AN INVESTIGATION INTO THE USE OF CHICKEN MANURE TO ENHANCE THE BIODEGRADATION OF TOTAL PETROLEUM HYDROCARBONS

Submitted in candidature for the degree of

Doctor of Philosophy

In the University of Wales

By

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Abstract

The use of chicken manure to enhance the biodegradation of total petroleum hydrocarbons (TPH) in composting bioremediation was investigated to help develop an improved understanding of the chemical, biological and toxicological processes involved. Treatability studies combined with an extensive suite of laboratory analyses were designed and undertaken whereby naturally contaminated oil refinery sludge was either amended with chicken manure or left unamended for a total duration of 90 days. The effects of chicken manure on the biodegradation of fractionated aliphatic and aromatic hydrocarbons, differentiation between biostimulation and bioaugmentation effects of chicken manure, and the potentially detrimental effects of chicken manure on the bioremediation process through the introduction and adverse proliferation of non-hydrocarbon degrading microorganisms and the potential introduction of compounds that may elicit toxic effects on hydrocarbon degrading microorganisms were monitored over the duration of the treatability studies using a combination of chemical, toxicity and microbial laboratory analyses. This study found that the addition of chicken manure enhanced the degradation of C_9-C_{12} aliphatic hydrocarbons. It was found that this reflects a combination of biostimulation and bioaugmentation effects and that volatilisation was minimal. This investigation also found that the addition of chicken manure can have positive effects on bioremediation as evident by the enhancement of conditions for microbial growth and/or activity, introduction and enhanced growth of potential hydrocarbon degrading bacterial populations, and the enhanced reduction in toxicity of methanol extractable hydrocarbons. However, it was found that the addition of chicken manure was seen to cause an increase in toxicity of total leachable compounds, which may present a risk to TPH biodegradation through potential toxic effects on hydrocarbon degrading microorganisms. It is concluded from this study that there is a potential for the use of chicken manure to enhance TPH biodegradation, but that this is likely restricted to low molecular weight hydrocarbons.

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"It is probably not unscientific to suggest that somewhere or other some organism exists which can, under suitable conditions, oxidise any substance which is theoretically capable of being oxidised"

E.F. Gale (1952)

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Chapter 1

Introduction

1.1 Introduction

In its recent policy document on sustainable development, 'Securing the Future', released in 2005, the UK Government states that 'the past 20 years have seen a growing realisation that the current model of development in the UK is unsustainable' (HM Government, 2005). Sustainable development is defined in this policy document as *"development which meets the needs of the present without compromising the ability of future generations to meet their own needs"*. In response to the Johannesburg World Summit on Sustainable Development held in 2002, the UK strategy for sustainable development has the key priorities of 1) sustainable consumption and production, 2) climate change and energy, 3) natural resource protection and environmental enhancement, and 4) sustainable communities (HM Government, 2005).

It is proposed by Yong (2003) that 'unless a sustainable geoenvironment is obtained, sustainable development will not be realised'. The geoenvironment includes air, water and soil and these resources sustain human life and provide the habitat for various land and aquatic biota (Yong, 2003). The UK Government has recognised that as well as tackling the pressures on the environment today, the legacy of degraded environment resulting from previous industrial and agricultural activities also needs to be addressed (HM Government, 2005; Environment Agency, 2006).

It is estimated by the UK Environment Agency that there are thousands of sites in the UK which could potentially be identified as contaminated land under Part IIA of the Environmental Protection Act (1990) (Environment Agency, 2006; Bardos et al,

2000a; BIOWISE, 2000), many of which are contaminated with petroleum hydrocarbons (Nathanail et al, 2001). Contaminated land can present a risk to human health, the immediate ecosystem, and the environment and it is recognised that these sites should be remediated or managed to prevent the occurrence of risk (Brar et al, 2006; Yong and Mulligan, 2004; Ladislao et al, 2004; SEPA, 2001). The management of contaminated land and the need for land remediation in the UK is based on risk assessment, and aims to reduce levels of risk to human health and the environment posed to levels deemed to be satisfactory based on the current land use of a site, and/or proposed future land use (Pollard et al, 2004a; Nathanail and Bardos, 2005).

Land remediation in the UK has been dominated by the disposal of contaminated land to landfill (Nathanail et al, 2001; Reisinger, 1995; BIOWISE, 2000; Vik and Bardos, 2002). Due to the recent implementation of the Landfill (England and Wales) Regulations in 2002, this 'dig and dump' strategy is now seen as unsustainable (Semple et al, 2001; Bardos et al, 2000b), and emphasis is now on the application of sustainable remediation technologies that conserve land and resources (Pollard et al, 2004a; BIOWISE, 2000).

Bioremediation is a biological treatment strategy that is receiving increasing attention as a cost effective and sustainable remediation technique, as not only does it have the potential to reduce contaminant concentrations, but also to reduce contaminant mobility and toxicity, and therefore risk (Loehr and Webster, 2000; Dobson et al, 2004; Semple et al, 2001; Boopathy, 2000; Bento, 2003; Sabate et al, 2004). Bioremediation relies on microorganisms to degrade organic contaminants such as petroleum hydrocarbons (Alexander, 1999), and involves optimisation of conditions for biodegradation through the addition of nutrients, oxygen and water (Atlas, 1995; Alexander, 1999; Brar et al, 2006; Loehr et al, 2001b).

The contribution of agriculture to the degradation of the environment is of increasing concern in the UK (Burton and Turner, 2003; Hooda et al, 2000). In particular, a number of environmental risks associated with the storage and handling of livestock manures (such as chicken manure) have been identified in the literature, and include greenhouse gas emissions, eutrophication and spreading of disease (SEPA, 2001; DEFRA, 2005; BiffaWard, 2002). The perception of livestock manures has changed

from being a valuable resource to being a waste (Burton and Turner, 2003; Merrington et al, 2002). A questionnaire survey carried out at the beginning of this study identified that there is a surplus of chicken manure in Wales, and the author proposes that finding sustainable uses for this organic product will help reduce the likelihood of environmental and human health risk occurring.

Owing to the high nutrient content of chicken manure (as reported by Nicholson et al, 1996) and diverse microbial community (as reported by Ijah and Antai, 2003; Atagana, 2004b; Ibekwe et al, 2006), there is a potential for the use of this organic resource to enhance the bioremediation of soils contaminated with organic compounds such as petroleum hydrocarbons, in a technique called '*composting bioremediation*'. Composting bioremediation is under increasing investigation and involves mixing contaminated soils with organic wastes and enhancing the simultaneous decomposition of the two materials through composting processes (Semple et al, 2001). This potential has been investigated by Ijah and Antai (2003), Atagana (2004b), Ibekwe et al (2006), and was found to be successful.

However, the author has identified a need to further the understanding of the chemical, biological and toxicological processes that take place during the composting bioremediation process. Owing to the nature of composting bioremediation in that the volume of contaminated soil is ultimately increased, failure of this bioremediation strategy could result in increased costs in the long run. The author therefore deems the understanding of these processes important.

This chapter introduces chicken manure and soil contaminated with petroleum hydrocarbons as two sources of environmental concern in the UK, and introduces the focus of this thesis which is to investigate the potential sustainable use of chicken manure as a co-composting organic amendment to enhance the biodegradation of petroleum hydrocarbons, and to help develop an improved understanding of the processes involved with this bioremediation strategy.

1.2 Chicken Manure

1.2.1 Introduction

The poultry industry in the UK comprises approximately 28 million birds (Pratt et al, 2002; DEFRA, 2002) producing approximately 3.5 to 4 million tonnes of poultry manure each year (MAFF, 1999; Nicholson et al, 1996). The flock size of the poultry industry had increased by 15% in the UK between 1990 and 1997, with an increase in bird density (measure of intensification of the poultry industry) of 70% over the same time period (Burton and Turner, 2003).

The most common use of livestock manure such as poultry manure is to apply it to agricultural land (landspreading) (Environment Agency, 2001; Burton and Turner, 2003; Merrington et al, 2002). According to Burton and Turner (2003), approximately 50% of poultry manure produced in the UK each year is applied to arable land, and a further 40% is applied to grassland.

Applications of livestock manure to agricultural land is regarded as beneficial to soil due to its high nutrient content, and therefore value as an organic fertiliser (Environment Agency, 2001; Burton and Turner, 2003; Merrington et al, 2002). Prior to the 20th century, livestock manure offered the only method of enriching the soil (Burton and Turner, 2003). Table 1.1 summarises the average nutrient content of poultry manure. These data are based on a study on 121 poultry manure samples collected from commercial holding in England and Wales undertaken by Nicholson et al (1996).

Table 1.1: Average nutrient content of poultry manure (Nicholson et al, 1996).

Nutrient	Content (% - dry matter basis)
Total Nitrogen	5.2 – 6.0
Ammonium Nitrogen	0.6 – 2.3
Uric-acid Nitrogen	0.3 – 2.1
Total Phosphorus	1.3 – 2.1
Total Potassium	1.7 – 2.8

The nutrient content of poultry manure is variable, as seen from Table 1.1. Such variation is reported in the literature to reflect variations in feed composition, bird characteristics (breed, sex, age and efficiency of feed conversion), and storage/handling practices adopted (Merrington et al, 2002; Nicholson et al, 1999; Shepherd and Gibbs, 2002). The storage and handling practices used in the management of poultry manure can have a significant impact on the nutrient content, due to losses of nitrogen through ammonia volatilisation or nitrate leaching during storage in heaps or during/after spreading on land (Shepherd and Gibbs, 2002; DEFRA, 2002; Tiquia and Tam, 2000; Pratt et al, 2002).

1.2.2 Waste or Resource?

The specialisation and intensification of agriculture during the latter half of the 20th century (in response to a doubling of the world population) has resulted in a land division between arable and livestock farming in the UK (Merrington et al, 2002; Burton and Turner, 2003). The predominance of livestock farming in the west of the UK and arable farming in the east of the UK has meant that the use of livestock manure as a fertiliser of agricultural land is increasingly restricted (Merrington et al, 2002). With subsequent limited local application, the perception of livestock manures has changed from being a valuable resource to being a waste (Burton and Turner, 2003; Hooda et al, 2000). Landspreading remains the most common use of livestock manure (Burton and Turner, 2003), however it is more frequently seen by farmers as a means of disposing of excess manure (Merrington et al, 2002).

This concept is supported by the results of a questionnaire survey of poultry farming in Wales (UK), which was carried out by the author at the beginning of this study, in 2003. The original aim of the questionnaire survey was to attain general data regarding the use of poultry manure in Wales and to identify any problems experienced by poultry farmers regarding the management of poultry manure. A total of 149 questionnaires were sent to poultry farmers in Wales, of which 28 responses were received. The results of this questionnaire are provided in Appendix 1; for confidentiality reasons the names and locations of the poultry farms are not included. The results of this questionnaire are summarised below. Figure 1.1 shows the uses of poultry manure adopted by the farmers contacted during this questionnaire survey.

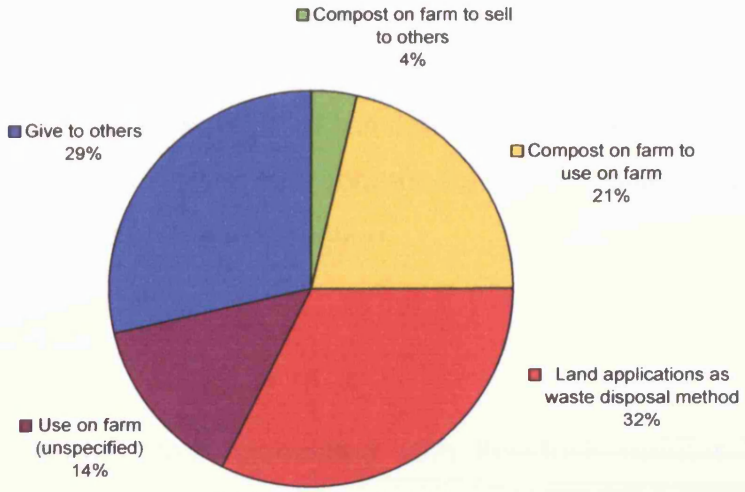


Figure 1.1: Uses of poultry manure in Wales, as recorded in 2003.

It can be seen from Figure 1.1 that of the 28 poultry farmers contacted during the questionnaire survey, 32% admitted to disposal of poultry manure through land applications. This value is higher than that recorded by Burton and Turner (2003) who suggested that 10% of manure is disposed of. In addition to uses of poultry manure, a total of 5 farmers took the opportunity to express identification of manure management problems. All 5 such responses stated that they had too much manure to store and too little land to beneficially apply it to.

The data recorded above, together with observations recorded in the literature, suggests that there is a surplus of poultry manure, which is not being beneficially applied to agricultural land. A large proportion of such manure is, however, being applied to agricultural land by means of waste disposal. Whereas such applications of livestock manures are seen as being beneficial to the agricultural industry (for example in reducing the quantity of inorganic fertilisers used, and improving soil quality), if undertaken inappropriately, land applications can lead to water, air, and soil pollution (BiffaWard, 2002; Sistani et al, 2003; Nicholson et al, 1996). Concerns regarding environmental pollution resulting from the storage and handling of livestock manure have increased over recent years (Burton and Turner, 2003; Hooda et al, 2000).

It is beyond the scope of this thesis to provide an extensive overview of the environmental risks posed by the storage and handling of livestock manures such as chicken manure, however a brief summary is provided in the following section. The recognition of agriculture as a source of environmental pollution has led to the development of legislation and guidance documents to minimise such risk. These documents are discussed later on in this section.

1.2.3 Environmental Risk

A number of environmental risks associated with livestock manures have been identified in the literature and result from the movement of nutrients, heavy metals, pathogens, and gases from the manure (both during storage or during/after land spreading) into the environment. The main transport pathways of such manure constituents are illustrated in Figure 1.2. The environmental risks are outlined below, and have been categorised into human health risks and ecological risks.

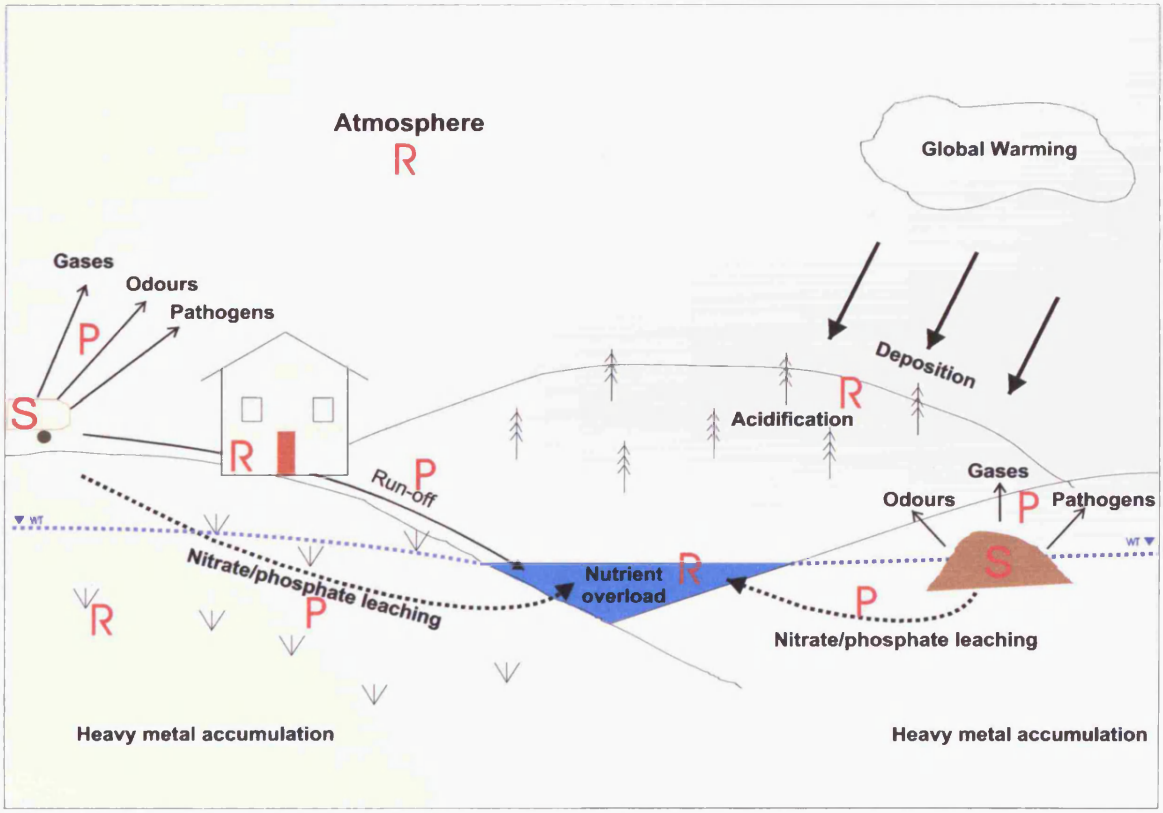


Figure 1.2: Potential pollution pathways from storage and landspreading of manure (adapted from Burton and Turner, 2003). *S = source; P = pathway; R = receptor*

1.2.3.1 Human Health Risks

Nitrates:-

Nitrates (NO_3^-) can be leached from livestock manure into surface and ground water bodies (Hooda et al, 2000; Merrington et al, 2002). Much of the UK's drinking water is sourced from rivers and ground waters (Merrington et al, 2002). In many areas of Europe the nitrate concentration of surface and ground waters has increased in recent decades (MAFF, 1999; DEFRA, 2005) and this is thought to reflect the intensification of agriculture (Merrington et al, 2002). Agriculture is the main source of nitrate in most UK rivers and ground waters (MAFF, 1999), supplying 60% nitrates and 43% phosphates (DEFRA, 2005). Extensive leaching of nitrates into these sources has resulted in many areas that approach or exceed the European maximum concentration limit of 50 mg l^{-1} (Merrington et al, 2002; Chambers et al, 2001). Such areas have been designated as Nitrate Vulnerable Zones (NVZ's), which are discussed further in Section 1.2.4.

Ingestion of large quantities of nitrate in drinking water may be harmful to humans due to the reduction of nitrates to nitrites in the mouth and gut (Merrington et al, 2002; Burton and Turner, 2003). The accumulation of nitrites in young babies (less than 6 months old) can lead to methaemoglobinaemia ('Blue Baby Syndrome') whereby the oxygen transporting capacity of blood is reduced (Hodgson and Levi, 1997; Shortle and Abler, 2001). The occurrence of this is considered to be rare in Western Europe (Shortle and Abler, 2001). A total of 14 cases were reported in the UK since 1945, with the last known case reported in 1972 (Skinner et al, 1997). There is also a suspected link between nitrates and risk of gastric cancer in adults owing to the formation of potentially carcinogenic nitrosamines, (Merrington et al, 2002; Hodgson and Levi, 1997; Shortle and Abler).

Pathogens:-

Livestock manures are potential carriers of pathogenic microorganisms such as *Escherichia coli* (*E-coli* O157), *Salmonellae*, *Camphylobacters* and *Cryptosporidium* (SEPA, 2001; Hooda et al, 2000; Merrington et al, 2002; Shepherd and Gibbs et al, 2002; Burton and Turner, 2003). Contamination of water and soil with such manures

therefore represents a potential health risk to humans (Hooda et al, 2000; Merrington et al, 2002; Burton and Turner, 2003). The pathways by which pathogens from livestock manure can potentially reach humans are illustrated in Figure 1.3. The prevalence of selected pathogens in fresh poultry manure from the UK is summarised in Table 1.2.

Table 1.2: The occurrence of selected pathogens in fresh poultry manure (Nicholson et al, 2000).

Pathogen	Occurrence (%)
Salmonella	<0.1
E.coli O157	0
Camphylobacter	75
Cryptosporidium	<0.1

The survival of these pathogens in soil after landspreading is uncertain (Jiang et al, 2002), however Hooda et al (2000) report that *Salmonellae* can survive in soil for between 5 and 968 days, with a survival in poultry manure of 120 days (Burton and Turner, 2003). It is reported in the literature that the survival of pathogens in manure and/or soil depends on meteorological conditions (temperature and moisture content), pH, and the presence of other microbial populations (i.e. competition for growth factors) (Burton and Turner, 2003).

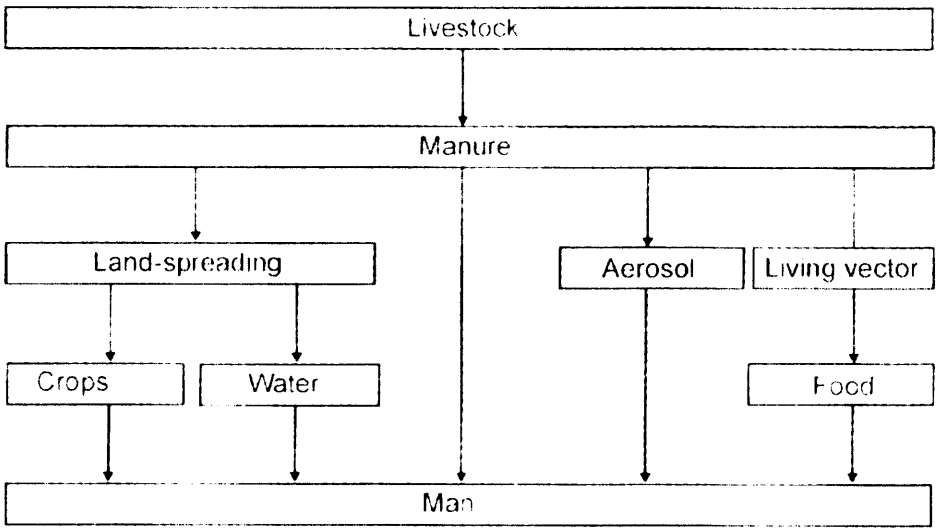


Figure 1.3: Pathways by which pathogens from livestock manure can reach humans (Burton and Turner, 2003).

1.2.3.2 Ecological Risks

Eutrophication:-

The enrichment of surface waters (rivers and lakes) with excessive quantities of nitrates and phosphates can lead to eutrophication of these waters (Shepherd and Gibbs, 2002; DEFRA, 2005; Shortle and Abler, 2001). Nitrates and phosphates can reach and accumulate in surface waters through leaching or surface run-off from livestock manures (Merrington et al, 2002; Abel, 1996). The risk of this occurring increases when:-

a) Livestock manures are spread on agricultural land during the autumn and winter months when rainfall is highest and crop requirements for nutrients are low (MAFF, 1995; Skinner et al, 1997).
b) Repeated spreading of manures increases the quantity of nitrates and phosphates in the soil, which increases their susceptibility to removal by surface run-off or leaching (MAFF, 1999; Shepherd and Gibbs, 2002).
c) Where manures are inappropriately stored, for example in heaps with no cover or leachate collection system, nitrates and phosphates are susceptible to removal from the manure through surface run-off and/or leaching (Hooda et al, 2000).

Changes in water nutrient levels may have a number of adverse effects, for example adverse changes in the species composition and diversity with a proliferation of algae; decomposing algae and weeds can deoxygenate water causing fish mortality; some algal species produce ecologically disruptive toxins which can also be hazardous to humans (Merrington et al, 2002; Burton and Turner, 2003; Abel, 1996).

Crop Yields:-

The excessive accumulation of nitrogen in soils can have detrimental effects on crop quality (Burton and Turner, 2003; Ihnat and Fernandes, 1996). For example, too much nitrogen may reduce the sugar content of sugar beet and produce crops with large thin-walled cells resulting in weaker stems and increased proneness to insect attack (Merrington et al, 2002).

Poultry manure is also reported to contain trace elements, as found by Nicholson et al (1999), and Ihnat and Fernandes (1996). The average content of trace elements is summarised in Table 1.3.

Table 1.3: Trace elemental composition of poultry manure.

	Concentration (mg kg^{-1} dry matter basis)	
Trace Element	**Nicholson et al, 1999**	**Ihnat and Fernandes, 1996**
Zinc	400.0	534.0 ± 18.0
Copper	80.0	53.0 ± 3.0
Nickel, Lead, Cadmium	<10.0	
Manganese		461.0 ± 23.0
Arsenic	<10.0; but 1 sample at 40.0	
Chromium	<10.0; but 1 sample at 70.0	

The trace elements present in livestock manure are derived from feeds and reflect the efficiency of feed conversion by the livestock (Nicholson et al, 1999). The soil acts as a long term sink for heavy metals, which can have residence times ranging from hundreds to thousands of years, depending on the retention characteristics of soils (Nicholson et al, 1999). Losses occur through uptake by crops or livestock, leaching, and soil erosion. Zinc and copper are of particular concern (SEPA, 2001). Copper can cause poor root development of crops, with subsequent difficulties in the uptake of nutrients (Merrington et al, 2002).

Air Pollution:-

Livestock production has been identified as a significant contributor to atmospheric pollution (Burton and Turner, 2003; Merrington et al, 2002; DEFRA, 2002). Air pollution from chicken manure includes odours and greenhouse gases.

Odours are a major cause of public complaint in the UK regarding poultry farming practices, and such complaints have increased with the intensification of livestock (Burton and Turner, 2003). A total of four poultry farmers contacted through the questionnaire survey carried out for this study during 2003 (Section 1.2.2) admitted to receiving odour complaints from neighbouring land users. DEFRA (2001a) also record that in 1995/1996, poultry installations were responsible for 30% of complaints

received from the public regarding odours in England and Wales, equating to 2,700 complaints.

The contribution of agriculture to gaseous emissions (ammonia, nitrous oxide, methane and carbon dioxide) in the UK has been of increasing concern (Burton and Turner, 2003).

Agriculture is the largest source of ammonia in the UK (Merrington et al, 2002; SEPA, 2001) and accounts for 90% of the total ammonia emissions in the UK (SEPA, 2001). Approximately 40% of this originates from livestock housing, and 30% from the spreading of livestock manures (Merrington et al, 2002). Poultry production itself is reported to contribute 10-14% of the total ammonia emissions in the UK (Pratt et al, 2002; DEFRA, 2002). Ammonia has a short residence time as a gas in the atmosphere, therefore is not considered to be a significant greenhouse gas (Merrington et al, 2002). Ammonia is, however, considered to present an environmental risk, as the deposition of ammonia and ammonium is a source of nitrogen which can enrich soils and waters (DEFRA, 2005) and contribute to soil acidification and eutrophication (DEFRA, 2002, SEPA, 2001; Merrington et al, 2002; Chambers et al, 2001).

Agriculture accounts for 7-8% of total greenhouse gas emissions in the UK (Merrington et al, 2002; DEFRA, 2005). The greenhouse gases released from livestock farming are methane, nitrous oxide and carbon dioxide. Agriculture accounts for approximately 1% of the UK carbon dioxide emissions (DEFRA, 2005). Carbon dioxide is the most important of greenhouse gases but has a lifespan of less than 2 years in the atmosphere (Merrington et al, 2002). Methane, however, has a longer residence time and may absorb 21 times as much infrared radiation that carbon dioxide (Merrington et al, 2002; DEFRA, 2005). Agriculture accounts for 47% of total methane emissions in the UK (DEFRA, 2005). Nitrous oxide has a global warming potential 200-300 times that of carbon dioxide (DEFRA, 2005), and agriculture accounts for 67% of total nitrous oxide emissions from the UK (DEFRA, 2005), with 17% from waste storage alone (Merrington et al, 2002).

1.2.4 Management of Poultry Manure in the UK

1.2.4.1 Handling and Storage – Legislation and Guidance

Animal manures such as chicken manure are not a controlled waste under the new Agricultural Waste Management (England and Wales) Regulations (2006) as long as they are used for agricultural benefit (Environment Agency, 2001). However, a number of regulations do apply to the handling and storage of livestock manures, as follows:

- Water Resources Act (1991) – regulates discharges to controlled waters; namely estuaries, coastal waters, lakes and ground waters.
- Groundwater Regulations (1998) – protects groundwater by controlling the discharge or disposal of potentially harmful and polluting materials.
- Nitrates Directive (1991) – implemented in the UK through the designation of Nitrate Vulnerable Zones (NVZ's) in 1996 to protect drinking waters from nitrate contamination. See below for further details.
- Pollution Prevention and Control (England and Wales) Regulations (2000) – aim for sustainable management of wastes to prevent and/or minimise environmental risk. For poultry farms with over 40,000 birds, they must apply for a permit to operate from 1st November 2006. Such poultry installations must also have a manure management plan to minimise pollution risks from the landspreading of manures (Environment Agency, 2004b).

The European Nitrates Directive (1991) was adopted as a result of the human health and ecological risks associated with excessive nitrate concentrations in soils and water (as was discussed in Section 1.2.3). This directive required that member states reduce nitrate pollution by introducing controls on agriculture in water catchments where the nitrate concentration in the water either exceeds the 50 mg l^{-1} limit or is at risk of doing so (MAFF, 1999).

In order to comply with the Nitrate Directive, 68 Nitrate Vulnerable Zones (NVZ's) were designated in England and Wales in 1996 (Figure 1.4), covering 600,000

hectares in total (MAFF, 1999; Merrington et al, 2002). An action programme of control measures for the 68 NVZ's came into effect from 19 December 1998 (MAFF, 1999). The rules for NVZ's set a limit for annual total nitrogen loadings of 250kg ha^{-1} on grassland, and 210 kg ha^{-1} on land for non-grass crops; after 4 years, the limit for land for non-grass crops reduces to 170 kg ha^{-1}.

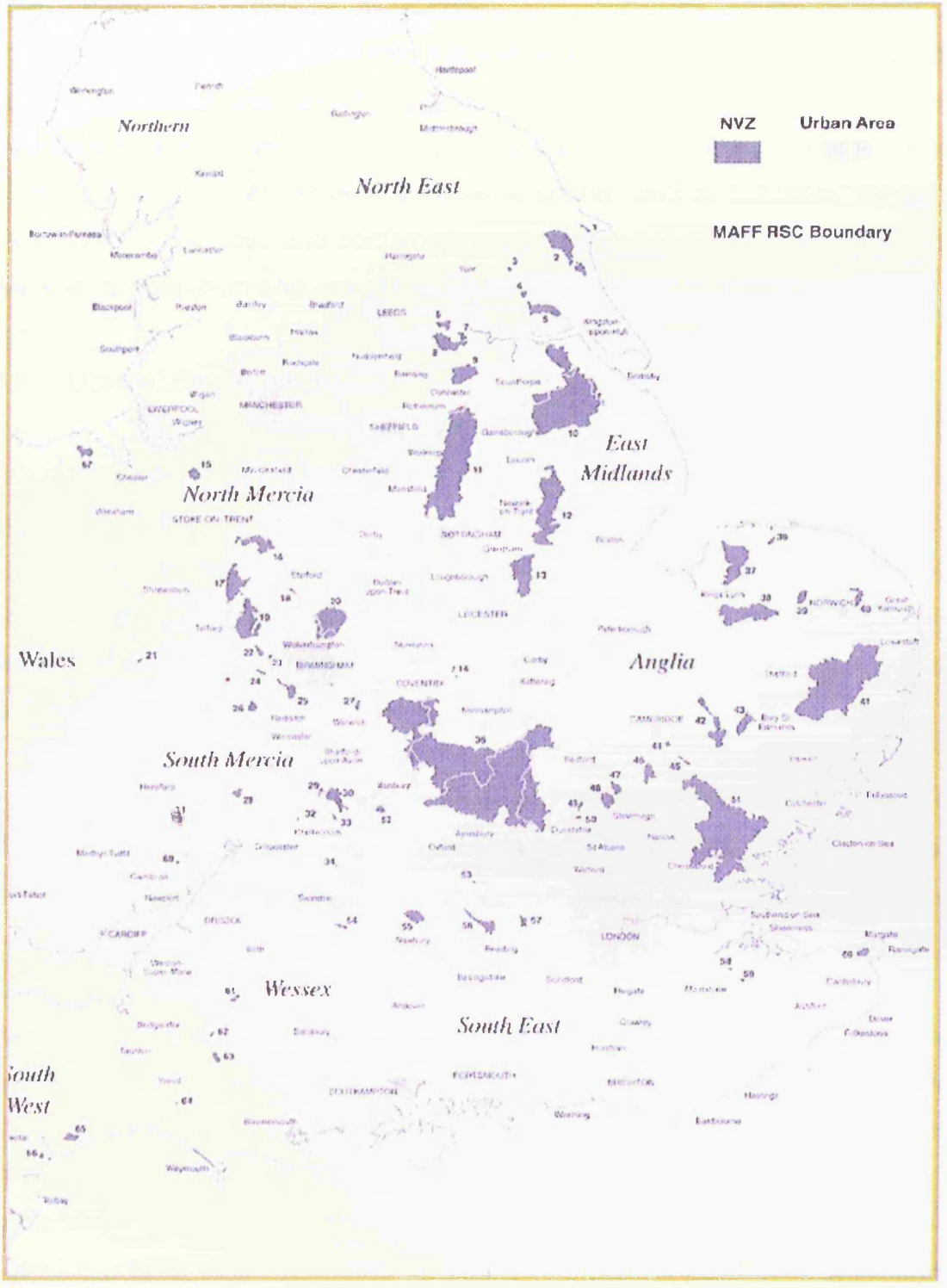


Figure 1.4: Location of Nitrate Vulnerable Zones in England and Wales (MAFF, 1999)

There is no statutory requirement for manure management plans other than in NVZ's or under PPC regulations. However, the UK Government has published various Codes of Good Agricultural Practice for the protection of Air, Water and Soil (DEFRA, 2001 a, b, c). These codes are not statutory codes but rather provide practical advice to help farmers avoid causing environmental pollution. It is beyond the scope of this thesis to detail the contents of these codes. Some practical advice in these codes includes: incorporation of manure into the soil as soon as possible to reduce odours, ammonia volatilisation and run-off; apply less than 50 tonnes of manure per hectare at any one time to reduce odours; avoid spreading within 10m of a ditch or watercourse and within 50m of a water source such as a spring, well or borehole; run-off from stores should be collected and contained; apply manure to fields when potential crop uptake is at its maximum and risk of losses at their lowest (i.e. during spring).

1.2.4.2 Uses of Poultry Manure

As indicated by the 2003 questionnaire (Section 1.2.2), even with effective nutrient management of manures, there remains a problem of too much poultry manure to store, and too little land to apply it to. This indicates that there is a surplus of poultry manure, at least in Wales. Therefore, in the author's opinion, and as expressed by Burton and Turner (2003), one solution to the environmental risks posed by poultry manure might be to remove the surplus.

The estimated fertiliser value of poultry manure in the UK is £50 million (Merrington et al, 2002). Awareness of the resource value of organic materials in the UK is growing, and the drivers for better utilisation of these materials are increasing (Environment Agency, 2001).

A potential method of dealing with the surplus of poultry manure might be to turn it into value added products and reduce the quantity of manure through composting. Composting is a natural treatment process that reduces the quantity of and stabilises organic materials such as livestock manure (Alberta, 1996-2006), and thus is recommended to farmers (Tiquia and Tam, 2000). The composting process is discussed in greater detail in Chapter 2, and is summarised here. During composting, organic materials are decomposed by microorganisms, therefore good composting

requires optimisation of conditions for microbial growth and activity; for example through nutrient balance, moisture content control, aeration, temperature control and improving substrate availability through turning (Burton and Turner, 2003). The lowest cost composting techniques available are static pile (whereby manures are formed into long narrow piles and left undisturbed) and windrows (same as static pile but manure is regularly turned to disperse oxygen, moisture, nutrients and microorganisms) (Burton and Turner, 2003; The Composting Association, 2006b). For poultry manure, nitrogen content is high but carbon is lacking, therefore it is usual practice to blend the manure with sawdust or straw to attain an optimum carbon to nitrogen ratio (Burton and Turner, 2003; Atkinson et al, 1996).

Although composting is seen to be a relatively simple waste management technique, it does require management, especially to optimise the composting process and to minimise risks to the environment posed by gaseous emissions and leachate (Peigne and Girardin, 2003). Bioaerosols (microorganisms suspended in air) may be released during the composting process, and there is increasing concern that they may pollute the environment (The Composting Association, 2006a). Therefore, even simple composting can be time consuming, costly, and it requires land space, which may not be practical for smaller farms. Also the issue of utilising the end product may still be a problem in areas lacking agricultural land to beneficially apply it to, unless the product meets composting standards for being sold as a value added product. Markets for such products are competitive and profit margins may be narrow (Burton and Turner, 2003).

For farms where controlled composting is impractical, the need to remove surplus manure still exists. One simple solution may be to transport the manure to areas of nutrient demand, however there are concerns regarding the potential for spreading disease (Burton and Turner, 2003; Merrington et al, 2002). An alternative use to landspreading is the generation of electricity from manure. Currently there is a power station in East Anglia (UK) that generates electricity from poultry manure (BiffaWard, 2002). The solid by-product of this process is an ash rich in potash and phosphate, which can be used as a fertiliser (BiffaWard, 2002).

An emerging use of livestock manure is as an organic amendment to enhance the bioremediation of land contaminated with organic compounds such as petroleum hydrocarbons. This land remediation technique is known as composting bioremediation, whereby the two materials are composted together resulting in the microbial decomposition of both the organic waste (manure) and the contaminants (Semple et al, 2001). The rationale behind this technique is that the nutrients and microbial community of the manure stimulate the biodegradation of the contaminants. Contaminated land and its remediation are outlined in the following section. The potential use of chicken manure in composting bioremediation is discussed later on in this chapter.

1.3 Contaminated Land

1.3.1 Introduction

There are thousands of sites which have been identified as being potentially contaminated in the UK; the Environment Agency (UK) recently estimated that there may be 300,000 hectares of contaminated land in the UK (Environment Agency, 2006; Potter, 2005; Environment Agency 2005a; BIOWISE, 2000). It is recognised in the literature that these contaminated sites are a legacy of previous industrial and waste disposal practices in the UK (Nathanail and Bardos, 2005; CL:AIRE, 2004). Contaminated land in the UK is defined under Part IIA of the Environmental Protection Act (1990) as "any land which appears to the Local Authority in whose area it is situated to be in such a condition, by reason of substances in, on or under the land that a) significant harm is being caused or there is a significant possibility of such harm being caused; or b) pollution of controlled waters is being, or is likely to be caused" (SEPA, 2001).

Under the UK strategy for sustainable development, the UK Government has set key goals for working towards a sustainable geo-environment; including the protection of natural resources and the environment (HM Government, 2005). Contaminated land presents a risk to human health, the immediate ecosystem, and the environment as a result of the movement and uptake of contaminants by receptors (Alloway and Ayres, 1993; SEPA, 2001; Tugan et al, 2003; Brar et al, 2006; Yong and Mulligan, 2004). It is

therefore recognised that such contaminated sites should be remediated or managed to prevent the occurrence of risk (Brar et al, 2006; Yong and Mulligan, 2004; Ladislao et al, 2004; SEPA, 2001).

The main driver for land remediation in the UK is the redevelopment of land for either residential or commercial use (Nathanail et al, 2001; NICOLE, 2002; Bardos et al, 2000; Pollard et al, 2004a). With greater geo-environmental awareness by the Government, there is an emphasis on the protection of greenbelt (previously undeveloped land) and agricultural land in the UK and therefore increased need for the redevelopment of brownfield sites (previously developed land) (CL:AIRE, 2004; Nathanail et al, 2001; SEPA, 2001; Pollard et al, 2004a; BIOWISE, 2000). For example, the Government has stated that it requires the construction of 2.4 million new residential houses by the year 2016, of which 60% are to be constructed on brownfield sites (CL:AIRE, 2004). Such brownfield sites are potentially contaminated and therefore unsuitable for residential use, thus remediation may be needed.

During the early 1980s few contaminated sites were recognised, and the risks to human health and the environment were not well understood (NICOLE, 2002; Cairney and Hobson, 1998; Nathanail and Bardos, 2005). However, it is now recognised that land contamination in the UK is widespread and it is viewed as an infrastructural problem (Nathanail and Bardos, 2005; Nathanail et al, 2001; NICOLE, 2002). Government and industry in the UK deem remediation of all contaminated sites unfeasible, and usually unnecessary when a full, site specific, risk assessment is carried out (NICOLE, 2002). The ultimate emphasis of land remediation is now on ensuring that a site is fit for its current or intended land-use (Nathanail and Bardos, 2005).

Remediation strategies in the UK are based on risk assessment, whereby courses of action to mitigate risks posed under specific circumstances are chosen based on the Source-Pathway-Receptor principle (NICOLE, 2002; Nathanail and Bardos, 2005; BIOWISE, 2000). This concept reflects the way in which a contaminant is likely to migrate to a receptor from a source through various environmental media and how the receptor may become exposed to the contaminant (NICOLE, 2002). For a contaminant to pose a risk it must first be made available to a receptor through

mobilisation and transport, and then must elicit an adverse response from the receptor due to exposure (Loehr, 1996). Remediation strategies aim to control, modify, or destroy pollutant linkages that present unacceptable risks (Nathanail and Bardos, 2005). This approach relies on applying a large and multidisciplinary knowledge base that straddles natural, physical, engineering and social sciences within a practical, commercial, regulatory and often community context (Pollard et al, 2004a). Risk based management is considered by most EU member states as the best available strategy for dealing with the problems posed by the land contamination (NICOLE, 2002).

1.3.2 Land Contaminated with Petroleum Hydrocarbons

Petroleum products from the refinery of crude oil are major global resources and are used in industrial and transportation sectors throughout the world (Singh et al, 2006). In a recent review of the UK oil refinery industry by the UK Petroleum Industry Association (UKPIA, 2006) it is reported that there are 9 oil refineries currently operational, supplying 90% of the transport fuels needed in the UK. In addition, there are approximately 1,500 miles of oil transportation pipelines and 9,750 service stations in the UK. The UK has the fourth largest refining capacity in Europe and at its peak had 19 oil refineries in operation in 1975 (UKPIA, 2006).

Petroleum hydrocarbons constitute a large portion of subsurface contamination throughout the world (Reisinger, 1995), and soil contamination by petroleum hydrocarbons is a widespread problem (Bundy et al, 2004; Simon et al, 2004). Singh et al, (2006) report that over 7.7 million cubic meters of petroleum products were released into the water and soil environments in the world between 1878 and 1992. A recent survey of remediation practice in the UK in 2001 was carried out by FirstFaraday (Nathanail et al, 2001) and found that, of 68 sites whose details were collected, 65% of these sites were contaminated with organic compounds, of which 80% were contaminated with petroleum hydrocarbons.

The widespread problem of soils contaminated with petroleum hydrocarbons reflects the numerous opportunities for release of these products into the environment due to leaks, spillages and transport accidents associated with the distribution of petroleum

products (Reisinger, 1995; Margesin and Schinner, 1997; Rahman et al, 2003). Many petroleum hydrocarbons potentially elicit adverse human health and environmental effects, therefore a need for their remediation frequently exists (Reisinger, 1995).

1.3.3 Legislation and Land Remediation

Current legislation in the UK governing the requirement for land remediation is Part IIA of the Environmental Protection Act (1990) (SEPA, 2001). Part IIA of the Environmental Protection Act (1990) defines contaminated land as:

"Contaminated land is any land which appears to the local authority in whose area it is situated to be in such a condition, by reason of substances in, on or under the land that:-

a) *Significant harm is being caused or there is a significant possibility of such harm being caused; or*
b) *Pollution of controlled waters is being, or is likely to be caused."*

Where:

"Harm means harm to the health of living organisms or other interference with the ecological systems of which they form part, and, in the case of man, includes harm to his property"

Part IIA addresses the problem of historically chemically contaminated land. Under these regulations Local Authorities are responsible for inspecting their areas to identify sites of contaminant land, and for ensuring that, where required, remediation is carried out (SEPA, 2001).

There are many techniques available for the remediation of contaminated soils. It is beyond the scope of this thesis to detail these technologies, but briefly such techniques can be grouped into physical (for example containment and excavation + disposal), chemical (for example dechlorination) and biological (bioremediation), which are carried out either singly or in combination (Brar et al, 2006; BIOWISE,

2000; Vik and Bardos, 2002). Remediation techniques are also grouped according to the location of where the remediation is taking place, i.e. *in-situ* (whereby contaminated land is treated in place), and *ex-situ* (whereby contaminated land is excavated and treated either on-site or off-site) (Vik and Bardos, 2002). The most commonly adopted remediation technique in the UK was removal of contaminated soils followed by their disposal to landfill (Reisinger, 1995; Nathanail et al, 2001; Paton, 2001; Petts et al, 2000; Vik and Bardos, 2002; Bardos et al, 2000b).

Recent changes in legislation in the UK have had a significant impact on the choice of remediation strategy. Of particular importance is the European Union Landfill Directive (99/31/EC) which is implemented in the UK through the Landfill (England and Wales) Regulations (2002) (Summersgill, 2006; CL:AIRE, 2004). The Landfill Regulations aim to prevent or reduce as far as possible the negative effects of landfilling on human health and the environment, and to encourage a reduction in reliance on the disposal of wastes to landfills, and a more sustainable approach to the management of wastes (Environment Agency, 2004a). Under the Landfill Regulations the co-disposal of hazardous (materials deemed to be hazardous according to the Hazardous Waste Directive 91/689/EC) and non-hazardous wastes is no longer permitted in the UK (Environment Agency, 2004a). Contaminated soils may be classified as hazardous or non-hazardous depending on the concentrations of contaminants present; therefore chemical analysis of soils is required under the Landfill Regulations.

Under the sustainable development agenda, the focus of remediation strategies is on application of sustainable remediation technologies that conserve land and resources, and the consideration of point and diffuse source of soil pollution over the long term (Pollard et al, 2004a; Vik and Bardos, 2002). Disposal of contaminated soils to landfill is now perceived as being the least desirable option (Semple et al, 2001; BIOWISE, 2000). Bioremediation is a biological treatment strategy that is receiving increasing attention as a cost effective and sustainable remediation technique, as not only does it have the potential to reduce contaminant concentrations, but also to reduce contaminant mobility and toxicity (Loehr and Webster, 2000; Dobson et al, 2004; Semple et al, 2001; Boopathy, 2000; Bento, 2003; Sabate et al, 2004). An overview of bioremediation is provided below.

1.3.4 Bioremediation

Bioremediation is the controlled use of microorganisms to degrade organic contaminants such as petroleum hydrocarbons, resulting in microbial cell mass, carbon dioxide and water (Loehr et al, 2001b; Brar et al, 2006; Alexander, 1999). Biodegradation can result in the reduction of complexity, toxicity and mobility of contaminants (Alexander, 1999; Loehr and Webster, 2000; Dobson et al, 2004; Semple et al, 2001; Boopathy, 2000; Bento, 2003; Sabate et al, 2004).

Microorganisms play a central role in the cycling of nutrients and therefore in supporting and maintaining life on Earth (Madigan et al, 2003). They possess metabolic systems enabling them to degrade a wide range of organic compounds, and therefore can play a central role in controlling the fate of organic contaminants in the environment (Alexander, 1999; Yong and Mulligan, 2004; Brar et al, 2006; Wellington and Larkin, 2004; Atlas, 1995).

Microorganisms need energy, carbon and nutrients in order to sustain their viability and growth (Alexander, 1999; Madigan et al, 2003), and are widely reported to be capable of metabolising hydrocarbons as a source of carbon and energy (Alexander, 1999; Hayes et al, 1995; Lovley, 2003; Wellington and Larkin, 2004; Semple et al, 2003; Brar et al, 2006; Ibekwe et al, 2006). Such hydrocarbon degrading microorganisms are ubiquitous in nature and the degradation of petroleum hydrocarbons by microorganisms is widely recognised (Röling et al, 2002; Ijah and Antai, 2003; Atlas, 1995; Simon et al, 2004), even as early as the 1940s (Atlas, 1981).

Bioremediation differs to conventional land remediation strategies such as landfilling in that it results in the partial or complete biotransformation of organic compounds rather than their transfer between environmental compartments (Sabate et al, 2004; Bento et al, 2003; Semple et al, 2001; Alexander, 1999; Dobson et al, 2004). Bioremediation is therefore regarded as a potentially sustainable remediation strategy (Semple et al, 2001).

The success of bioremediation relies on a complex array of factors, including contaminant biodegradability and bioavailability, presence of contaminant degrading

microorganisms and the ability of these microorganisms to function under prevailing environmental conditions (Alexander, 1999; Semple et al, 2003; Paton et al, 2003; Brar et al, 2006; Fetter, 1999; Loehr, 2001a; Boopathy, 2000). These factors are often site and compound specific (Sabate et al, 2004; Alexander, 1999), and are discussed in detail in Chapter 2. It is recognised that not all hydrocarbons are readily biodegraded by microorganisms, and that their biodegradation is dependent on the chemical structure and physical-chemical properties of the hydrocarbons, which control resistance to, and availability for microbial degradation (Alexander, 1999; Yong and Mulligan, 2004; Fetter, 1999; Loehr et al, 2001a; Pollard et al, 2004b). The chemical structure and physical-chemical properties of hydrocarbons are discussed in Chapter 2.

Bioremediation strategies involve enhancing conditions to enable microbial degradation to take place (Atlas, 1995; Alexander, 1999). Ex situ bioremediation strategies include solid phase treatment, most commonly through land-farming (contaminated soil is added to a surface soil layer; Hogan, 1998) and biopiles (contaminated soil are formed into piles/windrows; Mulligan, 2002) (Loehr et al, 2001b; Straube et al, 2003), whereby biodegradation is enhanced through the introduction of nutrients (biostimulation), oxygen and water (Quinn and Reinhart, 1997; Brar et al, 2006; Loehr et al, 2001b). Optimum biodegradation rates of petroleum hydrocarbons in soils are associated with aerobic conditions (Rhykerd et al, 1999; Alexander, 1999; Vasudevan and Rajaram, 2001; Huesemann and Truex, 1996; Brar et al, 2006); therefore ex-situ bioremediation strategies commonly introduce oxygen in order to enhance biodegradation. The addition of microorganisms known to have contaminant degrading abilities has been proposed to enhance the biodegradation of certain compounds, and is termed bioaugmentation (Zhu et al, 2004; Alexander, 1999). However, bioaugmentation is not widely practiced in the field (Thompson et al, 2005; Parsons, 2004; Kaplan and Kits, 2004; Zhu et al, 2004; Atlas, 1995). Biostimulation and bioaugmentation are detailed in Chapter 2.

An alternative to land-farming and biopiles is the use of composting techniques to enhance the biodegradation of petroleum hydrocarbons, a strategy known as composting bioremediation (Semple et al, 2001). This bioremediation strategy involves mixing contaminated soil with organic wastes and composting the materials

together. Composting bioremediation is thought to enhance biodegradation through increased microbial activity and diversity, and the achievement of higher pile temperatures (which is thought to enhance hydrocarbon bioavailability and biodegradability), and the introduction of nutrients. Composting bioremediation is still an emerging ex-situ bio-treatment technology (Ladislao et al, 2004) but has been under increasing investigation (Šašek et al, 2003; Hogan, 1998). Composting bioremediation is detailed in Chapter 2.

1.4 Chicken Manure and Composting Bioremediation

A surplus of chicken manure has been identified through a questionnaire of the poultry farming industry in Wales carried out in 2003, as was discussed in Section 1.2.2. The mismanagement of livestock manure such as chicken manure has been identified in the literature as a potential source of environmental and human health risk, as was discussed in Section 1.2.3. In order to bring about its sustainable management, and therefore reduction in environmental risks posed, there is a need to return the status of livestock manure (such as chicken manure) to that of a valuable resource rather than a waste.

One potentially sustainable use of organic wastes such as livestock manure is that of composting bioremediation. The use of chicken manure to enhance the biodegradation of petroleum hydrocarbons has been investigated by Ijah and Antai (2003), Atagana (2004b), Atagana (2003) and Ibekwe et al (2006), and was found to be successful. Microorganisms need carbon, essential nutrients, and micronutrients in order to maintain their growth and activity, and therefore potential degradation of compound such as petroleum hydrocarbons (Madigan et al, 2003; Alexander, 1999). Chicken manure is known to be rich in nitrogen and phosphorus, as was discussed in Section 1.2.1, and to contain trace elements such as magnesium (7,720 mg kg^{-1}), calcium (80,000 mg kg^{-1}) and iron (1,530 mg kg^{-1}) (Ihnat and Fernandes, 1996) which are reported to be important in enzyme production (Madigan et al, 2003; Mulligan, 2002). There is therefore a potential for the addition of chicken manure to soils contaminated with petroleum hydrocarbons to enhance the biodegradation of these compounds through biostimulation. This is discussed in greater detail in Chapter 2.

Also, chicken manure is reported to have a diverse microbial community (Ijah and Antai, 2003; Atagana, 2004b; Ibekwe et al, 2006). During composting, the activity of microorganisms leads to an increase in pile temperature (Peigne and Girardin, 2004). There is a potential for elevated temperatures to enhance the biodegradation of petroleum hydrocarbons by enhancing their biodegradability and bioavailability to compound degrading microorganisms (Gestel et al, 2003; Semple et al, 2001; Feitkenhauer et al, 2003; Coulon et al, 2005). The addition of chicken manure, and therefore a diverse microbial community, could therefore enhance the biodegradation of hydrocarbons through attaining elevated pile temperatures, which may not normally be attained in biopiles. This is discussed in detail in Chapter 2.

In addition, microorganisms with hydrocarbon degrading abilities have been identified in chicken manure by Ijah and Antai (2003). Therefore there is a potential that the addition of chicken manure could enhance hydrocarbon biodegradation through bioaugmentation. This is discussed in detail in Chapter 2.

However, while the author recognises that there is a need for additional investigation into the use of chicken manure, the author has concerns that the biological, chemical and toxicological processes involved during the composting bioremediation of soils contaminated with petroleum hydrocarbons using chicken manure (or other organic wastes) have not been sufficiently explored by previous authors. The author proposes that there is a risk that the addition of chicken manure could have a detrimental effect on the biodegradation process; therefore optimum biodegradation may be achieved through alternative bioremediation strategies. Of concern to this study is the potential for chicken manure to have a detrimental effect on biodegradation through adverse competition for growth factors between hydrocarbon degrading microorganisms and non-hydrocarbon degrading microorganisms introduced by the chicken manure, and the introduction of toxins such as trace elements, which may elicit adverse toxic effects on hydrocarbon degrading microorganisms. Owing to the nature of composting bioremediation in that the volume of contaminated soil is ultimately increased, and that therefore expansive land space may be required, failure of composting bioremediation could result in increased costs in the long run. These concerns are discussed further in Chapter 2.

1.5 Aims and Objectives

The aims and objectives of this thesis are as follows:

Aim: ***To investigate the use of chicken manure as a co-composting amendment to enhance the biodegradation of petroleum hydrocarbons and to help develop an improved understanding of the chemical, biological and toxicological processes involved.***

Objective One: - *To determine the potential for using chicken manure to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH).*

This is investigated through the use of laboratory based treatability studies combined with chemical analyses. Oil refinery sludge was either amended with chicken manure or left un-amended. TPH concentrations of solid samples taken throughout the treatability studies were determined by chemical analyses to give TPH degradation profiles. Comparisons of TPH degradation profiles are made between amended and un-amended oil refinery sludge to determine the extent to which chicken manure amendments enhance TPH biodegradation.

Objective Two: - *To determine the potential for using chicken manure to enhance the biodegradation of the more resistant TPH compounds.*

This is investigated through the use of treatability studies combined with chemical analyses as per Objective One above. Petroleum products contain a mixture of hydrocarbons of varying degrees of susceptibility to microbial attack owing to their chemical and physical properties (Yong and Mulligan, 2004). Chemical analyses were used to separate TPH compounds into fractions of similar properties (based on molecular weight and carbon number), and quantify concentrations of these fractions. Comparisons are made between amended and un-amended oil refinery sludge to determine the extent to which chicken manure addition enhances biodegradation of each fraction, with particular interest on higher molecular weight fractions deemed to be less biodegradable.

Objective Three: - *To determine the potential enhancement of environmental conditions for microbial growth and activity through the amendment of oil refinery sludge with chicken manure.*

The success of bioremediation is dependent on a number of inter-dependent factors including environmental conditions (water, oxygen, temperature, nutrients and pH) which affect microbial growth and activity (Alexander, 1999). The addition of chicken manure to oil refinery sludge could improve environmental conditions, particularly through the supply of nutrients. To determine the extent to which chicken manure enhances environmental conditions, two parameters were monitored; pile temperature and microbial activity. Comparisons are made between amended and un-amended oil refinery sludge.

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 1) bioaugmentation versus biostimulation, (Part 2) microbial population dynamics, and (Part 3) toxicological dynamics, resulting from the addition of chicken manure to TPH contaminated material.*

Organic wastes such as chicken manure contain nutrients and diverse microbial communities. The addition of nutrients and microorganisms through soil amendment with chicken manure could enhance TPH biodegradation through biostimulation and bioaugmentation respectively. This study aims to differentiate between the biostimulation and bioaugmentation effects of adding chicken manure through the use of laboratory analyses (environmental parameter monitoring, chemical, biological and toxicological analyses) and comparison of results between oil refinery sludge amended with chicken manure, and that amended with sterile chicken manure.

The addition of microorganisms, however, could adversely affect biodegradation through adverse competition with hydrocarbon degrading microorganisms for growth factors such as nutrients, carbon, oxygen, and water. This study aims to monitor microbial population dynamics through the use of biological analyses to determine the likely extent to which microbial populations from chicken manure dominate or interact with microbial populations from oil refinery sludge.

Also, chemical constituents of chicken manure may be toxic to hydrocarbon degrading microorganisms, thereby inhibiting or limiting TPH degradation. This study aims to assess toxicological dynamics through the use of in-vitro toxicity tests. Of additional interest is the use of these toxicity tests to assess the success or failure of composting bioremediation in reducing the toxicity (and therefore risk) of the oil refinery sludge.

Given that the addition of chicken manure to TPH contaminated materials ultimately increases the volume of contaminated material, failure of composting bioremediation processes could incur significant costs. Understanding the biological and toxicological processes involved with composting bioremediation is therefore, in the author's opinion, important.

1.6 Thesis Overview

An overview of the contents of this thesis is as follows:

Chapter 1 has introduced chicken manure and soils contaminated with petroleum hydrocarbons as two waste streams and sources of environmental risk in the UK. The environmental risks and the legislation associated with these waste streams have been outlined, and the potential use of chicken manure to enhance the remediation of soils contaminated with petroleum hydrocarbons was introduced. The aims and objectives of this study are stated.

Chapter 2 discusses the controls of biodegradation (in particular, biodegradability and bioavailability of contaminants, microbial consortia and environmental controls on microbial growth and activity) in detail; the main bioremediation techniques (biostimulation and bioaugmentation) employed during ex-situ bioremediation with example successes and failures; composting bioremediation and its controls; and areas of further research needed into the processes of composting bioremediation, which are to be addressed in this study.

Chapter 3 details the design of the treatability studies undertaken during this study, the reasoning behind the design adopted, and characterises the oil refinery sludge and chicken manure used in this study.

Chapter 4 presents data for environmental parameters (temperature and pH) and microbial activity which were monitored during the treatability studies. These parameters are used to indicate the extent to which the addition of chicken manure (live and sterile) stimulates total microbial activity, which is a key parameter in the biodegradation of organic contaminants such as petroleum hydrocarbons.

Chapter 5 presents the chemical methodology adopted for the TPH analyses, TPH concentration data (both total and fractionated), and headspace TPH concentration data. These data are used to determine the extent to which, a) the addition of chicken manure enhanced remediation of TPH compounds (with particular interest in the less biodegradable compounds), b) volatilisation likely contributed to TPH degradation, and c) the addition of chicken manure affected volatilisation.

Chapter 6 presents the molecular microbial methods adopted during this study, the baseline microbial population composition for chicken manure and oil refinery sludge, and microbial population dynamics data collected during the treatability studies. These data are used to determine the extent to which the addition of chicken manure affected microbial population dynamics in terms of microbial strains present, with particular reference to identification of microbial strains and their likely source (chicken manure or oil refinery sludge).

Chapter 7 presents the methodology used for the toxicity analyses and the toxicity data collected during the treatability studies. These data are used to determine any potential adverse effect on TPH biodegradation caused by the addition of chicken manure, and therefore potential toxic compounds, and to determine the success/failure of composting bioremediation in reducing toxicity (and therefore risk) of the oil refinery sludge.

Chapter 8 brings together the individual data sets (environmental parameters, microbial activity, chemical, biological, and toxicity) and discusses them. Conclusions

made based on the data and interpretation presented in this thesis are stated, and recommendations for further research are presented.

Chapter 9 provides the references for published documents referred to in this thesis.

Chapter 2

Bioremediation of Petroleum Hydrocarbons Using Chicken Manure

2.1 Introduction

This thesis investigates the potential use of chicken manure in the composting bioremediation of materials contaminated with Total Petroleum Hydrocarbons (TPH), and aims to help develop an improved understanding of the chemical, biological and toxicological processes involved.

Soil contamination by petroleum hydrocarbons is a world wide problem (Bundy et al, 2002; Bundy et al, 2004; Heiss-Blanquet et al, 2005; Simon et al, 2004). Bioremediation is receiving greater emphasis as a suitable technique for the remediation of such hydrocarbon contaminated soils (Sabate et al, 2004; Bento et al, 2003; Semple et al, 2001; Alexander, 1999; Dobson et al, 2004).

Composting bioremediation is an ex-situ solid phase bioremediation technique whereby contaminated soils are mixed with organic wastes and composted (Sasek et al, 2005; Hogan, 1998; Jorgensen et al, 2000; Semple et al, 2001). This remediation technique relies on a) the unique ability of microorganisms to metabolise and degrade organic compounds such as petroleum hydrocarbons (which in turn relies on contaminant biodegradability and bioavailability, presence of suitable microorganisms

and their ability to grow and be active under prevailing environmental conditions), and b) optimisation and maintenance of environmental conditions to enhance the growth and metabolic activity of microorganisms (through engineering practices such as aeration, water addition and nutrient addition) (Alexander, 1999; Semple et al, 2003; Paton et al, 2003; Brar et al, 2006; Fetter, 1999; Loehr, 2001a). These factors are complex and are often site and contaminant specific (Alexander, 1999; Boopathy, 2000).

Composting bioremediation is under increasing investigation and has been successfully applied to hydrocarbon contaminated soils, such as the work of Atagana (2003, 2004b), Namkoong et al (2002) and Ibekwe et al (2006). However, experimental design of such investigation appears inconsistent, and there is little information regarding the chemical, biological and toxicological processes involved. Given that composting bioremediation ultimately increases the quantity of contaminated material, failure of this technique could result in greater remediation costs in the long run. Therefore, in the author's opinion, the chemical, biological and toxicological processes involved with composting bioremediation should be understood.

This chapter outlines the characteristics of petroleum hydrocarbons, and discusses the factors affecting the biodegradation of petroleum hydrocarbons in general, ex-situ bioremediation strategies adopted to enhance biodegradation, and the process of composting bioremediation together with case studies. The likely advantages and disadvantages of using chicken manure in composting bioremediation, and the need for further investigation are discussed.

2.2 Petroleum Characteristics

Petroleum products are either fractions or blends of fractions from the distillation of crude oil. In a recent review of the UK oil refinery industry by the UK Petroleum Industry Association (UKPIA, 2006) it is reported that there are 9 oil refineries currently operational, approximately 1,500 miles of oil transportation pipelines and

9,750 service stations in the UK. The UK has the fourth largest refining capacity in Europe and at its peak had 19 oil refineries in operation in 1975 (UKPIA, 2006).

