plant species that would grow successfully in diesel fuel contaminated soil. This problem was investigated in more detail (Sections 2.6.1.3 and 2.6.1.5) and the results discussed in a previous chapter (Sections 6.2 and 6.4). At high levels of diesel fuel contamination (25g–50g diesel $kg^{-1}$ soil) a decrease in germination rate was observed in contaminated treatments compared to control soils. Plants grown in contaminated treatments were stunted compared to the corresponding plants in control soil and the overall shoot and root biomass was reduced in contaminated soil compared to control soil. A decrease in plant height and reduction in plant biomass are both symptoms of the chronic toxicity of diesel fuel on plant health. Despite these disadvantages, the visual condition of the plants was good. In low levels of contamination (5g–20g), reduction in germination rate, plant height and shoot and root biomass was again observed for the majority of the plant species tested. Westerwold's ryegrass growth however, appeared stimulated at low levels (5g diesel $kg^{-1}$ soil) of diesel fuel contamination and would be further investigated as a possible candidate for phytoremediation of diesel fuel contaminated soil. When growing legumes in low levels of diesel fuel contaminated soil, the microorganisms responsible for infecting plant roots to develop root nodules were found to be present and still active. One worry during this investigation was that diesel fuel would kill off the *Rhizobia* present naturally in the soil as *Rhizobia* are quite sensitive to contamination. This was not found to be the case in the pot experiments. In fact, more developed root nodules were found on the contaminated plants than on control plants. This unusual observation was studied in more detail in the later, larger scale greenhouse trials that will be discussed in the next chapter (Chapter 9). Finally, flowering and seeding in contaminated plants was delayed when compared to plants grown in control soil, suggesting a lag in plant development.

Variability did exist between Willow clones grown in diesel fuel contaminated soil. The initial stages of root and shoot development was suppressed in the Jorr and Jorrun clones but not in the Rosewarne white and Ulv clones. Shoot height, shoot diameter and plant biomass was reduced in all the clones grown in diesel fuel contaminated soil compared to uncontaminated soil. The Swedish variety, Jorr, had superior agronomic performance in diesel fuel contaminated soil compared to the other Willow clones investigated.

The small pot experiments have allowed a number of plant species to be chosen for the final large scale greenhouse trials to investigate the processes involved and the effect of phytoremediaiton on diesel fuel contaminated soil. The experiments have also shown what measurements need to taken to provide a thorough agronomic assessment of plant performance in diesel fuel contaminated soil.

# CHAPTER NINE

## PHYTOREMEDIATION OF DIESEL FUEL CONTAMINATED SOIL

A number of plant species that performed well in diesel fuel contaminated soil throughout the initial screening experiments and small pot experiments were selected for larger greenhouse trials to study the phytoremediation ability of these plants. A detailed investigation of agronomic performance of these plant species in varying levels of diesel fuel contaminated soil was carried out. In addition, the effect of planting and diesel fuel contamination on soil enzymatic activity was studied and the concentration of diesel fuel remaining in planted and unplanted soils was measured to determine if diesel fuel biodegradation was enhanced in planted soils.

Soil enzymes may originate from plants, animals, fungi and bacteria although it is generally agreed that the microbial component is the main source of enzymes in soil (Dick, 1997). Soil enzymes have a unique role in assessing soil health because soil as a whole (not only the biological component) can be thought of as a living biological system capable of carrying out certain biochemical reactions without viable cells (Dick, 1997). Because many enzymes are substrate specific and can be chosen from different functional groups, there is an opportunity to determine the potential of soil to carry out a whole range of reactions that may be critical to the functioning of the ecosystem. Soil enzyme activity can relate to plant productivity, microbial biomass, biogeochemical cycling, impacts of pollutants on soils or the status of remediated soil. By studying soil enzyme activities, a

clearer picture of the effects of diesel fuel contamination on the soil system as a whole and the influence of planting on the soil system was obtained.

The soil enzymes investigated during this study were dehydrogenase, acid and alkaline phosphatase and non specific proteases, lipases and esterases. These enzymes come from different functional groups and can therefore be used to determine the activity of different compartments of the soil system. Dehydrogenase enzymes reflect the total oxidative capabilities of soil microorganisms (Camiña et al., 1998, Casida, 1977). They are linked to respiration and show if the soil microorganisms are utilising diesel fuel as a substrate for growth. Phosphatase enzymes release plant available, inorganic phosphate from organic matter (Boero and Thien, 1979) which is essential for plant growth and would show if nutrient cycling was continuing in the contaminated soil. Finally, non specific proteases, lipases and esterases can be measured by a broad spectrum enzyme assay. This provides a general indication of soil hydrolytic activity which can be used to assess soil microbial activity (Schnürer and Rosswall, 1982, Swisher and Carroll, 1980, Adam and Duncan, in press). This will show whether microbial activity is affected by diesel fuel contamination and whether activity is increased under planted soils compared to unplanted soils.

## 9.1 Ryegrass versus Oil Seed Rape

Westerwold's ryegrass and oil seed rape were chosen to illustrate the phytoremediation potential of diesel fuel in a larger greenhouse experiment as these species consistently germinated and grew well in diesel fuel contaminated soil. A real soil (Arkleston) was chosen for this study to provide more realistic soil conditions with a natural population of soil microorganisms which would allow the effect of diesel fuel contamination on soil microbial activity and diesel fuel biodegradation to be studied. The Arkleston soil physical and chemical characteristics are given in Table 9.1.1.

| Textural properties | | Chemical properties | |
|---|---|---|---|
| % coarse sand | 32.7 | pH | 6.86 |
| % fine sand | 35.3 | LOI % | 6.5 |
| % silt | 16.3 | Total N % | 0.17 ± 0.01 |
| % clay | 15.7 | Ext. P mg $kg^{-1}$ | 26.7 ± 0.38 |
| Textural class | Sandy loam | Ext. K mg $kg^{-1}$ | 71.0 ± 0.24 |

Average values are given. LOI % ± SE, n = 3. Total N % ± SE, n = 5. Extractable P ± SE, n = 4 and Extractable K ± SE, n = 3.

Textural characteristics taken from Metwaly, Ph. D thesis, University of Glasgow, 1999.

Table 9.1.1. Physical and chemical characteristics of Arkleston soil.

The experimental design incorporated two plant species as well as unplanted soil at four different treatment levels (0, 5, 7.5 and 10g diesel $kg^{-1}$ soil). The concentrations of diesel fuel were lower than previously used to determine the effect on growth and breakdown of diesel fuel at lower soil contamination levels.

Germination rate measured after 1 week was high for both plant species in all diesel fuel treatment levels. Table 9.1.2 shows the germination rate of both plant species in 0, 5, 7.5 and 10g diesel $kg^{-1}$ soil. The Westerwold's ryegrass germinated well at all treatment levels. The Oil seed rape germination rate showed an almost linear decrease as diesel fuel concentration increased. Despite the reasonably high germination rate of Oil seed rape in diesel fuel contaminated soil, the plants became badly infected at the seedling stage. The health of the plant must have been reduced by growing in diesel fuel contaminated soil, making the plants more susceptible to infection. As plant growth was terribly stunted, seedlings were extremely close to the soil surface providing an easy transfer of infection from the soil to the plant. A fungus, possibly a powdery mildew, took over the Oil seed rape plants, which had a huge knockdown effect on the plant population. For this reason, the Oil seed rape treatments were removed from the pot experiment. The experiment was continued with Westerwold's ryegrass as the only plant species.

| | Av. Germination rate % | | | |
|---|---|---|---|---|
| | Treatment level g diesel $kg^{-1}$ soil | | | |
| Species | 0 | 5 | 7.5 | 10 |
| Westerwold's ryegrass | 66 ± 2.26 | 69 ± 3.43 | 66 ± 2.97 | 60 ± 3.88 |
| Oil seed rape | 84 ± 3.63 | 77 ± 3.52 | 68 ± 6.57 | 56 ± 7.11 |

Average values correspond to average of two levels of pots (2 month and 4 month replicates) at same treatment level, n = 6.

Table 9.1.2. Germination rate of Oil seed rape and Westerwold's ryegrass after 1 week in diesel fuel contaminated soil.

### 9.1.1 Agronomic Assessment

Germination rate and tallest shoot height were to be measured at 2 and 4 months. However, after the 2 month sampling date the remaining Westerwold's ryegrass plants, which would have been sampled at 4 months, became infected with Black Fly. The occurrence of disease on both plant species suggested the soil used for this experiment was a carrier of disease/pests to begin with. Growing plants in contaminated soil would reduce plant health making them more susceptible to attack but disease would have to be already present to cause such a widespread infection. This set back in the pot experiment was unfortunate but a lot of information could be gathered from the 2 month Westerwold's ryegrass samples when compared to the control and unplanted pots at the same treatment levels. Table 9.1.1.1 gives a summary of the results of the agronomic parameters assessed at the 2 month sampling date.

| Treatment g diesel $kg^{-1}$ soil | Av. germination (%) | Av. tallest shoot (cm) | Av. shoot biomass per plant (mg) | Av. root biomass per plant (mg) |
|---|---|---|---|---|
| 0 | 60 ± 1.67 | 93.3 ± 4.81 | 254 ± 1 | 3645 ± 150 |
| 5 | 68 ± 6.01 | 66.0 ± 2.00 | 45 ± 0 | 980 ± 150 |
| 7.5 | 55 ± 1.91 | 62.3 ± 4.06 | 47 ± 1 | 1089 ± 100 |
| 10 | 49 ± 6.27 | 63.7 ± 4.18 | 55 ± 1 | 1272 ± 150 |

Average values are given ± SE, n = 3. Biomass values are expressed as oven dry (75°C) weights.

Table 9.1.1.1. Summary of agronomic performance of Westerwold's ryegrass at the 2 month sampling date.

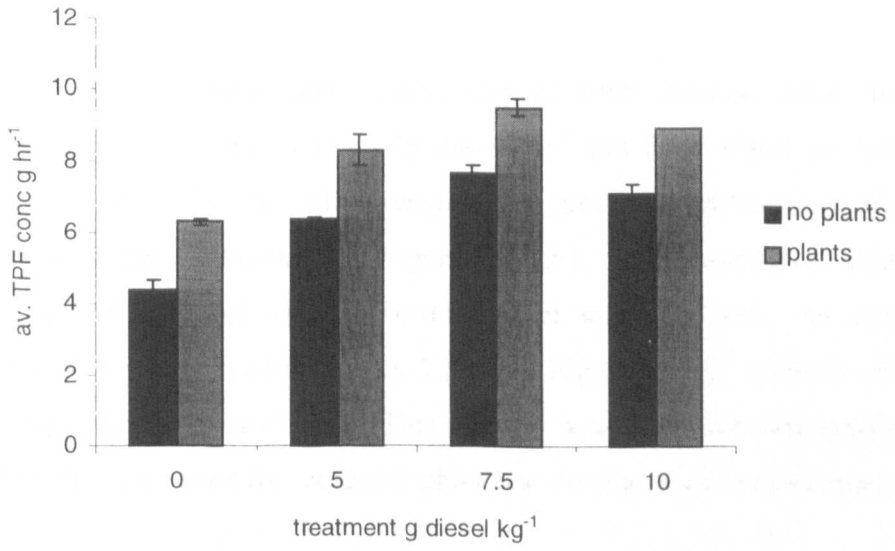
There was a slight decrease in the number of plants at each treatment level when compared to the original % germination results recorded at week 1. This decrease was more apparent at the higher diesel fuel contamination levels and was probably due to overcrowding in the pots causing the smaller, weaker plants to die out as well as the effect of diesel fuel contamination. The average shoot height of contaminated plants was stunted compared to control plants but very little difference in shoot height was observed between contaminated treatments. Contaminated plants shoot height was, on average, reduced by 32% compared to the controls average shoot height. A large difference in shoot and root biomass was observed between the control and contaminated plants. On average, shoot biomass decreased to 19% of the control plant biomass in contaminated plants and root biomass was reduced by 50% in contaminated plants. Within the contaminated treatments, a small increase in shoot biomass was observed with increasing diesel fuel contamination levels. This unusual trend is more apparent in the root biomass results where a significant increase is observed. An increase in the root biomass over the shoot biomass may suggest these plants are stressed and are not obtaining enough water for growth so they are allocating more of their energy to producing a larger root system to compensate. Addition of diesel fuel to soil has been shown to increase soil water repellency (Section 5.2) therefore the plant may be responding to this contamination by producing a larger root to shoot ratio to obtain essential water and nutrients.

## 9.1.2 Enzymatic Assessment of the Plant-Soil System

At the two month harvest, after the pots had been destructively sampled for plant biomass, the remaining soil was subsampled for enzymatic analysis. The subsamples were then analysed for phosphatase acitivity (as described in Section 2.5.1), dehydrogenase activity (as described in Section 2.5.2) and Fluorescein diacetate (FDA) activity (as described in Section 2.5.3).

### 9.1.2.1 Dehydrogenase Activity

Addition of diesel fuel to soil caused a significant increase in activity up to the 10g diesel $kg^{-1}$ soil level where a slight decrease was seen. Addition of diesel fuel caused a 54.7%, 86.0% and 72.3% increase in dehydrogenase activity compared to the control soils activity in 5g, 7.5g and 10g diesel $kg^{-1}$ soil as shown in Figure 9.1.2.1.1. All the contaminated soils dehydrogenase activities were significantly higher than the control soil with no diesel fuel addition which suggests proliferation of the natural microbial population by diesel fuel addition caused by the microbial population utilising diesel fuel as a food (carbon) source. Steubing (1967) found a similar increase in dehydrogenase activity in soils contaminated with heating oil compared to control soil. The higher results were explained by the increase in the hydrocarbon oxidising bacteria present.




Average values given with standard error bars.

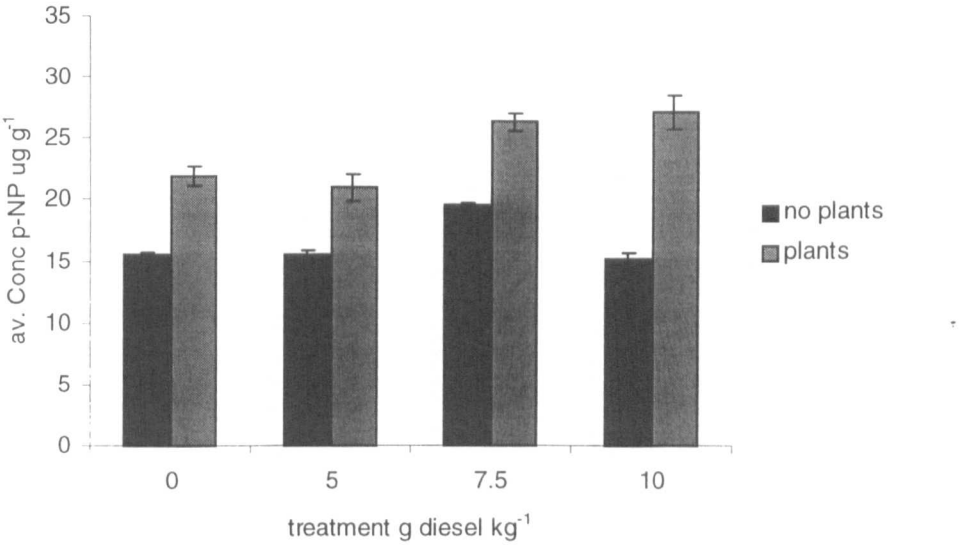
Figure 9.1.2.1.1 Dehydrogenase activity in unplanted and Westerwold's ryegrass planted soils at 0g, 5g, 7.5g and 10g diesel $kg^{-1}$ soil treatments.

By growing Westerwold's ryegrass in soil, an increase of 61% in the dehydrogenase activity was observed. The dehydrogenase levels increased following the same pattern as observed in the unplanted soil, up to the 10g diesel $kg^{-1}$ soil level, where dehydrogenase activity stayed the same. The dehydrogenase activity results clearly showed the increased microbial activity and microbial population under planted soils as opposed to unplanted soils. The decrease in dehydrogenase activity observed at the 10g diesel $kg^{-1}$ soil level in unplanted soil was not seen in planted soil at this contamination level. The plant rhizosphere effect may be protecting the microorganisms from the inhibitory effect of the diesel fuel as shown by the high level of dehydrogenase activity that was maintained.

### 9.1.2.2 Phosphatase Activity

Plant roots are major producers of acid phosphatase but do not produce alkaline phosphatase (Kramer and Green, 2000, Pant *et al.*, 1994). Acid phosphatase is closely tied to root growth activity and plant demand for phosphorus. Soil microorganisms can also produce acid phosphatase. Alkaline phosphatase originates entirely from soil bacteria, fungi and fauna (Frankenberger and Dick, 1983, Pant *et al.*, 1994, Tabatabai, 1994). Microorganisms can produce and release large quantities of extracellular phosphatase due to their large combined biomass, high metabolic capabilities and short life cycles. Again, because phosphatases are adaptive enzymes, the intensity of excretion by microorganisms will be determined by their need for phosphate (Skujinš, 1976).

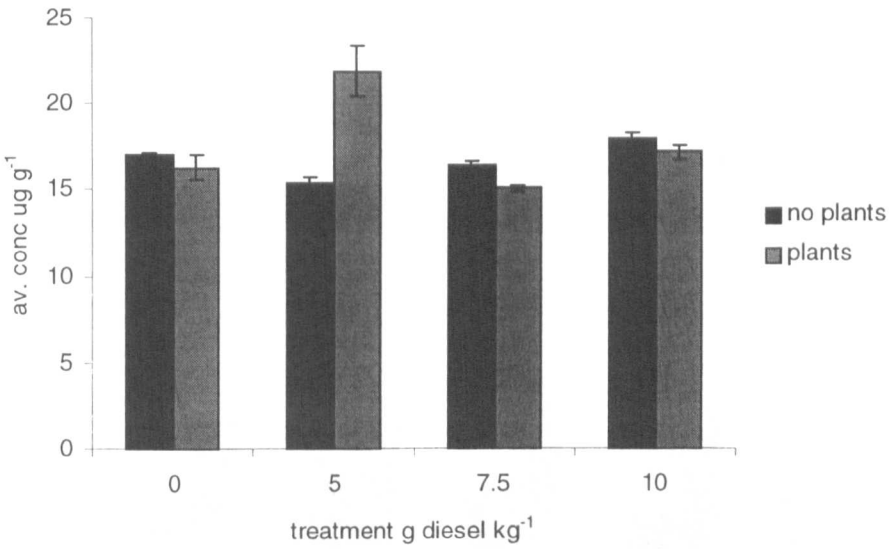
The acid phosphatase results showed little increase from the control level in unplanted soils except at the 7.5g diesel $kg^{-1}$ soil level where an increase of 26% was observed. When Westerwold's ryegrass was grown, an increase in activity in all treatment levels was seen as illustrated in Figure 9.1.2.2.1. Acid phosphatase activity in the control, planted soil increased 41% above the control, unplanted soil. An increase of 35%, 69% and 75% was observed in the 5g, 7.5g and 10g diesel $kg^{-1}$ planted soils compared to the corresponding unplanted soils. This increase in acid phosphatase activity is representative of the increased need for inorganic phosphate by plants and microorganisms for growth.




Average values given with standard error bars

Figure 9.1.2.2.1 Acid phosphatase activity in unplanted and Westerwold's ryegrass planted soils at 0g, 5g, 7.5g and 10g diesel $kg^{-1}$ soil treatments.

The alkaline phosphatase results showed increased production of enzymes by microorganisms at the 10g diesel $kg^{-1}$ soil level. The increase was small, being only 7% above the control activity, but it may suggest there was a lack of available phosphate which was required for microbial growth. A large increase was seen at the 5g diesel $kg^{-1}$ soil level under planted conditions as shown in Figure 9.1.2.2.2. This may suggest that at this diesel fuel treatment level, very little inorganic phosphate was available for microbial proliferation, possibly due to the plants using a lot of the available inorganic phosphate for growth. Therefore production of alkaline phosphatase was increased to enhance the availability of inorganic phosphate for microbial growth.




Average values given with standard error bars

Figure 9.1.2.2.2 Alkaline phosphatase activity in unplanted and Westerwold's ryegrass planted soils at 0g, 5g, 7.5g and 10g diesel $kg^{-1}$ soil treatments.

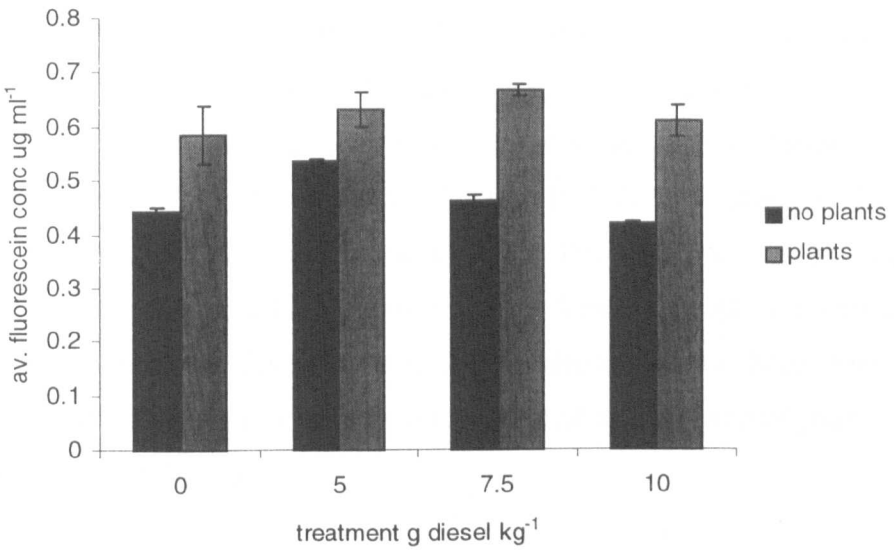
## 9.1.2.3 Fluorescein Diacetate (FDA) Activity

FDA hydrolysis is a broad spectrum enzyme assay as the enzymes responsible are non specific proteases, lipases and esterases. They are widespread in the soil environment and provide a general estimate of total microbial activity in the soil.

FDA activity was seen to increase in unplanted soil by addition of 5g diesel $kg^{-1}$ soil. This initial stimulation in activity tailed off at higher levels of diesel fuel addition as shown in Figure 9.1.2.3.1. FDA activity fell from 120% of the control activity in the 5g diesel $kg^{-1}$ soil treatment to 104% and 94% of the control activity in the 7.5g and 10g diesel $kg^{-1}$ soil treatments respectively. The results show that the total microbial activity was stimulated by diesel fuel, in the majority of treatments, in unplanted soil. At the 10g diesel $kg^{-1}$ soil treatment, FDA activity was suppressed slightly which suggests that this concentration of diesel fuel is beginning to have an inhibitory or even toxic effect on the soil microorganisms as their total activity is decreasing.

By growing Westerwold's ryegrass in the soil, FDA activity was increased by 32% compared to the unplanted soil. FDA activity was increased in diesel fuel contaminated,

planted soil by 9% on average, compared to the uncontaminated planted soil. Little difference was observed between FDA activities in the different diesel fuel treatments. This suggests in planted soils, the level of non specific proteases, lipases and esterases were increased compared to unplanted soil but the further addition of higher levels of diesel fuel had no effect on the levels of these enzymes. The increased level of microorganisms being supported by the added diesel fuel and the plants may have reached




its maximum.

Average values are given with standard error bars.

Figure 9.1.2.3.1 Fluorescein diacetate (FDA) activity in unplanted and Westerwold's ryegrass planted soils at 0g, 5g, 7.5g and 10g diesel $kg^{-1}$ soil treatments.

## 9.1.3 Analysis of Residual Diesel Fuel

After two months, the amount of diesel fuel remaining in the unplanted and Westerwold's ryegrass planted soil, at all diesel fuel treatment levels, was measured. The residual diesel fuel was extracted from the soil as described in Section 2.3.2 and analysed by GC-FID as described in Section 2.3.4. Total petroleum hydrocarbon (TPH) values were calculated for the residual diesel fuel in each soil treatment as well as for diesel fuel extracted at time zero to illustrate the % breakdown observed. A subsample of each diesel fuel treated soil was also stored in the cold room (4°C) for 2 months to provide a baseline value of the % diesel fuel lost through abiotic processes and not due to microbial or plant influence. The

results for each diesel fuel treatment level are presented in Tables 9.1.3.1, 9.1.3.3 and 9.1.3.5.

The 5g diesel $kg^{-1}$ soil treatment showed a large decrease in the amount of diesel fuel remaining after 2 months as shown in Table 9.1.3.1. The loss of diesel fuel from both planted and unplanted soils was very similar with % diesel fuel remaining in unplanted soil being 39.8% and % diesel fuel remaining in planted soil being 37.2%. The soil stored in the cold room over the 2 month period that the experiment was running showed a loss of approximately 28% compared to diesel fuel extracted from freshly contaminated soil (time zero). This loss was due to volatilisation and adsorption of diesel fuel components but not biological loss. The true breakdown rates were therefore calculated using the cold room TPH value as the starting point to demonstrate true biological breakdown. The breakdown rates due to microbial and plant influence were 45% and 48% in the unplanted and Westerwold's ryegrass planted soil. Only 3% more diesel was broken down under planting at the 5g diesel $kg^{-1}$ soil level. Figure 9.1.3.2 illustrates the diesel fuel components remaining in the planted and unplanted soils compared to the chromatogram of the original diesel extracted at time zero.

| treatment | TPH peak area | % difference between replicates | % diesel remaining |
|---|---|---|---|
| Fresh (time zero) | 34622.55 | 1.16 | 100 |
| Cold room | 24956.66 | 1.12 | 72.1 |
| 5g diesel, no plants | 13781.00 | 0.86 | 39.8 |
| | | | |
| Plants, 5g diesel 1 | 12195.28 | 1.58 | Average |
| Plants, 5g diesel 2 | 12433.23 | 0.65 | 37.2 |
| Plants, 5g diesel 3 | 13970.39 | 2.73 | |

TPH peak areas calculated from replicates with less than 5% difference between them, n = 3.

Table 9.1.3.1 Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 2 months for contaminated, unplanted and planted soils.

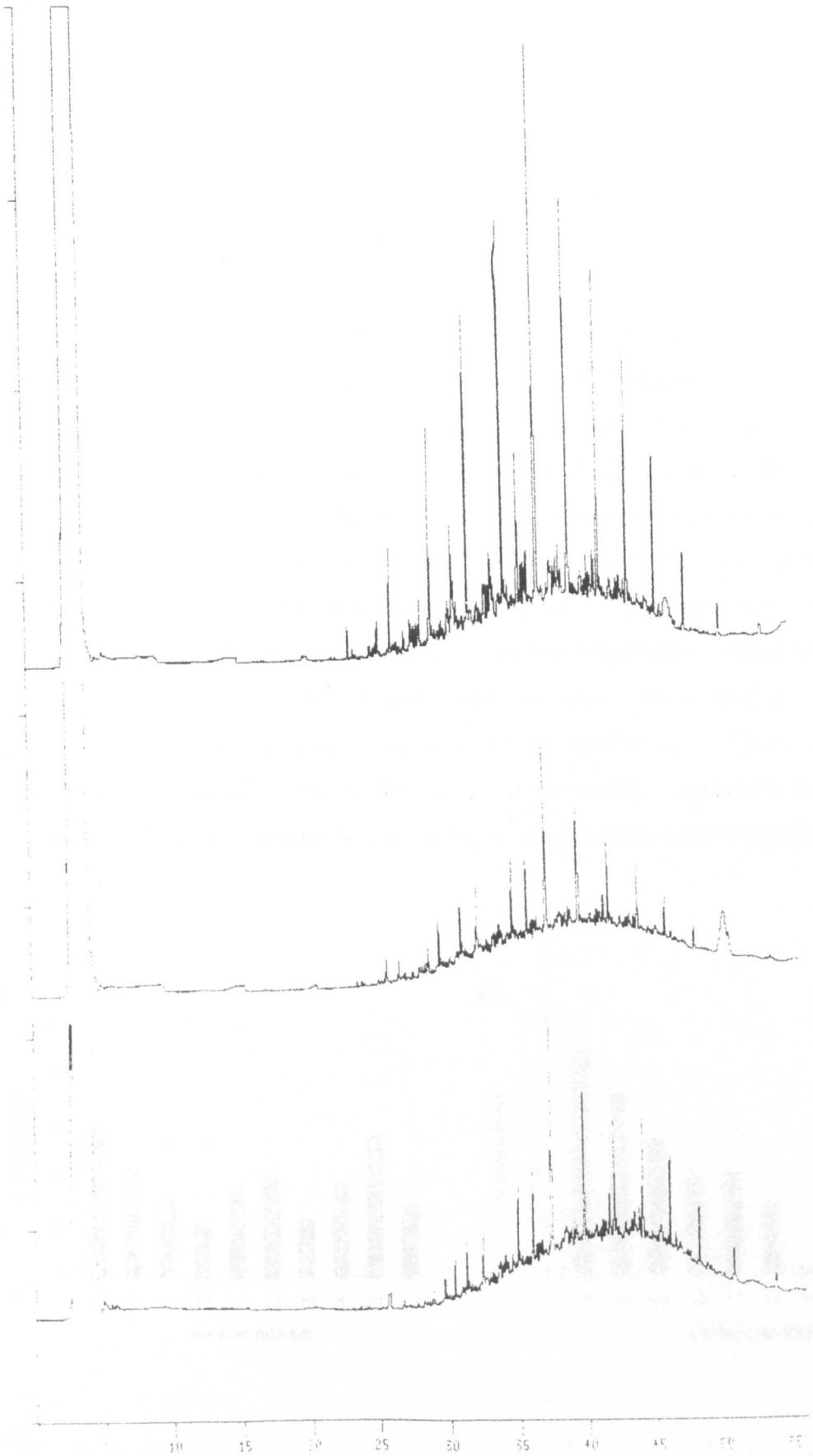



Figure 9.1.3.2 Chromatograms of 5g diesel $kg^{-1}$ soil original diesel fuel extracted at time zero and residual diesel fuel extracted from unplanted and planted soil (top to bottom) plotted together. Original chromatograms were set to the same scale.

Although the amount of diesel fuel remaining in the planted and unplanted soils was similar, the pattern of diesel fuel distribution was quite different. The difference cannot be seen clearly from the chromatograms in Figure 9.1.3.2 but if you compare the extent of breakdown of individual petroleum hydrocarbons, a pattern begins to emerge. n-alkanes ranging from carbon 13 to carbon 22 were used to illustrate this. Figure 9.1.3.3 shows the percentage breakdown of individual n-alkanes in unplanted and planted soils. The % breakdown is calculated by subtracting the peak area of the individual n-alkane remaining in the soil from the peak area of the same individual n-alkane extracted from the cold room sample. This gives the true amount of breakdown observed under the specific treatment. Figure 9.1.3.3 showed that breakdown in unplanted soil at the 5g diesel $kg^{-1}$ soil contamination rate was quite even for all the n-alkanes investigated. The smallest (C13) and slightly higher (C21) n-alkanes had the highest amount of breakdown, being 58% and 55%. The mid range n-alkanes (C17 and C18) also showed high % breakdown. A very different pattern of breakdown was observed for n-alkanes in planted soil. The highest % breakdown was seen for the smaller n-alkanes but the % breakdown tailed off rapidly after that. An almost linear decrease in degradation was seen with increasing carbon number (Figure 9.1.3.3). This increased breakdown of the smaller, lower molecular weight n-alkanes shows a definite shift in the microbial community responsible for diesel fuel degradation when compared to the even breakdown of n-alkanes in the unplanted soil.

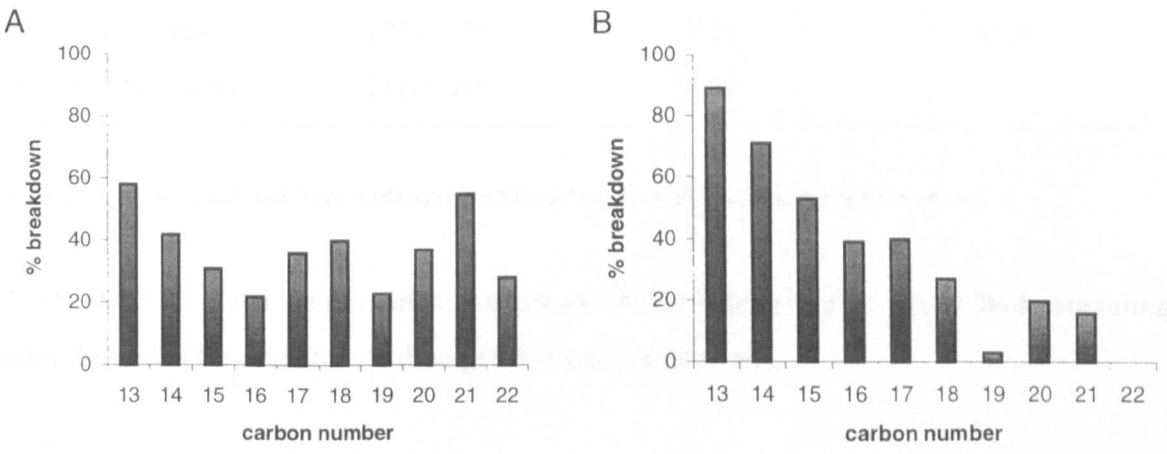



Figure 9.1.3.3 % degradation of individual n-alkanes in 5g diesel $kg^{-1}$ unplanted and Westerwold's ryegrass planted soil.

At the higher contamination level of 7.5g diesel $kg^{-1}$ soil the amount of diesel fuel remaining in the unplanted soil was much higher than previously observed in the 5g diesel $kg^{-1}$ soil treatment. 56.9% diesel fuel was left in the unplanted soil as shown by the results in Table 9.1.3.4. This higher level of diesel fuel may require a longer period of breakdown by soil microorganisms due to its toxicity at high concentrations or the increased quantity requiring breakdown. The amount of diesel fuel remaining in Westerwold's ryegrass planted soil was 34.3%. This large difference in residual diesel fuel content is attributed entirely to the influence of plants and their associated microorganisms on contaminant breakdown. The % breakdown of diesel fuel by purely biological processes in the unplanted and planted soils is 20% and 52% when loss by abiotic processes is taken into account. The increased breakdown under planted soil is clearly seen in Figure 9.1.3.5 where the unplanted and planted residual diesel fuel chromatograms are compared.

| treatment | TPH peak area | % difference between replicates | % diesel remaining |
|---|---|---|---|
| Fresh (time zero) | 60436.45 | 2.95 | 100 |
| Cold room | 42947.77 | 2.74 | 71.1 |
| 7.5g diesel, no plants | 34400.18 | 1.76 | 56.9 |
| Plants, 7.5g diesel 1 | 18128.55 | 3.03 | Average |
| Plants, 7.5g diesel 2 | 19841.09 | 1.79 | 34.3 |
| Plants, 7.5g diesel 3 | 24180.09 | 2.50 | |

TPH peak areas calculated from replicates with less than 5% difference between them, n = 3.

Table 9.1.3.4 Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 2 months for contaminated, unplanted and planted soils.

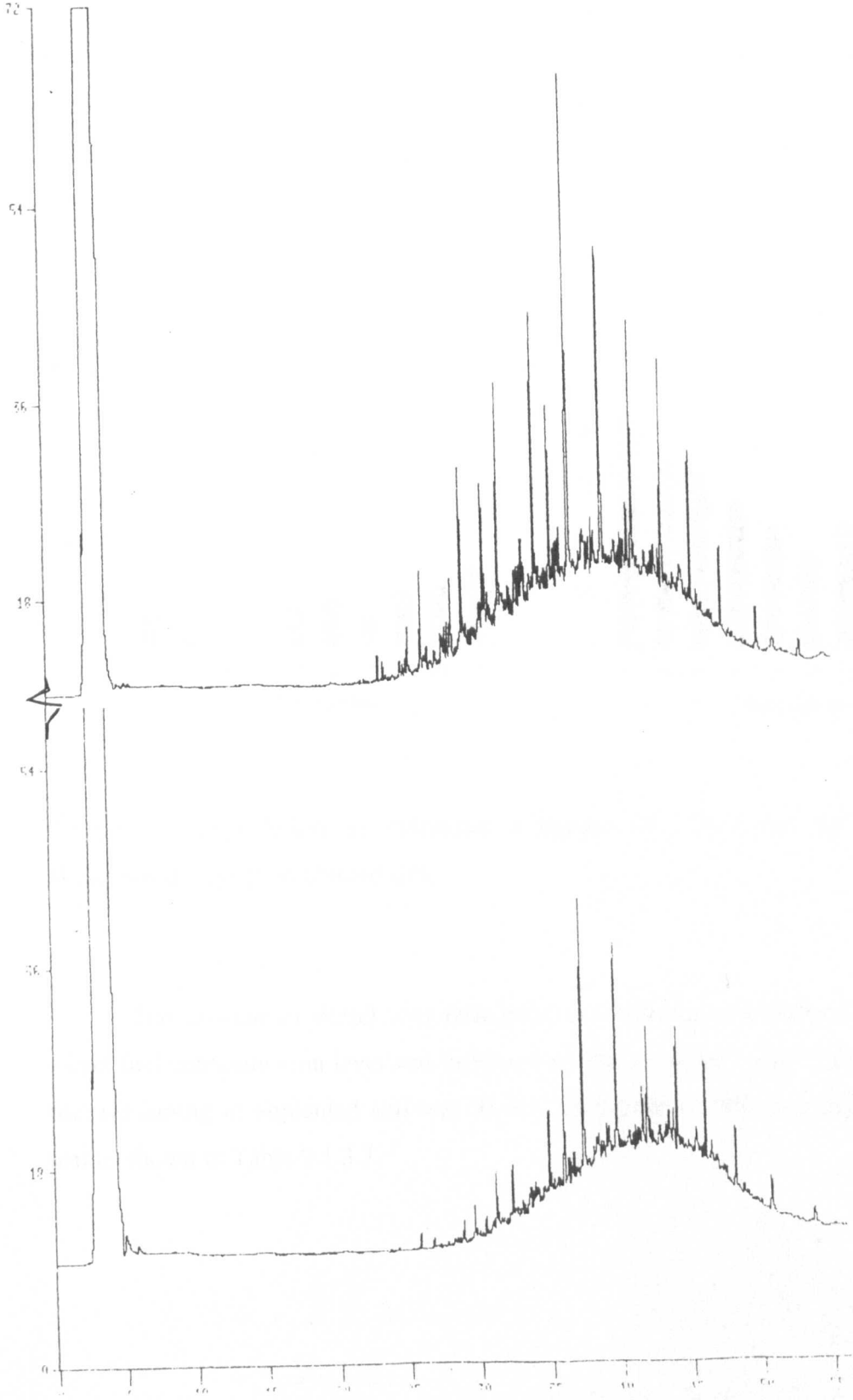
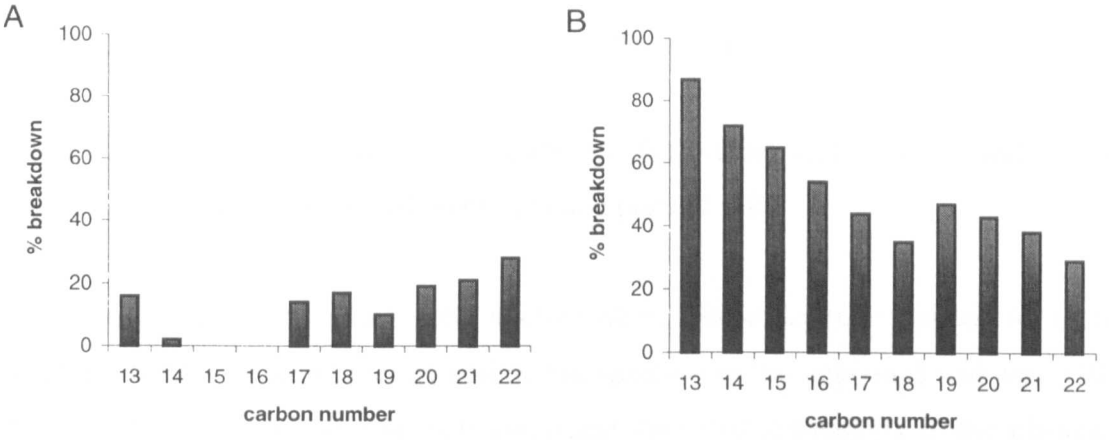


Figure 9.1.3.5. Chromatograms of residual diesel fuel extracted from 7.5g diesel $kg^{-1}$ soil unplanted and planted soil (top to bottom) plotted together.