Petroleum products comprise complex mixtures of hydrocarbons (Fetter, 1999; Potter and Simmons, 1998), heteroatoms and small concentrations of metallic constituents (Weisman, 1998; Bhattacharya et al, 2003). This thesis is concerned with the bioremediation of hydrocarbons only.

Hydrocarbons are organic compounds comprising carbon and hydrogen only (Fetter, 1999). They are separated into two groups; aliphatics and aromatics. These two groups differ from each other based on the bonding patterns between carbon atoms (Potter and Simmons, 1998). The basic structure of aliphatic and aromatic hydrocarbons is outlined in the following sections. The general composition of the main petroleum products is also outlined. Physical-chemical properties of hydrocarbons greatly influence their degradation by microorganisms (Alexander, 1999; Pollard et al, 2004b; Loehr et al, 2001a; Semple et al, 2003). These properties are outlined and discussed in Section 2.3 in relation to compound biodegradability and bioavailability.

2.2.1 Aliphatic Hydrocarbons

Aliphatic hydrocarbons are straight, branched or cyclic compounds, and are further divided into alkanes, alkenes and cycloalkanes (Potter and Simmons, 1998).

Alkanes

Alkanes have the general formula C_NH_{2N+2} and their carbon atoms are linked together by single bonds, while all other bonding sites are saturated with hydrogen atoms (Fetter, 1999; Patrick, 2000, Nathanail and Bardos, 2005). Alkanes are therefore referred to as *saturated hydrocarbons*. Alkanes are either straight-chained or branched, as illustrated in Figure 2.1. For the same chemical formula (e.g. C_5H_{12}) varying branched configurations can exist, known as structural isomers (Figure 2.1). Such structural isomers (isoalkanes) have different properties to their straight-chained counterpart, and are actually different compounds (Fetter, 1999).

Cycloalkanes

Cycloalkanes have the general formula C_NH_{2N} and differ from alkanes it that the carbon atoms are bonded together to form a ring structure (Potter and Simmons, 1998; Fetter, 1999; Patrick, 2000, Nathanail and Bardos, 2005) as illustrated in Figure 2.1. These hydrocarbons are also referred to as *saturated hydrocarbons* as all bonding sites are occupied (Fetter, 1999). Due to their ring structure, cycloalkanes are typically more stable than normal alkanes, and therefore less susceptible to biodegradation (Mulligan, 2002) as further discussed in Section 2.3.

Alkenes

Alkenes have the general formula C_NH_{2N} and differ from alkanes and cycloalkanes in that they have double bonds between at least two of the carbon atoms (Fetter, 1999; Mulligan, 2002; Nathanail and Bardos, 2005). Alkenes are therefore referred to as unsaturated hydrocarbons. Alkenes also comprise straight-chain and branched-chain configurations (Figure 2.1). The double bond between carbon atoms is shorter and stronger than single bonds of alkanes and cycloalkanes, therefore alkenes have greater structural stability and lower biodegradability (Patrick, 2000), as further discussed in Section 2.3.

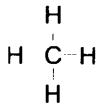
2.2.2 Aromatic Hydrocarbons

Aromatic hydrocarbons differ to aliphatic hydrocarbons in that they consist solely of ring structures (Fetter, 1999). The ring structure of aromatic hydrocarbons is based on that of benzene (Potter and Simmons, 1998; Fetter, 1999). Benzene comprises 6 carbon atoms which are joined to form a ring structure by 3 double bonds and 3 single bonds (Figure 2.2), and are therefore referred to as unsaturated hydrocarbons. The bonds are not held between specific pairs of atoms, but are free to move over the entire ring (Patrick, 2000). The benzene ring, and therefore aromatic hydrocarbons, has greater structural stability than aliphatic hydrocarbons, and therefore is of lower biodegradability (Mulligan, 2002; Nathanail and Bardos, 2005) as further discussed in Section 2.3.

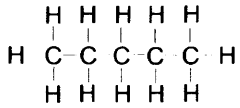
Benzene, Toluene, Ethylbenzene and Xylene (collectively known as BTEX compounds) are monoaromatics (they comprise single rings). In contrast, polycyclic

aromatic hydrocarbons (PAHs) comprise two or more benzene rings which are joined together (Fetter, 1999) as illustrated in Figure 2.2, and have the general formula $C_{4N+2}H_{2N+4}$ (Mulligan, 2002). The higher the number of benzene rings in a PAH compound, the greater its stability and therefore lower its biodegradability (Weisman, 1998; Mulligan, 2002) as further discussed in Section 2.3

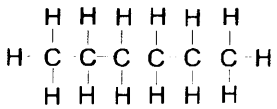
Alkanes



Methane (CH_4)

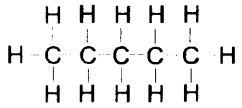


Pentane (C_5H_{12})

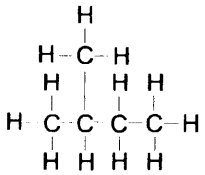


Hexane (C_6H_{14})

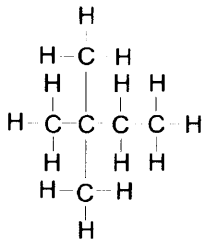
Isoalkanes (C_5H_{12})



Pentane (C_5H_{12})

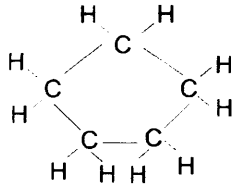


2 Methylbutane (C_5H_{12})

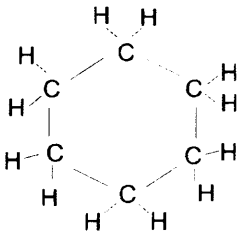


2,2 Dimethylpropane (C_5H_{12})

Cycloalkanes

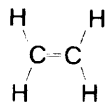


Cyclopentane (C_5H_{10})

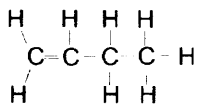


Cyclohexane (C_6H_{12})

Alkenes



Ethylene (C_2H_4)

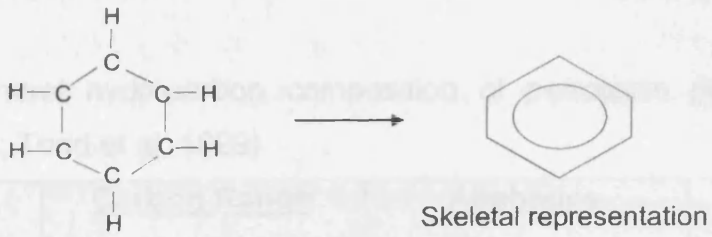


1-Butene ($C4H_8$)

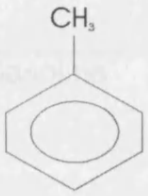
Figure 2.1: Aliphatic Hydrocarbons

Monoaromatics (BTEX)

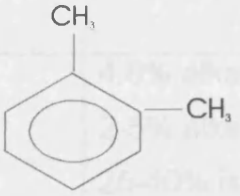
Benzene (C_6H_6)



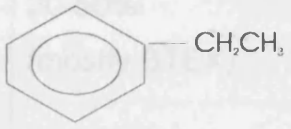
Skeletal representation



Toluene



Xylene

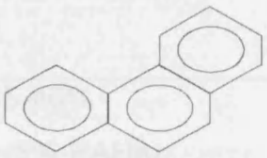


Ethylbenzene

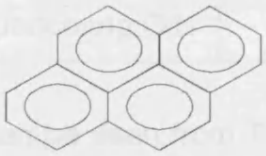
Polycyclic Aromatic Hydrocarbons (PAHs)

Napthalene

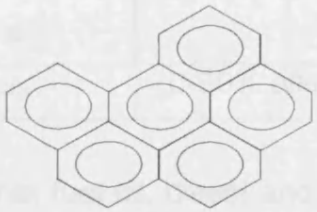
Anthracene



Phenanthrene



Pyrene



Benzo*(g,h,i)*pyrelene

Figure 2.2: Aromatic Hydrocarbons

2.2.3 Petroleum Products – General Composition

Petroleum is separated into fractions by distillation. The composition of each fraction is complex and depends on the source of the crude oil and the specific refining practices used (Todd et al, 1999). A brief compositional outline of the main petroleum

products is presented in Table 2.1. For detailed information, the reader is referred to the Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG), Volume 2, by Potter and Simmons, 1998.

Table 2.1: General hydrocarbon composition of petroleum products (Potter and Simmons, 1998; Todd et al, 1999)

Petroleum Product	**Carbon Range**	**Aliphatics**	**Aromatics**
Gasoline	C_4-C_{12}	4.8% alkanes 2-5% alkenes 25-40% isoalkanes 3-7% cycloalkanes	20-50% (mostly BTEX)
Jet Fuel (JP4)	C_4-C_{16}	32% alkanes 31% isoalkanes 16% cycloalkanes <5% alkenes	20-25%
Fuel Oil (#2)	C_{11}-C_{20}	60-65% (1-2% alkenes)	35-40% (<5% PAHs)
Diesel #1 #2	C_8-C_{17} C_8-C_{26}	60-90%	<40%
Lubricating Oils	C_{20}-C_{45+}	70-90% alkanes	10-30%

It can be seen from Table 2.1 that fuel oil, diesel and lubricating oils are heavier than gasoline and jet fuel, as shown by their higher carbon ranges. In general, a reverse relationship is seen between the molecular weight (i.e. carbon number) and biodegradability/bioavailability of a hydrocarbon (i.e. the higher the molecular weight of a hydrocarbon, the lower its biodegradability and bioavailability) (Alexander, 1999; Potter and Simmons, 1998). Therefore, it can be said that gasoline and jet fuel are more susceptible to biodegradation than fuel oil, diesel and lubricating oils. The relationship between molecular weight and biodegradability/bioavailability is further discussed in Section 2.3.

2.3 Biodegradation

Before we can look at the bioremediation of any given hydrocarbon contaminated soil, it is important to look at whether or not bioremediation will likely be successful. Bioremediation relies on (Alexander, 1999; Semple et al, 2003; Paton et al, 2003; Brar et al, 2006; Fetter, 1999; Boopathy, 2000):

a) Compound biodegradability (function of chemical structure and molecular weight).
b) Compound bioavailability (function of physical-chemical properties of a compound, compound concentration, and time).
c) Microbial consortia (whether or not suitable microorganisms with compound degrading abilities are present).
d) Environmental conditions (whether or not conditions are suitable for microbial growth and activity).

2.3.1 Biodegradability

Microorganisms need energy, carbon and nutrients in order to sustain their viability and growth (Alexander, 1999; Madigan et al, 2003). Petroleum hydrocarbons present a source of carbon, and the metabolism of these compounds yields energy. If the contaminant cannot be metabolised by microorganisms, it will not serve as a source of energy and carbon (Alexander, 1999).

Biodegradability of hydrocarbons is related to their physical and chemical properties (Alexander, 1999). Petroleum products contain a highly diverse range of hydrocarbons, as was outlined in Section 2.2. These hydrocarbons range from highly biodegradable to persistent (resistant to biodegradation) compounds (Alexander, 1999; Yong and Mulligan, 2004).

Generally, within each group of hydrocarbons (i.e. alkanes, alkenes, cycloalkanes, monoaromatics), resistance to microbial degradation increases with increasing molecular weight (Pollard et al, 2004b), which in turn increases with increasing number of carbon atoms and chain length (Potter and Simmons, 1998). Resistance

also increases with increasing branching (Yong and Mulligan, 2004), and in the case of PAHs, increasing number of aromatic rings (Fetter, 1999; Atagana, 2003; Yong and Mulligan, 2004; Alexander, 1999).

Trends in biodegradability of the various hydrocarbon groups are reported by Potter and Simmons (1998), Loehr et al (2001a), Fetter (1999), Nancarrow et al (2001), and Pollard et al (2004b), and are as follows:

n-alkanes
isoalkanes
low molecular weight cycloalkanes
alkenes
BTEX
high molecular weight cycloalkanes
PAHs (decreasing biodegradability with increasing number of rings)

decreasing biodegradability

While alkanes are widely considered to be the most biodegradable, Nancarrow et al (2001) reports that bioremediation is only suitable for those alkanes with less than 24 carbon atoms, and Todd et al (1999) reports on a upper limit of 22 carbon atoms. It is widely realised that there are many persistent, heavy, hydrocarbons for which bioremediation is likely unsuitable (Alexander, 1999; Boopathy, 2000; Dobson et al, 2004; Dua et al, 2002; Hogan, 1998). Such persistent hydrocarbons are reported to include pristine, phytane, hopanes, steranes (Pollard et al, 2004b), and PAHs with 4 rings or more (Loehr et al, 2001a).

The relationship between biodegradability and the physical properties of a hydrocarbon also coincides with changes in the behaviour of such compounds in the soil. Behaviour of compounds in the soil depends on their physical-chemical properties, and affects the availability of such compounds to microorganisms for biodegradation (Alexander, 1999). Such physical-chemical properties and bioavailability are discussed below.

2.3.2 Bioavailability

Even if a compound is structurally biodegradable, it must be available to microorganisms before it can be biodegraded (Alexander, 1999; Semple et al, 2001; Loehr et al, 2001a; Stroo et al, 2000; Semple et al, 2003; Head, 1998). The availability of hydrocarbons to microorganisms is therefore a major factor determining the success of bioremediation strategies, and is referred to as bioavailability.

Microorganisms use enzymes to degrade contaminants (Madigan et al, 2003). Although it is known that some microorganisms can produce extracellular enzymes (i.e. external to the microbial cell) to carry out such degradation, it is believed that the majority of compound degradation takes place using intracellular enzymes (Alexander, 1999). Therefore, in order for a compound (i.e. hydrocarbon) to be metabolised by microorganisms, it must be assimilated into the microbial cell. It is therefore widely believed that the majority of biodegradation takes place on compounds present in the aqueous phase, as these are readily available for assimilation into microbial cells (Reid et al, 2000; Alexander, 1999; Semple et al, 2001; Loehr and Webster, 2000).

Bioavailability is defined by Dobson et al (2004) as *'the proportion of contaminants that are available for rapid transfer to the aqueous phase'*. The quantity of hydrocarbons in the aqueous phase depends on solubility/hydrophobicity and partitioning between the solid and aqueous phase. These physical-chemical properties of hydrocarbons are presented in Table 2.2 and are discussed later on in this section. Most petroleum hydrocarbons are hydrophobic and preferentially partition onto solid surfaces, therefore rendered non-bioavailable to microorganisms (Alexander, 1999). Therefore, bioavailability is believed to be limited by the rate of desorption/mass transfer into the aqueous phase (Dobson et al, 2004; Semple et al, 2001).

Sorption of compounds to solid particles includes adsorption, whereby compounds adhere to solid surfaces, and absorption, whereby compounds become entrapped within particles (Alexander, 1999). Both processes can reduce the size of the

bioavailable portion of hydrocarbons in soils, thereby affecting the bioremediation of hydrocarbon contaminated soils (Semple et al, 2001).

Although it is believed that the majority of contaminant degradation takes place in the aqueous phase, it is possible that some microorganisms can utilise adsorbed compounds directly by adhering to the same surface (Alexander, 1999). This theory is supported by the work of Huesemann et al (2004) who investigated the effects of bioavailability on biodegradation rates. The data attained indicated that the biodegradation rates of alkanes were higher than their abiotic desorption rates. It was therefore concluded by Huesemann et al (2004) that alkanes do not have to be dissolved in the aqueous phase in order to be biodegraded by soil microorganisms. However, it is proposed by Alexander (1999) that, should a microorganism adsorb to the correct location, the microenvironment may, in some cases, be less favourable for microbial growth and activity than the surrounding solution. This may reflect adverse changes in pH, nutrient availability or presence of toxins (higher concentrations of contaminants may be toxic to microorganisms) (Alexander, 1999).

Various parameters are reported in the literature to control the sorption/bioavailability of hydrocarbons. These are a) physical-chemical properties and soil organic carbon content, b) contaminant concentration, and c) contaminant residence time in a soil. These factors are discussed below.

Physical-Chemical Properties:

Sorption of hydrocarbons onto solid surfaces reflects their polarity and hydrophobicity (Fetter, 1999). Hydrophobicity is a function of the degree to which organic compounds are attracted by the polar water molecules, and this depends on the polarity of the compound. Hydrocarbons are non-polar but have differing degrees of non polarity owing to differing bonding patterns (Potter and Simmons, 1998). The primary absorptive surface for hydrocarbons is the fraction of organic matter in the soil (Fetter, 1999). The extent to which hydrocarbons will adsorb onto the organic carbon fraction (f_{oc}) of a soil is measured by the water-organic carbon partition coefficient (k_{oc}). K_{oc} is calculated as per Equation 2.1 (taken from Fetter (1999) and Gustafson et al (1997).

$$K_{oc} = \frac{k_d}{f_{oc}}$$ Equation 2.1

Where:

$k_d = \frac{concentration\ in\ solid}{concentation\ in\ water}$

f_{oc} = fraction of soil organic carbon

The organic carbon content of soils (organic matter) has been reported to influence the size of the sorbed fraction of hydrocarbons by Semple et al (2001), Alexander (1999), Breedveld et al (2000), Macleod and Semple (2003), and Garcia-rivero et al (2002). Soils with higher organic matter contents have a larger capacity to sequester hydrocarbons, rendering them less/non-bioavailable (Macleod and Semple, 2003).

A common measure of hydrophobicity of hydrocarbons is solubility (S). Solubility of a compound is expressed in terms of mass per volume of water (mg l^{-1}) (Fetter, 1999). The lower the solubility of a hydrocarbon, the lower its bioavailability (Todd et al, 1999).

Also of importance to compound mobility is Henry's law constant (H). Henry's law constant is a water-air partition coefficient (Equation 2.2; Gustafson et al, 1997), and measures the volatility of an organic compound (Fetter, 1999; Todd et al, 1999; Yong and Mulligan, 2004). The volatility of a compound is a function of its vapour pressure, solubility and molecular weight (Todd et al, 1999). Henry's law constant (H) relates these three parameters. The higher the value of H, the higher the volatility of a compound (Fetter, 1999; Yong and Mulligan, 2004).

$$H = \frac{concentration\ in\ air}{concentration\ in\ water}$$ Equation 2.2

Physical-chemical properties of hydrocarbons are presented in Table 2.2. It is impossible to identify all individual hydrocarbons present in petroleum products, therefore hydrocarbons have been grouped into fractions of similar physical-chemical properties (and therefore behaviour in soil) based on carbon number by the Total

Petroleum Hydrocarbons Criteria Working Group (TPHCWG) (Weisman, 1998). It can be seen from Table 2.2 that:

1. Molecular weight increases with increasing number of carbon atoms.
2. Solubility decreases with increasing molecular weight, and aromatic hydrocarbons have higher solubility than their aliphatic hydrocarbon counterparts.
3. K_{oc} increases with molecular weight, and aromatic hydrocarbons have lower propensity to partition onto organic carbon fractions than their aliphatic hydrocarbon counterparts.
4. H increases with increasing molecular weight for aliphatic hydrocarbons, but decreases with increasing molecular weight for aromatic hydrocarbons. For each carbon fraction, aromatic hydrocarbons have lower volatility than aliphatic hydrocarbons.

Table 2.2: Physical-chemical properties of hydrocarbon fractions (taken from TPHCWG, Volume 3; Gustafson et al, 1997).

Fraction	BP (°C)	EC	MW (g/mole)	S (mg/L)	VP (atm)	H (cm^3/cm^3)	log K_{oc}
ALIPHATICS							
EC 5-6	5.1E+01	5.5E+00	8.1E+01	3.6E+01	3.5E-01	4.7E+01	2.9E+00
EC >6-8	9.6E+01	7.0E+00	1.0E+02	5.4E+00	6.3E-02	5.0E+01	3.6E+00
EC >8-10	1.5E+02	9.0E+00	1.3E+02	4.3E-01	6.3E-03	5.5E+01	4.5E+00
EC >10-12	2.0E+02	1.1E+01	1.6E+02	3.4E-02	6.3E-04	6.0E+01	5.4E+00
EC >12-16	2.6E+02	1.4E+01	2.0E+02	7.6E-04	4.8E-05	6.9E+01	6.7E+00
EC >16-21	3.2E+02	1.9E+01	2.7E+02	2.5E-06	1.1E-06	8.5E+01	8.8E+00
AROMATICS							
EC 5-7	8.0E+01	6.5E+00	7.8E+01	2.2E+02	1.1E-01	1.5E+00	3.0E+00
EC >7-8	1.1E+02	7.6E+00	9.2E+01	1.3E+02	3.5E-02	8.6E-01	3.1E+00
EC >8-10	1.5E+02	9.0E+00	1.2E+02	6.5E+01	6.3E-03	3.9E-01	3.2E+00
EC >10-12	2.0E+02	1.1E+01	1.3E+02	2.5E+01	6.3E-04	1.3E-01	3.4E+00
EC >12-16	2.6E+02	1.4E+01	1.5E+02	5.8E+00	4.8E-05	2.8E-02	3.7E+00
EC >16-21	3.2E+02	1.9E+01	1.9E+02	6.5E-01	1.1E-06	2.5E-03	4.2E+00
EC >21-35	3.4E+02	2.8E+01	2.4E+02	6.6E-03	4.4E-10	1.7E-05	5.1E+00

BP (boiling point); EC (equivalent carbon number); MW (molecular weight); S (solubility); VP (vapour pressure); H (Henry's Lay constant).

Contaminant Concentration: - it has been suggested that the biodegradation of organic compounds is growth linked, i.e. increases in microbial growth during a bioremediation process are indicators of increased biodegradation of contaminants (Alexander, 1999). In microbiology, microbial growth is defined as an increase in the number of cells (Madigan et al, 2003). Microorganisms need a base level of energy to maintain their viability (to stay alive). For microbial growth to result from the metabolism of contaminants, a threshold concentration is needed, above which

surplus energy is gained and the production of 'new' cells can occur (Alexander, 1999; Fetter, 1999). Therefore the concentration of a contaminant available to microorganisms will affect microbial growth (Macleod et al, 2000). However, it has been reported that sorption of contaminants increases with increasing concentration of the contaminant (Alexander, 1999; Semple et al, 2001). There appears to be a trade off between threshold concentration and increasing sorption; while a higher contaminant concentration is needed for biodegradation, its availability for degradation may be compromised (Semple et al, 2001).

Contaminant Residence Time: - it has been widely observed that there is a time dependent decline in bioavailability, i.e. the longer a contaminant remains in contact with soil, the lower its bioavailability (Semple et al, 2001, 2003; Garcia-rivero et al, 2002; Alexander, 1999; Macleod et al, 2000). This process has been termed 'ageing', and is thought to result from sorption onto organic matter and solid surfaces and diffusion into nanopores (Boopathy, 2000; Huesemann et al, 2004; Semple et al, 2003). The ultimate result of ageing is the movement of compounds from accessible soil compartments into less or inaccessible soil compartments, and an increase in the size of the non-bioavailable fraction (Reid et al, 2000; Semple et al, 2003) as illustrated in Figure 2.3. It has been reported that three soil associated chemical pools exist after ageing; a) one that is rapidly desorbed, b) one that is more slowly desorbed, and c) one termed bound residue or non-extractable (Semple et al, 2001).

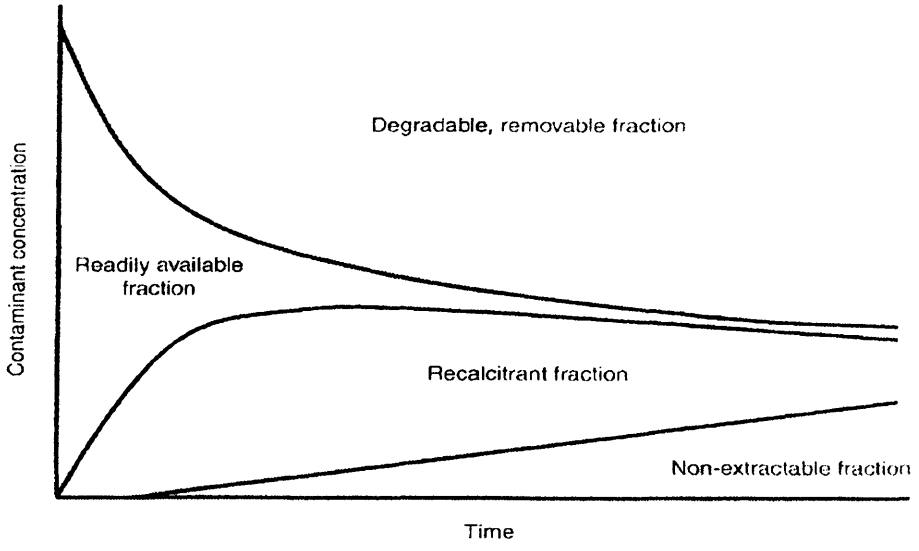


Figure 2.3: The influence of contact time on the extractability and bioavailability of a contaminant (taken from Semple et al, 2003).

Heavy hydrocarbons with strong hydrophobicity and sorption tendencies are often 'non-bioavailable' and therefore termed persistent (Reid et al, 2000; Semple et al, 2001; Alexander, 1999). These compounds resist microbial degradation and include high molecular weight and branched aliphatics (e.g. pristane and phytane; Alexander, 1999; Pollard et al, 2004b), hydrocarbons with greater than 22-24 carbon atoms (Nancarrow et al, 2001; Todd et al, 1999), and PAHs comprising greater than 4 rings (e.g. pyrene, benzo(a)pyrene and benzo(g,h,i)perylene; Alexander, 1999; Fetter, 1999; Todd et al, 1999). The presence of persistent/non-bioavailable hydrocarbons can limit attempts to remediate polluted sites through bioremediation (Alexander, 1999). It may therefore be necessary to encourage desorption of these compounds into the aqueous phase to increase biodegradation potential (Garcia-rivero et al, 2002).

However, bioavailability of a compound is not enough to enable its biodegradation. Huesemann et al (2004) studied the role of bioavailability limitations during the bioremediation of PAHs and alkanes in aged soils by directly comparing abiotic desorption rates and biodegradation rates. They found that desorption rates did not always control biodegradation rates, and that other controlling factors are equally important. For example 5 and 6 ring PAHs, despite being readily bioavailable (indicated by abiotic release rates), were still not biodegraded. Huesemann et al (2004) concluded that microbial factors rather than bioavailability limitations were responsible for the persistence of these PAHs. Microbial factors include the presence of suitable microorganisms, and their ability to grow and be active under prevailing environmental conditions. These factors are discussed in the following sections.

Bioavailability not only affects the extent and/or success of bioremediation, but can also affect the design of remediation strategies (i.e. concentration endpoints). Remediation strategies require that the concentration of a compound (i.e. hydrocarbon) is reduced to levels that are perceived to present no/acceptable risk to human health and the environment (Tugan et al, 2003). For a compound in a soil to pose a risk it must be made available for uptake by the receptor through mobilisation and transport, and then it must induce an adverse response from the receptor due to exposure (Loehr, 1996). Remediation strategies, and risk assessments, are based on contaminant concentration as determined by chemical analyses (Loehr, 1996).

Chemical analyses involve contaminant extraction steps which yield the determination of 'total' contaminant concentrations (Reid et al, 2000). However, such methods do not differentiate between 'total' and 'bioavailable' contaminant fractions. It is suggested by Loehr et al (2001a), Huesemann et al (2004), Alexander (1995), Tugan et al (2003) and Semple et al (2003) that non-bioavailable fractions do not pose risks and therefore can represent an environmentally acceptable endpoint; thus lowering cleanup targets in some cases.

However, bioavailability is not an all encompassing term; it can be organism and species specific (Stroo et al, 2000; Reid et al, 2000). Although, an investigation carried out by Loehr et al (2000) indicated that non-bioavailable fractions remain as such in the long term, there is still concern that non-bioavailable fractions may be released in the long term (Semple et al, 2001). Therefore, the removal of such contaminant fractions from risk analyses may not be appropriate.

2.3.3 Microbial Consortia

The mere presence of biodegradable and bioavailable hydrocarbons is not sufficient for their successful biodegradation. Suitable microorganisms possessing suitable enzymes for the biodegradation of these compounds must be present (Alexander, 1999; Semple et al, 2003; Paton et al, 2003; Brar et al, 2006; Fetter, 1999; Loehr, 2001b).

Microorganisms are widely reported to be capable of metabolising hydrocarbons as a source of carbon and energy (Alexander, 1999; Lovley, 2003; Wellington and Larkin, 2004; Semple et al, 2003; Brar et al, 2006; Ibekwe et al, 2006). Such hydrocarbon degrading microorganisms are ubiquitous in nature (Röling et al, 2002; Ijah and Antai, 2003; Atlas, 1995; Simon et al, 2004). Bacteria are considered to represent the predominant microbial agents for hydrocarbon degradation (Alexander, 1999; Röling et al, 2002; Riffaldi et al, 2005).

Individual bacteria likely only degrade a limited range of hydrocarbons. Therefore, due to the complexity of petroleum products, biodegradation usually requires the cooperation of more than one species (Riffaldi et al, 2005; Korda et al, 1997; Röling et

al, 2002; Baltzis and Lewandowski, 1998). Many genera of bacteria have been identified in the literature as being capable of metabolising hydrocarbons. These include *Alcanivorax* (Röling et al, 2002; Syutsubo et al, 2001; Liu and Shao, 2005; Heiss-Blanquet et al, 2005), *Rhodococcus* (Alexander, 1999; Behki, 1994; Irvine et al, 2000; Heiss-Blanquet et al, 2005), *Actinomycetes* (Margesin and Schinner, 1997), *Micrococcus* (Fetter, 1999; Ijah and Antai, 2003; Ibekwe et al, 2004), *Corynebacteria* (Yong and Mulligan, 2004), *Nocardia* (Fetter, 1999; Yong and Mulligan, 2004; Ibekwe et al, 2004), *Mycobacterium* (Yong and Mulligan, 2004; Fetter, 1999; Wang et al, 1996), *Bacteroides* (Kaplan and Kitts, 2004), *Xanthomonas* (Kaplan and Kitts, 2004), *Hydrocarboniphaga effusa* (Palleroni et al, 2003), *Syntrophus* (Dojka et al, 1998), *Bacillus* (Bento et al, 2003) and *Pseudomonas* (Ijah and Antai, 2003; Fetter, 1999; Straube et al, 2003; Kaplan and Kitts, 2004; Irvine et al, 2000; Ibekwe et al, 2004; Heiss-Blanquet et al, 2005; Bento et al, 2003).

Microorganisms require suitable enzymes for the degradation of different chemical compounds (Dua et al, 2002). Enzymes are catalysts that facilitate reactions (Madigan et al, 2003), and act on a series of closely related reactions or molecules (Alexander, 1999; Madigan et al, 2003). For aerobic degradation of petroleum hydrocarbons, such enzymes are called *oxygenases* and act to incorporate oxygen atoms into a hydrocarbon compound (Alloway and Ayres, 1994; Boopathy, 2003; Mulligan, 2002). There are two types of oxygenases; *monooxygenase* whereby only one oxygen atom from molecular oxygen (O_2) is incorporated into a hydrocarbon compound and *dioxygenase* whereby both oxygen atoms from molecular oxygen are incorporated into a hydrocarbon compound (Madigan et al, 2003; Alloway and Ayres, 1994; Boopathy, 2003; Mulligan, 2002).

2.3.4 Microbial Growth and Activity

Possessing the requisite enzymes to carry out contaminant transformation is not sufficient for the organism to succeed. Microbial populations are subject to a variety of factors that influence their growth, their activity and their very existence (Alexander, 1999). The persistence of hydrocarbon compounds may not always be a consequence of the absence of hydrocarbon degrading microorganisms, but rather

the absence of the full set of conditions necessary for the resident species to function (Deborger et al, 1978).

The main factors influencing, and even controlling, the growth and activity of microorganisms are temperature, water availability, oxygen availability, pH and nutrient availability. Every microorganism has a range of tolerances to these 'environmental parameters' (Alexander, 1999). The presence of toxins and predators can also affect contaminant degrading microbial populations (Alexander, 1999). These factors are discussed as follows.

Temperature: - temperature is of paramount importance to the growth and activity of microorganisms (Alexander, 1999; Coulon et al, 2005). Each species has a range of temperature tolerance, within which microbial growth and activity increases as temperature rises (Alexander, 1999; Madigan et al, 2003; Coulon et al, 2005). Microbial metabolism is reported to double with each 10°C rise in temperature from 10°C to 40°C (Coulon et al, 2005). The optimum temperature for biodegradation of hydrocarbons is 20-30°C (Margesin and Schinner, 1997; BIOWISE, 2000) although biodegradation at 0-10°C has been reported by Coulon et al (2005) and Margesin and Schinner (1997).

Water Availability: - microorganisms carrying out contaminant degradation (or any metabolic transformation) require adequate moisture for their growth and activity (Alexander, 1999; Hogan, 1998). Water also functions as a medium to transport microorganisms, nutrients and hydrocarbons around the soil (Hogan, 1998). An inadequate supply of water can severely restrict biodegradation in surface soils. The optimum moisture content will depend on soil properties, the compound in question, and whether the transformation is aerobic or anaerobic, but a general guideline of 50-70% of a soils water holding capacity is reported by Atagana (2003). The latter is of importance as water displaces oxygen from soil pores, and water logging can cause anaerobic environments to develop as oxygen diffusion through water is 10,000 times slower (Alexander, 1999; Hogan, 1998; Battelle, 1996).

Oxygen Availability: - microorganisms require an electron acceptor to carry out metabolic functions such as metabolism (oxidation) of organic compounds. For

petroleum hydrocarbons, the preferred electron acceptor is oxygen. Although anaerobic degradation of hydrocarbons is possible, such degradation in natural ecosystems proceeds very slowly, if at all (Alexander, 1999). The optimum biodegradation rates of hydrocarbons are aerobic (Alexander, 1999; Vasudevan et al, 2001). The oxygen content of soils depends on soil texture, water content and microbial activity. A low oxygen level in soil has been shown to limit bioremediation of soils contaminated with hydrocarbons (Vasudevan et al, 2001). In general an oxygen content of >2mg l^{-1} is recommended (BIOWISE, 2000).

pH: - at extremes of acidity or alkalinity, microbial activity declines. Biodegradation tends to be fastest at more moderate pH values. Most bacteria are neutrophiles therefore optimum pH is 6.0 to 8.0 (Alexander, 1999; Loehr et al, 2001a; Atagana, 2003), although a pH range of 5.0 to 9.0 is recommended by BIOWISE (2000).

Nutrients: - in addition to a source of carbon, microorganisms also need other nutrients such as nitrogen and phosphorus (macronutrients) (Alexander, 1999), and most often the nutrients that become limiting in hydrocarbon contaminated soils are nitrogen and phosphorus (Atagana, 2003). Nitrogen is an important element in the building of proteins, and nucleic acids (Madigan et al, 2003). Phosphorus is also required for the synthesis of nucleic acids (Madigan et al, 2003). Microorganisms also require much smaller quantities of micronutrients such as magnesium, copper and iron, which are important in the production of enzymes (Madigan et al, 2003).

Sorption, and therefore bioavailability, of nutrients, however, is a problem and can affect the potential growth and activity of microorganisms. However, if nutrients are sorbed to sites of contaminant sorption, such concentration at these sites may enhance the growth and activity of microorganisms, especially if the surrounding solution has a low concentration of nutrients (Alexander, 1999).

Toxicity: - many organic compounds are toxic to microorganisms and can therefore suppress microbial proliferation and metabolism. Petroleum products are mixtures of hydrocarbon compounds, and one or more of the mixture may be inhibitory to hydrocarbon degrading microorganisms (Alexander, 1999). Cyclic hydrocarbons are

reported to be particularly toxic to bacterial membranes (Jorgensen et al, 2000). This can adversely impact the population of contaminant degrading microorganisms.

Predators: - in many environments, as well as bacteria there will be predators or parasites. Protozoa are well known to affect biodegradation – they multiply by feeding on bacteria, and therefore can have a deleterious impact. However they may also facilitate the cycling of limiting inorganic nutrients. The impact of protozoa depends on their grazing rate and the rate of bacterial multiplication. If bacterial multiplication rates exceed those of grazing by protozoa, protozoa will have little or no effect, and vice versa (Alexander, 1999).

2.4 Enhanced Ex-Situ Bioremediation

Ex-situ bioremediation strategies typically involve forming contaminated soils into piles (termed engineered biopiles or windrows) above ground and enhancing biodegradation by stimulating aerobic microbial activity (BIOWISE, 2000; Battelle, 1996). Enhanced biodegradation of petroleum hydrocarbons is primarily achieved by optimising oxygen and nutrient availability (through a strategy commonly referred to as biostimulation), but also involves maintaining optimum moisture content, pH, and temperature (Battelle, 1996). An alternative strategy is that of bioaugmentation whereby microorganisms of known hydrocarbon degrading abilities are added to the contaminated soil (Zhu et al, 2004; Alexander, 1999). Biostimulation and bioaugmentation are discussed in the following sections, with examples of successes and failures for each.

2.4.1 Biostimulation

2.4.1.1 Introduction of Oxygen

Optimum biodegradation rates of petroleum hydrocarbons in soils are associated with aerobic conditions (Rhykerd et al, 1999; Alexander, 1999; Vasudevan and Rajaram, 2001; Huesemann and Truex, 1996; Brar et al, 2006). The oxygen content of soils depends on soil texture, water content and microbial activity (Vasudevan, 2001).

In ex-situ bioremediation contaminated soils are often formed into static engineered biopiles whereby the pile is aerated via a series of perforate pipes which are placed at various depths within the pile (Battelle, 1996; Vik and Bardos, 2002; Quinn and Reinhart, 1997). Aeration via these pipes can be passive (whereby aeration relies on convection currents through the pipes and pile), or forced (whereby air is pumped or sucked through the piles by use of a pump). In the case of passive aeration, bulking agents such as wood chips or sand can be mixed with the contaminated soils to improve oxygen circulation by increasing porosity (Rhykerd et al, 1999; Battelle, 1996).

Vasudevan et al (2001) looked at the effects of bulking agents and microbial inocula on hydrocarbon degradation. They found that inoculation with a bacterial consortium (microbial composition not specified) resulted in 40% degradation compared with 25% for the unamended control. The addition of wheat bran as a bulking agent, enhanced degradation further, with 72% hydrocarbon degradation, compared with the 40% for microbial inoculation. This suggests that the bulking agents enhanced hydrocarbon degradation.

An alternative to engineered biopiles is to form the contaminated soil into windrows and aerate the pile through physical disruption and mixing (tillage) of the material on a regular basis (much in the same way as aerobic composting, as discussed later on in this chapter) (Rhykerd et al, 1999).

To the author's knowledge, few studies have been published which compare the effects of aeration strategies on biodegradation. Rhykerd et al (1999) undertook a series of experiments whereby soils contaminated with petroleum oil (10% TPH) were treated over a period of 30 weeks with different bulking agents (none, sawdust, hay and vermiculite), and underwent either no aeration (static control), tillage or forced aeration.

Figure 2.4 shows the results taken from Rhykerd et al (1999). It can be seen that the addition of bulking agents enhanced TPH degradation when compared to the un-amended control. It can also be seen that forced aeration had little effect on TPH degradation when compared with that for the static control, throughout the 30 week

period. Tillage offered the best enhancement of TPH degradation; however the effect was more on degradation rates rather than TPH end points. Tillage offered only a minor enhancement of TPH end points with approximately 12% TPH remaining after 30 weeks compared with 15-20% for the static control, and 12-20% for the soil treated with forced aeration. More significant effects were seen at Week 12, whereby the combination of tillage with hay offered the greatest enhancement of TPH degradation with approximately 15% remaining after 12 weeks as compared with 35% static control (amended with hay) and 40% forced aeration (amended with hay).

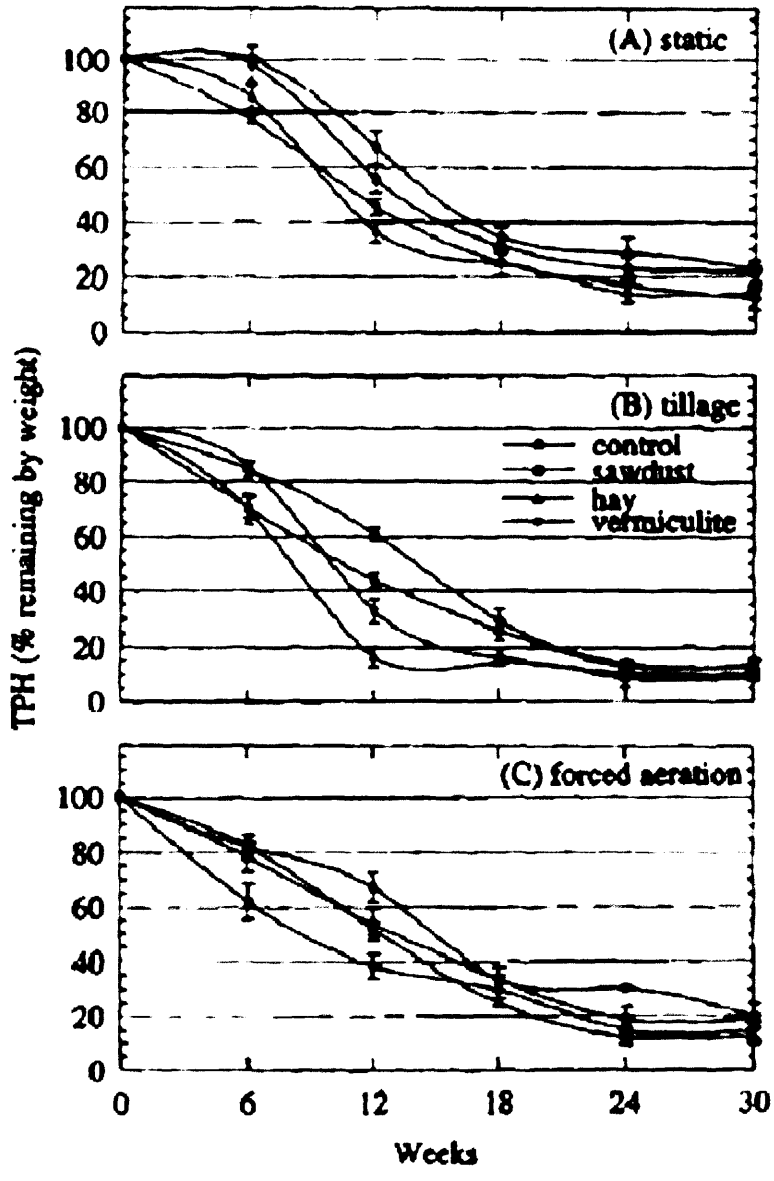


Figure 2.4: Influence of bulking agents on disappearance of TPH from drummed oily waste was (A) left static, (B) tilled once every two weeks, or (C) aerated daily. Initial TPH concentration was 10% in each treatment. Error bars represent standard deviation (Rhykerd et al, 1999).

2.4.1.2 Addition of Nutrients

The supply of nitrogen and phosphorus in soils usually exceeds the needs of indigenous microbial communities. Carbon is usually the growth limiting nutrient in natural environments (Alexander, 1999; Atagana, 2003) as organic matter typically provides the only source of carbon in these environments, and such organic matter is very slowly consumed by microorganisms if at all (Alexander, 1999).

However, where environments are polluted with petroleum hydrocarbons, this situation can be reversed as carbon becomes plentiful, whereas levels of other nutrients (particularly nitrogen and phosphorus) are insufficient to meet demand and therefore become limiting (Alexander, 1999; Atagana, 2003). The addition of nutrients is therefore frequently undertaken to enhance biodegradation, as is discussed in this section.

In the case of biopiles, addition of nutrients is achieved through percolation through the pile, or along a network of internal galleries/pipes (Vik and Bardos, 2002). In the case of windrows, nutrients are physically mixed in with the contaminated soils during tillage. This is considered to result in a more even distribution of nutrients (USEPA, 1994).

Numerous studies have been published in which the addition of nitrogen and/or phosphorus to soils contaminated with petroleum hydrocarbons has enhanced the biodegradation of these compounds. A selection of such studies are summarised in Table 2.3.

Bento et al (2003), however, reported that the addition of nutrients had little effect on the biodegradation of diesel oil in one soil, and in another soil such biostimulation actually reduced biodegradation (Table 2.3). Johnson and Scow (1999) also report that the effects of nutrients on pollutant degradation in soil are inconsistent, and that little effect on contaminant degradation or even a decline in degradation has been reported. It is believed in these instances, that bioavailability of nutrients is the limiting factor (Johnson and Scow, 1999). As mentioned in Section 2.3.4, sorption of nutrients to solid soil fractions is a problem and can reduce their bioavailability to

microorganisms requiring them, which is turn can adversely affect contaminant degradation. Johnson and Scow (1999) also hypothesised that the addition of nutrients may stimulate degradation of carbon compounds other than the desired pollutant (in their study, phenanthrene) or stimulate the proliferation of non hydrocarbon degrading microorganisms at the detriment of the hydrocarbon degrading microorganisms.

Rahman et al (2003) spiked soil with 10% and 20% refinery sludge (containing 87% oil and grease) and added an NPK fertiliser (nitrogen, phosphorus, potassium; unspecified quantities) to determine the effects of nutrient additions on hydrocarbon degradation as compared with an un-amended soil control. Hydrocarbons were separated according to carbon number. The results from this study are presented in Figure 2.5.

Rahman et al (2003) concluded that the addition of nutrients had a larger effect on hydrocarbon degradation when there was only 10% refinery sludge than when there was 20%. They concluded that this likely reflected a toxic effect of the higher hydrocarbon concentration on microorganisms, thus degradation was slower. This demonstrates that high concentrations of hydrocarbons may inhibit microbial degradation, as was discussed in Section 2.3.4.

It is evident from the case studies summarised in Table 2.3 that the addition of nutrients to hydrocarbon contaminated soils often results in enhanced hydrocarbon biodegradation when compared against un-amended controls. The increase in degradation seen in Table 2.3, however, is inconsistent, and varies between 4% (Sabaté et al, 2004) and 90% (Carmichael and Pfaender, 1997). In the work of Sabaté et al (2004), it is apparent that the effect of nutrient addition was to reduce the lag phase (time period during which biodegradation does not occur; Alexander, 1999) rather than to reduce the end point achieved. This could prove advantageous when there are time constraints on site remediation (for example due to site redevelopment deadlines). In the case of Breedveld et al (2000), the addition of nutrients not only enhanced PAH degradation, but also resulted in the biodegradation of 4 ring PAH compounds (which are deemed to be less biodegradation as discussed in Section 2.2.1) when compared against un-amended controls. This indicates that the addition

of nutrients can, in some cases, not only enhance the degradation of hydrocarbons, both in terms of time and endpoints, but also enhance the degradation of more persistent hydrocarbons, which may present a risk to human health and the environment in the long term.

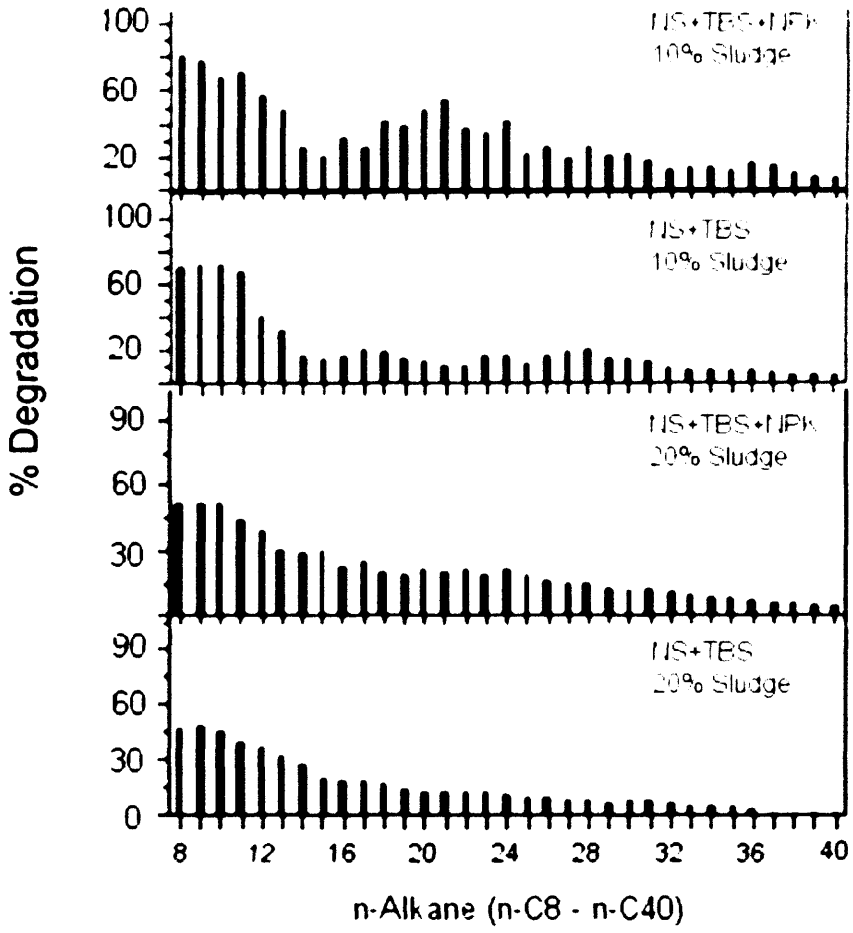


Figure 2.5: n-Alkane (nC_8 - nC_{40}) degradation with 10% and 20% of refinery sludge (NS+TBS) and nutrient (NS+TBS+NPK) on 56th day of treatment (adapted from Rahman et al, 2003).

However, it is also apparent from this section that the addition of nutrients does not always enhance hydrocarbon biodegradation. This could reflect inappropriate nutrient levels (i.e. concentration and balance), ineffective methods of introducing and distributing the nutrients and therefore low bioavailability of nutrients, and/or the occurrence of other limiting factors, for example environmental conditions (pH, moisture content, temperature), unsuitable microbial consortia, low compound

biodegradability and bioavailability. It is therefore clear that treatability studies should be undertaken prior to large scale bioremediation works, to reduce any cost implications of potential failure of this strategy.

Table 2.3: Nutrient additions - successes and failures.

Successes		
Author(s)	***Experiment description***	***Result***
Margesin et al, 2000	Soil was spiked with 5,000ppm diesel oil, and fertiliser (15% nitrogen, 5.5% phosphorus and 12% potassium) was added. Comparisons were made with an un-amended control.	Addition of fertiliser gave 88% diesel oil degradation over 88 days, compared with 72% for the control.
Coulon et al 2005	Spiked soil with crude oil (3%) or diesel (2.7%) and added Inipol EAP 22 commercial fertiliser Comparisons were made with unfertilised controls. Experiments lasted for 6 months, at 10°C. The effect of temperature was also investigated; see Section 2.6.4.	Addition of the fertiliser enhanced crude oil degradation with 12% remaining after 6 months, compared with 17% control; diesel degradation was also enhanced with 10% remaining after 6 months compared with 15% for the control.
Vasudevan et al, 2001	Added inocula, and inocula + inorganic nutrients (ammonium nitrate and dipotassium hydrogen phosphate) to soil spiked with oil refinery sludge to a level of 5% w/w.	Found that the addition of nutrients caused an additional 25% oil degradation over 90 days, (65% inocula + nutrients vs. 40% inocula only).
Sabaté et al 2004	Nutrients (NH_4NO_3 NS K_2HPO_4) were added to soil TPH contaminated soil (12,000ppm) to give a C:N:P ratio of 100:10:1. Comparisons were made with a control comprising aerated TPH contaminated soil. The experiments lasted for 360 days.	The addition of nutrients enhanced TPH degradation with 60% reduction with 50% reduction by Day 60, compared with 56% by Day 270 for the control. Found that the addition of nutrients encouraged degradation from day 1 whereas degradation was not seen in the control for the first month.
Carmichael and Pfaender 1997	Four soils were spiked with pyrene and a nutrient buffer (containing Na_2PO_4, K_2HPO_4, NH_4CL and $MgSO_4$) was added. Comparisons were made with an un-amended control.	Addition of the nutrient buffer enhanced pyrene degradation by 50-90% when compared with the un-amended control. When the nutrients were added separately it was found that phosphorus had no effect on pyrene degradation, suggesting that nitrogen was the most important nutrient in the buffer.
Riffaldi et al, 2005	Spiked soil with 10000ppm diesel oil, and treated with inorganic nutrients $(NH_4)H_2PO_4$ and NH_4NO_3 to give a C:N ratio of 4:1. Comparisons were made with an un-amended control. Experiments comprised 1kg batches, which were incubated at 25°C for 100 days.	Addition of inorganic nutrients gave a total diesel oil degradation of 84% over 100 days, compared with 49% for the control.

Table 2.3 continued...

Successes		
Author(s)	***Experiment description***	***Result***
Breedveld et al 2000	Added nitrogen (200mg NH_4^+ l^{-1}) and phosphorus (50mg PO_3^{4-} l^{-1}) to two soil types (topsoil and aquifer sand) contaminated with creosote (approx 6000ppm and 325ppm respectively). Comparisons were made with a control comprising creosote contaminated soil which was aerated and moisture added.	The addition of nutrients enhanced degradation of 4-ring PAH compounds in both soil types, whereas aeration only enhanced degradation of 2 and 3 ring PAH compounds. Pyrene concentrations fell by 50% and 90% for topsoil and aquifer sand respectively when nutrients were added, whereas concentrations increased by 30% for the topsoil, and fell by only 15% for the aquifer sand for the control. Similar relationships were found for fluoranthrene, benzo(a)anthracene and chrysene.
Straube et al, 2003	Added 2% dried blood (nitrogen) (w/w basis) to soil contaminated with 13,000ppm creosote. Experiments lasted for 11 months.	Addition of nitrogen resulted in 34% creosote degradation compared with 23% for un-amended control.
Atagana, 2003	Added mono-ammonium-phosphate fertiliser (1g kg^{-1}) to soil contaminated with 260g kg^{-1} creosote. Experiments were based on 25kg batches and lasted for 16 weeks.	Addition of fertiliser resulted in 62.9% creosote degradation compared with 58.4% for the unamended control.
Failures		
Author(s)	***Experiment description***	***Result***
Johnson and Scow, 1999	Spiked four soils with 50µg kg^{-1} ^{14}C labelled phenanthrene. Nitrogen (4.7mg kg^{-1} NH_4NO_3) and phosphorus (47mg kg^{-1} KH_2PO_4 and K_2HPO_4) were added, and comparisons were made with an un-amended control.	Nutrient additions did not result in faster phenanthrene biodegradation rates in any of the four soils.
Rahman et al, 2003	Spiked soil with 10% and 20% refinery sludge (with 87.4% oil and grease). Nutrients (NPK fertiliser – unspecified) were added and compared with un-amended controls. Experiments were 56 day duration.	Found that the NPK fertiliser had little effect on hydrocarbon degradation for the 20% refinery sludge content, but had more effect for the 10% refinery sludge content.
Bento et al, 2003	Evaluated the effect of biostimulation (250mg kg^{-1} $(NH_4)_2SO_4$, and 100mg kg K_2HPO_4) on diesel oil (C_{12}-C_{23}, C_{23}-C_{40}) degradation in two soils (A; 2800ppm C_{12}-C_{23}, 9,450ppm C_{23}-C_{40}; and B; 3,300ppm and 7,450ppm) naturally contaminated with diesel oil.	Addition of nutrients had no effect on degradation compared to the control (45.5% vs. 47.2% control) for Soil A and seems to have reduced degradation for Soil B, 16% vs. 23.3% for light fraction, and 6.2% vs. 7.5% for heavy fraction.

2.4.2 Bioaugmentation

An alternative bioremediation strategy to biostimulation is bioaugmentation (Zhu et al, 2004; Alexander, 1999). The principle behind bioaugmentation is that when indigenous microorganisms are unable, or of insufficient cell number, to degrade a hydrocarbon compound, the introduction of microorganisms known to have compound degrading abilities may enhance biodegradation (Alexander, 1999; Atlas, 1995; Zhu et al, 2004).

Commercial bioaugmentation products are available (Simon et al, 2004). The standard way of obtaining a microbial population for subsequent inoculation is to prepare an enrichment culture, whereby microorganisms are cultivated in the laboratory using the contaminant of interest as the sole source of carbon (Alexander, 1999).

Bioaugmentation is generally not practiced in the field, mostly because it has an unreliable performance record (Thompson et al, 2005; Parsons, 2004) and it is often thought that introduced microorganisms cannot compete with well adapted, indigenous microbial communities (Kaplan and Kits, 2004; Parsons, 2004; Zhu et al, 2004; Atlas, 1995). Also, inocula prepared through cultivation methods are done so under controlled conditions whereby nothing is limiting; this does not exist in natural environments (Alexander, 1999).

Bioaugmentation has, however, been widely investigated. Examples of successes and failures of bioaugmentation in enhancing hydrocarbon biodegradation are summarised in Table 2.4. However, it must be emphasised that such investigations are often based on small scale laboratory studies. Such studies are easier to control than natural contaminated environments, thus where such studies show success of bioaugmentation, it is often found to be unsuccessful in the field (Alexander, 1999; Simon et al, 2004).

The work of Bento et al (2003) highlights the importance of the ability of introduced microorganisms to survive and compete with indigenous microorganisms. Bento et al (2003) concluded that the best bioaugmentation effects can be achieved by the use of

hydrocarbon degrading microorganisms which are already present in the soil. Bioaugmentation in this sense results in increasing the abundance of indigenous hydrocarbon degrading microorganisms rather than that of non-indigenous species. Thompson et al (2005) also states that it is most likely better to increase the abundance of a microbial strain that is already prevalent in an environment as it is more likely to persist as an inoculum than one that is non-indigenous to the environment. The failures of bioaugmentation may reflect mistakes made during the initial strain selection step as up until now this has been focussed on microorganisms which are catabolically competent with little or no consideration for other essential features that are required for the microorganisms to be functionally active and persistent in target habitats (Thompson et al, 2005).

However, Venosa et al (1996) found that increasing the abundance of indigenous microorganisms may not always result in enhanced bioremediation. This highlights the importance of pre-adaptation of indigenous microorganisms to the pollutant in question. In the case of sudden polluting activities such as oil spills, indigenous microorganisms may not have had time to adapt to the 'new' carbon source, therefore increasing their abundance may have little effect. In the case of aged contaminated soils, indigenous microorganisms may have adapted to the presence of such contaminants, therefore increasing their abundance may well be successful in enhancing biodegradation.

Jorgensen et al (2000) found that species of a microbial inocula added to a hydrocarbon contaminated soil could not be isolated by traditional culturing methods, suggesting that they could not compete with indigenous microorganisms. However, this latter finding may also be explained by other unfavourable conditions such as nutrient availability and threshold contaminant concentration. Jorgensen et al (2000) concluded that it is more important to create suitable conditions for indigenous microorganisms than to introduce new species.

Table 2.4: Successes and failures of bioaugmentation.

Successes		
Author(s)	***Experiment description***	***Result***
Rahman et al, 2003	Spiked soil with 10% and 20% refinery sludge (containing 87.4% oil and grease), and added microbial inocula (containing *Micrococcus sp, Bacillus sp, Corynebacterium sp, Flavobacterium sp* and *Pseudomonas sp*. Comparisons were made with an un-amended soil control.	The microbial inocula enhanced the degradation of all hydrocarbon fractions (C8-C40) to various degrees (Figure 2.6) for both 10% and 20% refinery sludge contents over 56 days.
Vasudevan et al 2001	Added commercial inocula (unspecified) to TPH contaminated soil/sludge, based on 5kg microcosms incubated at 30°C for 90 days.	Inoculation enhanced TPH degradation giving 40% degradation compared with 25% for un-amended soil/sludge over a 90 day period.
Straube et al, 2003	Added *Pseudomonas aeruginosa* and nitrogen (2% dried blood (w/w)) to soil contaminated with 13,000ppm creosote. Experiments lasted for 11 months.	Addition of microbial inoculum together with nitrogen resulted in 86.6% creosote degradation, compared with 34.3% for nitrogen additions only, and 23% for un-amended control.
Atagana, 2003	Added microbial inocula (unspecified) to soil contaminated with 260g kg^{-1} creosote. Experiments were based on 25kg batches and lasted for 16 weeks.	Addition of microbial inocula resulted in 89% creosote degradation compared with 58.4% for the un-amended control.
Failures		
Author(s)	***Experiment description***	***Result***
Bento et al 2003	Took two diesel oil contaminated soils, A and B, (both soils received 250pm $(NH_4)_2SO_4$ and 100ppm K_2HPO_4) and added inocula previously isolated from Soil A.	Found that inoculation + biostimulation caused 75% degradation in Soil A vs. 45% biostimulation only, but no enhancement in Soil B.
Jorgensen et al 2000	Added two commercial inocula (A and B) to petroleum hydrocarbon contaminated soil which already had nutrients added.	No significant effect was observed, 72% inocula + nutrients, versus 67% nutrients only for inocula A, and 62% versus 67% for inocula B.
Sabaté et al 2004	Added nutrients and nutrients + inocula to TPH contaminated soil.	Found that the addition of nutrients and inocula gave no additional enhancement than nutrients alone (both gave 60% degradation vs. 56% un-amended control) thus suggesting that inoculation did not work.

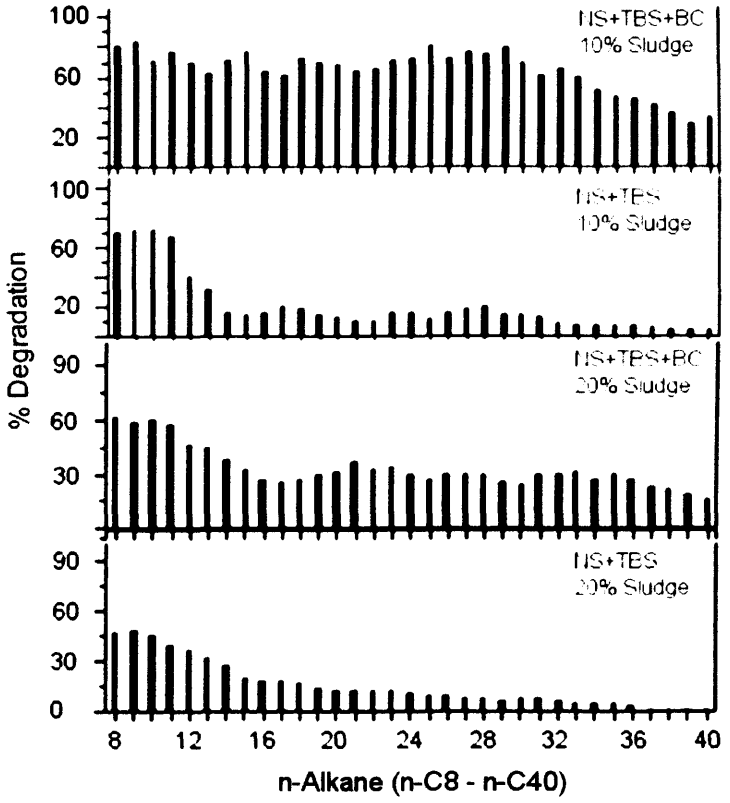


Figure 2.6: n-Alkane (nC_8 - nC_{40}) degradation with 10% and 20% of refinery sludge (NS+TBS) and microbial consortia (NS+TBS+MC) on 56th day of treatment (adapted from Rahman et al, 2003).

Failure of bioaugmentation in the field may reflect engineering difficulties associated with delivering the inocula, both due to problems with the transportation of viable microorganisms to a site, and with the actual application and uniform distribution of them onto the contaminated soils (Simon et al, 2004; Parsons, 2004; Zhu et al, 2004).

2.5 Composting Bioremediation

2.5.1 Introduction

Composting bioremediation is a strategy whereby soils contaminated with organic compounds are mixed with organic wastes and composted together, usually in windrows (Jorgensen et al, 2000; Semple et al, 2001; Beaudin et al, 1999). The rationale behind this strategy is that organic wastes such as livestock manure are

known to be rich in essential nutrients, and to harbour diverse microbial communities (Gestel et al, 2003; Namkoong et al, 2002; Barker and Bryson, 2002). Their addition to soils contaminated with petroleum hydrocarbons could therefore potentially enhance their bioremediation though biostimulation and bioaugmentation (Jorgensen et al, 2000; Barker and Bryson, 2002).

Standard composting management practices (pile aeration, moisture content control and nutrient balance) are applied during the composting bioremediation process (Hogan, 1998). The goal of such practices is to optimise environmental conditions for microbial growth and activity. An overview of composting and its management is presented below. Composting bioremediation differs to composting of non-hazardous wastes in that, whereas the primary goal of the latter is volume reduction and hygienisation, the goal of composting bioremediation is to maximise the rate and extent of contaminant biodegradation, and reduction of risks posed to human health and the environment (Kirchmann and Ewnetu, 1998; Hogan, 1998).

Of importance to the composting bioremediation process is the contaminated soil to organic waste ratio and pile temperature. These parameters can significantly affect the optimisation of contaminant biodegradation (Ladislao et al, 2005). These parameters are therefore discussed in this section.

The use of composting strategies in bioremediation is a relatively recent development (Semple et al, 2001; Ladislao et al, 2005) and its investigation has increased over the past decade, although published literature remains sparse. Case studies are presented and discussed in this section. The author has identified key gaps in the research, particularly regarding the chemical, biological and toxicological processes involved with composting bioremediation. The need for further research, and the basis of this study, is discussed further later on in this chapter.

2.5.2 Composting

Composting is a natural process whereby organic materials are decomposed by microorganisms. Composting relies on the growth and activity of microorganisms and as such decomposition rates can be increased by optimising conditions for

microorganisms, for example through nutrient balance (C:N ratio), and the addition of moisture and oxygen (USEPA, 1994). In this sense, composting is defined as the 'controlled microbial decomposition of organic matter' (USEPA, 1994). Composting can occur under aerobic or anaerobic conditions, but aerobic composting is much faster (USEPA, 1994). A generalised equation (taken from Peigne and Girardin, 2004) for aerobic composting is as follows:

Fresh organic matter + oxygen $\rightarrow$ *humus like substances* + CO_2 + H_2O + *heat*

Equation 2.3

The simplest composting is carried out in windrows (material is arranged into long piles of approximately 1.5-2m in height) (Burton and Turner, 2003). Pile management strategies are typically adopted whereby moisture content is optimised, and the piles are turned (typically using tractors), mainly to supply adequate oxygen (Burton and Turner, 2003). Turning also acts to promote uniform decomposition of composting materials as cooler outer layers of the compost pile are moved to inner layers where they are exposed to higher temperatures and more intensive microbial activity (USEPA, 1994). It is recognised by Korda et al (1997) that microbial populations often have a patchy distribution in soils, and that this likely reflects uneven distribution of growth factors such as nutrients, oxygen and moisture. Turning/mixing processes aids the even distribution of hydrocarbon degrading microorganisms and growth factors. As well as accelerating decomposition, pile management strategies also aim to minimise any environmental problems (such as eutrophication, odours, leachate production) that may ensue (USEPA, 1994).

The composting process is self heating as the microbial decomposition of organic matter generates heat (see Equation 2.3 above). The temperature of the composting pile is therefore related to microbial activity. The composting process can be subdivided into four main phases based on temperature (Potter et al, 1999; Šašek et al, 2003). The first is the mesophilic phase whereby the active microbial population within the pile is dominated by mesophilic microorganisms which operate at temperatures of between 10 and 45°C. These microorganisms decompose readily available organic matter, and in doing so generate heat (Peigne and Girardin, 2004).

If the pile is sufficiently large it will have insulation capacity and the heat will become trapped. The greatest microbial diversity has been observed in the mesophilic stage (Potter et al, 1999; Šašek et al, 2003). As temperatures rise above 45°C, thermophilic microorganisms take over, and the 'thermophilic phase' begins. This phase is said to be the most active phase of composting, where decomposition rates are at their highest, and can last from several days up to several weeks (Peigne and Girardin, 2004). The thermophiles continue decomposing the feedstock materials as long as growth factors are available. As temperatures rise above 60°C (Peigne and Girardin, 2004) and resources are used up, the thermophiles begin to die, pile temperatures drop and the 'cooling phase' begins (USEPA, 1994). This phase is dominated by mesophiles. The final phase is the 'maturation phase' during which pile temperatures fall to ambient temperatures (Peigne and Girardin, 2004).

2.5.3 Composting Bioremediation – Case Studies

Published examples of investigations into the composting bioremediation of soils contaminated with petroleum hydrocarbons are summarised in this section. The first four examples are of the use of poultry manure as the organic amendment. The amendments used in the remaining five examples vary, and include pig manure, horse manure, sewage sludge, biowaste and green waste. The experimental design of these examples also varies, and unfortunately Kirchmann and Ewnetu (1998) and Wong et al (2002) did not undertake an un-amended control. Therefore the effect of composting bioremediation on hydrocarbon degradation can only be inferred for these examples.

Example 1:

Ijah and Antai (2003) investigated the potential use of chicken droppings on the biodegradation of crude oil. Soil was spiked with 10% crude oil, and mixed with chicken droppings (collected with no bedding material, sun dried and ground) with a soil to amendment ratio of 10:1. Comparisons were made with an un-amended soil control. The experiments were incubated at 28 ± 2°C for 16 days. They found that the addition of chicken droppings gave a total crude oil degradation of approximately 68% over 16 days, compared with approximately 51% for the un-amended soil, thereby indicating that the addition of chicken droppings was successful in enhancing crude oil

degradation. Ijah and Antai (2003) also investigated the potential growth and utilisation of crude oil by bacteria from the chicken droppings through simple enumeration studies. Serial dilutions of the chicken droppings were spread inoculated onto petri dishes (growth plates) containing nutrients and crude oil as the sole carbon source. They found that out of 25 bacterial isolates identified, 12 were shown to grow on and utilise crude oil. These bacteria were identified as the genera *Pseudomonas*, *Bacillus*, *Micrococcus*, and *Proteus*.

Example 2:

Ibekwe et al (2006) investigated the potential use of poultry manure as a source of nutrients to enhance the bioremediation of soils contaminated with petroleum hydrocarbons. Soil was spiked with 10g kg^{-1} crude oil and four microcosms were established as follows: 100g soil + 30g manure, 100g soil + 60g manure, 100g soil + 90g manure, 100g soil only (control). The experiments were carried out and monitored over 7 weeks. It was found that the addition of 30g manure resulted in a 40% reduction of crude oil, 60g manure resulted in a 45% reduction of crude oil, and 90g manure resulted in a 49.5% reduction of crude oil, whereas only 29.5% reduction of crude oil was seen for the un-amended control. These differences were found to be statistically significant ($p<0.001$). Ibekwe et al (2006) therefore concluded that the addition of poultry manure enhanced crude oil biodegradation through biostimulation, and the larger the quantity of poultry manure added, the greater the degradation of crude oil.

Example 3:

Atagana (2003) investigated the use of various organic amendments (sewage sludge, cow manure and poultry manure; at a soil: waste ratio of 9:1) on the enhanced biodegradation of soil contaminated with creosote (260g kg^{-1}). The experiments were based on 25kg batches and lasted for 16 weeks. They found that the addition of sewage sludge resulted in 86% reduction of creosote, cow manure resulted in 85% reduction in creosote and poultry manure resulted in 84.5% reduction in creosote, compared with 58.4% reduction seen for the un-amended control. Atagana (2003) concluded that there was no statistical difference ($p=0.05$) between the organic amendments, but that the increase in creosote degradation seen compared to the un-amended control is significant.

Example 4:

Atagana (2004b) then investigated the use of poultry manure to enhance the bioremediation of soil contaminated with creosote (>30,000 mg kg^{-1}) at a soil to waste ratio of 4 parts contaminated soil + wood chips to 1 part poultry manure. The experiments were much larger for this investigation, and were based on 350kg batches and lasted for 19 months. Changes in PAH concentration were monitored and the results are presented in Tables 2.5 and 2.6. PAH degradation was significantly faster when poultry manure was added, and all PAHs were degraded to ≤1mg kg-1, including 4 ring PAHs such as chrysene, pyrene and fluoranthene, and the 5 ring PAH of benzo(a)pyrene. Such degradation endpoints were not attained in the un-amended control.

Example 5:

Namkoong et al (2002) investigated the effects of sewage sludge additions on the biodegradation of diesel oil contaminated soil (based on soil spiked with 10000mg kg^{-1} diesel oil). Various amendment ratios of (soil: sewage sludge; wet weight basis) 1:0.1, 1:0.3, 1:0.5, 1:1 were investigated and comparisons were made with an un-amended control. Experiments were carried out under a controlled temperature of 20°C over 30 days. The addition of sewage sludge enhanced TPH degradation with a total percentage degradation of 86% to 98% depending on soil: sewage sludge ratio (Table 2.7), compared with 65% total degradation for the soil control. Namkoong et al (2002) concluded that the addition of sewage sludge enhanced biodegradation; however, excessive quantities of the amendment retarded biodegradation. The soil to amendment ratio must therefore be optimised.

Table 2.5: Changes in PAH concentration (mean of 3 replicates; mg kg^{-1}) during amended composting (taken from Atagana, 2004b).

Months	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Naphthalene	159	120	65	0	0	0	0	0	0	0
Anthracene	72	62	34	1	1	1	0	0	0	0
Phenanthrene	257	201	101	3	1	1	1	0	0	0
Fluorene	68	56	12	4	3	2	1	1	1	1
Pyrene	182	149	135	95	87	40	15	8	5	4
Chrysene	93	80	69	64	58	44	35	23	15	8
Fluoranthene	189	165	115	110	105	54	24	5	2	1
Benzo(a)pyrene	68	61	42	38	34	26	13	9	4	3
Months	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Naphthalene	0	0	0	0	0	0	0	0	0	0
Anthracene	0	0	0	0	0	0	0	0	0	0
Phenanthrene	0	0	0	0	0	0	0	0	0	0
Fluorene	1	1	1	1	1	1	0	0	0	0
Pyrene	2	1	1	1	0	0	0	0	0	0
Chrysene	5	4	4	4	4	3	1	1	1	1
Fluoranthene	0	0	0	0	0	0	0	0	0	0
Benzo(a)pyrene	2	1	1	0	0	0	0	0	0	0

Table 2.6: Changes in PAH concentration (mean of 3 replicates; mg kg^{-1}) during un-amended composting (taken from Atagana, 2004b)

Months	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Naphthalene	159	123	115	111	96	90	88	85	81	79
Anthracene	72	68	64	61	59	56	53	53	51	51
Phenanthrene	257	219	210	208	205	203	202	201	200	198
Fluorene	68	58	57	56	56	55	55	54	54	53
Pyrene	182	153	146	138	135	133	130	124	112	107
Chrysene	93	84	79	79	78	78	78	76	72	70
Fluoranthene	189	169	159	137	129	123	115	110	107	104
Benzo(a)pyrene	68	64	58	55	54	52	50	49	48	47
Months	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Naphthalene	76	73	68	65	63	61	60	59	59	58
Anthracene	50	47	45	42	42	42	41	41	41	40
Phenanthrene	195	193	193	191	191	190	189	188	188	188
Fluorene	51	48	47	46	45	44	43	43	42	41
Pyrene	106	102	101	99	98	96	93	92	90	88
Chrysene	68	66	65	63	61	59	57	56	56	55
Fluoranthene	101	99	98	98	97	96	96	96	95	95
Benzo(a)pyrene	46	45	44	42	41	39	39	38	38	38

Table 2.7: Percentage TPH degradation over 30 days at 20°C. Taken from Namkoong et al (2002).

Mix Ratio (soil: sewage sludge)	Total % Degradation
1:0.1	86.0
1:0.3	98.1
1:0.5	98.1
1:1	94.6
Soil only	**64.5**

Example 6:

Gestel et al (2003) investigated the effect of biowaste (vegetable, fruit and garden waste) on the biodegradation of diesel oil. Three experiments were conducted; 1) 90kg biowaste was mixed with 10kg diesel contaminated soil (6g kg-1 diesel oil) and composted in a composting bin (named C+); 2) 10kg diesel contaminated soil incubated at room temperature (named S-RT), and 3) 10 kg diesel contaminated soil incubated under the same temperatures as the composting material (named S-CT). They found that total diesel oil degradation was 85% for both C+ and S-CT, whereas total diesel oil degradation for S-RT was 35%. Gestel et al (2003) concluded that temperature rather than composting related factors (nutrients, organic matter, and microbial diversity) played the key role in diesel oil degradation in this study. The observed pile temperatures for the C+ treatment rose from approximately 30°C on Day 1 to 75°C on Day 9, and then steadily fell to approximately 30°C by Day 20. However, it could be argued that the addition of organic waste caused the rise in temperature, and therefore the enhanced degradation seen.

Example 7:

Kirchmann and Ewnetu (1998) investigated the use of horse manure as a co-composting material to enhance the biodegradation of petroleum refinery sludge (32% oil content (wet basis); 608g kg^{-1} (dry weight basis) aliphatic hydrocarbons and 78.5g kg^{-1} (dry weight basis) aromatic hydrocarbons). Refinery sludge was added to horse manure to form 2.1% or 7.1% of the total material quantity (dry matter basis) and composted in composting bins. The composting bin receiving 7.1% refinery sludge received repeated additions of horse manure on days 61 and 128 (corresponding to 15% and 8% of the initial mass respectively). The duration of the two experiments differed (117 days for the 2.1% refinery sludge content, and 154 days for the 7.1% refinery sludge content) therefore direct comparison is complicated. Results (Table 2.8) are therefore discussed here based on hydrocarbon concentrations determined on Day 96 for the 2.1% content, and Day 100 for the 7.1% content, as these sampling points are the closest.

Total oil concentration was degraded by approximately 66% and 85% for the 2.1% and 7.1% sludge contents respectively. In addition, it was found that aliphatic hydrocarbons (despite an initial rise in concentration during the first 2 weeks, which

Kirchmann and Ewnetu (1998) reported as reflecting the faster decomposition of horse manure than hydrocarbons, thereby showing an 'false' increase in hydrocarbon concentration), were degraded by 59% and 85% for the 2.1% and 7.1% sludge contents respectively. Aromatic hydrocarbons were seemingly degraded by 85% for the 2.1% sludge content (although values fluctuated throughout the experiments thereby this apparent degradation may be erroneous; Table 2.8) and by 0% for the 7.1% sludge content. Kirchmann and Ewnetu (1998) concluded that composting of horse manure together with oil wastes was found to be a suitable environment to achieve a microbial oil breakdown and that repeated additions of horse manure enhanced oil decomposition. This infers an enhanced biodegradation effect of horse manure additions.

However, Kirchmann and Ewnetu (1998) made no comparison with an un-amended soil control; therefore the direct effect of horse manure on hydrocarbon degradation cannot be determined. Also as recognised in Section 2.4, the contaminant concentration can have an effect on the bioavailability of the contaminant and on the growth and activity of microorganisms degrading that contaminant. Kirchmann and Ewnetu (1998) did not address these issues in their experiments, therefore the higher hydrocarbon degradation seen when horse manure was repeatedly added may partially or wholly reflect the higher hydrocarbon concentration. Also, as seen in Table 2.8, pile temperature was inconsistent between the two treatments, with the 7.1% sludge content attaining 40°C whereas the 2.1% sludge content only attained 30°C (although it is unclear from the data presented in Table 2.8 how long these temperatures were sustained for). Temperature can have an effect on hydrocarbon degradation, as is discussed in Section 2.6.4. The repeated additions of horse manure appeared to affect pile temperature, which rose following each addition (Table 2.8). This may reflect increased microbial activity which in turn causes increases in pile temperature. Therefore it could be argued that the addition of horse manure enhanced conditions for microbial growth and activity, thereby likely enhancing biodegradation. However, it is not clear if the additional dilution effect of adding more horse manure was taken into consideration when reporting hydrocarbon concentrations recorded following these additions.

Table 2.8: Hydrocarbon degradation for 2.1% and 7.1% Sludge Content (adapted from Kirchmann and Ewnetu, 1998).

Day	Temperature (°C)	Total Oil Concentration (g kg^{-1} DM)	Aliphatic Hydrocarbon Concentration (g kg^{-1} DM)	Aromatic Hydrocarbon Concentration (g kg^{-1} DM)
Addition of 2.1% petroleum refinery residues:				
0	15	20.7	14.6	6.1
14	30	34.7	30.0	4.7
33	25	24.7	17.1	7.6
55	24	15.6	14.6	1.0
75	24	16.9	16.4	0.5
96	23	7.0	6.0	1.0
117	22	13.0	2.0	8.0
Addition of 7.1% petroleum refinery residues (repeated horse manure additions)				
0	15	71.5	70.5	1.0
12	30	82.1	81.1	1.0
33	25	27.1	26.1	1.0
54	24	33.2	32.2	1.0
61*	25	n/a	n/a	n/a
70	40	8.5	6.4	2.1
100	25	10.9	10.0	0.9
128*	25	n/a	n/a	n/a
131	35	12.3	10.3	2.0
154	25	6.9	5.9	1.0

** repeated addition of horse manure at 15% and 8% respectively.*
DM – dry matter

Example 8:

Wong et al (2002) investigated the effects of varying quantities of pig manure on the biodegradation of three PAH compounds (phenanthrene, anthracene and pyrene). Soil was spiked with 100mg kg^{-1} of each PAH compound, and mixed with varying amounts of pig manure; 12.5%, 25% and 50%. Experiments were based on 4kg (total weight) microcosms with 50 day duration. It was found that an increase in pig manure content from 12.5% to 25% gave an increase in total PAH degradation from approximately 85% to approximately 95%. No further increase in PAH degradation was gained with a higher pig manure content of 50%. Unfortunately, an un-amended control was not undertaken; therefore any enhanced biodegradation of PAHs through the addition of pig manure can only be inferred by these results. Wong et al (2002) concluded that a pig manure content of 25% (soil to waste ratio of 3:1) gave optimum PAH degradation.

Example 9:

Ladislao et al (2005) investigated the effect of various contaminated soil (aged coal tar soil) to green waste amendment ratios of 0.6:1, 0.7:1, 0.8:1 and 0.9:1 (dry weight basis) at three incubation temperatures of 38°C, 55°C and 70°C. The experiments comprised 65g (total weight) microcosms and had a duration of 56 days. The green waste amendment was artificially made from foodstuff, sawdust, leaves, grass and wheat straw. The results are presented in Table 2.9. It was concluded that increasing the content of green waste increased PAH degradation. Unfortunately an un-amended control was not undertaken, therefore it can only be inferred that PAH degradation would have been enhanced through the addition of green waste. Ladislao et al (2005) concluded that the optimum PAH degradation was attained at a ratio of 0.8:1 for all incubation temperatures. The effect of temperature on degradation is discussed in Section 2.5.5.

Table 2.9: Percentage PAH removal after 56 days (taken from Ladislao et al, 2005).

Mix ratio (soil : green waste)	38°C	55°C	70°C
0.6:1	61.2%	52.7%	21.7%
0.7:1	66.1%	50.4%	23.2%
0.8:1	75.1%	63.9%	22.6%
0.9:1	72.7%	54.2%	18.6%

2.5.4 Material Combination Ratios

It is important to optimise the ratio of hydrocarbon contaminated soil to organic waste (Ladislao et al, 2005). An inappropriate amendment ratio may retard or inhibit microbial activity, and therefore contaminant degradation (Namkoong et al, 2000). Nutrients (in particular nitrogen) may be toxic to microorganisms when present in excessive quantities (Atagana, 2003; Atagana, 2004a) therefore large doses of organic waste may be detrimental to the bioremediation process. Also, organic wastes may present a preferential source of carbon and energy for the microorganisms, therefore excessive doses of organic waste may retard biodegradation (Potter et al, 1999; Namkoong et al, 2000; Johnson and Scow, 1999).

Of the investigations summarised in Section 2.5.2, only the work of Wong et al (2002), Namkoong et al (2002), and Ladislao et al (2005) investigated variable soil to waste

ratios. A summary of the ratios found to be optimum by these investigations is presented in Table 2.10.

Table 2.10: Optimum Amendment Ratios Determined by Published Investigations

Author	Experiment Set Up	Optimum Ratio (soil: amendment)
Wong et al, 2002	Soils spiked with 100mg kg^{-1} each of phenanthrene, anthracene and pyrene. 4kg microcosms incubated for 50 days at unspecified temperature. Various pig manure contents investigated – 12.5%, 25% and 50% (weight basis unspecified).	3:1 (25% pig manure content)
Namkoong et al, 2002	Soil spiked with 10,000ppm diesel oil. Microcosms incubated at 20°C for 30 days. Microcosm size unspecified. Various soil: sewage sludge ratios investigated; 1:0.1, 1:0.3, 1:0.5, 1:1 (wet weight basis).	1:0.3 1:0.5
Ladislao et al, 2005	Soil naturally contaminated with PAH (100mg kg^{-1}). 65g total weight microcosms, duration of 56 days. Various green waste amendment ratios investigated; 0.6:1, 0.7:1, 0.8:1 and 0.9:1 (dry weight basis) incubated at 38°C, 55°C and 70°C.	0.8:1

The above investigations appear to have been based on randomly selected amendment ratios, with no regard for nutrient balance. Nutrient balance can be vital to microbial growth and activity, as excessive levels of particular nutrients may be toxic to microorganisms (Namkoong et al, 2002). Of particular importance to microbial growth and activity are the nutrients carbon and nitrogen. It is widely recognised in literature regarding composting of organic wastes that a carbon to nitrogen (C:N) ratio of between 20:1 and 30:1 provides optimum nutrient balance for microbial growth and activity (Peigne and Girardin, 2003; Utah State University, 1995; USEPA, 1994).

Although, to the author's knowledge, there appears to be no investigation on the effects of C:N ratio on the bioremediation of hydrocarbon contaminated soil, the work of Atagana (2003) can be used as a basis. Atagana (2003) investigated optimum C:N ratio for the bioremediation of creosote contaminated soil (>250,000mg kg^{-1} creosote). They amended the soil with nitrogen (NH_4NO_3) to attain C:N ratios of 25:1, 20:1, 15:1, 10:1 and 5:1. The results are presented in Table 2.11. It was concluded that optimum creosote degradation was attained at a C:N ratio of 25:1. The highest nitrogen

amendment (C:N 5:1) had a negative effect on creosote degradation when compared to the un-amended control. This could reflect a toxic effect of the nutrient.

Table 2.11: Total creosote degradation in different C:N ratios (taken from Atagana, 2003).

C:N ratio	Percentage creosote removal (%)
25:1	68.7
20:1	61.1
15:1	56.3
10:1	63.8
5:1	33
Un-amended control	**46.6%**

2.5.5 Temperature

As outlined in Section 2.5.1, the composting process is characterised by four phases based on temperature, whereby temperature increases with increasing microbial activity (and therefore decomposition of organic wastes). Such increases in temperature are not normally found in biopiles comprising contaminated soil only.

Elevated temperatures are reported to enhance petroleum hydrocarbon biodegradation, through enhancing hydrocarbon bioavailability mainly through increased solubility and diffusivity (which ultimately enhances mass transfer from sorbed phases to soluble phases within soils) (Gestel et al, 2003; Semple et al, 2001; Feitkenhauer et al, 2003; Coulon et al, 2005). Therefore it is argued that the use of composting strategies for bioremediation should prove advantageous (Semple et al, 2001).

Beaudin et al (1999) investigated the effects of temperature and length of thermophilic phase on the degradation of mineral oil and grease in a weathered hydrocarbon contaminated soil (17,000mg kg^{-1}) during composting bioremediation. The contaminated soil was mixed with a compost (alfalfa, maple leaves, calcium carbonate and mature compost), with the compost forming approximately 63% of the total material (contaminated soil formed 37%). In the first part of the experiment, the microcosms (based on 1 litre capacity jars) underwent a typical composting process

temperature profile with temperatures held at 23°C for 1 day, followed by a 5 day duration thermophilic phase, followed by 5 days whereby temperatures were allowed to gradually fall to 23°C, and then held at 23°C for 19 further days (total experiment duration of 30 days). The temperature of the thermophilic phase was varied, at 23°C, 40°C, 50 or 60°C. Beaudin et al (1999) found that hydrocarbon degradation was optimum at 23°C where total degradation of 56% was achieved, compared with 33% for 40°C, 47% for 50°C, and 23% for 60°C. In the second part of the experiment, the thermophilic phase was held at 50°C, but the duration of this phase was either 5 days or 30 days. Optimum hydrocarbon degradation was achieved under the 30 day duration thermophilic phase with 70% total degradation, compared to 47% for the 5 day thermophilic phase.

Beaudin et al (1999) therefore concluded that optimum total hydrocarbon degradation was achieved when the temperature profile was maintained at 23°C rather than imposing a 5 day thermophilic plateau. However, when thermophilic conditions were maintained for 30 days, hydrocarbon degradation was higher than at 23°C for 30 days. This suggests that higher temperatures achieved during composting can enhance hydrocarbon degradation, as long as temperatures are consistent throughout the contaminant degradation period.

As discussed in Section 2.5.2, Gestel et al (2003) also found that hydrocarbon degradation was enhanced by higher temperatures. They found that total diesel oil degradation was the same when hydrocarbon contaminated soil was composted with biowaste (C+) and when soil was incubated at temperatures (S-CT) matching those attained by the biowaste amended soil (total degradation of 85% for both C+ and S-CT), whereas total diesel oil degradation for soil held at room temperature (S-RT) was only 35%. Gestel et al (2003) therefore concluded that temperature played a significant role in enhanced hydrocarbon degradation.

However, high temperatures were found to have a negative effect on hydrocarbon degradation by Ladislao et al (2005). Three temperatures were investigated, 38°C, 55°C and 70°C. The results are shown in Table 2.9. Ladislao et al (2005) concluded that optimum PAH degradation was attained at a constant incubation temperature of 38°C. They also investigated the effects of a typical composting process temperature

profile, where pile temperatures were held at 38°C for 2 weeks, followed by 55°C for 3 weeks, 70°C for 1 week and finally 38°C for 2 weeks. This latter experiment was conducted with a soil to amendment ratio of 0.8:1. Total PAH degradation was approximately 60% where the temperature profile was imposed, compared to 75% where incubation temperatures were maintained at 38°C, Ladislao et al (2005) concluded that higher temperatures did not always enhance hydrocarbon degradation, and suggested that this reflected suppression of microbial growth and diversity at higher temperatures. Ladislao et al (2005) therefore recommended an extended mesophilic phase for composting bioremediation strategies. Where composting regulations need to be met, thermophilic conditions can be imposed once hydrocarbon degradation has been completed.

2.6 Potential Use of Chicken Manure

Although published investigation into the potential use of chicken manure in composting bioremediation is limited (to the author's knowledge) the information that is presented in this chapter is used in this section to indicate such a potential use. Evidence for and against the use of chicken manure in composting bioremediation is therefore discussed in the following sections.

2.6.1 Evidence For Chicken Manure Additions

The use of chicken manure to enhance the bioremediation of soils contaminated with petroleum hydrocarbons was found to be successful by Ijah and Antai (2003), Atagana (2004b), Atagana (2003) and Ibekwe et al (2006), as discussed earlier. Atagana (2004b) also reports that the enhancement of hydrocarbon degradation by the addition of chicken manure in soil-compost mixtures was previously reported by Hill and McCarty (1967) and Wilson et al (1983).

Chicken manure is known to be rich in nutrients, particularly nitrogen and phosphorus (Nicholson et al, 1996; Ijah and Antai, 2003; Atagana, 2004b). Numerous studies have shown that the addition of nutrients (based on inorganic fertilisers) can successfully enhance the biodegradation of petroleum hydrocarbons, as was

discussed in Section 2.4.1.2. In addition to macronutrients such as nitrogen and phosphorus, chicken manure is also reported to contain a diverse range of micronutrients such as magnesium, calcium and iron (Nicholson et al, 1999) which are reported to be important in enzyme production (Madigan et al, 2003; Mulligan, 2002). In the author's opinion, there is therefore a potential for the use of chicken manure to enhance hydrocarbon biodegradation through biostimulation (addition of nutrients).

Chicken manure is also reported to have a diverse microbial community (Ijah and Antai, 2003; Atagana, 2004b; Ibekwe et al, 2006). Ijah and Antai (2003) identified 12 bacterial isolates (through laboratory cultures) from chicken manure that were able to grow on and utilise crude oil. These 12 isolates were from the genera *Pseudomonas*, *Bacillus*, *Micrococcus* and *Proteus*. It was found that *Pseudomonas aeruginosa* and Bacillus were particularly active in crude oil degradation. These findings are summarised in Table 12 and indicate that there is a potential to use chicken manure to enhance hydrocarbon biodegradation through bioaugmentation. Bioaugmentation has been found successful by many authors as was discussed in Section 2.4.2.

Table 2.12: Extent of growth and degradation of crude oil by bacterial isolates from chicken droppings (taken from Ijah and Antai, 2003).

Bacterial isolates	Growth in crude oil medium	Amount of crude oil degraded (% weight loss)[a]
Pseuodomonas aeruginosa CDB-06	+++	68.5 ± 2.4
Bacillus sp. CDB-08	+++	65.3 ± 2.2
Bacillus sp. CDB-10	++	48.4 ± 3.2
Pseudomonas sp. CDB-15	++	45.6 ± 2.3
Bacillus sp. CDB-18	++	44.7 ± 1.5
Micrococcus roseus CDB-03	++	42.6 ± 1.6
Bacillus sp. CDB-11	+	27.8 ± 2.5
Bacillus sp. CDB-23	+	20.5 ± 1.2
Micrococcus sp. CDB-13	+	18.2 ± 1.8
Micrococcus sp. CDB-16	+	18.2 ± 1.6
Proteus sp. CDB-24	+	18.0 ± 1.4
Proteus sp. CDB-20	+	16.5 ± 2.1

+++ Heavy growth; ++ moderate growth; + little growth
[a] Mean of three determinations

2.6.2 Evidence Against Chicken Manure Additions

It is recognised in the literature that even if nutrients are balanced and available, the contaminant is both biodegradable and bioavailable, and the correct microbial consortia are present, biodegradation may not always proceed. This may be due to a number of reasons, including adverse competition for growth factors, presence of toxins, unsuitable contaminant concentration, and/or presence of a preferential source of carbon (Alexander, 1999). These factors may be established due to the addition of chicken manure, as follows:

1. Adverse competition for growth factors - hydrocarbon degrading microorganisms may have to compete for resources (i.e. nutrients, oxygen and water) with other microorganisms. Although chicken manure has been shown to contain hydrocarbon degrading microorganisms, it also contains a diverse population of non-hydrocarbon degrading microorganisms (Ijah and Antai, 2003). Such competition may retard the growth and activity of the hydrocarbon degrading microorganisms.
2. Presence of toxins - compounds may be present in concentrations which are toxic to hydrocarbon degrading microorganisms, for example nutrients, hydrocarbon compounds, or other compounds present such as trace elements (Namkoong et al, 2002; Alexander, 1999). Chicken manure is reported to have a high nutrient content and trace elements such as barium, calcium, cadmium, copper, iron, manganese, molybdenum, nickel, lead, rubidium, strontium, zinc, selenium, arsenic, chromium, mercury and aluminium (Nicholson et al, 1999; Ihnat and Fernandes, 1996) therefore may present a source of compounds toxic to microorganisms. There is also a potential for chicken manure to present a source of environmental risk in the long term due to the presence of these constituents.
3. Contaminant concentration – the addition of organic waste amendments such as chicken manure has a dilution effect on contaminant concentration. Therefore excessive dilution of contaminant concentration could cause levels to drop below the threshold concentration thereby inhibiting microbial growth and activity, and therefore biodegradation. The levels of chicken manure addition adopted by previous authors were 10% (Ijah and Antai, 2003; Atagana, 2003), 20% (Atagana, 2004b), and 47% (Ibekwe et al, 2006). Of these, only Ibekwe et al (2006)

investigated alternative amendment levels of 23% and 38%. They found that 47% chicken manure additions gave the optimum hydrocarbon degradation. These amendment levels were not reported by the associated authors to be based on nutrient balance (i.e. carbon to nitrogen ratio).

4. Preferential carbon source – chicken manure may present a preferential source of carbon which is easier to metabolise than the contaminant of interest, thereby slowing down or inhibiting contaminant degradation.

Of particular concern to this study is the potential for chicken manure to have a detrimental effect on TPH biodegradation through a) adverse competition for growth factors between hydrocarbon degrading microorganisms and non-hydrocarbon degrading microorganisms, and b) introduction of toxins. The potential dilution of TPH below threshold concentrations is beyond the scope of this study. However, avoidance of adverse effects resulting from dilution of TPH concentration is attempted in this study through nutrient balance (carbon to nitrogen ratio) which is reported to significantly affect microbial growth and activity (Peigne and Girardin, 2003; Utah State University, 1995; USEPA, 1994).

2.7 Discussion

It is clear from the information presented in this chapter that the factors controlling biodegradation are complex and therefore the success or failure of bioremediation strategies is complicated, often site and contaminant specific, and not always predictable (Alexander, 1999; Head, 1998).

There has been extensive investigation into the effects of biostimulation and bioaugmentation on petroleum hydrocarbon biodegradation, with the work of numerous authors discussed in Section 2.4. It is evident that the effects of biostimulation and bioaugmentation on hydrocarbon degradation are inconsistent, which likely reflects the complex array of factors affecting biodegradation discussed in Section 2.3. Therefore the success of bioremediation remains relatively unpredictable.

Composting bioremediation is a relatively new bioremediation strategy and is increasingly receiving attention, as outlined in Section 2.5. However, to the author's knowledge, published investigations remain sparse, and experimental design is inconsistent, particularly with regard to contaminated soil to amendment ratio and concentrations of hydrocarbons studied.

Using the information presented in this chapter it is evident that the addition of chicken manure could enhance biodegradation of petroleum hydrocarbons. There is a potential for either biostimulation or bioaugmentation, or a combination of both effects, due to the nutrient content and presence of hydrocarbon degrading microorganisms within chicken manure. However, as was discussed in Section 2.6.2, there are also many potential disadvantages associated with the addition of chicken manure, which could inhibit or limit enhancement of hydrocarbon biodegradation. Given the risk of increasing the volume of contaminated soil should composting bioremediation fail, the chemical and biological processes involved with composting bioremediation must be understood.

Of particular concern is microbial population dynamics during the composting bioremediation process, and how different microbial populations within chicken manure (i.e. hydrocarbon degraders vs. non hydrocarbon degraders) and microbial communities between chicken manure and contaminated soil, interact with each other. There is a potential for non-hydrocarbon degrading microorganisms to inhibit or limit the growth and activity of hydrocarbon degrading microorganisms through competition for growth factors such as nutrients, moisture and oxygen, thereby negatively affecting hydrocarbon biodegradation. Although published literature has often looked at changes in hydrocarbon degrading microbial communities through the use of enumeration studies, with particular regard to growth of such microorganisms (such as the work of Breedveld et al, 2000; Atagana et al, 2004a; Sabate et al, 2004; Rhykerd et al, 1990), total microbial community dynamics and interactions between microbial communities do not appear to have been assessed so far. Therefore, this thesis aims to address this gap in research concerning composting bioremediation.

In addition to microbial population dynamics, it is evident that the addition of chicken manure could enhance the bioremediation of TPH contaminated soils through

biostimulation and/or bioaugmentation. Differentiation between these two effects has not been undertaken by previous authors (to the author's knowledge). Given the costs likely associated with transportation of chicken manure to a site, and engineering costs associated with the prevention/minimisation of environmental risks associated with chicken manure, the author suggests that such differentiation is necessary to ensure a cost efficient bioremediation strategy is designed. Should any enhanced biodegradation be evident and is solely attributable to biostimulation or bioaugmentation, it may be more cost effective to adopt an alternative bioremediation strategy, i.e. the use of inorganic fertilisers. This thesis therefore aims to differentiate between biostimulation and bioaugmentation effects of the addition of chicken manure during composting bioremediation.

There also appears to be a lack of investigation into the use of organic amendments to enhance the biodegradation of more persistent, high molecular weight hydrocarbons. Of the composting bioremediation investigations reviewed in this chapter, Atagana (2003; 2004b) and Wong et al (2002) studied the effects of composting bioremediation on PAHs of varying ring number. Wong et al (2002) found that the addition of pig manure enhanced the biodegradation of 3 ring PAHs (phenanthrene and anthracene) but not of pyrene (4 ring PAH). Conversely Atagana (2004b) found that the addition of poultry manure enhanced the biodegradation of 2-5 ring PAHs, with all present at ≤1 mg kg-1 after 19 months. The majority of published investigations into composting bioremediation of hydrocarbon contaminated soils were based on total concentration values, rather than fractionated hydrocarbons. The TPHCWG recognise that, in the case of complex hydrocarbon mixtures such as those of petroleum products, changes in total TPH values do not present enough information for risk based remediation strategies. Different hydrocarbon fractions have differing behaviour in soils, differing toxicities and therefore risks. It is therefore important to study the concentrations of hydrocarbon fractions present so as to monitor changes in risk. This thesis aims to determine any effects of the addition of chicken manure in composting bioremediation on the degradation of varying hydrocarbon fractions, with particular interest on the higher molecular weight hydrocarbons.

Of additional interest to this study is the use of laboratory toxicity assays to a) identify any adverse toxic effects on potential hydrocarbon degrading microorganisms (and therefore potential adverse effects on TPH biodegradation) resulting from the addition of chicken manure, and b) to determine the potential success or failure of composting bioremediation in reducing the toxicity (and therefore risk) of oil refinery sludge. The reported use of toxicity tests in either context is limited.

2.8 Conclusions

Bioremediation is increasingly adopted as a remediation strategy for soils contaminated with petroleum hydrocarbons as it is deemed to be sustainable owing to the destruction and degradation of compounds rather than their transportation between environmental compartments (Boopathy, 2000; Semple et al, 2001; Sabaté et al, 2004; Bento et al, 2003; Alexander, 1999; Dobson et al, 2004). However, the bioremediation of soils contaminated with petroleum hydrocarbons is complex and controlled by a number of often site and compound specific factors (Alexander, 1999). Such factors include chemical-biological processes such as compound biodegradability, bioavailability, the presence of compound degrading microorganisms, and the ability of such microorganisms to function under prevailing environmental conditions (Alexander, 1999; Semple et al, 2003; Paton et al, 2003; Brar et al, 2006; Fetter, 1999; Loehr, 2001b); and engineering processes such as soil homogenisation, and introduction of growth factors to enhance environmental conditions for compound biodegradation (Straube et al, 2003; Parsons, 2004; Alexander, 1999).

Composting bioremediation is a relatively new bioremediation strategy, and has been increasingly investigated over the past decade. This strategy involves mixing contaminated soils with organic wastes and composting the mixture (Jorgensen et al, 2000; Semple et al, 2001). Bioremediation of organic compounds (such as hydrocarbons) under this strategy is thought to occur through biostimulation (addition of nutrients) and bioaugmentation (addition of compound degrading microorganisms) due to the nutrient content and microbial diversity of organic wastes (Gestel et al, 2003; Namkoong et al, 2002). Biodegradation is also reported to be enhanced by the

achievement of higher pile temperatures as compared with traditional ex-situ bioremediation strategies such as biopiles. Elevated pile temperatures are thought to improve compound biodegradability and bioavailability (Gestel et al, 2003; Semple et al, 2001; Feitkenhauer et al, 2003; Coulon et al, 2005).

Published investigations into composting bioremediation of soils contaminated with petroleum hydrocarbons are limited (to the author's knowledge) and experimental design of such investigations is inconsistent. The author therefore recognises that further investigation is needed, and proposes chicken manure as a suitable organic waste amendment due to its reported nutrient content (Nicholson et al, 1996) and potential possession of hydrocarbon degrading microorganisms as indicated by the work of Ijah and Antai (2003).

However, given that composting bioremediation ultimately increases the quantity of contaminated material to be dealt with, failure of this strategy could result in higher bioremediation costs in the long term. Two factors of particular concern have been identified by the author as potentially having an adverse effect on the bioremediation of petroleum hydrocarbon contaminated soils. These are the potential proliferation of non-hydrocarbon degrading microorganisms at the expense of hydrocarbon degrading microorganisms due to adverse competition for growth factors between such microbial populations; and the potential toxic effects of nutrients and trace elements present in the chicken manure on microbial growth and activity. Such factors have not been addressed by previous authors (to the author's knowledge) and the author deems it vital that further understanding of these processes is needed to help avoid wrongful choice of composting bioremediation (and the use of chicken manure as an organic amendment) as a remediation strategy.

The author has also identified a gap in the research associated with investigating the effects of composting bioremediation on the degradation of more persistent, high molecular weight hydrocarbons. This requires chemical analysis of hydrocarbon fractions (hydrocarbons with similar physical-chemical properties) rather than total TPH values. Such information enable a more informed risk based approach, and determination of the full effects of composting bioremediation of hydrocarbon degradation.

Of additional interest to the author is the differentiation between potential biostimulation and bioaugmentation effects of the addition of chicken manure to hydrocarbon contaminated soils. Such differentiation has not been undertaken by previous authors (to the author's knowledge) and the author suggests that this is necessary to make an informed, cost effective, choice of composting bioremediation over alternative bioremediation strategies.

The aims of this study, briefly, are therefore to investigate the potential use of chicken manure as a co-composting amendment to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH), with particular attention to the potential enhanced biodegradation of persistent, higher molecular weight hydrocarbons, and to help develop an improved understanding of the biological and toxicological processes involved.

Chapter 3

Treatability Studies – Experimental Design

3.1 Introduction

The success of bioremediation is governed by a complex series of physical, chemical and biological factors, as was discussed in Chapter 2. It is accepted that bioremediation is not suitable for all contaminated sites or indeed for all contaminants. In order to determine the likely success of bioremediation without incurring excessive costs, laboratory scale experiments are typically carried out (Atlas, 1995). These experiments are referred to as *microcosms* or *treatability studies* and involve comparison of soils treated with the proposed bioremediation strategy with untreated soils (un-amended controls).

There are relatively few published investigations into the use of composting bioremediation strategies to enhance the biodegradation of petroleum hydrocarbons, as was identified in Chapter 2. There is also a lack of investigation into the use of chicken manure for this purpose. The focus of this study is therefore to demonstrate the potential use of chicken manure as a co-composting material to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH), particularly of higher molecular weight hydrocarbon compounds which are deemed to be less biodegradable, as was discussed in Chapter 2. In addition to the lack of published investigations of chicken manure use in composting bioremediation, the author has recognised a need to further the understanding of the processes involved with this strategy. Therefore this study also aims to help develop an improved understanding of the biological (microbial population dynamics) and toxicological processes involved with composting bioremediation using chicken manure, and differentiation between

biostimulation (nutrient addition) and bioaugmentation (addition of microorganisms) effects potentially resulting from the addition of chicken manure on TPH biodegradation.

The aims of this study (stated in Chapter 1 and outlined in Section 3.2 below) were achieved through the use of bench-top scale treatability studies combined with an extensive suite of chemical, biological, and toxicological laboratory analyses. The potential use of chicken manure in composting bioremediation of TPH compounds was demonstrated on oil refinery sludge. Such oil refinery sludge provides a suitable study material as it contains a wide range of hydrocarbon compounds, including heavier compounds which are deemed to be less biodegradable (Yong and Mulligan, 2004; Alexander, 1999).

This chapter details the design of the treatability studies and introduces the laboratory analytical programme; comprising monitoring of environmental parameters, chemical, microbial and toxicological analyses. The methodology adopted in this study and the results of these analyses are detailed in Chapters 4 to 7. An overall discussion of the results is presented in Chapter 8 together with the conclusions.

3.2 Aims and Objectives

The treatability studies and analytical programme have been designed in order that the aims and objectives of this thesis can be satisfied, as follows:

Aim: ***To investigate the use of chicken manure as a co-composting amendment to enhance the biodegradation of petroleum hydrocarbons and to help develop an improved understanding of the chemical, biological and toxicological processes involved.***

Objective One: - *To determine the potential for using chicken manure to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH).*

This is achieved through the use of chemical analyses to determine changes in TPH concentration over the duration of the treatability studies and comparison of TPH degradation profiles for TPH contaminated material (oil refinery sludge) amended with chicken manure, with those from un-amended TPH contaminated material. Such experimental procedure whereby contaminated soils are amended as per the proposed bioremediation strategy and compared with un-amended contaminated soils forms the basic requirement of bioremediation studies, and has been adopted by numerous authors including Margesin et al (2000), Coulon et al (2005), Sabate et al (2004), Carmichael et al (1997), Breedveld et al (2000), Riffaldi et al (2005), Johnson and Scow (1999), Rahman et al (2003), Namkoong et al (2002), Ijah and Antai (2003), and Gestel et al (2003).

Objective Two: - *To determine the potential for using chicken manure to enhance the biodegradation of the more resistant TPH compounds.*

This is achieved through the use of chemical analyses (detailed in Chapter 5) to fractionate TPH compounds according to their molecular weight and carbon number (which can be used to indicate biodegradability, as was discussed in Chapter 2). Fractionated degradation profiles from samples of oil refinery sludge mixed with chicken manure are compared with those from un-amended oil refinery sludge samples to determine the extent to which the addition of chicken manure enhanced the biodegradation of various hydrocarbon fractions. Of particular interest is the enhanced biodegradation of those hydrocarbon fractions deemed to be less biodegradable (i.e. of higher molecular weight and carbon number).

Objective Three: - To determine the potential enhancement of environmental conditions for microbial growth and activity through the amendment of oil refinery sludge with chicken manure.

The growth and activity of microorganisms requires a suite of optimum environmental conditions, including water availability, oxygen availability, temperature, and pH. In this thesis, the term 'pile viability' is defined by the author as *'the ability of the pile to support microbial growth and activity'*. The key indicators of pile viability used in this thesis are pile temperature and microbial activity (respiration). These two parameters

were monitored throughout the treatability studies and comparisons are made between un-amended and amended oil refinery sludge to determine the effect of chicken manure additions on pile health.

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 1) bioaugmentation versus biostimulation, (Part 2) microbial population dynamics, and (Part 3) toxicological dynamics, resulting from the addition of chicken manure to TPH contaminated material.*

Part 1 – Differentiation between bioaugmentation and biostimulation effects of chicken manure addition is achieved through the comparison of pile viability, chemical, biological and toxicological data between oil refinery sludge amended with chicken manure, and oil refinery sludge amended with sterile chicken manure.

Part 2 - Determining the effects of chicken manure addition on microbial population dynamics (bacterial strain identification and bacterial diversity) is achieved through the use of microbial analyses, in particular molecular techniques whereby DNA fingerprinting of bacterial communities is achieved. Such data were collected throughout the treatability studies, and are compared with baseline data obtained for chicken manure and oil refinery sludge. These data are used to (a) identify potential hydrocarbon degrading bacteria present and their source (i.e. chicken manure or TPH contaminated material) to indicate bioaugmentation effects of chicken manure additions; and (b) indicate any potential predominance of non-hydrocarbon degrading bacteria over hydrocarbon degrading bacteria, and therefore any potential negative effect of chicken manure on hydrocarbon degrading bacterial populations.

Part 3 - Determining the effects of chicken manure on toxicological profiles is achieved through the use of acute in vitro toxicity analyses. These data are used to a) determine any potential adverse effect on TPH biodegradation caused by the addition of chicken manure, and therefore potential toxic compounds, and b) determine the success/failure of composting bioremediation in reducing toxicity (and therefore risk) of the oil refinery sludge.

3.3 Experimental Design

This section details the design of the treatability studies, with particular reference to the combination of oil refinery sludge and chicken manure, and the pile management strategy (aeration and addition of water to enhance conditions for biodegradation) adopted.

3.3.1 Material Source and Characterisation

Oil Refinery Sludge:-

Oil refinery sludge was chosen as the test material for this study as such sludge is reported to contain a variety of hydrocarbon compounds ranging from readily biodegradable to less biodegradable due to variations in chemical properties and structure (Yong and Mulligan, 2004; Alexander, 1999). By grouping these compounds into fractions of similar properties, the extent to which the addition of chicken manure enhances their biodegradation can be determined.

The oil refinery sludge for this study was collected from a former oil refinery site (single source) in the United Kingdom, and is in excess of 10 years in age. The sludge had previously been stockpiled for ex-situ bioremediation (proposed strategy unknown and data not available) on site, and some sludge was set aside (in large HDPE containers) for this study by the site engineer. The sludge was transferred into 5-litre HDPE buckets using a stainless steel shovel, and sealed with no headspace (to minimise the loss of volatiles) prior to storage.

Triplicate samples were sent to Alcontrol Geochem Ltd (UK) prior to storage, for determination of TPH concentrations and homogeneity, and thus the need for material homogenisation prior to the treatability studies. Analysis was by gas chromatography interfaced with a flame ionisation detector (GC-FID). This method was also adopted for the chemical analyses undertaken during the treatability studies, and is described in Chapter 5. The raw data are provided in Appendix 2. The results are summarised in Table 3.1.

Table 3.1: Chemical Characterisation of Triplicate Sludge Samples

	Total TPH mg kg^{-1} DW	Total Aliphatics mg kg^{-1} DW	Total Aromatics mg kg^{-1} DW
Sludge 1	51,000	46,300	4,700
Sludge 2	13,000	11,000	2,000
Sludge 3	11,600	10,000	1,850

Note: DW = Dry Weight Basis.

Hydrocarbon contaminated samples are reported to be subject to chemical (particularly volatilisation) and biological (biodegradation) changes during storage, therefore it is important to minimise these changes if samples are to be stored for any period of time by retarding chemical and biological activity (Korda et al, 1997). The oil refinery sludge samples were therefore stored in sealed HDPE containers (with no headspace) at approximately 4°C as recommended by Korda et al (1997) and British Standard 7755 Part 2.6 (1994), to minimise any chemical and biological changes to the samples during storage. Storage was for approximately 1 year before the treatability studies commenced. The oil refinery sludge was removed from storage and brought up to ambient temperature (10-15°C) for approximately 24 hours prior to further handling of these materials. Moisture content analyses were undertaken following storage, in accordance with BS 7755; Part 3.1 (1994), and calculated using Equation 3.1. The approximate moisture content of the sludge was 80% - 90%. Owing to the variable TPH concentrations given in Table 3.1, the sludge was homogenised prior to the treatability studies, as discussed in Section 3.3.2.

$$Moisture\,Content = \left(\frac{wet\ soil\ weight - dry\ soil\ weight}{wet\ soil\ weight} \right) \times 100\% \qquad \textbf{Equation 3.1}$$

Chicken Manure:-

Many chicken farming practices combine bedding materials such as hay and straw with the manure as an odour abatement strategy (Burton and Turner, 2003). Such bedding materials are also known to be applied to contaminated soils in biopile strategies in order to improve oxygen diffusion in the pile (in this case these materials are termed bulking agents), thereby enhancing biodegradation of hydrocarbons (Chapter 2). It was therefore decided for this study that such bedding materials should

be omitted from the chicken manure, in order to assess the bioremediation potential of chicken manure only.

Fresh chicken manure was collected from a deep-litter free range chicken farm in South Wales (UK). Nutrient content was determined by Direct Laboratories Ltd (UK); the results are discussed in Section 3.3.3.4. This source of chicken manure was chosen for the characteristics of the manure (i.e. without bedding materials), and willingness of the farmer to provide access to the chicken sheds, and unlimited sample collection.

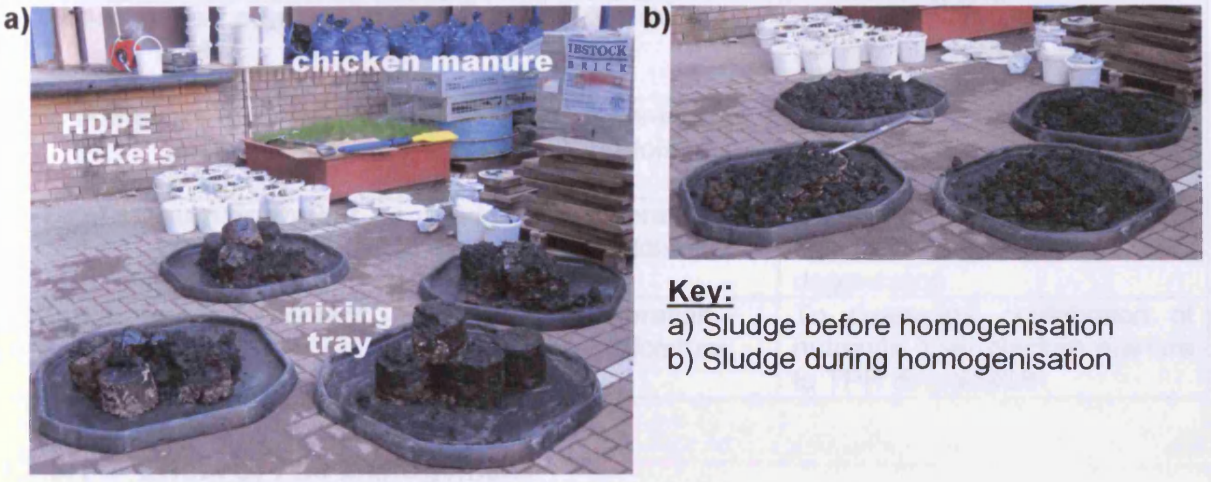
The chicken manure was collected approximately 2 weeks before the commencement of the treatability studies, and stored in HDPE sample bags in the dark, at ambient temperature (approximately 10-15°C). Moisture content analyses were undertaken following storage in accordance with BS 7755; Part 3.1 (1994), and calculated using Equation 3.1, indicating an approximate moisture content of 60 - 70%.

3.3.2 Material Preparation

Due to the variability in TPH concentrations indicated by preliminary chemical testing (Table 3.1), the sludge needed to be homogenised before its use in the treatability studies. Other authors have reported the use of various homogenisation methods, many of whom air dried the materials and sieved them (to between 2mm and 6mm diameter sized particles) (Macleod et al, 2003; Breedveld et al, 2000; Namkoong et al, 2002; Sabate et al, 2004; Vasudevan and Rajaram, 2001). However facilities for the air drying of such a large quantity of material were not available, therefore alternative methods were sought. Atagana (2003) and Potter et al (1999) reported using a concrete mixer to homogenise materials. A homogenisation trial was therefore carried out using a concrete mixer. Due to the high moisture content of the sludge, this method proved unsuccessful as the sludge kept balling into large clumps and coated the inside of the mixer.

The sludge was therefore homogenised on four concrete mixing trays by repeated turning and mixing (tilling) using a stainless steel shovel (Figure 3.1) for approximately

1 hour for the entire quantity of sludge. Rhykerd et al (1999) also reported material homogenisation by tilling.



Key:
a) Sludge before homogenisation
b) Sludge during homogenisation

Figure 3.1: Sludge Homogenisation

Due to the cost of chemical analyses, the material was deemed to be homogenous by visual inspection only. To minimise the effect of residual heterogeneity of materials used in the treatability studies, a 3kg composite sample was taken for each sampling event and homogenised by hand using a stainless steel scoop. From this composite sample, sub-samples were taken for each set of laboratory analyses.

3.3.3 Material Combination and Pile Management

Eight treatability studies were designed with four key combinations of materials and pile management (pile aeration and maintenance of moisture content), as detailed in Table 3.2.

Such design was chosen to enable determination of the contribution of the pile management strategy and the addition of chicken manure to TPH degradation, and differentiation between the potential biostimulation and bioaugmentation effects resulting from the addition of chicken manure. Each material and pile management combination was duplicated to assess repeatability. A total of 26kg material (wet weight basis) was placed in each tray.

Table 3.2: Treatability Study Material Content and Pile Management.

Tray	Material Content	Pile Management	Aim
Un-managed controls (UMC) Trays 1 and 2	Sludge	None	To determine contribution of aeration and water addition to TPH degradation.
Managed controls (MC) Trays 3 and 4	Sludge	Aeration + Moisture	
Total manure treatment (TMT) Trays 5 and 6	Sludge + Manure	Aeration + Moisture	To determine contribution of chicken manure to TPH degradation.
Sterile manure treatment (SMT) Trays 7 and 8	Sludge + Sterile Manure	Aeration + Moisture	To determine contribution of nutrients from chicken manure to TPH degradation.

3.3.3.1 Effect of Pile Management

As was discussed in Chapter 2, microbial degradation of organic contaminants, such as TPH compounds, relies on a number of pre-requisite conditions. In addition to compound bioavailability and biodegradability, microorganisms also need nutrients, oxygen, moisture, and optimal pH and temperature for growth and activity (Alexander, 1999; Madigan et al, 2003). During the treatability studies, a pile management strategy (detailed in Section 3.3.4) was adopted whereby moisture content was maintained at 40-60% and piles were aerated. Such management practices are commonly adopted during typical composting procedures as well as during standard ex-situ bioremediation strategies (Chapter 2), and alone are reported to enhance TPH biodegradation (such as the work of Rhykerd et al, 1999). It is therefore necessary to differentiate between the effects of chicken manure and the pile management strategy on TPH biodegradation.

Trays 1 to 4 comprise TPH contaminated sludge only. Trays 1 and 2 (un-managed control - UMC) benefited from no pile management strategy, whereas Trays 3 and 4 (managed control - MC) did. This was to determine the contribution of such environmental factors to any overall degradation of TPH compounds. Trays 3 to 8 (managed control (MC), total manure treatment (TMT) and sterile manure treatment (SMT)) received the same pile management to enable comparison between these trays.

3.3.3.2 Abiotic versus Biotic Degradation

The British Standard guidance on laboratory testing for biodegradation of organic chemicals in soils under aerobic conditions (BS 7755; Part 4.1.1; 1995) recommends that a sterile incubation should be performed to distinguish between abiotic and biotic degradation mechanisms.

There is a variety of methods available for the sterilisation of environmental samples (Trevors, 1996; McNamara et al, 2003). Autoclaving is widely used, and has been used for previous bioremediation investigations such as the work of Atagana (2004a), Potter et al (1999) and Bento et al (2003). Unfortunately autoclaving is reported to be very disruptive to soil properties (Trevors, 1996; McNamara et al, 2003). An alternative sterilisation method is that of gamma irradiation which is reported to be less disruptive to soil properties than autoclaving (Trevors, 1996; McNamara et al, 2003) and was used by Liste et al (2002) and Atagana (2003).

Trevors (1996) recognises that these methods have varying effectiveness, and total sterilisation of environmental samples is not always achievable, particularly with large quantities of material. In addition, the maintenance of sterile conditions is difficult, and that repeated sterilisation may be needed. In the case of autoclaving and gamma irradiation, this may not be practical or acceptable should negative effects on soil physical and chemical properties be found (for example nutrient content, as reported by McNamara et al (2003), which could give rise to erroneous data.

An alternative method is the use of microbial poisons which simply inhibit microbial activity rather than sterilise samples (Trevors, 1996). Huesemann et al (2004) and Margesin et al (2000) reported using sodium azide (NaN_3) as a microbial poison, whereas Margesin and Schinner (1997) used silver nitrate ($AgNO_3$). However, these poisons are also toxic to humans and require careful use. Given the location of the treatability studies (Section 3.3.4.2; Figure 3.2) and the use of this area by other people, the use of poisons was deemed impractical and unsafe. In addition, a large amount of poison would be needed given the large quantity of material used in these treatability studies, which could have been expensive. Also, there were no facilities available for the mixing of such poisons with the materials.

Given the large volume of material to be sterilised (a total of 208kg) and the likely difficulties in obtaining and maintaining sterile conditions, a completely sterile control was not used in this study. Also, the addition of chicken manure to TPH contaminated sludge could enhance abiotic degradation processes, particularly through volatilisation due to the potential achievement of higher pile temperatures, compared with the un-amended sludge (Chapter 2). The potential contribution of volatilisation to total TPH degradation, and the effects of chicken manure additions on volatilisation, was therefore determined through the use of headspace analyses. These analyses are introduced in Section 3.4.1 and detailed in Chapter 5.

3.3.3.3 Effect of Total Chicken Manure

Comparison of laboratory analyses results (chemical, biological and toxicological) between oil refinery sludge amended with chicken manure and un-amended oil refinery sludge enable the following objectives (discussed in Section 3.2) to be investigated:

Objective One: - *To determine the potential for using chicken manure to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH).*

Objective Two: - *To determine the potential for using chicken manure to enhance the biodegradation of the more resistant TPH compounds.*

Objective Three: - *To determine the potential enhancement of environmental conditions for microbial growth and activity through the amendment of oil refinery sludge with chicken manure.*

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 2) microbial population dynamics, and (Part 3) toxicological dynamics, resulting from the addition of chicken manure to TPH contaminated material.*
(Note: Objective Four Part 1 is addressed in Section 3.3.3.4).

The total manure treatment (TMT) combined fresh chicken manure with the TPH contaminated sludge. A concrete mixer was used to mix the sludge with the chicken manure. Due to the fibrous nature and lower moisture content of the chicken manure, this mixing method proved successful, based on visual inspection of the materials. The TMT treatment received the same pile management as the managed control (MC).

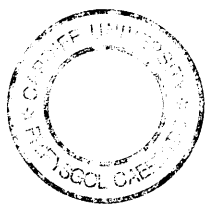
In deciding the ratio of oil refinery sludge to chicken manure, there are two options. Firstly, the amount of chicken manure as a percentage of the total material quantity could be varied to determine the effects of varying chicken manure contents; i.e. 25%, 50%, 75% etc, which is the strategy adopted by Namkoong et al (2002), Ladislao et al (2005), Gestel et al (2003), and Wong et al (2002), as was discussed in Chapter 2. Secondly, the ratio of chicken manure to sludge could be determined according to the optimum nutrient balance (carbon to nitrogen ratio; C:N ratio) for microbial growth and activity. Due to the financial budget of this study, the second option was chosen.

Various authors investigating the effect of nutrient supplementation (biostimulation) on hydrocarbon biodegradation have based their bioremediation investigations on the C:N ratio approach. A summary of these authors, and the C:N ratio used is presented in Table 3.3.

Table 3.3: Carbon to Nitrogen Ratios Used in Published Bioremediation Studies.

Author	Starting C:N
Atagana, 2003	25:1
Margesin et al, 2000	20:1
Sabate et al, 2004	10:1
Rhykerd et al, 1999	100:1
Wong et al, 2002	30:1

Of the investigations listed in Table 3.4, only Atagana (2003) investigated the effects of *varying* C:N ratio on hydrocarbon biodegradation, as was discussed in Chapter 2. Atagana (2003) estimated that a C:N ratio of 10:1 would be adequate to stimulate microbial growth and creosote degradation, based on the assumption that the amount of nitrogen needed for the biodegradation of hydrocarbons reflects the amount of hydrocarbon that must be incorporated into the biomass formed as the microbes use



the carbon source for growth. However, it is accepted that complete assimilation of hydrocarbons into the biomass is not achievable due to sorbed hydrocarbon compounds (Atagana, 2003), and immediate energy expenditure (i.e. to maintain cell viability) (Alexander, 1999). Therefore, to test the hypothesis that a C:N ratio of 10:1 would be optimum for hydrocarbon biodegradation, the creosote contaminated soil was amended with a range of C:N ratios above and below this value through the addition of inorganic nitrogen (NH_4NO_3) to obtain C:N ratios of 25:1, 20:1, 15:1, 10:1 and 5:1. The results are presented in Chapter 2, Section 2.5.3 and revealed that a C:N ratio of 25:1 was most effective in enhancing microbial growth and creosote removal.