The additional biological breakdown in unplanted soil was minimal as can be seen from Figure 9.1.3.6. Surprisingly, the higher molecular weight n-alkanes had the highest % breakdown in unplanted soil. The planted soil showed enhanced % breakdown for all the n-alkanes investigated. Breakdown was again higher for the low molecular weight n-alkanes and decreased as carbon number increased (as shown in Figure 9.1.3.6). The huge difference in breakdown illustrated can be clearly seen by comparing the chromatograms in Figure 9.1.3.5.




9.1.3.6 % degradation of individual n-alkanes in 7.5g diesel $kg^{-1}$ unplanted and Westerwold's ryegrass planted soil.

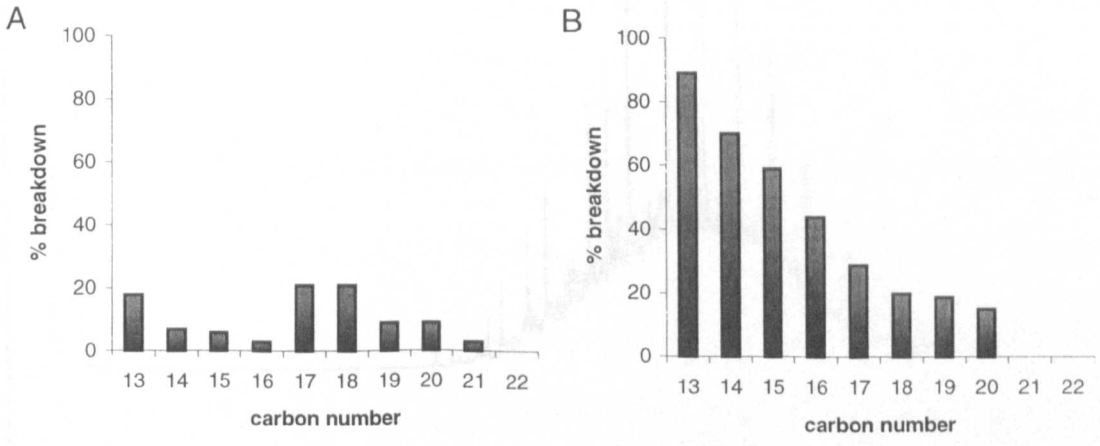
The amount of diesel fuel remaining in unplanted soil increased further as the diesel fuel contamination level was increased to 10g diesel $kg^{-1}$ soil. The amount of diesel fuel remaining in unplanted soil was 61.4%, compared to 46% remaining in the planted soil as shown in Table 9.1.3.7.

| treatment | TPH peak area | % difference between replicates | % diesel remaining |
|---|---|---|---|
| Fresh (time zero) | 82878.77 | 2.50 | 100 |
| Cold room | 65864.82 | 2.36 | 79.5 |
| 10g diesel, no plants | 50851.39 | 0.23 | 61.4 |
| | | | |
| Plants, 10g diesel 1 | 34175.84 | 2.39 | Average |
| Plants, 10g diesel 2 | 42003.71 | 2.16 | 46.0 |

TPH peak areas calculated from replicates with less than 5% difference between them, n = 3.

Table 9.1.3.7. Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 2 months for contaminated, unplanted and planted soils.

Again the cold room sample allowed abiotic losses to be accounted for therefore breakdown rates of diesel fuel by soil microorganisms in the unplanted soil was 33% and 42% breakdown by plants and their associated microbial community in the planted soil. Enhanced breakdown of individual n-alkanes was again observed under planted soil as opposed to unplanted soil and the lower molecular weight n-alkanes were again preferentially degraded in planted soil as shown in Figure 9.1.3.8. Figure 9.1.3.9 compares the unplanted and planted chromatograms from the 10g diesel $kg^{-1}$ soil treatments.




9.1.3.8 % degradation of individual n-alkanes in 7.5g diesel $kg^{-1}$ unplanted and Westerwold's ryegrass planted soil.

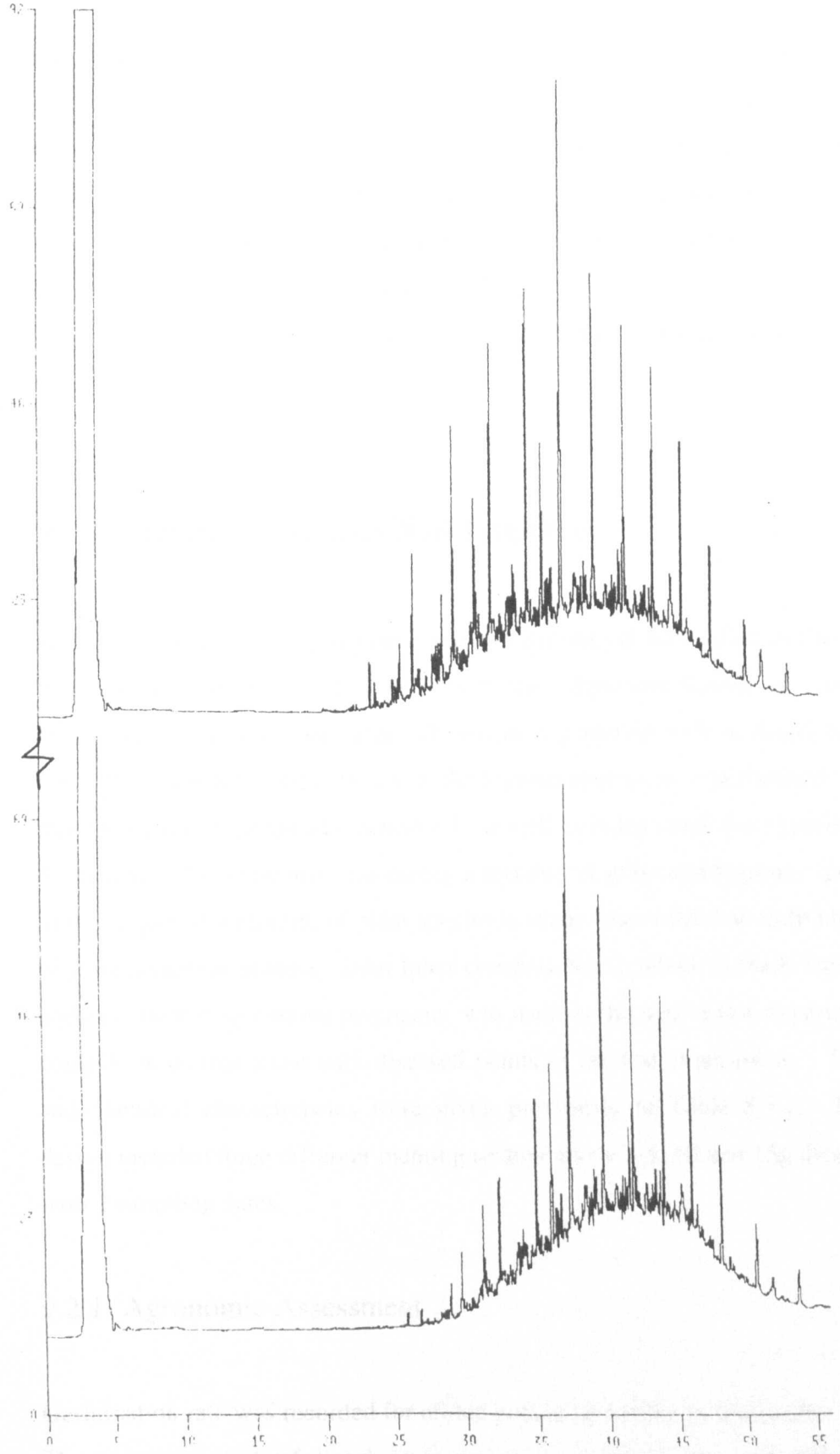


Figure 9.1.3.9 Chromatograms of residual diesel fuel extracted from 10g diesel $kg^{-1}$ soil unplanted and planted soil (top to bottom) plotted together.

The results clearly showed that at low levels of diesel fuel contamination (5g diesel $kg^{-1}$ soil), little advantage was seen in diesel fuel breakdown rates in planted soil as opposed to unplanted soil. At higher levels (7.5g and 10g diesel $kg^{-1}$ soil) a significant difference was observed between planted and unplanted soil. Degradation of individual n-alkanes in 5g diesel $kg^{-1}$ unplanted soil showed no bias as to what molecular weight range of n-alkanes were degraded. The microbial population that had proliferated in the Westerwold's ryegrass rhizosphere was seen to preferentially degrade the lower molecular weight alkanes. Breakdown of n-alkanes at the higher contamination levels (7.5g and 10g diesel $kg^{-1}$ soil) in unplanted soil was minimal.

Growing Westerwold's ryegrass clearly enhanced the degradation rate of diesel fuel in contaminated soil.

## 9.2 Legumes versus Non Legumes

A large pot trial involving legumes and non legumes at four different diesel fuel treatment levels was designed to further investigate phytoremediation of contaminated soil. Westerwold's ryegrass was again chosen as it performs well in diesel fuel contaminated soil. Common vetch was chosen as the legume species as it performed well in the initial plant screening experiment (section 6.1) as well as in the small pot experiment described in Section 8.2. Meadow mix, containing a mixture of grass and legumes species was chosen to investigate if a mixture of plant species is more successful than individual species in the phytoremediation process. John Innes compost No. 2, which is made from sterilised loam and heat treated to remove pathogens, was used as the soil in this experiment to avoid the complications that arose with diseased plants in the last experiment. The soil physical and chemical characteristics were given previously in Table 8.1.1. The experimental design included three different planting treatments in 0, 5, 10 and 15g diesel $kg^{-1}$ soil levels with 2 sampling dates.

### 9.2.1 Agronomic Assessment

Germination rate was recorded for all the pots at all treatment levels after 2 months growth. The germination rates for each plant species investigated were very different as illustrated in Table 9.2.1.1. Westerwold's ryegrass appeared to germinate more successfully at the 5g diesel $kg^{-1}$ soil level than in control soil (0g diesel $kg^{-1}$ soil). There was little difference

between germination rates in the control and 10g diesel $kg^{-1}$ soil but a definite decrease in germination was observed at the 15g diesel $kg^{-1}$ soil level. Common vetch germination appeared unaffected by the diesel fuel contamination levels chosen in this pot experiment. Meadow mix also appeared unaffected by contamination level until the 15g diesel $kg^{-1}$ soil treatment, where germination rate was reduced by 50%.

| Treatment g diesel $kg^{-1}$ soil | % germination |
|---|---|
| Westerwold's ryegrass | |
| 0 | 58 ± 0.88 |
| 5 | 67 ± 1.45 |
| 10 | 59 ± 1.53 |
| 15 | 44 ± 1.76 |
| Common vetch | |
| 0 | 60 ± 0.88 |
| 5 | 62 ± 1.76 |
| 10 | 64 ± 2.52 |
| 15 | 63 ± 1.25 |
| | Av. no. seedlings per pot |
| Meadow mix | |
| 0 | 10 ± 0.88 |
| 5 | 11 ± 1.76 |
| 10 | 11 ± 2.52 |
| 15 | 5 ± 1.15 |

Average % germination values are given for both Westerwold's rygrass and Common vetch whereas average number of seedlings per pot are given for Meadow mix, n = 3.

Average values correspond to two levels of pots (2 month and 4 month) at the same treatment level.

Table 9.2.1.1 Germination rate of Westerwold's ryegrass, Common vetch and Meadow mix at all treatment levels after 2 months growth.

After two months growth the Westerwold's ryegrass plants were growing extremely well in the 5g diesel $kg^{-1}$ soil treatment level, quite well in the 10g diesel $kg^{-1}$ soil treatment level but were poor at the 15g diesel $kg^{-1}$ soil treatment level. Figure 9.2.1.2 illustrates the health of each plant species grown and the production of shoot biomass in this pot experiment at the first harvest date (2 months). Although the Westerwold's ryegrass plants were growing well in the 5g diesel $kg^{-1}$ soil treatment level their overall

shoot biomass was reduced compared to the control. Table 9.2.1.3 shows the shoot and root biomass results of each plant species after 2 months growth.

Westerwold's ryegrass plants shoot biomass was reduced by 34%, 59% and 95% compared to the control plants average biomass in the 5g, 10g and 15g diesel $kg^{-1}$ soil treatment levels respectively. The root biomass results did not follow the same pattern, with little difference in root biomass observed until the 15g level, where root biomass actually increased. At the 5g and 10g diesel $kg^{-1}$ soil treatment levels, root biomass was reduced by 5% and 15% compared to the control. In the 15g diesel $kg^{-1}$ soil treatment level however, root biomass per plant was seen to increase 67% compared to the control. This unusual trend has been observed for Westerwold's ryegrass in a previous pot experiment (Section 8.3). The shoot to root ratio was extremely off balance in favour of root biomass which suggests the plant was allocating more of its energy and resources to producing a larger root system, possibly as a means of obtaining more water.

Common vetch plants grew quite successfully at the 5g and 10g diesel $kg^{-1}$ soil levels but again were poor at the 15g diesel $kg^{-1}$ soil level as shown in Figure 9.2.1.2. Although germination rate showed no distinct difference between control and contaminated treatments the shoot and root biomass results showed a clear difference, as illustrated in Table 9.2.1.3. Shoot biomass was reduced by 62% in both the 5g and 10g diesel $kg^{-1}$ soil treatments and by 93% in the 15g diesel $kg^{-1}$ soil treatment. This huge reduction in shoot biomass was not reflected in the root biomass results. Root biomass was reduced by 17%, 23% and 28% in the 5g, 10g and 15g diesel $kg^{-1}$ soil treatments compared to the control.

The Meadow mix plants grew well at the 5g diesel $kg^{-1}$ soil level, were poor at the 10g diesel $kg^{-1}$ soil level and barely grew at the 15g diesel $kg^{-1}$ soil level. Shoot biomass was moderately reduced by 28% in the 5g diesel $kg^{-1}$ treatment level and drastically reduced by 87% and 93% in the 10g and 15g diesel $kg^{-1}$ soil treatment level compared to the control. Root biomass was reduced by 67%, 73% and approximately 100% in the 5g, 10g and 15g diesel $kg^{-1}$ soil treatment levels compared to the control.

Plant height was also measured each month during the course of the experiment but these results will be given together with the 4 month results.


Figure 9.2.1.2 Growth of Westerwold's ryegrass, Common vetch and Meadow mix (from top to bottom) in 0g, 5g, 7.5g and 10g diesel $kg^{-1}$ soil (from left to right) after 2 months.

| | Shoot biomass oven dry wt $mg^{-1}$ per plant g diesel $kg^{-1}$ soil | | | | Root biomass oven dry wt $mg^{-1}$ per plant g diesel $kg^{-1}$ soil | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | 0 | 5 | 10 | 15 |
| Plant species Westerwold's ryegrass | 456 ± 63 | 302 ± 54 | 188 ± 41 | 23 ± 6 | 58 ± 16 | 53 ± 22 | 49 ± 6 | 97 ± 13 |
| Common Vetch | 181 ± 21 | 68 ± 35 | 69 ± 15 | 13 ± 3 | 18 ± 3 | 15 ± 6 | 14± 3 | 13 ± 3 |
| Meadow mix | 61 ± 16 | 44 ± 7 | 8 ± 1 | 4 ± 2 | 15 ± 7 | 5 ± 1 | 4 ± 1 | nd |

9.2.1.3. Shoot and root biomass results at the 2 month harvest.

After 4 months growth, the general health and biomass of plants grown in the lower levels of diesel fuel contaminated soil (5g and 10g) had greatly increased for all the plants investigated. The plants grown in 15g diesel $kg^{-1}$ contaminated soil were still not producing much bulk. Figure 9.2.1.4 illustrates Westerwold's ryegrass, Common vetch and Meadow mix in the four soil treatment levels at the 4 month harvest date. As you can see from the photographs, there is little difference between plants grown in 0g, 5g and 10g diesel $kg^{-1}$ contaminated soil for the three planting species tested. Figure 9.2.1.5 shows the increase in plant height for each plant species over the course of the experiment. The Westerwold's ryegrass plants showed a more defined difference in plant height between treatment levels at the end of the experiment than they did at the start. The Common vetch plants grown in 0g, 5g and 10g diesel $kg^{-1}$ contaminated soil showed little difference in plant height throughout the experiment and the plants grown in 5g and 10g diesel $kg^{-1}$ contaminated soil actually had taller shoot heights at the end of the experiment (4 months) than the control plants. Common vetch plants did not grow well in 15g diesel $kg^{-1}$ contaminated soil but their plant height did improve slightly over the course of the experiment. Meadow mix plants, like Westerwold's ryegrass, showed a more defined difference in plant height nearer the end of the experiment than at the start. From about the 2 month measurement, plants grown in 5g diesel $kg^{-1}$ contaminated soil had taller shoot heights than the control plants. Plants grown in 10g diesel $kg^{-1}$ soil closely followed the control plant heights until the 4 month measurement where the control plant height increased above the height of plants grown in 10g diesel $kg^{-1}$ soil. Plants grown in 15g diesel $kg^{-1}$ soil were much shorter than plants grown in the other treatments but plant height was seen to rapidly improve after 3 months.

Figure 9.2.1.4. Growth of Westerwold's ryegrass, Common vetch and Meadow mix (from top to bottom) in 0g, 5g, 7.5g and 10g diesel $kg^{-1}$ soil (from left to right) after 4 months.

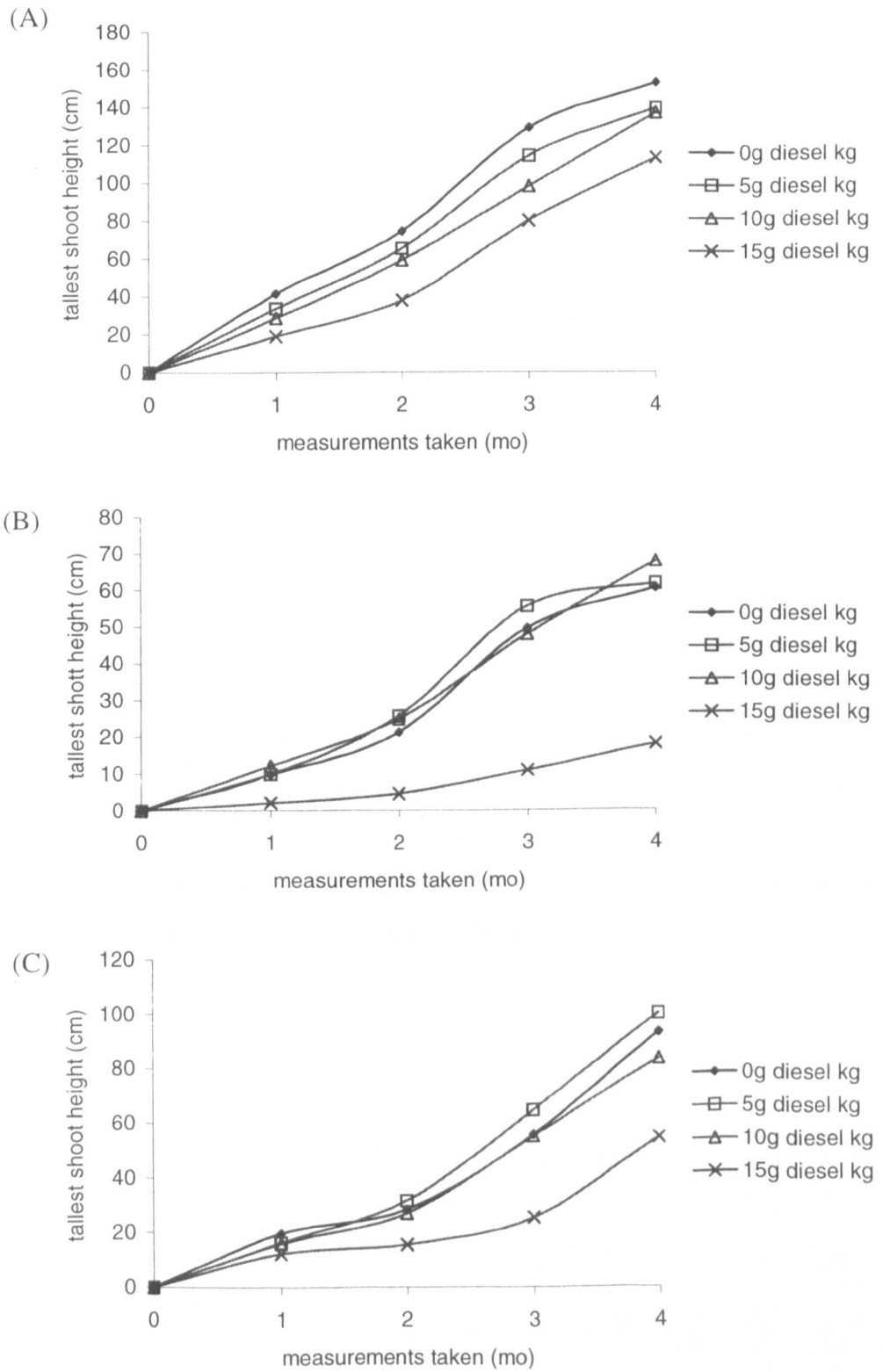



Figure 9.2.1.5 Plant height data over the course of the experiment for (A) Westerwold's ryegrass, (B) Meadow mix and (C) Common vetch in 0g, 5g, 7.5g and 10g diesel $kg^{-1}$ soil.

Shoot and root biomass was again collected at the 4 month harvest date. This collection of biomass differed from the first biomass collection at 2 months as a cutting regime was included whereby shoot biomass was cut back after 2 months growth and this biomass added to the biomass collected at the 4 month harvest. This cutting proved to be successful as healthy re-growth of all the plant species was observed.

Westerwold's ryegrass shoot biomass was still reduced when compared to the control plants shoot biomass after 4 months. Shoot biomass was reduced by 52%, 68% and 77% in 5g, 10g and 15g diesel $kg^{-1}$ soil compared to the control as shown in Table 9.2.1.6. Root biomass was reduced compared to the control by 47%, 67% and 68% in 5g, 10g and 15g diesel $kg^{-1}$ contaminated soil. No increase in root biomass was observed as seen previously at the 2 month harvest date. Common vetch plants grown in 0g, 5g and 10g diesel $kg^{-1}$ soil had similar plant heights but a noticeable difference in shoot biomass was observed. Shoot biomass was reduced by 38% and 55% in the 5g and 10g diesel $kg^{-1}$ soil treatments compared to the control. Common vetch plants grown in 15g diesel $kg^{-1}$ contaminated soil had a reduced shoot biomass of 90%. Meadow mix plants grown in 5g and 10g diesel $kg^{-1}$ contaminated soil produced slightly more shoot biomass than previously seen with any of the other plant species, when compared to the control biomass. Shoot biomass was reduced by 14% and 45% in 5g and 10g diesel $kg^{-1}$ contaminated soil compared to the control. At the 15g diesel $kg^{-1}$ soil level however, the shoot biomass was drastically reduced to 0.12% of the control biomass.

| | Shoot biomass oven dry wt per plant $mg^{-1}$ g diesel $kg^{-1}$ soil | | | | Root biomass oven dry wt per plant $mg^{-1}$ g diesel $kg^{-1}$ soil | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | 0 | 5 | 10 | 15 |
| Plant species Westerwold's ryegrass | 1388 ± 49 | 667 ± 2 | 442 ± 3 | 318 ± 8 | 238 ± 45 | 126 ± 13 | 78 ± 16 | 77 ± 12 |
| Common Vetch | 1230 ± 84 | 889 ± 52 | 552 ± 31 | 123 ± 50 | 55 ± 15 | 58 ± 8 | 49 ± 8 | 24 ± 18 |
| Meadow mix | 746 ± 84 | 644 ± 97 | 411 ± 102 | 9 ± 3 | 168 ± 23 | 275 ± 16 | 208 ± 26 | nd |

9.2.1.6. Shoot and root biomass results at the 4 month harvest.

## 9.2.2 Enzymatic Assessment of the Soil-Plant System

At two and four months, plants were harvested. After pots had been destructively sampled for plant biomass, the remaining soil was subsampled for enzymatic analysis. As before, subsamples were analysed for phosphatase activity (as described in Section 2.5.1), dehydrogenase activity (as described in Section 2.5.2) and fluorescein diacetate (FDA) activity (as described in Section 2.5.3). The results of the 2 and 4 month enzymatic analyses are discussed below.

### 9.2.2.1 Dehydrogenase Activity

Addition of diesel fuel to soil caused a huge increase in dehydrogenase activity. Dehydrogenase activity rose to 369%, 700% and 708% in 5g, 10g and 15g diesel $kg^{-1}$ in unplanted soil compared to control soil (0g diesel $kg^{-1}$ soil) as shown in Table 9.2.2.1.1. This large increase in activity was seen in the previous experiment (Section 9.1) and represented the increase in microbial numbers caused by the input of diesel fuel being utilised as a food source by the microorganisms. Little difference was seen, at this early stage, between planted and unplanted, uncontaminated soil dehydrogenase activities. However, an increase in dehydrogenase activity was observed in planted, contaminated soil as opposed to unplanted, contaminated soil. The largest increase in activity was seen under Common vetch planted soil as illustrated in Figure 9.2.2.1.2. Dehydrogenase activity rose from 97% of the unplanted, 5g diesel $kg^{-1}$ soil value to 136% and 203% above the 10g and 15g diesel $kg^{-1}$ unplanted soil values. Westerwold's ryegrass and Common vetch planted soils had dehydrogenase activities above the corresponding dehydrogenase values in unplanted soil. Meadow mix planted soil had dehydrogenase values below or just slightly over the corresponding dehydrogenase values in unplanted soil. This suggests the microbial population associated with Meadow mix plants is much smaller and less well developed than the microbial populations present under the other two plant species.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc formazan g $h^{-1}$ | conc formazan g $h^{-1}$ per pot | av conc formazan g $h^{-1}$ | conc formazan g $h^{-1}$ per pot | av conc formazan g $h^{-1}$ | conc formazan g $h^{-1}$ per pot | av conc formazan g $h^{-1}$ |
| 0 | 35.99 ± 2.65 | 33.54 ± 2.19<br>42.08 ± 4.41<br>25.57 ± 3.39 | 33.73 ± 4.77 | 38.64 ± 3.08<br>37.56 ± 2.18<br>46.98 ± 3.16 | 41.06 ± 2.98 | 40.51 ± 3.09<br>37.88 ± 5.52<br>40.37 ± 1.66 | 39.59 ± 0.85 |
| 5 | 132.92 ± 9.58 | 160.02 ± 8.33<br>211.05 ± 25.91<br>254.74 ± 20.48 | 208.61 ± 27.37 | 141.80 ± 8.44<br>132.90 ± 5.31<br>113.39 ± 6.07 | 129.36 ± 8.39 | 93.56 ± 5.68<br>107.24 ± 10.09<br>100.62 ± 5.76 | 100.47 ± 3.95 |
| 10 | 251.78 ± 17.23 | 456.78 ± 18.11<br>303.84 ± 20.64<br>473.39 ± 35.05 | 411.34 ± 53.96 | 256.57 ± 26.66<br>410.17 ± 30.68<br>362.64 ± 16.55 | 343.13 ± 45.40 | 145.89 ± 26.12<br>285.24 ± 20.89<br>256.41 ± 12.84 | 229.18 ± 42.47 |
| 15 | 254.72 ± 12.04 | 315.56 ± 17.91<br>317.78 ± 20.09<br>373.86 ± 33.42 | 335.75 ± 19.07 | 719.86 ± 34.86<br>361.28 ± 20.22<br>470.54 ± 7.23 | 517.23 ± 106.11 | 350.06 ± 32.28<br>316.83 ± 16.39<br>305.46 ± 15.19 | 324.12 ±13.38 |

Figure 9.2.2.1.1. Dehydrogenase activity results for planted and unplanted, contaminated and uncontaminated soil at the 2 month harvest. Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

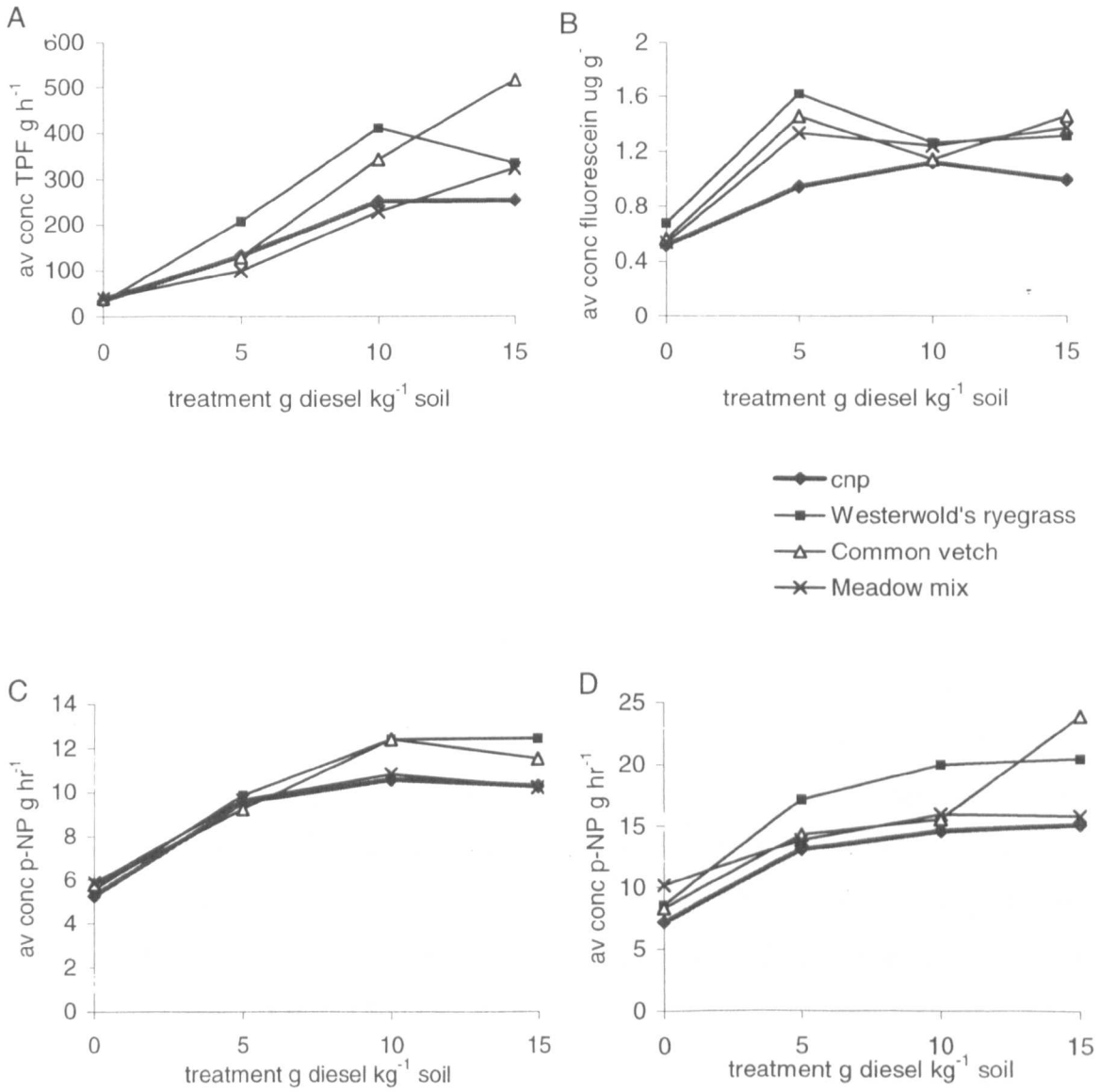



Figure 9.2.2.1.2. Enzymatic analyses for 2 month legumes vs nonlegumes soil samples. A) Dehydrogenase activity, B) FDA activity, C) Acid phosphatase activity and D) Alkaline phosphatase activity.

The initial increase in dehydrogenase activity observed at 2 months by addition of diesel fuel to soil had lessened in the 5g and 10g diesel $kg^{-1}$ soil treatments by the 4 month measurement. A huge increase in activity of 581% above the uncontaminated soil value was observed at the 15g diesel $kg^{-1}$ soil level as shown in Table 9.2.2.1.3. This high dehydrogenase activity may indicate that there is a lot of diesel fuel still present and being utilised as a food source by microorganisms in the 15g diesel $kg^{-1}$ soil. The quantity of diesel fuel remaining in the lower contamination levels may be low or the diesel fuel components left are very difficult to breakdown so the overall dehydrogenase activity of these treatments is low.

A difference in dehydrogenase activity was observed between planted and unplanted treatments at the 4 month sampling date which was not seen after 2 months. An increase of 67%, 40% and 11% was observed by growing Westerwold's ryegrass, Common vetch and Meadow mix compared to uncontaminated, unplanted soil. Figure 9.2.2.1.4 illustrates the difference in dehydrogenase activities of the planted and unplanted soil at all treatment levels. Westerwold's ryegrass planted soil dehydrogenase activity continued to increase with diesel fuel addition to 62%, 176% and 254% above the uncontaminated, planted soil in 5g, 10g and 15g diesel $kg^{-1}$ soil respectively. Common vetch planted soil dehydrogenase activity increased up to the 10g diesel $kg^{-1}$ soil, where it levelled off to the 15g diesel kg-1 soil. Meadow mix planted soil dehydrogenase activity again increased to the 10g diesel $kg^{-1}$ soil level, where a slight decrease in activity was observed at the 15g diesel $kg^{-1}$ soil level.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc formazan g $h^{-1}$ | conc formazan g $h^{-1}$ per pot | av conc formazan g $h^{-1}$ | conc formazan g $h^{-1}$ per pot | av conc formazan g $h^{-1}$ | conc formazan g $h^{-1}$ per pot | av conc formazan g $h^{-1}$ |
| 0 | 65.34 ± 2.56 | 115.76 ± 6.27<br>94.37 ± 2.81<br>117.57 ± 4.20 | 109.23 ± 7.45 | 81.10 ± 3.31<br>104.85 ± 14.64<br>87.98 ± 4.60 | 91.31 ± 7.06 | 69.19 ± 3.96<br>76.17 ± 7.00<br>71.13 ± 6.13 | 72.17 ± 2.08 |
| 5 | 100.38 ± 8.72 | 209.82 ± 14.85<br>138.96 ± 4.67<br>180.45 ± 11.67 | 176.41 ± 20.55 | 223.61 ± 24.01<br>292.02 ± 4.71<br>261.81 ± 8.02 | 259.15 ± 19.80 | 271.73 ± 15.48<br>233.68 ± 10.20<br>249.97 ± 8.97 | 251.77± 11.02 |
| 10 | 138.11 ± 7.04 | 346.27 ± 7.87<br>290.35 ± 10.60<br>267.94 ± 14.16 | 301.52 ± 23.29 | 278.35 ± 7.68<br>249.51 ± 8.19<br>409.75 ± 7.16 | 312.53 ± 49.32 | 354.13 ± 16.03<br>335.95 ± 9.20<br>326.65 ± 3.78 | 338.91 ± 8.07 |
| 15 | 445.43 ± 17.17 | 383.27 ± 10.11<br>289.01 ± 7.66<br>486.54 ± 22.73 | 386.27 ± 57.04 | 336.80 ± 10.93<br>303.67 ± 21.83<br>293.64 ± 15.53 | 311.37 ± 13.04 | 361.60 ± 17.06<br>326.83 ± 20.37<br>264.71 ± 19.84 | 317.71 ± 28.34 |

Table 9.2.2.1.3. Dehydrogenase activity results for planted and unplanted, contaminated and uncontaminated soil at the 4 month harvest. Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

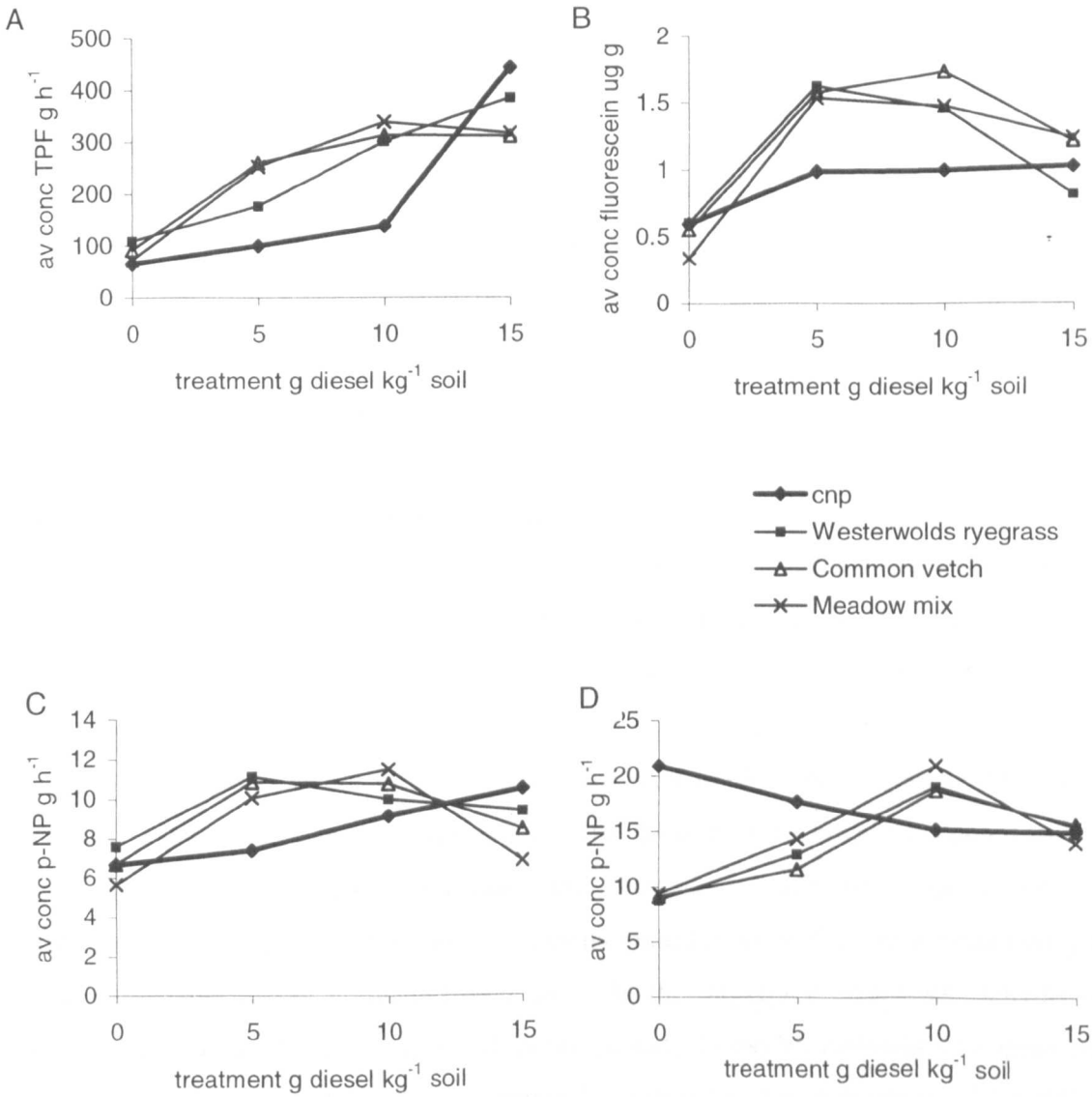



Figure 9.2.2.1.4. Enzymatic analyses for 4 month legumes vs nonlegumes soil samples. A) Dehydrogenase activity, B) FDA activity, C) Acid phosphatase activity and D) Alkaline phosphatase activity.