In addition to the work of Atagana (2003), guidelines for the composting of organic wastes also recommend an optimum C:N ratio of between 20:1 and 30:1 (Peigne and Girardin, 2004; Utah State University, 1995; USEPA, 1994; Burton and Turner, 2003). Therefore, for this study, a starting C:N of 25:1 was chosen.

Triplicate samples of the sludge and chicken manure were sent to Direct Laboratories Ltd (UK) to determine their C:N ratio. The full results are provided in Appendix 2. The C:N results (average of 3 replicate tests) were 6:1 (±0) for chicken manure (which complies with published data by Nicholson et al, 1996) and 84:1 (±3) for the sludge. Using the guidelines provided by Utah State University (1995), the ratio of chicken manure to sludge was then calculated to be 3:1 (by weight) in order to generate the desired C:N ratio of approximately 25:1, as follows:

3 parts chicken manure	= (6+6+6)
1 part oil refinery sludge	= (84)
Combined C:N ratio	= (6+6+6+84)/4
	= C:N ratio of 25.5:1

3.3.3.4 Effect of Sterile Chicken Manure

As stated in Section 3.2, Objective Four Part 1 of the treatability studies includes differentiation between TPH degradation due to biostimulation (resulting from the optimisation of nutrients, moisture, and oxygen) and bioaugmentation (resulting from

the introduction of a non-indigenous microbial population) resulting from the addition of chicken manure. Such differentiation does not, to the author's knowledge, appear to have been addressed by previous authors, as discussed in Chapter 2.

In order to do this, two options were considered. Firstly the option of adding nutrients to the sludge to mimic the nutrient content of chicken manure was explored. However, this option was dismissed as attaining the correct nutrient balance would be difficult, and the effect of chicken manure as a bulking agent would be lacking. The second, and chosen option, was to sterilise the chicken manure before it was added to the sludge.

As outlined in Section 3.3.3.2, there are various techniques for the sterilisation of environmental samples, and the most commonly used techniques are autoclaving, gamma irradiation, and addition of microbial poison. These options were explored for the purpose of sterilising chicken manure only, as discussed below.

Option One: *Sterilisation through the addition of microbial poison.*
Sodium azide (previously used by Margesin et al, 2000, and Huesemann et al, 2004) and silver nitrate (previously used by Margesin and Schinner, 1997) act as microbial poisons and can therefore be used to inhibit microbial activity in soils (Trevors, 1996). The use of microbial poison was considered for this study; however residual poison in the chicken manure could poison the microorganisms indigenous to the oil refinery sludge upon mixing. The option of washing the sodium azide out of the chicken manure following the sterilisation step was considered, however this would affect the soluble nutrients within the chicken manure. Also, there were no facilities for mixing such a large volume of material with sodium azide, and washing out the sodium azide afterwards. There were also concerns regarding the safe use of such poisons.

Option Two: *Sterilisation by autoclaving.*
As mentioned in Section 3.3.3.2, autoclaving has often been used by previous authors (Xie et al, 2003; Atagana, 2004a; Potter et al, 1999; Bento et al, 2003) for the sterilisation of materials and environmental samples. Published studies using this approach reported that environmental samples are autoclaved 3 times, for 20-30mins at 121°C on each occasion (Trevors, 1996; Xie et al, 2003). However, it is reported

that autoclaving is very disruptive to soil properties (Trevors, 1996; McNamara et al, 2003), although previous authors (bioremediation investigations) do not appear to have quantified any changes caused. A trial was therefore carried out within the School of Biosciences at Cardiff University. Six 500g (wet weight) samples of chicken manure were placed in individual sterile autoclave bags. Due to odour complaints received from other building users, autoclaving was not repeated on these samples, and could not be carried out within the university facilities on future occasions. Alternative autoclaving facilities were sought at external laboratory and sterilising facilities, however the facilities contacted would not accept the manure. Therefore sterilisation by autoclaving was not pursued any further in this study.

Option Three: *Sterilisation by Gamma Irradiation.*

Due to the complaints received during the autoclaving trial, and the lack of autoclaving facilities that would accept the chicken manure, sterilisation by gamma irradiation was chosen. As mentioned in Section 3.3.3.2, gamma irradiation is reported to cause minimal disturbance of the original physical and chemical characteristics of soil (Trevors, 1996; McNamara et al, 2003), and was adopted by Liste et al (2002) and Atagana (2003). Due to the cost of gamma irradiation, it was decided that a trial sterilisation with additional testing to determine any effect on nutrient content of the manure would not be carried out. Instead, the full quantity of chicken manure needed for sterile manure treatment (TMT) were sent for gamma irradiation at Isotron Plc in Oxford (UK).

A total of 22 HDPE sealed buckets containing approximately 2.4kg chicken manure each were exposed to a dose of 25-33kGy. McNamara et al (2003), states that a dose of 20-70kGy should be sufficient for killing bacteria. A method statement and results were provided by Isotron plc, and are presented in Appendix 2.

Triplicate samples of the gamma irradiated chicken manure along with three samples of non-sterilised chicken manure were sent to Direct Laboratories Ltd (UK) for nutrient testing to determine any changes in nutrient concentration resulting from the gamma irradiation process. The results are summarised in Table 3.4. The raw data are provided in Appendix 2.

As shown in Table 3.6, the nutrient content of the chicken manure is variable both before and after gamma irradiation. Based on the averages calculated, concentrations of Total Potassium, Total Nitrogen and Total Phosphorus do not appear to have been significantly affected by the sterilisation process. Ammonium-N, however, appears to have reduced by approximately 25% (based on average values presented in Table 3.6). The sterilised chicken manure did not receive additional Ammonium-N to compensate for the reduction seen. The C:N ratio increased during the gamma irradiation process, giving a combined C:N ratio of 26.2:1 (C:N ratio = (7+7+7+84)/4).

Table 3.4: Effect of Gamma Irradiation on Nutrient Content.

	AGI 1	AGI 2	AGI 3	A 1	A 2	A 3
C:N ratio	7	7	7	6	6	6
Total Potassium mg kg^{-1} 100% DM	20800	25800	24200	21900	23100	26100
	Average = 23,600 mg kg^{-1}			**Average = 23,700 mg kg^{-1}**		
Ammonium-N mg kg^{-1}100% DM	3790	3800	4810	5070	5200	6540
	Average = 4,133 mg kg^{-1}			**Average = 5,603 mg kg^{-1}**		
Total Nitrogen mg kg^{-1}100% DM	35500	29100	32200	30400	33400	36600
	Average = 32,266 mg kg^{-1}			**Average = 33,466 mg kg^{-1}**		
Total Phosphorus mg kg^{-1}100% DM	10900	13200	12500	12300	11500	12800
	Average = 12,200 mg kg^{-1}			**Average = 12,200 mg kg^{-1}**		

Note: AGI = gamma irradiated sample.
A = not gamma irradiated sample.

The sterile chicken manure was mixed with the sludge using a concrete mixer. The success of this in terms of homogeneity was determined by visual inspection only. The concrete mixer was cleaned thoroughly using a pressure washer, and then rinsed with sterile de-ionised water prior to mixing the materials together to minimise any introduction of non-indigenous microorganisms to the materials during the mixing process.

Trevors (1996), states that the verification of successful sample sterilisation is necessary. This is typically achieved through the use of standard laboratory cultivation procedures whereby soil dilutions are prepared in sterile buffer (saline solution or deionised water) and spread plated on a general growth media such as nutrient agar, and incubated at approximately 28°C for approximately 48 hours. The presence of

bacterial growth indicates unsuccessful sterilisation. Sterile chicken manure samples were sent to Direct Laboratories Ltd (UK) for this procedure. Triplicate samples were plate spread on nutrient agar and incubated at 30°C for 72 hours. A total aerobic colony count revealed <10 colony forming units per gram of soil (CFU g^{-1} soil). Raw data are provided in Appendix 2. In comparison, Ijah and Antai (2003) found 1.2 $x10^8$ CFU/g (based on incubation on nutrient agar for 48 hours at 30°C), while Lu et al (2003) found 10^9 CFU/g (based on chicken manure mixed with bedding material; incubation for 24 hours at 41.5°C). The results given by Direct Laboratories Ltd are therefore deemed to indicate sterile conditions, thus the gamma irradiation process was found to be satisfactory.

3.3.4 Pile Management / Monitoring of Environmental Parameters

Microbial growth and activity is influenced by nutrient levels and balance (C:N ratio), pH, temperature, moisture, and oxygen availability (Alexander, 1999; Madigan et al, 2003). Such environmental parameters can therefore have a significant effect on the degradation rates of organic compounds such as TPH. To enable direct comparison of biodegradation rates between the MC, TMT and SMT conditions, it is important that such environmental parameters are kept constant where possible.

3.3.4.1 Nutrient Balance and pH

As stated in Section 3.3.3.3 the starting C:N ratio for the total and sterile manure treatments (TMT and SMT) was set at 25:1. The C:N ratio or nutrient levels were not maintained or monitored during these treatability studies. Of the previous studies regarding composting bioremediation discussed in Chapter 2, Section 2.4.2, only Kirchmann et al (1998) reported repeated application of horse manure to remove risk of nutrient deficiency. However, as with the other investigations, they did not monitor changes in C:N ratio. Also, Kirchmann et al (1998) did not compare the effects of such repeated applications with a control whereby horse manure was applied only once.

The optimum pH range for microbial growth and activity is 6 to 8 (Composting Association, 2004; USEPA, 1994; Burton and Turner, 2003; BiffaWard, 2002). The pH of the materials was measured using a calibrated laboratory pH meter before the

treatability studies, and the pH levels (between 7.9 and 8.3 approx.) were found to be within the optimum pH range for microbial growth and activity. Adjustment of pH was therefore deemed to be unnecessary. pH levels were monitored throughout the treatability studies in accordance with BS 7755; 3.2 (1995). The pH data are presented and discussed in Chapter 4. It was not deemed necessary to adjust pH during the treatability studies as levels remained within the guidelines stated above.

3.3.4.2 Ambient and Pile Temperature

The treatability studies were carried out in a greenhouse within the School of Biosciences (Cardiff University) (Figure 3.2). Although the greenhouse was heated, it was not temperature controlled, thus maintaining constant ambient temperature over the 90 day period was not possible. Ambient temperature was therefore monitored on a regular basis. The treatability studies were carried out in two batches with each batch one week apart in order to cope with the analytical program. Therefore the batches were exposed to different ambient temperatures.

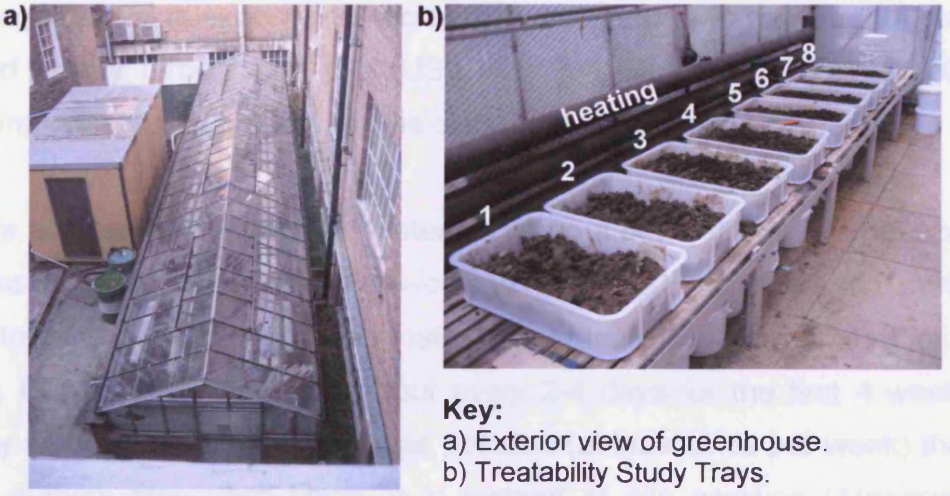
The treatability studies ran from August to November 2005, and a reduction in ambient temperature was encountered. The heating facilities within the greenhouse also failed. Two convector storage heaters were placed in the greenhouse to minimise the effect of falling ambient temperature on pile temperature.

Pile temperature was also monitored on a regular basis using a digital thermometer from Testo Ltd (UK) (accuracy of ± 0.1°C), at 5 equidistant points across each tray, at mid-pile depth (this depth is reported to have the maximum temperature; USEPA, 1994; Burton and Turner, 2003). The pile temperature profiles are presented and discussed in Chapter 4. All raw data are provided in Appendix 3.

Pile temperature has a direct, positive relationship with microbial activity (Madigan et al, 2003; Alexander, 1999; Miyatake and Iwabuchi, 2006), and is affected by numerous factors including amendment materials (and availability of readily decomposable carbon sources), microbial activity and size of pile (insulation capacity) (USEPA, 1994). Due to the small scale of these treatability studies, the maximum attainable temperature of the piles may be lower than that of full scale operation. As

pile temperature could affect biodegradation (Chapter 2, Section 2.5.4), these treatability studies may not reflect TPH degradation rates and/or endpoints attainable under field scale operations. Thus scale is a recognised limitation of these treatability studies, and of other such experiments (Alexander, 1999).

It is stated by British Standard 7755, Part 4.1.1 (1995) that pile temperatures of between 10°C and 25°C are representative of normal environmental conditions. Reported investigations on the bioremediation of hydrocarbon contaminated soils are typically based on laboratory scale microcosms whereby the test materials are incubated at constant temperatures, for example 20°C (Namkoong et al, 2002), 25°C (Riffaldi et al, 2005), and 28-30°C (Ijah and Antai, 2003; Vasudevan and Rajaram, 2001). Where larger scale experiments were undertaken and pile temperature was not controlled, pile temperatures of approximately 30-40°C was obtained by Kirchmann et al (1998) whereby horse manure was added to oil refinery sludge, and pile temperatures of approximately 75°C were reached where 10kg oil contaminated soil was co-composted with 90kg biowaste (Gestel et al, 2003).



Key:
a) Exterior view of greenhouse.
b) Treatability Study Trays.

Figure 3.2: Location of Treatability Studies

3.3.4.3 Moisture Content

The moisture content of the managed sludge (MC), total manure treatment (TMT), and sterile manure treatment (SMT) conditions was monitored on a fortnightly basis in accordance with BS 7755; Part 3.1, (1994), and calculated using Equation 3.1. Sterile

de-ionised water was added as necessary to maintain moisture content of between 40 and 60%, based on recommended optimum composting conditions (The Composting Association, 2005; Pace et al, 1995; Rynk, 2000). Between moisture content analyses, water was added when the material looked and felt dry (in accordance with Hansen et al, 1995).

Although the starting moisture content of the sludge and chicken manure were individually higher than 60%, it was decided not to air dry the samples prior to the treatability studies. This was because a) offensive odours were likely, and given the complaints received during the homogenisation trial, they would be a nuisance; and b) drying of the materials is deemed to be part of the composting bioremediation process.

3.3.4.4 Pile Aeration and Mixing

It is important to 'turn' composting piles on a regular basis to ensure a) sufficient oxygen supply to microorganisms to encourage aerobic activity, b) to ensure that all composting material is exposed to microorganisms, and c) to ensure that moisture is distributed evenly through the pile (USEPA, 1994; Burton and Turner, 2003; The Composting Association, 2005), as was discussed in Chapter 2.

Piles were aerated by 'turning' the materials using a large stainless steel scoop. The scoop was cleaned using sterile de-ionised water between aeration periods and between trays to avoid cross contamination. A separate scoop was used for the SMT condition. Pile aeration was carried out every 2-4 days for the first 4 weeks of the treatability studies, and then as often as possible (at least once per week) there after. Previous authors also used tilling as a method of pile aeration (Atagana, 2004a; Riffaldi et al, 2005; Vasudevan and Rajaram, 2005; Sabate et al, 2004; Margesin et al, 2000).

3.3.5 Odour Nuisance and Technical Problems

A trial treatability study of four week duration was undertaken to determine any potential technical problems with the treatability studies, such as excessive leachate production, low pile temperature and odour nuisance.

The trial comprised a mixture of chicken manure and sludge (ratio of 1:1; C:N ratio had not been determined for the materials at this time) with a total wet weight of 16kg. Leachate production was not identified as an issue during the trial treatability study, therefore adjustments to the experimental design were not made. Pile temperatures of between 18°C and 22°C were attained. Although this complies with BS 7755 Part 4.1.1 (1995) it was decided that the quantity of composting material should be increased to 26kg (wet weight) to encourage higher pile temperatures, and to minimise the effect of pile destruction due to sampling.

During the trial, odour nuisance was evident. An air treatment system was therefore constructed (Figure 3.3). An air pump was used to take air from the greenhouse to a drum of activated carbon via aluminium ducting. The odours were treated by the activated carbon, eliminating odour nuisance. Three glass panes were replaced with standard air vents to draw clean air into the greenhouse.

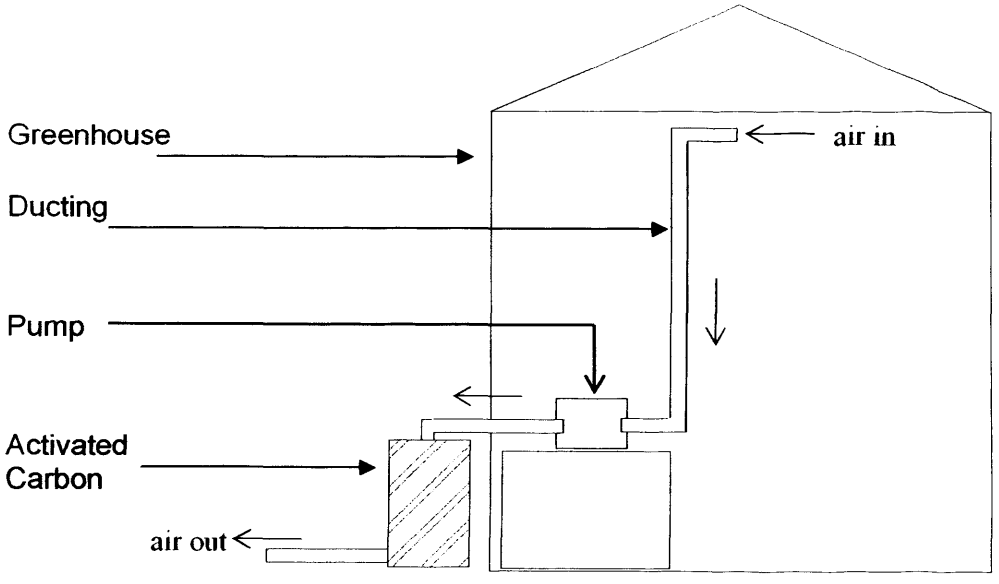


Figure 3.3: Air Treatment System

3.4 Laboratory Analyses Programme

This study relies on the combination of the treatability studies outlined in this chapter with laboratory analyses in order to achieve the objectives set out in Section 3.2. In this section, the design of the laboratory analysis programme undertaken is outlined. Detailed methodology is presented along with corresponding results and discussion in Chapters 4 to 8.

3.4.1 Chemical Analyses

Two suites of chemical analyses were carried out during the treatability studies. These are outlined as follows:

TPH Quantification

As stated in Section 3.2, Aims One, Two, and Four Part Two of this study are:

Objective One: - *To determine the potential for using chicken manure to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH).*

Objective Two: - *To determine the potential for using chicken manure to enhance the biodegradation of the more resistant TPH compounds.*

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 1) bioaugmentation versus biostimulation, resulting from the addition of chicken manure to TPH contaminated material.*

In order to satisfy these objectives, changes in TPH concentration (degradation profiles) over the duration of the treatability studies *must* be determined, for which chemical analyses are carried out.

For this study a method published by the Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG) was adopted (The Direct Method; TPHCWG, 1998). This

method is discussed in Chapter 5, but briefly uses solid phase separation to separate the aliphatic and aromatic hydrocarbon fractions, and gas chromatography interfaced with a flame ionisation detector (GC-FID) to quantify concentrations of various hydrocarbon fractions based on carbon number.

Through comparison of TPH degradation profiles for the four pile conditions (UMC, MC, TMT and SMT), the extent to which the addition of chicken manure enhanced TPH biodegradation, and biodegradation of more resistant TPH fractions, is determined.

The investigation of Objective Two is of particular interest as it is recognised that petroleum products span a wide variety of hydrocarbon compounds, not only of varying biodegradability, but also of varying toxicity and environmental risk. Where some bioremediation strategies do not appear to be having an effect on *total* TPH degradation, they may actually succeed in enhancing biodegradation of those compounds potentially deemed to present a higher environmental risk.

The majority of bioremediation investigations discussed in Chapter 2 measured only changes in total hydrocarbon concentrations (for example, Namkoong et al, 2002; Gestel et al, 2003; Ijah and Antai, 2003; Ladislao et al, 2005; Vasudevan and Rajaram, 2001; Sabate et al, 2004; Coulon et al, 2005; Margesin et al, 2000; Jorgensen et al, 2000). Very few authors have determined effects of bioremediation strategies on varying hydrocarbon fractions. Kirchmann et al (1998) divided hydrocarbons in aliphatic and aromatic fractions. Although aromatic hydrocarbon compounds are deemed to be less biodegradable due to their more stable ring structure (Alexander, 1999), separation of hydrocarbons on this basis does not enable a full idea of the success of bioremediation strategies in enhancing the biodegradation of more resistant compounds within both aliphatic and aromatic fractions. Rahman et al (2003) separated total hydrocarbons according to n-alkane carbon number, and Bento et al (2000) separated hydrocarbons into light (C_{12-23}) and heavy fractions (C_{23}-C_{40}), thus giving a better indication of enhanced biodegradation of more resistant compounds.

With regard to Objective Four Part 1, there is also an interest in the differentiation between bioaugmentation and biostimulation effects of chicken manure addition on TPH degradation; as such differentiation has not, to the author's knowledge, been addressed in previous composting bioremediation investigations. This is achieved through the comparison of TPH degradation profiles from the total manure and sterile manure treatments (TMT and SMT), and through the use of biological analyses (Section 3.4.3).

Headspace Analyses:

Headspace analyses were undertaken to indicate the potential contribution of volatilisation processes to TPH degradation, and the effect of adding chicken manure and the composting bioremediation process on volatilisation. Elevated temperatures may be attainable through the composting bioremediation process, and these elevated temperatures may enhance petroleum hydrocarbon biodegradation, potentially through increasing volatilisation, as was discussed in Chapter 2. The headspace analyses undertaken are detailed in Chapter 5, but briefly involved sampling headspace volatiles using a solid phase micro-extraction (SPME) fiber, and quantifying relative concentrations of total volatiles using gas chromatography interfaced with a mass spectrometer (GC-MS). Through comparison of volatilisation profiles between the eight treatability study trays, the extent to which the addition of chicken manure affects hydrocarbon volatilisation is indicated.

An indication of the potential for volatilisation is also attained through comparison of baseline TPH fraction data with the physical-chemical properties of these fractions outlined in Chapter 2.

3.4.2 Pile Viability

As stated in Section 3.2, Aim Three of this study is:

Objective Three: - *To determine the potential enhancement of environmental conditions for microbial growth and activity through the amendment of oil refinery sludge with chicken manure.*

As outlined in Section 3.3.4, microorganisms require optimum environmental conditions (pH, nutrients, water, oxygen, temperature) for their growth and activity (Madigan et al, 2003; Alexander, 1999), and microbial growth and activity affects hydrocarbon biodegradation (Alexander, 1999). In this thesis, the term *pile viability* is used and is defined by the author as 'the ability of the pile to support microbial growth and activity', as outlined in Section 3.2.

Typical bioremediation strategies, as outlined in Chapter 2 (Section 2.4), include the addition of oxygen, water and nutrients to enhance conditions for hydrocarbon biodegradation, such as the work of Margesin et al (2000), Coulon et al (2005), Sabate et al (2004), Vasudevan et al (2001), Breedveld et al (2000) and Riffaldi et al (2005).

In this study, nutrient addition was achieved through the addition of chicken manure. A pile management strategy was also adopted whereby moisture content and oxygen levels were maintained, as outlined in Section 3.

The success of pile management strategies/bioremediation strategies in enhancing conditions for microbial growth and activity can be monitored through measuring pile temperature and/or microbial activity. These two parameters are said to have a direct relationship (Madigan et al, 2003; Alexander, 1999; Miyatake and Iwabuchi, 2006), i.e. as microbial activity increases pile temperature increases, and vice versa. Pile temperature is also affected by pile size and insulation capacity, frequency of aeration and surface cooling (USEPA, 1994). Therefore monitoring pile temperature alone may not give a strong indication of relative pile viability.

These two parameters were therefore measured during the treatability studies. As outlined in Section 3.3.4.2, pile temperature was monitored using a digital thermometer. For microbial activity there are several methods available as discussed in Chapter 4. This study measured microbial activity through the quantification of carbon dioxide evolution rates. This method is detailed in Chapter 4, but briefly involves headspace sampling using a gas tight syringe, and subsequent analysis by gas chromatography interfaced with a thermal coupled detector (GC-TCD).

By comparing microbial activity and pile temperature profiles, the effects of chicken manure additions on environmental conditions can be determined. It is important to clarify that this is based on total microbial activity and does not directly indicate any changes in hydrocarbon degrading microbial activity.

3.4.3 Microbial Analyses

As stated in Section 3.2, Aim Four of this study is:

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 1) bioaugmentation versus biostimulation, (Part 2) microbial population dynamics, and (Part 3) toxicological dynamics, resulting from the addition of chicken manure to TPH contaminated material.*

Part 1:-
As stated in Section 3.4.1, of interest to this study is the differentiation between bioaugmentation and biostimulation effects of chicken manure addition on TPH degradation. Such differentiation has not, to the author's knowledge, been addressed in previous composting bioremediation investigations. By fingerprinting the microbial populations and comparing these data with baseline data attained for chicken manure and oil refinery sludge, the source (i.e. chicken manure or refinery sludge) of microorganisms over time can be indicated. These data are used to differentiate between bioaugmentation and biostimulation effects resulting from the addition of chicken manure.

Part 2: -
It was identified in Chapter 2 that there is a lack of investigation into the biological processes taking place during the composting bioremediation of TPH contaminated soils. Of particular concern is the potential negative effect of non-hydrocarbon degrading bacteria present within chicken manure on hydrocarbon degrading bacteria (either from chicken manure itself or from the sludge) due to competition for growth factors such as nutrients, oxygen, and water.

There are two main approaches for studying microbial population dynamics, and these approaches are detailed in Chapter 6. Briefly they are separated into culture-dependent methods, whereby microorganisms are cultured in the laboratory on growth media containing nutrients, and culture-independent methods (also known as molecular techniques), whereby microorganisms are studied at the molecular level (based on their DNA).

For this study, culture independent methods were chosen. The reasons for this choice are detailed in Chapter 6, but briefly molecular techniques were chosen because they are reported to enable a more complete and representative study of microbial communities, and enable simultaneous analysis of multiple samples thus enabling the study of community dynamics. They are also time and cost efficient in comparison to culture-dependent methods.

3.4.4 Toxicity Analyses

As stated in Section 3.2, Aim Four, Part 3 of this study is:

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 3) toxicological dynamics, resulting from the addition of chicken manure to TPH contaminated material.*

It was identified in Chapter 2 that, although chicken manure has the potential to enhance TPH biodegradation through biostimulation (due to nutrient content) and bioaugmentation (due to likely content of hydrocarbon degrading microorganisms), there is also a risk that chemical constituents of chicken manure could have a toxic effect on microorganisms, thereby potentially inhibiting or limiting TPH biodegradation. Due to the risk that addition of chicken manure to contaminated soils ultimately increases the quantity of contaminated material should the composting bioremediation strategy fail, all processes which could inhibit TPH biodegradation need to be understood.

The use of toxicological analyses in bioremediation investigations is limited, and was not adopted in the composting bioremediation investigations discussed in Chapter 2. Toxicity analyses have been previously used to determine the potential use toxicity tests as an alternative method of indicating contaminant biodegradation, such as the work of Phillips et al (2000) and Potter et al (1999).

This study aims to assess toxicological dynamics through the use of two acute in-vitro toxicity tests. Of additional interest is the use of these toxicity tests to assess the success or failure of composting bioremediation to reduce the toxicity (and therefore risk) of the oil refinery sludge.

3.5 Conclusions

The success of bioremediation strategies is governed by a complex series of physical, chemical and biological factors, and therefore is not suitable for all contaminated sites or indeed for all contaminants (Alexander, 1999). In order to determine the likely success of a proposed bioremediation strategy, feasibility assessments are needed. Normally these begin with laboratory scale experiments, and then proceed to pilot scale tests depending on the results (Alexander, 1999; Atlas, 1995).

In this study, eight treatability studies whereby oil refinery sludge were either amended with chicken manure (either live chicken manure or sterile chicken manure) or left un-amended (either managed or un-managed) were carried out over a duration of 90 days. These treatability studies were combined with an extensive laboratory analysis program including chemical, biological and toxicological analyses.

Chapter 4

Pile Viability

4.1 Introduction

Biodegradation of petroleum hydrocarbons (and other organic compounds) depends on several factors, as was discussed in Chapter 2. One such factor is the presence of suitable environmental conditions for the growth and activity of microorganisms, including the parameters of pH, temperature, water availability, oxygen availability, and nutrient availability (Alexander, 1999). As stated in Chapter 3, the term 'pile viability' is used in this thesis, and is defined by the author as *'the ability of the pile to support microbial growth and activity'*.

In this study, nutrients were introduced to the oil refinery sludge by means of amendment with chicken manure. In addition to this, a pile management strategy was adopted whereby oxygen and water availability were maintained, as was discussed in Chapter 3. Initial material pH levels were measured and deemed to be within ranges (pH levels 6-8) recommended in the literature (USEPA, 1994; Burton and Turner, 2003; BiffaWard, 2002; The Composting Association, 2004; Battelle, 1996) therefore adjustments were not deemed necessary. Monitoring of pH levels was undertaken during the treatability studies to ensure suitable levels continued.

To determine the extent to which the addition of chicken manure and the pile management strategy enhanced environmental conditions / pile viability for microbial growth and activity (and therefore TPH biodegradation), two parameters were monitored; pile temperature and microbial activity. These two parameters are reported to be directly related; as microbial activity within a soil pile increases, pile temperature also increases, and vice versa (Madigan et al, 2003; Alexander, 1999; Miyatake and Iwabuchi, 2006).

This chapter discusses the methodology available for determining microbial growth and activity, and presents the methods chosen for measuring this parameter and those of temperature and pH. The results obtained are presented and discussed in this chapter. These data are further discussed in Chapter 8.

4.2 Aims and Objectives

As stated in Chapters 1 and 3, Objective Three of this study is as follows:

Objective Three: - *To determine the potential enhancement of environmental conditions for microbial growth and activity through the amendment of oil refinery sludge with chicken manure.*

This is investigated through the determination of pile temperature and microbial activity throughout the treatability studies and comparisons made between the four pile conditions (un-managed sludge (UMC), managed sludge (MC), total manure treatment (TMT), and sterile manure treatment (SMT)).

4.3 Methodology

4.3.1 pH

For this study, pH levels were measured in accordance with British Standard 7755 Part 3.2 (1995a). Briefly, replicate samples per sampling event were dried in a ventilated oven at 30±2°C for approximately 18 hours, and then passed through a 2mm aperture funnel. Using a measuring spoon (as suggested by BS 7755; 3.2), 5ml of sample was taken and mixed vigorously with 20ml deionised water. The solution was left for approximately 18 hours, and the soil re-suspended in the water (using a glass rod) before a pH measurement was taken with a calibrated glass electrode pH meter.

4.3.2 Temperature

Both ambient greenhouse temperature and pile temperature were measured throughout the treatability studies.

Ambient temperature was recorded using two max/min thermometers which were placed at tray level. Recordings were made between the hours of 9am and 11am on each occasion.

Pile temperature was measured using a digital thermometer from Testo Ltd (UK) (accuracy of ± 0.1°C). Measurements were taken at 5 equidistant points across each tray, at mid-pile depth (this depth is reported to have the maximum temperature; USEPA, 1994; Burton and Turner, 2003). Measurements were taken prior to aeration/mixing.

4.3.3 Microbial Growth and Activity

In this study, microbial growth and activity is used to indicate the success of amendment of oil refinery sludge with chicken manure and the pile management strategy adopted in enhancing pile viability. There are various methods available for measuring microbial growth and activity, and these are discussed below. These methods were considered for this study as discussed in Section 4.3.3.3. The method (and method development) adopted in this study is discussed in Section 4.3.3.4.

4.3.3.1 Microbial Growth – Available methods

It is often assumed that the mineralization of organic compounds is characteristic of growth-linked biodegradation (Alexander, 1999). In microbiology population growth is defined as an increase in the number of microbial cells (Madigan et al, 2003). According to Alexander (1999), several studies have demonstrated that the number of microbial cells or biomass acting on a particularly contaminant increases as degradation proceeds, therefore indicating that mineralization reflects population changes.

Many bioremediation studies have included assessments of microbial growth as an indication of enhanced microbial activity through bioremediation strategies (i.e. biostimulation and/or bioaugmentation), such as the work of Margesin et al (2000), Breedveld and Sparrevik (2000), Atagana (2004a) and Rahman et al (2003). Two methods are available, viable plate counts and most probable number; as outlined (according to Madigan et al, 2003) below.

Viable Plate Counts: -

A viable cell is defined as one that is able to divide and form offspring. A viable plate count determines the number of microbial cells in a sample which are capable of forming colonies. Such cells are termed Colony Forming Units (CFU's).

The viable plate count method relies on the cultivation of microorganisms under laboratory conditions. Briefly, the method (Figure 4.1) comprises a number of steps whereby a solid sample (i.e. soil) is suspended in a suitable media (such as phosphate buffered saline (PBS; pH 7.2) as used by Lu et al, 2006) and serial dilutions are made. Samples from each dilution is then incubated on a petri dish containing a growth media (typically nutrient agar; containing nutrients) until CFU's are visible (usually a minimum of 24-48 hours).

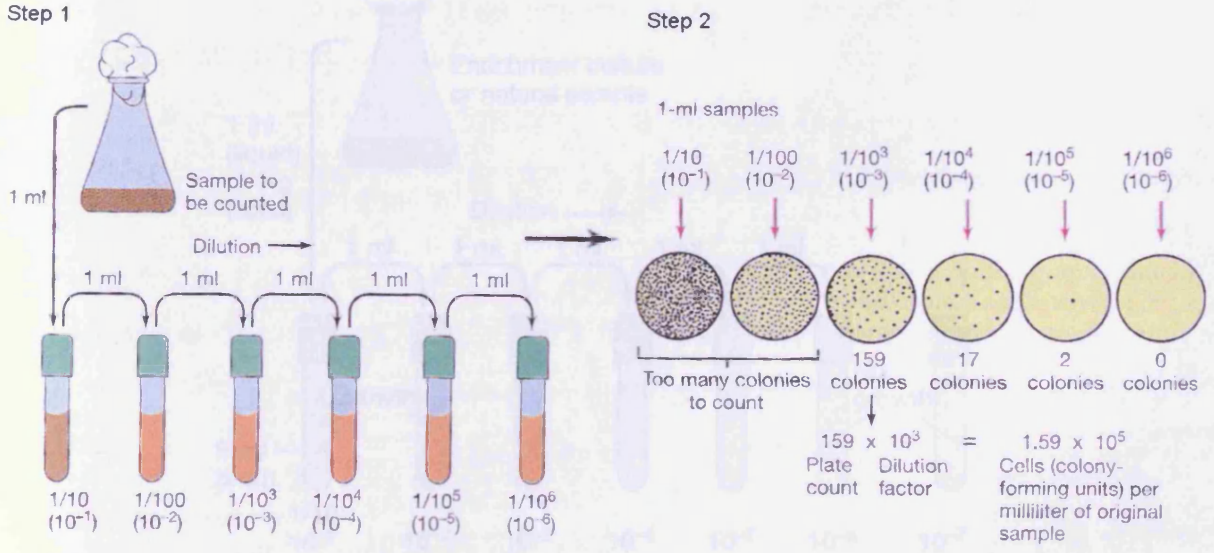


Figure 4.1: Plate Count Method (taken from Madigan et al, 2003).

Dilutions are made as it is important that there are not too many CFU's in the sample as this will cause overcrowding of the petri dish and could lead to erroneous counts. As it is not normally known how many viable cells are present within a sample, serial dilutions are made. Following incubation the petri dishes are then visualised under a microscope and the dilution for which CFU's can be counted is assessed. The number of CFU's is multiplied by the dilution factor to attain CFU's per millilitre of original sample, which can then be converted into CFU's per gram soil (dry weight).

The viable plate count method has been used during previous bioremediation investigations, such as the work of Margesin et al (2000), Breedveld and Sparrevik (2000), Atagana (2004a) and Rahman et al (2003).

Most Probable Number:

The most probable number (MPN) method is similar to the viable plate count method in that it relies on the cultivation of microorganisms in growth media under laboratory conditions. In this method, however, a soil sample is added to a growth media (i.e. nutrient agar) and serial dilutions are made (Figure 4.2). Rather than counting the number of CFU's, an estimation of cell number (MPN) is defined as the highest dilution showing growth (for the example shown in Figure 4.2, this is 10^{-5}).

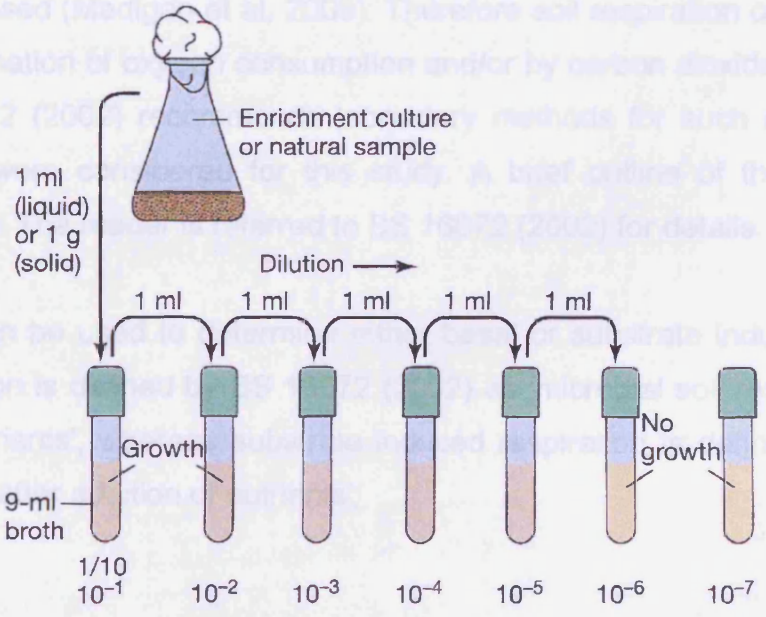


Figure 4.2: Most Probable Number (MPN) Method (taken from Madigan et al, 2003).

The MPN method has been widely used in bioremediation studies, such as the work of Margesin and Schinner (1997), Venosa et al (1996), Bento et al (2005), Coulon et al (2005) and Vasudevan et al (2001).

Total Bacterial Counts versus Hydrocarbon Degraders: -

Both the Viable Plate Count and Most Probable Number methods have been adapted by many authors to determine likely counts of hydrocarbon degrading bacteria only, for example by Bento et al (2005), Margesin et al (2000), Coulon et al (2005), Breedveld and Sparrevik (2000) and Atagana (2004a). In these adaptations, the growth media used contains the hydrocarbon(s) of interest as the sole source of carbon, thereby discriminating against those bacteria which cannot grow on or utilise the test compound. This allows determination of changes in number of hydrocarbon degrading microorganisms over time and between bioremediation strategies.

4.3.3.2 Microbial Activity – Available Methods

Microbial activity can be determined through measuring microbial soil respiration. Microbial soil respiration results from the metabolism of organic compounds, during which (in the case of aerobic respiration) oxygen is consumed and carbon dioxide and water are released (Madigan et al, 2003). Therefore soil respiration can be measured by the determination of oxygen consumption and/or by carbon dioxide release. British Standard 16072 (2002) recommends laboratory methods for such determination, a few of which were considered for this study. A brief outline of these methods is provided below. The reader is referred to BS 16072 (2002) for details.

All methods can be used to determine either basal or substrate induced respiration. Basal respiration is defined by BS 16072 (2002) as 'microbial soil respiration without addition of nutrients', whereas substrate induced respiration is defined as 'microbial soil respiration after addition of nutrients'.

Oxygen Consumption

Pressure Measurement in a Static System: -

The process of soil respiration results in oxygen consumption and simultaneous formation of carbon dioxide. When soil is placed in a closed, static system (Figure 4.3), and an absorbent (e.g. calcium hydroxide, potassium hydroxide or sodium hydroxide solution) for carbon dioxide is available, the consumption of oxygen during microbial respiration (over a period of 24 hours) will cause a reduction in gas pressure within the system. This pressure drop can be measured by connecting a manometer to the reaction vessel (Figure 4.3).

This method has not been adopted during the bioremediation studies discussed in Chapter 2, but was deemed to be a relatively quick and cost effective method; therefore a trial was carried out prior to the treatability studies, as discussed in Section 4.3.3.3.

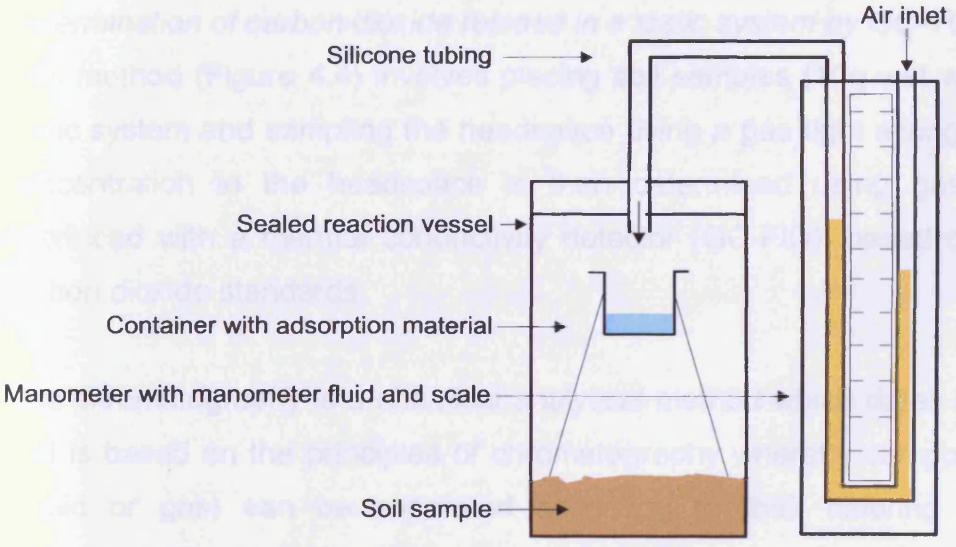


Figure 4.3: Determination of Oxygen Consumption in a Closed Static System (adapted from BS: 16072 (2002)).

Carbon Dioxide Release

Determination of carbon dioxide release by titration in a static system:-

Based on the same principles of Figure 4.3, this method quantifies carbon dioxide absorbed (evolved) rather than oxygen uptake. Sodium hydroxide solution is used as the carbon dioxide absorbent and after incubation at room temperature (22 ± 2°C) for 24 hours, barium carbonate is added to the sodium hydroxide to precipitate the carbon dioxide absorbed. An indicator solution (phenolphthalein) is added to the remaining sodium hydroxide, which is then titrated with hydrochloric acid. The amount of carbon dioxide released is then calculated. Controls are also undertaken whereby no soil is added to the vessel.

This method has been widely adopted by previous authors, including Margesin and Schinner (1997), Johnson and Scow (1999), Atagana (2004a), Namkoong et al (2002) and Riffaldi et al (2006).

Determination of carbon dioxide release in a static system by GC-TCD:-
This method (Figure 4.4) involves placing soil samples (10g wet weight) in a closed static system and sampling the headspace using a gas tight syringe. Carbon dioxide concentration in the headspace is then determined using gas chromatography interfaced with a thermal conductivity detector (GC-FID), based on calibration with carbon dioxide standards.

Gas chromatography is a chemical analytical method which dates back to the 1940s and is based on the principles of chromatography whereby compounds in a mixture (liquid or gas) can be separated according to their differing physical-chemical properties (Rouessac and Rouessac, 2004). Briefly (according to Rouessac and Rouessac, 2004), a gas chromatograph comprises three main components; an injection port, a column, and a detector (Figure 4.5). A sample is injected (usually using a gas-tight syringe) into the injection port of the GC, where it is vaporised and mixed with a carrier gas (typically hydrogen or helium). The carrier gas (containing the sample) is then loaded onto a column which is internally coated with a thin layer of stationary phase, and housed within a thermostated oven. The compounds within the

mixture are separated according to differing partitioning behaviour between the carrier gas and the stationary phase. As the compounds exit the compound they are detected and measured according to their concentration in the sample. A chromatogram is produced which shows a series of peaks representing compounds and their concentration within a mixture (Figure 4.5). The peaks rise from a baseline which is the chromatogram trace obtained in the absence of compounds.

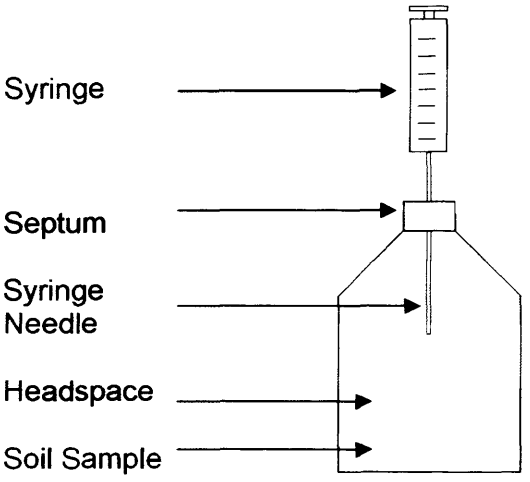


Figure 4.4: Determination of CO_2 Release in a Closed Static System (adapted from BS: 16072, 2002).

There are various detectors available. For the measurement of carbon dioxide in headspace samples, a thermal conductivity detector (TCD) was used, as specified by BS 16072 (2002). Briefly (according to Rouessac and Rouessac, 2004), the operating principle of the TCD relies on the thermal conductivity of gaseous mixtures. A TCD comprises two thermisters; one is flushed with clean carrier gas (i.e. before it is mixed with the samples), and the other is flushed with carrier gas eluting from the GC column. When a compound elutes from the column, there is a change in the composition of the carrier gas, and thus in the thermal conductivity. This results in a deviation from thermal equilibrium between the two thermisters; the deviation is proportional of the concentration of the compound.

Beaudin et al (1999) adopted this principle in their investigation into the effect of temperature and nutrient addition on degradation of mineral oil and grease degradation.

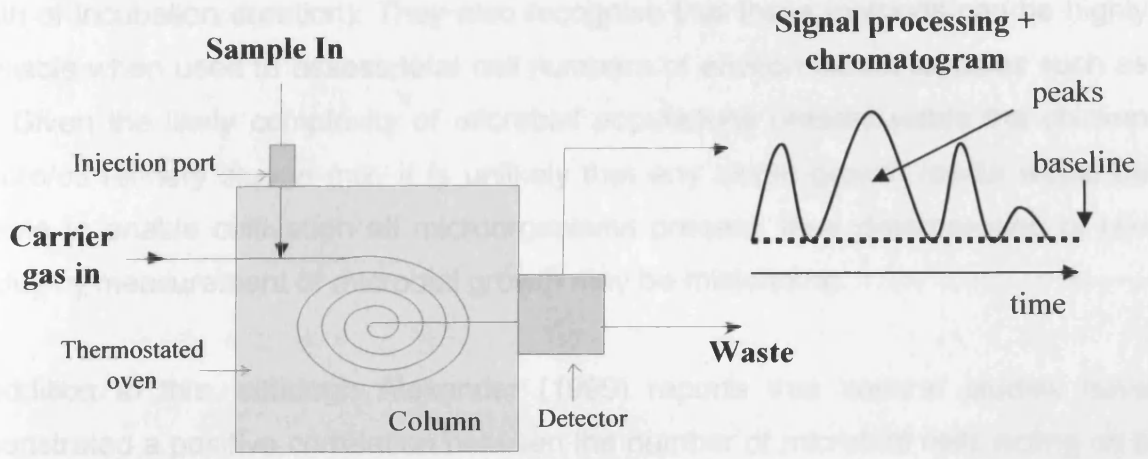


Figure 4.5: (a) main components of a GC (adapted from Rouessac and Rouessac, 2004), and (b) example chromatogram.

4.3.3.3 Discussion and Method Trials

The methods outlined above were considered for this study. The chosen method was that of microbial respiration by determination of carbon dioxide evolution by GC-TCD. The reasons for not choosing the alternative methods are presented as follows:

Microbial Growth (viable plate count or MPN):-

Although these methods have been widely used by previous authors, as discussed earlier, there is one major limitation; they rely on the cultivation of microorganisms under laboratory conditions. It is widely recognised in microbiology literature that culture dependent techniques cannot be used to grow all microorganisms:-

"... 99% of all microorganisms in nature can not be isolated in pure cultures mainly due to our ignorance of the culture conditions under which these microorganisms thrive in their natural environment." G. Muyzer. 1999

" ... it is now generally accepted that the traditional, culture based approach to analysis of bacterial diversity is able to detect only a small portion, often less than 1% of the bacteria from natural habitats". M Howeler et al. 2002.

Madigan et al (2003) also recognise that the number of colonies obtained from these methods depends not only on the number of viable cells present in a sample, but also on the suitability of the growth media and the incubation conditions (temperature and

length of incubation duration). They also recognise that these methods can be highly unreliable when used to assess total cell numbers of *environmental samples* such as soil. Given the likely complexity of microbial populations present within the chicken manure/oil refinery sludge mix, it is unlikely that any single growth media would be suitable to enable cultivation all microorganisms present, thus determination of pile viability by measurement of microbial growth may be misleading.

In addition to this, although Alexander (1999) reports that several studies have demonstrated a positive correlation between the number of microbial cells acting on a particular contaminant and contaminant degradation (therefore indicating that mineralization reflects population changes), he also states that it is likely that compound degradation in nature occasionally results from non proliferating (non growing) populations. Several authors have reported a lack of correlation between microbial counts and hydrocarbon degradation, and, more importantly to this chapter, between microbial counts and bioremediation strategies (for example, nutrient addition versus aeration), for example Bento et al (2003), Venosa et al (1996), Jorgensen et al (2000), and Breedveld and Sparrevik (2000).

Venosa et al (1996) hypothesised that the lack of correlation between microbial count, hydrocarbon degradation and bioremediation strategy may have reflected that the contaminant degrading populations were already at their maximum field capacity, thus other factors limited contaminant degradation.

Margesin et al (2000) found that the total microbial count remained almost constant during their experiment, whereas the number of hydrocarbon degrading microorganisms increased with time. Despite this, increases in hydrocarbon degraders were found to occur irrespective of treatment. They recognise that the quantification of viable soil microbes alone gives no information regarding the efficiency of the populations.

These methods were therefore dropped from the treatability studies due to a) inconsistent data regarding the relationship between bioremediation treatments and microbial counts, b) the limitation regarding culture-dependent methods, and c) the

fact that this method is highly time consuming. It was decided for this study that microbial activity rather than microbial growth should be measured.

Microbial Soil Respiration by Oxygen Uptake:-

Although, to the author's knowledge, this method has not been used during previous bioremediation investigations, it was the first choice for this study as it appeared simple, time and cost-effective.

A basal respiration trial was carried out prior to the treatability studies in accordance with the methodology described by BS 16072 (2002). A chicken manure/oil refinery sludge mix (taken from the refrigerator) was used (1:1 ratio) and sodium hydroxide was used as the carbon dioxide absorbent. A manometer was connected to the reaction vessel, and the change in pressure was monitored over a 24 hour period. Small pressure drops of 0.5 hectopascals were recorded. It was deemed that this may have reflected the long term refrigeration of the mixture; therefore the method was undertaken during the first week of the treatability studies. However, over a 24 hour period, no changes in pressure were detected. It was deemed that this either reflected complete saturation of the sodium hydroxide with carbon dioxide, or a lack of sensitivity of this method. This method was therefore dropped from the treatability studies due to time constraints for method development.

Microbial Soil Respiration by Carbon Dioxide Evolution:-

As the oxygen uptake method (above) was found to be unsuitable for this study, an alternative was needed. Although carbon dioxide determination by titration with hydrochloric acid has been widely used by previous authors (Section 4.3.3.2), carbon dioxide determination by GC-TCD was considered first as equipment was available, enabling immediate commencement of measurements. The author also deemed this method to give a more accurate measurement of carbon dioxide concentration.

A trial of this method was successfully carried out during the first week of the treatability studies, whereby 10g moist soil was placed in a bottle sealed with a septum, and incubated for 24 hours at 22 ± 2°C to determine basal microbial soil

respiration. It was found that the soil was so active that the majority of oxygen within the bottles was consumed over a 24 hour incubation period, as shown in Figure 4.6.

It was clear that this method was highly sensitive, therefore it was chosen for this study. The methodology stipulated by BS 16072 (2002) was amended slightly as discussed in Section 4.3.3.4.

4.3.3.4 Carbon Dioxide Evolution by GC-TCD

In this study, microbial activity was determined by measuring basal soil microbial respiration. The method chosen was carbon dioxide evolution by GC-TCD as this was shown to be highly sensitive during a trial (as discussed in Section 4.3.3.3), and equipment was available within close proximity to the remaining laboratory analyses. The method used was adapted from that stipulated by BS 16072 (2002), as discussed in this section.

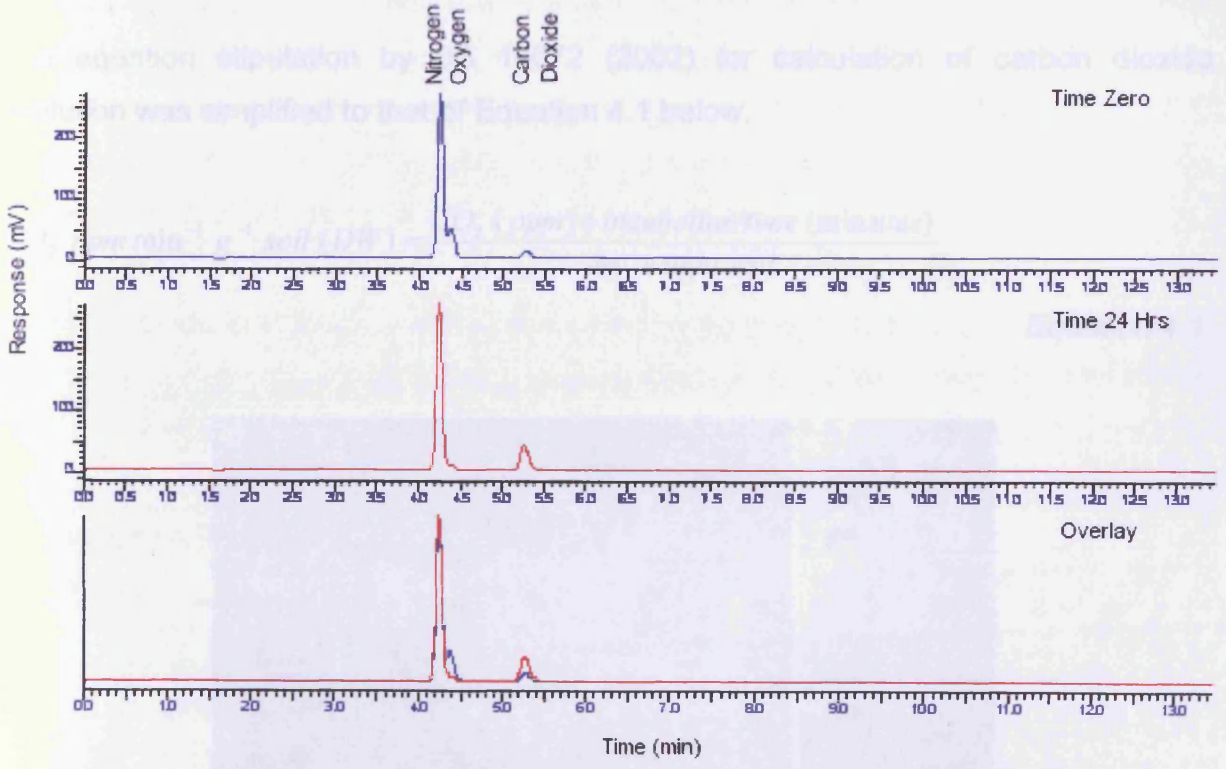


Figure 4.6: Chromatogram showing oxygen uptake and CO_2 evolution over 24 hours (TMT, Day 1 sample).

As shown in Figure 4.6, oxygen levels were nearly fully utilised over a 24 hour incubation period, thus a shorter incubation time of 5 hours was applied, to avoid development of anaerobic conditions. An alternative would have been to use larger serum bottles; however such bottles were not immediately available. Another alternative would have been to use smaller sample, however it was felt that this would have significantly reduced representative sampling.

The 10g (wet weight) sample was placed in a 120ml serum bottle, sealed with a septum, and incubated at 22±2°C for 5 hours (Figure 4.7). Using a gas tight syringe, a 1ml sample of the headspace was removed from the bottle after the 5 hour incubation period, and injected into a gas chromatograph interfaced with a Thermal Conductivity Detector (GC-TCD). Starting carbon dioxide concentrations were assumed to be at ambient concentration (assumed to be 350ppm; Merrington et al, 2002). A separate sample was analysed for dry matter content in accordance with BS 7755; Part 3.1 (1994).

The equation stipulation by BS 16072 (2002) for calculation of carbon dioxide evolution was simplified to that of Equation 4.1 below.

$$CO_2\ ppm\ \text{min}^{-1}\ g^{-1}\ soil\ (DW) = \frac{CO_2\ (ppm) \div incubation\ time\ (\text{min}utes)}{dry\ weight\ soil}$$

Equation 4.1

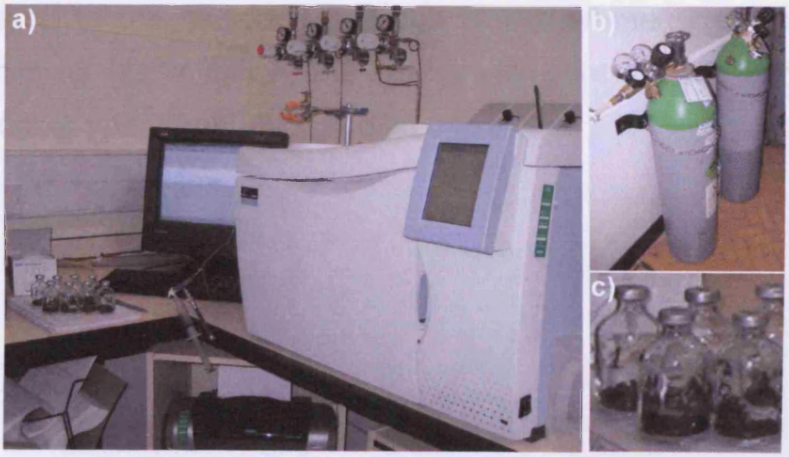


Figure 4.7: Determination of Carbon Dioxide Release from a Static System (a) GC-TCD with computer; b) Gas tanks for CO_2 control; c) 120 ml serum bottles with soil samples.

The measurements were made using the Natural Gas Analyser (GC-TCD) of the School of Earth, Ocean and Planetary Sciences (Cardiff University). Measurements were made as regularly as possible, but depended on equipment availability and commitments to the other laboratory analyses undertaken during these treatability studies. The gas chromatograph was calibrated using CO_2 calibration gas (calibration data is provided in Appendix 3), and a 5000ppm CO_2 standard was run with each batch of samples to ensure repeatability between sample batches. The chromatographs were automatically integrated using TotalChrom Version 6.2.1 software from PerkinElmer and baseline positioning was checked by hand for each chromatograph, and adjusted as necessary.

Due to the limited availability of the Natural Gas Analyser (NGA), and commitments to the other analyses, only one sample was carried out per tray. However excellent repeatability of results between the replicate trays was found for the first reading (Figure 4.10; Section 4.4.3), therefore one sample per tray per sampling event was deemed satisfactory. In order to maximise representative sampling, samples were taken from a 3kg sample batch which was homogenised by hand using a sterile stainless steel scoop. Measurements were taken on the day of sampling; where this was not possible, data points were omitted, as the effect of refrigeration on carbon dioxide evolution was not quantified during this study.

Carbon dioxide was likely already present in the pore spaces of the chicken manure amended and managed oil refinery sludge (MC, TMT, SMT), due to their higher porosity compared to the compacted sludge of the un-managed sludge condition (UMC). To minimise this limitation, the bottles were not shaken during the 5 hour incubation period, or prior to headspace extraction.

4.4 Results

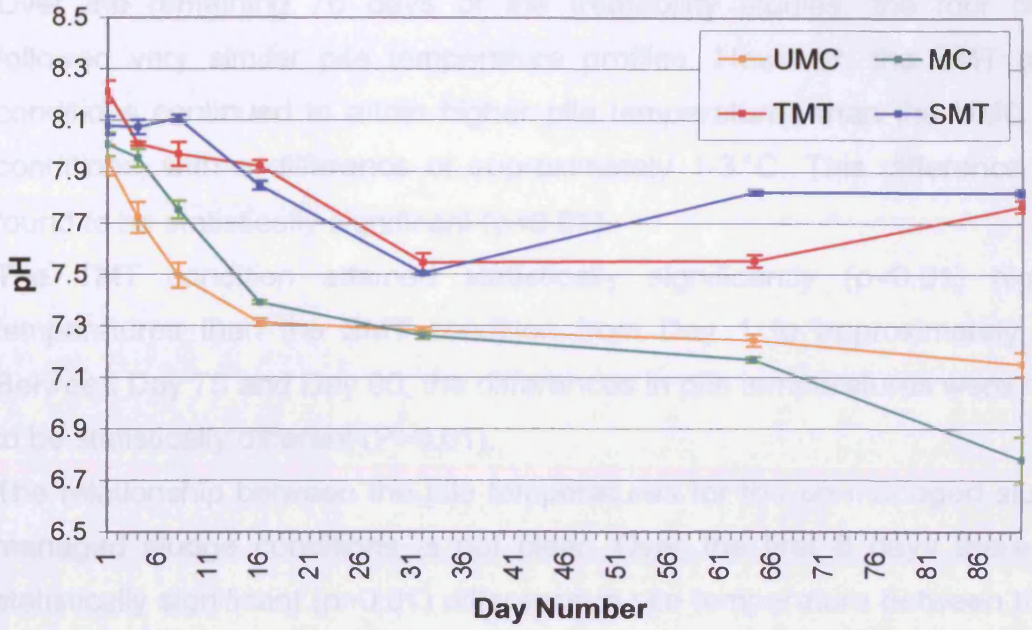
For each environmental parameter monitored (temperature, pH and microbial activity), the data are graphically presented and summarised in the following sub-sections. These data are discussed in Section 4.5. All raw data are provided in Appendix 3. Statistical analyses were carried out to identify any significant differences between the pile conditions (un-managed sludge (UMC), managed sludge (MC), total manure treatment (TMT), sterile manure treatment (SMT)) (using two-tailed Z-test for two means in Microsoft Excel, and a significance level of 1% (p=0.01)), and correlation between microbial activity (carbon dioxide evolution rate) and pile temperature (using Pearson correlation coefficient in Microsoft Excel). These statistical analyses were carried out in accordance with Schmuller (2005).

4.4.1 pH

Figure 4.8 shows the pH measurements taken during the treatability studies for the four conditions. The values plotted are mean pH (mean of four data points per condition, for each sampling event), and the error bars plotted show plus and minus one standard error (± 1 SE). Observations are as follows:-

1. pH for all four treatment conditions stayed within the recommended levels of 6 to 8 (USEPA, 1994; Burton and Turner, 2003; BiffaWard, 2002; The Composting Association, 2004), with the exception of initial values for the TMT and SMT conditions, which varied between 8.1 and 8.2.
2. The UMC and MC conditions shared similar pH profiles, with a gradual reduction in pH from 7.9 – 8.0 (approx) to 6.8 – 7.2 (approx) by Day 90.
3. The TMT and SMT conditions shared similar pH profiles whereby pH level were relatively consistent (7.5-8.0 approx.), but maintained higher (approximately 0.2-0.5) pH values than the UMC and MC conditions.

Figure 4.8: pH Measurements



4.4.2 Temperature

Figure 4.9 shows the pile temperature profiles for the four treatment conditions. The values plotted are mean pile temperature (mean of 5 data points for each sampling event), and the error bars plotted show plus and minus one standard error (± 1 SE). Observations are as follows:-

1. All conditions attained pile temperatures within the 10-25 °C range recommended by BS 7755, Part 4.1.1 (1995), with the exception of TMT and SMT conditions, which exceeded this range during the first 14 days of the treatability studies.
2. The pile temperature profiles are divided into two time frames; the first 14 days (where large differences between the treatments are seen), and the remaining 76 days (where smaller differences between the treatments are seen). Over the first 14 days of the treatability studies it is clear from Figure 4.9 that the TMT and SMT conditions attained higher pile temperatures than the UMC and MC conditions. The greatest difference in pile temperatures between the four conditions was seen on Days 2 and 3, where the manure amended sludge attained pile temperatures of approximately 45-50 °C compared with 21-23 °C

for the un-amended sludge. This difference was found to be statistically significant ($p<0.01$).

3. Over the remaining 76 days of the treatability studies, the four conditions followed very similar pile temperature profiles. However, the TMT and SMT conditions continued to attain higher pile temperatures than the UMC and MC conditions, with a difference of approximately 1-3°C. This difference was still found to be statistically significant ($p<0.01$).
4. The TMT condition attained statistically significantly ($p<0.01$) higher pile temperatures than the SMT condition from Day 1 to approximately Day 75. Between Day 75 and Day 90, the differences in pile temperatures were not found to be statistically different ($P>0.01$).
5. The relationship between the pile temperatures for the un-managed sludge and managed sludge conditions is not clear. Over the first 8 days there was no statistically significant ($p>0.01$) difference in pile temperature between these two conditions. From Day 9 onwards, the relationship appears almost cyclical whereby higher pile temperatures were attained by the managed sludge from Day 8 to Day 15, then by the un-managed sludge until Day 55, then by the managed sludge until Day 70, and finally by the un-managed sludge until Day 90. This is discussed in Section 4.5.

Figures 4.10 and 4.11 show the mean pile temperatures for each treatment condition (mean of 10 data points from the duplicate trays for each treatment condition) against mean greenhouse ambient temperature. Observations are as follows:-

1. The commencement of the UMC and TMT conditions were offset from the MC and SMT conditions by 1 week, therefore the ambient temperature was also offset by 1 week.
2. The TMT and SMT conditions attained pile temperatures higher than ambient temperature over the first 40-45 days of the treatability studies. The greatest difference in temperature (of approx 20-22°C) was seen over the first 7-9 days.

Figure 4.9: Pile Temperature Profiles

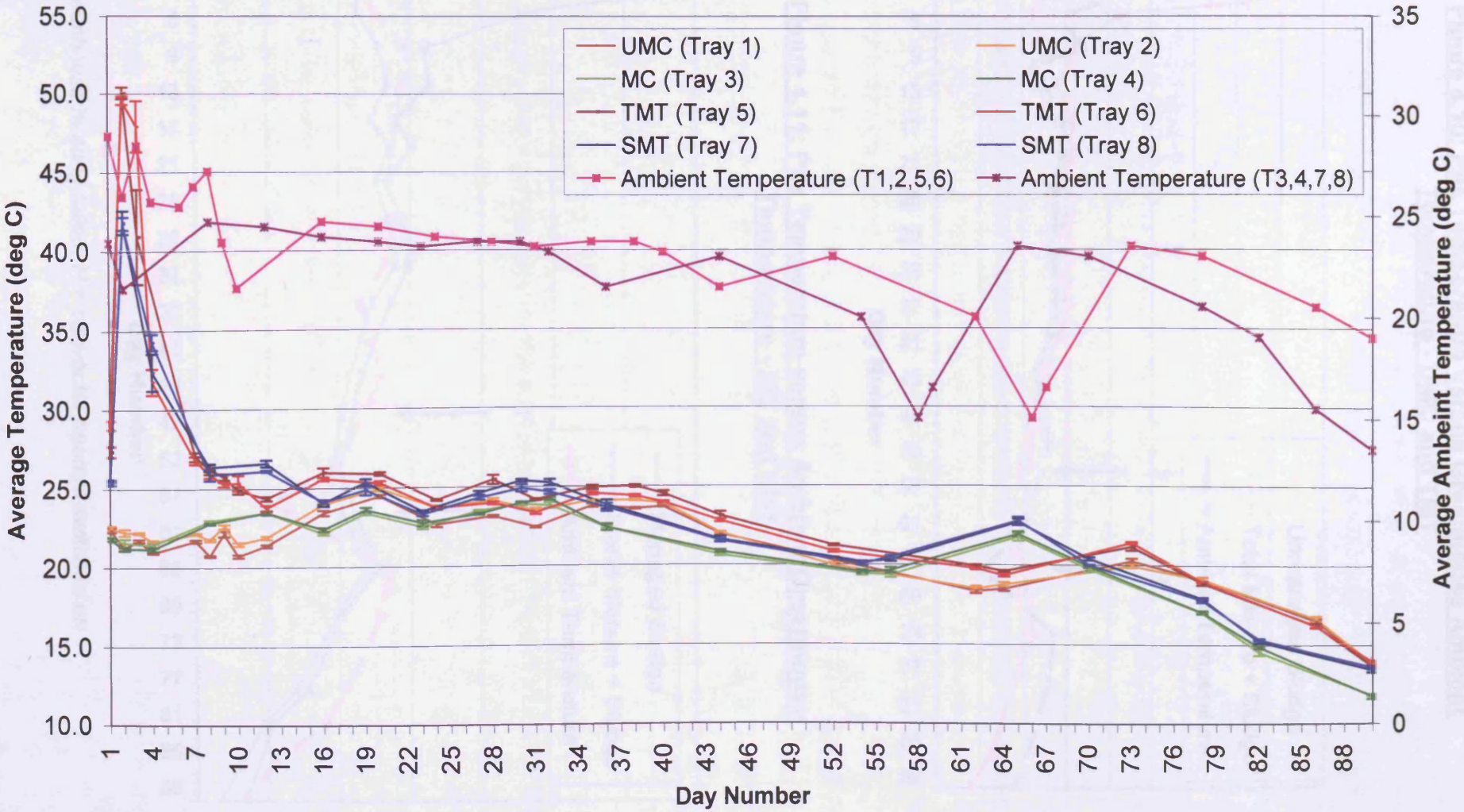


Figure 4.10: Pile Temperature Versus Greenhouse Ambient Temperature - UMC and TMT

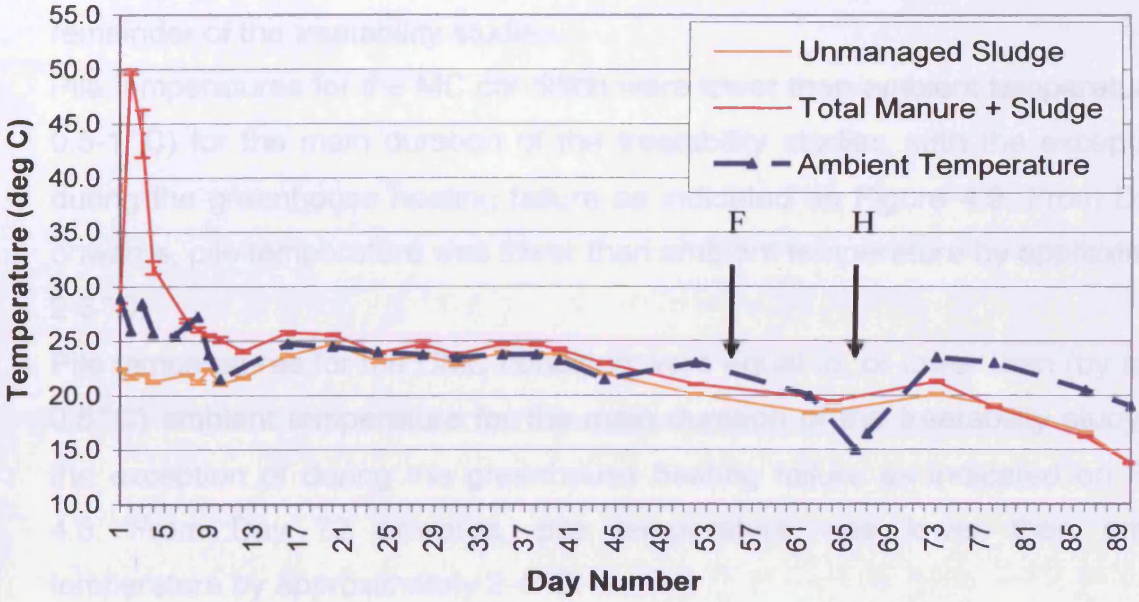
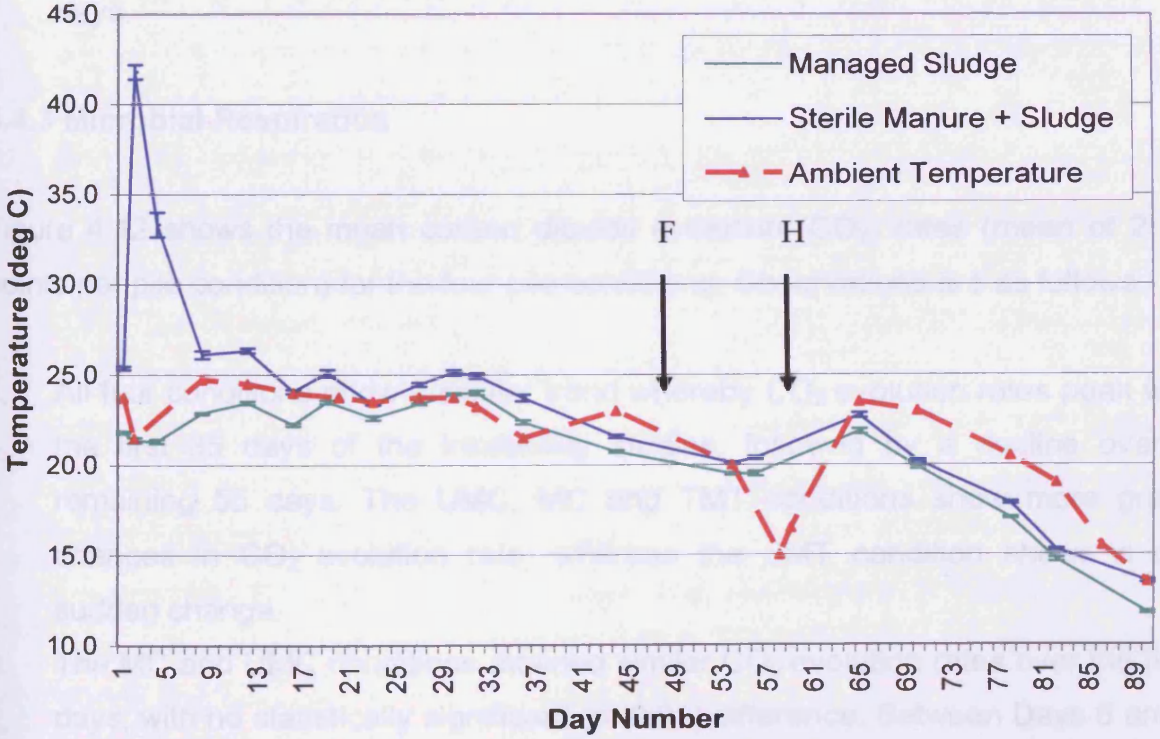


Figure 4.11: Pile Temperature versus Ambient Greenhouse Temperature - MC and SMT



F = Greenhouse heating failed; H = convector heaters switched on

Following this the difference narrowed to approximately 1-2°C higher than ambient temperature. With the exception of during the greenhouse heating failure (indicated on Figures 4.8 and 4.9), pile temperatures for these treatment conditions were lower than ambient (by approximately 0.5-2°C) for the remainder of the treatability studies.

3. Pile temperatures for the MC condition were lower than ambient temperature (by 0.5-1°C) for the main duration of the treatability studies, with the exception of during the greenhouse heating failure as indicated on Figure 4.9. From Day 65 onwards, pile temperature was lower than ambient temperature by approximately 2-3°C.
4. Pile temperatures for the UMC condition were equal to, or lower than (by approx 0.5°C) ambient temperature for the main duration of the treatability study, with the exception of during the greenhouse heating failure as indicated on Figure 4.8. From Day 72 onwards, pile temperature was lower than ambient temperature by approximately 2-4°C.
5. Pile temperatures for the UMC remained at approximately 22°C for longer than those for the MC condition (9 days compared with 5 days, after which pile temperatures rose), despite a higher ambient temperature over the first 7-10 days.

4.4.3 Microbial Respiration

Figure 4.12 shows the mean carbon dioxide evolution (CO_2) rates (mean of 2 data points per pile condition) for the four pile conditions. Observations are as follows:-

1. All four conditions show a similar trend whereby CO_2 evolution rates peak within the first 35 days of the treatability studies, followed by a decline over the remaining 55 days. The UMC, MC and TMT conditions show more gradual changes in CO_2 evolution rate, whereas the SMT condition shows a more sudden change.
2. The MC and UMC conditions attained similar CO_2 evolution rates over the first 8 days, with no statistically significant ($p>0.01$) difference. Between Days 8 and 28 it appears that the MC attained higher CO_2 evolution rates (19 ppm min^{-1} g^{-1} soil

dry weight) than the UMC (15 ppm min^{-1} g^{-1} soil dry weight). This relationship is reversed between Days 29 and 52. However, there are fewer data points for the UMC over this time period; therefore caution is advised during data interpretation. Between Days 58 and 90, it is observed that the MC attained higher CO_2 evolution rates (9 ppm min^{-1} g^{-1} soil dry weight) than the UMC (1 ppm min^{-1} g^{-1} soil dry weight).

3. The TMT condition attained statistically significantly ($p<0.01$) higher CO_2 evolution rates than the UMC and MC conditions over the course of the treatability studies, with a difference of approximately 10-20 ppm min^{-1} g^{-1} soil dry weight. However, by Day 90, no statistically significant ($p>0.01$) difference between the TMT and MC conditions were recorded with CO_2 rates of approximately 9 ppm min^{-1} g^{-1} soil dry weight.
4. The SMT condition attained statistically significantly ($p<0.01$) higher CO_2 evolution rates than the UMC and MC conditions over the duration of the treatability studies, with the exception of between Days 58 and 90 where no statistically significant ($p>0.01$) difference between the SMT and MC conditions was recorded. On the whole, this difference was small (approximately 2-5 ppm min^{-1} g^{-1} soil dry weight), although a significant difference of approximately 45 ppm min^{-1} g^{-1} soil dry weight was attained on Day 24. By Day 58, the SMT and MC conditions attained the same CO_2 evolution rates (approximately 9 ppm min^{-1} g^{-1} soil dry weight), compared with 1 ppm min^{-1} g^{-1} soil dry weight for the UMC.
5. The TMT condition attained higher carbon dioxide evolution rates than the SMT condition, with a difference of approximately 15 ppm min^{-1} g^{-1} soil dry weight, except for between Days 16 and 34 where the SMT condition attained a difference of approximately 20-30 ppm min^{-1} g^{-1} soil dry weight higher. However, there were fewer data points for the TMT between Days 8 and 32, thus a peak could have been missed. By Day 90, it appears that the TMT and SMT conditions attained the same carbon dioxide evolution rate, of approximately 9ppm/min/g soil dry weight.
6. On a whole, the total manure + sludge, and sterile manure + sludge treatment conditions attained higher carbon dioxide evolution rates than the un-managed sludge and managed sludge treatment conditions.

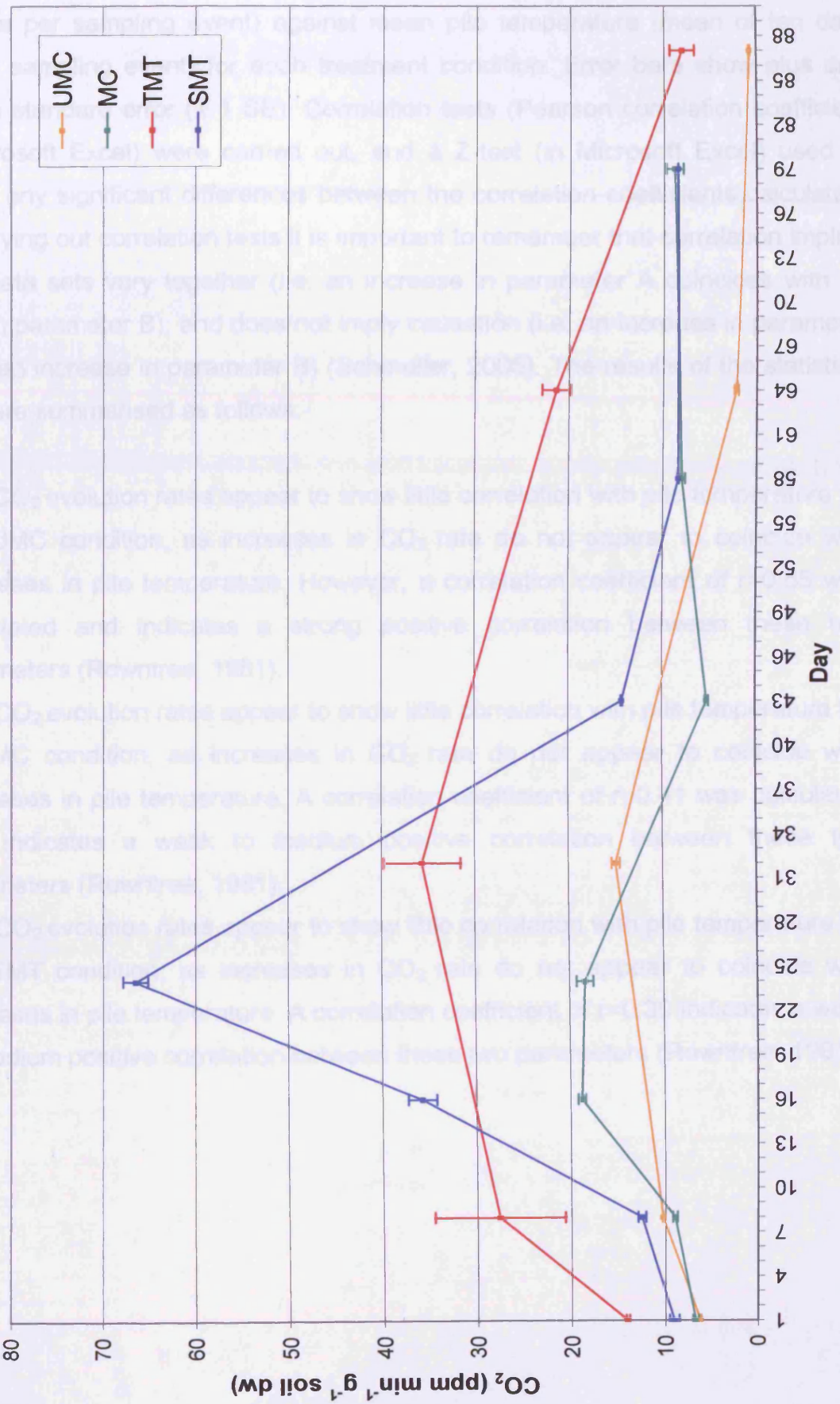


Figure 4.12: Carbon Dioxide Evolution Rates

Figure 4.13 and 4.14 show the mean carbon dioxide evolution rates (mean of two data points per sampling event) against mean pile temperature (mean of ten data points per sampling event) for each treatment condition. Error bars show plus and minus one standard error (± 1 SE). Correlation tests (Pearson correlation coefficient (r) in Microsoft Excel) were carried out, and a Z-test (in Microsoft Excel) used to determine any significant differences between the correlation coefficients calculated. When carrying out correlation tests it is important to remember that correlation implies that two data sets vary together (i.e. an increase in parameter A coincides with an increase in parameter B), and does not imply causation (i.e. an increase in parameter A causes an increase in parameter B) (Schmuller, 2005). The results of the statistical analyses are summarised as follows:-

1. The CO_2 evolution rates appear to show little correlation with pile temperature for the UMC condition, as increases in CO_2 rate do not appear to coincide with increases in pile temperature. However, a correlation coefficient of r=0.85 was calculated and indicates a strong positive correlation between these two parameters (Rowntree, 1981).
2. The CO_2 evolution rates appear to show little correlation with pile temperature for the MC condition, as increases in CO_2 rate do not appear to coincide with increases in pile temperature. A correlation coefficient of r=0.41 was calculated and indicates a weak to medium positive correlation between these two parameters (Rowntree, 1981).
3. The CO_2 evolution rates appear to show little correlation with pile temperature for the TMT condition, as increases in CO_2 rate do not appear to coincide with increases in pile temperature. A correlation coefficient of r=0.39 indicates a weak to medium positive correlation between these two parameters (Rowntree, 1981).

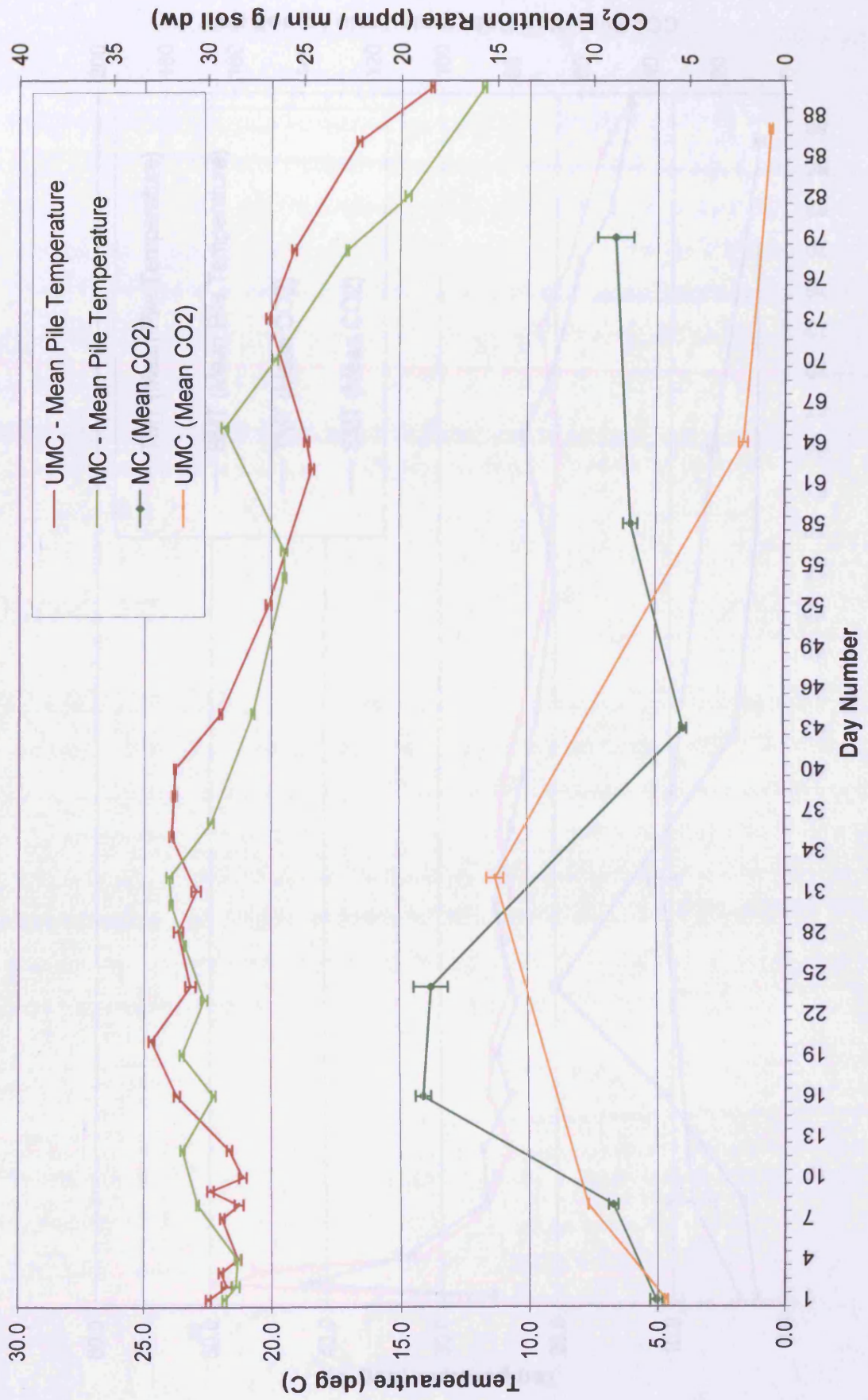


Figure 4.13: Carbon Dioxide Evolution versus Pile Temperature UMC and MC

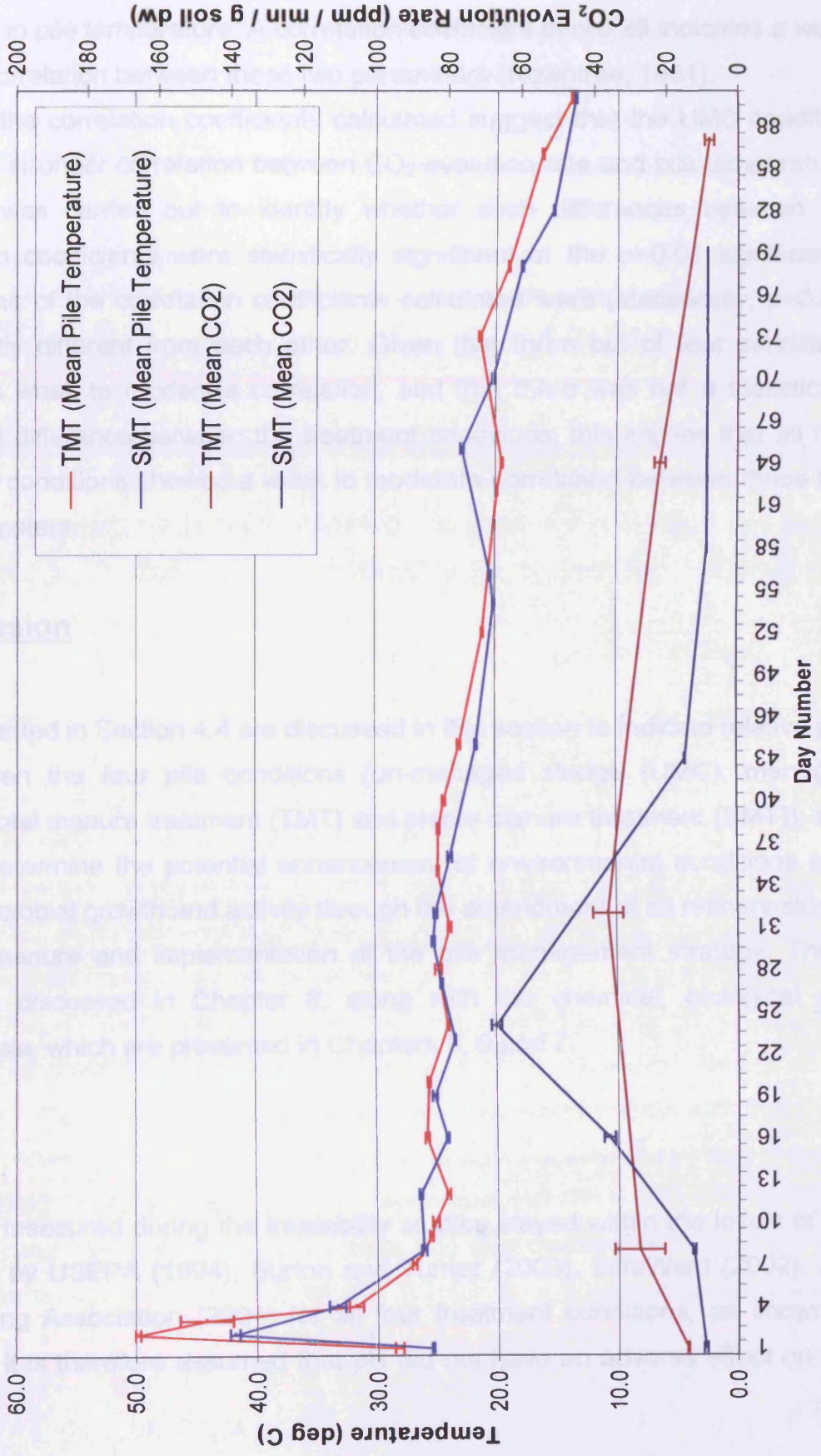


Figure 4.14: Carbon Dioxide Evolution Rate versus Pile Temperature TMT and SMT

4. The CO_2 evolution rates appear to show little correlation with pile temperature for the SMT condition, as increases in CO_2 rate do not appear to coincide with increases in pile temperature. A correlation coefficient of r=0.23 indicates a weak positive correlation between these two parameters (Rowntree, 1981).
5. Although the correlation coefficients calculated suggest that the UMC condition showed a stronger correlation between CO_2 evolution rate and pile temperature, a Z-test was carried out to identify whether such differences between the correlation coefficients were statistically significant at the p=0.01 significance level. None of the correlation coefficients calculated were (statistically, p>0.01) significantly different from each other. Given that three out of four conditions showed a weak to moderate correlation, and that there was not a statistically significant difference between the treatment conditions, this implies that all four treatment conditions showed a weak to moderate correlation between these two test parameters.

4.5 Discussion

The data presented in Section 4.4 are discussed in this section to indicate relative pile viability between the four pile conditions (un-managed sludge (UMC), managed sludge (MC), total manure treatment (TMT) and sterile manure treatment (SMT)); and ultimately to determine the potential enhancement of environmental conditions (pile viability) for microbial growth and activity through the amendment of oil refinery sludge with chicken manure and implementation of the pile management strategy. These data are also discussed in Chapter 8, along with the chemical, biological and toxicological data, which are presented in Chapters 5, 6 and 7.

4.5.1 pH

The pH levels measured during the treatability studies stayed within the levels of 6-8 recommended by USEPA (1994), Burton and Turner (2003), BiffaWard (2002), and The Composting Association (2004) for all four treatment conditions, as shown in Section 4.4.1. It is therefore assumed that pH did not have an adverse effect on pile viability.

4.5.2 Pile Temperature

Differences in pile temperatures between the conditions are used in this section to indicate differences in pile viability. Comparison of pile temperatures between the conditions enables differentiation between the likely contribution of pile management strategy, and the addition of nutrients (through amendment of oil refinery sludge with chicken manure). Comparison between the TMT and SMT treatment conditions also indicates whether or not the addition of chicken manure enhances the activity of microorganisms indigenous to the oil refinery sludge alone, or whether it simply reflects the activity of microorganisms indigenous to the chicken manure, or the activity of both microbial populations.

Pile temperature profiles for the four conditions are divided into two time periods; the first 14 days (where large differences between the treatment conditions are seen), and the remaining 76 days (where smaller differences between the treatment conditions are seen).

The first 14 days:

Pile Management Strategy:

The UMC and MC treatment conditions did not attain (statistically, $p>0.01$) significantly different pile temperatures over the first *8 days* of the treatability studies. Given that the MC treatment condition received aeration during this time period, the lack of significant difference between these treatment conditions could reflect the high moisture content (>60%) of the oil refinery sludge, and/or the lack of nutrients (or unsuitable C:N ratio of 86:1). Over the next 6 days, a statistically significant ($p<0.01$) difference in pile temperature was recorded for these treatment conditions. This could indicate enhancement of pile viability through the pile management strategy adopted for the MC treatment condition. However, it is also seen that the ambient temperature during these 6 days was higher for the MC condition than for the UMC condition. The effect of the pile management strategy on enhancing pile viability is therefore unclear at this stage.

Addition of Chicken Manure:

Over the first 14 days it is apparent from Section 4.4.2 that the TMT and SMT conditions attained pile temperatures approximately 20-22°C higher than those of the UMC and MC treatment conditions. This difference was found to be (statistically, $p<0.01$) significantly different. These data indicate that the addition of chicken manure (sterile or non-sterile) enhanced pile viability, presumably through the addition of nutrients and/or microorganisms, above that of the pile management strategy.

Microbial Population Contributions to Total Microbial Activity:

The TMT treatment condition attained pile temperatures approximately 7°C higher than those of the SMT treatment condition. This difference was found to be (statistically, $p<0.01$) significantly different. These data indicate that the addition of chicken manure (non-sterile) enhances pile viability, but that the increase in pile temperature reflects both the microbial populations indigenous to the chicken manure and those indigenous to the oil refinery sludge. It can therefore be inferred that:

a) The enhancement of soil viability through the addition of sterile chicken manure, assuming total sterility of the manure (as indicated by <10 CFU g^{-1} soil; Chapter 3), reflects the stimulation of microbial growth and activity of the microorganisms indigenous to the oil refinery sludge, presumably through the addition of nutrients.
b) The enhancement of soil viability through the addition of chicken manure (non-sterile) reflects the stimulation of both microbial populations (i.e. microorganisms indigenous to the oil refinery sludge, and microorganisms indigenous to the chicken manure).

The remaining 76 days:

Pile Management Strategy:

Over the remaining 76 days of the treatability studies, it is seen that the effect of the pile management strategy on pile viability remains unclear. From Day 9 onwards, the relationship appears almost cyclical whereby higher pile temperatures were attained by the MC from Day 8 to Day 15, then by the UMC until Day 58, then by the MC until Day 70, and finally by the UMC until Day 90. Where pile temperatures for the MC

treatment condition exceed those for the UMC treatment condition (Days 58-70), they coincide with higher ambient temperatures for the MC treatment condition, thus may reflect ambient temperature rather than microbial activity. These pile temperature data therefore suggest that the pile management strategy did not enhance pile viability (i.e. failed to stimulate the activity of microorganisms indigenous to the oil refinery sludge). It may be that pile aeration (as part of the pile management strategy) caused temperatures to fall below those of the UMC condition (which can be assumed to represent baseline level), therefore a better indication of enhanced pile viability through the pile management strategy may be attained from the microbial respiration data (Section 4.5.4).

Addition of Chicken Manure:

Over the remaining 76 days of the treatability studies, the differences in pile temperature between the manure amended sludge (TMT and SMT) and un-amended sludge (UMC and MC) narrowed to approximately 1-3°C. However the differences recorded over this time period were still found to be statistically significant ($p<0.01$) thus suggesting that the addition of chicken manure continued to enhance pile viability.

Between Days 76-90, no (statistically, $P>0.01$) significant difference in pile temperature was seen between the TMT and the UMC treatment conditions. By Day 90 of the treatability studies, there was no statistically significant difference ($p>0.01$) between the manure amended sludge (total manure and sterile manure) and the UMC treatment conditions. This indicates that pile viability fell to baseline levels (i.e. those of UMC condition).

Microbial Population Contributions to Total Microbial Activity:

Over the remaining 76 days, the differences in pile temperature between TMT and SMT also narrowed to approximately 1-3°C, but these differences were still found to be statistically significant. For the main duration of this time period, TMT continued to attain higher pile temperatures than SMT indicating that both microbial populations indigenous to the chicken manure and those indigenous to the oil refinery sludge continued to contribute to total microbial activity. There was, however, one period (Days 58-69) where the pile temperatures attained by SMT exceeded those attained

by TMT. This could indicate that the introduction of microorganisms indigenous to the chicken manure had a deleterious/negative effect on the microorganisms indigenous to the oil refinery sludge. However, this likely reflects the changes in ambient temperature (SMT>TMT) over this time period.

4.5.3 Pile Temperature versus Ambient Temperature

It is apparent from Section 4.4.2 that the pile temperature profiles for the four treatment conditions appear to reflect the ambient temperature profiles for the main duration of the treatability studies.

However, a few interesting points can be made about the comparison of pile temperature with ambient temperature. The manure amended sludge (total manure and sterile manure) attained pile temperatures which exceeded the ambient temperature for the first 40-45 days of the treatability studies, with the greatest difference (of 20-22°) seen on Days 3 and 4. In contrast, the un-amended sludge (UMC and MC) attained pile temperatures which were either equal to or less than ambient temperature for the main duration of the treatability studies (with the exception of during the greenhouse heating failure, whereby pile temperatures were higher than ambient temperature; this is likely to reflect insulation capacity of the pile).

These data could therefore indicate that the manure amended sludge pile conditions were acting independently of ambient temperature conditions, whereas the un-amended sludge treatment conditions were not. Pile temperature is a function of microbial activity, aeration frequency and pile size (insulation capacity) (Burton and Turner, 2003). Given that pile size was uniform between the treatment conditions, it is likely that the higher pile temperatures (in excess of ambient temperature) reflect microbial activity, thereby suggesting that pile viability was enhanced through the addition of chicken manure. The rapid reduction in pile temperature between Days 3-8 seen for the manure amended sludge treatment conditions likely reflects the aeration frequency and small pile size (and thus likely low insulation capacity), but could also indicate a reduction in microbial activity. This latter argument is deemed unlikely given the microbial respiration levels which were higher than those for the un-amended sludge, as discussed in Section 4.5.4.

4.5.4 Microbial Respiration

Differences in microbial respiration between the four pile conditions are used in this section to indicate relative pile viability. As with pile temperature, the comparison of microbial respiration between the treatment conditions enables differentiation between the likely contributions of the pile management strategy, and the addition of nutrients (through amendment of oil refinery sludge with chicken manure). Comparison between the TMT and SMT conditions also indicates the likely extent to which the microorganisms indigenous to the chicken manure contribute to total microbial respiration.

Pile Management Strategy:

It is apparent from Figure 4.11, and Section 4.4.3, that there is not a clear difference in microbial respiration between the UMC and MC conditions, for the main duration of the treatability studies. Over the first 30 days of the treatability studies it appears that microbial respiration was higher (statistically significant difference, $p<0.01$) for the MC condition than for the UMC condition, with a difference of approximately 5 ppm min^{-1} g^{-1} soil dry weight) therefore indicating that the pile management strategy enhanced pile viability. However, it is also recognised that there are fewer data points over this period for the UMC condition (owing to exclusion of data points obtained after refrigeration of samples), therefore a comparative 'peak' may have been missed. In addition to this, the relationship is reversed from Day 31 to Day 53 whereby microbial respiration was higher for the UMC condition than for the MC condition (by approximately 5 ppm min^{-1} g^{-1} soil dry weight). Again it is recognised that there are fewer data points over this time period for the UMC condition, thus a comparative 'trough' in microbial respiration may have been missed. From Day 53 onwards, microbial respiration was (statistically; $p<0.01$) significantly higher for the MC condition, than for the UMC condition (by approximately 8 ppm min^{-1} g^{-1} soil dry weight), indicating that the pile management strategy enhanced pile viability over this time period. However, for the main duration of the treatability studies it is not clear from the microbial respiration measurements whether or not the pile management strategy enhanced soil viability. A lack of enhancement likely reflects a lacking in another environmental parameter which affects microbial growth and activity, i.e. lack of nutrients.

Addition of Chicken Manure:

The TMT condition consistently attained (statistically, $p<0.01$) significantly higher microbial respiration levels than the un-amended sludge (UMC and MC) conditions throughout the treatability studies. A maximum difference of approximately 25-30 ppm min^{-1} g^{-1} soil dry weight was attained for the main duration of the treatability studies (Days 5-70), but a slightly lower difference of 5-15 ppm min^{-1} g^{-1} soil dry weight was attained over the first 4 days and last 20 days (approx.). This comparison indicates that the addition of chicken manure enhanced soil viability.

The SMT condition also attained higher microbial respiration levels than the un-amended sludge (un-managed and MC) conditions. The maximum difference seen was approximately 65 ppm min^{-1} g^{-1} soil dry weight on Day 23. However, for the main duration of the treatability studies (Day 1-8, and Day 43-58), the difference (approx 3-7 ppm min^{-1} g^{-1} soil dry weight) was much lower than that between the TMT and un-amended sludge conditions (although this difference was still found to be statistically significant, $p<0.01$). From Day 58 to Day 90, there was no (statistically, $p>0.01$) significant difference between microbial respiration levels for SMT and MC treatment conditions.

These data indicate that soil viability was enhanced through the addition of chicken manure.

Microbial Population Contribution to Total Microbial Activity:

When the microbial respiration levels are compared between the TMT and SMT conditions, it can be seen that, with the exception of between Days 16 and 36, that microbial respiration levels were higher for the TMT than for the SMT condition. Between Days 16 and 36, it is observed that there are fewer data points for the TMT condition, therefore a peak comparable to that of the SMT condition over this time period may have been missed. Nonetheless it is apparent that, for the main duration of the treatability studies, higher microbial respiration levels were attained for the TMT treatment condition than for the SMT treatment condition.

Given that there was a lower difference (3-7 ppm min^{-1} g^{-1} soil dry weight) in microbial respiration levels between the SMT and un-amended sludge (UMC and MC)

conditions compared with the larger difference (25-30 ppm min^{-1} g^{-1} soil dry weight) between the TMT and un-amended sludge (UMC and MC) conditions for the main duration of the treatability studies, these data indicate that the enhanced pile viability seen to result from the addition of chicken manure likely reflects activity of the microorganisms indigenous to the chicken manure more so than those indigenous to the oil refinery sludge. The role of the microorganisms introduced by the chicken manure cannot be inferred by these data, but rather by the microbial analyses presented in Chapter 6. Given that previous authors (Ijah and Antai, 2003) identified the presence of hydrocarbon degrading microorganisms in chicken manure, these data do not necessarily indicate that the stimulation of microorganisms indigenous to the chicken manure has a negative or positive impact on TPH biodegradation. This is ultimately determined through the chemical analyses (Chapter 5) and microbial analyses (Chapter 6). These data sets are collectively discussed in Chapter 8.

4.5.5 Microbial Respiration versus Pile Temperature

Madigan et al (2003) report that there is a positive relationship between microbial activity and pile temperature, as an increase in one parameter coincides with an increase in the other. Correlation tests were therefore carried out on paired data points obtained for these two parameters, as discussed in Section 4.4.3. A positive correlation was identified; however such correlation was concluded to be weak to moderate (Section 4.4.3).

However, it can be seen from the discussions in Section 4.5 that both sets of data agree with other in their indications regarding the contributions of pile management strategy (i.e. that the data does not indicate a clear enhancement of pile viability due to the pile management strategy) and amendment of oil refinery sludge with chicken manure (i.e. the addition of chicken manure appears to have enhanced pile viability).

The two data sets do, however, appear to disagree over the extent to which microbial activity reflects the microbial population indigenous to the oil refinery sludge, or the microbial population indigenous to the manure. It appears from the pile temperature data that the TMT condition attained pile temperatures only slightly above those of the SMT condition, compared to the difference between the TMT and un-amended sludge

conditions. The pile temperature data therefore suggests that, although both microbial populations are active, the pile temperature is increased mainly through the stimulation of the microorganisms indigenous to the oil refinery sludge, presumably due to the introduction of nutrients from the chicken manure. In contrast, however, the microbial respiration data indicates that, for the main duration of the treatability studies, the microorganisms indigenous to the chicken manure made a greater contribution to total microbial respiration than those indigenous to the oil refinery sludge. This is indicated by the greater difference seen between the TMT condition and un-amended sludge (approximately 25-30 ppm min^{-1} g^{-1} soil dry weight) than between the TMT condition and un-amended sludge (approximately 3-7 ppm min^{-1} g^{-1} soil dry weight).

Given that previous authors (Ijah and Antai, 2003) identified the presence of hydrocarbon degrading microorganisms in chicken manure, the fact that the microbial respiration data indicates a prevalence of the microbial population indigenous to the chicken manure, these data do not necessarily indicate that the activity of microorganisms indigenous to the chicken manure has a negative impact on TPH biodegradation. This is discussed further in Chapter 8, through comparison with the results of the microbial population dynamics analyses.

4.5.6 Summary

The data and discussions presented in this Chapter are summarised as follows:

pH

- The pH levels for all four treatment conditions stayed within the 6-8 levels (recommended by USEPA, 1994; Burton and Turner, 2003; BiffaWard, 2002; The Composting Association, 2004; Battelle, 1996). It is therefore assumed that pH did not have an adverse effect on pile viability.

Pile Temperature

- The pile temperature data indicates that the pile management strategy did not enhance soil viability. This is reflected in the low differences in pile temperature between the two treatment conditions, the cyclical relationship between the two treatment conditions and that periods of supposed enhanced pile viability through the pile management strategy coincide with periods of higher ambient temperature, thus likely reflect ambient temperature rather than microbial activity.

- The pile temperature data indicates that the addition of chicken manure enhanced pile viability, as indicated by the higher pile temperatures attained by the TMT and SMT treatment conditions; and that:

  a. Both microbial populations indigenous to the chicken manure and those indigenous to the oil refinery sludge contributed to total microbial activity.
  b. The microbial population indigenous to the oil refinery sludge contributed to total microbial activity more so than those indigenous to the chicken manure.

  This is reflected in the greater difference between SMT and un-amended sludge treatment conditions than between SMT and TMT treatment conditions.

- The pile temperatures appear to reflect ambient temperatures for the main duration of the treatability studies. However, the manure amended sludge (TMT and SMT) conditions attained pile temperatures which exceeded the ambient temperature for the first 40-45 days of the treatability studies. In contrast, the un-amended sludge (UMC and MC) conditions did not attain pile temperatures which exceeded the ambient temperature. Such data indicate that the pile temperatures attained in the manure amended sludge treatment conditions were acting independently of the ambient temperature, whereas the un-amended sludge treatment conditions were not. The maximum difference in temperature attained by the manure amended sludge was approximately 25-27°C on Days 2 and 3 (pile temperature of 42-50°C). The pile temperature for these treatment conditions fell to approximately 25°C by Day 8. It is likely that this drop in temperature reflects

the aeration frequency (as part of the pile management strategy) and pile size (and therefore incubation capacity) rather than a drop in microbial activity. These data therefore indicate that if a larger pile size was constructed, a longer duration thermophilic phase may be attained. The high temperatures attained in these treatability studies, and the potential elongated thermophilic phase should a larger pile be construction may or may not enhance TPH biodegradation. Elevated temperatures are reported to enhance petroleum hydrocarbon biodegradation (Gestel et al, 2003; Semple et al, 2001; Feitkenhauer et al, 2003; Coulon et al, 2005), therefore it is argued that the use of composting strategies for bioremediation should prove advantageous (Semple et al, 2005), as discussed in Chapter 2. Such positive effects of high temperature on hydrocarbon biodegradation were found by Beaudin et al (1999) and Gestel et al (2003). However Ladislao et al (2005) found that temperatures above 38°C had a negative effect on hydrocarbon biodegradation, as discussed in Chapter 2. Pilot scale treatability studies would need to be undertaken to confirm this.

Microbial Respiration

- The microbial respiration data indicates that the pile management strategy did not enhance soil viability for the main duration of the treatability studies. This is reflected in the low differences in carbon dioxide evolution rates between the two treatment conditions, and the uncertainty regarding comparable data points. During the last 37 days of the treatability studies, it appears that the MC attained higher carbon dioxide evolution rates than the UMC treatment condition. The difference between these two treatment conditions over this time period (approximately 8 ppm min^{-1} g^{-1} soil dry weight) was found to be statistically significant ($p<0.01$). A stronger indication of enhancement of pile viability through the pile management strategy may be given through comparison with the microbial analyses results, as discussed in Chapter 8.

- The microbial respiration data indicates that the addition of chicken manure enhanced pile viability, as reflected in the higher carbon dioxide evolution rates attained by the TMT and SMT conditions, and that:

a. Both microbial populations indigenous to the chicken manure and those indigenous to the oil refinery sludge contributed to total microbial respiration.
b. The microbial population indigenous to the chicken manure contributed to total microbial respiration more so than those indigenous to the oil refinery sludge.

This is reflected in the greater difference between TMT and un-amended sludge treatment conditions than between SMT and un-amended sludge treatment conditions.

Pile Temperature versus Microbial Respiration

- The microbial respiration data did not show a strong correlation with the pile temperature data, however both data sets indicate that the pile management strategy unlikely enhanced pile viability, and that addition of chicken manure did enhance pile viability.
- These two data sets however did disagree over the likely extent to which the microorganisms indigenous to the chicken manure contributed to total microbial activity; the pile temperature data indicates that such microorganisms contributed less to total microbial activity than those indigenous to the oil refinery sludge whereas the opposite was indicated by the microbial respiration data. Such inferences can be strengthened through comparison of these data with those of the chemical and microbial analyses.

4.6 Conclusions

Pile temperature and microbial respiration (by carbon dioxide evolution) were monitored throughout the treatability studies and the data used to indicate relative pile viability between the treatment conditions, with the ultimate aim of determining the likely extent to which a) the pile management strategy and b) the addition of chicken manure enhanced environmental conditions (pile viability) for microbial growth and activity.

The following conclusions have been made based on these data alone:

1. The pile temperature and microbial respiration data do not show a clear indication of pile viability enhancement for the main duration of the treatability studies through the adoption of the pile management strategy. However, the microbial respiration data indicates a slight enhancement of pile viability due to the pile management strategy over the last 37 days of the treatability studies, as indicated by statistically significantly ($p<0.01$) higher carbon dioxide evolution rates (approximately 8ppm/min/g soil dry weight higher) than those attained by the un-managed sludge (UMC) pile condition.

2. The pile temperature and microbial respiration data indicate that the addition of chicken manure to the oil refinery sludge enhanced pile viability, as shown by higher pile temperatures and carbon dioxide evolution rates for the manure amended sludge (TMT and SMT) compared with those for the un-amended sludge (UMC and MC) pile conditions.

3. The pile temperature and microbial respiration data indicate that the activity of both the microorganisms indigenous to the chicken manure and those indigenous to the oil refinery sludge were enhanced through the amendment of the oil refinery sludge with chicken manure. The pile temperature data indicated that the microorganisms indigenous to the chicken manure contributed less to total microbial activity than the microorganisms indigenous to the oil refinery sludge. In contrast, the microbial respiration data indicate that the microorganisms indigenous to the chicken manure contributed more to total microbial activity than the microorganisms indigenous to the oil refinery sludge.

Chapter 5

Chemical Analyses

5.1 Introduction

To assess the success of bioremediation it is necessary to determine loss of parent compound(s) over time (Joergensen et al, 1995; Battelle, 1996; Atlas, 1995; USEPA, 1991). Such determination is recommended under the British Standard on laboratory testing for biodegradation of organic chemicals (BS, 1995b).

The majority of previous bioremediation investigations, such as those discussed in Chapter 2, determined changes in total TPH (Total Petroleum Hydrocarbons) concentrations as an indication of successful bioremediation. However, it is recognised that petroleum products are complex mixtures of hydrocarbons of varying physical-chemical properties and toxicity, as was discussed in Chapter 2. Determination of changes in total TPH concentration has been criticised as it does not provide sufficient information on the variety of hydrocarbons present in a samples, and therefore does not enable a full risk assessment (Bundy, 2004; Sadler and Connell, 2003; Weisman, 1998). The same concentration of TPH may represent very different compositions and therefore different risks to human health and the environment (Weisman, 1998).

The Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG) of the United States have developed an approach to monitoring changes in TPH concentrations whereby TPH mixtures are subdivided into aliphatic and aromatic hydrocarbon fractions, and then these fractions are further subdivided into fractions based on carbon number (Weisman, 1998). The hydrocarbons fractions were chosen by the TPHCWG based on physical-chemical properties and therefore mobility in the environment (Weisman, 1998).

The UK Environment Agency has recognised the benefit of the TPHCWG fractionated TPH approach to enable a more defined risk assessment, and recently reported that this approach is already widely used in the UK (Askari and Pollard, 2005). The TPHCWG approach, and methodology, was therefore adopted for this study.

In addition to this, headspace analyses were carried out to indicate the potential contribution of volatilisation to TPH degradation and the effect of composting bioremediation (pile management strategy and addition of chicken manure) on volatilisation. Such differentiation between biodegradation and volatilisation is recommended by Loehr et al (2001b).

This chapter outlines the sampling programme and methodology adopted for determination of changes in total and fractionated TPH concentration, and volatilisation over the duration of the treatability studies. The results of these analyses are presented and discussed. Further discussion of these results is presented in Chapter 8.

5.2 Aims and Objectives

As stated in Chapter 1 and 3, Objectives One, Two, and Four Part 2 of this study are as follows:

Objective One: - *To determine the potential for using chicken manure to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH).*

Objective Two: - *To determine the potential for using chicken manure to enhance the biodegradation of the more resistant TPH compounds.*

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 1) bioaugmentation versus biostimulation, resulting from the addition of chicken manure to TPH contaminated material.*

The investigation of these objectives is achieved through the use of chemical analyses to quantify changes in total and fractionated TPH concentration over the duration of the treatability studies, and comparison of TPH degradation profiles for the four pile conditions (un-managed control (UMC), managed control (MC), total manure treatment (TMT), and sterile manure treatment (SMT)). Headspace analyses were also undertaken to indicate changes in concentration of headspace TPH over the duration of the treatability studies. These data are used to indicate the potential extent to which volatilisation contributed to TPH degradation, and the potential effect of the composting bioremediation process and addition of chicken manure on volatilisation.

5.3 Sampling Programme

A sampling programme was designed in accordance with British Standard 7755, Part 4.1.1 (1995b). Bulk samples (of approximately 3kg wet weight) were taken from the eight treatability study trays on Days 1, 2, 4, 8, 16, 32, 64, and 90, and homogenised by hand using a sterile stainless steel scoop (homogenisation deemed by visual inspection). A sample of approximately 200g (wet weight) was removed from each bulk sample and stored in amber glass jars with a Teflon lined screw cap, with no headspace, and refrigerated. Duplicate sub-samples were taken for chemical analyses comprising solid and headspace TPH quantification.

5.4 Methodology

5.4.1 TPH Analyses

The TPH analyses undertaken during the treatability studies were carried out by the Analytical Services Group of the School of Biosciences, Cardiff University. The analyses were based on the Association for Environmental Health and Services (AEHS) Direct Method (2000) developed by the Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG). A full copy of the Direct Method is provided in Appendix 4, and an overview of this method is given below. The method was modified by the Analytical Services Group in order to optimise its sensitivity to the samples tested during this study. A summary of such method modification is provided below.

5.4.1.1 The Direct Method – Overview

The Direct Method comprises three steps, as outlined below:

Step One: Extraction of Petroleum Hydrocarbons

Extraction of petroleum hydrocarbons from a soil is achieved through the addition of n-pentane and vigorous mixing by vortex. The eluate is then collected.

Step Two: Solid Phase Separation

The eluate from the extraction step is separated into aliphatic and aromatic hydrocarbon classes by means of silica gel columns, as illustrated in Figure 5.1.

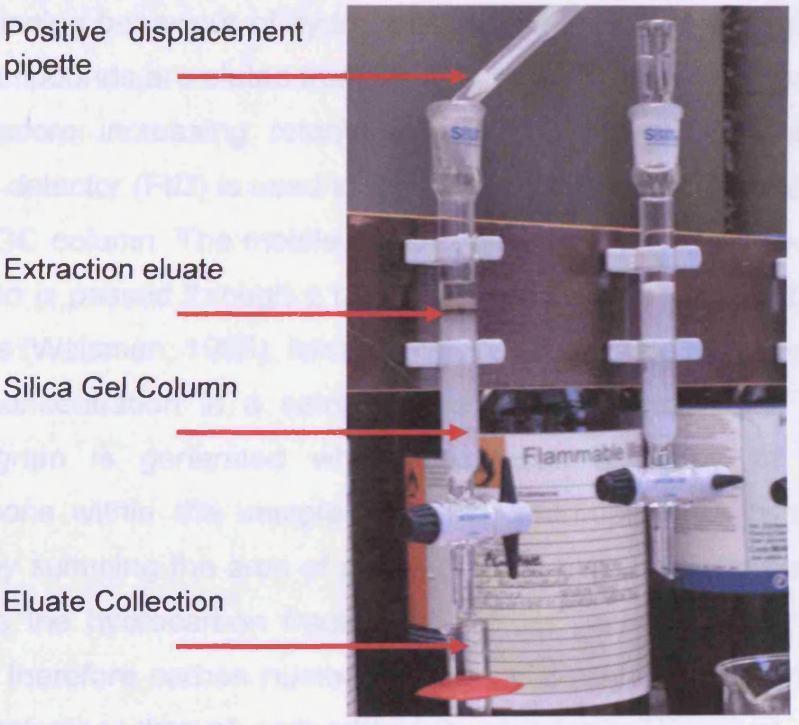


Figure 5.1: Solid Phase Separation by Silica Gel Column

During this step, 1ml of extraction eluate is gradually loaded onto the silica gel column, and washed through the column using n-pentane until 6.5ml of eluate have been collected. This fraction represents the aliphatic hydrocarbon fraction, and is transferred immediately to the freezer until required for quantification as per Step Three below. The aromatic hydrocarbon fraction is collected by passing acetone: dichloromethane (1:1 ratio) through the column until 6.5ml eluate has been collected.

This solid phase separation step underwent method development by the Analytical Services Group, as summarised in Section 5.4.1.2.

Step Three: Quantification by GC-FID

The final step is to further divide the aliphatic and aromatic hydrocarbon eluates into hydrocarbon fractions based on carbon number by gas chromatograph, and to quantify these fractions using a flame ionisation detector (FID).

The principles of gas chromatography (GC) were outlined in Chapter 4, and was shown to involve the separation of compounds within a mixture according to differences in partitioning behaviour between a mobile phase and a stationary phase as they travel through a chromatographic column (Rouessac and Rouessac, 2004). The partitioning behaviour of hydrocarbons is related to their boiling point (Weisman, 1998). Compounds are eluted from the GC column in order of increasing boiling point (and therefore increasing retention time) (Kealey and Haines, 2002). A flame ionisation detector (FID) is used to quantify the petroleum hydrocarbons as they elute from the GC column. The mobile phase (carrying the hydrocarbons) eluting from the GC column is passed through a hydrogen flame which ionises the hydrocarbons and yields ions (Weisman, 1998). Ionised molecules produce a current that is proportional to their concentration in a sample. The current is recorded as a signal, and a chromatogram is generated which comprises a series of peaks representing hydrocarbons within the sample. The concentration of a hydrocarbon fraction is attained by summing the area of peaks present over the retention time interval which represents the hydrocarbon fraction of interest (retention time is related to boiling point and therefore carbon number; Fetter, 1999) and converting these area values into concentrations through calibration with standards (Weisman, 1998).

5.4.1.2 Method Development

An Agilent 6890N GC-FID was used with Chemstation software and 7683 autosampler. The GC was equipped with a HP5 MS 30m x 0.25mm column, with a 0.25µm stationary phase. The temperature programme was set to 30°C for 4 minutes, then increased at 15°C/minute to 320°C; and held at 320°C for 20 minutes.

The Direct Method protocol was slightly modified by the Analytical Services Group, as summarised below:

Extraction Step: -

10g (accuracy of 0.001g) solid sample was mixed with anhydrous sodium sulphate to produce a free flowing sample, as per the Direct Method protocol. Due to the nature of the samples, the optimum quantity of sodium sulphate was found to be 20g, rather than the 10g stipulated by the Direct Method protocol. The mixture was then vortexed with 15ml rather than 10ml n-pentane for 1 minute to extract the hydrocarbons. The larger volume of pentane was necessary due to the increased quantity of sodium sulphate used.

Fractionation Step:-

Silica gel columns (Figure 5.1) were prepared as per the Direct Method protocol, and 1ml extract sample was loaded onto the gel. Separation of the aliphatic and aromatic fractions was achieved by passing n-pentane through the column and collecting 6.5ml of the eluent containing the aliphatic fraction. The Direct Method protocol suggests collecting 8-12ml eluent before flushing the aromatics through the column. However, the Analytical Services Group found that if more than 6.5ml was collected, the eluate would contain aliphatic and some aromatic fractions.

5.4.2 Headspace Analyses

Duplicate solid samples from the treatability studies were placed in sealed glass vials, and volatile compounds were allowed to equilibrate between the headspace and the solid sample. The headspace was then sampled by Solid Phase MicroExtraction (SPME) and analysed by gas chromatography interfaced with a mass spectrometer (GC-MS) to semi-quantify changes in concentration of headspace volatile hydrocarbons over the duration of the treatability studies. Method development was undertaken prior to the treatability studies to optimise detection of the volatile hydrocarbons. The method development is detailed in Section 5.4.2.2. The protocol adopted is provided in Section 5.4.2.3

5.4.2.1 Method Overview

SPME comprises a fused silica fiber that is coated with chemical adsorbents or particles, and is housed in a syringe-like assembly, as illustrated in Figure 5.2 (Supelco, 2004a). When the fiber is exposed to a sample (solid, liquid or gaseous), compounds that have affinity to the chemical coating are retained on the fiber by passive adsorption. The amount of compound adsorbed onto the fiber is proportional to the concentration of the compound in the sample (Supelco, 2004b).

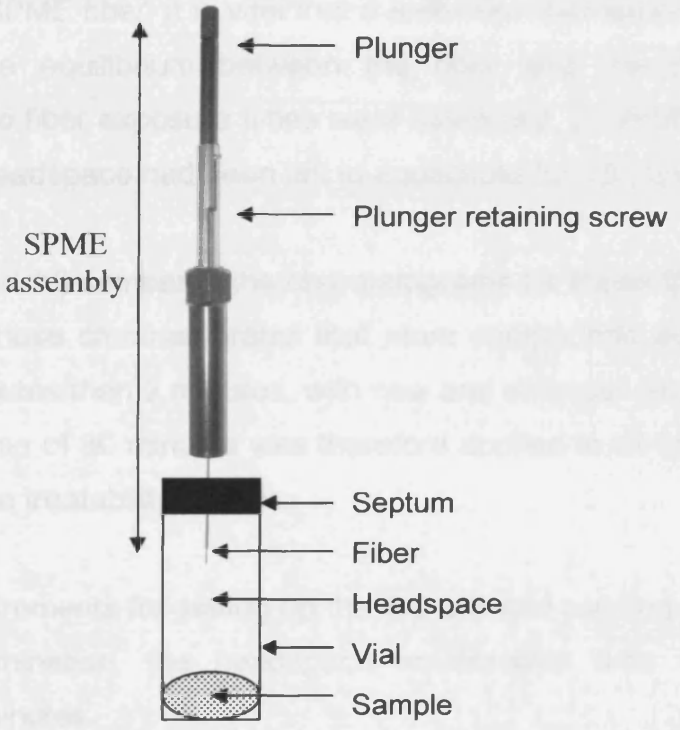


Figure 5.2: SPME assembly and headspace sampling.