When the dehydrogenase activities for each treatment at the 2 month sampling date and the 4 month sampling date were compared, the increase or decrease in activity over the length of the experiment, could be determined.

In the uncontaminated, unplanted treatments, the dehydrogenase activity continued to increase from the 2 to 4 month sampling date. This would be expected as the microbial population present would utilise the organic matter and nutrients present in the soil and continue to proliferate. In the 5g and 10g diesel $kg^{-1}$ soil, the dehydrogenase activity decreased over the 4 month period. The initial flourish of activity caused by the input of a small amount of useable substrate (diesel fuel) would quickly go, causing a decrease in dehydrogenase activity sometime after the 2 month measurement. The microorganisms would use the easily degradable diesel fuel components first, which would cause the large, initial increase in dehydrogenase activity observed. The remaining diesel fuel may not be as suitable a substrate for microbial degradation hence the microbial activity and the number of microorganisms the soil could support would fall causing the observed decrease in dehydrogenase activity. At the 15g diesel $kg^{-1}$ soil level the dehydrogenase activity was increasing which suggests the remaining diesel fuel was still being utilised effectively by soil microorganisms.

In the planted soil, three different situations were found under the three different planting regimes used in this experiment. The Westerwold's ryegrass planted treatments followed the same pattern of increases and decreases in dehydrogenase activity as the unplanted soil. Dehydrogenase activity was increased more in the uncontaminated, planted treatments than in the unplanted treatment. In the unplanted treatment, dehydrogenase activity increased 80% over the 2 to 4 month period. In the Westerwold's ryegrass planted treatment, an increase of 320% was observed over the 2 to 4 month period. This difference illustrates the influence of plant exudates input and the presence of the growing plant on microbial numbers and dehydrogenase activity. The same increase in dehydrogenase activity over the 2 to 4 month period was observed for Common vetch and Meadow mix uncontaminated treatments compared to the uncontaminated, unplanted treatment. Whereas dehydrogenase activity was seen to decrease in the 5g diesel $kg^{-1}$ unplanted and Westerwold's ryegrass planted treatments, an increase was observed in the Common vetch and Meadow mix planted treatments. The reason for this difference may be that the microbial populations developing under the Common vetch and Meadow mix planted soils are different from the populations present in the unplanted and Westerwold's ryegrass planted soil. The microbial community may have developed at a slower rate which may indicate there was still easily degradable diesel fuel left for microbial utilisation or the microorganisms may have been more varied in the substrates they could degrade, allowing

higher molecular weight diesel fuel components to be used which may have been avoided by the microorganisms present in the unplanted and Westerwold's ryegrass planted soils. At the 10g and 15g diesel $kg^{-1}$ soil level, dehydrogenase activity was seen to decrease in the Common vetch planted treatments. The initial high activities observed at the 2 month sampling date imply the majority of useable diesel fuel components would have been used up initially, with the more specialised microbial component surviving to the 4 month sampling date. The Meadow mix planted soil dehydrogenase activity continued to increase in the 10g diesel $kg^{-1}$ soil before levelling off at an activity that was maintained in the 15g diesel $kg^{-1}$ soil. This suggests the microbial population that was supported in the 15g diesel $kg^{-1}$ Meadow mix planted soil had reached its maximum level of activity and still had enough substrate for growth to maintain this high level of activity.

### 9.2.2.2 Phosphatase Activity

An increased need for inorganic phosphate caused an increase in acid phosphate activity in the diesel fuel contaminated soil compared to uncontaminated soil. In unplanted soil, acid phosphate activity increased 81%, 100% and 93% in 5g, 10g and 15g diesel $kg^{-1}$ soil above the 0g diesel $kg^{-1}$ soil value. Table 9.2.2.2.1 shows the acid phosphatase results under each treatment at the 2 month harvest date. In planted treatments, the Meadow mix planted soil showed a similar pattern of acid phosphatase activity as the uncontaminated, unplanted soil as shown in Figure 9.2.2.1.2. The other two plant species phosphatase activities rose above the control, unplanted and Meadow mix planted soils only at the higher levels of diesel fuel contamination (10g and 15g diesel $kg^{-1}$ soil). There was little difference between the activities of Westerwold's ryegrass and Common vetch. The small increase in acid phosphatase activity suggested the plants had sufficient inorganic phosphate for growth hence they did not need to produce vast quantities of additional acid phosphatase enzymes to breakdown organic phosphate to inorganic phosphate at this stage.

The alkaline phosphatase results, which illustrate phosphatases produced only from micoorganisms, showed a slightly different pattern of activity. Alkaline phosphatase activity was increased by the addition of diesel fuel, as observed in the acid phosphatase results, but also by growing plants on the soil. Alkaline phosphatase activity values were increased by 20% in Westerwold's ryegrass planted soil, 16% in Common vetch planted soil and 42% in Meadow mix planted soil compared to unplanted, uncontaminated soil as shown in Table 9.2.2.2.2. Diesel fuel contamination also caused an increase in alkaline phosphatase activity under planted soil. On average an increase of 123%, 115% and 52%

was seen in diesel fuel contaminated Westerwold's ryegrass, Common vetch and Meadow mix planted soil compared to uncontaminated, planted soil. Figure 9.2.2.1.2 illustrates the pattern of alkaline phosphatase activity at each treatment level. The larger difference in alkaline phosphatase activity in planted soils compared to acid phosphatase activity may be due to the plants utilising the majority of inorganic phosphate available with the microorganisms responding to this by producing more alkaline phosphatase which would in turn release more inorganic phosphate for microbial growth.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc p-NP released ug $g^{-1}$ | conc p-NP released ug $g^{-1}$ per pot | av conc p-NP released ug $g^{-1}$ | conc p-NP released ug $g^{-1}$ per pot | av conc p-NP released ug $g^{-1}$ | conc p-NP released ug $g^{-1}$ per pot | av conc p-NP released ug $g^{-1}$ |
| 0 | 5.29 ± 0.043 | 5.79 ± 0.150<br>5.66 ± 0.102<br>5.33 ± 0.156 | 5.59 ± 0.14 | 5.81± 0.247<br>5.73 ± 0.111<br>5.88 ± 0.075 | 5.81 ± 0.04 | 6.26 ± 0.203<br>5.60 ± 0.155<br>5.79 ± 0.112 | 5.88 ± 0.20 |
| 5 | 9.57 ± 0.260 | 8.18 ± 0.092<br>11.71 ± 0.105<br>9.76 ± 0.247 | 9.88 ± 1.02 | 9.74 ± 0.246<br>9.31 ± 0.255<br>8.70 ± 0.141 | 9.25 ± 0.30 | 9.20 ± 0.215<br>9.59 ± 0.477<br>10.23 ± 0.202 | 9.67 ± 0.30 |
| 10 | 10.57 ± 0.249 | 12.12 ± 0.187<br>11.93 ± 0.313<br>13.11 ± 0.464 | 12.39 ± 0.37 | 12.03 ± 0.078<br>13.68 ± 0.479<br>11.49 ± 0.225 | 12.40 ± 0.66 | 10.95 ± 0.071<br>11.00 ± 0.196<br>10.52 ± 0.184 | 10.82 ± 0.15 |
| 15 | 10.23 ± 0.189 | 11.59 ± 0.246<br>12.02 ± 0.087<br>13.55 ± 0.118 | 12.39 ± 0.59 | 9.99 ± 0.199<br>11.78 ± 0.290<br>12.69 ± 0.206 | 11.49 ± 0.79 | 10.08 ± 0.671<br>9.93 ± 0.281<br>10.54 ± 0.194 | 10.18 ± 0.18 |

Figure 9.2.2.2.1. Acid phosphatase activity results for planted and unplanted, contaminated and uncontaminated soil at the 2 month harvest. Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ | av conc p-NP released ug $ml^{-1}$ |
| 0 | 7.19 ± 0.270 | 8.24 ± 0.196<br>8.44 ± 0.324<br>9.09 ± 0.345 | 8.59 ± 0.257 | 8.06 ± 0.157<br>7.84 ± 0.116<br>9.14 ± 0.187 | 8.34 ± 0.403 | 10.65 ± 0.243<br>10.61 ± 0.303<br>9.30 ± 0.309 | 10.19 ± 0.446 |
| 5 | 13.10 ± 0.159 | 13.24 ± 0.178<br>20.22 ± 0.660<br>18.05 ± 0.573 | 17.17 ± 2.064 | 16.31 ± 0.169<br>13.78 ± 0.204<br>12.82 ± 0.178 | 14.31 ± 1.041 | 13.28 ± 0.391<br>13.97 ± 0.216<br>14.35 ± 0.228 | 13.87 ± 0.314 |
| 10 | 14.67 ± 0.178 | 20.50 ± 0.677<br>16.99 ± 0.086<br>22.61 ± 0.744 | 20.03 ± 1.638 | 14.40 ± 0.215<br>17.22 ± 0.208<br>15.14 ± 0.241 | 15.59 ± 0.845 | 16.27 ± 0.310<br>15.86 ± 1.076<br>15.79 ± 0.314 | 15.97 ± 0.149 |
| 15 | 15.14 ± 0.241 | 17.92 ± 0.564<br>19.77 ± 0.442<br>23.67 ± 0.206 | 20.46 ± 1.695 | 30.26 ± 0.628<br>23.03 ± 0.338<br>18.40 ± 0.361 | 23.89 ± 3.453 | 15.44 ± 0.134<br>13.23 ± 0.086<br>16.86 ± 0.180 | 15.81± 1.055 |

Figure 9.2.2.2.2. Alkaline phosphatase activity results for planted and unplanted, contaminated and uncontaminated soil at the 2 month harvest. Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

The increase in acid phosphatase activity caused by the addition of diesel fuel that was observed at the 2 month sampling date was continued to the 4 month sampling date. Acid phosphatase activity increased 10%, 37% and 57% above the uncontaminated soil in 5g, 10g and 15g diesel $kg^{-1}$ soil treatments as shown in Table 9.2.2.2.3. Acid phosphatase activity was also seen to increase slightly in Westerwold's ryegrass and Meadow mix planted soils when compared with unplanted soil, as illustrated in Figure 9.2.2.1.4. Westerwold's ryegrass planted soil demonstrated a large increase in acid phosphatase activity in 5g and 10g diesel $kg^{-1}$ soil treatments compared to the corresponding unplanted treatments. This suggests the plants and microorganisms required more inorganic phosphate for growth than was currently available in the soil therefore an increase in acid phosphatase activity was seen to counteract this problem. The level of activity fell at the 15g diesel $kg^{-1}$ soil level. Figure 9.2.2.1.4 illustrates the pattern of acid phosphatase activity under Common vetch and Meadow mix planted soil, which is similar to the pattern observed under Westerwold's ryegrass planted soil.

The pattern of alkaline phosphatase activity observed after 4 months was extremely different in the unplanted and planted soil treatments as shown in Table 9.2.2.2.4 and Figure 9.2.2.1.4. In unplanted soil, alkaline phosphatase activity started at a high value in the uncontaminated soil and slowly decreased to the 15g diesel $kg^{-1}$ soil value. The planted soils had the opposite effect, with an increase in all plant species being observed from the uncontaminated soil to the 10g diesel $kg^{-1}$ soil. A further decrease in activity was observed for all plant species in the 15g diesel $kg^{-1}$ soil. The need for inorganic phosphate by microorganisms was obviously much more important to the microorganisms present in the planted soil than in the unplanted soil. The majority of the inorganic phosphate would be used by the plants for growth leaving little available for microbial growth.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ per pot | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ per pot | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ per pot | av conc p-NP released ug $ml^{-1}$ |
| 0 | 6.68 ± 0.160 | 8.37 ± 0.120<br>8.12 ± 0.215<br>6.28 ± 0.149 | 7.59 ± 0.66 | 6.49 ± 0.083<br>7.53 ± 0.078<br>6.13 ± 0.174 | 6.72 ± 0.42 | 5.20 ± 0.180<br>5.70 ± 0.188<br>6.14 ± 0.433 | 5.68 ± 0.27 |
| 5 | 7.39 ± 0.191 | 10.53 ± 0.170<br>10.47 ± 0.166<br>12.37 ± 0.162 | 11.13 ± 0.62 | 10.01 ± 0.429<br>9.71 ± 0.228<br>12.84 ± 0.331 | 10.85 ± 1.00 | 11.54 ± 0.231<br>10.56 ± 0.166<br>8.07 ± 0.096 | 10.06 ± 1.03 |
| 10 | 9.13 ± 0.159 | 11.41 ± 0.147<br>8.68 ± 0.164<br>9.92 ± 0.289 | 10.00 ± 0.79 | 11.01 ± 0.225<br>10.13 ± 0.259<br>11.14 ± 0.301 | 10.76 ± 0.32 | 12.23 ± 0.370<br>10.77 ± 0.372<br>11.45 ± 0.265 | 11.48 ± 0.42 |
| 15 | 10.50 ± 0.654 | 9.11 ± 0.098<br>7.91 ± 0.061<br>11.10 ± 0.436 | 9.37 ± 0.93 | 9.21 ± 0.170<br>9.10 ± 0.212<br>7.08 ± 0.162 | 8.46 ± 0.69 | 8.05 ± 0.201<br>6.62 ± 0.127<br>5.92 ± 0.104 | 6.86 ± 0.63 |

Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

Table 9.2.2.2.3. Acid phosphatase activity results for planted and unplanted, contaminated and uncontaminated soil at the 4 month harvest.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ per pot | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ per pot | av conc p-NP released ug $ml^{-1}$ | conc p-NP released ug $ml^{-1}$ per pot | av conc p-NP released ug $ml^{-1}$ |
| 0 | 20.92 ± 0.691 | 8.35 ± 0.180<br>9.23 ± 0.111<br>8.85 ± 0.442 | 8.81 ± 0.25 | 8.44 ± 0.638<br>8.93 ± 0.191<br>9.92 ± 0.223 | 9.10 ± 0.44 | 9.58 ± 0.591<br>10.21 ± 0.364<br>8.31 ± 0.362 | 9.37 ± 0.56 |
| 5 | 17.67 ± 0.412 | 13.78 ± 0.263<br>11.44 ± 0.345<br>13.53 ± 0.121 | 12.92 ± 0.74 | 10.97 ± 0.377<br>11.36 ± 0.302<br>12.28 ± 0.365 | 11.53 ± 0.39 | 14.44 ± 0.741<br>12.22 ± 0.438<br>16.36 ± 0.153 | 14.34 ± 1.20 |
| 10 | 15.18 ± 0.544 | 22.67 ± 0.321<br>18.50 ± 0.348<br>15.90 ± 0.107 | 19.02 ± 1.97 | 18.65 ± 0.282<br>17.74 ± 0.294<br>19.68 ± 0.876 | 18.69 ± 0.56 | 22.43 ± 0.313<br>19.97 ± 0.719<br>20.32 ± 0.163 | 20.91 ± 0.77 |
| 15 | 14.92 ± 0.246 | 16.07 ± 0.177<br>12.81 ± 0.131<br>17.32 ± 0.209 | 15.40 ± 1.34 | 16.78 ± 0.180<br>15.48 ± 0.206<br>14.67 ± 0.312 | 15.64 ± 0.61 | 16.23 ± 0.580<br>13.40 ± 0.142<br>12.21 ± 0.125 | 13.95 ± 1.19 |

Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

Table 9.2.2.2.4. Alkaline phosphatase activity results for planted and unplanted, contaminated and uncontaminated soil at the 4 month harvest.

When the phosphatase activities were compared at the 2 and 4 month sampling dates, conclusions could be drawn about the role of phosphatase enzymes in the contaminated soil system. In unplanted soils, addition of diesel fuel was used as a substrate by microorganisms for growth, creating a need for additional inorganic phosphate, which resulted in an increase in both acid and alkaline phosphatase activity. By growing plants in soil, the plants are removing inorganic phosphate for growth, which is taking available phosphate away from the microbial component. In response, an increase in predominantly alkaline phosphatase, with some acid phosphatase, was observed to release inorganic phosphate from organic phosphate for use by the microorganisms.

### 9.2.2.3 Fluorescein Diacetate (FDA) Activity

Addition of diesel fuel had a larger effect on FDA hydrolysis activity than planting had. Addition of 5g, 10g and 15g of diesel fuel to unplanted soil caused a 82%, 116% and 92% increase in FDA activity compared to control soil (0g diesel $kg^{-1}$ soil, unplanted). Table 9.2.2.3.1 shows the FDA activity results in unplanted soil and under different planting treatments. A large increase in FDA activity was observed for all plant species in the 5g diesel $kg^{-1}$ soil treatment as illustrated in Figure 9.2.2.1.2. This suggests, at this level of contamination, the microbial population associated with each plant species is working at a similar level of total activity and is not being hindered either by the quantity of diesel fuel or its toxicity. When diesel fuel contamination is increased to 10g diesel $kg^{-1}$ soil, a decrease in the level of total activity is observed. This level of activity is held in the 15g diesel $kg^{-1}$ soil for all the planted soils except Common vetch planted soil where the FDA activity appears to rise again.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc fluorescein released ug $ml^{-1}$ | conc fluorescein released ug $ml^{-1}$ | av conc fluorescein released ug $ml^{-1}$ | conc fluorescein released ug $ml^{-1}$ | av conc fluorescein released ug $ml^{-1}$ | conc fluorescein released ug $ml^{-1}$ | av conc fluorescein released ug $ml^{-1}$ |
| 0 | 0.520 ± 0.018 | 0.712 ± 0.028<br>0.725 ± 0.028<br>0.607 ± 0.017 | 0.681 ± 0.038 | 0.518 ± 0.016<br>0.508 ± 0.022<br>0.676 ± 0.008 | 0.567 ± 0.054 | 0.633 ± 0.016<br>0.436 ± 0.008<br>0.551 ± 0.011 | 0.540 ± 0.080 |
| 5 | 0.945 ± 0.015 | 1.165 ± 0.033<br>1.880 ± 0.026<br>1.819 ± 0.048 | 1.621 ± 0.229 | 1.534 ± 0.013<br>1.546 ± 0.021<br>1.304 ± 0.028 | 1.461 ± 0.079 | 1.383 ± 0.040<br>1.332 ± 0.029<br>1.300 ± 0.022 | 1.338 ± 0.024 |
| 10 | 1.121 ± 0.046 | 1.465 ± 0.095<br>1.224 ± 0.031<br>1.106 ± 0.018 | 1.265 ± 0.106 | 1.227 ± 0.021<br>1.190 ± 0.020<br>1.012 ± 0.055 | 1.143 ± 0.067 | 1.379 ± 0.034<br>1.184 ± 0.036<br>1.160 ± 0.050 | 1.241 ± 0.069 |
| 15 | 0.988 ± 0.026 | 1.041 ± 0.021<br>1.152 ± 0.034<br>1.742 ± 0.070 | 1.312 ± 0.217 | 1.389 ± 0.039<br>1.212 ± 0.020<br>1.763 ± 0.039 | 1.455 ± 0.163 | 1.270 ± 0.031<br>1.437 ± 0.033<br>1.408 ± 0.025 | 1.372 ± 0.051 |

Figure 9.2.2.3.1. FDA activity results for planted and unplanted, contaminated and uncontaminated soil at the 2 month harvest.

Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

After 4 months, FDA activity in unplanted soil at all treatment levels was much lower than the corresponding planted treatment as shown in Figure 9.2.2.1.4. The highest level of FDA activity was observed in the 5g and 10g diesel $kg^{-1}$ soil treatments for the three plant species investigated, with activity decreasing in the 15g diesel $kg^{-1}$ soil treatment. Table 9.2.2.3.2 shows the FDA activities for unplanted and planted soil at all treatment levels. At the two month measurement, FDA activities in unplanted and planted soil were similar except at the 5g diesel $kg^{-1}$ soil level. Over the course of the experiment, FDA activities of planted soils became more distinguishable from the unplanted soil activities. The FDA activity of planted soil, which represents the total microbial activity of the soil, was increased well above the activity of unplanted soil in the majority of treatments. Common vetch planted soil had the highest activity after 4 months growth of all the plant species investigated.

Measurement of FDA activity showed the level of total microbial activity was much higher in diesel contaminated soil compared to uncontaminated soil and that planted soils had a much higher level of FDA activity than unplanted soils.

| | No plants | Westerwold's ryegrass | | Common vetch | | Meadow mix | |
|---|---|---|---|---|---|---|---|
| treatment diesel g $kg^{-1}$ | av conc fluorescein released ug $ml^{-1}$ | conc fluorescein released ug $ml^{-1}$ per pot | av conc fluorescein released ug $ml^{-1}$ | conc fluorescein released ug $ml^{-1}$ per pot | av conc fluorescein released ug $ml^{-1}$ | conc fluorescein released ug $ml^{-1}$ per pot | av conc fluorescein released ug $ml^{-1}$ |
| 0 | 0.489 ± 0.020 | 0.564 ± 0.017<br>0.524 ± 0.007<br>0.402 ± 0.010 | 0.497 ± 0.048 | 0.383 ± 0.013<br>0.489 ± 0.023<br>0.487 ± 0.006 | 0.453 ± 0.035 | 0.261 ± 0.007<br>0.276 ± 0.015<br>0.232 ± 0.053 | 0.257 ± 0.013 |
| 5 | 0.806 ± 0.014 | 1.416 ± 0.029<br>1.157 ± 0.028<br>1.405 ± 0.040 | 1.326 ± 0.085 | 1.226 ± 0.097<br>1.066 ± 0.020<br>1.571 ± 0.009 | 1.287 ± 0.149 | 1.344 ± 0.055<br>1.237 ± 0.025<br>1.184 ± 0.010 | 1.255 ± 0.047 |
| 10 | 0.813 ± 0.050 | 1.340 ± 0.030<br>0.985 ± 0.028<br>1.251 ± 0.037 | 1.192 ± 0.107 | 1.324 ± 0.030<br>1.410 ± 0.023<br>1.527 ± 0.032 | 1.420 ± 0.059 | 1.381 ± 0.027<br>1.349 ± 0.027<br>0.894 ± 0.022 | 1.208 ± 0.157 |
| 15 | 0.837 ± 0.039 | 0.714 ± 0.011<br>0.631 ± 0.033<br>0.650 ± 0.020 | 0.665 ± 0.025 | 1.014 ± 0.039<br>0.955 ± 0.026<br>0.995 ± 0.063 | 0.988 ± 0.017 | 1.171 ± 0.029<br>0.963 ± 0.057<br>0.891 ± 0.014 | 1.008 ± 0.084 |

Average values are given per pot ± SE, n = 5, and average values per treatment ± SE, n = 3.

Table 9.2.2.3.2. FDA activity results for planted and unplanted, contaminated and uncontaminated soil at the 4 month harvest.

## 9.2.3 Analysis of Residual Diesel Fuel

A huge amount of breakdown was observed over the 2 month period before the first harvest. The treatments containing 5g diesel $kg^{-1}$ soil had diesel fuel residues below the measurable range, in both the planted and unplanted treatments. This huge decrease in residual diesel fuel content was not unexpected, as all the plant species investigated grew extremely well at this level of contamination and the level of enzymatic activity was high. The 10g and 15g diesel $kg^{-1}$ soil treatments also had small residual diesel fuel concentrations as shown in Tables 9.2.3.1 and 9.2.3.2. At the 10g diesel $kg^{-1}$ soil level, the lowest % diesel fuel remaining was found under Common vetch planted soil (2.1% remaining), with Westerwold's ryegrass (3.1% remaining) and Meadow mix (4.7% remaining) planted soil closely following. At the 15g diesel $kg^{-1}$ soil level, this pattern changed with Meadow mix planted soil performing slightly better than Westerwold's ryegrass planted soil. After 2 months, the % diesel fuel remaining was less than 5% of the diesel fuel added, in all treatments, which is an extremely promising result.

| treatment | TPH peak area | % difference between replicates | Av. TPH peak area | % diesel remaining |
|---|---|---|---|---|
| Fresh (time zero) | 74312 | 1.1 | | |
| | 66196 | 2.1 | | |
| | 71776 | 1.0 | 70761 | 100 |
| Ryegrass 10g 1 | 1422 | 5.6 | | |
| Ryegrass 10g 2 | 1636 | 3.8 | | |
| Ryegrass 10g 3 | 3444 | 3.3 | 2168 | 3.1 |
| Vetch 10g 1 | 1354 | 3.6 | | |
| Vetch 10g 2 | 1544 | 5.5 | 1449 | 2.1 |
| M. mix 10g 1 | 5207 | 4.4 | | |
| M. mix 10g 2 | 1415 | 5.9 | 3311 | 4.7 |

TPH peak areas calculated from replicates, n = 3.

Table 9.2.3.1 Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 2 months for 10g diesel $kg^{-1}$ contaminated, planted soils.

| treatment | TPH peak area | % difference between replicates | Av. TPH peak area | % diesel remaining |
|---|---|---|---|---|
| Fresh (time zero) | 114748 | 1.3 | | |
| | 104327 | 3.6 | | |
| | 112156 | 3.6 | 110410 | 100 |
| Ryegrass 15g 1 | 4201 | 4.6 | | |
| Ryegrass 15g 2 | 8784 | 2.6 | | |
| Ryegrass 15g 3 | 2959 | 4.8 | 5314 | 4.8 |
| Vetch 15g 1 | 3752 | 4.5 | | |
| Vetch 15g 2 | 3130 | 4.0 | | |
| Vetch 15g 3 | 1826 | 5.4 | 2903 | 2.6 |
| M. mix 15g 1 | 1485 | 4.9 | | |
| M. mix 15g 2 | 3262 | 4.1 | | |
| M. mix 15g 3 | 5635 | 1.9 | 3460 | 3.2 |

TPH peak areas calculated from replicates, n = 3.

Table 9.2.3.2 Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 2 months for 15g diesel $kg^{-1}$ contaminated, planted soils.

After 4 months, the amount of diesel fuel remaining had decreased even further. In the 10g diesel $kg^{-1}$ soil treatment the amount of diesel fuel residue left under Common vetch, Meadow mix and Westerwold's ryegrass planted soil was 1.6%, 1.9% and 2.1% as shown in Table 9.2.3.3. The overall rate of biodegradation was high in the Common vetch planted soil and the amount of diesel fuel remaining in this soil was the lowest of the three planting treatments. Meadow mix and Westerwold's ryegrass planted soil were not far behind. At the 15g diesel $kg^{-1}$ soil level, the same pattern of breakdown was observed as illustrated in Table 9.2.3.4. Common vetch planted soil had the lowest diesel fuel residue level, with Meadow mix and Westerwold's ryegrass planted soil in second and third place. The difference between the amount of diesel fuel remaining in each planting treatment was much larger in the 15g diesel $kg^{-1}$ treatment than in the 10g diesel $kg^{-1}$ soil treatment. There was, on average, a 0.4% difference between the residue levels of Common vetch and the other two plant species at the 10g diesel $kg^{-1}$ soil level. This difference rose to 1.5%

between the residue levels of Common vetch and the other two plant species at the 15g diesel $kg^{-1}$ soil level.

| treatment | TPH peak area | % difference between replicates | Av. TPH peak area | % diesel remaining |
|---|---|---|---|---|
| Ryegrass 10g 1 | 1179 | 2.9 | | |
| Ryegrass 10g 2 | 2051 | 2.2 | | |
| Ryegrass 10g 3 | 1274 | 2.3 | 1501 | 2.1 |
| Vetch 10g 1 | 1191 | 1.3 | | |
| Vetch 10g 2 | 850 | 4.5 | | |
| Vetch 10g 3 | 1311 | 4.5 | 1117 | 1.6 |
| M. mix 10g 1 | 1119 | 1.4 | | |
| M. mix 10g 2 | 1353 | 1.7 | | |
| M. mix 10g 3 | 1536 | 1.4 | 1336 | 1.9 |

TPH peak areas calculated from replicates, n = 3.

Table 9.2.3.3 Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 4 months for 10g diesel $kg^{-1}$ contaminated, planted soils.

| treatment | TPH peak area | % difference between replicates | Av. TPH peak area | % diesel remaining |
|---|---|---|---|---|
| Ryegrass 15g 1 | 4677 | 0.4 | | |
| Ryegrass 15g 2 | 2237 | 1.2 | | |
| Ryegrass 15g 3 | 4515 | 2.2 | 3810 | 3.5 |
| Vetch 15g 1 | 2653 | 1.4 | | |
| Vetch 15g 2 | 1924 | 3.8 | | |
| Vetch 15g 3 | 1712 | 1.1 | 2096 | 1.9 |
| M. mix 15g 1 | 3690 | 6.8 | | |
| M. mix 15g 2 | 3898 | 5.5 | | |
| M. mix 15g 3 | 3629 | 1.0 | 3738 | 3.4 |

TPH peak areas calculated from replicates, n = 3.

Table 9.2.3.4 Total petroleum hydrocarbon (TPH) values and % diesel fuel remaining after 4 months for 15g diesel $kg^{-1}$ contaminated, planted soils.

The results of the large scale greenhouse trials illustrate how selected plant species can be successfully used to enhance the degradation of diesel fuel contaminated soil. This observation has been noted by many authors for a variety of petroleum hydrocarbon contaminants such as PAHs (Ferro *et al.*, 1998, Qui *et al.*, 1998, Wetzel *et al.*, 1998, Binet *et al.*, 2000), crude oil and fuels (Song *et al.*, 1990, Carman *et al.*, 1998).

The residual diesel fuel concentration was found to be different under different planting species which suggests there is a difference in the microbial consortia associated with certain plant species. This is not unexpected as plant species release differing quantities and composition of root exudates which in turn, support a consortia of microorganisms. Enzymes studies were used to try and clarify the difference in microbial activity observed under different plant species and if this information could be related to the total amount of diesel fuel degraded. The most useful enzymes system studied was dehydrogenase activity. Dehydrogenase enzymes are only found in live microbial cells therefore they represent a measure of the microbial biomass and microbial activity. The results from the dehydrogenase enzyme assays showed a clear picture of how diesel fuel was affecting the microorganisms present in the soil and how microbial activity changed in planted soils as opposed to unplanted soils. This is in agreement with work carried out by Bauer *et al* (1991) who assessed the sensitivity of different soil microbiological and enzymological methods and concluded that dehydrogenase activity was the most sensitive assay for assessing biological effects of oil contamination on soil.

Phosphatases measured in soils reflect the activity of enzymes bound to soil colloids and humic substances, free phosphatases in soil solution and phosphatases associated with living and dead plant or microbial cells. It has been suggested that phosphatase enzymes can be a good indicator of the organic phosphorus mineralisation potential and biological activity of soils (Kramer and Green, 2000). An increase in acid phosphatase activity was observed in planted soils as opposed to unplanted soils which holds with the fact that acid phosphatases are produced from plants and microorganisms. This strengthens the observation of increased activity in planted soils as opposed to unplanted soils and shows that the phosphorus mineralisation cycle was continuing in diesel fuel contaminated soils. Apart from these points, measurement of phosphatase activities did prove useful in assessing the effect of diesel fuel on the soil ecosystem.

FDA hydrolysis activity was used to measure total microbial activity and again illustrated the increased activity in planted soils versus unplanted soils. It also helped to distinguish between total microbial activities under different planting species. Little difference in FDA activity was observed at the low levels of diesel fuel contamination but distinct differences was seen at the higher levels of contamination. The highest FDA

activity was observed in Common vetch planted soil. The highest rate of diesel fuel degradation was also found under Common vetch planted soil. This observation was also noted by Wang and Bartha (1990) who found FDA hydrolysis to be inversly correlated with diesel fuel persistence.

# CHAPTER TEN

## GENERAL DISCUSSION AND CONCLUSIONS

### 10.1 Analysis of Diesel Fuel

The methods developed in Chapter 3 for the extraction of diesel fuel from soil and the analysis of diesel fuel extracts allowed the changes in diesel fuel composition, after it had been released into the soil environment, to be followed. The extraction techniques employed gave more than acceptable recovery values and the improved separation of diesel fuel components using the modified GC-FID programme allowed a clear picture of diesel fuel in the soil to be obtained. Information on the composition of diesel fuel was difficult to find when this project began. Therefore, an integral part of this study was to thoroughly characterise diesel fuel to obtain detailed information on the individual hydrocarbon components that make up the diesel fuel chromatogram. Differences in the composition of diesel fuel depend on the source of crude oil and the refinery processes involved in producing the diesel fuel product. Clear differences in the aliphatic and aromatic fraction of two different diesel fuels was apparent thus it was concluded that studies should be carried out using the same source of diesel fuel (i.e. from an Esso petrol station) to minimise differences in the resulting chromatograms. The majority of work in this thesis used the same batch of diesel fuel to ensure any differences detected was due to the influence of the soil environment and not due to differences in the diesel fuel itself. If a new batch of diesel fuel was required, the fuel

was again characterised to identify any changes in hydrocarbon content from the last diesel fuel used.

Capillary GC-FID analysis produced a very distinctive chromatogram. The majority of diesel fuel components, providing the backbone of the chromatogram, were n-alkanes ranging from n-nonane ($C_9$) to tricosane ($C_{23}$). This is in agreement with the suggested carbon range of diesel fuel (S.C.L.F., 1998, Holtzclaw *et al.*, 1991). These n-alkane components were well separated by GC-FID and easy to identify using external standards. However, beneath the backbone of n-alkanes, lay a whole range of peaks, some of which were separated whilst others were small and indistinguishable from adjacent peaks. Some branched cyclohexanes, branched benzenes and branched naphthalenes were identified by GC-FID but it became apparent that another method of analysis would have to be used to provide the in depth information of diesel fuel composition required. To provide positive identification of the lesser hydrocarbon peaks found in the diesel fuel profile, pure diesel fuel was separated by capillary GC-FID then the individual peaks analysed by mass spectrometry (MS). This proved an enormous task as 177 peaks were resolved and required positive identification. GC-MS is an extremely powerful tool for identifying unknown compounds, but the complexity of diesel fuel made interpretation of the mass spectra difficult. Nevertheless, out of the 177 peaks resolved, 106 were positively identified. And it should be emphasized that out of the 61 unidentified peaks, only 7 are truly unknown. The other 54 peaks were identified into the appropriate hydrocarbon class using the mass spectra data. Capillary GC-FID analysis provided excellent separation of the major components in the diesel fuel chromatogram and was sufficient for carrying out total petroleum hydrocarbon (TPH) analysis on petroleum samples. However, capillary GC-MS is required if any in depth analysis is required. Without GCMS analysis, a lot of the more interesting components of diesel fuel would not have been identified. As expected, the majority of components were n-alkanes, branched alkanes and diaromatic hydrocarbons. However, a less obvious homologous series of branched cyclohexanes was identified by GC-MS. This series ranged from methyl cyclohexane, which is extremely volatile to hexadecyl cyclohexane, which is completely non volatile. There are no accounts of this class of cyclic alkanes being present in diesel fuel and the majority of toxicity and degradation information centres on alkanes and aromatics. The identification of this class of compounds was an important find and was investigated in more detail. The results of this investigation will be discussed later.

The detailed analysis of diesel fuel by GC-MS was time consuming but provided valuable and much needed information on the composition of diesel fuel. When the results of the diesel fuel analysis were summarised into specific hydrocarbon fractions, a clear picture of the hydrocarbon classes present in diesel fuel emerged. The alkane fraction made up 57% of the total diesel fuel, with n-alkanes being 25%, branched alkanes (and alkenes) being 24% and cyclic alkanes being 7%. This high percentage of branched alkanes and cyclic alkanes would make the diesel fuel more difficult to degrade than if it contained only n-alkanes. The aromatic fraction was 27% of the total diesel fuel, with 5% attributed to monoaromatics, 19% diaromatics and 3% polyaromatics (PAHs). Again, this high level of PAHs would make the diesel fuel more resistant to degradation. The trend of results of the hydrocarbon class data were in agreement with work published by Song *et al* (1990) and Bundt *et al* (1991) and represent a realistic measure of the percentage hydrocarbon class distribution in a typical diesel fuel.

## 10.2 Behaviour of Diesel Fuel in the Soil Environment.

### 10.3.1 Weathering Effects

Changes occur in diesel fuel composition after it has been released into the environemnt due to abiotic processes such as volatilisation, leaching and adsorption and biotic processes, namely biodegradation.

#### 10.2.1.1 Volatilisation

Non-biological change in diesel fuel composition was caused mainly by volatilisation. Diesel fuel is a complex mixture of hydrocarbons containing both volatile and non volatile components. It was important to determine which compounds would volatilise under normal conditions and how this affected the diesel fuel chromatogram. GC-FID analysis illustrated that compounds up to approximately 20 minutes on the diesel fuel chromatogram would volatilise under normal conditions. The components were

identified by GC-MS as mainly BTEX (benzene, toluene, ethyl benzene and xylenes), n-alkanes ($C_9$-$C_{12}$), branched alkanes, branched benzenes and branched cyclohexanes (methyl to butyl). On average, the volatile fraction of diesel fuel made up approximately 10% of the total diesel fuel product.

### 10.2.1.2 Leaching

Leaching of diesel fuel on entering the soil system from an above ground source was found to be minimal, due to the hydrophobic properties of diesel fuel and the adsorptive capacity of the surface soil components. Organic matter and silt and clay content played an extremely important role in retaining diesel fuel in the top 30cm of the soil. This factor is extremely beneficial to the phytoremediation of diesel fuel contaminated sites as the contaminant is held within the rhizosphere of the plant where degradation takes place. It also reduces the possibility of diesel fuel contaminating ground water. If diesel fuel were to enter from an underground source, for example from a leaking underground storage tank, this scenario would be completely different. The amount of organic matter is greatly reduced in subsurface soils therefore diesel fuel entering would not be retained and would migrate more readily. This may lead to possible groundwater contamination. The contaminant would also be out with the rooting zone of most plant species and the number of microorganisms is greatly reduced in subsurface soils, making biodegradation unlikely. It was therefore concluded that phytoremediation of diesel fuel contaminated soils would only be applicable to surface contamination, not underground spills.

### 10.2.1.3 Adsorption

During the leaching experiments it was observed that diesel fuel components, particularly the higher molecular weight aromatic compounds, could be adsorbed onto soil components such as organic matter. It was therefore not unexpected that samples contaminated with diesel fuel then stored for 2 months, had lower recoveries of diesel fuel components by soxhlet extraction than freshly contaminated soil. A subsample of each contaminated soil was stored at 4°C to provide a baseline value of the percentage diesel fuel lost through abiotic processes and not due to microbial breakdown or plant

influence. Although volatilisation would be considerably slower at 4°C, the method of soxhlet extraction would remove the readily volatile components. The contaminated soil was stored in bags which prevented leaching from occurring. The samples would not represent the loss of diesel fuel through abiotic processes entirely, but loss through volatilisation and adsorption. On average, 30% of the diesel fuel fraction added at the start of the experiment was not recovered after 2 months storage at 4°C. It has been shown that approximately 10% of this fraction would be readily lost through volatilisation therefore only 20% is unaccountable for. If the diesel fuel components were adsorbed to the soil surface, then the 6 hour hot solvent soxhlet extraction would be expected to remove them efficiently. This was not the case, which suggests the diesel fuel components have diffused into the soil organic matter matrix and possibly become irreversibly bound. The organic fraction of soils is responsible for the sorption of many compounds, particularly those that are hydrophobic (Alexander, 1994) such as the majority of diesel fuel components. Sorption of organic contaminants to soils often entails an initially rapid and reversible process followed by a period of slow sorption occurring over weeks, months or even years and this slow sorption leads to a fraction that resists desorption (Hatzinger and Alexander, 1995) hence resists extraction by 'normal' methods. This desorption resistant fraction is often persistent in the soil environment as it is less available to microbial and enzymatic breakdown. PAHs have been found to become 'trapped' in the humic polymer of soil organic matter (Eschenbach et al., 1993, Alexander, 1994, Hatzinger and Alexander, 1995) which increases their environmental persistence. The fact that approximately 20% of my diesel fuel became unavailable was worrying from a remediation point of view as I wanted to induce microbial degradation of the contaminants. If a large proportion of the contaminant was unavailable to the microbial population, complete remediation of diesel fuel contaminated soil would not be feasible. However, in soil systems with high clay and particulate matter content the majority of bacterial cells are themselves sorbed (Alexander, 1994). It is likely, that bacteria active in degradation are retained on solids, a view that is supported by the finding that as naphthalene is being metabolised in the soil, the population of naphthalene degraders that are sorbed are two orders of magnitude above those present in the water phase (Di Grazio *et al.*, 1990). In addition, exoenzymes released from microbial cells and plants become sorbed to soil components and play and important role in contaminant degradation. So although sorption may reduce the rate and extent of biodegradation, it does not necessarily prevent it.