Once the compounds have sorbed onto the fiber, the syringe assembly is inserted into the injection port of a GC and the fiber exposed (Supelco, 2004a). The compounds are thermally desorbed from the fiber and passed through a GC column. The compounds in the sample are separated in the GC column as per Section 5.4.1.1. As they elute from the column they are detected and quantified using a mass spectrometer (MS). Briefly, compounds eluting from the GC column are ionised by collision with free electrons (which are generated in an ion source within the MS). The ions are separated according to their mass and charge and are detected by means of an electrical charge which is proportional to their concentration in the sample (Kealey and Haines, 2002).

5.4.2.2 Method Development

Trials were carried out to determine the optimum GC oven temperature gradient for compound separation and the optimum headspace sampling protocol. A petrol and diesel mix was used as the sample.

Headspace Sampling:-
Due to the nature of competition between volatile compounds to adsorb to and be retained by the SPME fiber, it is vital that a sufficient fiber exposure time is applied in order to achieve equilibrium between the fiber and the headspace (C.Muller, pers.comm.). Two fiber exposure times were assessed, 2 minutes and 30 minutes. In both cases the headspace had been left to equilibrate for 15 minutes.

Figure 5.3 ('a' and 'b') compares the chromatograms for these fiber exposure times. It is evident from these chromatograms that more compounds adsorbed to the SPME fiber after 30 minutes than 2 minutes, with new and stronger peaks being identified. A fiber exposure time of 30 minutes was therefore applied to all samples analysed over the duration of the treatability studies.

Due to time requirements for setting up the GC-MS and running a blank fiber to check for cross contamination, the headspace equilibration time was increased to a minimum of 45 minutes.

For comparison reasons only, headspace was also sampled using an air-tight syringe, which is an alternative sampling technique. As seen in Figure 5.3c, this sampling technique was not as sensitive as the SPME method (Figure 5.3b), and therefore was not used in this study.

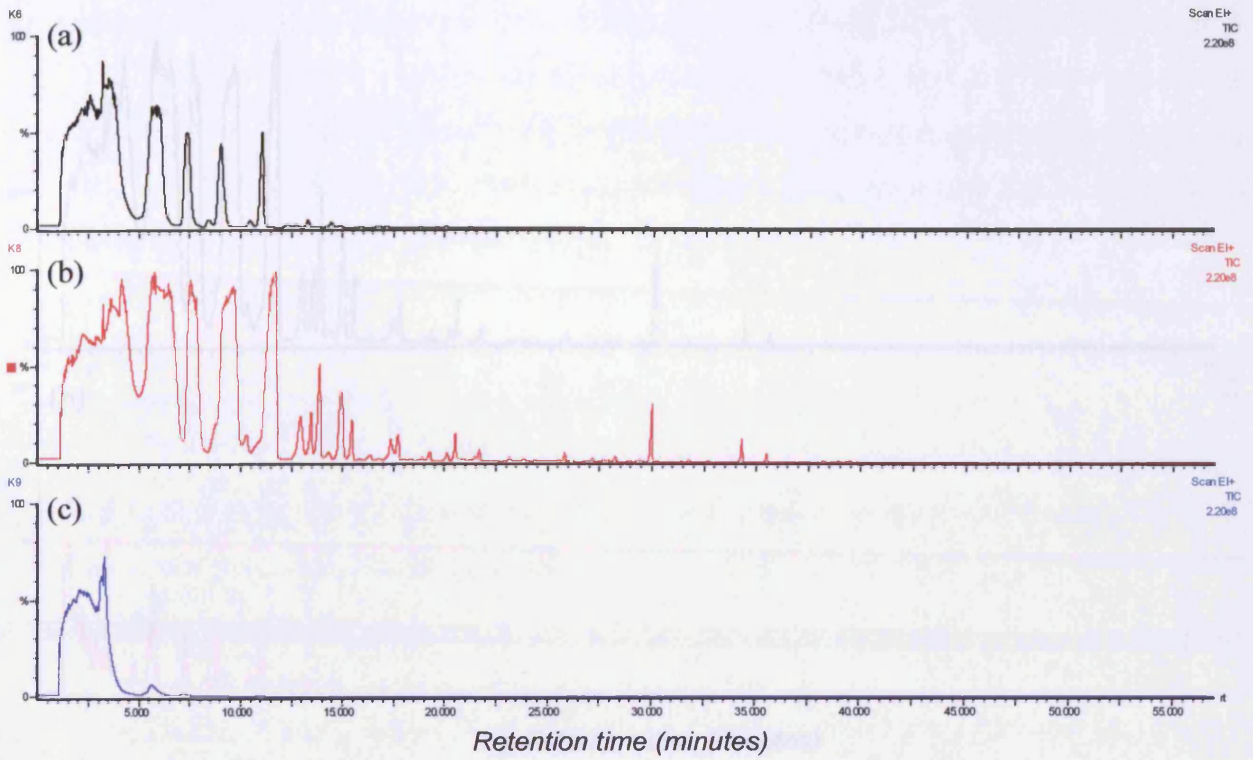


Figure 5.3: Chromatograms showing the effect of longer SPME fiber exposure time and sampling technique *(a) 2 minutes SPME fiber exposure, (b) 30 minutes SPME fiber exposure, (c) air tight syringe.*

Temperature Profile:-

Due to the likely complexity of the TPH compounds found in the oil refinery sludge, a temperature gradient was applied to the GC column to enable better separation of the hydrocarbons.

An oven temperature gradient of 2.5°C/min from 40°C to 160°C was set, and was found to give relatively good peak separation (Figure 5.4a). However, the sample was overloading the column for the first 5 minutes. A split injection was therefore adopted, whereby only 1% of the sample enters the column to prevent overloading. Peak separation was optimised in this way (Figure 5.4b) and was adopted for initial samples from the treatability studies, until detection of peaks reduced, at which point a split-less injection was adopted whereby the entire sample (100%) entered the column.

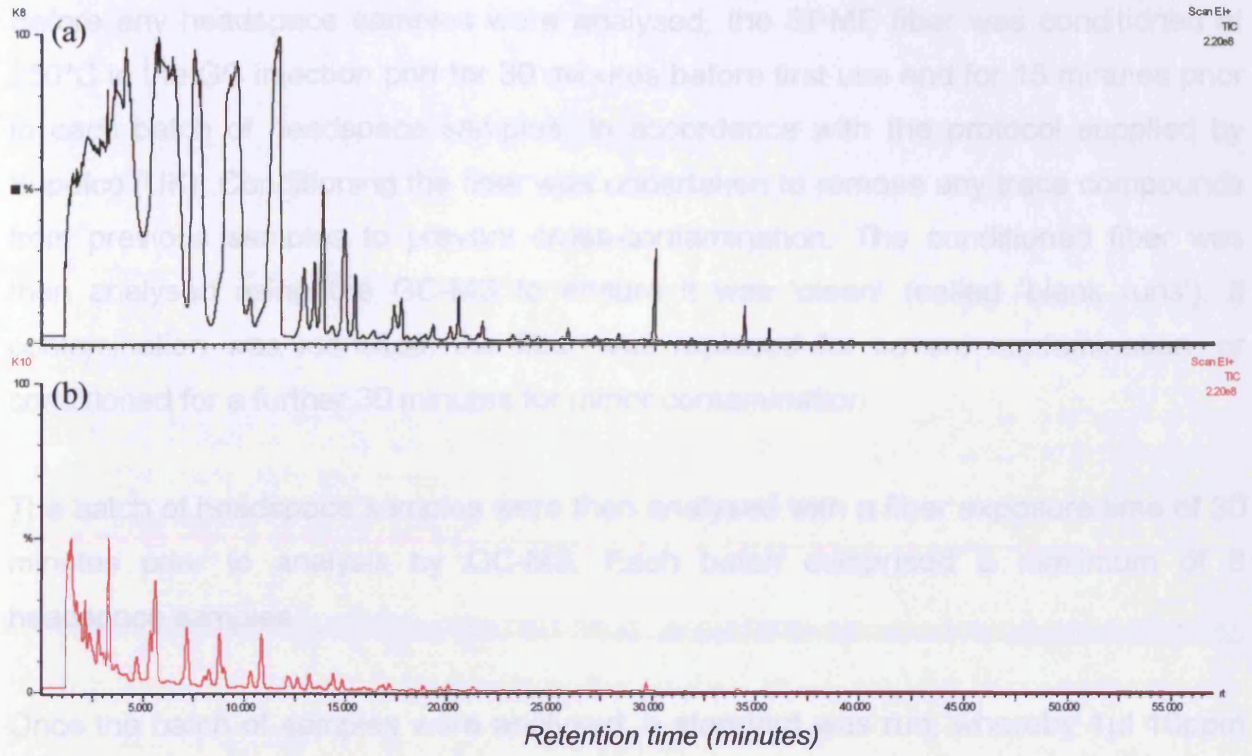


Figure 5.4: Sample injection mode (a) split-less injection, (b) split injection.

5.4.2.3 Protocol

For this study, a polydimethysiloxane (PDMS) coated SPME fiber (100µm) from Supelco (UK) was used to sample the headspace. A Fisons GC8000 with Finnigan MD800 MS was used, with Masslab software. A Varian Factor Four CF-23MS 30m x 0.25mm column with a 0.25µm phase was used.

A GC temperature programme was held at 40°C for 5 minutes, then increased by 2.5°C/min from 40°C to 160°C and held at 160°C for 5 minutes. This temperature programme was set and applied to all headspace samples and blanks.

Duplicate solid samples (of equal volume) from each treatability study tray were placed in 20ml glass vials and sealed with silicon septa and PTFE screw caps to prevent loss of volatiles. The volatiles were allowed to equilibrate with the headspace for a minimum of 45 minutes.

Before any headspace samples were analysed, the SPME fiber was conditioned at 250°C in the GC injection port for 30 minutes before first use and for 15 minutes prior to each batch of headspace samples, in accordance with the protocol supplied by Supelco (UK). Conditioning the fiber was undertaken to remove any trace compounds from previous samples to prevent cross-contamination. The conditioned fiber was then analysed using the GC-MS to ensure it was 'clean' (called 'blank runs'). If contamination was identified, the fiber was replaced for severe contamination, or conditioned for a further 30 minutes for minor contamination.

The batch of headspace samples were then analysed with a fiber exposure time of 30 minutes prior to analysis by GC-MS. Each batch comprised a maximum of 8 headspace samples.

Once the batch of samples were analysed, a standard was run, whereby 1µl 10ppm hexadecane in n-pentane was loaded onto the GC column using an air-tight syringe. This was to monitor any changes in sensitivity of the GC-MS. A separate GC temperature profile was used for the hexadecane standard. This temperature profile was as follows; 40°C for 5 minutes, followed by an increase to 80°C at a rate of 2.5°C/min, and then held at 80°C for 5 minutes.

Calibration of SPME headspace data is usually achieved through comparison with gas standards which are subjected to the same GC temperature profile as the samples being tested (Namieśnik et al, 1998). It was intended to calibrate total headspace TPH compounds with a petrol/diesel gas standard. The gas standard would have been created by allowing a known quantity of petrol/diesel mix to evaporate in a closed chamber of known volume. This would have been sampled using an SPME fiber in the same way as the samples taken from the treatability studies, and run on the GC-MS using the same conditions. Unfortunately, however, there were technical difficulties with the GC-MS following the data collection period, therefore such calibration could not be undertaken during this study. The potential contribution of volatilisation to TPH degradation is therefore indicated through comparison with the TPH degradation profiles, as is discussed in Section 5.6.

5.5 Results

5.5.1 Baseline Chemical Characterisation of Oil Refinery Sludge and Biodegradation Potential

Oil refinery sludge was chosen as the test material for this study as such sludge is reported to contain a variety of hydrocarbon compounds ranging from readily biodegradable to less biodegradable, reflecting variations in chemical properties and structure. The oil refinery sludge was collected from a former oil refinery site in the UK and is believed to be in excess of 10 years in age.

The distribution of fractionated aliphatic and aromatic hydrocarbons (as a percentage of total TPH concentrations) determined for Day 1 samples of the four pile conditions (UMC, MC, TMT, SMT) is summarised in Table 5.1.

Table 5.1: Distribution of fractionated aliphatic and aromatic hydrocarbons in Day 1 samples of the treatability studies.

	Fractionated Aromatic Hydrocarbons (% total TPH)				
	C_9-C_{10}	**C_{10}-C_{12}**	**C_{12}-C_{16}**	**C_{16}-C_{22}**	**C_{22}-C_{36}**
UMC	0.14	0.96	6.14	12.09	9.14
MC	0.13	0.91	6.88	11.19	4.69
TMT	0.06	0.33	5.59	12.69	8.48
SMT	0.07	0.55	6.88	13.01	6.09
	Fractionated Aliphatic Hydrocarbons (% total TPH)				
	C_9-C_{10}	**C_{10}-C_{12}**	**C_{12}-C_{16}**	**C_{16}-C_{22}**	**C_{22}-C_{36}**
UMC	1.75	5.41	17.93	32.98	13.47
MC	1.78	5.66	23.03	29.52	16.22
TMT	0.74	4.24	21.26	31.34	15.27
SMT	1.24	5.05	26.58	27.67	12.85
	Total Fractionated Hydrocarbons (% total TPH)				
	C_9-C_{10}	**C_{10}-C_{12}**	**C_{12}-C_{16}**	**C_{16}-C_{22}**	**C_{22}-C_{36}**
UMC	1.88	6.37	24.07	45.07	22.60
MC	1.91	6.56	29.91	40.71	20.90
TMT	0.80	4.57	26.85	44.03	23.75
SMT	1.31	5.60	33.46	40.68	18.95

It is evident from the data presented in Table 5.1 that the oil refinery sludge was dominated by high molecular weight hydrocarbons and that aliphatic hydrocarbons constituted a greater percentage of total TPH compounds than aromatic hydrocarbons.

It is reported by Loehr et al (2001a) that predictions can be made regarding the potential for biodegradation of soils contaminated with petroleum hydrocarbons based on the ratio of aliphatic hydrocarbons to aromatic hydrocarbons, and on the composition of the petroleum hydrocarbons with regards to molecular weight (and carbon number). Loehr et al (2001a) report that a ratio of aliphatic to aromatic hydrocarbons of 2.9:1 indicates a high potential for biodegradation, whereas a ratio of 1.3:1 indicates a low potential for biodegradation. Loehr et al (2001) also report that soils with petroleum hydrocarbons dominated by the C_{16}-C_{35+} range will have a lower potential for biodegradation than those dominated by the $<C_{16}$ range. These criteria are based on the physical-chemical properties of hydrocarbons discussed in Chapter 2, whereby in general aromatics are more resistant to biodegradation than aliphatics, and higher molecular weight (and higher carbon number) hydrocarbons are more resistant to biodegradation than lower molecular weight (and lower carbon number) hydrocarbons.

The oil refinery sludge used in this study had an aliphatic to aromatic hydrocarbon ratio of 2.79 (± 0.15 SE) to 1, based on mean ratios attained for the four pile conditions. This indicates a relatively high potential for biodegradation. However, C_{16}-C_{35+} hydrocarbons account for approximately 64% of total TPH and $<C_{16}$ hydrocarbons account for approximately 36% of total TPH. Therefore, the dominance of higher molecular weight hydrocarbons in the oil refinery sludge indicates a low potential for biodegradation.

5.5.2 TPH Analyses

The results of the total and fractionated TPH analyses attained over the duration of the treatability studies for the four pile conditions (UMC, MC, TMT, SMT) are presented in this section. All raw data is provided in Appendix 4. The data have been plotted as a series of graphs in Figures 5.5 to 5.15 to show TPH degradation curves. The graphs show changes in mean TPH concentration. TPH concentrations have been converted to percentage TPH remaining over time to account for variations in initial TPH concentration between the pile conditions for easier comparison of results. Error bars shown represent plus and minus one standard error (± 1SE). Statistical analyses were carried out using two-tailed Z-test for two means in Microsoft Excel

(significance level of 1%; p=0.01), in accordance with Schmuller (2005). Observations are presented in this section. These data are discussed in Section 5.6.

5.5.2.1 Total TPH

The total TPH degradation profiles are plotted in Figure 5.5. Observations are as follows:-

1. The UMC condition showed a reduction in total TPH concentration of approximately 35% between Days 1 and 16, however this reduction was not found to be statistically significant (p>0.01). Between Days 16 and 64, total TPH concentration increased by approximately 30%, however this increase was not found to be statistically significant (p>0.01). Between Days 64 and 90, total TPH concentration fell, giving a total reduction in total TPH concentration of approximately18% over the duration of the treatability studies. However, this loss of TPH was not found to be statistically significant (p>0.01).
2. The MC condition showed a reduction in total TPH concentration of approximately 16% over the duration of the treatability studies. However, this loss of TPH was not found to be statistically significant (p>0.01).
3. The TMT condition showed a reduction in total TPH concentration of approximately 22% over the duration of the treatability studies. However, this loss of TPH was not found to be statistically significant (p>0.01).
4. The SMT condition showed an increase in total TPH concentration of approximately 35% between Days 1 and 32, however this increase was not found to be statistically significant (p>0.01). This was followed by a reduction in total TPH concentration of approximately 37% between Days 32 and 90, giving a total reduction in total TPH concentration of approximately 2% over the duration of the treatability studies. However, this loss of TPH was not found to be statistically significant (p>0.01).
5. No statistically significant (p>0.01) differences in total TPH concentrations on Day 90 of the treatability studies was recorded between the four pile conditions (UMC, MC, TMT and SMT).

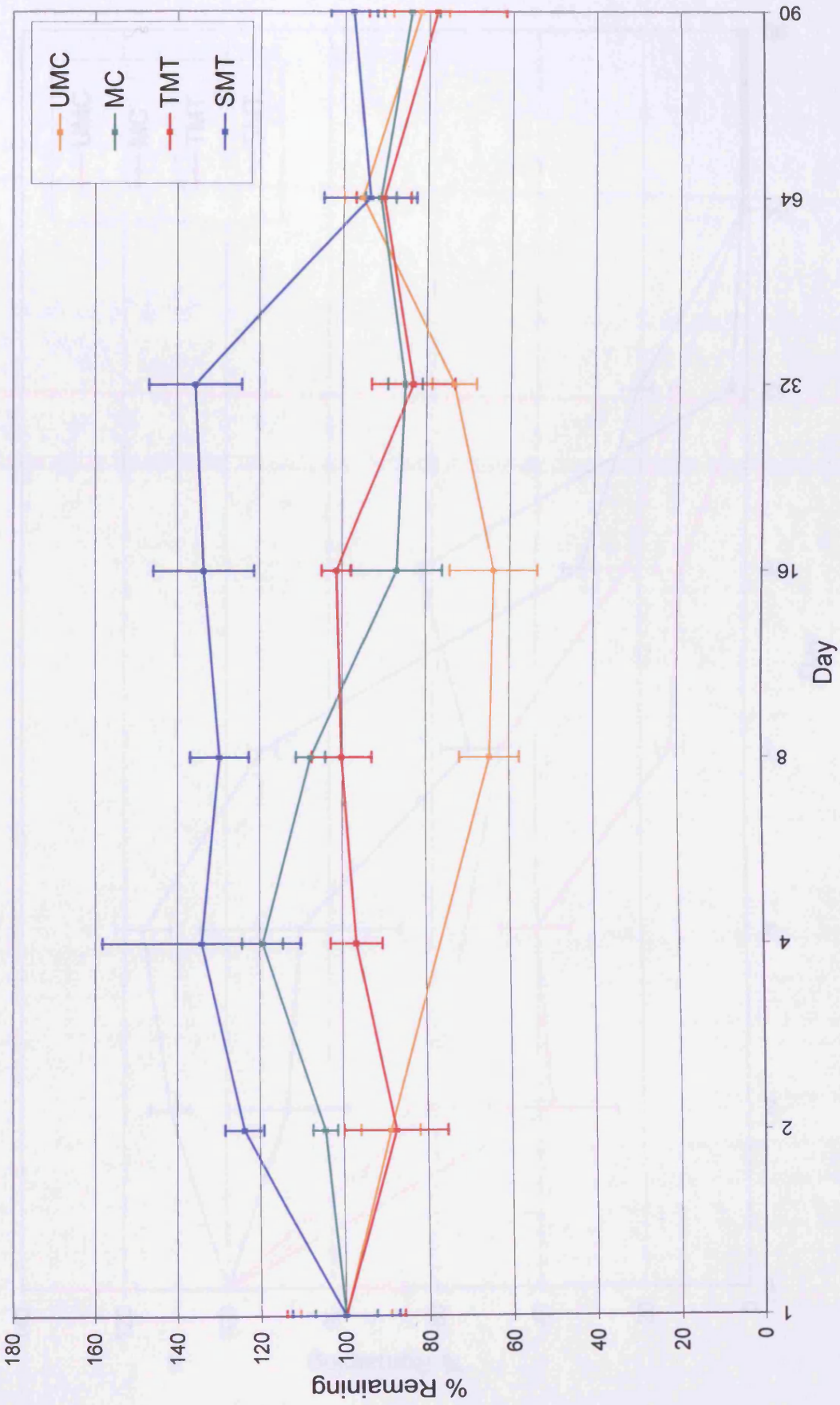


Figure 5.5: Percentage Total TPH Remaining

Figure 5.6: Percentage Degradation Aliphatic Hydrocarbons C_9-C_{10}

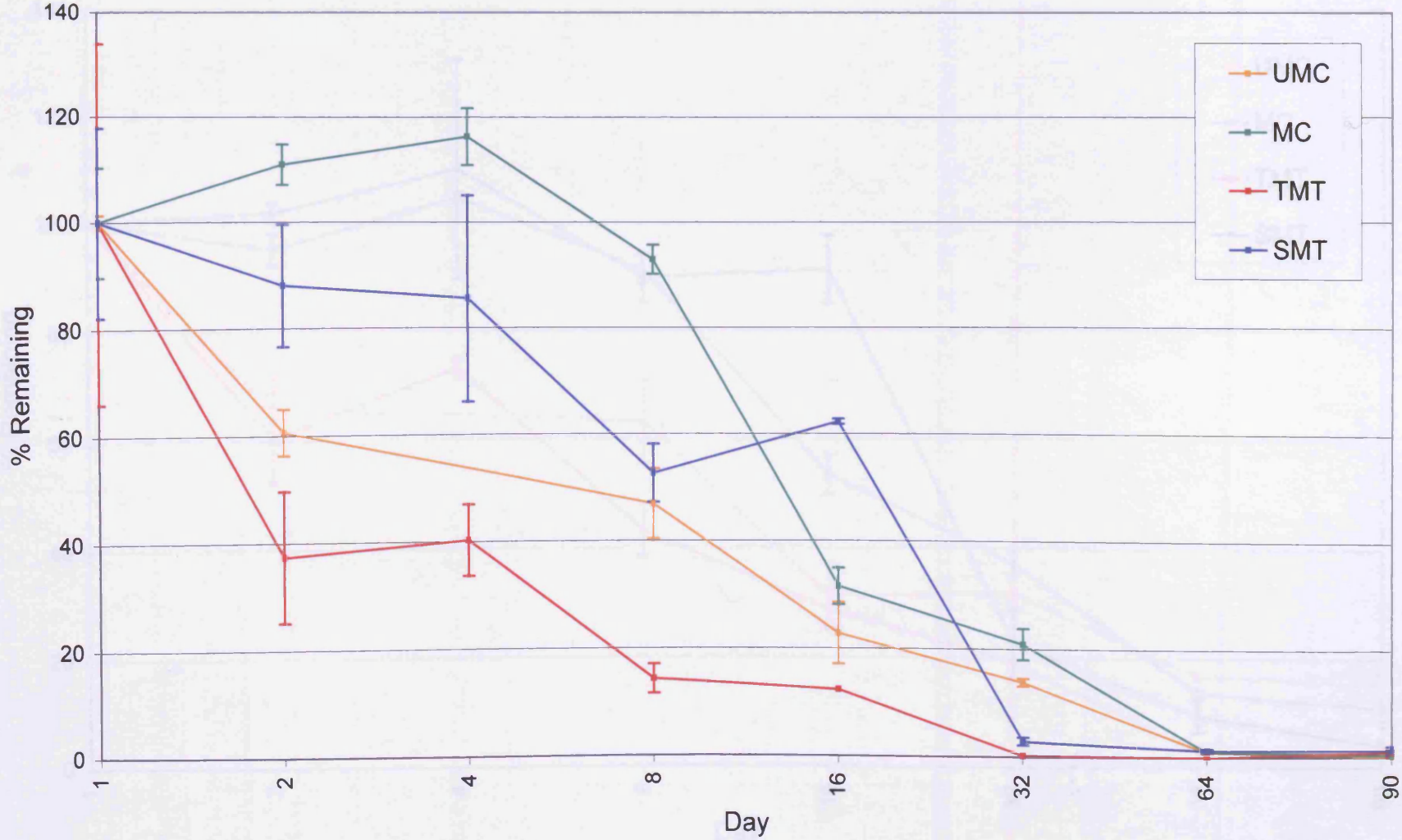


Figure 5.7: Percentage Degradation Aliphatic Hydrocarbons C_{10}-C_{12}

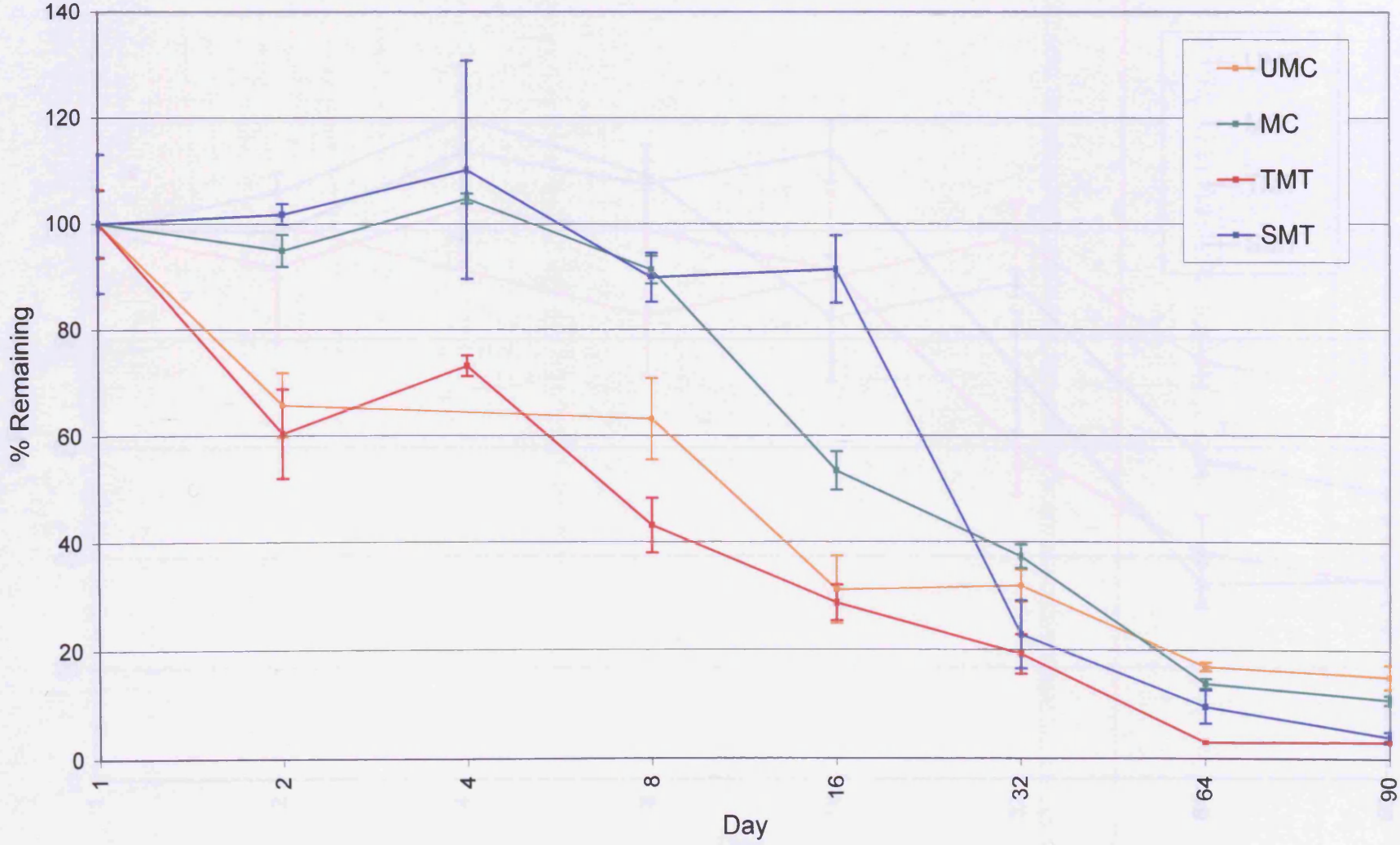


Figure 5.8: Percentage Degradation Aliphatic Hydrocarbons C_{12}-C_{16}

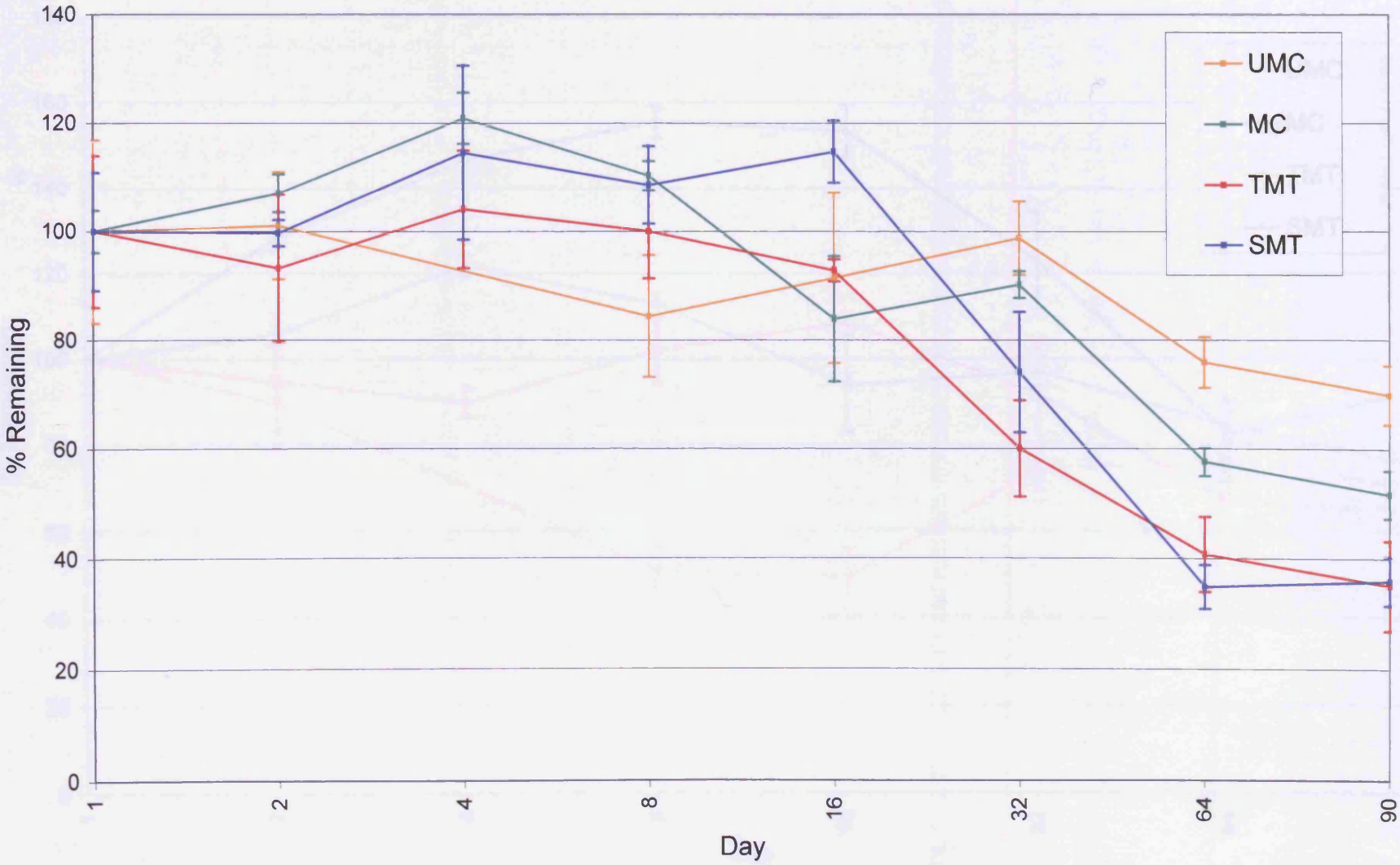


Figure 5.9: Percentage Degradation Aliphatic Hydrocarbons C_{16}-C_{22}

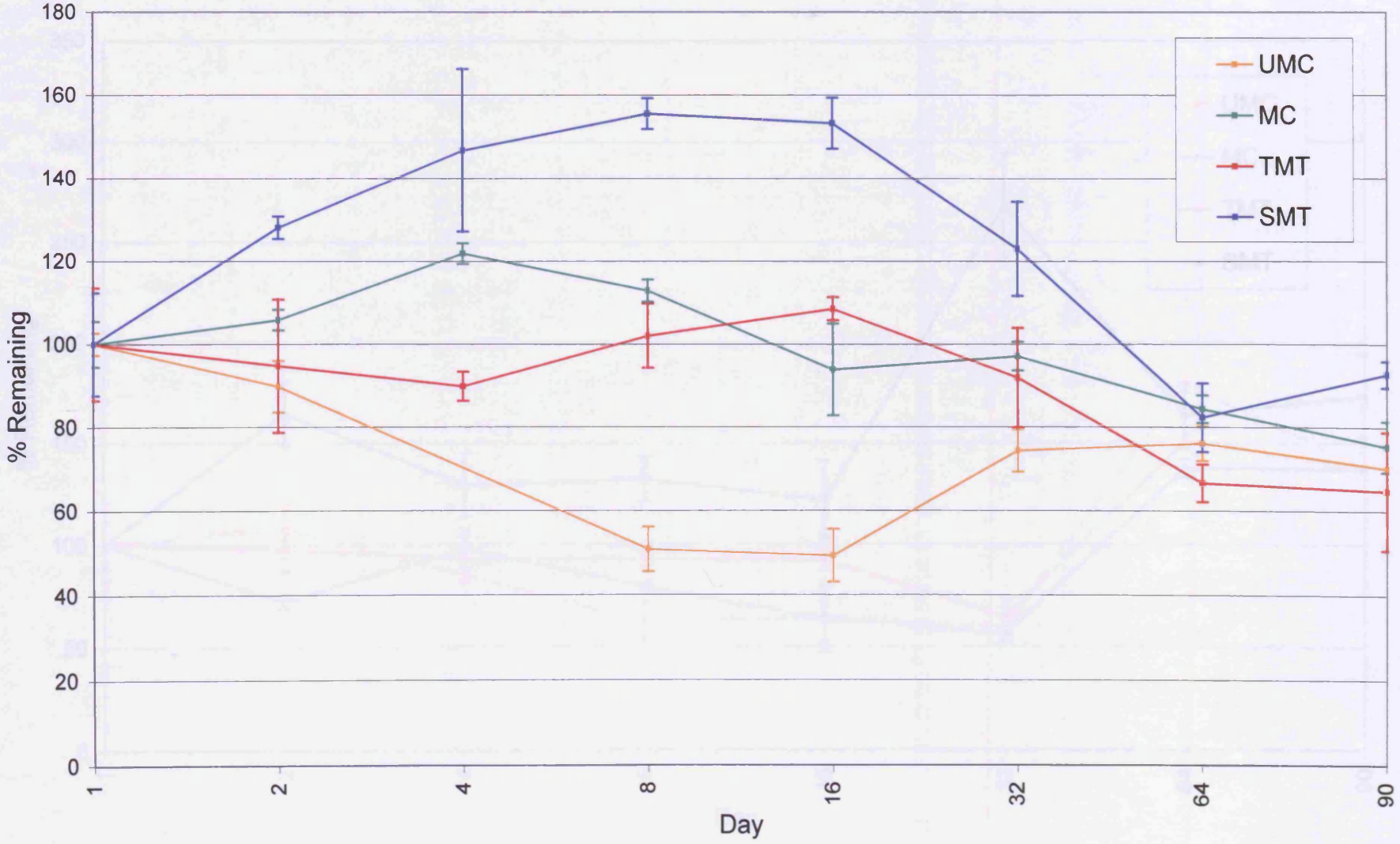


Figure 5.10: Percentage Degradation Aliphatic Hydrocarbons C_{22}-C_{36}

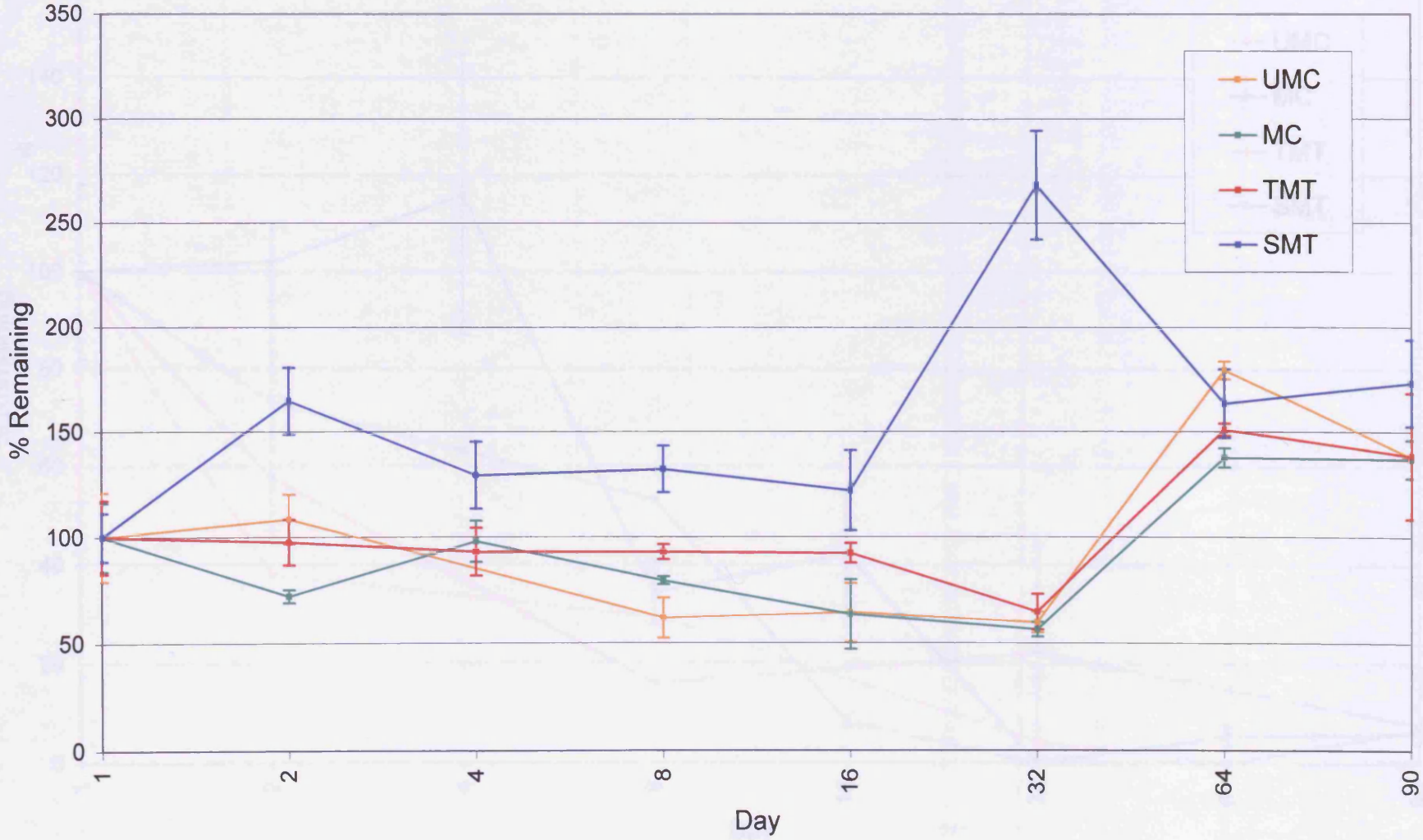
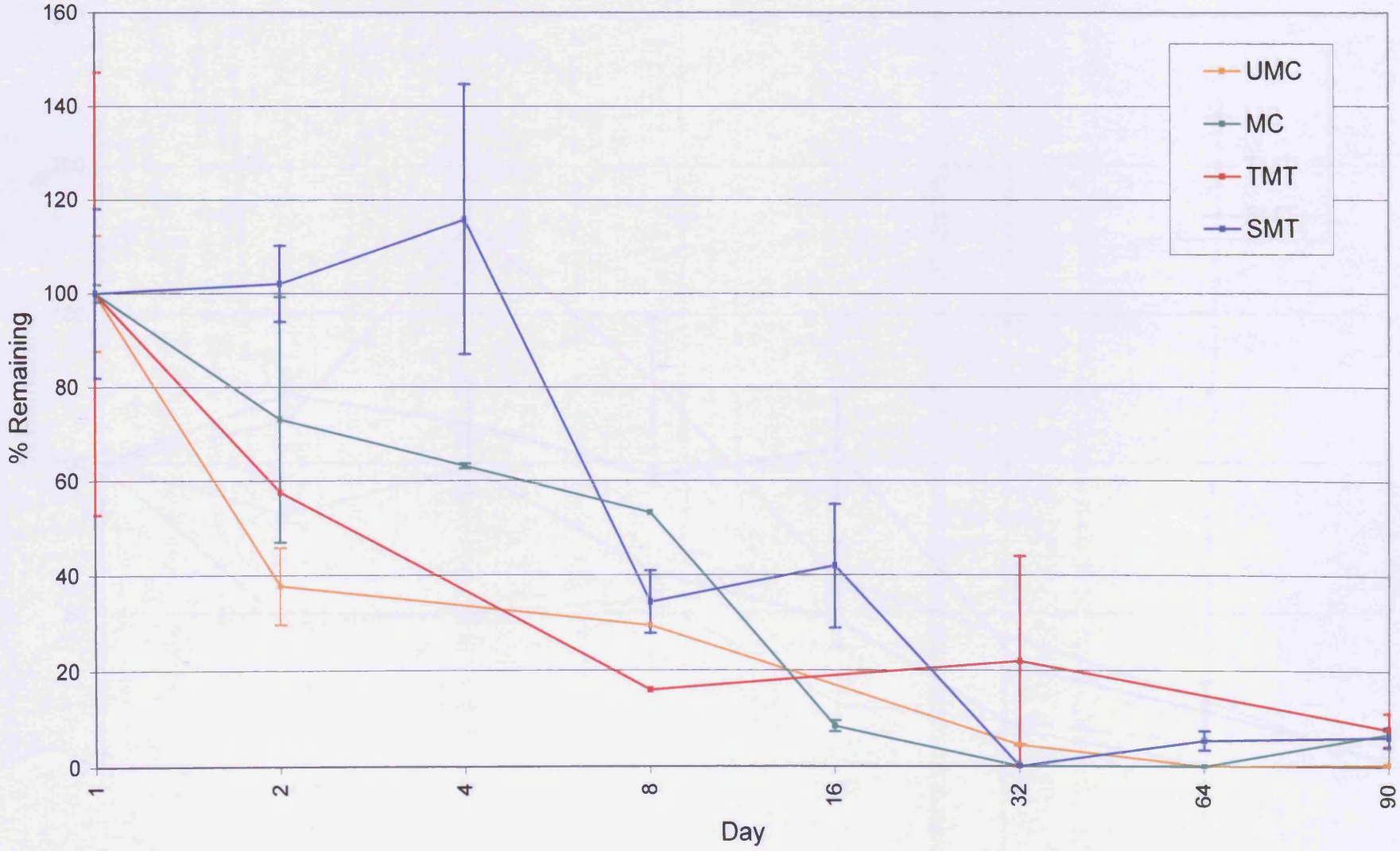


Figure 5.11: Percentage Degradation Aromatic Hydrocarbons C_9-C_{10}



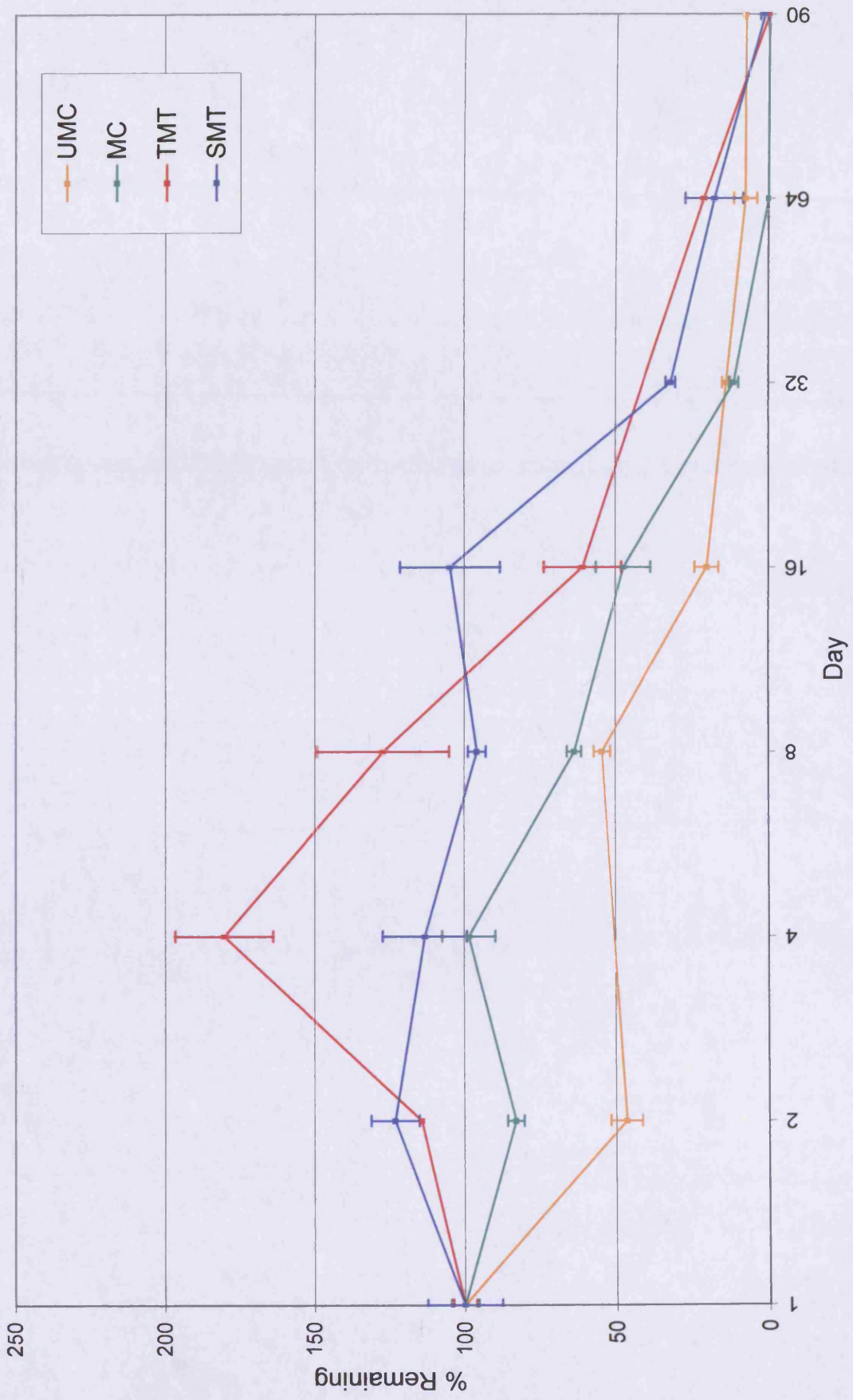


Figure 5.12: Percentage Degradation Aromatic Hydrocarbons C_{10}-C_{12}

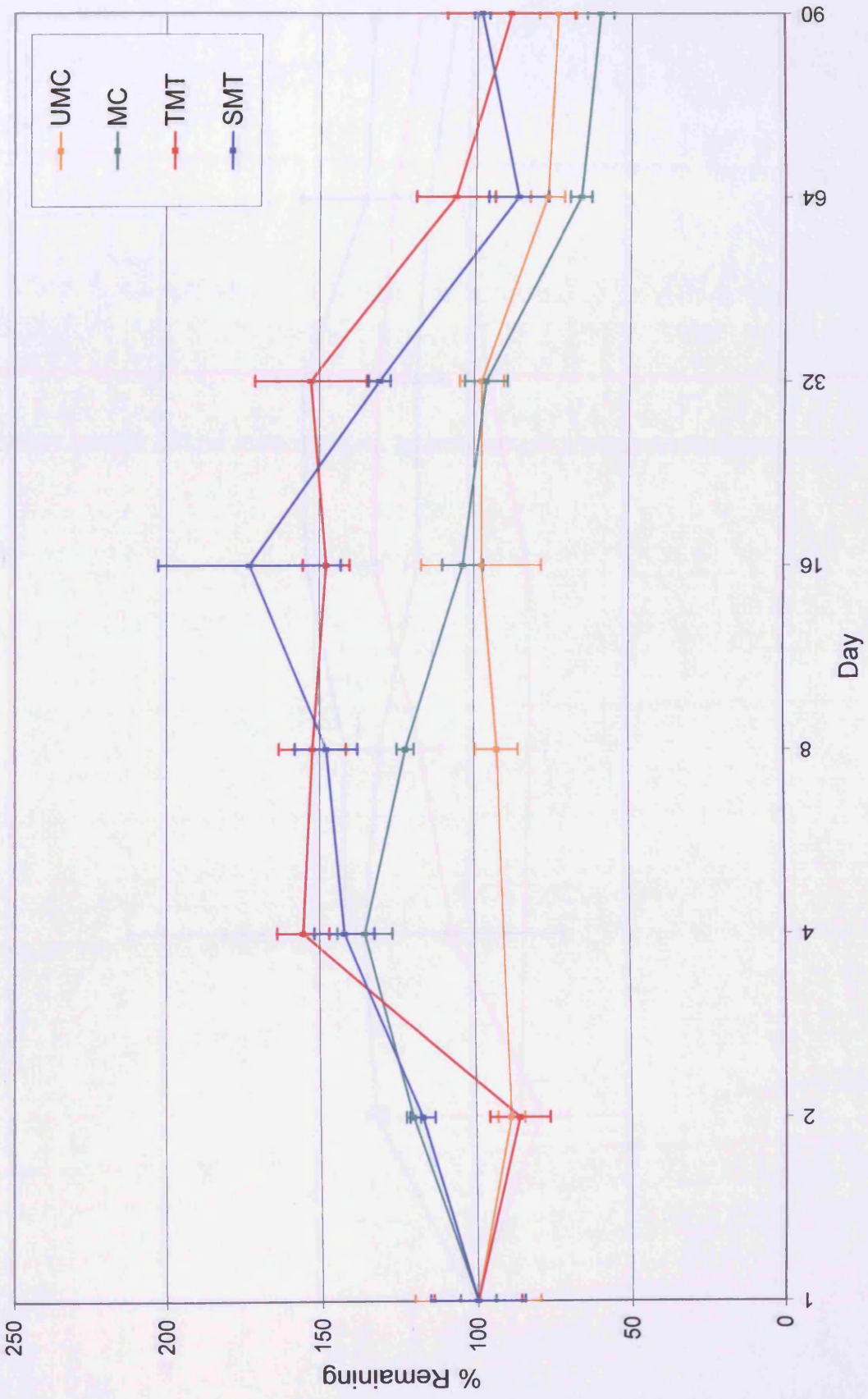


Figure 5.13: Percentage Degradation Aromatic Hydrocarbons C_{12}-C_{16}

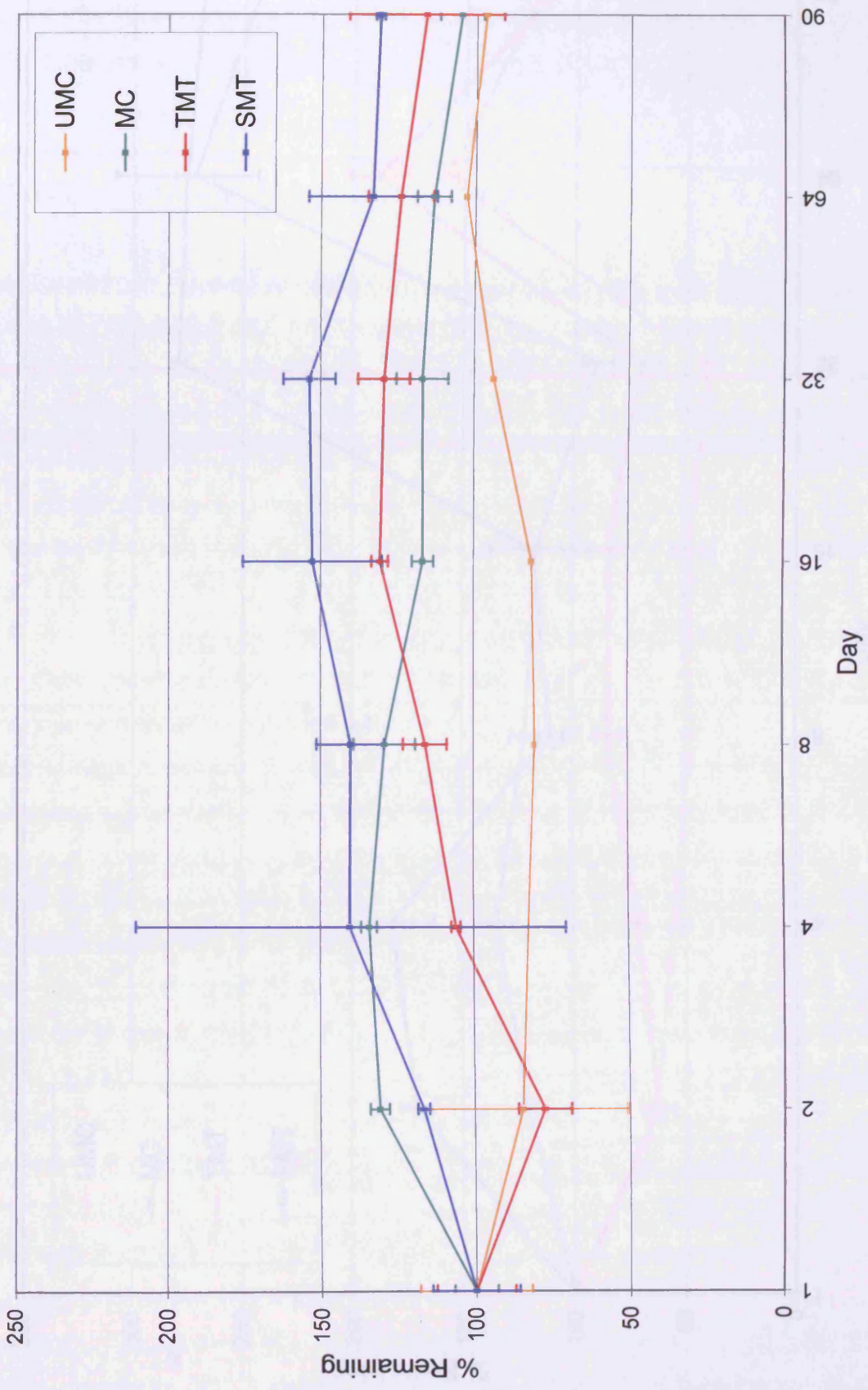
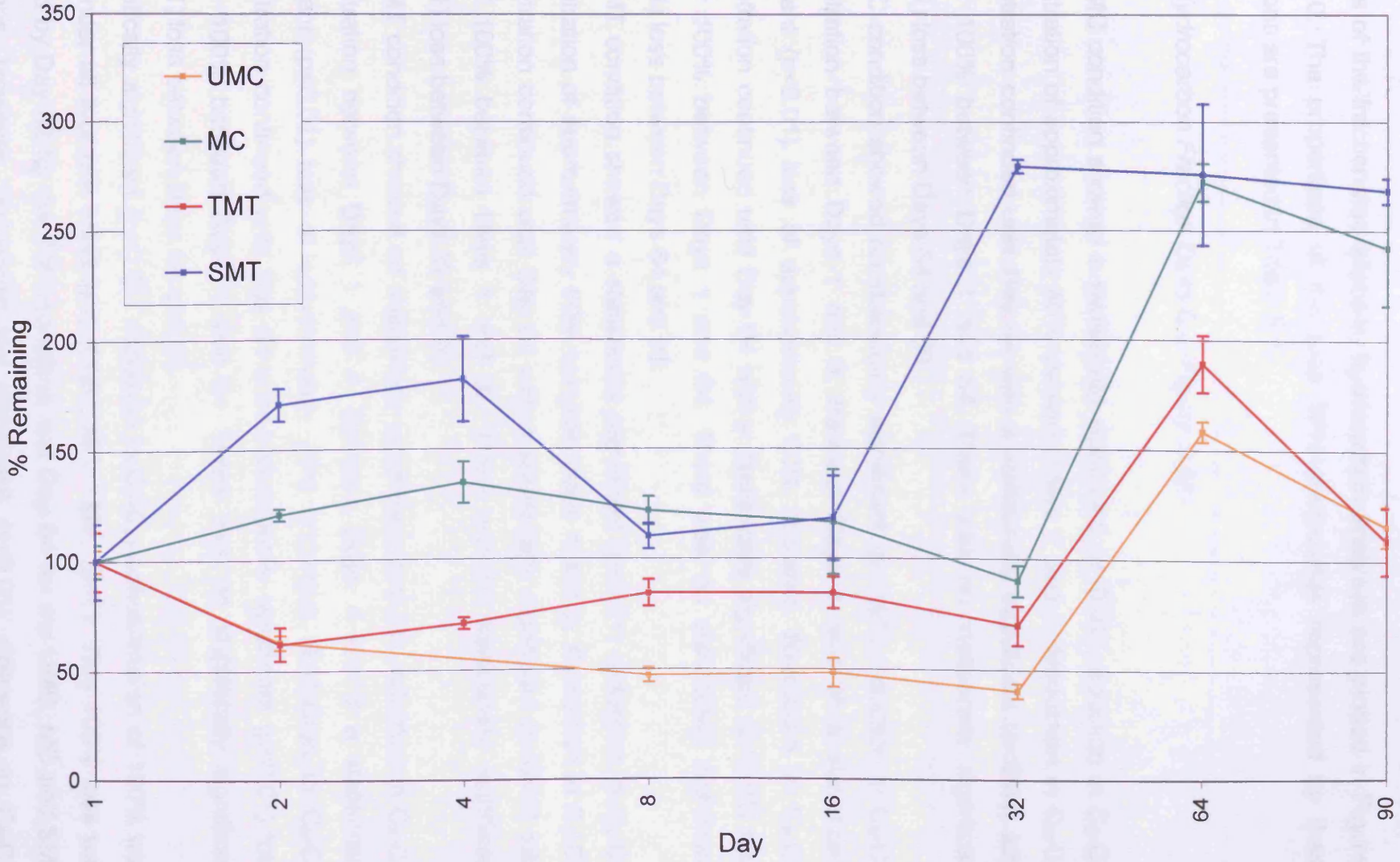


Figure 5.14: Percentage Degradation Aromatic Hydrocarbons C_{16}-C_{22}

Figure 5.15: Percentage Degradation Aromatic Hydrocarbons C_{22}-C_{36}



5.5.2.2 Fractionated Aliphatic Hydrocarbons

The results of the fractionated aliphatic hydrocarbon analyses are plotted in Figures 5.6 to 5.10. The proportions of the total TPH compounds represented by these hydrocarbons are presented in Table 5.1.

Aliphatic Hydrocarbon Fraction C_9 to C_{10} (Figure 5.6):-

1. The UMC condition showed a statistically significant ($p<0.01$) reduction in C_9-C_{10} concentration of approximately 40% between Days 1 and 2. Reduction in C_9-C_{10} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 100% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
2. The MC condition showed no statistically significant ($p>0.01$) reduction in C_9-C_{10} concentration between Days 1 and 8. Between Days 8 and 16 a statistically significant ($p<0.01$) loss of approximately 60% occurred. Reduction in C_9-C_{10} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 100% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
3. The TMT condition showed a statistically significant ($p<0.01$) reduction in C_9-C_{10} concentration of approximately 60% between Days 1 and 2. Reduction in C_9-C_{10} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 100% between Days 1 and 32. There was no statistically significant ($p>0.01$) loss between Days 32 and 90.
4. The SMT condition showed no statistically significant ($p>0.01$) reduction in C_9-C_{10} concentration between Days 1 and 4. Between Days 4 and 8 a statistically significant ($p<0.01$) loss of approximately 25% occurred. Reduction in C_9-C_{10} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 100% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
5. A statistically significant ($p<0.01$) reduction in C_9-C_{10} concentration of 100% was seen under all four pile conditions (UMC, MC, TMT, SMT). This 100% loss was attained by Day 32 for the TMT condition and Day 64 for the UMC, MC and SMT conditions. However, no statistically significant ($p>0.01$) difference in C_9-C_{10}

concentration between the TMT and SMT conditions on Day 32 was found, therefore it is assumed that 100% C_9-C_{10} loss was attained by Day 32 under both the TMT and SMT conditions. Statistically significant ($p<0.01$) differences between the manure amended oil refinery sludge (TMT and SMT conditions) and un-amended oil refinery sludge (UMC and MC conditions) were found on Day 32.

6. C_9-C_{10} concentrations were statistically significantly ($p<0.01$) lower between Days 2 and 16 under the TMT condition than under the UMC, MC and SMT conditions.

Aliphatic Hydrocarbon Fraction C_{10} to C_{12} (Figure 5.7):-

1. The UMC condition showed a statistically significant ($p<0.01$) reduction in C_{10}-C_{12} concentration of approximately 35% between Days 1 and 2. Reduction in C_{10}-C_{12} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of approximately 80% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
2. The MC condition showed no statistically significant ($p>0.01$) reduction in C_{10}-C_{12} concentration between Days 1 and 8. Between Days 8 and 16 a statistically significant ($p<0.01$) loss of approximately 45% occurred. Reduction in C_{10}-C_{12} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 80% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
3. The TMT condition showed a statistically significant ($p<0.01$) reduction in C_{10}-C_{12} concentration of approximately 40% between Days 1 and 2. Reduction in C_{10}-C_{12} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 95% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
4. The SMT condition showed no statistically significant ($p>0.01$) reduction in C_{10}-C_{12} concentration between Days 1 and 16. Between Days 16 and 32 a statistically significant ($p<0.01$) loss of approximately 70% occurred. Reduction in C_{10}-C_{12} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 90% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
5. The UMC and TMT conditions shared a similar degradation profile for C_{10}-C_{12}. No statistically significant ($p>0.01$) differences in C_{10}-C_{12} concentration were recorded

between these pile conditions between Days 1 and 32. However, between Days 32 and 90, C_{10}-C_{12} concentration levels were statistically significantly ($p<0.01$) lower under the TMT condition than under the UMC condition.

6. The MC and SMT conditions shared a similar degradation profile for C_{10}-C_{12}. No statistically significant ($p>0.01$) differences in C_{10}-C_{12} concentration were recorded between these pile conditions, with the exception of on Day 16 where C_{10}-C_{12} concentrations were statistically significantly ($p<0.01$) higher under the SMT condition compared with the MC condition. However, on Day 90, C_{10}-C_{12} concentration levels were statistically significantly ($p<0.01$) lower under the SMT condition than under the MC condition.
7. The C_{10}-C_{12} concentration levels were statistically significantly ($p<0.01$) lower under the TMT condition than under the MC condition for the main duration of the treatability studies, including on Day 90.
8. The C_{10}-C_{12} concentration levels were not statistically significantly ($p>0.01$) different between the UMC and MC conditions between Days 32 and 90.
9. The C_{10}-C_{12} concentration levels were not statistically significantly ($p>0.01$) different between the UMC and SMT conditions between Days 32 and 90.
10. The C_{10}-C_{12} concentration levels were statistically significantly ($p<0.01$) lower under the TMT condition than under the SMT, with the exception of between Days 32 and 90.

Aliphatic Hydrocarbon Fraction C_{12} to C_{16} (Figure 5.8):-

1. The UMC condition showed no statistically significant ($p>0.01$) change in C_{12}-C_{16} concentration between Days 1 and 32. A reduction in C_{12}-C_{16} concentration of approximately 30% between Days 32 and 90, but this was not found to be statistically significant ($p>0.01$). A total reduction in C_{12}-C_{16} concentration of approximately 30% between Days 1 and 90 was recorded, but this was not found to be statistically significant.
2. The MC condition showed no statistically significant ($p>0.01$) reduction in C_{12}-C_{16} concentration between Days 1 and 32. Between Days 32 and 90 a statistically significant reduction in C_{12}-C_{16} concentration of approximately 40% was recorded. A total reduction in C_{12}-C_{16} concentration of approximately 40% between Days 1 and 90 was recorded, and this was found to be statistically significant.

3. The TMT condition showed no statistically significant ($p>0.01$) change in C_{12}-C_{16} concentration between Days 1 and 16. A reduction in C_{12}-C_{16} concentration of approximately 60% between Days 16 and 90, and this was found to be statistically significant ($p<0.01$). A total reduction in C_{12}-C_{16} concentration of approximately 65% between Days 1 and 90 was recorded, and this was found to be statistically significant ($p<0.01$).
4. The SMT condition showed no statistically significant ($p>0.01$) change in C_{12}-C_{16} concentration between Days 1 and 16. A reduction in C_{12}-C_{16} concentration of approximately 60% between Days 16 and 90, and this was found to be statistically significant ($p<0.01$). A total reduction in C_{12}-C_{16} concentration of approximately 65% between Days 1 and 90 was recorded, and this was found to be statistically significant.
5. There were no statistically significant ($p>0.01$) differences in C_{12}-C_{16} concentrations between the four pile conditions over the duration of the treatability studies, with the exception of SMT C_{10}-C_{12} concentrations were statistically significantly ($p<0.01$) lower that UMC condition on Day 64.

Aliphatic Hydrocarbon Fraction C_{16} to C_{22} (Figure 5.9):-

1. The UMC condition showed a statistically significant ($p<0.01$) reduction in C_{16} to C_{22} concentration of approximately 50% between Days 1 and 16. However, C_{16} to C_{22} concentration increased between Days 16 and 32 (although this increase was not found to be statistically significant ($p>0.01$)). No statistically significant reduction in C_{16} to C_{22} concentration was recorded between Days 1 and 90.
2. The MC condition showed no statistically significant ($p>0.01$) reduction in C_{16} to C_{22} concentration between Days 1 and 90.
3. The TMT condition showed no statistically significant ($p>0.01$) reduction in C_{16} to C_{22} concentration between Days 1 and 4. Between Days 4 and 16 C_{16} to C_{22} concentrations increased by approximately 20%, however this increase was not found to be statistically significant ($p>0.01$). Between Days 16 and 90, C_{16} to C_{22} concentrations decreased by approximately 45% (giving a total reduction in C_{16} to C_{22} concentrations of approximately 35%), however this decreased was not found to be statistically significant ($p>0.01$). The TMT condition showed no statistically significant ($p>0.01$) reduction in C_{16} to C_{22} concentration between Days 1 and 90.

4. The SMT condition showed a gradual increase in C_{16} to C_{22} concentration of approximately 55% between Days 1 and 16, however this increase was not found to be statistically significant ($p>0.01$). Between Days 16 and 90 a reduction in C_{16} to C_{22} concentration of approximately 60 (giving a total reduction in C_{16} to C_{22} concentration of approximately 8%) was recorded, however this was not found to be statistically significant ($p>0.01$). The SMT condition showed no statistically significant ($p>0.01$) reduction in C_{16} to C_{22} concentration between Days 1 and 90.

Aliphatic Hydrocarbon Fraction C_{22} to C_{36} (Figure 5.10):-

1. The UMC condition showed no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 1 and 32. Between Days 32 and 64, C_{22} to C_{36} concentrations increased by approximately 80%, however this increase was not found to be statistically significant ($p>0.01$). Overall, there was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentrations over the duration of the treatability study.
2. The MC condition showed no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 1 and 32. Between Days 32 and 64, C_{22} to C_{36} concentrations increased by approximately 40%, however this increase was not found to be statistically significant ($p>0.01$). Overall, there was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentrations over the duration of the treatability study.
3. The TMT condition showed no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 1 and 32. Between Days 32 and 64, C_{22} to C_{36} concentrations increased by approximately 50%, however this increase was not found to be statistically significant ($p>0.01$). Overall, there was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentrations over the duration of the treatability study.
4. The SMT condition showed no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 1 and 16. Between Days 16 and 32, C_{22} to C_{36} concentrations increased by approximately 170%. This increase was found to be statistically significant ($p>0.01$). Between Days 32 and 64, C_{22} to C_{36} concentrations reduced by approximately 100% to a level of approximately 160%. This reduction was not found to be statistically significant ($p>0.01$). Overall, there

was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentrations over the duration of the treatability study, when comparing Day 1 values with Day 64 and Day 90 values.

5.5.2.3 Fractionated Aromatic Hydrocarbons

The results of the fractionated aromatic hydrocarbon analyses are plotted in Figures 5.11 to 5.15. The proportions of the total TPH compounds represented by these hydrocarbons are presented in Table 5.1.

Aromatic Hydrocarbon Fraction C_9 to C_{10} (Figure 5.11):-

1. The UMC condition showed a statistically significant ($p<0.01$) reduction in C_9-C_{10} concentration of approximately 60% between Days 1 and 2. Reduction in C_9-C_{10} concentration continued until Day 64 with a statistically significant ($p<0.01$) total loss of 100% between Days 1 and 64. There was no statistically significant ($p>0.01$) loss between Days 64 and 90.
2. The MC condition showed no statistically significant ($p>0.01$) reduction in C_9-C_{10} concentration between Days 1 and 8. Between Days 8 and 16, a statistically significant ($p<0.01$) reduction of approximately 90% was recorded. By Day 32, C_9-C_{10} concentrations were reduced to 0%. There was no statistically significant ($p>0.01$) change in C_9-C_{10} concentration between Days 32 and 90.
3. The TMT condition showed no statistically significant ($p>0.01$) reduction in C_9-C_{10} concentration between Days 1 and 2. By Day 8, C_9-C_{10} concentrations were reduced by approximately 85%, this reduction was found to be statistically significant ($p<0.01$). There was no statistically significant ($p>0.01$) change in C_9-C_{10} concentration between Days 8 and 90. A total loss of 90% was recorded between Days 1 and 90, and this was found to be statistically significant ($p<0.01$).
4. The SMT condition showed no statistically significant ($p>0.01$) reduction in C_9-C_{10} concentration between Days 1 and 4. Between Days 4 and 8, a statistically significant ($p<0.01$) reduction of approximately 60% was recorded. This was followed by no statistically significant ($p>0.01$) change in C_9-C_{10} concentration between Days 8 and 16, but a statistically significant ($p<0.01$) reduction in C_9-C_{10} concentration to 0% was recorded by Day 32. There was no statistically significant

($p>0.01$) change in C_9-C_{10} concentration between Days 32 and 90. A total loss of 90% was recorded between Days 1 and 90, and this was found to be statistically significant ($p<0.01$).

5. There was no statistically significant ($p>0.01$) difference in C_9-C_{10} concentration between the 4 pile conditions (UMC, MC, TMT, SMT) between Days 32 and 90 therefore it is assumed that all pile conditions reached the same endpoint concentration of C_9-C_{10}.

Aromatic Hydrocarbon Fraction C_{10} to C_{12} (Figure 5.12):-

1. The UMC condition showed a statistically significant ($p<0.01$) reduction in C_{10}-C_{12} concentration of approximately 50% between Days 1 and 2. There was no statistically significant ($p>0.01$) change in C_{10}-C_{12} concentration between Days 2 and 8. Between Days 8 and 16 a further statistically significant ($p<0.01$) reduction of approximately 30% was recorded. Between Days 1 and 90 C_{10}-C_{12} concentration had been reduced by approximately 90%. This was found to be statistically significant ($p<0.01$).
2. The MC condition showed no statistically significant change in C_{10}-C_{12} concentration between Days 1 and 4. Between Days 4 and 32, a statistically significant ($p<0.01$) reduction in C_{10}-C_{12} concentration of approximately 90% was recorded. By Day 64, C_{10}-C_{12} concentration was reduced by 100%. Overall, between Days 1 and 64, a statistically significant ($p<0.01$) reduction in C_{10}-C_{12} concentration of 100% was recorded.
3. The TMT condition showed a statistically significant ($p<0.01$) increase in C_{10}-C_{12} concentration of approximately 80% between Days 1 and 4. Following this increase, C_{10}-C_{12} concentration levels gradually reduced by 100% by Day 90. This reduction was found to be statistically significant ($p<0.01$).
4. The SMT condition showed no statistically significant ($p>0.01$) reduction in C_{10}-C_{12} concentration between Days 1 and 16. Between Days 16 and 90, a statistically significant ($p<0.01$) reduction in C_{10}-C_{12} concentration of approximately 100% was recorded.
5. There was no statistically significant ($p>0.01$) difference in C_{10}-C_{12} concentration levels between the 4 pile conditions (UMC, MC, TMT, SMT) between Days 64 and 90.

Aromatic Hydrocarbon Fraction C_{12} to C_{16} (Figure 5.13):-

1. The UMC condition showed no statistically significant ($p>0.01$) change in C_{12} to C_{16} concentration over the duration of the treatability studies.
2. The MC condition showed no statistically significant ($p>0.01$) change in C_{12} to C_{16} concentration between Days 1 and 32. However, by Day 90, a statistically significant ($p<0.01$) reduction in C_{12} to C_{16} concentration of approximately 40% was recorded.
3. The TMT condition showed an increase in C_{12} to C_{16} concentration between Days 1 and 4 of approximately 50%. However, this increase was not found to be statistically significant ($p>0.01$). The TMT condition showed no statistically significant ($p>0.01$) change in C_{12} to C_{16} concentration over the duration of the treatability studies.
4. The SMT condition followed a similar profile to that of the TMT condition, and showed no statistically significant ($p<0.01$) change in C_{12} to C_{16} concentration over the duration of the treatability studies.
5. Although the MC condition showed a statistically significant ($p<0.01$) reduction in C_{12} to C_{16} concentration of approximately 40% by Day 90, there was no statistically significant ($p>0.01$) difference in C_{12} to C_{16} concentration levels between the 4 pile conditions (UMC, MC, TMT, SMT) on Day 90. Therefore it is assumed that all pile conditions attained a similar endpoint concentration of C_{12} to C_{16}.

Aromatic Hydrocarbon Fraction C_{16} to C_{22} (Figure 5.14):-

1. The UMC condition showed no statistically significant ($p>0.01$) change in C_{16} to C_{22} concentration over the duration of the treatability studies.
2. The MC condition showed no statistically significant ($p>0.01$) change in C_{16} to C_{22} concentration over the duration of the treatability studies.
3. The TMT condition showed no statistically significant ($p>0.01$) change in C_{16} to C_{22} concentration over the duration of the treatability studies.
4. The SMT condition showed a gradual increase in C_{16} to C_{22} concentration over the duration of the treatability studies, however this was not found to be statistically significant ($p>0.01$).

Aromatic Hydrocarbon Fraction C_{22} to C_{36} (Figure 5.15):-

1. The UMC condition showed a statistically significant ($p<0.01$) reduction in C_{22} to C_{36} concentration of approximately 40% between Days 1 and 2. There was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 2 and 32. However, between Days 32 and 64, there was a statistically significant ($p<0.01$) increase in C_{22} to C_{36} concentration of approximately 100%, resulting in C_{22} to C_{36} concentration levels approximately 60% higher than Day 1 values. This ultimate increase in C_{22} to C_{36} concentration between Day 1 and Day 64 was found to be statistically significant. Between Days 64 and 90, C_{22} to C_{36} concentrations were reduced to approximately 115%, however this reduction was not found to be statistically significant ($p>0.01$). Overall, between Days 1 and 90, there was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration.
2. The MC condition showed no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 1 and 32. However, between Days 32 and 64 a statistically significant ($p<0.01$) increase in C_{22} to C_{36} concentration of approximately 170% was recorded. Between Days 64 and 90, C_{22} to C_{36} concentrations were reduced by 30%, giving an overall statistically significant ($p<0.01$) increase in C_{22} to C_{36} concentration from Day 1 to Day 90 of approximately 140%.
3. The TMT condition showed no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 1 and 32. However, between Days 32 and 64 a statistically significant ($p<0.01$) increase in C_{22} to C_{36} concentration of approximately 90% (to 190%) was recorded. Between Days 64 and 90, C_{22} to C_{36} concentrations were reduced by 90% (to 100%). Overall there was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration over the duration of the treatability studies.
4. The SMT condition showed a statistically significant ($p<0.01$) increase in C_{22} to C_{36} concentration of approximately 70% between Days 1 and 2. Between Days 2 and 4 there was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration. Between Days 4 and 8, C_{22} to C_{36} concentration levels was reduced to approximately 100%, which is not statistically significantly ($p>0.01$) different to Day 1 C_{22} to C_{36} concentration levels. Between Days 8 and 16 there was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration. However, between Days

16 and 32 there was a statistically significant ($p<0.01$) increase in C_{22} to C_{36} concentration of approximately 160% (to 260%). There was no statistically significant ($p>0.01$) change in C_{22} to C_{36} concentration between Days 32 and 90. From Day 1 to Day 90, there was a statistically significant ($p<0.01$) increase in C_{22} to C_{36} concentration of approximately 160%.

5. C_{22} to C_{36} concentration levels under the SMT condition on Day 32 were statistically significantly ($P<0.01$) higher than under the UMC, MC and TMT conditions.
6. On Day 64, C_{22} to C_{36} concentration levels under the SMT and MC conditions were statistically significantly ($P<0.01$) higher than under the UMC and TMT conditions.
7. There was no statistically significant ($p>0.01$) difference in C_{22} to C_{36} concentration levels between the UMC and TMT conditions between Days 32 and 90.

5.5.3 Headspace Analyses

5.5.3.1 Peak Integration and Data Manipulation

Chromatograms from un-amended sludge samples (UMC and MC conditions) were compared with those of manure-amended sludge samples (TMT and SMT conditions) to identify any erroneous peaks which may not have been of TPH origin. There were no peaks identified that were not of TPH origin.

Each chromatogram was then integrated using MassLab software, and the peak areas combined to give a total area value. The baseline parameters were the same for each chromatogram. Although some noise was also included in the integration, it was deemed that such peak areas would be insignificant in terms of total area. Peak integration was also carried out for the hexadecane standards, and the results are shown in Table 5.2.

Comparison of total area values over time enables determination of relative changes in headspace TPH concentrations. Comparison of headspace TPH concentration profiles with the TPH degradation profiles (solid samples) indicates the potential contribution of volatilisation to TPH degradation.

As seen in Table 5.2, the hexadecane area values vary. Correction factors were therefore calculated as per Table 5.2 using Hexadecane Batch 1 as the reference point and were applied to the relevant data values for the treatment conditions, as recommended by the Analytical Services Group of the School of Biosciences, Cardiff University (C.Muller, pers.comm.).

Table 5.2: Hexadecane (10ppm) Peak Integration Values and Correction Factors

Hexadecane Batch	Peak Area	Reference Point	Correction Factor
1	13836635	13836635	1
2	23144442		0.60
3	25285092		0.55
4	37946860		0.36
5	29475660		0.47
6	13469432		1.03
7	27516732		0.50
8	27385048		0.51
9	32806102		0.42
10	17986296		0.77
11	386389472		0.04

Corrected data for the treatment conditions are presented in Section 5.5.2.2. Day 1 area values were set at 100 units (arbitrary units) and subsequent area values are expressed as a percentage of this to semi-quantify changes in headspace TPH concentration over time for each pile condition. This is used to indicate the potential contribution of volatilisation to TPH degradation, as discussed in Section 5.6.

5.5.3.2 Results

Figure 5.16 presents the volatilisation profiles for Trays 1 to 8. The graph shows changes in mean headspace TPH over the duration of the treatability studies. Headspace TPH concentrations have been converted to percentage remaining over time. The error bars represent plus and minus one standard deviation (± 1SE). Statistical analyses were carried out using two-tailed Z-test for two means in Microsoft Excel (significance level of 1%; p=0.01), in accordance with Schmuller (2005). Observations are presented in this section. These data are discussed in Section 5.6.

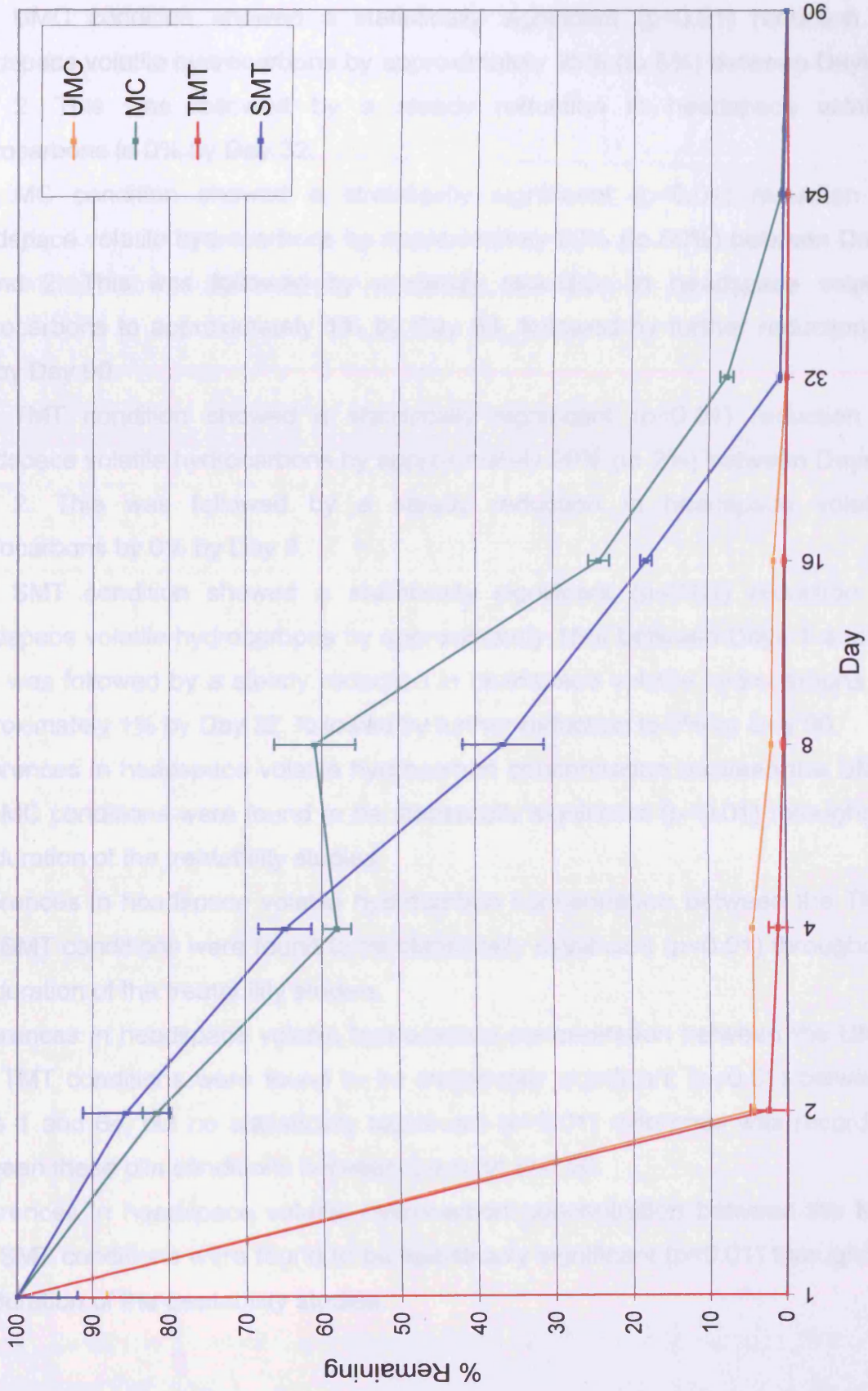


Figure 5.16: Percentage Remaining Headspace Volatile Hydrocarbons

Observations are as follows:

1. The UMC condition showed a statistically significant ($p<0.01$) reduction in headspace volatile hydrocarbons by approximately 95% (to 5%) between Days 1 and 2. This was followed by a steady reduction in headspace volatile hydrocarbons to 0% by Day 32.
2. The MC condition showed a statistically significant ($p<0.01$) reduction in headspace volatile hydrocarbons by approximately 20% (to 80%) between Days 1 and 2. This was followed by a steady reduction in headspace volatile hydrocarbons to approximately 1% by Day 64, followed by further reduction to 0% by Day 90.
3. The TMT condition showed a statistically significant ($p<0.01$) reduction in headspace volatile hydrocarbons by approximately 98% (to 2%) between Days 1 and 2. This was followed by a steady reduction in headspace volatile hydrocarbons by 0% by Day 8.
4. The SMT condition showed a statistically significant ($p<0.01$) reduction in headspace volatile hydrocarbons by approximately 15% between Days 1 and 2. This was followed by a steady reduction in headspace volatile hydrocarbons to approximately 1% by Day 32, followed by further reduction to 0% by Day 90.
5. Differences in headspace volatile hydrocarbon concentration between the UMC and MC conditions were found to be statistically significant ($p<0.01$) throughout the duration of the treatability studies.
6. Differences in headspace volatile hydrocarbon concentration between the TMT and SMT conditions were found to be statistically significant ($p<0.01$) throughout the duration of the treatability studies.
7. Differences in headspace volatile hydrocarbon concentration between the UMC and TMT conditions were found to be statistically significant ($p<0.01$) between Days 1 and 64, but no statistically significant ($p>0.01$) difference was recorded between these pile conditions between Days 64 and 90.
8. Differences in headspace volatile hydrocarbon concentration between the MC and SMT conditions were found to be statistically significant ($p<0.01$) throughout the duration of the treatability studies.

A mixture of aliphatic standards was also subjected to the same GC-MS temperature profile as the SPME samples, to identify the positions of hydrocarbon fractions according to retention time, as illustrated in Figure 5.17. It can be seen that the TPH compounds detected in the headspace cover hydrocarbons ranging from C_9 to C_{28}, with the majority of C_9-C_{10} hydrocarbons. Based on this, the contribution of the hydrocarbon fractions identified in the solid sample TPH analyses to the total headspace TPH concentration was calculated for Day 1 samples from each of the pile conditions (by summing the area of peaks detected within the appropriate retention times). A summary of these results is presented in Table 5.3.

Table 5.3: Fractionated headspace TPH compounds.

Hydrocarbon Fraction	% Total Headspace TPH	Standard Error
$<C_9$	34.1 %	± 2.64
C_9-C_{10}	60.4 %	± 2.73
C_{10}-C_{12}	4.1 %	± 0.25
C_{12}-C_{16}	1.2 %	± 0.09
C_{16}-C_{22}	0.09 %	± 0
C_{22}-C_{28}	0 %	± 0

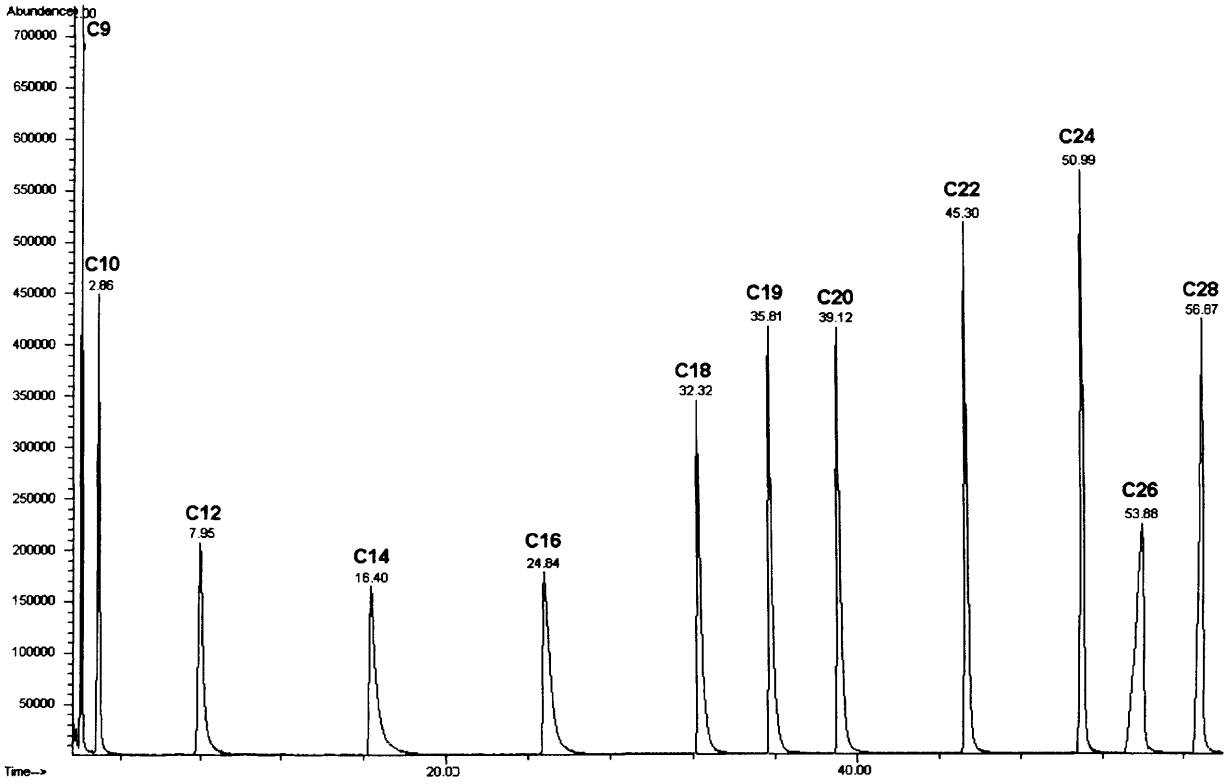


Figure 5.17: Retention times of C_9 to C_{28} under the GC-MS temperature profile used for the headspace analyses.

5.6 Discussion

5.6.1 TPH Analyses

5.6.1.1 Total TPH

It is evident from the data presented in Section 5.5.1.1 that there was no statistically significant ($p>0.01$) change in total TPH concentration over the duration of the treatability studies under any of the four pile conditions (UMC, MC, TMT, SMT). These data indicate that the bioremediation strategy (pile management strategy and the addition of chicken manure) did not have a statistically significant ($p>0.01$) effect on total TPH degradation.

The oil refinery sludge used in this study had an aliphatic to aromatic hydrocarbon ratio of 2.79 (± 0.15 SE) to 1, as was discussed in Section 5.5.1. This indicates a relatively high potential for biodegradation. However, C_{16}-C_{35+} hydrocarbons account for approximately 64% of total TPH and $<C_{16}$ hydrocarbons account for approximately 36% of total TPH. Therefore, the dominance of higher molecular weight hydrocarbons in the oil refinery sludge indicates a low potential for biodegradation.

The lack of statistically significant change in total TPH concentration over the duration of the treatability studies under any of the four pile conditions may reflect:

1. The starting distribution of fractionated aliphatic and aromatic hydrocarbons. In order for a statistically significant ($p<0.01$) reduction in total TPH concentration to occur, degradation of the higher molecular weight hydrocarbons would be needed as these constitute a larger percentage of total TPH than the lower molecular weight hydrocarbons (Table 5.1). Such hydrocarbons are deemed to be of lower biodegradability and bioavailability, as was discussed in Chapter 2.
2. Heterogeneity of samples, and therefore high variance in sample concentration recorded.
3. Insufficient nutrient availability to potential hydrocarbon degrading microorganisms.

4. Adverse competition between non-hydrocarbon and hydrocarbon degrading microorganisms resulting in the proliferation of non-hydrocarbon degrading microorganisms. This is discussed further in Chapter 8.
5. Adverse toxic effects resulting from the addition of chicken manure on potential hydrocarbon degrading microorganisms. This is discussed further in Chapter 8.

5.6.1.2 Fractionated Aliphatics

The data presented in Section 5.5.1.2 indicate that:

1. The pile management strategy did not enhance the degradation of C_9 to C_{36} as the UMC and MC conditions did not attain statistically significant ($p>0.01$) differences in concentrations of C_9 to C_{36} by Day 90.
2. The addition of chicken manure enhanced the loss of C_9 to C_{10} as 100% loss of this hydrocarbon fraction was attained by Day 32 under the TMT condition compared with Day 64 under the UMC and MC conditions. The enhanced loss of C_9 to C_{10} hydrocarbons resulting from the addition of chicken manure potentially reflects biostimulation of hydrocarbon degrading microorganisms indigenous to the oil refinery sludge only. This is evident from the lack of statistically significant ($p>0.01$) difference in C_9 to C_{10} concentration between the TMT and SMT conditions on Day 32, indicating that bioaugmentation effects resulting from the addition of chicken manure did not contribute to the enhanced loss seen. However, between Days 1 and 2 there was a statistically significant ($p<0.01$) reduction in C_9 to C_{10} concentration under the TMT condition, but not under the SMT condition. This could indicate enhanced loss through bioaugmentation effects resulting from the addition of chicken manure. From Days 2 to 90, the rate of degradation under the TMT condition was equal to or less than that under the SMT condition, indicating biostimulation effects only.
3. The addition of chicken manure enhanced the loss of C_{10} to C_{12} as statistically significantly ($p<0.01$) lower concentrations were recorded under the TMT condition than under the UMC condition between Days 32 and 90, and under the MC condition between Days 1 and 90. The enhanced loss potentially resulting from the addition of chicken manure may reflect biostimulation effects only. This is evident from the lack of statistically significant ($p>0.01$) difference in C_{10} to C_{12}

concentration between the TMT and SMT conditions between Days 32 and 90. However, between Days 1 and 16 there was a statistically significant ($p<0.01$) reduction in C_{10} to C_{12} concentration under the TMT condition, but not under the SMT condition. This could indicate enhanced loss through bioaugmentation effects resulting from the addition of chicken manure. From Days 32 to 90, the rate of loss under the TMT condition was less than that under the SMT condition, indicating biostimulation effects only.

4. The addition of chicken manure did not enhance the degradation of C_{12} to C_{36} hydrocarbons as seen by the lack of statistically significant ($p>0.01$) differences in concentration between the TMT and SMT conditions compared with the UMC and MC conditions.

5.6.1.3 Fractionated Aromatics

The data presented in Section 5.5.1.3 indicate that:

1. The pile management strategy did not enhance the degradation of C_9 to C_{36} aromatic hydrocarbons as the UMC and MC conditions did not attain statistically significant ($p>0.01$) differences in concentrations by Day 90.
2. The addition of chicken manure (both non-sterile and sterile) did not enhance the degradation of C_9 to C_{36} aromatic hydrocarbons as the TMT and SMT conditions did not attain statistically significant ($p>0.01$) differences in concentrations by Day 90, compared to the un-amended oil refinery sludge (UMC and MC conditions).
3. There was an increase in C_{22} to C_{36} aromatic hydrocarbon concentrations of approximately 60% under the UMC condition, and 140% under the MC condition between Days 32 and 64. Between Days 64 and 90, the concentration returned to Day 1 concentration values under the UMC condition but remained the same under the MC condition. This indicates that the pile management strategy had a potentially adverse effect on C_{22} to C_{36} concentrations. There was an increase in C_{22} to C_{36} concentrations of approximately 60% under the TMT condition between Days 32 and 64. These conditions were returned to Day 1 concentration values between Days 64 and 90. When the TMT and MC conditions are compared it appears that the addition of chicken manure aided the degradation of C_{22} to C_{36} between Days 64 and 90 when the pile management strategy did not. The SMT

condition showed an increase in C_{22} to C_{36} concentrations of approximately 160% between Days 16 and 32 and these concentrations remained the same until Day 90. It may therefore be that the reduction in concentration of C_{22} to C_{36} between Days 64 and 90 under the TMT condition reflect bioaugmentation effects resulting from the addition of chicken manure, as such reductions were not seen under the MC or SMT conditions. However, there was no statistically significant ($p>0.01$) difference between the TMT and UMC conditions, suggesting that the addition of chicken manure had no effect on C_{22} to C_{36} concentrations.

5.6.2 Headspace Analyses

It is evident from the data presented in Section 5.5.5.2 that:

1. The headspace TPH compounds detected were of the range C_9 to C_{28}, with approximately 98.6% of the $<C_{12}$ range. Therefore the changes in headspace TPH quantity seen most likely reflect changes in concentration of the $<C_{12}$ hydrocarbon fraction.

2. The UMC and MC conditions showed different headspace TPH profiles, whereby the percentage of TPH compounds detected in the headspace was statistically significantly ($p<0.01$) less under the UMC condition than under the MC condition for each sampling event. Several possible explanations for this difference were considered, as follows:

    a. This difference could reflect the adoption of the pile management strategy under the MC condition whereby the oil refinery sludge was regularly physically disturbed whereas under the UMC condition the oil refinery sludge was undisturbed over the duration of the treatability studies. Therefore, under the MC condition, fresh material from which loss of volatile compounds could occur would have been continually exposed. This would mean that the 'shallower' headspace TPH profile of the MC condition reflects a replenishment of headspace TPH compounds on each sampling occasion. This would imply that losses of TPH compounds would be greater under the MC condition than under the UMC condition. However, this was not the case seen in Section 5.5.1.2

whereby no statistically significant ($p>0.01$) reduction in C_9 to C_{12} over the first 8 days of the treatability studies, compared with statistically significant ($p<0.01$) loss of this hydrocarbon range under the UMC condition over the same duration of the treatability studies. Therefore the 'shallower' headspace TPH profile under the MC condition most likely indicates that the pile management strategy may have slowed the rate of loss of volatile TPH compounds. Also, the sampling strategy adopted for all pile conditions was the same, whereby a bulk composite sample was removed from the trays on each sampling occasion, and homogenised prior to removal of a sub-sample for laboratory analyses. Therefore fresh material would also have been exposed during the sampling procedure under the UMC condition.

b. Volatilisation is affected by moisture content, whereby high moisture contents can lead to retention of volatile compounds (Yong and Mulligan, 2004). The MC condition received water throughout the treatability studies as part of the pile management strategy whereby moisture content was maintained at levels between 40% and 60% (as discussed in Chapter 3). Therefore it may be expected that volatile TPH compounds could be retained within the oil refinery sludge of the MC condition as opposed to that of the UMC condition. However, this does not explain the statistically significant ($p<0.01$) differences between these two conditions over the first week of the treatability studies. The moisture content of the oil refinery sludge in both conditions was the same over this duration.

c. Volatilisation is also affected by temperature (Yong and Mulligan, 2004). The pile temperature of the UMC and MC conditions were not statistically significantly ($p>0.01$) different, as was discussed in Chapter 3. Therefore it is unlikely that pile temperature had an effect on differences in potential volatilisation between these pile conditions.

d. It was considered that, due to the heterogeneity of the oil refinery sludge, it may be that there was a greater proportion of C_9 to C_{12} under the MC condition than under the UMC condition, and that this could affect rates of losses of such compounds. However, the proportion of C_9 to C_{12} of total TPH under the MC condition was only marginally higher than under the UMC condition, with 1.91% and 1.88% respectively. Therefore the author feels that this alone is unlikely to

account for the difference seen in headspace TPH profiles under these conditions.

e. The only difference between the UMC and MC conditions was that the there was a 7 day period between the commencement of the UMC and the commencement of the MC conditions. During this time period, ambient greenhouse temperatures were approximately 3-7°C higher over the first week of the UMC condition than the MC condition. This, in the author's opinion, is the only potentially viable explanation for the differences seen, but this cannot be concluded from these data.

3. The SMT and MC conditions shared a similar headspace TPH profile, however from Day 8 until Day 32 (inclusive), headspace TPH levels were statistically significantly ($p<0.01$) lower under the SMT condition than under the MC condition. This potentially indicates an enhanced loss of volatile TPH compounds resulting from the addition of sterile chicken manure. Possible reasons for such an effect are as follows:

a. The enhanced loss of volatile TPH compounds may reflect the statistically significantly ($p<0.01$) higher pile temperatures which were attained under the SMT condition over the duration of the treatability studies, with the largest difference in pile temperature seen over the first 14 days (as was discussed in Chapter 4).
b. Pile temperature and microbial activity are reported by Alexander (1999), Madigan et al (2003) and Miyatake and Iwabuchi (2006) to have a positive relationship. Statistically significantly ($p<0.01$) higher microbial activity (measured through carbon dioxide evolution) was recorded under the SMT condition than the MC condition for the first 58 days of the treatability studies. Therefore, the potentially enhanced loss of volatile TPH compounds under the SMT condition may reflect, at least in part, biotic rather than abiotic processes.
c. The SMT condition had a lower proportion of C_9 to C_{12} hydrocarbons of 1.3% of the total TPH concentration on Day 1, compared with 1.91% under the MC condition. This may account for the faster loss of these compounds; however this cannot be concluded from these data.

d. The MC and SMT conditions were simultaneously established, therefore changes in ambient temperature does not account for the differences seen.

4. The TMT and MC conditions showed different headspace TPH profiles, whereby the percentage of TPH compounds detected in the headspace was statistically significantly ($p<0.01$) lower under the TMT condition than under the MC condition for each sampling event. This indicates an enhanced loss of volatile TPH compounds resulting from the addition of chicken manure. The possible reasons for such an effect discussed above for the SMT condition also apply here as the TMT condition also attained statistically significantly ($p<0.01$) higher pile temperature and microbial activity than the MC condition. However, given the differences in headspace TPH profile between the TMT and SMT conditions whereby the percentage of TPH compounds detected in the headspace was statistically significantly ($p<0.01$) less under the TMT condition than under the SMT condition for each sampling event, it may be that the potentially enhanced loss of volatile TPH compounds resulting from the addition of chicken manure reflects biostimulation and bioaugmentation effects. This is supported by the statistically significantly ($p<0.01$) higher pile temperatures and microbial activity attained under the TMT condition as opposed to those under the SMT condition over the main duration of the treatability studies, as was discussed in Chapter 4. Such effects could have resulted in enhanced biodegradation of these compounds, or may simply have resulted in increased pile temperature and therefore higher potential for volatilisation of these compounds. However, the proportion of C_9 to C_{12} of total TPH concentration was 0.8% under the TMT condition, compared with 1.3% under the SMT condition. This may account for the faster loss of these compounds; however this cannot be concluded from these data.

5. It is apparent from the data presented in Section 5.5.2.2 that the TMT and MC conditions shared a similar headspace TPH profile, while the MC and SMT conditions also shared a similar headspace TPH profile. There are statistically significant differences between the UMC and TMT conditions, and between the MC and SMT conditions, indicating that the addition of chicken manure did enhance the potential loss of volatile TPH compounds. However the pattern does imply that the ambient temperature had an effect on volatile TPH compounds.

5.6.3 Potential Contribution of Volatilisation to TPH Degradation

It is evident from the data presented in Section 5.5.2.2 that the total headspace TPH compounds constituted (approximately) 34.1% $<C_9$, 60.4% C_9-C_{10}, 4.1% C_{10}-C_{12}, and 2.1% C_{12}-C_{28}. Over the duration of the treatability studies, the quantity of total TPH compounds detected in the headspace reached 0% of the Day 1 levels, indicating loss of these volatile TPH compounds. Such loss potentially reflects volatilisation. Therefore volatilisation may have contributed to any loss of TPH compounds. Given the proportions of hydrocarbon fractions detected in the headspace, the largest loss was of $<C_{10}$ hydrocarbons.

It is evident from Section 5.6.1 that statistically significant ($p<0.01$) degradation of TPH compounds under the four pile conditions (UMC, MC, TMT, SMT) were only recorded for the C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbon fractions. The potential contribution of volatilisation to the degradation of these hydrocarbon fractions is discussed as follows:

5.6.3.1 Aliphatic Hydrocarbon Fraction C_9-C_{10}

UMC Condition:

The fastest rate of C_9-C_{10} loss was seen between Days 1 and 2, with approximately 40% reduction in the concentration of this fraction. This significant loss coincides with the greatest reduction in headspace TPH compounds of approximately 95% between Days 1 and 2. The C_9-C_{10} hydrocarbon fraction constituted approximately 60.4% of the total headspace TPH compounds detected on Day 1, therefore the reduction in headspace TPH compounds most likely reflects a significant loss of this hydrocarbon fraction, potentially through volatilisation. It is therefore likely that volatilisation played a significant role in the loss of C_9-C_{10} hydrocarbons between Days 1 and 2. However, it is unlikely that the C_9-C_{10} hydrocarbons detected in the headspace represent the total C_9-C_{10} hydrocarbons recorded in the solid samples, as reduction of this hydrocarbon fraction from the solid samples continued beyond Day 2 with a further reduction of approximately 60% by Day 64. Although a reduction in headspace total TPH compounds also continued over this time period, the rate was much lower (5% reduction over 30 days) than that seen for the solid samples (45% reduction over 30

days). Also, a reduction of C_9-C_{10} of approximately 15% was recorded between Days 32 and 64 for the solid samples, whereas no TPH compounds were detected in the headspace over this time period. Therefore the author concludes that volatilisation likely contributed to C_9-C_{10} loss between Days 1 and 2, but that biodegradation was likely the dominant process over the remaining duration of the treatability studies.

MC Condition:

The MC condition showed no statistically significant ($p<0.01$) reduction in C_9-C_{10} between Days 1 and 8, whereas a steady reduction in headspace TPH compounds was recorded over this time period, with a total potential loss of 40% TPH compounds. The only possible explanation for this is that the quantity of C_9-C_{10} potentially lost through volatilisation constitutes a minor fraction of total C_9-C_{10} in the solid samples. The author concludes that volatilisation unlikely made a significant contribution to total degradation of C_9-C_{10} under this pile condition.

TMT Condition:

The fastest rate of C_9-C_{10} loss was seen between Days 1 and 2, with approximately 60% reduction in the concentration of this fraction. This significant loss coincides with the greatest reduction in headspace TPH compounds of approximately 98% between Days 1 and 2. The C_9-C_{10} hydrocarbon fraction constituted approximately 60.4% of the total headspace TPH compounds detected on Day 1, therefore the reduction in headspace TPH compounds most likely reflects a significant loss of this hydrocarbon fraction, potentially through volatilisation. It is therefore likely that volatilisation played a significant role in the loss of C_9-C_{10} hydrocarbons between Days 1 and 2. However, it is unlikely that the C_9-C_{10} hydrocarbons detected in the headspace represent the total C_9-C_{10} hydrocarbons recorded in the solid samples, as reduction of this hydrocarbon fraction from the solid samples continued beyond Day 2 with a further reduction of approximately 40% by Day 8. Although a reduction in headspace total TPH compounds also continued over this time period, the rate was much lower (2% reduction between Days 2 and 8) than that seen for the solid samples (25% reduction between Days 2 and 8). Also, a reduction of C_9-C_{10} of approximately 15% was recorded between Days 8 and 32 for the solid samples, whereas no TPH compounds were detected in the headspace over this time period. Therefore the author concludes that volatilisation likely contributed to C_9-C_{10} loss between Days 1 and 2, but that

biodegradation was likely the dominant process over the remaining duration of the treatability studies.

SMT Condition:

The SMT condition showed no statistically significant ($p<0.01$) reduction in C_9-C_{10} between Days 1 and 4, whereas a steady reduction in headspace TPH compounds was recorded over this time period, with a total potential loss of 35% TPH compounds. The only possible explanation for this is that the quantity of C_9-C_{10} potentially lost through volatilisation constitutes a minor fraction of total C_9-C_{10} in the solid samples. The author concludes that volatilisation unlikely made a significant contribution to total degradation of C_9-C_{10} under this pile condition.

5.6.3.2 Aliphatic Hydrocarbon Fraction C_{10}-C_{12}

UMC Condition:

The fastest rate of C_{10}-C_{12} loss was seen between Days 1 and 2, with approximately 35% reduction in the concentration of this fraction. This significant loss coincides with the greatest reduction in headspace TPH compounds of approximately 95% between Days 1 and 2. The C_{10}-C_{12} hydrocarbon fraction constituted approximately 4.1% of the total headspace TPH compounds detected on Day 1, therefore the reduction in headspace TPH compounds potentially reflects a significant loss of this hydrocarbon fraction, potentially through volatilisation. It is therefore likely that volatilisation played a significant role in the loss of C_{10}-C_{12} hydrocarbons between Days 1 and 2. However, it is unlikely that the C_{10}-C_{12} hydrocarbons detected in the headspace represent the total C_{10}-C_{12} hydrocarbons recorded in the solid samples, as reduction of this hydrocarbon fraction from the solid samples continued beyond Day 2 with a further reduction of approximately 85% by Day 64. Although a reduction in headspace total TPH compounds also continued over this time period, the rate was much lower (5% reduction over 62 days) than that seen for the solid samples (55% reduction over 62 days). Also, a reduction of C_{10}-C_{12} of approximately 10% was recorded between Days 32 and 64 for the solid samples, whereas no TPH compounds were detected in the headspace over this time period. Therefore the author concludes that volatilisation likely contributed to C_{10}-C_{12} loss between Days 1 and 2, but that biodegradation was likely the dominant process over the remaining duration of the treatability studies.

MC Condition:

The MC condition showed no statistically significant ($p<0.01$) reduction in C_{10}-C_{12} between Days 1 and 8, whereas a steady reduction in headspace TPH compounds was recorded over this time period, with a total potential loss of 40% TPH compounds. The only possible explanation for this is that the quantity of C_{10}-C_{12} potentially lost through volatilisation constitutes a minor fraction of total C_{10}-C_{12} in the solid samples. The author concludes that volatilisation unlikely made a significant contribution to total degradation of C_{10}-C_{12} under this pile condition.

TMT Condition:

The fastest rate of C_{10}-C_{12} loss was seen between Days 1 and 2, with approximately 40% reduction in the concentration of this fraction. This significant loss coincides with the greatest reduction in headspace TPH compounds of approximately 98% between Days 1 and 2. The C_{10}-C_{12} hydrocarbon fraction constituted approximately 4.1% of the total headspace TPH compounds detected on Day 1, therefore the reduction in headspace TPH compounds most potentially reflects a significant loss of this hydrocarbon fraction, potentially through volatilisation. It is therefore likely that volatilisation played a role in the loss of C_{10}-C_{12} hydrocarbons between Days 1 and 2. However, it is unlikely that the C_{10}-C_{12} hydrocarbons detected in the headspace represent the total C_{10}-C_{12} hydrocarbons recorded in the solid samples, as reduction of this hydrocarbon fraction from the solid samples continued beyond Day 2 with a further reduction of approximately 58% by Day 64. Although a reduction in headspace total TPH compounds also continued over this time period, the rate was much lower (2% reduction between Days 2 and 32) than that seen for the solid samples (25% reduction between Days 2 and 32). Also, a reduction of C_{10}-C_{12} of approximately 18% was recorded between Days 32 and 64 for the solid samples, whereas no TPH compounds were detected in the headspace over this time period. Therefore the author concludes that volatilisation likely contributed to C_{10}-C_{12} loss between Days 1 and 2, but that biodegradation was likely the dominant process over the remaining duration of the treatability studies.

SMT Condition:

The SMT condition showed no statistically significant ($p<0.01$) reduction in C_{10}-C_{12} between Days 1 and 16, whereas a steady reduction in headspace TPH compounds

was recorded over this time period, with a total potential loss of 35% TPH compounds. The only possible explanation for this is that the quantity of C_{10}-C_{12} potentially lost through volatilisation constitutes a minor fraction of total C_{10}-C_{12} in the solid samples. The author concludes that volatilisation unlikely made a significant contribution to total degradation of C_{10}-C_{12} under this pile condition.

5.6.3.3 Summary

It is apparent from the data discussed in this section that volatilisation likely contributed to the loss of C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbon fractions under the UMC and TMT conditions between Day 1 and 2, but that biodegradation was likely the dominant process over the remaining duration of the treatability studies. In contrast, it was concluded that volatilisation unlikely made a significant contribution to the loss of these hydrocarbon fractions under the MC and SMT conditions over the duration of the treatability studies as while losses of these compounds from the headspace were recorded, simultaneous loss of these compounds from the solid samples was not recorded.

Although the headspace TPH compound data presented in this chapter is only semi-quantitative as calibration of the headspace samples could not be carried out due to technical difficulties with the GC-MS, it is apparent that the total chromatogram peak area for Day 1 samples from the MC condition was approximately 2.7% of that from the UMC condition, and that the total chromatogram peak area for Day 1 samples from the SMT condition was approximately 1.2% of that from the TMT condition. This data confirms that losses of headspace TPH compounds were far greater under the UMC and TMT conditions than under the MC and SMT conditions, and therefore volatilisation potentially made a greater contribution to TPH loss under these conditions than under the MC and SMT conditions, although such volatilisation was most likely only significant between Days 1 and 2 as discussed above.

5.7 Conclusions

Changes in total and fractionated TPH concentrations were recorded over the duration of the treatability studies, and the data were used to indicate the potential use of chicken manure to enhance the degradation of these hydrocarbons, and differentiation between biostimulation and bioaugmentation effects resulting from the addition of chicken manure. In addition, changes in headspace TPH concentrations were monitored to indicate any potential contribution of volatilisation to TPH degradation, and the potential effect of the composting bioremediation process on volatilisation.

The following conclusions have been made based on these data alone:

1. The oil refinery sludge had a low potential for further biodegradation as shown by the dominance of high molecular weight hydrocarbons (>C_{16} hydrocarbons accounted for approximately 64% of total TPH) over low molecular weight hydrocarbons (<C_{16} hydrocarbons accounted for approximately 36% of total TPH).
2. The pile management strategy and the addition of chicken manure (i.e. composting bioremediation) did not statistically significantly ($p<0.01$) enhance the degradation of *total* TPH compounds.
3. The pile management strategy did not statistically significantly ($p<0.01$) enhance the degradation of any aliphatic or aromatic hydrocarbon fractions (C_9-C_{36}).
4. The addition of chicken manure statistically significantly ($p<0.01$) enhanced the degradation of C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbon fractions and this was most likely a result of biostimulation effects only, with the exception of between Days 1 and 2 and Days 1 and 16 where enhanced degradation of C_9-C_{10} and C_{10}-C_{12} respectively appears to have resulted from bioaugmentation effects only.
5. The addition of chicken manure did not statistically significantly ($p>0.01$) enhance the degradation of C_{12}-C_{36} aliphatic hydrocarbons or C_9-C_{36} aromatic hydrocarbons.
6. The TPH compounds detected in the headspace were of C_9-C_{28} with the majority (approximately 60%) being C_9-C_{10}.

7. Statistically significant ($p<0.01$) differences in percentage TPH compounds detected in the headspace (as a percentage of Day 1 headspace TPH levels) were recorded, in increasing order of TMT<UMC<SMT<MC. The UMC and TMT conditions shared a similar headspace TPH profile, which was statistically significantly ($p<0.01$) different to the profile shared by the MC and SMT conditions. It is considered likely that this difference reflects the 7 day period between the commencement of the UMC and TMT conditions and the commencement of the MC and SMT conditions, during which ambient greenhouse temperatures were approximately 3-7°C higher. However, headspace TPH concentrations were lower, and the reduction of such concentrations was faster under the TMT condition as opposed to the UMC condition, and under the SMT condition as opposed to the MC condition. This potentially indicates that the addition of chicken manure may have enhanced the reduction in headspace TPH compounds. Such reduction could reflect either biodegradation of these compounds, or simply volatilisation owing to the higher pile temperatures attained by these conditions. Such higher pile temperatures likely reflect biostimulation and bioaugmentation effects resulting from the addition of chicken manure.
8. Although the exact contribution of potential volatilisation to TPH degradation cannot be determined from these data, it is concluded that volatilisation likely contributed to the degradation of C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbon fractions between Days 1 and 2 under the UMC and TMT conditions, but that such volatilisation did not cause the entire degradation of these hydrocarbons seen over this time period. It is also concluded that biodegradation was likely the dominant degradation process over the remaining duration of the treatability studies (Days 2 to 90) under the UMC and TMT condition. In contrast, it is concluded that volatilisation unlikely contributed to the degradation of the C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbon fractions under the MC and SMT conditions over the duration of the treatability studies. It is also concluded that volatilisation likely made a greater contribution to the degradation of these hydrocarbons under the UMC and TMT conditions than under the MC and SMT conditions as Day 1 headspace TPH concentrations were statistically significantly ($p<0.01$) greater than under the MC and SMT conditions.

Chapter 6

Microbial Analyses

6.1 Introduction

Chicken manure is reported by Ijah and Antai (2003) and Lu et al (2003) to possess a diverse microbial community, and has been found to harbour bacteria capable of growing on and degrading hydrocarbons by Ijah and Antai (2003). Such findings indicate a potential for the use of chicken manure to enhance TPH biodegradation through bioaugmentation, as was discussed in Chapter 2.

Bioaugmentation has been extensively investigated as a bioremediation strategy to enhance hydrocarbon degradation, but has not always been found to be successful, as found by Bento et al (2003), Jorgensen et al (2000) and Sabate et al (2004) (as was discussed in Chapter 2). It has been suggested by Kaplan and Kitts (2004), Bento et al (2003) and Jorgensen et al (2000) that a plausible reason for the failure of bioaugmentation to enhance hydrocarbon biodegradation may reflect competition between microorganisms indigenous to the contaminated soil and those introduced in microbial inocula for growth factors such as nutrients, water and oxygen, and that indigenous non-hydrocarbon degrading microorganisms may proliferate at the expense of the hydrocarbon degrading microbial inocula.

Therefore of concern regarding the amendment of TPH contaminated materials with chicken manure is the potential for non-hydrocarbon degrading microorganisms originating from the chicken manure to proliferate at the expense of hydrocarbon degrading microorganisms (originating from either TPH contaminated material or chicken manure). Microbial community dynamics with particular focus on interactions between two sources of microbial communities have not, to the author's knowledge,

been addressed in the literature regarding composting bioremediation, as was discussed in Chapter 2.

Microbial analyses were therefore undertaken during this study to a) determine the bacterial composition of chicken manure and thus potential use as a source of hydrocarbon degrading bacteria (bioaugmentation), and b) to help develop an improved knowledge of the interactions between two bacterial communities and community dynamics during the composting bioremediation of hydrocarbon contaminated material amended with chicken manure.

This chapter details the aims and objectives of the microbial analyses undertaken, the sampling programme adopted, and methodology of the microbial analyses. Baseline bacterial fingerprints for chicken manure and oil refinery sludge, and bacterial community dynamics data are presented and discussed in this Chapter. These data are further discussed in Chapter 8.

6.2 Aims and Objectives

Objective Four, Part One of the treatability studies is as follows:

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 2) microbial population dynamics resulting from the addition of chicken manure to TPH contaminated material.*

In order to satisfy this objective, microbial analyses were undertaken:-

1. To attain baseline data on the bacterial fingerprint of chicken manure and oil refinery sludge, to indicate the presence of potential hydrocarbon degrading bacteria in both materials through DNA sequencing, and for comparison with bacterial community dynamics data (for source determination of bacterial strains detected) under the total manure treatment (TMT) condition.

2. To determine the effects of pile management strategy on bacterial community dynamics through comparison of data for the un-managed control (UMC) condition with those for the managed control (MC) condition.

3. To determine the effects of chicken manure addition to oil refinery sludge on bacterial community dynamics, through comparison of data for the total manure treatment (TMT) condition with those for the managed control (MC) condition, and through comparison with baseline bacterial composition data for chicken manure and oil refinery sludge; in particular:

   a. To determine the likely survival of bacteria indigenous to the chicken manure.
   b. To determine the likely survival of bacteria indigenous to the oil refinery sludge.
   c. To indicate any predominance of potentially non-hydrocarbon degrading bacteria (likely indigenous to the chicken manure) over potentially hydrocarbon degrading bacteria (indigenous to the oil refinery sludge, and potentially chicken manure), thus presenting a potentially adverse effect on TPH degradation.

6.3 Sampling Programme

For the baseline bacterial composition analyses, 4 samples of chicken manure and 4 samples of oil refinery sludge were randomly taken from the stored material sources immediately prior to use of these materials in the treatability studies, using aseptic techniques. The samples (of approximately 50g wet weight) were stored in glass vials with Teflon lined screw caps and immediately transferred to a freezer (-20°C), where they were stored until required for analysis.

For the bacterial community dynamics analyses, solid samples were taken from those treatability study trays representing the un-managed control (UMC), managed control (MC), and total manure treatment (TMT). Due to financial and time restrictions it was decided that such analyses would be undertaken on samples taken from only one tray for each pile condition. The sampling programme for this work was designed in accordance with British Standard 7755 Part 4.1.1 (1995b). Bulk samples

(approximately 3kg wet weight) were taken from the relevant treatability study trays on Days 1, 2, 8, 16, 32, 64 and 90 and homogenised using a sterilised stainless steel scoop. Duplicate sub samples (approximately 1.5-2g wet weight) were placed in sterile 1.5ml eppendorfs (sealed test tubes), and transferred immediately to a freezer (-20°C), where they were stored until required for analysis. Such sample storage (for molecular microbial analyses) protocol was also adopted by Vinas et al (2005).

Samples for baseline and bacterial community dynamics analyses subsequently underwent a series of microbial analyses to determine their likely bacterial community structure. The methods used for these analyses are detailed in Section 6.5. Results are presented in Section 6.6 and discussed in Section 6.7. These data are further discussed in Chapter 8.

6.4 Microbial Analyses – Two Approaches

There are two key approaches to studying microbial communities; culture-dependent analyses and culture-independent (molecular) analyses (Madigan et al, 2003; Lukow et al, 2000). These two approaches are outlined and discussed in the following sections.

6.4.1 Culture Dependent Microbial Analyses.

The most common approach to analysing microbial communities has been the enrichment culture technique whereby microorganisms from environmental samples are cultivated in the laboratory (Madigan et al, 2003). Environmental samples such as soils are typically suspended in a buffer such as PBS (phosphate buffered saline, pH 7.2; Lu et al, 2006) and subsequently applied to a petri dish containing growth media (solution containing nutrients needed for microbial growth), and then incubated until colonies are formed. The method of application to the petri dish is commonly streaking (Figure 6.1). Individual microbial strains can be subsequently isolated in pure culture by repeat streaking (Madigan et al, 2003). Once cultivated, the isolated microorganisms can be characterised and identified. This is mainly achieved through the use of a microscope and/or staining techniques whereby dyes are used to

differentiate between bacteria (Madigan et al, 2003), although molecular techniques can be used to identify isolates through DNA sequencing (Section 6.5.7).

The culture dependent approach relies on the selection of a suitable growth media and incubation conditions (i.e. temperature and duration) for growth of microorganisms representative of the microbial community within the environmental sample being analysed (Madigan et al, 2003; Alexander, 1999). This approach enables observation of growth behaviour (thus has commonly been used to assess microbial growth, as was discussed in Chapter 4), nutritional requirements and physical characteristics of the microorganisms cultivated. These observations can then be used to identify particular microbial strains likely present within an environmental sample.

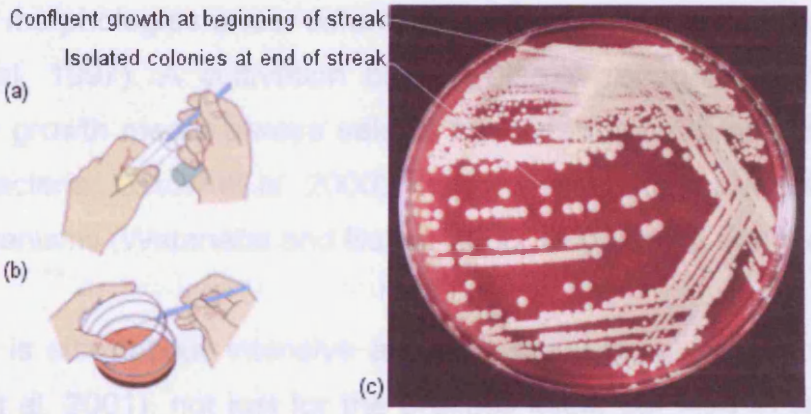


Figure 6.1: Isolation of Microorganisms in Pure Culture – The Streak Plate Method (taken from Madigan et al, 2003) (a) Sample is removed from enrichment. (b) Streak is made over a sterile agar plate, spreading out the microorganisms. Following the initial streak, subsequent streaks are made at angles to it. (c) The plate is then incubated and pure cultures can be obtained from the isolated colonies.

The culture dependent approach, however, has limitations. One such limitation is that the study of microbial communities using this approach is limited to microorganisms which can be cultured in the laboratory (Muyzer, 1999). This approach relies on the establishment of artificial conditions under which only the fittest of microorganisms will successfully compete, therefore the vast majority of microorganisms remain uncultivated (Hugenholtz and Goebel. 2001). Cultivation methods cannot therefore be directly applied to analyses of microbial diversity in entirety (Lu et al, 2006; Kirk et al,

2004). Other authors have also reported that not all microorganisms can be cultured in the laboratory:

"Traditional microbiological techniques such as microscopy and cultivation have only a limited use for classification and identification of microorganisms. 99% of all microorganisms in nature can not be isolated in pure cultures mainly due to our ignorance of the culture conditions under which these microorganisms thrive in their natural environment. Thus other techniques, which complement the microbiological approach, are necessary". Muyzer, 1999.

" ... it is now generally accepted that the traditional, culture based approach to analysis of bacterial diversity is able to detect only a small portion, often less than 1% of the bacteria from natural habitats". Howeler et al, 2002.

Additional limitations associated with microscopy and cultivation techniques are that 1) cell morphology is generally too simple to enable reliable identification of microbial strains (Muyzer, 1999; Watanabe and Baker, 2000), and 2) microorganisms may adopt different morphologies under different growth conditions (Watanabe and Baker, 2000; Liu et al, 1997). A cultivation bias is also a recognised limitation of this approach. The growth media always selects for certain microorganisms (usually the fast growing bacteria; Lukow et al, 2000), thus the results are always biased toward these microorganisms (Watanabe and Baker, 2000; Hugenholtz and Goebel, 2001).

This approach is also labour intensive and time consuming (Röling and Verseveld, 2002; Heuer et al, 2001), not just for the process itself, but also in terms of method development. For total analysis of microbial communities present in an environmental sample, growth media and incubation conditions need to be optimised for maximum recovery of microorganisms. Given the likely complexity of microbial communities within environmental samples, it is likely that several growth media and incubation conditions would be needed.