## 10.3 Physical Effects of Diesel Fuel on Soil

When considering the overall effect of contamination on a soil system, change to the soil's physical as well as biological characteristics must be investigated. The water holding capacity of soil was affected by diesel fuel contamination. Fresh diesel fuel contamination at high levels (50g diesel $kg^{-1}$ soil) increased water holding capacity by approximately 14% compared to uncontaminated soil. This observation may be caused by the presence of more hydrophilic, aromatic compounds, that are still present in freshly contaminated soil, attracting water. As the diesel fuel contaminated soil aged, this large increase in water holding capacity was not observed. After 4 months, a slight increase was found between the contaminated soils and uncontaminated soils water holding capacity. This small increase was probably due to the small residual diesel fuel content of the soil and the influence of soil microorganisms and plant growth on soil organic carbon content.

It seems that soil freshly contaminated with diesel fuel will attract and hold water more readily than uncontaminated or aged diesel fuel contaminated soil. This may be an additive factor as to why diesel fuel does not leach far through soil, but stays in the surface soil. Apart from the fuel components being adsorbed to soil organic matter and physically impeded from moving, if diesel fuel contaminated soil was holding water but not draining, the usual percolation of water through the soil profile would be affected.

A more important physical affect caused by diesel fuel contamination of soil was observed when contaminated soils, left in the greenhouse to age, were used in a pot experiment. These soils were seeded then the seeds thoroughly watered in. However, it was extremely difficult to wet the soil in these pots. The water pooled on the soil surface and seemed to sink into the soil very slowly. This phenomenon is indicative of water repellency. Soil water repellency is attributed to the presence of hydrophobic organic substances forming a coating over the surface of normally hydrophillic soil particles. The process of aging diesel fuel would have left the heavier, more hydrophobic hydrocarbons as a residue which caused the soil to develop water repellency. This phenomenon has been observed by other authors (Li *et al.*, 1997, Brown *et al.*, 1982). The formation of repellent soil may result in impaired plant-soil water relations as it would strongly influence the soil's ability to maintain appropriate

moisture conditions. This factor is extremely important, as provision of an adequate water supply is essential in optimising plant growth and maintaining good plant cover.

## 10.4 Effect of Diesel Fuel on Plant Growth and Development

### 10.4.1 Acute Toxicity

Diesel fuel contaminated soil had a huge effect on germination. The inhibition of germination generally increased with increasing diesel fuel concentration. The germination response however, varied greatly between plant species and even within subspecies. The most obvious example of this differential sensitivity to diesel fuel was found with the grasses. Some grass species germinated well (e.g. Westerwold's ryegrass) whereas other would not germinate at all (e.g. Couchgrass). Differences were also seen in plants within subspecies (e.g. Fescue). The grasses which appeared less tolerant of diesel fuel were weed species which was not surprising as diesel fuel was used, in the past, as a post emergence herbicide to remove weed grasses in vegetable crops (Gauvrit and Cabanne, 1993). Despite the low germination rates observed in diesel fuel contaminated soil initially, most of the plant species screened germination rates improved which suggested whatever had affected germination was short lived. Acute toxicity caused by the lighter fraction of diesel fuel was delaying seed emergence and reducing germination rate. When seeds were germinated in a more open environment where volatile diesel fuel components could dissipate or aged diesel fuel contaminated soils where the majority of volatile components had volatilised, the germination rate was much higher. To determine which compounds present in the volatile fraction of diesel fuel were causing this effect on germination rate, the data from the GC-MS analysis of diesel fuel were studied. The most predominant volatile hydrocarbons found in diesel fuel were the isomers of xylene (m-, o- and p-), short chained n-alkanes ($C_9$-$C_{12}$) and branched benzenes. There were also low levels of toluene, branched cyclohexanes (methyl to butyl) and alkenes. N-alkanes ($C_6$-$C_{12}$) are non toxic to plants (Crafts and Reiber, 1948) and introducing a double bond to the structure only increases toxicity slightly. These components of the volatile diesel fuel fraction were therefore unlikely to be causing the effect on germination observed.

Crafts and Reiber (1948) also stated that toxicity increased as the side chain length was increased. This statement was verified by the work of Currier (1951), who found toxicity increased in the order benzene > toluene > xylene. All these compounds were present in the volatile diesel fuel fraction and were probably having an effect on germination rate.

The most interesting components identified in the volatile diesel fuel fraction were branched cyclohexanes as little work has been carried out on these compounds. Cyclohexane was found to be more phytotoxic than methyl cyclohexane when applied as a spray diluted in paraffin oil (Crafts and Reiber, 1948). As these cyclohexanes were present as pure compounds in air, they are likely to be 3,000 times more phytotoxic than when they were present in paraffin oil (Currier, 1951). An unusual pattern of germination was apparent when these compounds were tested, with length of cyclohexane branching determining toxicity to seeds. The ethyl and butyl branched cyclohexanes had a huge detrimental effect on germination with practically no seeds germinating in any of the ethyl cyclohexane concentrations and only the lowest level of butyl cyclohexane allowed normal seed germination. The methyl and propyl cyclohexanes, on the other hand, had an almost insignificant effect on germination rate. A similar observation was noted by Crafts and Reiber (1948) concerning the toxicity of substituted benzenes. The mono and tri substitutions were low in toxicity and the di and tetra substitutions were high in toxicity. This pattern of toxicity according to the placement of selected branches forms the basis of herbicide toxicity as branches placed at certain positions on the herbicide molecule will be readily metabolised, whereas other positions will prevent metabolism and induce toxicity. Lightweight, volatile cyclic alkanes and aromatic hydrocarbons were shown to be the most likely candidates influencing the germination of seeds in diesel fuel contaminated soil. Although these volatile hydrocarbons had the largest effect in delaying and reducing seed emergence, it was not the only factor inhibiting germination.

### 10.4.2 Physical Impedance

Germination rate of seeds grown in diesel fuel contaminated soil with minimal volatile diesel fuel components did show an increase in germination rate when compared with the germination rate in freshly contaminated soil but the germination rate was never as high as the germination rate observed in uncontaminated soil. This implies the

remaining diesel fuel had some level of toxicity to seeds. Inhibition of germination was observed in seeds soaked in diesel fuel but the seeds themselves were still viable and capable of germinating. A delay in emergence was observed when these soaked seeds were planted in uncontaminated soil. This delay in emergence increased with increased soaking time of the seeds. As the embryos within the seeds were not killed outright by soaking in diesel fuel, the diesel fuel must have been asserting a physical effect on the seed. This leads us back to the physical hydrophobic property of diesel fuel and the fact that diesel fuel is water repellent. Seeds present in diesel fuel contaminated soil would probably be coated with a thin film of oil which may act as a physical barrier, preventing or reducing both water and oxygen from entering thus 'suffocating' the seed. Both water and oxygen are essential in initiating seed germination therefore a delay in germination would be expected if water and oxygen were prevented from reaching the internal seed structure. Amakiri and Onofeghara (1984) showed that seeds of *Capsicum frutescens* retained almost 100% viability after 32 weeks pre-soaking in crude oil but a lag phase preceding germination was observed.

### 10.4.3 Plant Hormone-Type Influence

During a pot trial involving Oil seed rape, an extremely low germination rate was observed in diesel fuel contaminated soil. When the seeds were removed from the soil after the pot experiment had ended, it became apparent what had caused the low germination rate. The majority of seeds had begun germinating and split their seed coats but the root and shoot tips failed to orient properly and were shrivelled and unable to emerge from the soil. The root tips grew horizontally or curled upwards whereas the shoot tips were frequently found orienting downwards. This disruption in geotropism has been noted before with Soybeans grown in oily sludge material (Bossert and Bartha, 1985) and is likely to be caused by hydrocarbon residues in the soil with plant hormone-type effects. Gudin and Harada (1974a and 1974b) found the presence of compounds with plant hormone activity in crude oil and petroleum fractions. Naphthenic acids and phenyl acetic acids illustrated certain developmental effects on vegetation, with phenyl acetic acid having a significant effect on geotropism (Gudin and Harada, 1974b).

Naphthenic acid is used in commercial powders to initiate root production in cut stems. Considerable branching of certain plant species was observed in diesel fuel

contaminated soil where no branching was apparent on the corresponding plants grown in uncontaminated soil. This substantiates the presence of certain compounds present in diesel fuel contaminated soil that possess plant hormone-type effects. Naphthalene derivatives were tested for their effect on root branching as a number of branched naphthalenes were identified in the diesel fuel fraction by GC-MS. Naphthalene showed no significant increase in root branching but root branching did appear more frequently as the number of substitutions on the naphthalene structure increased. 1,4 dimethyl naphthalene caused an increase in branching above that observed on plant roots grown in naphthalene. 2,3,5 trimethyl naphthalene further increased the number of plants illustrating branched roots.

The hormonal effects induced by diesel fuel may be harmful as shown by the effect on geotropism on Oil seed rape emergence or have no adverse effects or even beneficial effects, as illustrated by the increased root branching on selected plant species. This effect would be beneficial as it provides a larger surface area for microbial colonisation which would increase the rhizosphere effect created by plants and their associated microorganisms.

### 10.4.4 Effect on the Spatial Distribution of Roots

In order for phytoremediation to have a beneficial effect on diesel fuel breakdown, a large root mass would have to develop in the contaminated areas. Diesel fuel was proving to be inhibitory to many stages of plant development and uncertainty arose as to whether plant roots would actually grow in diesel fuel contaminated soil or whether diesel fuel would retard the growth of roots to such an extent that the plant would be damaged. In order to answer these questions, an experimental glass box system was set up which enabled the pattern of root development of plant species to be followed. Plant root growth was retarded when faced with a subsurface layer of diesel fuel. The roots stopped growing, just short of the diesel fuel contaminated horizon. However, once all the uncontaminated surface soil was packed with roots and there was no more available uncontaminated soil to grow into, the roots began to penetrate the contaminated area. This result was extremely promising, as plant roots would have to penetrate contaminated soil for rhizodegradation to occur. The plant roots passed through this contaminated area very quickly and were not as bulky as the roots at the top and the

bottom of the glass box. All the same, roots did grow and fill the contaminated area with a sizeable root mass. Variations on this experiment were conducted with the results illustrating that plant roots would tackle diesel fuel contaminated areas only when the majority of uncontaminated soil has been filled, unless the level of diesel fuel contamination is very low (5g – 10g diesel $kg^{-1}$ soil). The likely reasons for this avoidance of contaminated areas is obviously the toxicity of diesel fuel but also the oxygen levels in these contaminated areas would be low, causing the searching roots to avoid these areas. The contaminated patches would be colonised by hydrocarbon utilising microorganisms which would compete with plant roots for oxygen.

### 10.4.5 Effect on Nodulation of Leguminous Plants

When petroleum hydrocarbons contaminate soil, the carbon:nitrogen (C:N) ratio of the soil is altered. The added carbon stimulates microbial numbers but causes an imbalance in the C:N ratio which may result in immobilisation of soil nitrogen by the microbial biomass, leaving none available for plant growth. As members of the *Leguminosae* fix atmospheric nitrogen to produce their own nitrate for growth, they may prove more successful at growing on diesel fuel contaminated soil. To further support this statement, species of *Leguminosae* have been found to be the most abundant reinhabitors of petroleum contaminated sites (Gudin and Syratt, 1975).

An important factor in the legumes success would be dependant on the development of functional root nodules. Decrease in nodule formation has been noted by other authors in soils contaminated with heavy metals (Porter and Sheridan, 1981, Casella *et al.*, 1988), agrochemicals (Mårtensson, 1992) and polyaromatic hydrocarbons (Wetzel and Werner, 1995), however no work has been carried out on nodulation in diesel fuel contaminated soil.

During harvesting of pot experiments containing leguminous plants, a recurring difference in the number and formation of root nodules present on diesel fuel contaminated and uncontaminated plants was observed. The average total number of root nodules per plant was drastically reduced in contaminated plants compared to control plants and observation of nodule sections by light microscopy illustrated clear differences in their structure. Unexpectedly, the nodules taken from contaminated plants were more developed than the corresponding nodules on uncontaminated plants.

An explanation for this observation is the addition of diesel fuel to the soil system acted as a huge input of carbon, thereby causing an imbalance in the C:N ratio of the soil. This may have initiated a response in the plant, as it required more available nitrogen, where chemical signals were given out, attracting the appropriate species of *Rhizobium* to the plant root surface. At the root surface, the *Rhizobia* would infect root hairs and penetrate into the root structure, initiating the development of the root nodule. Although the number of nodules were reduced on plants grown in diesel fuel contaminated soil, they were well developed and effectively fixing atmospheric nitrogen which indicates legumes would be a potentially useful plant species to grow in diesel fuel contaminated soils.

### 10.4.6 Chronic Toxicity

Signs of chronic toxicity induced by diesel fuel contamination became apparent once the plant was more developed. Chronic toxicity is evident from slow development of injury with long lasting effects including yellowing of leaves, stunting of plant height and eventual death (Currier, 1951). Reduction in plant height was apparent for all plant and tree species grown in diesel fuel contaminated soil. This reduction in plant height caused further reductions in shoot and root biomass except at low levels of diesel fuel contaminated soil where one species of grass, appeared to have increased shoot and root biomass. The species of legumes investigated during this study seemed less affected by diesel fuel contamination. A larger decrease in shoot and root biomass was observed for non leguminous plants compared to leguminous plants which may be due to the legumes' symbiotic relationship with nitrogen fixing bacteria providing additional nitrogen for plant growth. Another observation made during this study indicating chronic toxicity was the onset of maturation was slower in contaminated plants when compared with control plants. Plants grown in uncontaminated soil began to seed and flower at an earlier stage than plants grown in contaminated soil. The lag phase observed in flowering/seeding of the plant species investigated suggests a chronic toxic effect of diesel fuel which is affecting the plants' physiology. The fact that the contaminated plants did eventually flower/seed suggests diesel fuel is delaying the developmental process rather than preventing it.

## 10.5 Effect of Diesel Fuel on Soil Microorganisms and Enzymic Activity

Addition of diesel fuel to the soil system would have an effect on the biological component of the soil. Diesel fuel may prove harmful to the higher organisms commonly found in soil such as earthworms and insects. This effect was not investigated however during this study. Attention was given to the effect of diesel fuel on the microbial component of soil as the microorganisms would be the main degraders of diesel fuel. It is known that nearly all soils contain populations of microorganisms capable of degrading petroleum hydrocarbons (Bossert and Bartha, 1984). Therefore, diesel fuel was not expected to have a deleterious effect on the microbial component, except perhaps at very high concentrations. The usual methods of measuring a change in microbial composition are microbiological methods such as most probable number (MPN) determinations and plating techniques. A more important measurement to be made during this study was the change in microbial activity not in microbial composition therefore another approach was taken, away from the usual microbiological techniques. Soil enzymology can be used to assess the biochemical reactions taking place in a soil system. Enzymes catalyse many degradative processes in soil and originate from plants, animals, fungi and bacteria although the microbial component is the main source of these enzymes. By studying some of the important soil enzyme systems, information could be provided on the degradation and cycling processes occurring in the soil and also provide an estimate of total microbial activity.

Addition of diesel fuel to soil caused a significant increase in dehydrogenase activity. As the dehydrogenase enzymes responsible for dehydrogenase activity are found only in intact microbial cells, this result indicates proliferation of the natural microbial community and/or a huge increase in the oxidative capabilities of the existing microbial population. Fresh diesel fuel contains a number of readily utilisable compounds such as n-alkanes, therefore it is likely that the existing microbial community used this huge carbon input as a food source which would mean a large increase in the oxidative behaviour of the microbial population but because there was such a large input of carbon, microbial proliferation would also have occurred. There would be nothing to limit the growth of the microbial population initially. This initial increase in dehydrogenase activity was seen to lessen in the lower diesel fuel

contaminated treatments (5g and 10g diesel $kg^{-1}$ soil) with time suggesting the readily degraded hydrocarbons were less abundant.

Degradation of organic carbon by microorganisms also requires an input of nutrients, particularly nitrogen (N) and phosphorus (P). The amount of plant available nitrogen decreased in the diesel fuel contaminated soils, as discussed previously, which resulted in the formation of nitrogen fixing root nodules on leguminous plants. The amount of phosphorus would also be expected to become limiting as the majority of P would be used by microorganisms during the degradation of diesel fuel. Acid and alkaline phosphatase activity was seen to increase with the addition of diesel fuel which supports the previous statement. The activities of soil phosphatases convert unavailable, organic P to available, inorganic P which suggests additional inorganic P was required in the diesel fuel contaminated soil.

A non specific enzyme assay was also carried out which measured the fluorescein diacetate (FDA) hydrolysing ability of proteases, lipases and esterases. The ability to hydrolyse FDA is widespread in the soil environment therefore FDA activity can also be used as a measure of total microbial activity. FDA activity differs from dehydrogenase activity in that FDA hydrolysing enzymes are found as both exoenzymes and within microbial cells whereas dehydrogenase enzymes are found only in live microbial cells. Addition of diesel fuel showed a large increase in FDA activity as expected suggesting the total metabolic activity of the FDA hydrolysing enzymes was increased to breakdown the added diesel fuel.

The enzyme analyses illustrate that diesel fuel is used as a food source by soil microorganisms which causes increased microbial activity and microbial proliferation. An additive effect of this diesel fuel utilisation is the increased need for nutrients which is supported by the increase in phosphatase activity in the soil and the production of root nodules by leguminous plants.

## 10.6 Effect of Diesel Fuel on Soil Microorganisms and Enzymic Activity in Planted Soils

The same series of enzyme assays were used to assess the level of enzyme activity in soils planted with selected plant species to try and illustrate plant influence on soil microbial communities.

Dehydrogenase activity increased under planted soil as opposed to unplanted soil which shows the larger microbial population and the increased activity of this population associated with the rhizosphere of the plant. An increase in dehydrogenase activity was also observed in diesel fuel contaminated, planted soil. Differences in dehydrogenase activity was also seen under different plant species which illustrates that plants themselves, through differences in root exudation and root biomass, influence the population of microorganisms that populate their rhizosphere.

The phosphatase activity results in planted soils were not as useful as the other measured enzymes assays. No clear trend was apparent but generalisations could be made. Increased phosphatase activity (both acid and alkaline phosphatase) was observed in planted soils as opposed to unplanted soils which is representative of the increased need for inorganic P for plant growth as well as for microbial growth.

FDA activity in planted soils, which represents the total microbial activity of the soil, was increased well above the activity in unplanted soils and this increase in activity did not decrease with time.

The enzyme assay results clearly illustrate the increased activity in planted soils as opposed to unplanted soils which suggests the degradative capabilities of rhizosphere microorganisms and enzymes should be greater than non-rhizosphere microorganisms and enzymes.

## 10.7 Enhanced Degradation of Diesel Fuel in Planted Soil

The enhanced activity under planting was reflected in the residual diesel fuel content of planted soils as opposed to unplanted soils. The amount of diesel fuel remaining in soil after phytoremediation treatment was lower than without treatment. At low levels of diesel fuel contamination (5g diesel $kg^{-1}$ soil) little enhancement was seen using phytoremediation when compared to natural attenuation (biodegradation by indigenous microorganisms without the addition of fertilisers, inoculants etc). Only 3% more diesel was broken down under planting. As the diesel fuel concentrations in the soil increased however, the benefits of phytoremediation on residual diesel fuel content was observed. The planted soils consistently degraded more diesel fuel leaving a smaller residual fraction than unplanted soil. On average, 15% less diesel fuel remained in

planted soils compared to unplanted soils. The microbial population associated with planted soil was seen to preferentially degrade different hydrocarbon compounds than the microorganisms in unplanted soil. This again substantiates the idea that the microbial community of the rhizosphere changes by plant influence. Differences in the amount of diesel fuel remaining in soil was also noted under different plant species with the leguminous species planted soil degrading more diesel fuel than either the grass species or the mix of grasses and legumes.

## 10.8 Implications for the Phytoremediation of Diesel Fuel Contaminated Sites

The results from this study allow various conclusions to be drawn on the potential of phytoremediation for diesel fuel contaminated sites. Firstly, the hydrophobic behaviour of diesel fuel prevents this contaminant from leaching far through the soil profile. This is a benefit as contamination of ground water is less of a risk and the contaminant is held within the rooting zone of the plant making rhizodegradation possible. The hydrophobic property of diesel fuel, particularly diesel fuel residues, causes the soil to become slightly water repellent which affects the plants ability to maintain appropriate moisture conditions. Growing plants may be affected initially by this but plant roots provide channels for water infiltration as they push their way through the soil profile and the additional root biomass will add to the organic carbon content of the soil which should increase the ability of soil to hold water.

The rhizosphere community that develops under plant species is greater in size, more diverse and more metabolically active than the microbial community found in unplanted soils. This is the major reason why many authors have found increased organic contaminant degradation in planted soils as opposed to unplanted soils. The release of exudates from plant roots may also aid in co-metabolism of the more resistant compounds found in diesel fuel which would not be possible in unplanted soils. The living plant exerts a definite influence on the surrounding microbial community and encourages the degradation of diesel fuel.

The greatest problem with establishing a plant population on diesel fuel contaminated soil was the toxicity of the volatile diesel fuel fraction. The volatile diesel

fuel fraction had a huge inhibitory effect on seed germination. It is therefore advised to leave a site contaminated with a fresh diesel fuel spill for at least 2 weeks before planting to allow the diesel fuel volatiles to dissipate. The majority of sites will contain aged hydrocarbons and this will not be a problem.

Plants respond very differently to diesel fuel contamination therefore the choice of plant species to phytoremediate diesel fuel contaminated sites is extremely important. The agricultural, ley grasses such as Westerwold's ryegrass were more tolerant of diesel fuel than any of the weed grass species. Grasses are useful in phytoremediation practices as they have extensive, fibrous root systems which allows a huge surface area for microbial colonisation. They also have considerable rooting depths, with Westerwold's ryegrass shown to root to a depth of >1m which is more than sufficient for treatment of contaminants in the surface soil. The roots of most plants were also shown to grow into diesel fuel contaminated areas after an initial acclimatisation period. Another successful species of plant found to grow in diesel fuel contaminated soil was the legumes. Legumes were less affected by growing in diesel fuel contaminated soil than the grasses. This is probably due to their ability to fix atmospheric nitrogen to produce nitrate for growth. This process was found to be unaffected and even enhanced in diesel fuel contaminated soils. If extremely high levels of diesel fuel are present on site then willow trees may be grown, as they are more tolerant of high levels of diesel fuel than lower plant species. Again, the selection of willow clone is very important as they also have differential sensitivities to diesel fuel contamination.

Increased bioremediation of diesel fuel contaminated soil was seen in planted soils compared to unplanted soils which suggests phytoremediation should be considered as an alternative remediation technique to the destructive and expensive techniques favoured for the clean up of petroleum hydrocarbon contaminated land. The practical implications that can be taken from this study are low levels of diesel fuel contamination can be successfully remediated, on a short time scale, using a cover of plants. Leguminous plants were particularly successful and would be of benefit as fertiliser addition is unnecessary, although an input of phosphate would increase plant biomass and may enhance degradation rates further. If the move to add ethanol to fuels in the UK was adopted, the remedial situation would change significantly. In the case of diesel fuel, its movement in the soil profile would increase, possibly taking the contaminant out of reach of the rooting zone, making rhizodegradation ineffective. There would also be a greater threat of groundwater contamination. Another alternative phytoremediation process may have to be considered.

# REFERENCES


Adam, G. and Duncan, H. (2001). Development of a sensitive and rapid method for the measurement of total microbial activity using Fluorescein Diacetate (FDA) in a range of soils. *Soil Biology and Biochemistry*, **33**, (7/8), 943-951.

ADAS. (1986). PH and lime requirement of mineral soil, Method 32. The Analysis of Agicultural Materials Reference Book 427, Ministry of Agriculture, Fisheries and Food, 3rd Edition. Pp 98-99.

ADAS. (1986). Particle size distribution in soil, Method 57. The Analysis of Agicultural Materials Reference Book 427, Ministry of Agriculture, Fisheries and Food, 3rd Edition. pp175-178.

ADAS. (1986). Phosphorus, extractable, Method 59. The Analysis of Agicultural Materials Reference Book 427, Ministry of Agriculture, Fisheries and Food, 3rd Edition. pp183-185.

ADAS. (1986).Potassium, extractable, Method 63. The Analysis of Agicultural Materials Reference Book 427, Ministry of Agriculture, Fisheries and Food, 3rd Edition. pp193-194.

Akhlaq, M. S. (1995). Rapid group type analysis of crude oils using high performance liquid chromatography and gas chromatography. *Journal of Chromatography*, **644**, 253-258.

Alexander, M. (1994). Biodegradation and Bioremediation. Academic Press, Ltd, London, UK.

Amakiri, J. O. and Onofeghara, F. A. (1984). Effects of crude oil pollution on the germination of *Zea mays* and *Capsicum frutescens*. *Environmental Polltuion*, **35**, 159-167.

Anderson, M. A., Hung, A. Y. C., Mills, D., Scott, M. A. (1995). Factors affecting the surface tension of soil solutions and solutions of humic acids. *Soil Science*, **160 (2)**, 111-116.

Aprill, W. and Sims, R. C. (1990). Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. *Chemosphere*, **20**, 253-265.

Atlas, R. M. and Bartha, R. (1981). Microorganisms and some novel pollution problems. In: Microbial Ecology: fundamentals and applications. Addison-Wesley Publishing Company, Inc., Philippines. Pp. 422-427.

Atlas, R. M. and Bartha, R. (1992). Hydrocarbon biodegradation and oil spill bioremediation. In: (Marshall, K. C.). Advances in Microbial Ecology, 12. Plenum Press, New York, USA. pp. 289-338.

Baker, J. M. (1970). The effects of oils on plants. *Environmental Pollution*, **1**, 27-44.

Battin, T. J. (1997). Assessment of fluorescein diacetate hydrolysis as a measure of total esterase activity in natural stream sediment biofilms. *The Science of the Total Environment*, **198**, 51-60.

Berry, K. A. T. and Burton, D. L. (1997). Natural attenuation of diesel fuel in heavy clay soil. *Canadian Journal of Soil Science*, **77**, 469-477.

Binet, P., Portal, J. M., Leyval, C. (2000). Dissipation of 3-6 ring polycyclic aromatic hydrocarbons in the rhizosphere of ryegrass. *Soil Biology and Biochemistry*, **32**, 2011-2017.

Bjurman, J. (1993). Determination of microbial activity in moulded wood by the use of fluorescein diactetate. *Material und Organismen*, **28**, 1-16.

Boero, G. and Thien, S. (1979). Phosphatase activity and phosphorus availability in the rhizosphere of corn roots. In : Hartley, J. L. and Russel, S. R. (Eds), The Soil-Root Interface, Academic Press Inc, Ltd., London.

Böhm, W. (1979). Root parameters and their measurement. In : Billings, W. D., Golley, F., Lange, O. L., Olson, J. S. (Eds). Methods of Studying Root Systems, Ecological Studies 33. Springer-Verlag, Germany.

Bossert, I. And Bartha, R. (1984). The fate of petroleum in soil ecosystems. In: Atlas, R. M. (Ed.). Petroleum Microbiology, MacMillan Publishing Company, USA. pp. 435-473.

Bossert, I. And Bartha, R. (1985). Plant growth in soils with a history of oily sludge disposal. *Soil Science*, **140 (1)**, 75-77.

Bremner, J. M. and Mulvaney, C. S. (1982). Total nitrogen. In : Methods of Soil Analysis. Page, A. L., Miller, R. H., Keeney, D. R. (Eds.). American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Wisconsin, USA. pp 1119-1123.

Breeuwer, P., Drocourt, J. L., Bunschoten, N., Zwietering, M. H., Rombouts, F. M., Abee, T. (1995). Characterisation of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescein product. *Applied and Environmental Microbiology*, **61**, 1614-1619.

Brewin, N. (1991). The legume root nodule. *Annual Review of Cell Biology*, **7**, 191-226.

Brock, T. D. and Madigan, M. T. (1991). Microbial Ecology. In : 6th Ed Biology of Microorganisms, 667-672.

Brown, K. W., Brawand, H., Thomas, J. C., Evan, G. B. (1982). Impact of simulated land treatment with oily sludges on ryegrass emergence and yield. *Agronomy Journal*, **74** (2), 257-261.

Brown, J. L., Syslo, J., Lin, Y. H., Getty, S., Vemuri, R., nadeau, R. (1998). On-site treatment of contaminated soils: an approach to bioremediation of weathered petroleum compounds. In: Andes, R. P., Barkan, C. P. L., Calabreses, E. J., Kostecki, P. T. (Eds.). Principles and Practices of Diesel Contaminated Soils, Volume VII. Association of American Railroads, Amherst Scientific Publishers, Massachusetts, USA.

Bundt, J., Herbel, W., Steinhart, H. Franke, S., Francke, W. (1991). Structure type separation of diesel fuels by solid phase extraction and identification of the two and three ring aromatics by capillary GC-mass spectrometry. *Journal of High Resolution Chromatography*, **14**, 91-98.

Burken, J. G. and Schnoor, J. L. (1997). Uptake and metabolism of Atrazine by Poplar trees. *Environmental Science and Technology*, **31**, 1399-1406.

Camiña, F., Trasar-Cepeda, C., Gil-Sotres, F., Leirós, C. (1998). Measurement of dehydrogenase activity in acid soils rich in organic matter. *Soil Biology and Biochemistry*, **30** (8/9), 1005-1011.

Carman, E. P., Crossman, T. L., Gatliff, E. G. (1998a). Phytoremediation of No.2 fuel oil contaminated soil. *Journal of Soil Contamination*, **7** (4), 455-466.

Carman, E. P., Crossman, T. L., Gatliff, E. G. (1998b). Phytoremediation of fuel oil contaminated soil. In: Andes, R. P., Barkan, C. P. L., Calabreses, E. J., Kostecki, P. T. (Eds.). Principles and Practices of Diesel Contaminated Soils, Volume VII. Association of American Railroads, Amherst Scientific Publishers, Massachusetts, USA.

Carr, R. H. (1919). Vegetative growth in soils containing crude petroleum. *Soil Science*, 66-69.

Casella, S., Frassinetti, S., Lupi, F., Squartini, A. (1988). Effect of cadmium, chromium and copper on symbiotic and free living *Rhizobium leguminosarum* biovar *trifolii*. *FEMS Microbiology Letters*, **49**, 343-347.

Casida, Jr., L. E., Klein, D. A., Santoro, T. (1964). Soil dehydrogenase activity. *Soil Science*, **98**, 371-376.

Chaîneau, C. H., Morel, J. L., Oudot, J. (1997). Phytotoxicity and plant uptake of fuel oil hydrocarbons. *Journal of Environmental Quality*, **26**, 1478-1483.

Chen, W., Hoitink, A. J., Schmitthenner, A. F., Tuovinen, O. H. (1998). The role of microbial activity in suppression of damping-off caused by *Pythium ultimum*. *Phytopathology*, **78**, 314-322.

Crafts, A.S. and Reiber, H. G. (1948). Herbicidal properties of oils. *Hilgardia*, **18 (2)**, 77-156.

Cunningham, S. D., Anderson, T. A., Schwab, A. P., Hsu, F. C. (1996). Phytoremediation of soils contaminated with organic pollutants. *Advances in Agronomy*, **56**, 55-113.

Cunningham, S. D., Shann, J. R., Crowley, D. E., Anderson, T. A. (1997). Phytoremediation of soil and water contaminants. In: Kruger, E. L., Anderson, T. A., Coats, J. L. (Eds.). ACS Symposium Series 664. American Chemical Society, Washington D. C., USA.

Curl, E. A. and Truelove, B. (1986). The rhizosphere. Springer-Verlag, Berlin, Germany.

Currier, H. B. (1951). Herbicidal properties of benzene and certain methyl derivatives. *Hilgardia*, **20 (19)**, 383-406.

Dibble, J. T. and Bartha, R. (1979a). Rehabilitation of oil-inundated agricultural land: a case history. *Soil Science*, **128** (1), 56-60.

Dibble, J. T. and Bartha, R. (1979a). Effect of environmental parameters on the biodegradation of oil sludge. *Applied and Environmental Microbiology*, **37** (4), 729-739.

Dick, R. P. (1997). Soil enzyme activities as integrative indicators of soil health. In : Pankhurst, C. E., Doube, B. M., Gupta, V. V. S. R. (Eds), Biological Indicators of Soil Health. CAB International, Wallingford, Oxon, UK.

D. Glass Associates, Inc. (1998) The 1998 United States Market for Phytoremediation. Phytoremediation report: executive summary. www.channel.co/dglassassoc/

Di Grazio, P. M., Blackburn, J. W., Bienkowski, P. R., Hilton, B., Reed, G. D., King, J. M. H., Sayler, G. S. (1990). *Applied Biochemistry and Biotechnology*, **24/25**, 237-252.

Environment Agency : National Groundwater and Contaminated Land Centre. (1999). The fuel additive MTBE – a groundwater protection issue? Reference Booklet.

Environment Times. (1998). Oil storage. Environment Times, **5** (1), 33-37.

Erickson, L. E., Banks, M. K., Davis, L. C., Schwab, A. P. Muralidharan, N., Reilley, K., Tracey, J. C. (1998). Using vegetation to enhance *in situ* bioremediation. www.engg.ksu.edu.

Eschenbach, A., Kästner, M., Bierl, R., Schaefer, G., Mahro, B. (1994). Evaluation of a new, effective method to extract polycyclic aromatic hydrocarbons from soil samples. *Chemosphere*, **28** (4), 683-692.

Fayad, N. M. and Overton, E. (1995). A unique biodegradation pattern of the oil spilled during the 1991 Gulf war. *Marine Pollution Bulletin*, **30** (4), 239-246.

Federle, T. W., Ventullo, R. M., White, D. C. (1990). Spatial distribution of microbial biomass, activity, community structure and the biodegradation of linear alkylbenzene sulfonate (LAS) and linear alcohol ethoxylate (LAE) in the subsurface. *Microbial Ecology*, **20**, 297-313.

Ferro, A., Kennedy, J., Rock, S. A. (1998). Phytodegradation of PCP and PAH contaminated soil using perennial ryegrass. In: Andes, R. P., Barkan, C. P. L., Calabreses, E. J., Kostecki, P. T. (Eds.). Principles and Practices of Diesel Contaminated Soils, Volume VII. Association of American Railroads, Amherst Scientific Publishers, Massachusetts, USA.

Fletcher, J. S. and Hedge, R. S. (1995). Release of phenols by perennial plant roots and their potential importance in bioremediation. *Chemosphere*, **31**, 3009-3016.

Fontvieille, D. A., Outagueroine, A., Thevenot, D. R. (1992). Fluorescein diacetate hydrolysis as a measure of microbial activity in acquatic systems : application to activated sludges. *Environmental Technology*, **13**, 531-540.

Frankenberger, Jr., W. T. and Dick, W. A. (1983). Relationship between enzyme activities and microbial growth and activity indices in soil. *Soil Science Society of America Journal*, **47**, 945-951.

Frankenberger, Jr., W. T. and Johnson, J. B. (1993). Factors affecting invertase activity in soils. *Plant and Soil*, **74**, 313-323.

Gudin, C and Harada, H. (1974a) Physiologie végétale – Présence de substances de type auxinique dans le pétrole. *C. R. Academy Science, Paris*, **278 Serie D**, 1229-1231.

Gudin, C and Harada, H. (1974b) Physiologie végétale – Présence dans le pétrole d'inhibiteurs de la croissance des plantes, de la séries des phtalates. *C. R. Academy Science, Paris*, **278 Serie D**, 1361-1364.

Gudin , C. and Syratt, W. J. (1975). Biological aspects of land rehabilitation following hydrocarbon contamination. *Environmental Pollution*, **8**, 107-112.

Gauvrit, C. and Cabanne, F. (1993). Oils for weed control : uses and mode of action. *Pesticide Science*, **37**, 147-153.

Gibson, D. T. and Subramanian, V. (1994). Microbial degradation of organic compounds. In: (Gibson, D. T.). Microbiology Series, 13. Marcel Dekker, Inc., New York. pp. 181-212.

Gill, N. T. and Vear, K. C. (1969). Agricultural Botany. Unwin Brothers Limited, Surrey, England.

Gramss, G., Voight, K. D., Kirsche, B. (1999). Oxidoreductase enzymes liberated by plant roots and their effects on soil humic material. *Chemosphere*, **38** (7), 1481-1494.

Grant, R., Brown, C. J., Birse, E.L. (1992). Map Sheet 14, Ayr. Soil Survey of Scotland. The Macualay Institute for Soil Research, Aberdeen.

Gumprecht, G., Gerlach, H., Nehrkorn, A. (1995). FDA hydrolysis and resazurin reduction as a measure of microbial activity in sediments from the south-east Atlantic. *Helogoländer Meeresuntersuchungen*, **49**, 189-199.

GWRTAC. (1996). Phytoremediation. In: Groundwater Remediation Technologies Analysis Centre, Technology Overview Report TO-96-03.

Hatzinger, P. B. and Alexander, M. (1995). Effect of aging of chemicals in soil on their biodegradability and extractability. *Environmental Science and Technology*, **29**, 537-545.

Higgins, I. J. and Burns, R. G. (1975). Hydrocarbons. In: The Chemistry and Microbiology of Pollution, Academic Press, Inc, London, UK. Pp. 111-139.

Hodgson, J. M. (1976). Soil survey field handbook. Soil Survey Technical Monograph No. 5, Rothamstead Experimental Station, Harpenden.

Holtzclaw, H. F. Jr., Robinson, W. R., Odom, J. D. (1991). Organic chemistry. In: General Chemistry, 9th Edition. DC Heath and Company, Massachusetts, USA.

Jørgensen, K. S., Puustinen, J., Suortti, A. M. (2000). Bioremediation of petroleum hydrocarbon-contaminated soil by composting biopiles. *Environmental Pollution*, **107**, 245-254.

King, P. M. (1981). Comparison of methods for measuring severity of water repellence of sandy soils and assessment of some factors that affect it measurement. *Australian Journal of Soil Research*, **19**, 275-285.

Kiss, S. (1998). Enzymology of oil-contaminated soils. In: Kiss, S., Pasca, D. and Dragan-Bularda, M (Eds.). Enzmology of Disturbed Soils, Developments in Soil Science 26, Elseveir, Amsterdam, The Netherlands.

Khan, M. Q. (1987). Studies on the measurement of extractable and mineralizable nitrogen in soil. Ph.D. thesis, University of Glasgow.

Kolb, B., Ettre, L. S. (1997). Theoretical background of headspace-gas chromatography and its applications. In: Static Headspace Gas Chromatography: Theory and Practice, Wiley-VCH, Inc., New York, USA.

Kramer, D. N. and Guilbault, G. G. (1963). A substrate for the fluoremetric determination of lipase activity. *Analytical Chemistry*, **35**, 588-589.

Krämer, S. and Green, D. M. (2000). Acid and alkaline phosphatase dynamics and their relationship to soil microclimate in a semiarid woodland. *Soil Biology and Biochemistry*, **32**, 179-188.

Li, X., Feng, Y., Sawatsky, N. (1997). Importance of soil-water relations in assessing the endpoint of bioremediated soils. I. Plant Growth. *Plant and Soil*, **192**, 219-226.

Lindsay, S. (1992). Retention amd peak dispersion. In: Barnes, J (Ed.) High Performance Liquid Chromatography, 2nd Edition, John Wiley and Sons, England. pp 17-19.

Loser, C., Seidel, H., Hofffmann, P., Zehnsdorf, A. (1999). Bioavailability of hydrocarbons during microbial remediation of a sandy soil. *Applied Microbiology and Biotechnology*, **51**, 105-111.

MacNaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y. J., White, D. C. (1999). Microbial population changes during bioremediation of an experimental oil spill. *Applied and Environmental Microbiology*, **65** (8), 3566-3574.

Manahan, S. E. (1994). Technology, Resources and Energy. In:. Environmental Chemistry, 6th Edition, CRC Press, Inc., Florida, USA. pp 509-512.

Margesin, R. and Schinner, F. (1997a). Efficiency of indigenous and inoculated cold-adapted soil microorganisms for biodegradation of diesel oil in alpine soils. *Applied and Environmental Microbiology*, **63** (7), 2660-2664.

Margesin, R. and Schinner, F. (1997b). Laboratory bioremediation experiments with soil from a diesel-oil contaminated site – significant role of cold-adapted microorganisms and fertilisers. *Journal of Chemical Technology and Biotechnology*, **70**, 92-98.

Mårtensson, A. M. (1992). Effects of agrochemicals and heavy metals on fast growing rhizobia and their symbiosis with small-seeded legumes. *Soil Biology and Biochemistry*, **24**, 435-445.

Ma'shum, M. and and Farmer, V. C. (1985). Origin and assessment of water repellency of a sandy south Australian soil. *Australian journal of Soil Research*, **23**, 23-626.

Massad, E., Böhm, G. M., Saldiva, P. H. N. (1993). Ethanol fuel toxicity. In: Handbook of Hazardous Materials. Academic Press, Inc., pp. 265-275.

Metwaly, H. A. H. (1999). A study of groundwater contamination and bioremediation treatment using natural soil and vegetation. Ph. D. thesis, University of Glasgow.

Negri, M. C. and Hinchman, R. R. (1996). Plants that remove contaminants from the environment. *Laboratory Medicine*, **27** (1), 36-40.

Nicolotti, G. and Egli, S. (1998). Soil contamination by crude oil: impact on the mycorrhizosphere and on the revegetation potential of forest trees. *Environmental Pollution*, **99**, 37-43.

Nichols, T. D., Wolf, D. C., Rogers, H. B., Beyrouty, C. A., Reynolds, C. M. (1997). Rhizosphere microbial populations in contaminated soils. *Water, Air and Soil Pollution*, **95**, 165-178.

Oudot, J., Ambles, A., Bourgeois, S., Gatellier, C., Sebyera, N. (1989). Hydrocarbon infiltration and biodegradation in a landfarming experiment. *Environmental Pollution*, **59**, 17-40.

Pant, H. K., Vaughan, D., Edwards, A. C. (1994). Molecular size distribution and enzymatic degradation of organic phosphorus in root exudates of spring barley. *Biology and Fertility of Soils*, **18**, 285-290.

Porter, J. R., Sheridan, R. P. (1981). Inhibition of nitrogen fixation in alfalfa by arsenate, heavy metals, fluoride and simulated acid rain. *Plant Physiology*, **68**, 143-148.

Prince, R. C. (1994). Monitoring the efficacy of shoreline bioremediation after the Exxon Valdez oil spill. OECD Documents, Bioremediation: The Tokyo '94 Workshop.

Qui, X., Leland, T. W., Shah, S. I., Sorensen, D. L., Kendall, E. W. (1998). Field study: grass remediation for clay soil contaminated with polycyclic aromatic hydrocarbons. In: Andes, R. P., Barkan, C. P. L., Calabreses, E. J., Kostecki, P. T. (Eds.). Principles and Practices of Diesel Contaminated Soils, Volume VII. Association of American Railroads, Amherst Scientific Publishers, Massachusetts, USA.

Ragg, J. M., Shipley, B. M., Duncan, N. A., Bibby, J. S., Mervilees, D. W. (1976). Map Sheet 31, Airdrie, Soil Survey of Scotland. The Macaulay Institute for Soil Research, Aberdeen.

RHS Encyclopedia of Gardening. (1996).

Rogers, H. B., Beyrouty, C. A., Nichols, T. D., Wolf, D. C., Reynolds, C. M. (1996). Selection of cold-tolerant plants for growth in soils contaminated with organics. *Journal of Soil Contamination*, **5 (2)**, 171-186.

Rotman, B. and Papermaster, B. W. (1996). Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proceedings of the National Academy of Science, USA*, **55**, 134-141.

Rovira, A. D. and McDougall, B. M. (1967). Microbiological and Biochemical Aspects of the Rhizosphere. In: McLaren, A. D., Peterson, G. H. (Eds.). Soil Biochemistry. Edward Arnold Publishers, Ltd., London, UK. Pp. 417-463..

Roy, J. L., McGill, W.B. (1997). Characterisation of disaggregated nonwettable surface soils found at old crude oil spill sites. *Canadian Journal of Soil Science*, **78**, 331-344.

Roy, R. and Greer, C. W. (2000). Hexadecane mineralization and denitrification in two diesel fuel-contaminated soils. *FEMS Microbiology Ecology*, **32**, 17-23.

Salisbury, F. B. and Ross, C. W. (1991). Hormones and growth regulators : Auxins and gibberellins. In : ($4^{th}$ Ed), Plant Physiology, Wadsworth Publishing Company, California, pp. 357-381.

Salt, D. E., Smith, R. D., Raskin, I. (1998). Phytoremediation. *Annual review of plant physiology and plant molecular biology*, **49**, 643-668.

Schwab, A. P., Su, J., Wetzel, S., Pekarek, S., Banks, M. K. (1999). Extraction of petroleum hydrocarbons from soil by mechanical shaking. *Environmental Science and Technology*, **33**, 1940-1945.

Schwab, A. P., Al-Assi, A. A., Banks, M. K. (1998). Adsorption of naphthalene onto plant roots. *Journal of Environmental Quality*, **27**, 220-224.

Schnoor, J. L., Licht, L. A., McCutcheon, S. C., Wolfe, N. L., Carreira, L. H. (1995). Phytoremediation of organic and nutrient contaminants. *Environmental Science and Technology*, **29** (7), 318A-323A.

Schnürer, J., Rosswall, T. (1982). Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. *Applied and Environmental Microbiology*, **43**, 1256-1261.

Scottish Contaminated Land Forum (SCLF). (1998). Analysing for total petroleum hydrocarbons (TPH) in soil. Information sheet No. 1.

Scottish Environmental Protection Agency (SEPA). (1997). Annual report and accounts.

Scottish Environmental Protection Agency (SEPA). (1998). Annual report and accounts.

Šepic, E., Trier, C., Leskovšek, H. (1996). Biodegradation studies of selected hydrocarbons from diesel oil. *Analyst*, **121**, 1451-1456.

Skujinš, J. J. (1967). Enzymes in soil. In: MacLaren, A. D., Peterson, G. H. (Eds.). Soil Biochemistry, Volume 1. Marcel Dekker, New York. pp 371-414.

Skujinš, J. (1976). Extracellular enzymes in soil. *CRC Critical Reviews in Microbiology*, 383-421.

Smith, F. E. (1951). Tetrazolium salt. *Science*, **113**, 751-754.

Snyder, L. R. (1961). Application of linear elution adsorption chromatography to the separation and analysis of petroleum. I. Compound class separation over alumina and silica. *Analytical Chemistry*, **33**, 1527-1534.

Song, H. G., Wang, X., Bartha, R. (1990). Bioremediation potential of terrestrial fuel spills. *Applied and Environmental Microbiology*, **56** (3), 652-656.

Steubing, T. (1998). Enzymology of oil-contaminated soils. In: Kiss, S., Pașca, D. and Drăgan-Bularda, M (Eds.). Enzmology of Disturbed Soils, Developments in Soil Science 26, Elseveir, Amsterdam, The Netherlands.

Stomp, A. M., Han, K. H., Wilbert, S., Gordon, M. P. Genetic improvement of tree species for remediation of hazardous wastes. *In vitro Cell Developmental Biology*, **29**(p), 227-232.

Stubberfield, L. C. F. and Shaw, P. J. A. (1990). A comparison of tetrazolium reduction and FDA hydrolysis with other measurements of microbial activity. *Journal of Microbiological Methods*, **12**, 151-162.

Song, H. G., Wang, X., Bartha, R. (1990). Bioremediation potential of terrestrial fuel spills. *Applied and Environmental Microbiology*, **56 (3)**, 652-656.

Swisher, J. and Carroll, G. C. (1980). Fluorescein diacetate hydrolysis as an estimator of microbial biomass on coniferous needle surfaces. *Microbial Ecology*, **6**, 217-226.

Tabatabai, M. A., Bremner, J. M. (1982). Phosphomonoesterases (Acid and Alkaline phosphatases). Soil Enzymes. In : Page, A. L., Miller, R. H., Keeney, D. K. (Eds). Methods of Soil Analysis Part 2 : Chemical and Microbiological Properties, 2nd Edition. American Chemical Society of Agronomy, Inc, Soil Science Society of America, Inc., Wisconsin, USA. pp 926-929.

Ururahy, A. F. P., Marins, M. D. M., Vital, R. L., Gabardo, I. T., Pereira, N. Jr. (1999). Effect of aeration on biodegradation of petroleum waste. *Revista de microbiologia*, 1-10.

US EPA. (1986). Method 8100 Polynuclear Aromatic Hydrocarbons. www.epa.gov.

van Overbeek, J. and Blondeau, R. (1954). Mode of action of Phytotoxic oils. *Weeds*, 55-65.

Walton, B. T. and Anderson, T. A. (1990). Microbial degradation of Trichloroethylene in the rhizosphere: potential application to biological remediation of waste sites. *Applied and Environmental Microbiology*, **56**(4), 1012-1016.

Walton, B. T., Guthrie, E. A., Hoylman, A. M. (1994a). Toxicant degradation in the rhizosphere. In: Anderson, T. A. and Coats, J. R. (Eds.). Bioremediation through rhizosphere technology, ACS Symposium Series 563, American Chemical Society, Washington D. C., USA. pp. 11-26.

Walton, B. T., Hoylman, A. M., Perez, M. M., Anderson, T. A., Johnson, T. R., Guthrie, E. A., Christman, R. F. (1994b). Rhizosphere microbial communities as a plant defence against toxic substances in soils. In: Anderson, T. A. and Coats, J. R. (Eds.). Bioremediation through rhizosphere technology, ACS Symposium Series 563, American Chemical Society, Washington D. C., USA. pp. 82-92.

Wang, X., Yu, X., Bartha, R. (1990). Effect of bioremediation on polycyclic aromatic hydrocarbon residues in soil. *Environmental Science and Technology*, **24**, 1086-1089.

Wang, Z., Fingas, M., Sergy, G. (1994). Study of 22 year old Arrow oil samples using biomarker compounds by GC/MS. *Environmental Science and Technology*, **28**, 1733-1745.

Warner, M. L., Sauer, R. H., Carlile, D. W. (1983). Barley growth in coal liquid and diesel liquid fuels from coal and oil : a comparison of potential toxic effects on barley. *Water, Air and Soil Pollution*, **22**, 47-55.

Watson, C. L., Letey, J. (1970). Indices for characterising soil-water repellency based on contact angle surface tension relationships. *Soil Science*, **108**, 58-63.

Wetzel, A. and Werner, D. (1995). Ecotoxicological evaluation of contaminated soil using legume root nodule symbiosis as effect parameter. *Environmental Toxicology and Water Quality*, **10**, 127-133.

Wetzel, S. C., Banks, M. K., Schwab, A. P. (1998). Rhizosphere effects on the degradation of pyrene and anthracene in soil. In: Andes, R. P., Barkan, C. P. L., Calabreses, E. J., Kostecki, P. T. (Eds.). Principles and Practices of Diesel Contaminated Soils, Volume VII. Association of American Railroads, Amherst Scientific Publishers, Massachusetts, USA.

Whittaker, M., Pollard, S. J. T., Fallick, T. E. (1995). Characterisation of refractory wastes at heavy oil-contaminated sites: a review of conventional and novel analytical methods. *Environmental Technology*, **16**, 1009-1033.

Widrig, D. L. and Manning, J. F. Jr. (1995). Biodegradation of No. 2 fuel in the vadose zone: a soil column study. *Environmental Toxicology and Chemistry*, **14** (11), 1813-1822.

Xu, J. G. and Johnson, J. L. (1995). Root growth, microbial activity and phosphatase activity in oil-contaminated, remediated and uncontaminated soils planted to barley and field pea. *Plant and Soil*, **173**, 3-10.

Xu, J. G. and Johnson, R. L. (1997). Nitrogen dynamics in soils with different hydrocarbon contents planted to barley and field pea. *Canadian Journal of Soil Science*, **77**, 453-458.

Yuste, L., Corbella, M. E., Turiégano, M. J., Karlson, U., Puyet, A., Rojo, F. (2000). Characterisation of bacterial strains able to grow on high molecular mass residues from crude oil processing. *FEMS Microbiology Ecology*, **32**, 69-75.

Zablotowicz, R. M., Locke, M. A., Smeda, R. J. (1998). Degradation of 2,4 D and Fluometuron in cover crop residues. *Chemosphere*, **37**, 87-101.

# Appendix i

Table showing settling times of silt plus clay and clay particles at selected temperatures.

| Temperature °C | Settling time at 20 cm<br>Silt plus clay<br>(equivalent settling diameter <0.06 mm) | Settling times at 10 cm<br>Clay<br>(equivalent settling diameter < 0.002 mm) |
|---|---|---|
| 10 | 83 seconds | 625 minutes |
| 11 | 81 | 608 |
| 12 | 79 | 590 |
| 13 | 77 | 575 |
| 14 | 75 | 559 |
| 15 | 73 | 544 |
| 16 | 71 | 530 |
| 17 | 69 | 517 |
| 18 | 67 | 503 |
| 19 | 65 | 491 |
| 20 | 64 | 479 |
| 21 | 62 | 467 |
| 22 | 61 | 456 |
| 23 | 59 | 446 |
| 24 | 58 | 435 |
| 25 | 57 | 426 |

# Appendix ii

**Published Papers**

Effect of diesel fuel on growth of selected plant species. (1999).
Adam, G. and Duncan, H.
*Environmental Geochemistry and Health*, **21**, 353-357.

Development of a sensitive and rapid method for the measurement of total microbial activity using Fluorescein Diacetate (FDA) in a range of soils. (2001).
Adam, G. and Duncan, H.
*Soil Biology and Biochemistry*, **33**, (7/8), 943-951.

# Effect of diesel fuel on growth of selected plant species

**G. Adam and H. Duncan**

*Department of Agricultural, Food and Environmental Chemistry, Joseph Black Building, University of Glasgow, GLASGOW G12 8QQ, Scotland.*

**Abstract**

Diesel oil is a complex mixture of hydrocarbons with an average carbon number of C8-C26. The majority of components consist of alkanes, both straight chained and branched and aromatic compounds including mono-, di- and polyaromatic hydrocarbons. Regardless of this complexity, diesel oil can be readily degraded by a number of soil microorganisms making it a likely candidate for bioremediation. The concept of using plants to enhance bioremediation, termed phytoremediation, is a relatively new area of scientific interest. It is particularly applicable to diesel oil contamination as diesel oil generally contaminates the top few metres of soil (surface soil) and contamination is not uniform throughout the site. By encouraging plants to grown on diesel oil contaminated soil, conditions are improved for the microbial degradation of the contaminant. During this study, establishing plants on diesel oil contaminated soil proved difficult. Diesel oil is phytotoxic to plants at relatively low concentrations. At concentrations below this phytotoxic level, the development of plants grown in diesel oil contaminated soil differs greatly from plants grown in uncontaminated soil. Tolerance of plants to diesel oil and ability to germinate in diesel oil contaminated soil varied greatly between plant species as well as within plant species. The broadest differences in germination were seen within the grasses with certain species thriving in low levels of contamination (e.g. Creeping bent) while others were intolerant of diesel oil contamination (e.g. Rough meadow grass). The herbs, legumes and commercial crops screened appeared to be largely unaffected by low levels of diesel oil contamination (25g diesel $kg^{-1}$). At the higher level of contamination (50g diesel $kg^{-1}$), half of the twenty two plant species screened failed reach a germination rate equal to 50% of the control rate. Two species of grass failed to germinate at all at this contamination level. Plant species that successfully germinated and grew were studied further to determine the effect of diesel oil contamination on the later stages of plant development. This work investigates the effect of diesel oil on plant growth and development.

**Keywords** : phytoremediation, petroleum hydrocarbons, PAHs, plant performance.

## 1. Introduction

Phytoremediation, or the use of green plants and their associated microbiota to remediate environmental contaminants, has recently become an area of intense study (Cunningham *et al.*, 1996). Plants have been shown to encourage organic contaminant reduction principally by providing an optimal environment for microbial proliferation in the root zone (rhizosphere) (Kruger *et al.*, 1997). These degradative processes are influenced not only by rhizosphere microorganisms, but also by unique properties of the host plant (Walton *et al.*, 1994). This often leads to enhanced breakdown of organic contaminants in soils that are vegetated, compared to non vegetated soils. If plants can be successfully established on polluted soils, then the plant - microbial interaction in the rhizosphere may provide an economical method for enhancing microbial degradation of complex organic contaminants. Diesel oil is one such contaminant that should, in theory, be remediated by a mixed community of microorganisms under these conditions. In practice however, there are many problems associated with establishing a beneficial plant cover on diesel oil contaminated soil.

Diesel oil is a complex mixture of petroleum hydrocarbons containing everything from volatile, low molecular weight alkanes which are potentially phytotoxic, to naphthalenes which may interfere with normal plant development. In addition, polycyclic aromatic hydrocarbons (PAHs) found in diesel spills are of particular concern as they are relatively persistent in the soil environment. Of the medium distillate fuel oils used in terrestrial situations, diesel oil has the highest content of PAHs and total aromatics (Wang *et al.*, 1990) which makes it increasingly more difficult to remediate. Within the framework of a larger study on diesel oil phytoremediation, attention was given to the effect of diesel oil on plant performance. This included the effect of diesel oil on seed germination, the spatial distribution of plant roots grown in diesel oil contaminated soil and changes in plant root morphology observed in the presence of diesel. Examples are given for a variety of different plant species including grasses, herbs, legumes and commercial crops.

## 2. Germination and Seed Emergence

Twenty two plant species including grasses, herbs, legumes and commercial crops were screened for their ability to germinate in diesel oil contaminated soil. At relatively low levels of diesel oil, delayed seed emergence and reduced germination rates were observed for a variety of plant species (Table 1).

*Table 1* Germination rates (%)[a] of plant species exposed to varying concentrations of diesel oil, measured 14 days after planting at 20°C.

| Plant species | | Germination Rate (%) | | |
|---|---|---|---|---|
| | | Diesel concentration (g/kg) | | |
| Common name | Latin name | 0 | 25 | 50 |
| Grasses | | | | |
| Cocksfoot | *Dactylis glomerata* | 53 | 20 | 0 |
| Creeping Bent [b] | *Agrostis stolonifera* | 30 | 38 | 5 |
| Highland Bent [b] | *Agrostis castellana* | 85 | 50 | 46 |
| Black Grass | *Alopecurus myosuroides* | 60 | 30 | 3 |
| Sweet Vernal Grass [b] | *Anthoxanthum odoratum* | 90 | 60 | 15 |
| Rough Meadow Grass [b] | *Poa trivialis* | 55 | 10 | 0 |
| Westerwold's Ryegrass | *Lolium multiflorum* | 78 | 64 | 50 |
| Sheep's Fescue | *Festuca ovina* | 58 | 38 | 24 |
| Strong Creeping Red Fescue | *Festuca rubra ssp. rubra* | 82 | 88 | 40 |
| Chewing's fescue | *Festuca rubra ssp. commutata* | 48 | 50 | 20 |
| Annual Canary Grass | *Phalaris canariensis* | 72 | 60 | 10 |
| Herbs and legumes | | | | |
| Black Medick | *Medicago lupulina* | 20 | 20 | 24 |
| Fodder Burnet | *Sanguisorba minor ssp muricata* | 18 | 16 | 2 |
| Common Vetch | *Vicia sativa* | 64 | 60 | 42 |
| Red Clover | *Trifolium pratense* | 56 | 56 | 40 |
| White Clover | *Trifolium album* | 68 | 36 | 12 |
| Little Yellow Trefoil | *Trifolium dubium* | 40 | 36 | 18 |
| Lucerne | *Medicago sativa* | 74 | 84 | 66 |
| Commercial crops | | | | |
| Oil Seed Rape cv. Rocket | *Brassica napus var. olifera* | 100 | 100 | 95 |
| Oil Seed Rape cv. Martina | *Brassica napus var. olifera* | 100 | 100 | 95 |
| Flax cv. Viking | *Linum usitatissimum* | 74 | 66 | 38 |
| Flax cv. Elise | *Linum usitatissimum* | 94 | 96 | 98 |

[a] 100% germination rate equals every seed planted germinating and producing a sizeable shoot (> 2mm).

[b] These seed species were planted at a sow rate of 100 per replicate. The remaining species were planted 25 seeds per replicate

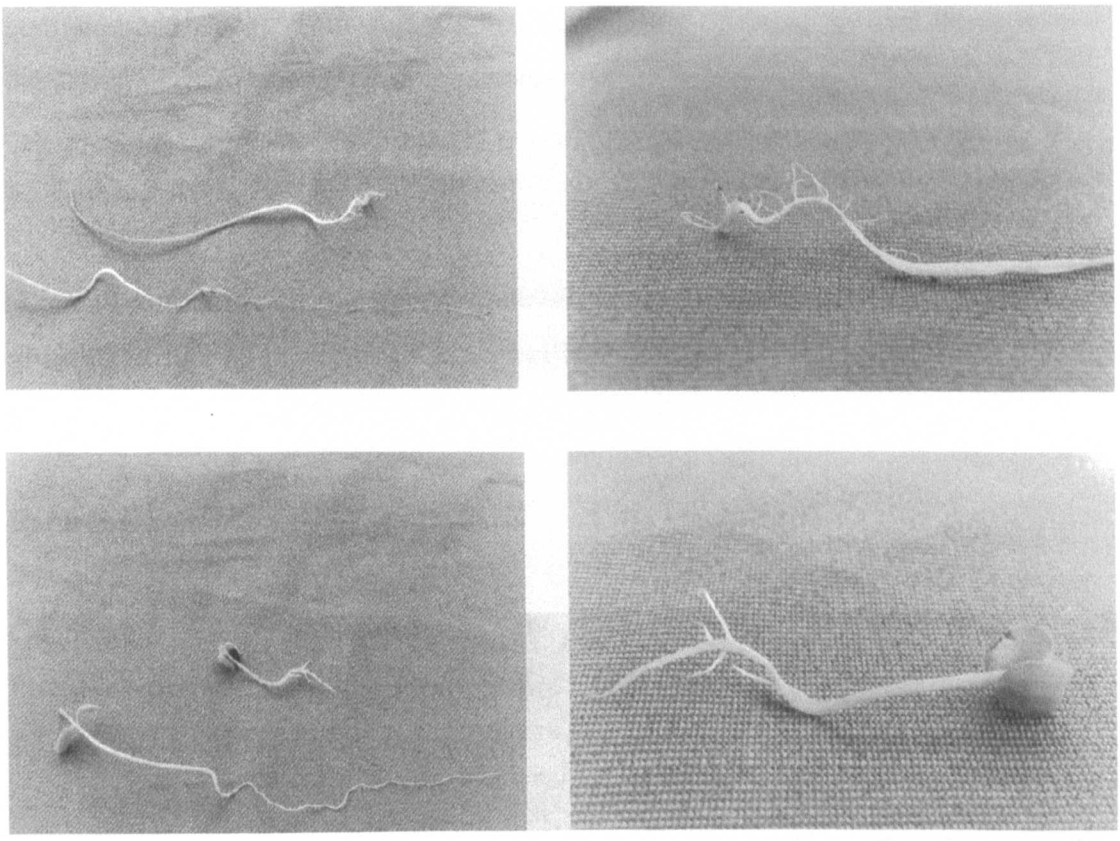
## 3. Plant Performance

The overall heights of plants grown in diesel oil contaminated soil were stunted compared to control plants grown in uncontaminated soil. This effect cannot be attributed directly to delayed seed emergence as some plant species germinated as successfully as the controls, yet their development was impaired by the presence of diesel. For example, the oil seed rape cultivar Martina germinated well in the presence of diesel (Table 1) but the production of top growth was noticeably reduced to 17.8% and 16.6% of the control top growth in 25g diesel/kg soil and 50g diesel/kg soil treatments respectively. The same pattern was observed for root biomass with reductions falling to 21% and 20% of the control biomass for the two treatments (unpublished work).

## 4. Root Morphology

Plants grown in diesel oil contaminated soil exhibit formation of adventitious roots (root structures which arise in unusual positions) as illustrated in Figure 1. Photographs (a) and (b) show the formation of adventitious roots on the stem of a Canary grass seedling where no such structures are found on the control seedling. Photographs (c) and (d) show increased lateral roots present on a Flax seedling grown in contaminated soil as opposed to the control seedling.

(a) (b)



(c) (d)

*Figure 1* Effect of diesel on root formation of two week old seedlings.
(a) Annual canary grass - seedlings grown in contaminated (top) and uncontaminated (bottom) soils ; (b) Annual canary grass - enlargement of seedling grown in contaminated soil ; (c) Flax - seedlings grown in contaminated (top) and uncontaminated (bottom) soil and (d) Flax - enlargement of seedling grown in contaminated soil.

## 5. Spatial Distribution of Roots

An experimental system was set up which enabled the pattern of root development of selected plant species to be followed in a model soil system contaminated with diesel oil. Initial observations indicate plant roots avoid diesel oil contaminated areas completely if they have uncontaminated soil to grow into. If there is no available uncontaminated soil, roots will grow through contaminated regions until they find an area of uncontaminated soil. However, at lower contamination levels (up to 10g diesel/kg soil) roots will enter the contaminated area after an acclimation period. This

observation is also seen with concentrated patches of diesel oil. Once the majority of the surrounding uncontaminated soil has been utilised, the roots begin to move into the contaminated patch. This suggests that degradation of diesel oil may be enhanced by the action of rhizosphere microorganisms.

## 6. Further work

Work is continuing on the effects of diesel on plant development with attention being given to attributing these effects to a specific fraction or fractions of diesel oil.

## References


Cunningham, S. D., Anderson, T. A., Schwab, A. P. and Hsu, F. C. 1996. Phytoremediation of soils contaminated with organic pollutants. *Advances in Agronomy.* **56** : 55 – 113.

Kruger, E. L., Anderson, T. A. and Coats, J. R. 1997. Phytoremediation of contaminated water and soil. In Kruger, E. L., Anderson, T. A. and Coats, J. R. (eds.), *Phytoremediation of soil and water contaminants.* ACS Symposium Series **664**. American Chemical Society, Washington, D. C. pp 2 – 17.

Walton, B. T., Guthrie, E. A. and Hoylman, A. M. 1994. *Toxicant degradation in the rhizosphere.* In Anderson, T. A. and Coats, J. R. (eds.), *Bioremediation through rhizosphere technology.* ACS Symposium Series **563**. American Chemical Society, Washington, D. C. pp 11 – 26.

Wang, X., Yu, X., and Bartha, R. 1990. Effect of bioremediation on PAH residues in soil. *Environmental Science and Technology.* **24** : 1086 – 1089.

# Development of a sensitive and rapid method for the measurement of total microbial activity using Fluorescein Diacetate (FDA) in a range of soils

Gillian Adam * and Harry Duncan

*Department of Environmental, Agricultural and Analytical Chemistry, Joseph Black Building, University of Glasgow, GLASGOW G12 8QQ, Scotland, UK.*

**Abstract**

Fluorescein diacetate (FDA) hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in a range of environmental samples, including soils. Colourless fluorescein diacetate is hydrolysed by both free and membrane bound enzymes, releasing a coloured end product fluorescein which can be measured by spectrophotometry. The current method for measuring FDA hydrolysis in soils is limited in its application. FDA activity was very low in sandy and clayey soils. The low activity observed for these soil types was made difficult to measure by the original authors' choice of solvent for terminating the hydrolysis reaction. Acetone (50% v/v) was found to be most efficient at stopping the hydrolysis reaction. During this study acetone (50% v/v) was found to cause a decrease of approximately 37% in the absorbance of fluorescein produced by the soil samples measured. Although this colour loss is independent of initial fluorescein concentration, it makes the measurement of FDA hydrolytic activity extremely difficult in soils with low microbial activity i.e. sandy and/or clayey soils. Chloroform/methanol (2:1 v/v) was found to successfully stop the hydrolysis reaction for up to 50 min in a range of soil samples without causing the loss of colour observed with acetone. By changing the solvent used for terminating the hydrolysis reaction, low activity soils could be measured successfully. Other parameters of the hydrolysis reaction were optimised for the measurement of soil samples including effect of pH, optimum temperature of incubation, amount of soil, time of incubation, amount of substrate and preparation of suitable standards. A new, more sensitive method is proposed adapted from the original method, which provides a more accurate determination of FDA hydrolysis in a wide range of soils.

*Keywords:* Fluorescein diacetate (FDA), enzymic hydrolysis, total microbial activity, soil.

## 1. Introduction

The use of fluorescein esters as a measure of enzyme activity was first noted by Kramer and Guilbault (1963) where a simple procedure was described for the assay of lipase activity in the presence of other esterases. It was not until 1980 that the use of fluorescein esters as a measure of microbial activity was applied to environmental samples. Swisher and Carroll (1980) demonstrated that the amount of fluorescein produced by the hydrolysis of fluorescein diacetate (FDA) was directly proportional to the microbial population growing on Douglas Fir foliage and a standardised method was developed. This method was later evaluated by Schnürer and Rosswall (1982) who used FDA hydrolysis to determine total microbial activity in soil and straw litter as well as cell density in pure microbial cultures.

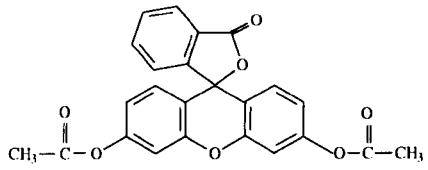
Fluorescein diacetate (3′ 6′ -diacetyl-fluorescein) is a fluorescein conjugated to two acetate radicals. This colourless compound is hydrolysed by both free (exoenzymes) and membrane bound enzymes (Stubberfield and Shaw, 1990), releasing a coloured end product, fluorescein. Fig. 1 illustrates the enzymic conversion of FDA to fluorescein which appears to be primarily a hydrolysis followed by a dehydration reaction. This end product absorbs strongly in the visible wavelength (490 nm) and can be measured by spectrophotometry. The enzymes responsible for FDA hydrolysis are plentiful in the soil environment. Non-specific esterases, proteases and lipases, which have been shown to hydrolyse FDA, are involved in the decomposition of many types of tissue. The ability to hydrolyse FDA thus seems widespread, especially among the major decomposers, bacteria and fungi (Schnürer and Rosswall, 1982). Generally more than 90% of the energy flow in a soil system passes through microbial decomposers, therefore an assay which measures microbial decomposer activity will provide a good estimate of total microbial activity.

The FDA method was also shown to correlate well with some of the most accurate measures of microbial biomass such as ATP content and cell density studies (Stubberfield and Shaw, 1990) and radio-labelled thymidine incorporation into microbial DNA (Federle *et al.*. 1990). Whereas these methods are time consuming and difficult to perform, enzyme assays are generally rapid and simple.

Since 1982, FDA hydrolysis has been used to measure total microbial activity in a range of samples from mould growth on wood and other building materials (Bjurman, 1993), to plant residues (Zablotowicz *et al.*, 1998), to stream sediment biofilms (Battin,

1997), activated sludge (Fontvieille, 1992) and deep sea clay and sand sediment profiles (Gumprecht *et al.*, 1995).

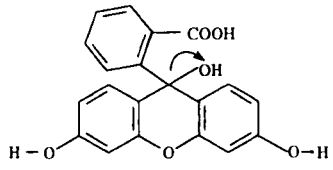
The advantage of this method being simple, rapid and sensitive, coupled with the widespread acceptance of FDA hydrolysis as a measure of total microbial activity, suggests this would be a good method to optimise to include a wide range of soils. The original Schnürer and Rosswall (1982) method, which most authors use today, is limited in its application. Schnürer and Rosswall found that FDA activity was very low in sand and clay samples. The low activity observed for these soil types was made more difficult to measure by the authors' choice of solvent for terminating the hydrolysis reaction. Because of the rapidity of FDA hydrolysis, it is necessary when working with many samples to find a way of terminating hydrolysis at a specific time. Schnürer and Rosswall found acetone (50% v/v) to be most efficient, totally stopping hydrolysis in a soil sample for 2 h. However, a substantial decrease in the absorbance of fluorescein produced by the soil samples was observed when acetone was added. This dramatic colour loss is independent of initial fluorescein concentration but makes the measurement of FDA hydrolytic activity very difficult in soils with low microbial activity i.e. sandy and/or clayey soils. Therefore, a new, more sensitive method is proposed adapted from the original Schnürer and Rosswall method which will provide a more accurate determination of FDA hydrolysis in a wide range of soils.



+ $3H_2O$ HYDROLYSIS →

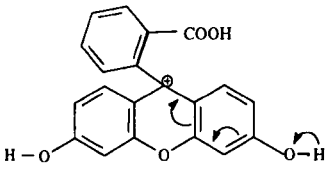
step 1

fluorescein diacetate – colourless



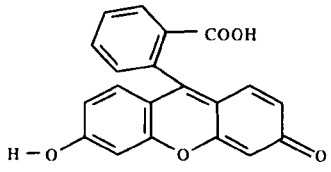
+ $2CH_3CO_2H$ DEHYDRATION →

step 2



→

step 3



+ $H_2O$

fluorescein – coloured acid yellow

visible 490 nm

Fig. 1 Enzymic conversion of fluorescein diacetate (FDA) to fluorescein.

The enzymic conversion of fluorescein diacetate (FDA) to fluorescein appears to be primarily a hydrolysis reaction followed by a dehydration reaction. The two acetate groups are hydrolysed at their ester linkage and the lactone part of the structure is cleaved at its internal ester link (step 1). The resultant OH group leaves, creating a positively charged bond (step 2). This charge must be satisfied so the above intermediary step occurs starting from a loss of H at the terminal position (step 3). This results in an overall loss of water.

## 2. Material and Methods

### 2.1 Soils

A total of five surface soils and one manufactured soil were selected to obtain a range of textural properties within the sandy and /or clayey textural class and cover a range of enzymic activities. Particle size analysis for the determination of textural class was carried out as described by the modified method of Khan (PhD thesis, University of Glasgow 1987) from the ADAS method 57 (1981b). Dehydrogenase activity was assessed by the method of Casida *et al* (1964). Total nitrogen content of the soils was determined by the digestion method of Bremner and Mulvaney (1982) coupled to an automated determination of nitrogen in the digests. Soil pH was determined according to ADAS method 32 (1981a). Organic matter content was assessed by loss on ignition (LOI). 5 g soil was weighed, in triplicate, into silica basins and dried overnight at 105°C. The soils were reweighed to obtain the oven dry soil weight. The soils were then placed in a muffle furnace and ignited at 500°C for 6 h. The samples were then reweighed and the weight of ignited soils calculated. The % organic matter by loss on ignition was calculated by subtracting the weight of ignited soil from the weight of oven dry soil. This value was then divided by the weight of oven dry soil and the resulting value multiplied by 100 to obtain % LOI. Table 1 shows the textural, chemical and biological properties of the six soils chosen.

Table 1A Textural properties and Table 1B Chemical and Biological properties of the soils used in the study.

| Soils | % coarse sand | % fine sand | % silt | % clay | Textural class |
|---|---|---|---|---|---|
| Barassie† | 77.7 | 14.2 | 4.1 | 4.4 | Sand |
| Bargour‡ | 39.4 | 27.5 | 11.3 | 21.8 | Sandy loam |
| Caprington‡ | 29.2 | 22.0 | 25.7 | 23.1 | Sandy clay loam |
| Dreghorn† | 32.7 | 35.3 | 16.3 | 15.7 | Sandy loam |
| Garscube | 51.7 | 20.8 | 12.8 | 15.2 | Loamy sand |
| John Innes compost | 72.2 | 17.6 | 4.9 | 8.4 | Sand |

Coarse sand > 0.18 mm, fine sand 0.18 – 0.05 mm, silt 0.05 – 0.002, clay < 0.002 mm.

Table 1B

| Soils | pH (water) | LOI (%) | Total N (%) | Dehydrogenase activity (μg TPF $g^{-1}$ oven dry soil $24\ h^{-1}$) |
|---|---|---|---|---|
| Barassie† | 7.20 | 5.7 | 0.20 | 173.59 |
| Bargour‡ | 5.46 | 6.9 | 0.18 | 145.79 |
| Caprington‡ | 6.46 | 10.5 | 0.28 | 151.16 |
| Dreghorn† | 6.86 | 6.5 | 0.17 | 67.80 |
| Garscube | 7.23 | 9.6 | 0.35 | 224.70 |
| John Innes compost | 7.06 | 10.2 | 0.19 | 76.88 |

† Textural properties and Total N (%) taken from Metwaly (Ph.D. thesis, University of Glasgow, 1999).

2.2 Reagents

60 mM potassium phosphate buffer pH 7.6

8.7 g $K_2HPO_4$ (Riedel-de Haën, Sigma-Aldrich Co. Ltd., Analar) and 1.3 g $KH_2PO_4$ (Merck, BDH Analar) were dissolved in approximately 800 ml deionised water. The contents were made up to 1 l with deionised water. The buffer was stored in the fridge (4°C) and pH checked on day of use.

2:1 chloroform/methanol

666 ml chloroform (Fisher Scientific UK Limited, analytical grade) was added to a 1 l volumetric flask. The flask was made up to 1 l with methanol (Fisher Scientific UK Limited, analytical grade) and the contents mixed thoroughly.

1000 µg FDA $ml^{-1}$ stock solution

0.1 g fluorescein diacetate (3′ 6′-diacetyl-fluorescein., Sigma-Aldrich Co. Ltd.) was dissolved in approximately 80 ml of acetone (Fisher Scientific UK Limited, analytical grade) and the contents of the flask made up to 100 ml with acetone. The solution was stored at - 20°C.

2000 µg fluorescein $ml^{-1}$ stock solution

0.2265 g fluorescein sodium salt (Merck, BDH Analar) was dissolved in approximately 80 ml of 60 mM potassium phosphate buffer pH 7.6 and the contents made up to 100 ml with buffer.

20 µg fluorescein $ml^{-1}$ standard solution

1 ml of 2000 µg fluorescein $ml^{-1}$ stock solution was added to a 100 ml volumetric flask and the contents made up to the mark with 60 mM potassium phosphate buffer pH 7.6. 1-5 µg $ml^{-1}$ standards were prepared from this standard solution by appropriate dilution in 60 mM potassium phosphate buffer pH 7.6.

2.3 Methods

The individual parameters of the fluorescein diacetate hydrolysis reaction were studied to optimize the assay for the measurement of soil samples. These factors included effect of pH, amount of soil, amount of substrate, time of incubation, optimum temperature of incubation, choice of solvent for terminating the hydrolysis reaction and preparation of suitable standards. The results from each parameter studied were

culminated to produce the final assay procedure. To determine the effect of each parameter on the FDA hydrolysis reaction, changes were made to the final procedure but these changes will be explained where appropriate in the results and discussion section.

2.4 Final Procedure

2 g soil (fresh weight, sieved < 2 mm) was placed in a 50 ml conical flask and 15 ml of 60 mM potassium phosphate buffer pH 7.6 added. 0.2 ml 1000 μg FDA $ml^{-1}$ stock solution was added to start the reaction. Blanks were prepared without the addition of the FDA substrate along with a suitable number of sample replicates. The flasks were stoppered and the contents shaken by hand. The flasks were then placed in an orbital incubator (Gallenkamp Orbital Incubator, 100 rev $min^{-1}$) at 30°C for 20 min.

The following steps involving chloroform/methanol were carried out in a fume cupboard. Once removed from the incubator, 15 ml of chloroform/methanol (2:1 v/v) was added immediately to terminate the reaction. Stoppers were replaced on the flasks and the contents shaken thoroughly by hand. The contents of the conical flasks were then transferred to 50 ml centrifuge tubes and centrifuged at 2000 rev $min^{-1}$ for approximately 3 min (MSE Scientific Instruments, Coolspin 2 centrifuge). The supernatant from each sample was then filtered (Whatman, No 2) into 50 ml conical flasks and the filtrates measured at 490 nm on a spectrophotometer (Hitachi U – 1100 spectrophotometer).

The concentration of fluorescein released during the assay was calculated using the calibration graph produced from 0 – 5 μg fluorescein $ml^{-1}$ standards which were prepared from a 20 μg fluorescein $ml^{-1}$ standard solution. The 0 μg $ml^{-1}$ fluorescein standard was used to zero the spectrophotometer before each set of blanks and samples were read.

2.5 Statistical analysis

Sample standard deviations were used to assess standard error and replicate variability was measured by the coefficient of variation (CV) using an EXCEL statistical package (Microsoft). One-way analysis of variance (ANOVA) was prepared using MINITAB (for WINDOWS 10.1). Probability values were set at 0.05 level for all statistical

measures. FDA hydrolysis values were expressed as μg fluorescein released $g^{-1}$ oven dry (105°C) soil unless stated otherwise.

## 3. Results and Discussion

### 3.1 Effect of pH

The rate of hydrolysis of fluorescein compounds reaches a maximum between pH 7.0 and 8.0 (Guilbault and Kramer, 1964). Fluorescein diacetate was found to exhibit a maximum rate of hydrolysis at pH 7.6 (Swisher and Carroll, 1980). Carrying out the enzymic reaction at this pH was advantageous for many reasons. At high and low pHs, solubilisation of organic matter in the soil samples caused interference problems with the measurement of fluorescein released, by creating blanks with very high background absorbances. Carrying out the reaction at pH 7.6 removed this interference problem. Spontaneous hydrolysis of fluorescein esters is known to occur at high pHs (Guilbault and Kramer, 1964). At pH 7.6 no spontaneous hydrolysis of fluorescein diacetate was observed. Finally, the product of FDA hydrolysis, fluorescein, exhibits a maximum fluorescence at about pH 8.0 (Guilbault and Kramer, 1964). This was verified when standards were prepared in buffers at different pHs. The absorbance values measured for the same concentration of fluorescein standards were more than double in the pH 7.6 potassium phosphate buffer compared with the pH 5.6 buffer. This illustrates that fluorescein is near its maximum absorbance at pH 7.6.

### 3.2 Effect of temperature

The rate of hydrolysis of a substrate by an enzyme depends on the temperature of incubation. A study of FDA activity in soil as a function of temperature showed maximum activity occurred at 30°C. This is in agreement with findings by Breeuwer *et al* (1995) who observed maximum FDA activity by yeast esterases at this temperature. The activity rapidly decreased just above 30°C suggesting inactivation of the enzymes involved at this elevated temperature (Fig 2). At high temperatures considerable spontaneous hydrolysis of fluorescein esters can occur (Guilbault and Kramer, 1964), adversely affecting the accuracy and reproducibility of the method. No spontaneous hydrolysis of FDA occurred between 20-40°C which covers the range around the temperature chosen for this assay.

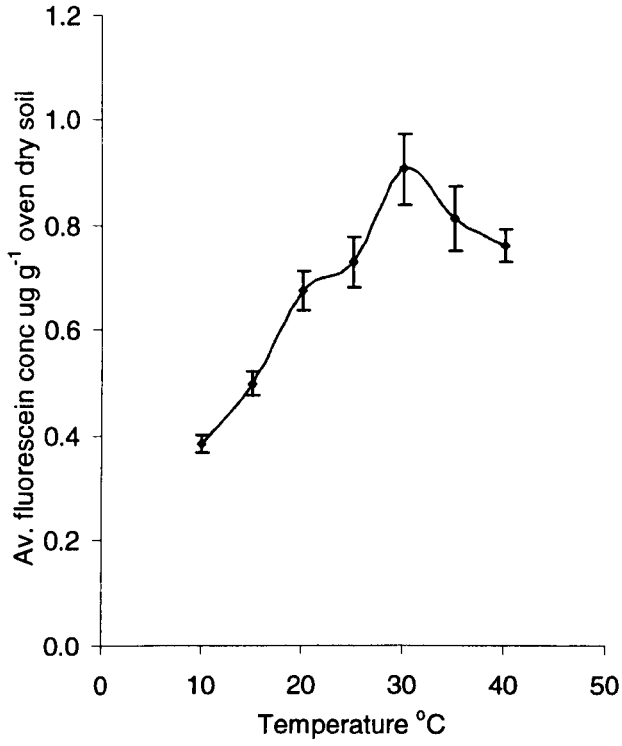



Fig. 2 Effect of temperature on FDA hydrolysis by enzymes present in a sandy soil (Barassie). Mean values with standard error bars, n = 4.

3.3 Amount of soil

The initial reaction rates of soil enzymes are usually proportional to the amounts of soil added to the assay (Frankenberger and Johanson, 1983). Soil weights (fresh weight, sieved < 2 mm) ranging from 0.5-5 g were incubated to discover the optimal amount of soil required for the reaction to proceed at a steady rate without substrate becoming limiting. A linear relationship was observed between soil weight and fluorescein released up to 2.5 g (Fig. 3). The deviation from linearity when soil weights greater than 2.5 g were used indicates substrate concentration was probably becoming a limiting factor.

A soil weight of 2 g was used for the final assay. This allowed the reaction to proceed at a steady rate, for all the soils tested, without substrate becoming limiting. It also ensured that the amount of fluorescein hydrolysed during the assay fell within the sensitivity range of the spectrophotometer (0.1-1.0). The three soils chosen to illustrate this in Fig. 3 were: (a) a manufactured compost (John Innes Compost No 2) which represents a sandy soil with low microbial activity; (b) Dreghorn which represents a

sandy loam with an intermediate microbial activity and (c) Garscube soil which is a loamy sand with high microbial activity. The low activity soil released enough fluorescein during the assay to be measured accurately by spectrophotometry (absorbance values greater than 0.1) and the high activity soil released enough fluorescein to lie within the range of the spectrophotometer without dilution (absorbance values below 1.0). These three soil types hopefully represent the range of microbial activities encountered in most soils.

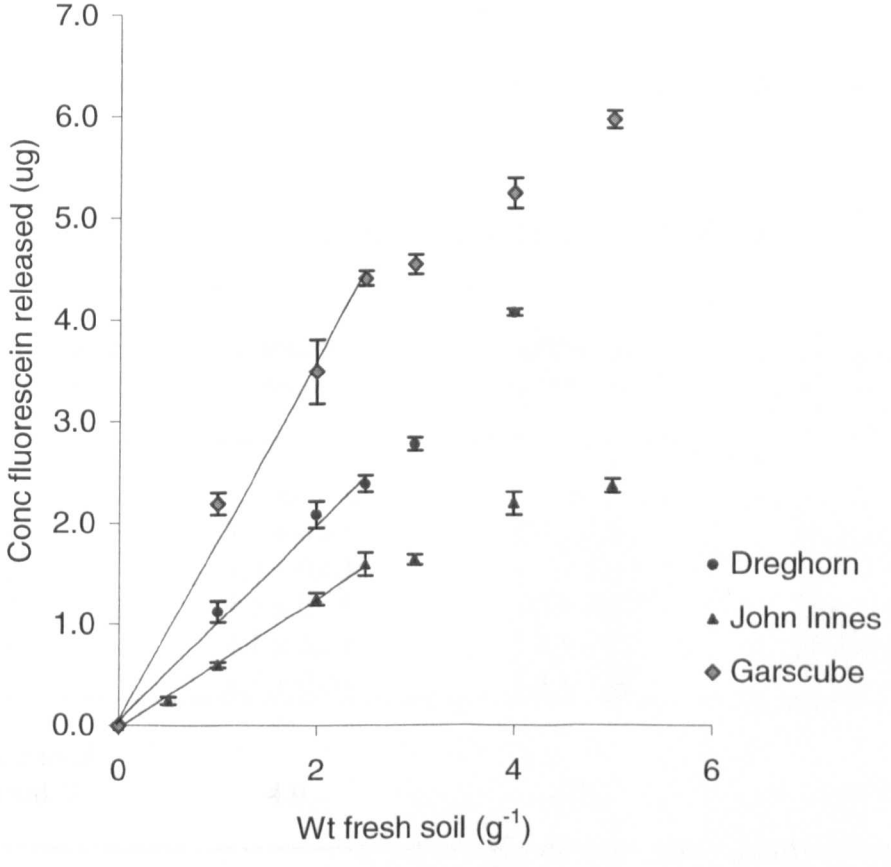



Fig. 3 Relationship between soil weight and FDA hydrolysis. Mean values with standard error bars, n = 3.

### 3.4 Adsorption of Fluorescein onto soil

The amount of fluorescein adsorbed onto soil was considered before carrying out the enzyme assay. When a new soil is investigated, the amount adsorbed onto soil should be calculated so the values obtained for the assay can be corrected for the loss. Soil samples were incubated using the conditions described for the final assay procedure in 15 ml fluorescein standard at each concentration (0-5 $\mu g\ ml^{-1}$) (Table 2). Blanks were

prepared without the addition of soil. Samples and blanks were centrifuged and filtered as described in the final assay procedure. The amount of fluorescein adsorbed at each concentration is shown as % fluorescein adsorbed and an average total value is given for each textural class. Generally the amount of fluorescein adsorbed is less than 5%. This observation was noted by the original authors who found the adsorption of fluorescein to soil did not exceed 7% and was mostly lower than 5% (Schnürer and Rosswall, 1982). A soil with a high silt–clay ratio and high organic matter content, such as Caprington can however adsorb up to 13.7% fluorescein, a large proportion of the total released.

Table 2 Adsorption of fluorescein onto soils with differing textural properties.

| | % fluorescein ($\mu g\ ml^{-1}$) adsorbed by soil | | |
|---|---|---|---|
| Fluorescein conc ($\mu g\ ml^{-1}$) in blank | Barassie sand | Dreghorn sandy loam | Caprington sandy clay loam |
| 0 | 0 | 0 | 0 |
| 1 | 5.7 ± 0.83 | 3.3 ± 0.69 | 20.2 ± 0.47 |
| 2 | 4.3 ± 0.60 | 7.1 ± 2.57 | 14.6 ± 0.39 |
| 3 | 3.2 ± 0.15 | 5.8 ± 0.27 | 11.1 ± 0.93 |
| 4 | 4.1 ± 0.29 | 3.3 ± 0.74 | 10.5 ± 0.26 |
| 5 | 2.7 ± 0.24 | 2.4 ± 1.07 | 11.7 ± 1.53 |
| Average total adsorbed % | 4.0 | 3.6 | 13.7 |

Given are means ± S.E., n = 3.

### 3.5 Time of incubation

It has been suggested that an assay for soil enzymes should not require a long incubation time because the risk of error through microbial proliferation increases with increasing incubation time. Part of this error can be minimised by the addition of toluene as a bacteriostat to enzyme assays although many authors do not favour the use of toluene for this purpose. Toluene has been shown to inhibit some enzymes as well as having an activating effect on others due to increased permeability of the cell membrane

in the presence of toluene, allowing entry of the substrate (Skujins, 1967). In this study the use of toluene as a bacteriostat in the assay of FDA hydrolysing enzymes was dismissed as it was found to inhibit FDA hydrolysis in the soil samples investigated by approximately 35%. It was therefore decided to keep the incubation time as short as possible.

The hydrolysis reaction was linear with time up to 40 min for the soils investigated using the conditions described for the final assay procedure (Fig. 4). The assay was not limited by substrate concentration over this time period.

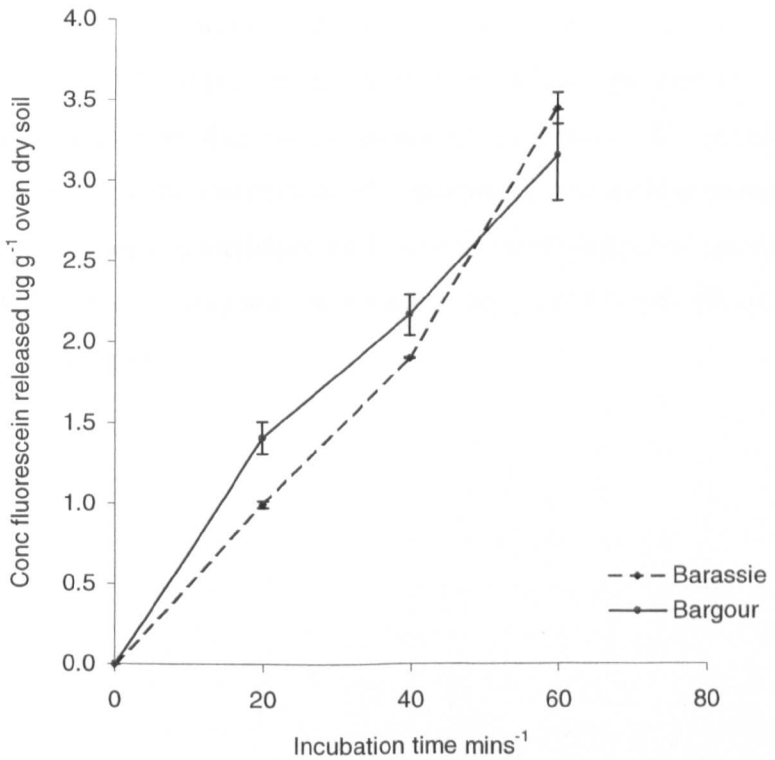



Fig. 4 Determination of optimum incubation time. Means and standard error bars, n = 3.

An incubation time of 20 min was chosen for the final assay procedure to allow the concentration of substrate hydrolysed to lie within the range of the spectrophotometer for all the soil types investigated without the need for dilution.

3.6 Choice of solvent for terminating hydrolysis

Schnürer and Rosswall found acetone (50% v/v) to be most efficient, totally stopping hydrolysis in a soil sample for 2 h. The addition of acetone to the soil samples did

terminate hydrolysis but it also caused a decrease in the amount of fluorescein measurable by spectrophotometry. Although the drop in colour was by the same ratio each time (ranged from 39.8-41.0% in 1-5μg $ml^{-1}$ fluorescein standards where acetone was added), samples with low microbial activity which only release a small amount of fluorescein were made increasingly more difficult to measure. This decrease in colour, which was more than a dilution effect, was, on average, 37% of the colour developed in a sandy soil. Table 3 illustrates the decrease in fluorescein concentration of samples where acetone has been used to stop the hydrolysis reaction compared to samples where chloroform/methanol (2:1 v/v) has been used. The samples sometimes dropped below the range of the spectrophotometer when acetone was added. The absorbance values for the samples where chloroform/methanol (2:1 v/v) has been used to terminate hydrolysis lie just above 0.1, the minimum absorbance that can be measured accurately by spectrophotometry. The sample values where acetone has been used to stop the reaction have fallen below the range that can be measured accurately. The relative precision of the method, defined by the coefficient of variation of replicate measurements, is also increased when chloroform/methanol (2:1 v/v) is used instead of acetone. Therefore chloroform/methanol (2:1 v/v) was proposed as the new solvent for terminating FDA hydrolysis in soil samples.

Table 3 Decrease in fluorescein concentration (μg $ml^{-1}$) of Barassie soil in acetone terminated samples compared with chloroform/methanol (2:1 v/v) terminated samples.

| | chloroform/methanol (2:1 v/v) | | Acetone (50% v/v) | | |
|---|---|---|---|---|---|
| replicate | abs 490 nm | conc (μg $g^{-1}$) soil | abs 490 nm | conc (μg $g^{-1}$) soil | Corrected conc (μg $g^{-1}$) soil |
| 1 | 0.129 | 0.543 | 0.060 | 0.249 | 0.332 |
| 2 | 0.125 | 0.5.33 | 0.063 | 0.268 | 0.356 |
| 3 | 0.109 | 0.467 | 0.061 | 0.255 | 0.340 |
| 4 | 0.109 | 0.455 | 0.043 | 0.180 | 0.239 |
| 5 | 0.133 | 0.568 | 0.068 | 0.288 | 0.383 |
| 6 | 0.124 | 0.518 | 0.052 | 0.221 | 0.294 |
| Final vol. of filtrate (ml) | | 20 | | 30 | 20 |
| av. conc (μg $g^{-1}$) soil | | 0.514 | | 0.244 | 0.324 |
| CV | | 8.61 | | | 15.74 |

All replicate absorbance values and concentration values in μg fluorescein $g^{-1}$ oven dry soil are given. The final volumes of filtrate collected were 20 ml in the chloroform/methanol (2:1 v/v) samples and 30 ml in the acetone samples. Due to the differences in the final volumes a conversion factor of 0.33 was used on the acetone values to counteract this dilution effect. The corrected concentration values for the acetone samples give fluorescein concentrations (μg $g^{-1}$ oven dry soil) in a 20 ml final volume. These values can be compared directly to the values obtained for the chloroform/methanol (2:1 v/v) samples. CV = coefficient of variation.

3.7 Change in hydrolysis over time

Changing from acetone to another means of terminating the hydrolysis reaction involved finding a substitute that would stop hydrolysis successfully without causing the same loss of colour observed with acetone. A 2:1 ratio of chloroform/methanol (v/v) was most efficient, stopping hydrolysis from continuing for up to 50 min after its addition (Fig. 5). The length of time the reaction was terminated for was sufficient to allow the measurement of a large number of samples without changes occurring in the samples. In addition, chloroform will help solubilise cell membranes, as acetone did, facilitating the extraction of fluorescein. FDA, being non-polar, readily penetrates into the cell and is hydrolysed to fluorescein. The polarity of fluorescein impedes its transport back through the cell membrane causing intracellular accumulation. Fluorescein is liberated into the environment only after the storage capacity of the cell has been exceeded and the excess is excreted (Rotman and Papermaster, 1966). Chloroform will help solubilise cell membranes aiding the extraction of fluorescein. The presence of methanol will help the chloroform interact with the moist soil hence increasing its ability to terminate the reaction effectively. Fluorescein released during the incubation also moves preferentially into the more polar potassium phosphate buffer/methanol phase, which increases the efficiency of the extraction procedure. Acetone also removed a lot of dissolved organic matter from the samples producing blanks with very high background absorbances whereas the chloroform/methanol (2:1 v/v) does not. All these advantages make chloroform/methanol a more beneficial solvent to use in terminating the hydrolysis reaction.

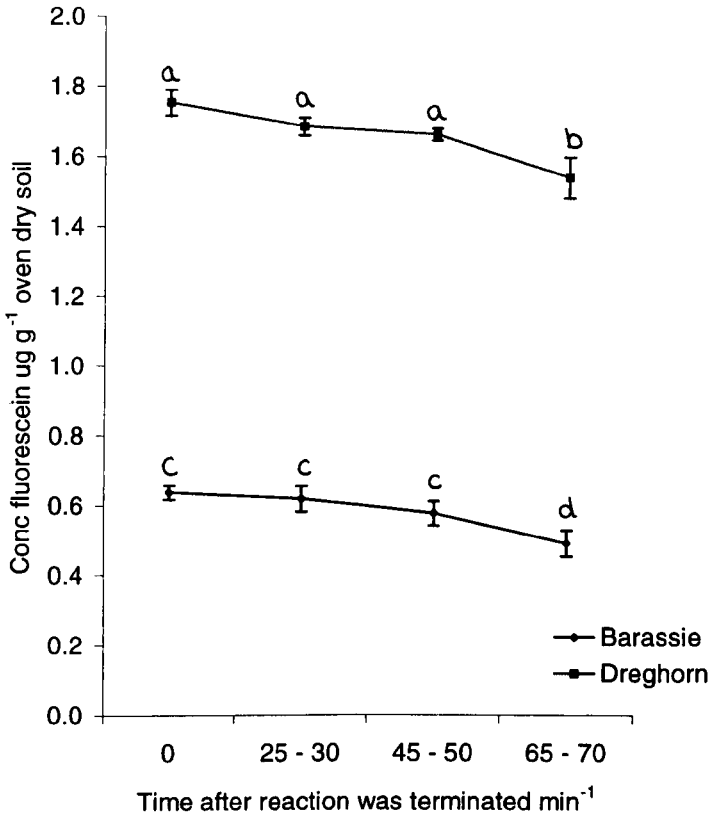



Fig. 5 Change in fluorescein concentration over time after termination of the hydrolysis reaction. Mean values and standard error bars, n = 5. Means followed by the same letter are not significantly different at $P < 0.05$.

3.8 Substrate concentration

2000 µg $ml^{-1}$ fluorescein diacetate (FDA) solution was used by most authors as the substrate for the reaction. By adding 0.2 ml of 2000 µg $ml^{-1}$ FDA, 400 µg FDA was achieved in each replicate. This amount was unnecessarily high for the conditions chosen for the final procedure. High concentrations of FDA should be avoided as FDA is poorly soluble in water and other polar solutions (Breeuwer *et al.*, 1995). Even in acetone, high concentrations of FDA produce slightly cloudy solutions suggesting not all the FDA added is in solution, hence available to the microorganisms. Instead a 1000 µg $ml^{-1}$ FDA solution was chosen to start the reaction. This supplied 200 µg FDA to each replicate which can, in turn, release a maximum of 160 µg of fluorescein. This 160 µg of fluorescein is diluted in 15 ml 60 mM potassium phosphate buffer pH 7.6 then a further 5 ml of methanol (from 15 ml chloroform/methanol (2:1 v/v) only the methanol is added to the filtrate). This gives a final possible fluorescein concentration

of 8 μg $ml^{-1}$. The maximum concentration of fluorescein is never released by the conditions described for the final procedure therefore standards are prepared covering a range of 0-5 μg fluorescein $ml^{-1}$. All the soils investigated, using the conditions set for the assay, were within the range described by the standards.

3.9 Preparation of standards

The original method stated that standards should be prepared using hydrolysed fluorescein diacetate (FDA). This was achieved by boiling FDA solutions of known concentrations in a water bath for 30 min (Schnürer and Rosswall, 1982). Other authors increased the boiling time to 60 min (Chen *et al.*, 1988). This method for obtaining reproducible standards proved too variable. 0-200 μg concentrations of FDA were added to 5 ml of 60 mM potassium phosphate buffer pH 7.6 in screw top vials (Fig 6A). The lids were replaced and the standards placed in a boiling water bath for a set time (30 or 60 min). Once cool a further 10 ml of 60 mM potassium phosphate buffer pH 7.6 was added to keep all volumes the same as the final procedure. 15 ml of chloroform/methanol (2:1 v/v) was added and the standards were centrifuged and filtered as described by the final procedure. The standards were measured at 490 nm and the results plotted to produce a standard calibration graph. The results differed for both hydrolysis times. Fluorescein diacetate can in fact be hydrolysed for up to 6 h in a water bath, although a slight plateau is reached after 4 h. Fig 6B illustrates the continued hydrolysis of a 100 μg FDA standard. Replicate 100 μg FDA standards in 5 ml 60 mM potassium phosphate buffer pH 7.6 were placed in a boiling water bath. A duplicate set of replicates were removed every hour and the fluorescein released measured as described above. Whether FDA is continuing to be hydrolysed or whether it is being degraded in the prolonged heating is unclear but the method for preparing standards is clearly unacceptable.

Sodium fluorescein salt was chosen instead to prepare the standards. Sodium fluorescein salt released the same acid yellow coloured fluorescein as FDA released allowing direct measurement of fluorescein released from FDA in soil by a standard calibration graph prepared from sodium fluorescein salt. Certain pure fluorescein preparations should be avoided as this compound is generally solvent yellow coloured and adds an error to the absorbance measurements. The fluorescein salt can be weighed accurately and known concentrations of fluorescein obtained so standard results rarely differ. Standards prepared by this method did not alter significantly over three months.

By using the boiling water bath method for preparing standards the amount of fluorescein hydrolysed by the samples and the amount of FDA hydrolysed during standard preparation was continually underestimated and was not always consistent. Preparing standards from sodium fluorescein salt is a much more accurate method.

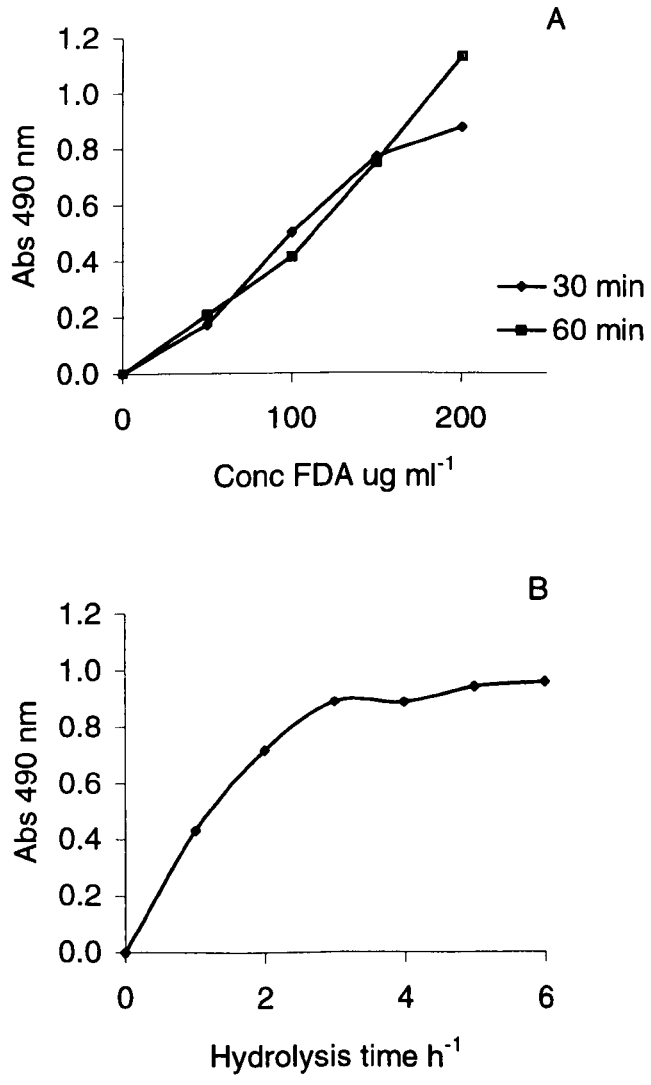



Fig. 6 (A) Fluorescein diacetate (FDA) hydrolysed in boiling water bath (diluted 1 : 1 in 60 mM potassium phosphate buffer pH 7.6) and (B) continued hydrolysis of 100 µg FDA standard in boiling water bath.

## 4. Conclusions

The potential of fluorescein diacetate (FDA) hydrolysis as a measure of total microbial activity has been recognised by many authors and used on a wide range of samples. The most frequently used method for measuring FDA activity in soil was found to be limited in the range of soil types it could measure successfully. The method described in this study critically assessed each individual parameter of the FDA hydrolysis assay and optimised each one for the measurement of a wide range of soils.

The most important parameter assessed during this study was the choice of solvent for terminating the reaction. By changing the solvent from acetone (50% v/v) to chloroform/methanol (2:1 v/v) low activity soils, such as sandy and clayey soils, could be measured successfully. This increased sensitivity was achieved as no loss of colour was observed when chloroform/methanol (2:1 v/v) was used.

## Acknowledgements

We thank Dr David Morris, Department of Chemistry at the University of Glasgow for his suggestions on the enzymic pathway of FDA to fluorescein. We also thank Dr. Hugh Flowers, Department of Environmental, Agricultural and Analytical Chemistry at the University of Glasgow for his help with the soil nutrient analysis.

## References

ADAS, 1981a. Method 32. pH and lime requirement of mineral soil. In: The analysis of agricultural materials. Reference book 427. 2nd ed. . Ministry of Agriculture, Fisheries and Food, HMSO, London. pp. 98-99.

ADAS, 1981b. Method 57. Particle size distribution in soils. In: The analysis of agricultural materials. Reference book 427. 2nd ed. . Ministry of Agriculture, Fisheries and Food, HMSO, London. pp. 175-180.

Battin, T. J., 1997. Assessment of fluorescein diacetate hydrolysis as a measure of total esterase activity in natural stream sediment biofilms. The Science of the Total Environment 198, 51-60.

Bjurman, J., 1993. Determination of microbial activity in moulded wood by the use of fluorescein diacetate. Material und Organismen 28, 1-16.

Breeuwer, P., Drocourt, J. L., Bunschoten, N., Zwietering, M. H., Rombouts, F. M., Abee, T., 1995. Characterisation of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescein product. Applied and Environmental Microbiology 61, 1614-1619.

Bremner, J. M., Mulvaney, C. S., 1982. Nitrogen – Total. In: Page, A. L., Miller, R. H., Keeney, D. K. (Eds). Methods Of Soil Analysis. Part 2 – Chemical and Microbiological Properties. Soil Science of America, Inc., Wisconsin. Pp 595-616.

Casida, L. E. Jr., Klein, D. A., Santoro, T., 1964. Soil dehydrogenase activity. Soil Science 98, 371 – 376.

Chen, W., Hoitink, A. J., Schmitthenner, A. F., Tuovinen, O. H., 1988. The role of microbial activity in suppression of damping–off caused by *Pythium ultimum*. Phytopathology 78, 314-322.

Federle, T. W., Ventullo, R. M., White, D. C., 1990. Spatial distribution of microbial biomass, activity, community structure and the biodegradation of Linear Alkylbenzene Sulfonate (LAS) and Linear Alcohol Ethoxylate (LAE) in the subsurface. Microbial Ecology 20, 297-313.

Fontvieille, D. A., Outaguerouine, A., Thevenot, D. R., 1992. Fluorescein diacetate hydrolysis as a measure of microbial activity in aquatic systems : application to activated sludges. Environmental Technology 13, 531-540.

Frankenberger Jr, W. T., Johanson, J. B., 1993. Factors affecting invertase activity in soils. Plant and Soil 74, 313-323.

Guilbault, G. G., Kramer, D. N., 1964. Fluoremetric determination of lipase, acylase, alpha- and gamma-chymotrypsin and inhibitors of these enzymes. Analytical Chemistry 36, 409-412.

Gumprecht, G., Gerlach, H., Nehrkorn, A., 1995. FDA hydrolysis and resazurin reduction as a measure of microbial activity in sediments from the south-east Atlantic. Helgoländer Meeresuntersuchungen 49, 189-199.

Kramer, D. N., Guilbault, G. G., 1963. A substrate for the fluorimetric determination of lipase activity. Analytical Chemistry 35, 588-589.

Rotman, B., Papermaster, B. W., 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proceedings of the National Academy of Science, USA 55, 134-141.

Schnürer, J., Rosswall, T., 1982. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. Applied and Environmental Microbiology 43, 1256-1261.

Skujins, J. J., 1967. Enzymes in soil. In: McLaren, A. D. and Peterson, G. H. (Eds.), Soil Biochemistry. Vol. 1. Marcel Dekker, New York. pp 371-414.

Stubberfield, L. C. F., Shaw, P. J. A., 1990. A comparison of tetrazolium reduction and FDA hydrolysis with other measurements of microbial activity. Journal of Microbiological Methods 12, 151-162.

Swisher, R., Carroll, G. C., 1980. Fluorescein diacetate hydrolysis as an estimator of microbial biomass on coniferous needle surfaces. Microbial Ecology 6, 217-226.

Zablotowicz, R. M., Locke, M. A., Smeda, R. J., 1998. Degradation of 2,4-D and Fluometuron in cover crop residues. Chemosphere 37, 87-101.

# Appendix iii

**Submitted Manuscripts**

Effect of alcohol addition on the movement of petroleum hydrocarbon fuels in soil.
Adam, G., Gamoh, K., Morris, D. G. and Duncan, H.
Submitted to *The Science of the Total Environment*, April 2001.

The effects of cationic surfactants on marine biofilm growth.
Smith, M. J., Adam, G., Duncan, H. J. and Cowling, M. J.
Submitted to Estuarine, Coastal and Shelf Science, May 2001.

The effect of diesel fuel on Common vetch (*Vicia sativa*) plants.
Adam, G. and Duncan, H.
Submitted to *Environmental Geochemistry and Health*, May 2001.

# Effect of alcohol addition on the movement of petroleum hydrocarbon fuels in soil

**Gillian Adam* [1], Keiji Gamoh [2], David G. Morris [1] and Harry Duncan [1]**

[1] *Department of Chemistry, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. Tel. (+44) 0141 330 6571 Fax. (+44) 0141 330 4888*

[2] *Faculty of Education, 5 – 1 Akebono-cho-2, Kochi University, Kochi 780-8520, Japan. Tel & Fax (+81) 88 844 8411*

** corresponding author : present address Scottish Universities Environmental Research Centre, Scottish Enterprise Technology Park, Rankine Avenue, East Kilbride, Glasgow G75 0QF. Tel : (+44) 01355 270 135 e-mail : gilliana@chem.gla.ac.uk*

**Abstract**

Groundwater contamination by fuel spills from aboveground and underground storage tanks has been of growing concern in recent years. This problem has been magnified by the addition of oxygenates, such as ethanol and methyl-tertiary-butyl ether (MtBE) to fuels to reduce vehicular emissions to the atmosphere. These additives, although beneficial in reducing atmospheric pollution, may however increase groundwater contamination due to cosolvency of petroleum hydrocarbons and by provision of a preferential substrate for microbial utilisation. With the introduction of ethanol to diesel fuel imminent and the move away from MtBE use in many states of the USA, the environmental implications associated with ethanol additive fuels must be thoroughly investigated. Diesel fuel movement was followed in a 1 m soil column and the effect of ethanol addition to diesel fuel on this movement determined. Addition of 5 % ethanol to diesel fuel was found to enhance the downward migration of the diesel fuel components, thus increasing the risk of groundwater contamination. A novel method using soil packed HPLC columns allowed the influence of ethanol on individual aromatic hydrocarbon movement to be studied. An aqueous ethanol concentration above 10 % was required for any movement to occur. At 25 % aqueous ethanol the majority of hydrocarbons were mobilised and the retention behaviour of the soil column lessened. At 50 % aqueous ethanol, all the hydrocarbons were

found to move unimpeded through the columns. The retention behaviour of the soil was found to change significantly when both organic matter content and silt/clay content was reduced. Unexpectedly, sandy soil with low organic matter and low silt/clay was found to have a retentive behaviour similar to sandy subsoil with moderate silt/clay but little organic matter. It was concluded that sand grains might have a more important role in the adsorption of petroleum hydrocarbons than first realised. This method has shown that soil packed HPLC columns can be used to provide a quick estimate of petroleum hydrocarbon, and possibly other organic contaminant, movement in a variety of different soil types.

*Keywords* : Diesel, Aromatic hydrocarbons, Ethanol, Mobilisation, Groundwater.

## Introduction

Since the petroleum crisis of the early 1970's, Brazil is the only country that has attempted a large scale programme to substitute a non-renewable fuel source by an entirely renewable one (i.e., hydrated ethanol) (Massad et al., 1993). A large proportion of cars are fuelled exclusively by ethanol and the remaining gasoline sold in petrol stations contains 24% ethanol. Like any other large-scale energy programme the advantages and disadvantages of ethanol fuel usage have been investigated. As well as the inherent economic advantages, ethanol addition to fuel is environmentally appealing as it is one of the oxygenates, along with methyl-tertiary-butyl ether (MtBE), used to reduce vehicular emissions of carbon monoxide and ozone precursors to the atmosphere. However MtBE, which is the most common oxygenate added to reformulated gasoline in North America, has been shown to be persistent in subsurface systems and to create taste and odour problems. In March 1999, California announced plans to phase out MtBE use by 2003 following a number of incidents involving the loss of groundwater supplies due to MtBE pollution (Environment Agency, 1999). Ethanol is therefore considered as a possible substitute. Several studies have been carried out on gasoline-ethanol fuels but little work has been done on other fuels. With the introduction of 3 % ethanol to diesel fuel, which is currently under revision by the Brazilian authorities and the move away from MtBE use in many states in the USA, the environmental implications associated with ethanol additive fuels must be thoroughly investigated.

Theoretically, the addition of ethanol to gasoline should enhance the downward migration of this contaminant in soil due to increased solubilisation of gasoline components in ethanol

and the wetting effect of ethanol on the more hydrophobic soil components. If this is true, risk of contamination of ground water by gasoline is greatly increased when ethanol is present. Gasoline comprises low molecular weight alkanes (C5 – C10) and aromatics (mainly benzenes and naphthalenes) with very little polyaromatic hydrocarbons (PAHs). A large proportion of gasoline is made up of BTEX (benzene, toluene, ethylbenzene and m-, o- and p-xylene) components, which are relatively soluble and would degrade readily under the right environmental conditions. The presence of ethanol along with gasoline may however retard the degradation of gasoline components, with microorganisms preferentially utilising ethanol over gasoline (personal communication). This increases the residence time of gasoline in the soil, which may cause further ground water contamination problems.

A blend of ethanol with other additives was developed for use with diesel engines. The above implications can be implied for the diesel-additive ethanol situation. Diesel however, contains a higher percentage of aromatics, which can include up to 3% PAHs. PAHs are of specific concern as they are more persistent in the environment and some PAHs have adverse health effects. Action levels for PAHs in ground water are very low and contamination by diesel fuel components would pose a serious threat to ground water quality. The higher concentration of aromatics in diesel may prevent the downward migration of the contaminant due to lower solubility of the aromatics and increased adsorption of the aromatics on soil components. However, the possibility of movement into groundwater must be investigated.

A laboratory study was undertaken to investigate the movement of diesel fuel and various aromatic compounds found commonly in gasoline (toluene, naphthalene) and diesel fuel (naphthalene, 1-ethyl naphthalene, 2,5 dimethylnaphthalene, phenanthrene, anthracene, pyrene and chrysene) through a soil column and to determine if the movement of these compounds is enhanced by ethanol. The effect of soil components on contaminant movement was also investigated.

## Materials and Methods

### Movement of diesel fuel through soil column

### Leaching column set up

Polythene drain pipe was cut into sections (L 10 cm x ID 4 cm). The sections were sealed together using waterproof tape to provide an airtight seal at the joins. Ten sections were fitted together to create a column 1 m in length. As the sections were fitted together, the column was filled with John Innes compost No. 2 by tapping the soil into each section to create an evenly packed column. John Innes Compost No. 2 was chosen as a substitute soil as it is prepared from sterilised loam which provided a low microbial activity soil (Adam and Duncan, in press). The column was built up, section by section, in this way. The bottom section of the column had a fine Nylon mesh covering the lower end to prevent the soil from escaping, but allowing the leachate to freely drain away. An extra section was placed on the top of the column to provide a collar for the water reservoir. The column was run at a temperature of approximately 15°C to reduce microbial activity in the column. The complete set up of the column is illustrated in Fig. 1.

### Procedure

10 ml of diesel fuel oil was added, using a 50 ml syringe, to the top of the column. The diesel fuel was allowed to penetrate into the soil for approximately 30 min. After this time, 50 ml of deionised water was poured in to wet the column, then 2 l of deionised water was added by inverting a 2.5 l plastic bottle into the top of the column. This acted as a reservoir allowing a constant supply of water to leach through the column. The flow rate depended entirely on gravity flow and the density of the soil packed column. It took approximately 24 h for 2 l of deionised water to leach through the column, therefore a fresh 2 l reservoir was set up each day. This process was continued for 5 days and provided a total of 10 l of water leached through the column.

The column was dismantled one section at a time and a 40 g subsample was taken from each section. The samples were then extracted separately to determine the amount of diesel fuel present in each section. This procedure was repeated using 10 ml of diesel fuel oil with 5 % ethanol additive. 10 l of water was again leached through the column and the sections dismantled for diesel analysis as above.

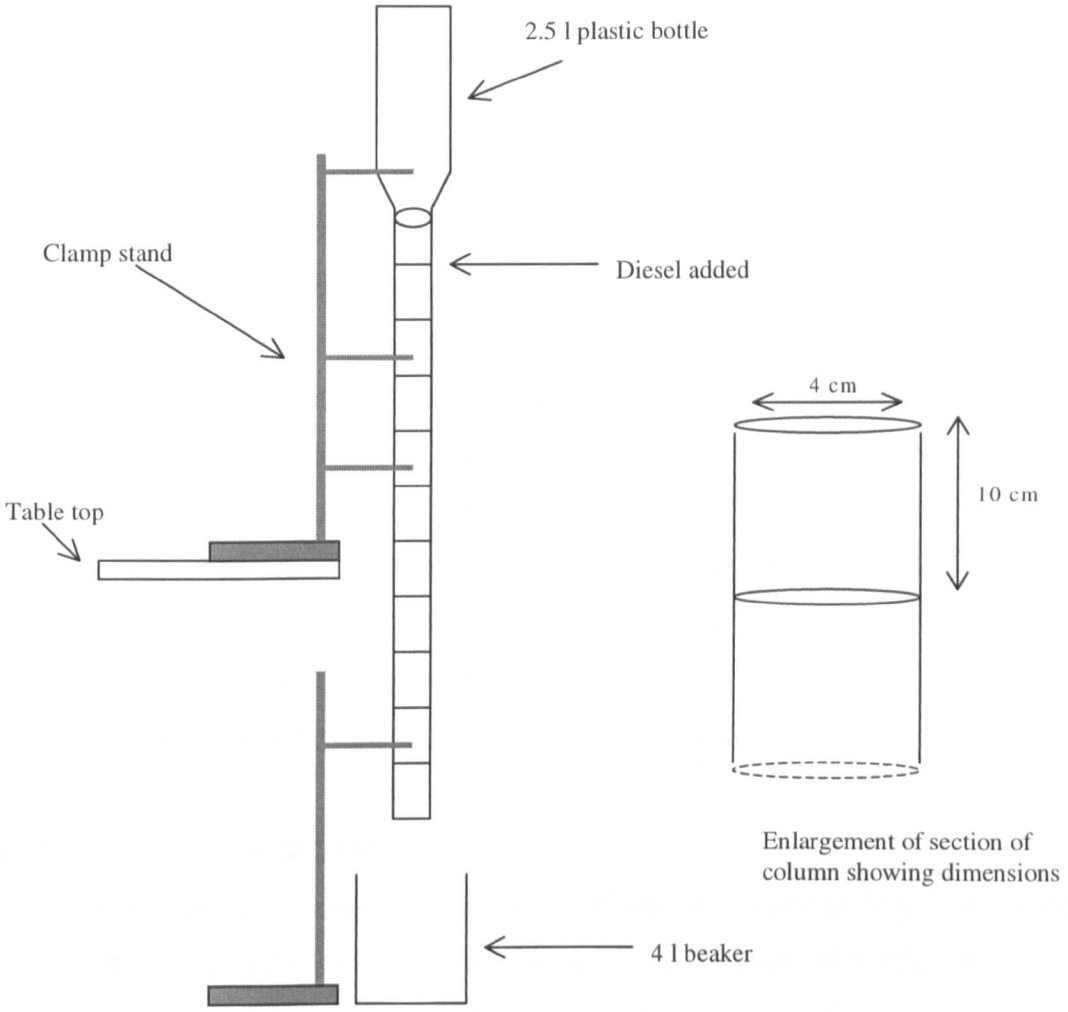



Fig. 1. Diagrammatic representation of the leaching column set up. The column consists of 11 sections (one extra section on top of the column to act as a collar for the reservoir) supported by clamp stands. The reservoir is a 2.5 l plastic bottle containing 2 l of water. A 4 l beaker collects the leachate beneath the column.

**Diesel fuel extraction**

Due to the short residence time of diesel fuel in the soil column, a method of extraction was developed that allowed the volatile, lighter fuel components to be removed effectively, followed by extraction of the heavier fuel components. A cold shaking extraction method was developed from the mechanical shaking method of Schwab *et al* (1999). 40 g of fresh soil (sieved < 2 mm) was extracted for 30 min in 100 ml 1:1 acetone: dichloromethane in an orbital incubator (15 °C, 200 rev $min^{-1}$). The extract was filtered (Whatman, No. 2)

into a 100 ml volumetric flask and the volume made up with 1:1 acetone: dichloromethane. This extract was analysed by GC-FID using the conditions described below and the total petroleum hydrocarbon (TPH) value calculated.

The 40 g soil sample was left to air dry overnight and was then transferred to a cellulose thimble for Soxhlet extraction. The Soxhlet method was modified from the US EPA method 3540C for non volatile and semi volatile organic compounds and the method of Song *et al* (1990). 5 g of anhydrous sodium sulphate was added to the bottom of a cellulose extraction thimble then the air dried soil sample added. The thimble was plugged with glass wool then placed into the Soxhlet apparatus. 100 ml of 1:1 acetone : dichloromethane was added and the sample extracted for 6 h. Once cool the extract was transferred, with washings, to a 100 ml volumetric flask and the volume made up to 100 ml with 1:1 acetone: dichloromethane. The extract was analysed by GC-FID as described below and the residual TPH value calculated. The TPH values obtained for each step of the extraction were summed to provide a total TPH value for the sample. Dilution of the original diesel fuel in dichloromethane served as a quantitative analytical standard.

**Diesel fuel analysis by GC-FID**

The method for diesel fuel analysis by capillary GC-FID was modified from the US EPA method 8100 for the analysis of polynuclear aromatic hydrocarbons (US EPA, 1986). The chromatographic conditions were as follows. Analyses were carried out with a Hewlett-Packard 5890A gas chromatograph and Flame Ionisation detector (FID). The GC was interfaced with a Hewlett-Packard Chemstation data system. Helium carrier gas was adjusted to the recommended linear flow velocity of 20 cm $sec^{-1}$ using the non-retained compound butane. Separations were performed on a SGE BPX 5 polysilphenylene-siloxane capillary column (25m x 0.32mm I.D. x 0.5µm). 0.5µl of sample was injected at 35 °C with a temperature hold of 3 min. The temperature increased 5 °C $min^{-1}$ up to 250 °C with a 10 min hold at the end of the run. The injector temperature was 260 °C and the detector temperature 270 °C.

**Statistical analysis**

Triplicate injections of each extract with no more than a 5 % difference in total peak area were obtained for each sample. Retention times and peak areas of each replicate injection were tabulated using Microsoft Excel to allow comparison of individual diesel fuel components. Ten assigned peaks were used for verification of acceptable replicate analysis.

The average total peak area from the three replicates was calculated and used to work out the total TPH content of that section.

**Movement of individual aromatic hydrocarbons through HPLC column**

**Preparation of column packing material**

The packing materials used in the study were prepared by sieving the initial samples to < 150 μm. This provided material, with an acceptable particle size range and narrower particle size distribution, for packing into a HPLC column that would give constant back pressure values and good chromatographic conditions.

Column G-01 packing material was prepared entirely from sieved Barassie soil. Column G-05 material is the sieved subsoil from the Barassie series. This provides the same soil matrix with lower organic matter, silt and clay content. Column G-05F material is Barassie soil that has been placed in a furnace at 500 °C for 6 h to burn off all the organic matter. For control purposes a column packed with acid washed sand (G-06) and silica gel columns (G-07 and G-08) were included.

Particle size analysis for the determination of size distribution was carried out by mechanical analysis as described by the modified method of Khan (Ph. D. thesis, University of Glasgow, 1987) from ADAS Method 57 (1981). Organic matter content was assessed by loss on ignition (LOI). 5 g soil was weighed, in triplicate, into silica basins and dried overnight at 105 °C. The soils were weighed to obtain the oven dry soil weight. The soils were then placed in a muffle furnace and ignited at 500 °C for 6 h. The samples were re-weighed and the weight of ignited soils calculated. The % organic matter by loss on ignition was calculated by subtracting the weight of ignited soil from the weight of oven dry soil. This value was then divided by the weight of oven dry soil and the resulting value multiplied by 100 to obtain % LOI. Table 1 shows the physical characteristics of the six packing materials used in the study.

Table 1 Physical characteristics of the packing materials used in this study

| | | | Particle size distribution | | | |
|---|---|---|---|---|---|---|
| Column | Description | Organic matter LOI % | Coarse + medium sand % | Fine sand % | Silt % | Clay % |
| G – 01 | Sandy soil | 16.68 | 1.68 | 38.86 | 21.56 | 24.78 |
| G – 05 | Sandy subsoil | 5.01 | 36.00 | 45.33 | 10.17 | 6.97 |
| G – 01F | Sandy soil (OM removed) | 0.00 | 1.95 | 44.75 | 24.80 | 28.50 |
| G – 06 | acid washed sand (Fisher Scientific Chemicals) | 0.00 | 100 ◄——► | | 0.00 | 0.00 |
| G – 07 | Matrex silica 60 (Fisher Scientific Chemicals) | | | 100 ◄——► (0.070 - 0.0035 mm) | | |
| G – 08 | Silica Gel 60H (Merck BDH) | | | | 100 ◄——► (0.0015 mm) | |

% sand values are based on one replicate measurement and % silt and clay are based on duplicate measurements.
Mechanical analysis recoveries for G – 01 and G – 05 were 103.56 % and 103.48 % respectively. Particle size measurements are based on : coarse sand > 0.18 mm, fine sand 0.18 – 0.05 mm, silt 0.05 – 0.002 mm, clay < 0.002 mm.

## Column preparation

The prepared material was packed into an empty stainless steel HPLC column (L 100 mm x ID 4.6 mm) by dry tapping. The packed column was attached to a HPLC pump and 50% aqueous ethanol flowed through (0.1 ~ 1.5ml min $^{-1}$) as a packing solvent. After 30 min, a small portion of soil, saturated in 50% aqueous ethanol, was added to the column head to fill up the crack left by the material shifting during packing. Once the column was successfully packed, 50% aqueous ethanol was run through overnight at 0.1 ml min $^{-1}$. Acetone was used to measure the void volume.

**Petroleum hydrocarbon standards**

100 mg $l^{-1}$ solutions of toluene (Fisher Scientific International Company, UK., 99 +% Analar), naphthalene, 1-ethylnaphthalene, 2,5-dimethylnaphthalene, anthracene, phenanthrene, pyrene and chrysene (Sigma-Aldrich Co. Ltd., UK., 99 +%) were prepared in acetone (Riedel-de Haën, Sigma-Aldrich Co. Ltd., UK., Analar).

**Procedure**

Each petroleum hydrocarbon standard was added to the selected column individually and varying % aqueous ethanol concentrations used as the mobile phase with isocratic elution.

The hydrocarbon standards were injected into the column at 1.6 ml $min^{-1}$ and detected by UV at 254, 285 and 335 nm. Flow rate was set for each column at a rate that provided a constant back pressure. The chromatographic conditions used for each column are outlined in Table 2. The trend in retention of the hydrocarbons on each column showed good linearity with carbon number, suggesting the column was performing successfully. A good recovery of the petroleum hydrocarbon analytes, from each column, under these conditions was achieved when the absorbance of the eluent from each column was measured by UV spectrophotometry and compared to the corresponding standard solution. The recoveries for mono- and di-aromatic analytes were >99 % and >94 % for polyaromatic analytes.

Table 2 Chromatographic conditions used for individual packed columns.

| | Mobile phase | | | | | |
|---|---|---|---|---|---|---|
| | 25 % aqueous ethanol | | | | water | |
| Column | G-01 | G-05 | G-01F | G-06 | Silica A | Silica B |
| Packing weight $g^{-1}$ | 1.6 | 2.6 | 2.4 | 2.2 | 1.2 | 0.85 |
| Flow ml $min^{-1}$ | 1.6 | 1.6 | 1.6 | 1.6 | 0.8 | 1.2 |
| Pressure psi | 90 | 600 | 110 | 20 | 100 | 2000 |

Pressure measure in $lb/in^2$ (psi). Metric conversion – 1 psi ~ 7 kPa

## Results

### Vertical Movement of Diesel Fuel Through a Soil column

The results from the 1 m soil columns suggested that the downward migration of diesel fuel in the soil profile was enhanced by ethanol addition. Fig. 2 shows the percentage distribution of diesel fuel in 1 m soil columns leached with 10 l of water, where only diesel fuel has been added and where diesel fuel with 5 % ethanol has been added. Little movement of diesel fuel was observed in the diesel fuel only column with diesel fuel distribution decreasing evenly from the top of the column (Section 1) to a depth of 30 cm (Section 3). Negligible amounts of diesel fuel were found below this depth. Diesel fuel with 5 % ethanol, on the other hand, was seen to migrate to a depth of 40 cm and the pattern of diesel fuel distribution in the soil profile was very different from that seen in the diesel fuel only column. Again, the largest percentage of diesel fuel was found in the top 10 cm (Section 1). The percentage of diesel fuel in Section 2 and 3 decreased, as before, to 22.5 % and 13.8 %. However, the percentage of diesel fuel in Section 4 rose to 24.1 % of the total diesel fuel added. No diesel fuel was found in sections below this depth.

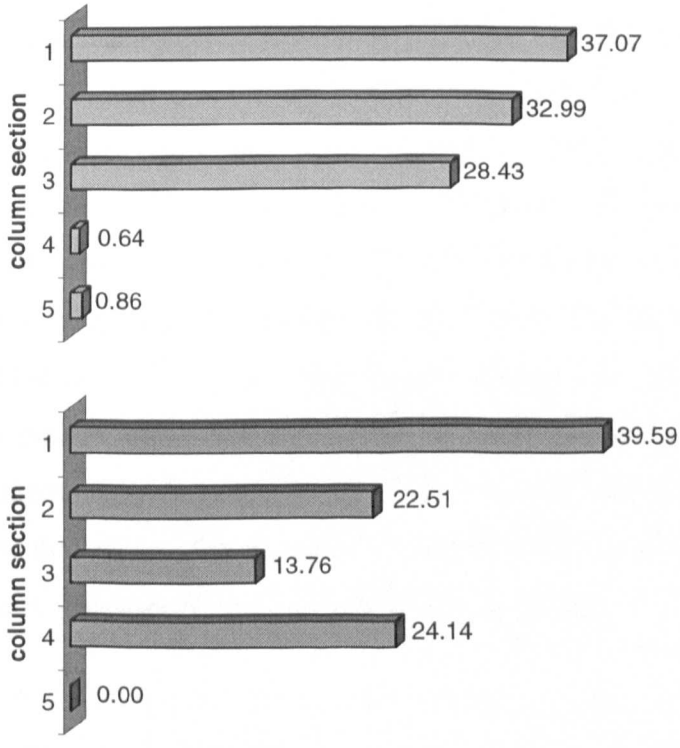



Fig. 2. % distribution of diesel in 1 m soil columns for diesel only (top) and diesel plus 5% ethanol (bottom). Only sections 1 –5 are shown as no diesel was found in the lower sections of the column (sections 6-10).

**Effect of ethanol on aromatic hydrocarbon movement in surface soil.**

The concentrations of ethanol required to enhance movement of aromatic hydrocarbons into the subsurface was investigated by a novel method using soil packed HPLC columns. A soil (Barassie series : 40.5% sand, 21.5% silt and 25.0% clay) with an average organic matter content (16.7%) was assessed. Aqueous ethanol concentrations ranging from 0 - 50 % ethanol were used as the mobile phase in this column (G-01). An aqueous ethanol concentration of above 10 % was required before any movement of aromatic hydrocarbons was observed. At a 25 % aqueous ethanol concentration, the lighter, more soluble aromatic hydrocarbons eluted slowly from the column whereas the larger aromatics (1,5 dimethyl naphthalene, phenanthrene, anthracene, pyrene, chrysene) were retained on the column (Table 3). Toluene, naphthalene and 1-ethyl naphthalene had retention times of 2.49, 9.86 and 28.00 min respectively. The length of time taken for these hydrocarbons to be eluted from column G-01 suggests the soil packing has hydrophobic sites capable of retaining aromatic hydrocarbons but the adsorption of the lighter hydrocarbons on these sites can be overcome by 25 % aqueous ethanol. When the aqueous ethanol concentration of the mobile phase was raised further to 50 % ethanol, all the aromatic hydrocarbons added could be eluted from the column and the time taken for elution was much less than with 25 % ethanol. This implies increasing concentrations of ethanol lessened the retentive behaviour of the soil column. This result is not unexpected as ethanol breaks the surface tension of repellent soil, allowing increased penetration (King, 1981). Table 3 shows the retention time ($t_r$) and capacity factor (k') of hydrocarbons on the sandy soil column (G-01) using different ethanol mobile phase concentrations. Capacity factors are included as although a peak can be identified by its retention time, this varies with column length and mobile phase flow rate (Lindsay, 1992). The same column lengths are used throughout this experiment but the mobile phase flow rates differ. By using capacity factors instead of retention times, a direct comparison can be drawn between different column results. The results clearly show that ethanol enhances hydrocarbon mobility and increasing ethanol concentration, in turn, increases the mobility of the hydrocarbons.

Table 3 Retention time ($t_R$) and capacity factor (k') of hydrocarbons on the Barassie soil column (G-01) using different aqueous ethanol mobile phase concentrations.

| | Mobile phase | | | |
|---|---|---|---|---|
| | 25% EtOH | | 50% EtOH | |
| Hydrocarbon | $t_R$ min | k' | $t_R$ min | k' |
| Acetone | $t_o = 0.78$ | | $t_o = 0.78$ | |
| Toluene | 2.49 | 2.19 | 1.40 | 0.79 |
| Naphthalene | 9.86 | 11.64 | 1.45 | 0.86 |
| 1 Ethyl naphthalene | 28.00 | 34.89 | 2.35 | 2.01 |
| 1, 5 Dimethyl naphthalene | - | - | 2.61 | 2.35 |
| Phenanthrene | - | - | 4.28 | 4.49 |
| Anthracene | - | - | 4.55 | 4.83 |
| Pyrene | - | - | 6.69 | 7.58 |
| Chrysene | | | 18.00 | 22.08 |

Capacity factor $k' = \frac{t_R - t_o}{t_o}$ where $t_R$ is the analyte peak retention time and $t_o$ is the peak of the unretained solvent front

**Influence of Soil Components on Aromatic Hydrocarbon Movement**

To determine what effect various soil components had on the adsorption of aromatic hydrocarbons, a series of soil columns were prepared with varying ratios of organic matter, sand, silt and clay. Column G-05 was prepared from the subsurface soil of the Barassie series used in column G-01. This subsurface soil consisted of a very large proportion of sand (approximately 81 %) with low organic matter content (approximately 5%), silt (approximately 10%) and clay (approximately 7 %). Column G-01F was packed from the Barassie soil used in column G-01 that had been ignited for 6 hours in a 500 °C furnace to remove all the organic matter. Column G-06 is a manufactured sand (Fisher Scientific Chemicals, 40 – 100 mesh) which provided a measure for the mineral fraction of soil and finally, columns G-07 and G-08 which are packed from silica of varying particle size. Column G-07 contains particles in the fine sand to silt range whereas column G-08 contains only silt sized particles. Fig. 3 shows the trend in retention of hydrocarbons on various soil columns. 25 % ethanol was used as the mobile phase as it was found to enhance hydrocarbon mobility in the original soil column (G-01) and is an important environmental

value as fuel in Brazil and many states in the USA contain 24 % ethanol (Massad et al., 1993).

The original soil column (G-01) which contained average organic matter, sand, silt and clay contents had the highest adsorptive capacity for petroleum hydrocarbons. This is shown in Figure 3 by the trend in retention lying above all the other column values as increasing ln k' values indicate increased retention of hydrocarbons due to increased adsorption to soil sites.

The sandy subsoil (column G-05), which contained high levels of sand but low levels of organic matter, silt and clay, had the next highest adsorptive capacity. The presence of organic matter appeared to be the most important factor in the adsorption of petroleum hydrocarbons because when all the organic matter was removed, as in column G-01F, the retentive behaviour of the soil column was drastically reduced. Petroleum hydrocarbons with a carbon number below 14 were not retained at all on the column but eluted along with the solvent front. However, some retention of the larger petroleum hydrocarbons, such as phenanthrene, was observed suggesting other factors were involved in retaining hydrocarbons on soil. Column G-06, which is a manufactured sand of narrow particle size range, also showed signs of retaining larger aromatic hydrocarbons suggesting sand particles themselves have some retentive behaviour.

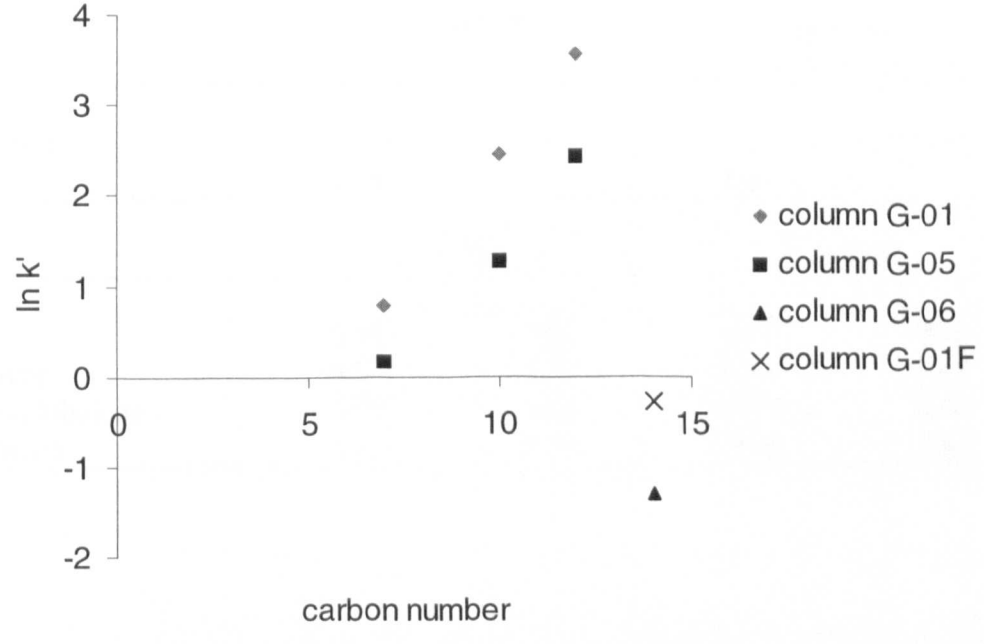



Fig. 3. Trend in retention of hydrocarbons on various soil columns.

Carbon number relates to: C7–Toluene, C10–Naphthalene, C12–1 Ethyl naphthalene and C14–Phenanthrene.

**Influence of Sand Particle Size on Aromatic Hydrocarbon Movement.**

Two silica columns were tested to distinguish between the influence of particle size on hydrocarbon retention. The results are given in Table 4. The silica used as packing material in these columns (G-07 and G-08) had no surface coatings hence they had little adsorptive capacity. Because of this water was used as the mobile phase. Column G-07 had a larger particle size distribution (diameter ranging from 0.0035 mm to 0.70 mm) than column G-08 (0.0015 mm particle size diameter). This was reflected by the capacity factors of the aromatic hydrocarbons on each column. The time taken for elution of each hydrocarbon was almost double on column G-08 compared with column G-07. These results show that mineral particles such as sand, may influence the adsorption of hydrocarbons even when they are not coated with organic matter or other active functional groupings.

Table 4 Retention times and capacity factors for petroleum hydrocarbons on silica columns with varying particle size ranges.

| | Mobile phase : water | | | |
|---|---|---|---|---|
| | Column G-07 | | Column G-08 | |
| Hydrocarbon | $t_R$ min | k' | $t_R$ min | k' |
| Acetone | $t_o = 1.47$ | | $t_o = 2.86$ | |
| Toluene | 2.10 | 0.42 | 4.81 | 0.68 |
| Naphthalene | 3.81 | 1.19 | 7.83 | 1.73 |
| 1 Ethyl naphthalene | 5.97 | 2.43 | 15.72 | 4.49 |
| Phenanthrene | 6.17 | 2.54 | 18.95 | 5.62 |

**Discussion**

An important factor on diesel fuel entering a soil system is its subsequent movement, both lateral and vertical, in the soil profile. Rainfall can encourage contaminant leaching through the soil profile which can lead to surface water and groundwater contamination. Diesel fuel, due to its hydrophobic character, should not move far in the soil profile. However, this statement is highly dependent on the characteristics of the soil that the diesel fuel is contaminating and whether diesel fuel is contaminating from an aboveground or underground source. The difference in the surface and subsurface soil characteristics allow diesel fuel, on entering these systems, to behave very differently.

The pattern of diesel fuel distribution in the 1m soil column clearly showed the enhancement of diesel fuel movement through the soil column due to ethanol addition. Gas chromatographic analysis of diesel fuel extracted from each soil section resulted in traces whose pattern of hydrocarbon distribution was very similar. There was no indication that specific components or fractions of the diesel fuel were being mobilised and moving further down the soil profile than other components or fractions of the diesel fuel. This implies that the effect ethanol has on enhancing diesel fuel movement in the soil may be due to the 'wetting' effect of ethanol on the soil components allowing mass movement of diesel fuel. Hydrophobic, soil organic matter components such as humic, fulvic and fatty acids, impart water repellent character to a soil (Anderson et al., 1995). Ethanol has been used in many studies on water repellent soils (King, 1981, Roy and McGill, 1997) as it can break the surface tension of repellent soil, allowing increased infiltration. Ethanol present within diesel fuel would therefore enhance infiltration of diesel fuel into the soil profile by lessening the adsorptive capacity of the hydrophobic sites of soil organic matter components. This allows diesel fuel to move further down the soil profile. By further increasing the concentration of ethanol in diesel fuel, enhanced movement of diesel fuel into the subsurface would be observed. This conclusion was verified by the results from the soil packed HPLC columns. Individual petroleum hydrocarbon movement was seen to increase with increasing ethanol content.

The characteristics of the contaminated soil were also important in influencing the movement of petroleum hydrocarbons. The percentage organic matter present was extremely important in retaining hydrocarbons. Organic matter is found at high levels in the surface soil and decreases to small amounts in subsurface soils. Therefore, petroleum hydrocarbons contaminating from an aboveground source would not migrate far in the soil

profile due to adsorption by organic matter. If petroleum hydrocarbons were contaminating from an underground source, the amount of organic matter in subsurface soils is minimal therefore increased movement of contaminants would be expected. This theory was verified by the results obtained from the subsurface soil column (G-05) and the column with no organic matter (G-01F). However, the results obtained during this investigation using various sandy soils and surrogate sand columns indicate that other factors may be involved in the adsorption of petroleum hydrocarbons. Other authors have found that sandy soil can bind hydrocarbons adsorptively although neither silty material nor significant amounts of organic matter was present. Loser et al, (1999) proposed that soil particles were covered with micropores, which enlarge the soil surface area in comparison with the macroscopic surface area. This microporosity is the reason for hydrocarbons being more strongly adsorbed to sandy soils than expected (Loser et al., 1999). This theory seems a likely explanation for the slightly retentive behaviour of both the silica columns tested (G-07 and G-08) and the sandy soil column with no organic matter present (column G-01F).

**Conclusion**

These results suggest a greater possibility of groundwater contamination by petroleum hydrocarbons in ethanol additive petrol and diesel fuel spills occurring from both underground storage tanks and aboveground spills. Surface soil components such as soil organic matter, as well as silt and clay, play an extremely important role in retaining petroleum hydrocarbons near the soil surface. However, ethanol was shown to enhance the movement of both individual aromatic hydrocarbons and diesel fuel by lessening the adsorptive capacity of the surface soil components. If these petroleum hydrocarbons reach the subsurface level, the low organic matter content and lower silt and clay content will allow hydrocarbon migration to occur more freely. In addition, an underground petroleum hydrocarbon spill, which would normally migrate and contaminate groundwater quite readily, would be further enhanced by the addition of ethanol.

This work has shown the benefits of using soil packed HPLC columns to better understand the role of soil components in determining movement of petroleum hydrocarbons. The method could be used to provide a quick estimate of petroleum hydrocarbon and other organic contaminant movement in a variety of differing soil types.

**Acknowledgements**

Prof. Gamoh would like to thank The Japanese Society of Promotion of Science for their financial support during his stay in Glasgow.

**References**

Adam G, Duncan H. Development of a sensitive and rapid method for the measurement of total microbial activity using Fluorescein Diactetate (FDA) in a range of soils. *Soil Biology and Biochemistry*, **33**, (7/8), 943-951.

ADAS. Particle size distribution in soil, Method 57. The Analysis of Agricultural Materials Reference Book 427. Ministry of Agriculture, Fisheries and Food. 1986, pp. 175-178.

Anderson M A, Hung A Y C, Mills D, Scott M A. Factors affecting the surface tension of soil solutions and solutions of humic acids. Soil Science 1995; 160 (2): 111-116.

Environment Agency : National Groundwater and Contaminated Land Centre. The fuel additive MTBE – a groundwater protection issue? 1999; Reference Booklet.

King P M. Comparison of methods for measuring severity of water repellence of sandy soils and assessment of some factors that affect its measurement. Australian Journal of Soil Research 1981; 19: 275-285.

Lindsay S. Retention and peak dispersion. In: Barnes, J editor. High Performance Liquid Chromatography. John Wiley and Sons, Chichester,England 1992, pp. 17-19.

Loser C, Seidel H, Hofffmann P, Zehnsdorf A. Bioavailability of hydrocarbons during microbial remediation of a sandy soil. Applied Microbiology and Biotechnology 1999; 51: 105-111.

Roy J L, McGill W B. Characterisation of disaggregated nonwettable surface soils found at old crude oil spill sites. Canadian Journal of Soil Science 1997; 78: 331-344.

Schwab A P, Su J, Wetzel S, Pekarek S, Banks M K. Extraction of petroleum hydrocarbons from soil by mechanical shaking. Environmental Science and Technology 1999; 33: 1940-1945.

Song H G, Wang X, Bartha R. Bioremediation potential of terrestrial fuel spills. Applied and Environmental Microbiology 1990; 56 (3): 652-656.

US EPA. Method 8100 Polynuclear Aromatic Hydrocarbons. Revised September 1986. www.epa.gov

# The Effects of Cationic Surfactants on Marine Biofilm Growth

**M.J. Smith [a], G. Adam [b], H.J. Duncan [b] and M.J. Cowling [a].**

[a] Glasgow Marine Technology Centre, University of Glasgow, Glasgow, G12 8QQ, UK.

[b] Department of Chemistry, University of Glasgow, Glasgow, G12 8QQ, UK.

Tel +44 (0)141 330 4336, e-mail m.smith@mech.gla.ac.uk

## Abstract

A method for the quantification of biofilm formation on hydrogel protective coatings for optical sensors and cameras has been developed using fluorescein diacetate (FDA) hydrolysis. In conjunction with these measurements the release of the fouling resistant cationic surfactants benzalkonium chloride, tallowbenzyldimethylammonium chloride and dicocodimethylammonium chloride was measured using high performance liquid chromatography (HPLC) to enable correlation to be made between release and biofilm resistance and thus determine the active lifetime of such coatings. Results indicate that the twin-chained material, dicocodimethylammonium chloride, produced superior biofouling resistance as, at the 12 week time point, little fouling was detected on this coating. The long-chained tallowbenzydmethylammonium chloride (mainly $C_{16}$ and $C_{18}$ chains) was the next best fouling resistant material, withstanding biofilm formation for 9 weeks. This correlates with the fact that these materials had an extremely slow to zero release rate compared to the shorter chained benzalkonium chloride (mainly $C_{12}$ and $C_{14}$), which showed signs of biofilm formation at the 3 week time point.

Keywords: biofilm, surfactants, fluorescein diacetate, marine, optical sensors

**Introduction**

Biofilm formation on marine underwater structures, vehicles, sensors etc is an ongoing problem leading to macrofouling and surface corrosion. It occurs in any situation where there is a solid/liquid, liquid/gas or solid/gas interface (Wimpenny, 1996). Biofilms form in many areas and in most cases their presence is unwelcome. However, there are areas, such as medical implants, where biofilm colonisation is essential to prevent rejection by the body. A biofilm is commonly composed of bacteria, diatoms, protozoans, microalgae and macroalgae (Anderson, 1995). Surfaces are initially colonised by bacteria and diatoms creating the "slime" layer, the biofilm, which is highly hydrated. When established, the biofilm is able to confer a defence for the organisms within it thus making the task of biofilm resistant materials more difficult (Costerton & Lashen, 1984).

The use of marine monitoring equipment has increased in recent years and long-term monitoring from remote buoys has necessitated biofilm resistant strategies to be developed in order that accurate data can be collected. Research into formation of biofilms on marine underwater sensors and camera lenses has shown that 1-2 weeks in temperate waters would result in useless data and poor camera images (Kerr *et al.*, 1998). At present, work to create a transparent fouling resistant coating for marine optical sensors and cameras continues.

Hydrogels coatings containing cationic surfactants have been shown to prevent biofouling formation on sensors and cameras increasing their underwater deployment time up to 20 weeks. (Cowling *et al.*, 1998). Cationic surfactants possess two characteristics, a hydrophillic head and a hydrophobic tail thus making them useful in many areas of industry. Their antimicrobial properties are utilised in products such as eyedrops, mouthwashes and laundry agents. Their surface active properties are important as lubricators, constituents for polishes and in corrosion inhibition. However, it is their dual properties that make them useful in biofouling resistance.

In order to understand the relationship between release of cationic surfactants from the hydrogel and biofilm formation it has been necessary to find a method for quantitative biofilm determination. The hydrogel used were poly- (hydroxyethyl methacrylate) with an equivalent water content (E.W.C.) of 40%. Current methods for testing biofouling resistant chemicals either test the response of a test organism, usually a diatom, to the substance under investigation (Callow & Willingham, 1996; Wigglesworth-Cooksey & Cooksey, 1996) or estimate biofilm metabolic or

physiological activities by targeting either the heterotrophic or autotrophic compartment. These methods generally require removal of the biofilm from the substrate, which results in considerable loss in precision. Staining techniques followed by light microscopy are not useful as the hydrogel is also stained, making counting of bacteria impossible. Biofouling accumulation can also be measured by UV transmission (Parr *et al.*,1998; Marrs *et al.*, 1999). However, such methods are only capable of investigating small areas at a time, which results in many measurements having to be taken on each sample. The methods described above are limited as they do not realistically model a biofilm population which is, at the initial stages, a mix of bacteria (heterotrophic) and algae (autotrophic). In addition, these methods are time consuming. A quick and reliable estimation of biofilm metabolic activity was required to measure the effectiveness of potential fouling resistant hydrogels in the marine environment.

Fluorescein diacetate (3′ 6′ -diacetyl-fluorescein) hydrolysis was used to quantify biofilm formation. This colourless compound is hydrolysed by both free and membrane bound enzymes (Stubberfield & Shaw, 1990) releasing a coloured end product, fluorescein, which can be measured by spectrophotometry. Fluorescein diacetate (FDA) hydrolysis has been used to assess microbial activity in marine (Gumprecht *et al*, 1995 Poremba, 1995) and freshwater sediments (Battin, 1997), activated sludge (Fontvielle *et al.*, 1992) as well as in pure cultures of bacteria (Schnürer & Rosswall, 1982) and algae (Gilbert *et al.* 1992). Only recently has this method been applied to biofilm estimation on surfaces (De Rosa *et al.*, 1998). This work follows the development of marine biofilms on submerged, surfactant treated hydrogels using FDA hydrolysis and monitors the release of cationic surfactants from the hydrogel. The advantages of the FDA method are the whole sample can be measured, the biofilm remains attached to its substrate, it can be used on opaque samples where light transmission microscopy is not useful and it measures over a wide range of biofilm thickness'. The method provides a more accurate and sensitive estimation of biofilm activity and is easy and rapid to perform.

## Materials and methods

### *Preparation of the Hydrogels*

The hydrogels used were transparent and contained 40% water. They were prepared in 250mm x 200mm poly- (methyl methacrylate ) PMMA moulds to a thickness of 1-2mm. They were stored in distilled water until required. The details of there preparation can be found elsewhere (Smith, 1997, Refojo, 1966).

### *Cationic Surfactants*

The three cationic surfactants used were quaternary ammonium compounds, their structures are shown in Figure 1. They were benzalkonium chloride (BAC) (Aldrich), tallowbenzyldimethylammonium chloride (Akzo Nobel) DMHTB-75™ and dicocodimethylammonium chloride (Akzo Nobel) 2C-75™. The hydrogels were loaded with surfactant by soaking them in 5%w/v solutions for 14 days (MAST II, 1995). In the case of the DMHTB-75™ material it was loaded at the hydrogel production stage due its relative insolubility. Previous work had shown (Smith, 1997) that release from hydrogels soaked in DMHTB-75™ to be the same as those with DMHTB-75™ included at the preparation stage. However, hydrogels prepared with DMHTB-75™ included at the production stage were found to be more reproducible.

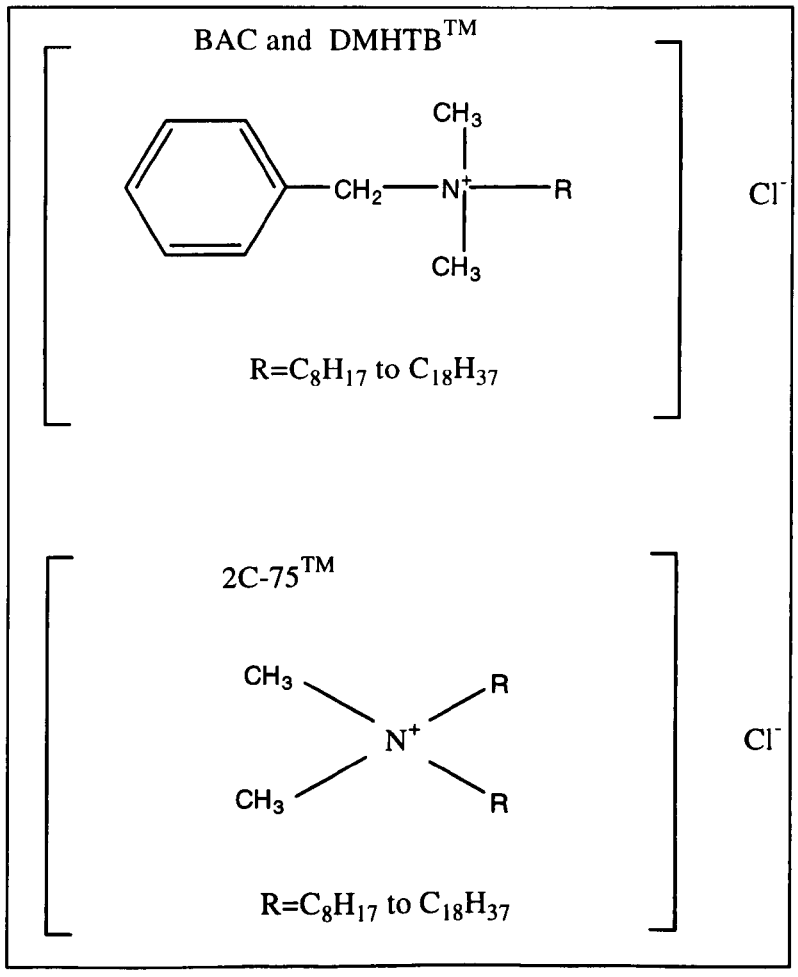



Figure 1 Structures of cationic surfactants

***Marine Exposure Trials***

The trials were carried out at the University Marine Biological Station, which is on the island of Cumbrae in the Firth of Clyde on the West Coast of Scotland. The hydrogels were held on a PMMA frame. There were 16 samples exposed, 4 containing benzalkonium chloride, 4 containing DMHTB-75™, 4 containing 2C-75™ and 4 unloaded hydrogels. They were arranged in a Latin-square formation. The exposed area was 60mm x 80mm. The rack was suspended from Keppel Pier in a vertical orientation to a depth of 3 metres in the sea. Figure 2 shows the layout of the test coatings. Keppel Pier is 30-40 metres from the shoreline. The trial was begun in July 2000 and was run for a 12 week period. Samples for both surfactant

quantification and biofilm detection were taken at, 3 weeks, 6 weeks, 9 weeks and 12 weeks. Quantitative analysis of cationic surfactant content was carried out on the BAC and the DMHTB-75™ at zero time.

The temperature in this area during the period of the trials ranged from 13°C to 15°C.

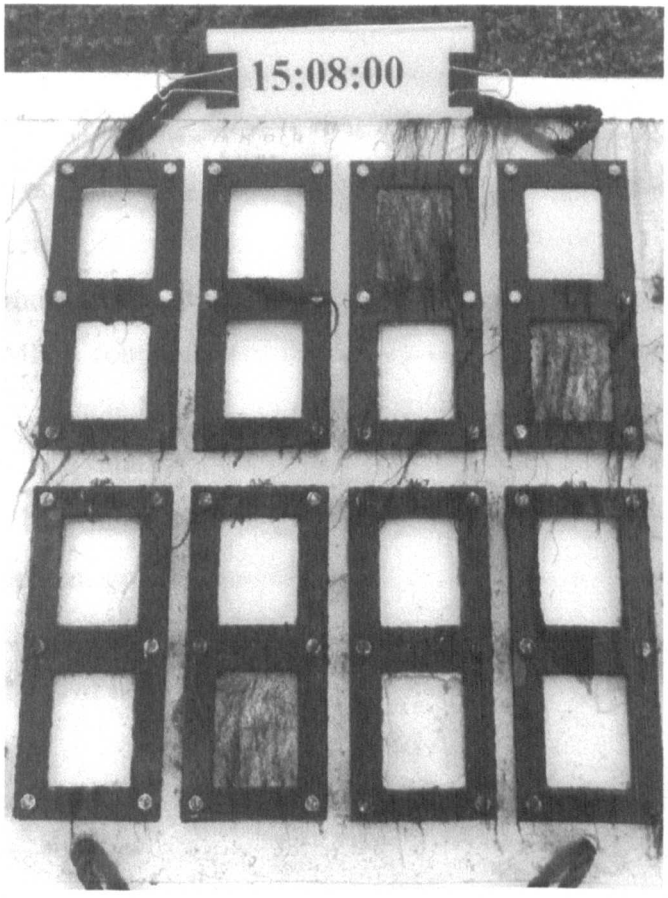



Figure 2 Hydrogel coatings in PMMA frame at 6 week time point.

***Quantitative Analysis***

The BAC and the DMHTB-75™ materials were quantitatively analysed using high performance liquid chromatography (HPLC) coupled with UV detection at 214nm (Guilfoyle *et al*, 1990). The results were reported as weight percent of BAC of dry gel weight. A dry weight was calculated at each time-point to account for any physical changes in the gel during the timescale of the experiment. The 2C-75™ material was analysed using a method described by Huang, 1987. The 2C-75™ is transparent in the UV range therefore a method of Indirect Photometric

Chromatography (IPC) is used where the analyte displaces the UV active species, in this case p-toluenesulphonic acid, from the mobile phase. This results in a negative baseline, which represents the UV transparent species. Sample discs were taken using a cork borer with a diameter of 20mm. Three were cut from each gel at each time point. The surfactants were extracted from the hydrogel using a method previously described (MAST II, 1995).

***Optimisation of Hydrogel Dimensions, Sampling and Fluorescein Diacetate Method***

A preliminary experiment was conducted to determine optimum surface area sampling size and length of submersion of hydrogels to obtain a measurable biofilm and also the optimum conditions for carrying out the FDA hydrolysis method. Varying sizes of PMMA coupons were submerged for differing lengths of time in the sea at UMBSM. The sample PMMA coupons were collected and transferred to sealed plastic containers with a small amount of seawater to prevent the biofilms drying out. The samples were then transported in a cool box to the laboratory where they were stored at 4 °C, to prevent growth from the time of sampling, and analysed within 24 hours of collection. Marine biofilm estimation was performed as described below except differing lengths of incubation were used.

***Estimation of Marine Biofilm Activity using Fluorescein Diacetate (FDA)***

Estimation of marine biofilm activity was carried out using a modified method based on the Adam and Duncan (2001) method.

Four cores (30mm diameter) were cut from each hydrogel section (60 x 80 mm) and placed into individual 60 ml glass powder jars. 15 ml of 60 mM potassium phosphate buffer pH 7.6 (8.7 g $K_2HPO_4$ : 1.3 g $KH_2PO_4$ made up to 1 litre in deionised water) was added to each jar and 0.2 ml of 1000 µg fluorescein diacetate (3′6′ - diacetyl-fluorescein, Sigma-Aldrich Co. Ltd) $ml^{-1}$ acetone solution added to start the reaction. One jar from each treatment was retained as a blank without the addition of the FDA substrate. The lids were replaced on the jars and the jars then placed in an orbital incubator (Gallenkamp orbital incubator, 100 rev $min^{-1}$) at 10 °C ± 1 °C for 1 hour. The following steps involving chloroform/methanol were carried out in a fume cupboard. Once removed from the incubator, the 30 mm diameter cores were taken

out of the buffer/FDA solution and 15 ml of chloroform/methanol (2:1 v/v) added immediately to terminate the hydrolysis reaction. The lids were replaced on the jars and the contents shaken thoroughly by hand. The contents of each jar were filtered (Whatman, No. 2) into 100 ml conical flasks and the filtrates measured at 490 nm on a spectrophotometer (Hitachi U-1100 spectrophotometer). The blank from each treatment was used to zero the spectrophotometer before reading the sample absorbance.

The concentration of fluorescein released during the assay was calculated using the calibration graph produced from 0-5 μg fluorescein $ml^{-1}$ standards which were prepared from a 20 μg fluorescein (fluorescein sodium salt, Merck-BDH, Analar) $ml^{-1}$ standard solution by appropriate dilution in 60 mM potassium phosphate buffer pH 7.6.

## Results

### *Application to Marine Biofilm Detection*

The results from the preliminary experiment indicated a surface area sampling size between 20 $mm^2$ and 50 $mm^2$ and a submersion period of > 14 days was sufficient to allow development of a sizeable marine biofilm. The optimum length of incubation using the conditions described below was 1 hour, which allowed a measurable amount of fluorescein to be released without substrate becoming limiting. Rather than performing the analyses at 30 °C, which is the temperature at which maximum FDA hydrolysis occurs (Adam and Duncan, 2001) or any other arbitrary temperature, monthly average sea temperatures were obtained from University Marine Biological Station. A temperature of 10 °C was chosen to provide realistic environmental conditions for carrying out the analyses. This temperature was a mean of twelve months temperature recordings that included winter months as it was decided to develop a method that would apply in all seasons. By keeping the incubation time short and the incubation temperature low, changes that could occur to the biofilm population would be minimised.

Biofilm FDA hydrolytic activity was measured, on each surfactant treated hydrogel as well as the untreated hydrogel (blank), at 3, 6, 9 and 12 week time points. The results are given in Table 1 and expressed in Figure 3 to illustrates biofilm development on the hydrogels investigated over time, as indicated by an increase in the amount of fluorescein released during the course of the incubation. The untreated hydrogel (blank) was quickly colonised by bacteria and the biofilm well established by the 3 week time point. At 3 weeks the blank hydrogel's FDA activity had reached a maximum of 1.393 μg fluorescein released $h^{-1}$ after which, FDA activity was seen to decrease steadily to 0.884 μg fluorescein released $h^{-1}$ at the 12 week time point. This reduction in activity, indicating a decrease in the amount of biofilm detected, is due to larger organisms, such as invertebrate larvae, grazing off bacteria on the biofilm as well as nutrient scavenging by algae, making it patchy (Anderson, 1995). A similar biofilm growth pattern was seen for the BAC treated hydrogel except a lag phase of 3 weeks was seen before an increase in FDA activity was observed. FDA activity increased from the 3 week time point to a maximum of 1.568 μg fluorescein released $h^{-1}$ at 9 weeks. The activity then decreased rapidly, as seen in the blank hydrogel curve, due to bacterial grazing. The DMHTB-75™ and 2C-75™ treated hydrogels showed very different patterns of biofilm growth. The DMHTB-75™ hydrogel showed little FDA activity over the 3 - 9 week sampling period suggesting little biofilm growth had occurred. It was not until the 9 week time point that an increase in FDA activity was observed. This activity rose steeply to it's highest measured value of 1.447 μg fluorescein released $h^{-1}$ at 12 weeks. The 2C-75™ hydrogel maintained a low level of FDA activity throughout the course of the experiment. For the first 6 weeks an extremely low FDA activity of 0.053 μg fluorescein released $h^{-1}$ (average value) was measured. This value rose to 0.327 μg fluorescein released $h^{-1}$ on average, during weeks 9 - 12. As shown in Figure 3, this level of FDA activity was extremely low compared to the other hydrogels tested and indicates that little biofilm growth occurred on this hydrogel.

Biofilm formation on the various surfactant treated hydrogels was also visually assessed at each sampling time point. Figure 2 illustrates the various hydrogels held on a PMMA frame after the 6 week exposure time. The untreated (blank) hydrogels are easily recognisable by the extensive algal fouling present on the hydrogel surface.

The other hydrogels retained their transparent appearance with little to no apparent fouling.

| | μg fluorescein released $h^{-1}$ | | | |
|---|---|---|---|---|
| hydrogel treatment | unloaded | BAC | 2C-75™ | DMHTB-75™ |
| Sampling time (weeks) | | | | |
| 3 | 1.393 ± 0.448 | 0.053 ± 0.002 | 0.043 ± 0.003 | 0.066 ± 0.013 |
| 6 | 1.313 ± 0.372 | 1.374 ± 0.116 | 0.063 ± 0.003 | 0.124 ± 0.011 |
| 9 | 1.042 ± 0.135 | 1.568 ± 0.663 | 0.325 ± 0.154 | 0.342 ± 0.114 |
| 12 | 0.884 ± 0.125 | 1.149 ± 0.337 | 0.330 ± 0.054 | 1.447 ± 0.265 |

Average values ± standard error (SE), n = 3

Table 1 Biofilm activity

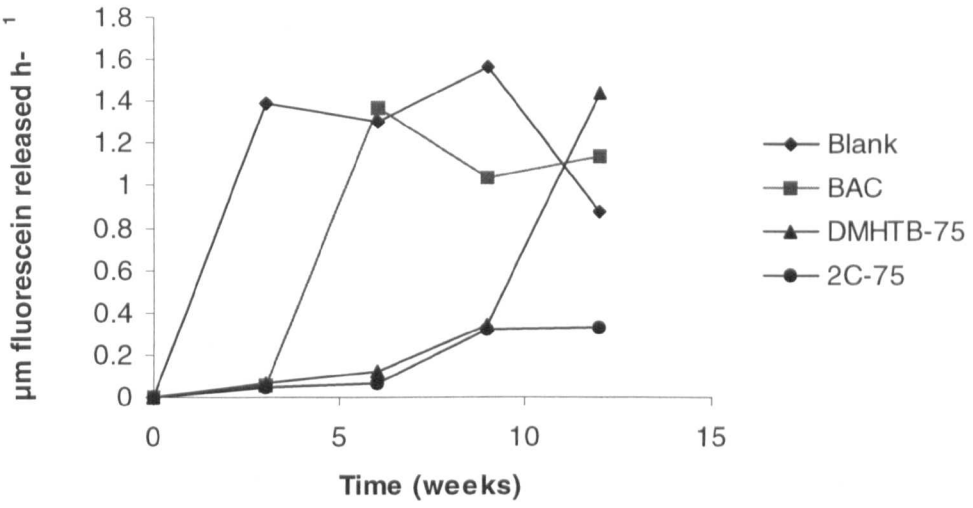



Figure 3 Estimation of biofilm formation measured by FDA hydrolysis activity over time.

***Release of Surfactants***

The hydrogels were quantitatively analysed using HPLC as described in the methods section. Table 2 shows the levels found. The amount of 2C-75™ found is greater relative to that found in the BAC and DMHTB™ although the soaking time was the same. This may be attributed to its twin structure. Figure 4 shows the release of BAC and DMHTB-75™ over the 12 week period. It can be seen that the BAC releases in a linear fashion over the period while the DMHTB-75™ is practically all retained in the hydrogel. BAC with its predominately $C_{12}$ and $C_{14}$ chains is water-soluble. It can therefore be contained mainly in the pore water of the hydrogel with a small amount becoming irreversible bound to the hydrophobic part of the hydrogel [Smith et al, 2000]. The DMHTB-75™ is mainly composed of $C_{16}$ and $C_{18}$ and is fairly insoluble at room temperature. As it is a much more hydrophobic material than BAC it is likely to attach itself to the more hydrophobic parts of the hydrogel i.e. not the pore water. Such attachment would be fairly irreversible, in addition to DMHTB-75™'s reluctance to release into the polar seawater environment, would result in DMHTB-75™ being retained in the hydrogel.

The 2C-75™ is mainly composed of $C_{12}$ and $C_{14}$ chains. It is water soluble but separates into two layers when left standing. It is a twin-chained material and has a

more hydrophobic character than the single chained BAC thus it is able to attach by hydrophobic interaction to the non-water portions of the hydrogel. From the quantitative analysis over the 12 weeks of the study it appears to be retained by the hydrogel thus suggesting despite its water solubility it is predominately held in the non-polar areas of the hydrogel.

| | % cationic surfactant (wt/wt) | | |
|---|---|---|---|
| hydrogel treatment | BAC | 2C-75™ | DMHTB-75™ |
| Sampling time (weeks) | | | |
| 0 | 11.163±0.094 | Not done | 12.622±0.273 |
| 3 | 8.898±0.5722 | 29.701±9.319 | 12.111±0.241 |
| 6 | 6.889±0.224 | 19.776±1.631 | 13.984±0.020 |
| 9 | 4.166±0.347 | 25.110±4.276 | 11.261±0.118 |
| 12 | 2.222±0.217 | 28.709±3.822 | 10.137±0.320 |

Average values ± standard error (SE), n = 3

Table 2 Average release of cationic surfactants.

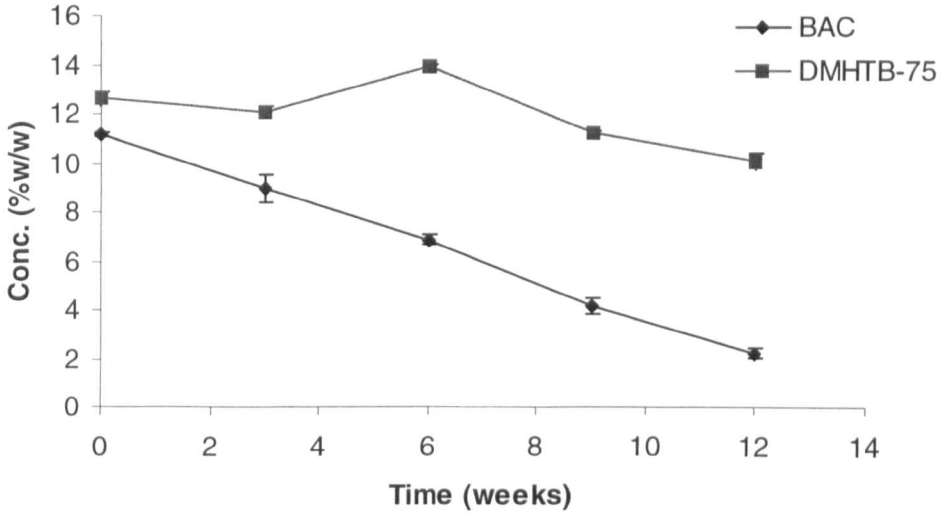



Figure 4 Release of BAC and DMHTB-75™ from the hydrogel over the 12 week period.

**Discussion**

The estimation of biofilm growth provided by the FDA hydrolysis method correlated well with release rates of the fouling resistant cationic surfactants. The FDA hydrolysis results showed that the BAC treated hydrogels had a sizeable biofilm forming by 3 weeks suggesting the active lifetime of this coating was short. This statement was verified by the release rate of BAC from the hydrogel. BAC content of the hydrogel decreased linearly over the course of the experiment from 11.16 % w/w to approximately 2 % w/w at the 12 week time point. The decrease in BAC content within the hydrogel diminished the surfactants fouling resistance, allowing bacterial attachment and biofilm growth. The DMHTB-75™ hydrogel showed little biofilm formation until after the 9 week time point where FDA hydrolytic activity rose sharply to the 12 week time point. Somewhere, shortly after the 9 week sample was taken, the release of DMHTB-75™ was sufficient as to allow biofilm growth to increase. The release rate of DMHTB-75™ from the hydrogel was extremely slow and the concentration of DMHTB-75™ was only slightly reduced over the course of

the experiment. Initially the concentration of DMHTB-75™ was 12.5 % w/w and by week 12 it had fallen to 10.2% w/w. Although the reduction in DMHTB-75™ concentration does not appear to be significant, the decrease was enough to allow the small biofilm population already present on this hydrogel to expand rapidly. The antifouling properties of the DMHTB-75™ surfactant appear to be lost at this point therefore the active lifetime of this coating is approximately 9 weeks. Finally, the FDA hydrolysis results for the 2C-75™ hydrogel indicated little biofilm activity that again corresponded well with the results obtained from the release of 2C-75™ from the hydrogel. Almost no 2C-75™ was released from the hydrogel over the 12 week exposure period suggesting the level of antifouling resistance the hydrogel held at the start of the trial was the same as the resistance imparted after 12 weeks. A slight increase in FDA activity was observed at the 9 week time point, indicating a change in the biofilm but this increase was minimal. The active lifetime of 2C-75™ was >12 weeks and was the greatest of all the cationic surfactants tested.

## Conclusions

The FDA method for the quantifying of biofilms on the hydrogel coatings is successful and rapid. The quantitative analysis of release of surfactant demonstrated that retention of the surfactant extends the lifetime of the coating. It can be postulated that the primary defence of DMHTB™ and 2C-75™ is a surface active phenomenon. BAC is releasing and can therefore thought to be acting as an antimicrobial until the levels of release become low and not effective.

## References

Adam, G., Duncan, H. J. 2001. Development of a more sensitive and rapid method for the measurement of total microbial activity using Fluorescein Diacetate (FDA) in a range of soils. *Soil Biology and Biochemistry,* 33, (7/8), 943-951.

Akzo Nobel Surface Chemistry, Code 97.02.11121, BTB Communication

Anderson, M.J. 1995 Variations in Biofilms Colonizing Artificial Surfaces. *Journal of the Marine Biological Association* **75**, 705-714.

Battin, T. J. 1997 Assessment of fluorescein diacetate hydrolysis as a measure of total esterase activity in natural stream sediment biofilms. *The Science of The Total Environment* **198**, 51 – 60.

Callow, M. E., Willingham, G. L. 1996 Degradation of antifouling biocides. *Biofouling* **10** (1-3), 239 – 249.

Costerton, J.W. and Lashen, E.S. Influence of Biofilm on Efficacy of Biocides on Corrosion-Causing Bacteria. National Association of Corrosion Engineers. February 1984.

Cowling, M.J., Hodgkiess, T., Parr, A.C.S., Smith, M.J., Kerr, A., Beveridge, C.M., Clegg, M. and Menlove, R., 'The Effects of Biofouling on Imaging Underwater, including Possible Remedies', Proceedings Underwater Optics III, Applied Optics and Optoelectronics, Institute of Physics, Brighton, March 1998.

De Rosa, S., Sconza, F., Volterra, L. 1998 Biofilm amount estimation by fluorescein diacetate. *Water Research*, **32** (9), 2621 – 2626.

EEC MAST II contract number MAS2-CT91-0009. Final Report June 1995. Glasgow Marine Technology Centre, University of Glasgow, Glasgow, G12 8QQ.

Fontvieille, D. A., Outagueroine, A., Thevenot, D. R. 1992 Fluorescein diacetate hydrolysis as a measure of microbial activity in acquatic systems: application to activated sludges. *Environmental Technology* **13**, 531-540.

Gilbert, F., Galgani, F., Cadiou, Y. 1992. Rapid assessment of metabolic activity in marine microalgae:application in ecotoxicological tests and evaluation of water quality. *Marine Biology* **112**, 199 - 205.

Guilfoyle D.E., Roos R. and Carito S.L. 1990 An evaluation of preservative adsorption onto nylon. *Journal of Parenteral Science and Technology* **44**(6), 314-319.

Gumprecht, G., Gerlach, H., Nehrkorn, A. 1995 FDA hydrolysis and resazurin reduction as a measure of microbial activity in sediments from the south-east Atlantic. *Helogoländer Meeresuntersuchungen* **49**, 189-199.

Holmström, C., James, S., Egan, S., Kjelleberg, S. 1996 Inhibition of common fouling organisms by marine bacteria isolates with special reference to the role of pigmented bacteria. *Biofouling*, **10** (1-3), 251 – 259.

Huang, C.B. 1987 Determination of Dialkyldimethylammonium Salt in Rolling Oil. *Journal of Liquid Chromatography* **10**, (6) 1103-1125.

Kerr, A., Head, R.M., Cowling, M.J., Davenport, J., Beveridge, C.M., Smith, M.J., Parr, A.C.S. and Hodgkiess, T. 1998 The Early Stages of Marine Biofouling and its Effect on Two Types of Optical Sensors. *Environment International* **24**, (3), 331-343,.

Marrs, S.J., Head, R.M., Cowling, M.J., Hodgkiess T. and Davenport, J.1999 Spectrophotometric Evaluation of Biofouling on Marine Optical Windows. *Estuarine and Coastal Shelf Science,* **48**, 137-141.

Parr, A.C.S., Smith, M.J., Beveridge, C.M., Kerr, A., Cowling, M.J. and Hodgkiess, T 1998 Optical Assessment of A Fouling-Resistant Surface (PHEMA/Benzalkonium Chloride) After Exposure to a Marine Environment. *Advanced Materials for Optics & Electronics*, **8**, 187-193

Poremba, K. 1995 Hydrolytic enzymatic activity in deep sea sediments. *FEMS Micrbial Ecology*, **16,** 213-222.

Refojo, M.F., 1966 Perparation of Water through Some Hetrogeneous Hydrophilic Membranes. *Journal of Applied Polymer Science* **10**, 185-190.

Schnürer, J., Rosswall, T. 1982. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. *Applied and Environmental Microbiology* **43**, 1256-1261.

Smith, M.J. The Use of Cationic Surfactants in Marine Anti-fouling applications. *M.Sc. Thesis*, 1997, University of Glasgow.

Smith, M.J., Flowers, T.H., Parr, A.C.S., Cowling, M.J. and Hodgkiess, T. 2000. Salinity and Temperature Effects on the Release of Benzalkonium Chloride from Hydrogel Material. *Polymers and Polymer Composites* **8**, 101-105.

Stubberfeild, L.C.S. and Shaw, P.J.A. 1990 A Comparison of Tetrazolium Reduction and FDA Hydrolysis With Other Measurements of Microbial Activity. *Journal of Microbiological Methods* **12**, 151-162.

Swisher, J. and Carroll, G. C. 1980 Fluorescein diacetate hydrolysis as an estimator of microbial biomass on coniferous needle surfaces. *Microbial Ecology* **6**, 217-226.

Wimpenny, J. 1996 Ecological Determinants of Biofilm Formation. *Biofouling* **10**(1-3), 43-63.

Wigglesworth-Cooksey, B., Cooksey, K. E. 1996. A computer based image analysis system for biocide screening. *Biofouling*, **10** (1-3), 225 – 237.

# THE EFFECT OF DIESEL FUEL ON COMMON VETCH (*VICIA SATIVA*) PLANTS

GILLIAN ADAM* AND HARRY DUNCAN

*Department of Environmental, Agricultural and Analytical Chemistry, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK.*

*corresponding author: present address Department of Chemistry, Scottish Universities Environmental Research Centre (SUERC), Scottish Enterprise Technology Park, East Kilbride, G75 0QF, Scotland, UK.*

*E-mail : gilliana@ chem.gla.ac.uk. Tel. No. 01355 270 135 Fax 01355 229 898*

**Abstract**

When petroleum hydrocarbons contaminate soil, the carbon:nitrogen (C:N) ratio of the soil is altered. The added carbon stimulates microbial numbers but causes an imbalance in the C:N ratio which may result in immobilization of soil nitrogen by the microbial biomass, leaving none available for plant growth. As members of *Leguminosae* fix atmospheric nitrogen to produce their own nitrate for growth, they may prove more successful at growing on petroleum hydrocarbon contaminated sites. During a wider study on phytoremediation of diesel fuel contaminated soil, particular attention was given to the performance of legumes versus other plant species. During harvesting of pot experiments containing leguminous plants, a recurring difference in the number and formation of root nodules present on control and contaminated Common vetch (*Vicia sativa*) plants was observed. The total number of nodules per plant was drastically reduced in contaminated plants compared to control plants but nodules on contaminated plants were more developed than corresponding nodules on control plants. Plant performance of Common vetch and Westerwold's ryegrass (*Lolium multiflorum*) was compared to illustrate any difference between the ability of legumes and grasses to grow on diesel fuel contaminated soil. Common vetch was less affected by diesel fuel and performed better in low levels of diesel fuel contaminated soil than Westerwold's ryegrass. The total amount of diesel fuel remaining after four months in Common vetch planted soil was slightly less than in Westerwold's ryegrass planted soil.

**Keywords** : diesel fuel, legumes, root nodules, phytoremediation, plant performance.

## 1. Introduction

When petroleum hydrocarbons contaminate soil, the added carbon stimulates microbial numbers but causes an imbalance in the C:N ratio which may result in immobilization of soil nitrogen by the microbial biomass, leaving none available for plant growth. Members of *Leguminosae* fix atmospheric nitrogen to produce their own nitrate for growth therefore, they may prove more successful at growing on petroleum hydrocarbon contaminated sites. To further support this statement, species of *Leguminosae* have been found to be the most abundant reinhabitors of petroleum hydrocarbon contaminated sites (Gudin and Syratt, 1975).

During a wider study on the phytoremediation of diesel fuel contaminated soil, particular attention was given to the performance of legumes versus other plant species. An important factor of the legumes success would be dependent on the development of root nodules. Decrease in nodule formation has been noted by other authors in soils contaminated with heavy metals (Porter and Sheridan, 1981, Casella *et al.*, 1988), agrochemicals (Mårtensson, 1992), acid rain (porter and Sheridan, 1981) and PAHs (Wetzel and Werner, 1995), however no work has been carried out on nodulation in diesel fuel contaminated soil. This work investigates nodule formation on Common vetch plants grown in diesel fuel contaminated soil. Plant performance of Common vetch and Westerwold's ryegrass was compared to illustrate any difference between the ability of legumes and grasses to grow on diesel fuel contaminated soil.

## 2. Materials and Methods

**Agronomic Assessment**. Germination rate, tallest shoot height and total shoot and root biomass was measured after two and four months growth. The number of root nodules per plant was assessed at the two month sampling date.

**Root Nodule Embedding and Microscopy.** Plants were shaken free from soil then washed thoroughly under running water. After the root sample was washed, the number of nodules was determined on a per plant basis. The freshly washed root nodules were then fixed in 0.2M cacodylate buffer for 6 hours then dehydrated through an ethanol series. The dehydrated nodules were then infiltrated with LR white resin for 48 hours and polymerized at 60°C overnight. Sections (2 μm) were cut using a glass knife on a LKB ultratone III and dried onto glass slides over a hotplate at 60°C. The sections were stained for 10 seconds with 1% toluidine blue in

1% sodium tetraborate. The sections were visualized at 40x and 100x magnification on a Leica ATC™ 2000 compound microscope with digital camera attachment.

**Diesel Fuel Extraction.** Diesel fuel remaining in the soil after four months was extracted using a hot solvent Soxhlet extraction. 40g of soil sample was added to a cellulose extraction thimble containing 5g anhydrous sodium sulphate then extracted for 6 hours in 100ml of acetone:dichloromethane (1:1 v/v). The extract was transferred to a 100ml volumetric flask and made to volume with acetone:dichloromethane (1:1 v/v). Extracts were analysed by GC-FID.

**GC-FID Analysis of Extracts.** Analyses were carried out on a Hewlett-Packard 5890A gas chromatograph (GC) with flame ionization detector (FID). The GC was interfaced with a Hewlett-Packard Chemstation data system. Helium carrier gas was adjusted to the recommended linear flow velocity of 20cm $sec^{-1}$ using the non-retained compound butane. Separations were performed on a SGE BPX5 polysilphenylene siloxane capillary column (25m x 0.32mm x I. D. 0.5µm). 0.5µl of sample was injected at 35°C with a temperature hold of 3 minutes. The temperature was increased 5°C $min^{-1}$ up to 250°C with a 10 minute hold at the end of the run. The injector temperature was 260°C and the detector temperature was 270°C.
% diesel fuel remaining was calculated from the total petroleum hydrocarbon (TPH) value of each sample extract subtracted from the TPH value of diesel fuel extracted from the soil at time zero. TPH values were calculated from total peak areas of triplicate injections with less than 5% difference between them.

## 3. Results and Discussion

During harvesting of pot experiments containing leguminous plants, a recurring difference in the number and formation of root nodules present on control and diesel fuel contaminated Common vetch plants was observed. The average total number of nodules per plant was drastically reduced in contaminated plants compared to control plants, with numbers falling from 8 nodules per plant in the controls, to 3, 4 and <1 in the 5g, 10g and 15g diesel $kg^{-1}$ contaminated soil respectively.
Observation of nodule sections by light microscopy illustrated clear differences between nodules of control Common vetch plants and plants grown in diesel fuel contaminated soil. The majority of nodules from control plants were spherical and

appeared to be at the initial stages of nodule differentiation. Few bacteroids were present within the central body of the nodule suggesting the nodule was immature and would therefore not be effectively fixing atmospheric $N_2$ (Figures 1A and 1B). Nodules taken from contaminated plants were elongate or club-shaped and the central mass of tissue showed successive stages of host cell invasion and differentiation by rhizobium. The bacteroids were numerous and filled most of the central nodule body as illustrated in Figure 1C and 1D. The results suggest at low levels of diesel fuel contamination (5-10g diesel $kg^{-1}$ soil), nodules formed on contaminated plants are actually more developed than the corresponding nodules on control plants.

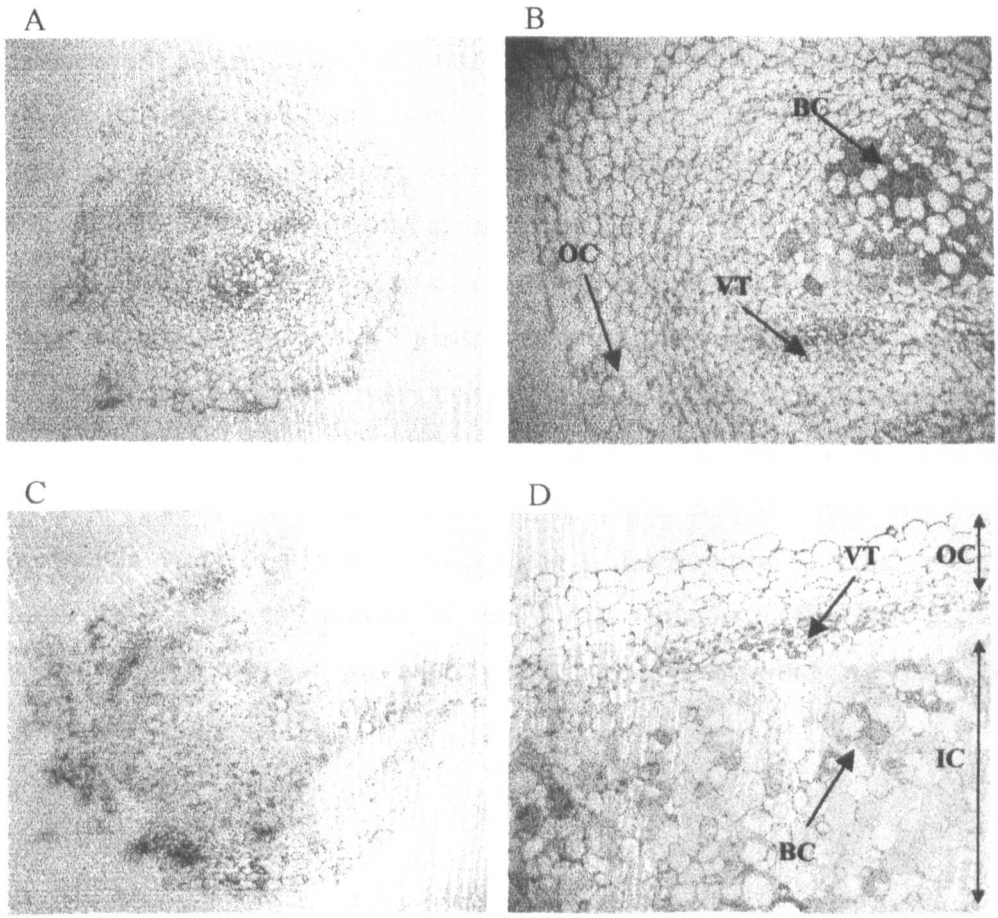



Figure 1. Light microscopy section (2μm) of a Common vetch nodule grown in (A) uncontaminated soil (magnification x40), (B) enlargement of this nodule (x100) and (C) diesel fuel contaminated soil (x40) and (D) enlargement of this nodule (x100). VT – vascular tissue, BC – bacteroid cluster, OC – outer cortex and IC – inner cortex.

This finding is in agreement with Carr who first noted the apparent stimulation of legume root nodules by low levels of hydrocarbon contamination on Soybean plants in 1919. Carr concluded that small amounts of oil may even be desirable in nodule development in Soybean plants and where the amount of oil was increased to the extent of damaging the plant, there was still some nodule development (Carr, 1919). An explanation for the apparent stimulation of nodule development in diesel fuel contaminated soil may be the additional carbon added to the soil in the form of diesel fuel, changed the soils C:N ratio. The addition of a huge carbon source, such as diesel fuel, would widen the C:N ratio which in turn, would leave less N available for plant uptake. This has been observed in soils contaminated with petroleum hydrocarbons (Xu and Johnson, 1997) with N becoming immobilised in microbial biomass leaving less N available for plant uptake. This may have caused the Common vetch plants growing in contaminated soil to nodulate quicker than plants grown in uncontaminated soil which would explain why contaminated nodules appeared more developed and at a later stage of differentiation than control nodules even though the seeds were planted at the same time.

When the agronomic performance of Common vetch plants was compared with a non-leguminous species such as Westerwold's ryegrass, a difference in shoot and root biomass was observed. Although germination rate and shoot height was not badly affected by growing in low levels (5-10g) of diesel fuel contaminated soil in either plant species, a larger decrease in shoot biomass was observed for Westerwold's ryegrass (Table 1) suggesting a deterioration in plant health and performance. The total amount of diesel fuel remaining after four months in Common vetch planted soil was slightly less than in Westerwold's ryegrass planted soil (Table 1).

## 4. Conclusion.

The results suggest Common vetch were less affected by diesel fuel and performed better in low levels of diesel fuel contaminated soil than Westerwold's ryegrass. This advantage over Westerwold's ryegrass may have been due to the presence of well developed root nodules on the contaminated plants.

| Plant species | Treatment (g diesel $kg^{-1}$ soil) | Av. Shoot biomass per plant (mg) | Av. Root biomass per plant (mg) | Germination (%) | Tallest shoot length (cm) | % diesel fuel remaining |
|---|---|---|---|---|---|---|
| Common vetch | 0 | 1230 ± 84 | 55 ± 15 | 55 ± 6 | 64 ± 3 | |
| | 5 | 889 ± 52 | 58 ± 8 | 66 ± 7 | 68 ± 4 | nd |
| | 10 | 552 ± 31 | 49 ± 8 | 60 ± 8 | 56 ± 5 | 1.58 |
| | 15 | 123 ± 50 | 24 ± 18 | 48 ± 4 | 39 ± 7 | 1.90 |
| Westerwold's | 0 | 1388 ± 49 | 55 ± 2 | 55 ± 2 | 79 ± 4 | |
| ryegrass | 5 | 667 ± 2 | 62 ± 6 | 62 ± 6 | 75 ± 8 | nd |
| | 10 | 422 ± 3 | 63 ± 7 | 63 ± 7 | 79 ± 5 | 2.12 |
| | 15 | 318 ± 8 | 38 ± 2 | 38 ± 2 | 76 ± 9 | 3.45 |

Table 1. Summary of agronomic assessment and diesel fuel breakdown in Common vetch and Westerwold's ryegrass planted soils after 4 months.
Average values are given ± standard error, n = 3. Values indicating % diesel fuel remaining had <5% difference between replicate areas, n = 3.

**Acknowledgements**

We would like to thank Mr Eoin Robertson of the IBLS Electron Microscopy Unit, University of Glasgow for preparation of the root nodule sections for microscopic examination and Mr Michael Beglan, Department of Environmental, Agricultural and Analytical Chemistry, University of Glasgow for his help in photographing the sections.

**References**

Carr, R. H.: 1919, Vegetative growth in soils containing crude petroleum, *Soil Science* 66-69.

Casella, S., Frassinetti, S., Lupi, F., Squartini, A.: 1988, Effect of cadmium, chromium and copper on symbiotic and free living *Rhizobium leguminosarum* biovar *trifolii*, *FEMS Microbiology Letters* **49**, 343-347.

Gudin,C., Syratt, W. J.: 1975, Biological aspects of land rehabilitation following hydrocarbon contamination, *Environmental Pollution* **8**, 107-112.

Mårtensson, A. M.: 1992, Effects of agrochemicals and heavy metals on fast growing rhizobia and their symbiosis with small-seeded legumes, *Soil Biology and Biochemistry* **24**, 435-445.

Porter, J. R., Sheridan, R. P.: 1981, Inhibition of nitrogen fixation in alfalfa by arsenate, heavy metals, fluoride and simulated acid rain, *Plant Physiology* **68**, 143-148.

Wetzel, A., Werner, D.: 1995, Ecotoxicological evaluation of contaminated soil using legume root nodule symbiosis as effect parameter, *Environmental Toxicology and Water Quality* **10**, 127-133.

Xu, J. G., Johnson, R. L.: 1997, Nitrogen dynamics in soils with different hydrocarbon contents planted to barley and field pea, *Canadian Journal of Soil Science* **77**, 453-458.