6.4.2 Culture Independent Microbial Analyses.

To overcome the limitations of the culture-dependent approach, molecular techniques have been developed which enable microbial communities to be studied without the need for cultivation (Watanabe and Baker, 2000; Kirk et al, 2004). Molecular techniques enable differentiation between and identification of microbial populations.

This is achieved through the detection and analysis of biochemical cell components (Rochelle, 2001; Schwieger and Tebbe, 1998), as discussed later on.

Through the use of molecular techniques it has been realised that microbial diversity is much greater than previously anticipated (Muyzer, 1999; Rochelle, 2001; Muyzer and Smalla, 1998; Watanabe, 1998) and that culture dependent techniques are insufficient to explore such diversity (Muyzer, 1999; Muyzer and Smalla, 1998). The use of molecular techniques allows microbial communities to be studied in a more realistic manner (Watanabe and Baker, 2000).

The focus of many molecular techniques used for the study of microbial community structure and dynamics is the detection and analysis of nucleic acids (Rochelle, 2001) extracted directly from an environmental sample such as soil. Nucleic acids provide the genetic information of microorganisms (Madigan et al, 2003). The most common approach to microbial community analyses using molecular techniques is through the analysis of the 16S rRNA (rDNA) gene (Liu et al, 1997; Dunbar et al, 1999). This gene is discussed further in Section 6.5.1.

By analysing the 16S rRNA gene, a bacterial community fingerprint can be produced for an environmental sample. Through comparison of fingerprints over time/ between samples, community dynamics can be assessed. In addition, nucleic acid sequences can be determined, and bacterial populations present in an environmental sample can be identified through comparison to 16S rRNA sequence databases (Kirk et al, 2004).

Fingerprinting methods enable the simultaneous analysis of multiple samples. This enables the determination of the effects of varying environmental conditions (i.e. bioremediation treatment conditions) on microbial communities and determination of community dynamics over time (Muyzer, 1999; Muyzer and Smalla, 1998; Kent et al, 2003).

6.4.3 Chosen Approach

The aims of the microbial analyses used in this study are to determine the bacterial composition of chicken manure and oil refinery sludge, and the effect of treatment conditions (pile management strategy and addition of chicken manure) on bacterial community dynamics over the course of the treatability studies.

It is apparent from the previous sections that molecular techniques, in particular *fingerprinting*, are more suited to the needs of these analyses. This is because they are reported to enable a more complete study of microbial communities and simultaneous analysis of multiple samples, thus enabling the study of community dynamics.

There are a number of steps involved with the production of a bacterial community fingerprint and subsequent identification of bacterial populations through sequencing. These steps are outlined in Section 6.5.2, and detailed in the following sections. Firstly, however, it is essential that basic microbial genetics is understood in order to understand the principles behind the molecular techniques used. An overview of microbial genetics is therefore presented in Section 6.5.1.

6.5 Methodology – Molecular Microbial Analyses

6.5.1 Overview of Microbial Genetics

Microorganisms exist as single cells or cell clusters. The cell (Figure 6.2) is the fundamental unit of life, and each cell, bound by a cell wall, contains a variety of structures (nucleus/nucleoid, and cytoplasm) and chemicals (proteins, nucleic acids, lipids and polysaccharides) that enable the cell to function (Madigan et al, 2003). The living processes of all cells are controlled by their genetic makeup (i.e. their full complement of genes – the genome). Organisms are divided into Prokaryotes (comprising *Archaea* and *Bacteria*) and Eukaryotes (Madigan et al, 2003). This study is concerned with *Bacteria*, as they are reported to be the dominant microorganisms

involved with the biodegradation of organic compounds (Alexander, 1999). Therefore eukaryote genetics are not discussed in this study.

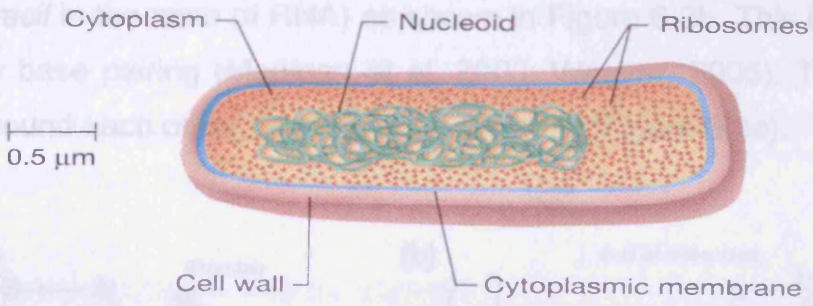


Figure 6.2: Prokaryotic Cell Structure (taken from Madigan et al, 2003)

Genes carry genetic information which is stored in Deoxyribonucleic Acids (DNA) and Ribonucleic Acids (RNA) (Madigan et al, 2003). In the case of prokaryotic cells, DNA and RNA are contained in one chromosomal mass known as the nucleoid (Figure 6.2). DNA carries the blueprint for the cell, whereas RNA acts as an intermediary molecule to convert the blueprint into functional entities (mostly proteins (play key roles in cell function; composed of amino acids) but also new RNA molecules) (Madigan et al, 2003). The process of synthesis of functional entities from DNA will be discussed later on in this section.

Nucleic acids such as DNA and RNA are macromolecules (polymers) of nucleotides. A nucleotide has 3 units (Weaver, 2005; Madigan et al, 2003); a) a five-carbon sugar (either deoxyribose for DNA or ribose for RNA), b) a nitrogen base, and c) a molecule of phosphate. The chain is formed by linking the sugars to one another through their phosphate groups. The backbone of DNA and RNA is a polymer in which sugar and phosphate molecules alternate. There are four nitrogen bases (Figure 6.3a), and their sequence along the nucleotide chain is the store of genetic information. These nitrogen bases are divided into two groups (Madigan et al, 2003; Weaver, 2005):

Purine Base:	Adenine (A)	***Pyrimidine Bases:***	Thymine (T; only present in DNA)
	Guanine (G)		Cytosine (C)
			Uracil (U; only present in RNA)

DNA is made up of two strands of nucleotides linked together by hydrogen bonds (Madigan et al, 2003). The most stable hydrogen bonding occurs between the nitrogen bases *Cytosine* and *Guanine* and between the nitrogen bases *Adenine* and *Thymine* (or *Uracil* in the case of RNA) as shown in Figure 6.3b. This is referred to as complementary base pairing (Madigan et al, 2003; Weaver, 2005). The two strands are wrapped around each other, forming a double helix (Figure 6.3c).

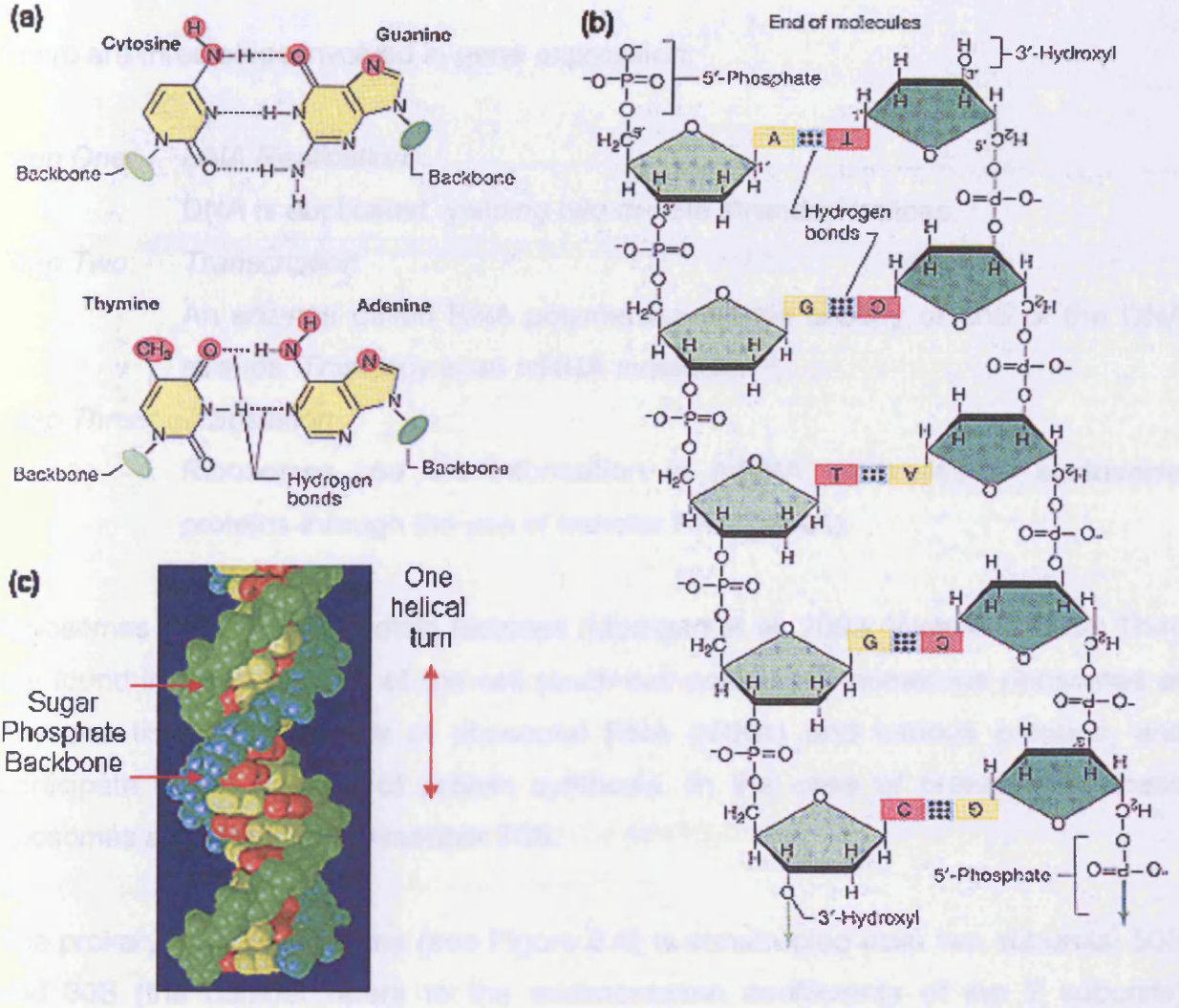


Figure 6.3 Structure of DNA (adapted from Madigan et al, 2003). *(a) Hydrogen bonding between Purine and Pyrimidine bases. (b) DNA structure; sugar and phosphate backbone with nitrogen bases. (c) DNA double helix; one sugar phosphate backbone shown in blue, and the other in green. Pyrimidine bases are shown in red, purine bases are shown in yellow.*

Proteins and other functional entities (i.e. RNA) are synthesised from the instructions in a DNA gene by a process called *gene expression* (Weaver, 2005; Madigan et al, 2003). This process is achieved through RNA, which is complementary in base sequence to DNA, and as such is a carrier of genetic information. There are three types of RNA; messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). These RNA molecules play important roles in nucleotide and protein synthesis (Madigan et al, 2003).

There are three steps involved in gene expression;

Step One: *DNA Replication*
DNA is duplicated, yielding two double stranded helices.

Step Two: *Transcription*
An enzyme called RNA polymerase makes a copy of one of the DNA strands. This copy is an mRNA molecule.

Step Three: *Translation*
Ribosomes use the information in mRNA molecules to synthesise proteins through the use of transfer RNA (tRNA).

Ribosomes are the cell's protein factories (Madigan et al, 2003; Weaver, 2005). They are found in the cytoplasm of the cell (each cell possesses numerous ribosomes at any one time), and consist of ribosomal RNA (rRNA) and various proteins, and participate in the process of protein synthesis. In the case of prokaryotes, these ribosomes are assigned the number 70S.

The prokaryote 70S ribosome (see Figure 6.4) is constructed from two subunits; 50S and 30S (the number refers to the sedimentation coefficients of the 2 subunits) (Madigan et al, 2003; Weaver, 2005). The 30S subunit consists of one molecule of rRNA, known as the 16S rRNA, and 21 ribosomal proteins. The 50S subunit comprises two rRNAs (23S and 5S) and 34 proteins.

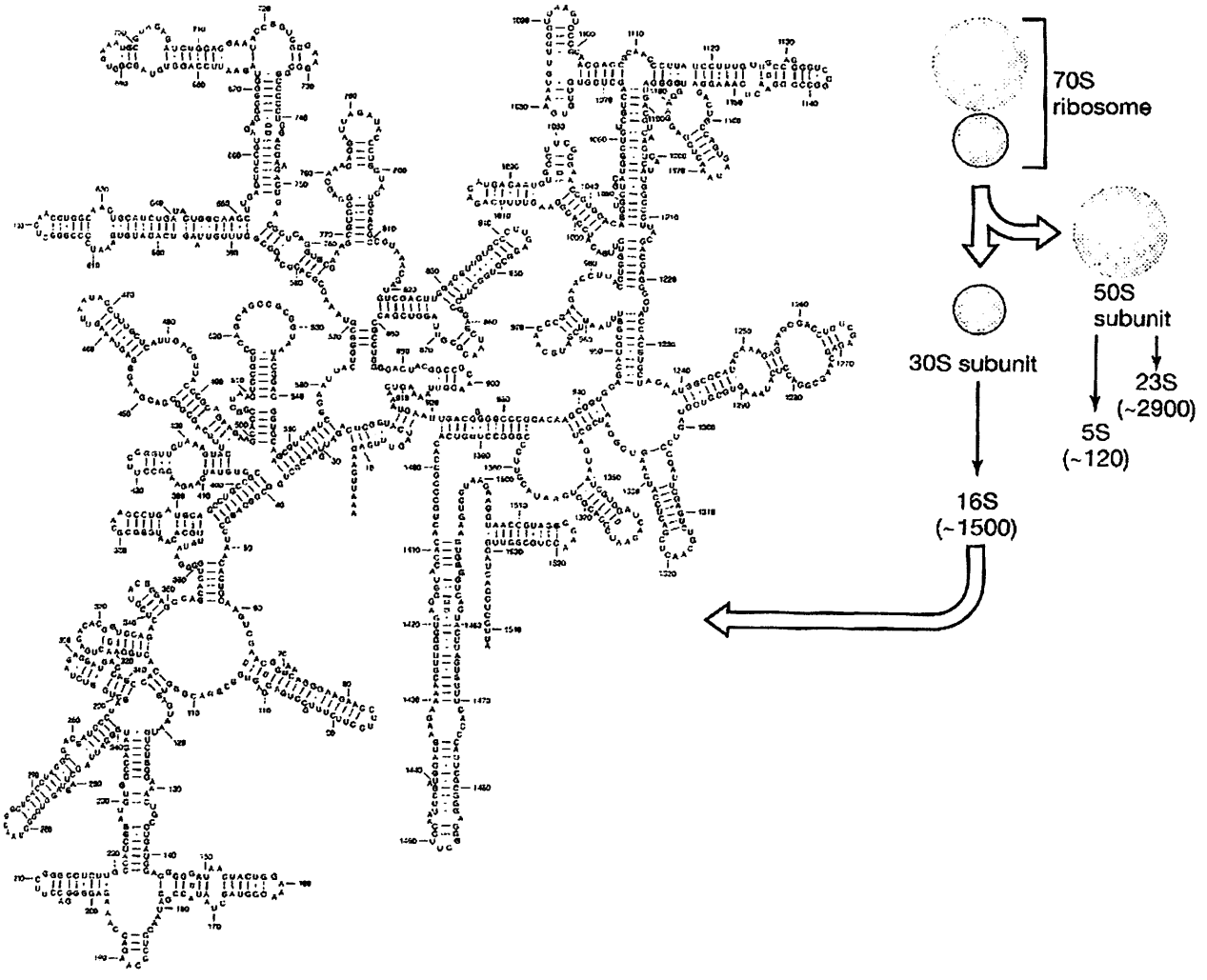


Figure 6.4: Structure of a 16S rRNA Prokaryotic Ribosome (taken from Madigan et al, 2003).

Ribosomal RNAs are excellent molecules for distinguishing between microorganisms. The reasons (Madigan et al, 2003) for this are:-

1. They are universally distributed across *Bacteria*.
2. They are functionally constant.
3. They have several regions of highly conserved sequence as well as regions of sequence variability, thus can be used to distinguish between microbial strains.

Of the three rRNA molecules in prokaryotes, the 16S rRNA (approximately 1500 base pairs) was first recognised as a measure of phylogenetic relatedness by Carl Woese in the 1970s, and has been extensively used in microbial community analyses

(Madigan et al, 2003). Therefore extensive databases of the variations in the 16S rRNA sequence exist and through comparison of genetic sequences obtained from molecular analyses with such databases, microorganisms from various environmental samples can be identified.

The 16S rRNA sequence for *Bacteria* is therefore the focus of the microbial community molecular analyses undertaken in this study.

6.5.2 Overview of Molecular Techniques

Figure 6.5 outlines the sequence in which 16S rRNA molecular techniques are used to generate a bacterial community fingerprint and identify bacterial populations through sequencing; this sequence (Madigan et al, 2003; Rochelle, 2001; Walker and Rapley, 2000) is summarised as follows. The first step is to extract and isolate total community genomic material (nucleic acids from all bacterial populations present in the sample) from the environmental sample. The next step is to identify the microorganisms of interest (in this study, *Bacteria*) and isolate specific genes (in this study a portion of the gene encoding 16S ribosomal RNA) and amplify them using the Polymerase Chain Reaction (PCR). Following this, the PCR products (copies of the target gene from all bacterial populations present in the sample) need to be separated to generate a community fingerprint. For this study, Denaturing Gradient Gel Electrophoresis (DGGE) was the chosen fingerprinting method, although alternative methods are available, as outlined in Section 6.5.6. Following fingerprinting, bacterial populations can be isolated and identified through sequence analyses.

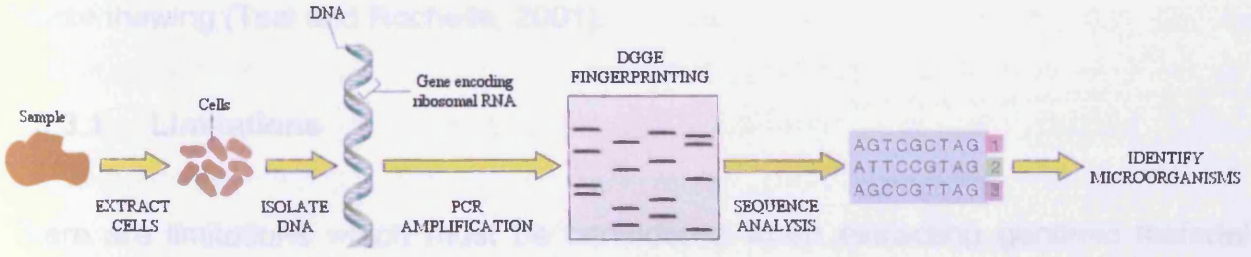


Figure 6.5: Molecular Analyses (adapted from Madigan et al, 2003).

Molecular techniques are not without their limitations. However, as stated by Rochelle (2001) it is worth remembering that we now know far more about microbes in their natural environments than would ever have been possible if these molecular methods had not been developed. The following sections describe the molecular techniques and their limitations in greater detail.

6.5.3 Step 1: Extraction of Total Community Genomic Material

As shown in Figure 6.5 the first step in molecular analyses of microbial communities is to extract and isolate total community genomic material from the environmental sample itself. This extraction step is critical as the genomic material obtained forms the backbone of the subsequent molecular analyses, therefore the quality of the genomic material extracted needs to be optimal (Tsai and Rochelle, 2001). There is a variety of commercially available extraction kits for varying types of environmental samples (i.e. water, soil etc), however, there is no single extraction technique that has been developed which can be used to extract genomic material from all types of environmental samples (Tsai and Rochelle, 2001). In general, extraction methods comprise a number of steps whereby cells are lysed (opened), nucleic acids are extracted, and then the extracted nucleic acids are purified (Tsai and Rochelle, 2001).

For this study, a commercially available extraction kit for soils was used, called Fast DNA Spin Kit for Soil, from Q Biogene (UK). This kit comprises a series of reagents and filters. An extraction protocol was provided with the FastDNA Spin Kit for Soil, and is provided in Appendix 5. This method relies on bead beating to lyse cells. Bead beating methods of DNA extraction are common. They physically disrupt and lyse bacterial cells, and are reported to yield more DNA than alternative methods such as freeze thawing (Tsai and Rochelle, 2001).

6.5.3.1 Limitations

There are limitations which must be considered when extracting genomic material from environmental samples. These are summarised as follows:

a) Contamination of environmental sample:

One of the greatest challenges in the application of molecular techniques is maintaining the integrity and pristine nature of the sample prior to and during extraction (Rochelle, 2001). Microorganisms are found everywhere and therefore it is easy to contaminate an environmental sample with microorganisms which are not indigenous to that sample (Madigan et al, 2003). It is therefore vital that aseptic approaches are adopted when carrying out the DNA extraction step; i.e. wearing gloves and changing them often, changing pipette tips between samples and reagents, sterilising sampling tools between samples, and keeping sample containers sealed as much as possible.

b) Potentially biased extraction:

Extraction of genomic material relies on cell lysis. Lysis efficiency of bacterial cells varies, whereby Gram negative bacteria are more easily lysed than Gram positive bacteria (Kirk et al, 2004). If the extraction method is not harsh enough, extraction may be biased toward Gram negative bacteria, whereas if the extraction method is too harsh, DNA itself may be sheared and therefore damaged (Kirk et al, 2004). Commercially available kits (such as the one used in this study) are optimised to minimise such bias (Madigan et al, 2003).

c) Co-extraction of PCR inhibitors:

A problem faced during extraction of nucleic acids from environmental samples is the co-extraction of PCR reaction inhibitors (Tsai and Rochelle, 2001). The most common inhibitor is humic-acid type materials, but other compounds such as heavy metals are also known to be inhibitory to the PCR process (Hugenholtz and Goebel, 2001). Removal of such inhibitors prior to PCR is therefore important. Commercial extraction kits, such as the one used in this study, are usually designed to minimise the co-extraction of such inhibitors (Madigan et al, 2003). In addition to this, the genomic material extracted can be 'cleaned' using commercially available filter systems to remove inhibitors if the PCR is found to fail. Such cleaning was not deemed to be necessary in this study, as the PCR was found to be successful (PCR images are provided in Appendix 5).

6.5.4 Step 2: The Polymerase Chain Reaction

Once genetic material has been extracted from an environmental sample, the next step is to isolate the target genes from the bulk nucleic acids (Hugenholtz and Goebel, 2001), and to generate sufficient quantities of these genes for subsequent molecular analyses.

The Polymerase Chain Reaction (PCR) is a technique by which a selected region of DNA (i.e. the genomic material extraction product) is isolated and amplified (copied) a million-fold (Madigan et al, 2003). Such amplification is carried out *in vitro* (in test tubes).

The target gene for this study is that encoding the 16S rRNA as this gene is reported to be excellent for community analyses of *Bacteria* as it is functionally constant and has regions of both conserved (consistent among *Bacteria*) and non-conserved (variable between *Bacteria*) base sequences.

The 16S rRNA gene is isolated from the bulk genomic material extracted from the environmental sample by adding *primers* (short pieces of DNA), which are designed to be complementary to base sequences (i.e. conserved regions of DNA) flanking either side of the selected region (Madigan et al, 2003). The base sequences of the primers used in this study are provided in Table 6.2. DNA synthesis is initiated through the selected region, resulting in copies of both DNA strands. This process is catalysed by an enzyme called *taq polymerase*. Amplification of the target gene is achieved by the PCR through a series of repeat cycles, each doubling the copies of DNA (Madigan et al, 2003; Walker and Rapley, 2000; Weaver, 2004). Each cycle consists of a series of steps, as illustrated in Figure 6.6.

Several reagents are needed for the PCR as detailed in Table 6.1. The reagents are mixed together in 0.2ml PCR tubes, and placed in a thermal cycler (Figure 6.6), which is an automated instrument programmed to perform multiple cycles (Madigan et al, 2003). For this study, a cycle programme was recommended by the School of Biosciences, Cardiff University (G.Webster; pers.comm.).

Table 6.2 provides the base sequences of the primers used in this study. The forward (F) primer and the reverse (R) primer used were 357F and 518R respectively. The 16S rRNA base sequence is approximately 1500 base pairs long (Figure 6.4), running from reference point 1 to 1500. The number assigned to the primers used corresponds to the location along the 16S rRNA base sequence to which the primers anneal.

Table 6.1: PCR reagent mixture used in this study

Reagent	Volume per tube (µl)
Buffer	5
dNTPs (25mM)	0.5
BSA (10mg ml^{-1})	1
$MgCl_2$ (50mM)	1.5
Forward Primer (20pmol μl^{-1})	0.5
Reverse Primer (20pmol μl^{-1})	0.5
Taq Polymerase (5U μl^{-1})	0.25
dDH_2O	39.75
DNA template	1
	Total volume per tube = 50µl

dNTPs = A,T,G,C bases
BSA = bovine serum albumin
dDH_2O = double distilled water

Table 6.2: Base sequence of PCR primers used in this study

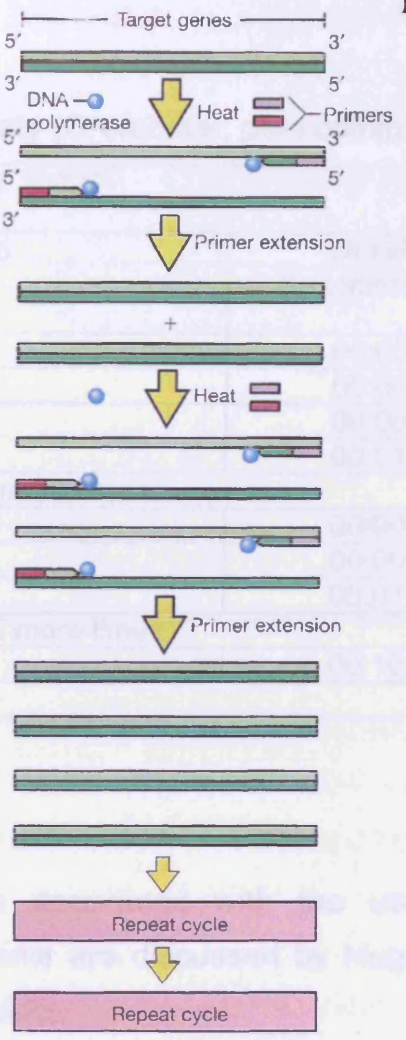
Primer	Base Sequence
357F	GC ***CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G***CC TAC GGG AGG CAG CAG Note: base sequences in underlined bold italics = GC clamp (Section 6.5.6)
518R	ATT ACC GCG GCT GCT GG

(a) Template DNA is heated to separate the strands. Primers flanking the target DNA are added along with DNA polymerase. The mixture is cooled to allow the primers to anneal to the target DNA.

(b) The reaction temperature is raised. DNA polymerase extends the primers yielding one copy or DNA.

(c) The next cycle begins with heat denaturation of the DNA strands, and the above steps are repeated.

Each cycle doubles the amount of targeted template DNA.



PCR Cycle	DNA Copies
0	1
1	2
2	4
3	8
4	16

Figure 6.6: The Polymerase Chain Reaction (Madigan et al, 2003).

Figure 6.7: PCR Cycler

The temperature cycles and their durations used in this study are summarised in Table 6.3.

Table 6.3: PCR cycles used in this study (G.Webster; pers.comm.).

Lid control mode: constant at 100°C		
Step	**Action**	**Duration (hrs:mins:secs)**
1	Incubate at 95.0°C	00:05:00
2	Incubate at 94°C	00:00:30
3	Incubate at 55°C	00:00:30
4	Incubate at 72°C	00:01:00
5	Cycle to Step 2 for 9 more times	
6	Incubate at 92°C	00:00:30
7	Incubate at 52°C	00:00:30
8	Incubate at 72°C	00:01:00
9	Cycle to Step 6 for 25 more times	
10	Incubate at 72°C	00:10:00
END		

6.4.5.2 Limitations of the PCR

There are a number of limitations associated with the use of the PCR on environmental samples. These problems are discussed by Hugenholtz and Goebel (2001), and are summarised as follows.

a) Inhibition of the PCR

Successful PCR amplification is dependent on the amount and, more importantly, the quality of the template DNA (extraction product) used. As mentioned in Section 6.4.4, PCR inhibitors such as humic acids and heavy metals can be co-extracted with genomic material from environmental samples. A number of approaches can be taken to prevent PCR inhibition, for example DNA cleaning (as mentioned in Section 6.4.4) and by trying different DNA polymerases as PCR inhibition can be polymerase specific. Such approaches were not deemed to be necessary for this study as high quality PCR products were attained.

b) Quantitation Issues

PCR can distort initial template DNA ratios (i.e. ratios of DNA extracted from individual microorganisms) due to differential amplification. This is thought to result from variable template concentration, primer annealing efficiency, variable denaturation and reannealing of templates. PCR based fingerprints are therefore best used as a qualitative measure of community structure only.

c) PCR Artefacts

PCR can result in amplification artefacts that do not accurately reflect the starting DNA template sequences. Chimeric sequences are the most problematic artefacts with environmental samples. Chimeric sequences are thought to form due to reannealing of an incompletely transcribed rRNA product to a different template, which is then transcribed to completion. This chimeric sequence suggests the existence of a microorganism which does not actually exist in the sample. The risk of chimera formation can be reduced by using high molecular weight template DNA (the DNA extraction kit used in this study was optimised for the recovery of high molecular weight DNA), and a low number of PCR cycles.

d) PCR Contamination

Due to the sensitivity of the PCR, the process is susceptible to contamination (i.e. non-indigenous microorganisms introduced in the laboratory). The risk of contamination can be minimised by changing gloves often, and changing pipette tips between samples and reagents. A negative control (autoclaved deionised water) should also be run to identify any contamination of the PCR reaction. Should contamination be found, the PCR samples should be rejected.

6.5.5 Step 3: Gel Electrophoresis

Gel electrophoresis enables the separation of charged molecules, such as DNA and RNA, according to their size (number of base pairs), when exposed to an electric field (Madigan et al, 2003). Nucleic acids have an overall negative charge at neutral pH owing to the phosphates in the DNA backbone, and will therefore migrate towards a positive charge (Weaver, 2005). Gel electrophoresis was used in this study to check

the quality and potential contamination of the PCR reaction to ensure the subsequent analytical steps could be undertaken.

In this study, agarose gels are used to check the quality of the PCR products. These are prepared by dissolving agarose (a polysaccharide obtained from seaweed; Pingoud et al, 2002) on heating in water. On cooling, the agarose forms a 3D network of double helical structures (Pingoud et al, 2002). The pore sizes formed are determined by the concentration of agarose used. High agarose concentrations generate small pores, and are used for small DNA fragments, and vice versa. In this study, a 1.2% agarose gel was used for the PCR products. A buffer (tris-borate) was also added to the gel mixture, to give a pH of 7.5 to 8.5.

In brief, as summarised by Pingoud et al (2002), a gel is formed by allowing the gel mixture to cool to approximately 40°C, and pouring it into a gel casting tray. A sample comb is inserted on the cathode side to form wells, as shown in Figure 6.8, and removed to leave wells once the gel has set. The tray is placed in an electrode chamber, which is filled with tris-borate buffer (of the same concentration as that used in the gel mixture). The PCR products are mixed with a loading dye and loaded into the gel wells (loading dye is used to enable the analyst to see the sample during loading). The electrode chamber is then connected to a power pack, and a voltage of approximately 70v is applied for approximately 40-50 minutes (based on a 50ml agarose gel size).

Nucleotide molecules of different sizes are separated due to friction as they migrate through the agarose gel (Madigan et al, 2003). Small nucleotide molecules endure little frictional drag from the gel and buffer, thus migrate further in a given time period than large nucleotide molecules which suffer more frictional drag. To enable identification of nucleic acid base length, a 'ladder' is also loaded onto the gel (the ladder used in Figure 6.8b was a 100base pair DNA ladder from Promega (USA); Figure 6.9c) (Pingoud et al, 2002). The ladder comprises varying fragment sizes of nucleic acids of known length.

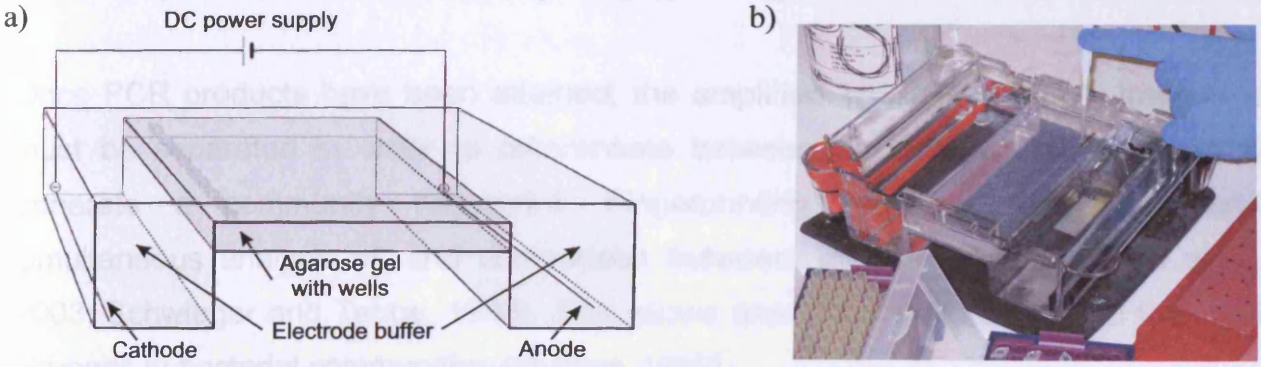


Figure 6.8: (a) Gel Electrophoresis Apparatus (taken from Pingoud et al, 2002); (b) Loading an Agarose Gel.

Once the samples have electrophoresed, the gel tray is removed from the chamber and placed on a UV transilluminator (light table; Figure 6.9a), and the gel image recorded by computer based image capture (Pingoud et al, 2002). The end result is a gel image which shows a series of bands, as shown in Figure 6.9b. In order to see the bands, ethidium bromide is added during the electrophoresis process, both to the gel mixture and to the buffer in the electrode chamber (Pingoud et al, 2002). Ethidium bromide is a red dye which fluoresces under UV light when it binds to DNA.

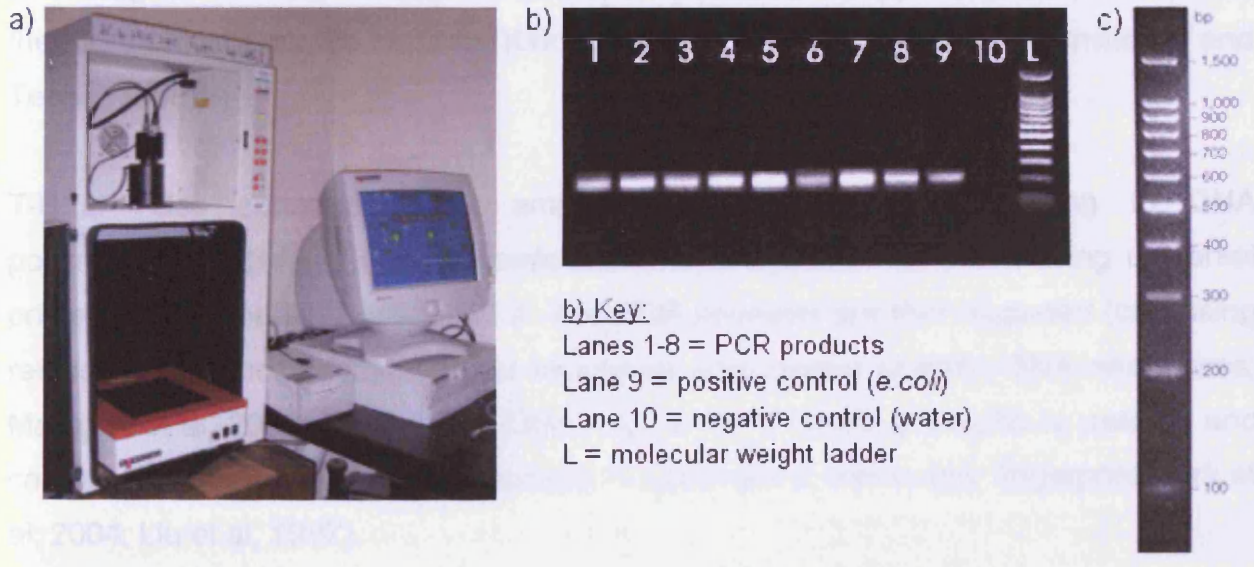


Figure 6.9: (a) Gel Image Capture; (b) Example Gel Image; (c) 100bp Ladder

6.5.6 Step 4: Bacterial Community Fingerprinting

Once PCR products have been attained, the amplified 16S rRNA (rDNA) fragments must be separated in order to differentiate between bacterial populations and to generate a community fingerprint. Fingerprinting methods offer rapid and simultaneous analysis of, and comparison between, multiple samples (Kent et al, 2003; Schwieger and Tebbe, 1998). This allows assessment of spatial and temporal changes in bacterial communities (Muyzer, 1999).

Three methods are commonly used for bacterial community fingerprinting; Single Strand Conformation Polymorphism (SSCP) (Schwieger and Tebbe, 1998; Tebbe et al, 2001), Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Lukow et al, 2000; Kent et al, 2003; Liu et al, 1997; Dunbar et al, 1999, Neilan, 2001) and Denaturing/Thermal Gradient Gel Electrophoresis (D/TGGE) (Heuer et al, 1999; Muyzer, 1999; Heuer et al, 2001; Macnaughton and Stephen, 2001).

SSCP is an electrophoretic method whereby PCR amplified DNA fragments of the same length but of differing base sequence are separated. Separation is achieved according to differing folding conformations determined by base sequence, and therefore electrophoretic mobility (Kirk et al, 2004; Tebbe et al, 2001; Schwieger and Tebbe, 1998).

T-RFLP also separates PCR amplified DNA fragments according to DNA polymorphisms. Briefly, total genomic material is amplified by PCR using universal primers, as outlined in Section 6.5.4. The PCR products are then digested (cut) using restriction enzymes (enzymes that recognise and cleave specific DNA sequences; Madigan et al, 2003). A series of DNA fragments of differing lengths is yielded, and can be separated by gel electrophoresis to generate a community fingerprint (Kirk et al, 2004; Liu et al, 1997).

D/TGGE uses gel electrophoresis to separate PCR amplified DNA fragments of the same length but of differing base sequence. Separation is based on the decreased electrophoretic mobility of partially melted double stranded DNA molecules in

polyacrylamide gels containing a linear gradient of DNA denaturant (urea and formamide) or a linear temperature gradient. Fragments with differing base sequences will have a different melting behaviour and will therefore stop migrating at different positions in the gel, yielding a fingerprint (Muyzer, 1999; Muyzer and Smalla, 1998).

For this study, DGGE was the chosen fingerprinting method owing to a) the extensive resources available in the School of Biosciences (Cardiff University), b) because DGGE is the most commonly used fingerprinting technique (Lukow et al, 2000), and c) DGGE is reported to be reliable, reproducible, rapid and inexpensive (Kirk et al, 2004). DGGE was first used for bacterial community analyses by Muyzer et al in 1993, and is established as a standard method in molecular microbial ecology (Heuer et al, 2001).

The electrophoresis takes place in a vertically placed polyacrylamide gel (Figure 6.10) containing a gradient of DNA denaturant (typically a mixture of urea and formamide) (Heuer et al, 2001; Madigan et al, 2003). When a DNA fragment (PCR product) moving through the denaturing gradient gel reaches a region containing sufficient denaturant, the double stranded fragment begins to 'melt' (the two strands begin to separate), at which point migration stops (Madigan et al, 2003; Muyzer, 1999; Muyzer and Smalla, 1998). Base sequence (i.e. hydrogen bond strength between nitrogen bases) controls the melting behaviour of DNA fragments, and therefore PCR products of the same size, but of differing base sequences, will have differing melting behaviour (Madigan et al, 2003). Complete melting of the double strands is not necessary to stop migration and is undesirable as samples that are fully denatured will have equivalent mobility resulting in poor resolution. Therefore GC clamps (GC rich sequences of 40-45 bases) are attached to one of the primers used during the PCR (Table 6.2) (Heuer et al, 2001). G+C bases have three hydrogen bonds between them compared to A+T bases which have two hydrogen bonds, thus are more difficult to separate (Madigan et al, 2003). In this study, a mixture of urea and formamide was used as the DNA denaturant with a gradient of 30% to 60%. The gradient was achieved by using a gradient former/delivery system with a peristaltic pump, in accordance with the protocol provided in Appendix 5.

Once the electrophoresis step is complete, the gel is stained, and then imaged on a UV transilluminometer. In this study imaging was performed using Syngene Genesnap (Synoptics Ltd, Cambridge, UK).

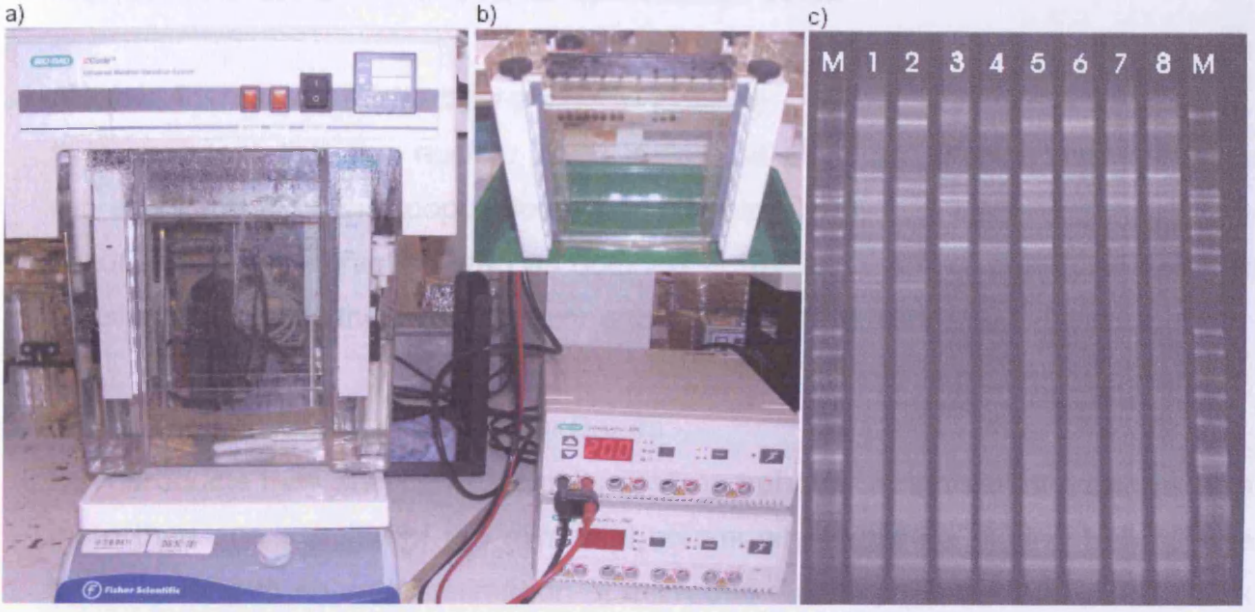


Figure 6.10: DGGE Apparatus and Example DGGE Image: (a) DGGE gel chamber in tank connected to power supply; (b) DGGE gel chamber with samples loaded before placed in tank; (c): M=microbial strain marker; Lanes 1-8 = microbial community fingerprint for environmental samples.

The DGGE image produced shows a pattern of distinct bands, as illustrated in Figure 6.10 (c). This pattern (or fingerprint) represents the bacterial community structure of a sample, in particular relative abundance of the main microbial species detected (Fromin et al, 2002). The basis of interpretation of DGGE images is that each discrete band represents a unique 'sequence type', which in turn is assumed to represent a discrete bacterial population (i.e. species/strain) (Fromin et al, 2002). In this way the DGGE image indicates bacterial diversity of a sample, and comparison of community structure can be made across multiple samples (Muyzer and Smalla, 1998) (i.e. over time or between environmental conditions or even bioremediation treatment conditions). However, there are limitations with DGGE, as discussed in Section 6.5.6.1. Further basis of DGGE interpretation is discussed in Section 6.6.

6.5.6.1 Limitations of DGGE

The main limitation associated with DGGE fingerprinting is the potential for misinterpretation of the original microbial community. There are a number of considerations associated with this, as summarised below.

a) *Nucleic Acid Biases (Extraction and PCR Biases)*
It is important that the nucleic acids extracted from an environmental sample represent the microbial populations actually present in that sample. Biases may occur during the extraction step as outlined in Section 6.4.4 or during preferential amplification during the PCR (Muyzer and Smalla, 1998; Kirk et al, 2004).

b) *Electrophoretic Mobility and Hidden Bands*
PCR products may also have a similar melting behaviour. In this way, bands from more than one species of the microbial community might be hidden behind one band, leading to an underestimation of microbial diversity (Heuer et al, 2001; Kirk et al, 2004).

c) *Multiple Operons*
Microorganisms often have more than one operon (a cluster of genes; Madigan et al, 2003) coding for 16S rRNA, and that the base sequence of these operons can differ (Heuer et al, 2001). This could lead to several bands which actually represent the same microorganism. There is therefore a risk that the microbial diversity can be overestimated.

6.5.7 Step 5: DGGE Band Excision and Sequencing

In addition to the community dynamics information yielded from fingerprinting, particular populations can be identified through DNA sequencing and comparison to 16S rRNA sequence databases. There are two principal methods of isolating populations for sequencing; cloning in a vector and excision/PCR re-amplification of DGGE bands. Due to time restraints for method development, the latter method was

chosen for this study. It is beyond the scope of this thesis to discuss the former method.

Once DGGE has been performed, individual bands can be excised from the DGGE gel (Madigan et al, 2003); it is therefore important that the gel is treated with care. Bands are excised using sterile blades, and stored in PCR tubes. In order to excise the bands, the gel is transferred to a UV transilluminometer. It is vital that excision is carried out as quickly as possible, as exposure of the DGGE gel to UV light causes degradation of the bands. It is therefore usual practice to excise the feint bands first, and then the stronger bands, as the stronger the band the more successful sequencing is likely to be.

The excised bands are then prepared for sequencing in order to identify the microbial strains present in the community analysed. A number of steps (as recommended by the School of Biosciences, Cardiff University; G.Webster, pers.comm.) are carried out to prepare the DGGE bands for sequencing, as follows:

Step One: 100µl of polished water was added to the DGGE bands, and left in a PCR tube for 10 minutes, during which the excised band was hydrated. After 10 minutes, excess polished water was removed, and the band left to air dry. Once dried, the bands were finely mashed using a pipette tip (tips were changed between samples to prevent cross contamination). 15µl polished water was then added, and the PCR tubes placed in the freezer for 1-2 hours. After this time, a PCR (Section 4.4.7) was carried out, using the hydrated DGGE band as template DNA.

Step Two: The PCR products were filtered using Microcon YM-50 filters according to the protocol supplied by the manufacturer. Gel electrophoresis was carried out on the filtered samples. The gel image was captured, and band densitometry carried out using Syngene Genetools (Synoptics Ltd, Cambridge, UK).

Step Three: 10µl of the remaining PCR products were aliquoted into fresh PCR tubes. A sample of the reverse primer was also placed into a separate PCR tube at a concentration of 1.6pmoles μl^{-1}.

In this study, the band samples were then sent to the DNA Sequencing Core within the School of Biosciences at Cardiff University. DNA sequencing is a process of determining the exact order of the bases A, T, C and G in a piece of DNA. The method used is an automated version of the method developed by Frederik Sanger in 1975, called the Sanger Chain Termination Method (Weaver, 2005). Briefly (Weaver, 2005), double stranded DNA produced from the DGGE bands are separated into single strands, and divided between four reaction tubes. To each tube, a primer is added which is complementary to a known section of the DNA sample (i.e. the primer used during PCR) along with deoxynucleotide bases (A, T, C and G) and chain terminating nucleotides (called di-deoxynucleotide bases). Four chain terminating nucleotides are used, each tagged with a different coloured fluorescent dye, to represent the four bases (A, T, C and G). Only one chain terminating nucleotide is added to each reaction tube.

The primer is extended through DNA polymerase to generate a new single DNA strand complementary to the template. However, primer extension is terminated wherever a particular base occurs due to annealing of the corresponding chain terminating nucleotides. The annealing of these nucleotides is random; therefore at the end of the reaction DNA fragments of different sizes will be generated. The four reaction mixtures are combined and run through a gel electrophoresis to separate the fragments based on size (with a resolution of one base size difference). As the sample passes through the gel, the fluorescent signal from the chain terminating nucleotides is detected and the base identified. The base sequence of the DNA sample is therefore determined.

The process results in a chromatogram comprising four colours representing each base. An example chromatogram is shown in Figure 6.11. These files are processed to enable comparison with a database of 16S rRNA sequences, as outlined in Section 6.5.8.

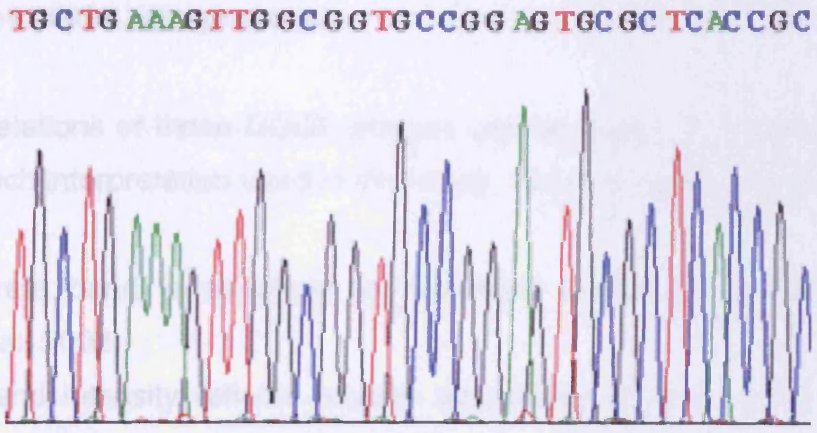


Figure 6.11: Example DNA sequencing chromatogram (taken from Weaver, 2005).

6.5.8 Step 6: Identification of Microbial Strains

The base sequences returned from the sequencing step are edited to exclude primer-binding sites, and converted into FASTA files, in this study, using *Chroma* software. The sequences were analysed using BLAST (Basic Local Alignment Search Tool) – which is a nucleotide sequence database (GenBank) from NCBI (National Center for Biotechnology Information), to enable identification of microbial strains. When using BLAST it is important to record the length and percentage of bases matched against database entries as an indication of reliability. Where the sequences are un-identified, an indication of the environment from which these bacteria were isolated can be taken from the subject area recorded for each entry, it is therefore important to record these details as well (G.Webster; pers.comm.).

6.6 Results and Discussion

The DGGE images (microbial community fingerprints) for the un-managed sludge (UMC), managed sludge (MC) and total manure treatment (TMT) pile conditions are presented along with the baseline DGGE images for chicken manure and oil refinery sludge and discussed in this section. Raw data (including gel electrophoresis images (PCR and DGGE) and sequence data) are presented in Appendix 5.

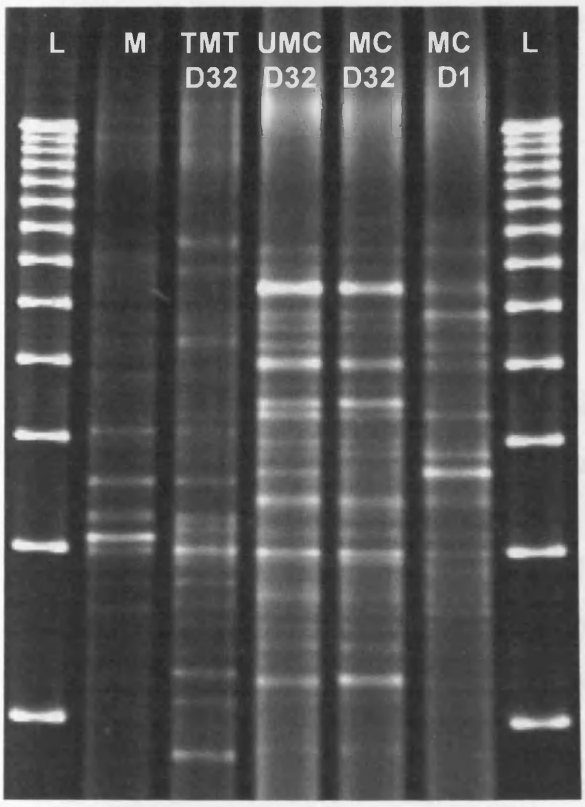
6.6.1 Basis of DGGE Interpretation

Before interpretations of these DGGE images can be made, it is important to outline the basis of such interpretation used in this study, which is summarised as follows:

a) Each discrete band is assumed to represent a discrete bacterial population (Fromin et al, 2002).
b) Relative band intensity reflects relative abundance of the bacterial populations detected in a sample (Fromin et al, 2002). Therefore bright bands represent the most dominant bacterial populations present in a sample. This assumption implies that no bias was obtained during the DNA extraction and PCR steps (Fromin et al, 2002).
c) Bands that share a common position between samples (termed 'common bands' in this thesis) are assumed to represent the same bacterial population (Fromin et al, 2002).
d) Changes in band intensity of a common band between samples (i.e. over time in this study) reflect changes in relative abundance and therefore dominance of bacterial populations. Changes in relative abundance could reflect changes in the abundance of the population represented, or changes in the abundance of another bacterial population (G.Webster; pers.comm.).
e) The use of reference patterns (ladders) enables comparison of multiple DGGE gels. However, comparisons between gels in terms of relative intensity of one band (i.e. is the bacterial species represented more enriched in one environment than in another) is complicated by the fact that samples with a high diversity, of equally abundant bacterial populations will generate DGGE images comprising lots of feint bands, rather than lots of strong bands (Heuer et al, 2002). For this reason, only fingerprint structure and changes in fingerprint structure are compared between the treatment conditions, to indicate changes in dominance (and likely growth/decline) of bacterial populations.

6.6.2 Alignment of DGGE Images

A total of three DGGE images were produced, each representing one of the three pile conditions assessed (UMC, MC, and TMT). To enable comparison of the DGGE images, the images were aligned. Alignment is normally carried out according to reference markers (ladders) which are run alongside each batch of samples. A 100bp molecular ladder was recommended (A.Weightman; pers.comm.) for this study, however it became apparent that it did not give consistent results, therefore could not be used as a reference marker for alignment of the three gels. To avoid repeating the DGGE technique using a different marker, a selection of samples from the three gels were loaded onto a separate gel, as shown in Figure 6.11, and used to align the DGGE images for the three pile conditions (Figure 6.13).



Key:

L = molecular weight ladder

M = chicken manure sample

TMT D32 = Total Manure Treatment Day 32

UMC D32 = Un-managed Control Day 32

MC D32 = Managed Control Day 32

MC D1 = Managed Control Day 1

Figure 6.11: Alignment reference for DGGE images.

6.6.3 Baseline Microbial Composition

The baseline DGGE images for chicken manure and oil refinery sludge are presented in Figure 6.12. Figure 6.12a shows the positions of bands identified as being common to both the chicken manure and the oil refinery sludge, and Figure 6.12b shows the positions of DGGE bands which were excised and sequenced. The sequencing results are presented in Table 6.4. Observations are presented and discussed in the following sections.

Lanes 1 to 7 were loaded on the same DGGE gel therefore alignment is based on the molecular weight (100bp) ladder used. Lanes 8 and 9, however, were loaded on a separate DGGE gel, thus alignment between these samples and those of Lanes 1 to 7 is based on the DGGE image presented in Figure 6.11.

6.6.3.1 Chicken Manure

1. The chicken manure samples possessed numerous discrete bacterial populations, with a bacterial community diversity of 27 populations (based on the total number of visible bands).

2. A total of 7 bands were excised from the chicken manure samples and sequenced. These bands represent 5 discrete bacterial populations. These populations show strong sequence similarity (89-99%) with bacteria belonging to *Gamma Proteobacteria* (Bands 1, 2, 3 and 14 (Figure 6.12a) belong to *Ectothiorhodospiraceae*, and Bands 4 and 5 (Figure 6.12a) are unidentified *Gamma Proteobacteria*) and *Actinobacteria* (Band 6 (Figure 6.12a) belongs to *Rhodococcus*) based on the BLAST sequence results in Table 6.4. A direct link between *Ectothiorhodospiraceae* and hydrocarbon degradation has not been identified in the literature, but can be inferred from the research areas recorded for these sequence matches (Table 6.4). *Rhodococcus* is reported by Alexander (1999), Irvine et al (2000), Heiss-Blanquet et al (2005), and Behki (1994) as having hydrocarbon degrading capabilities, and can be inferred from the research area recorded for this sequence match (Table 6.4).

3. A further 4 discrete microbial populations with potential hydrocarbon degrading capability can be identified in the chicken manure samples as they are represented by bands identified as being common to chicken manure and oil refinery sludge (common bands B, C, D and E; Figure 6.12a). Common band B represents bacteria belonging to *Hydrocarboniphaga effusa*, which was identified by Palleroni et al (2004) from soil contaminated with fuel oil and showed ability to utilise aliphatic hydrocarbons from C_9-C_{19}. Common band C represents bacteria belonging to *Pseudomonas*. *Pseudomonas* has been identified by Ijah and Antai (2003) in chicken manure and was seen to be most effective in the biodegradation of crude oil (68.5% crude oil loss, approx). *Pseudomonas* is also reported as being a petroleum degrader by Kaplan and Kitts (2004), Fetter (1999), Straube et al (2003), Irvine et al (2000), Ibekwe et al (2004), Heiss-Blanquet et al (2005), and Bento et al (2003). Common band D represents bacteria belonging to *Marinobacter* (*Gamma Proteobacteria*). The identity of bacteria represented by common band E could not be concluded (Table 6.5). The research areas recorded for these sequence matches infer a link between these bacteria and hydrocarbon environments and hydrocarbon degradation.

4. In addition to the 9 (5 confirmed by chicken manure samples and 4 from oil refinery sludge samples) potential hydrocarbon degrading bacterial populations identified in chicken manure (as per points 3 and 4 above), a further 7 discrete bacterial populations represented by bands identified as common to both chicken manure and oil refinery sludge have been identified. Unfortunately bands representing these populations were not excised; therefore their identity cannot be confirmed through sequencing. It is proposed by the author that these microbial populations are capable of growing in the presence of, or utilisation of, hydrocarbons based on their presence in the oil refinery sludge.

It can be seen from the data presented in Section 6.6.3 and the observations summarised above, that the chicken manure possessed bacterial populations potentially capable of hydrocarbon biodegradation, including *Ectothiorhodospiraceae*, *Rhodococcus*, *Pseudomonas, Marinobacter* and *Hydrocarboniphaga effusa*.

The presence of such suitable bacterial consortia is a significant factor affecting total biodegradation potential of an organic contaminant, as discussed in Chapter 2. Other significant factors are the biodegradability and bioavailability of the contaminant, and the suitability of environmental conditions to support microbial growth and activity, as was discussed in Chapter 2. The data presented in this section corroborate with the findings of Ijah and Antai (2003), which also identified the presence of bacteria in chicken manure with the ability to utilise hydrocarbons. This indicates that there is a potential for the use of chicken manure to enhance TPH biodegradation through bioaugmentation.

Ijah and Antai (2003) identified bacteria belonging to *Pseudomonas*, *Bacillus*, *Micrococcus* and *Proteus*. Of these bacteria, only *Pseudomonas* was identified in the baseline chicken manure samples for this study. However, not all bands were excised for sequencing as they were feint and/or insufficiently separated for precise excision. Therefore sequencing of such bands was deemed most likely unsuccessful (G.Webster; pers.comm.). The identity of the bacterial populations represented by these bands is therefore unknown. It may be that other hydrocarbon degrading bacteria, possibly including *Bacillus*, *Micrococcus* and *Proteus*, were also present in the chicken manure and represented by the un-sequenced bands.

Although potential hydrocarbon degrading bacterial populations have been identified in the chicken manure samples, it must be remembered that DGGE results are based on total genomic material extracted from an environmental sample, including that from active and non-active bacterial cells. Therefore these data cannot be used to infer the activity of these populations. Through the comparison of DGGE profiles over time and between the treatment conditions, an indication of active bacterial populations and the effect of treatment conditions on such populations can be attained. Such data is presented and discussed in Section 6.6.4.

As mentioned above, many bands were not excised as sequencing was deemed most likely unsuccessful. It is possible that some of these un-sequenced bands represent non-hydrocarbon degrading bacteria. Of concern to the composting bioremediation process is the potential proliferation of such bacteria at the expense of hydrocarbon

degrading bacteria upon mixing with the oil refinery sludge. This could have an adverse effect on TPH biodegradation. Whether or not this occurs during the treatability studies can be indicated by the bacterial community dynamics data presented and discussed in Section 6.6.4.

6.6.3.2 Oil Refinery Sludge

1. The oil refinery sludge samples possessed numerous discrete bacterial populations, with a bacterial community diversity of 28 populations (based on the total number of visible bands).
2. A total of 7 bands were excised from the oil refinery sludge samples and sequenced. These bands represent 6 discrete bacterial populations. These populations show strong sequence similarity (81-99%) with bacterial populations belonging to *Gamma Proteobacteria* (Band 7 belongs to *Hydrocarboniphaga effusa*, Band 8 belongs to *Pseudomonas*, and Band 9 belongs to *Marinobacter*), and *Delta Proteobacteria* (Bands 11 and 12 belong to *Syntrophus*) based on the BLAST sequence results in Table 6.4. As outlined in Section 6.6.3.1, *Hydrocarboniphaga effusa* has been identified by Palleroni et al (2004) as having the ability to utilise aliphatic hydrocarbons from C_9-C_{19}, and *Pseudomonas* is widely reported as being a petroleum degrader by Kaplan and Kitts (2004), Fetter (1999), Straube et al (2003), Irvine et al (2000), Ibekwe et al (2004), Heiss-Blanquet et al (2005), and Bento et al (2003). *Syntrophus* has been identified by Dojka et al (1998) as having the ability to degrade hydrocarbons. A direct link between *Marinobacter* and hydrocarbon biodegradation is not reported in the literature, but can be inferred from the research areas recorded for these sequence matches (Table 6.4).
3. A further 2 discrete microbial populations with potential hydrocarbon degrading capability can be identified in the chicken manure samples as they are represented by bands identified as being common to chicken manure and oil refinery sludge (common bands A and H, Figure 6.12a). Common band A represents bacteria belonging to *Ectothiorhodospiraceae*, and common band H represents bacteria belonging to unidentified *Gamma Proteobacteria*. A direct link

between these populations and hydrocarbon degradation can only be inferred from the research areas recorded for these sequence matches (Table 6.4).

4. In addition to the 8 potential hydrocarbon degrading bacterial populations identified in the oil refinery sludge (as per points 3 and 4 above), a further 8 discrete bacterial populations represented by bands identified as common to both chicken manure and oil refinery sludge have been identified. Unfortunately bands representing these populations were not excised; therefore their identity cannot be confirmed. It is proposed by the author that these microbial populations are capable of growing in the presence of, or utilisation of, hydrocarbons based on their presence in the oil refinery sludge. However, the author recognises that there is a possibility that these populations are non-hydrocarbon degrading bacteria and therefore may pose a risk to TPH degradation through adverse competition for growth factors with hydrocarbon degrading bacterial populations.

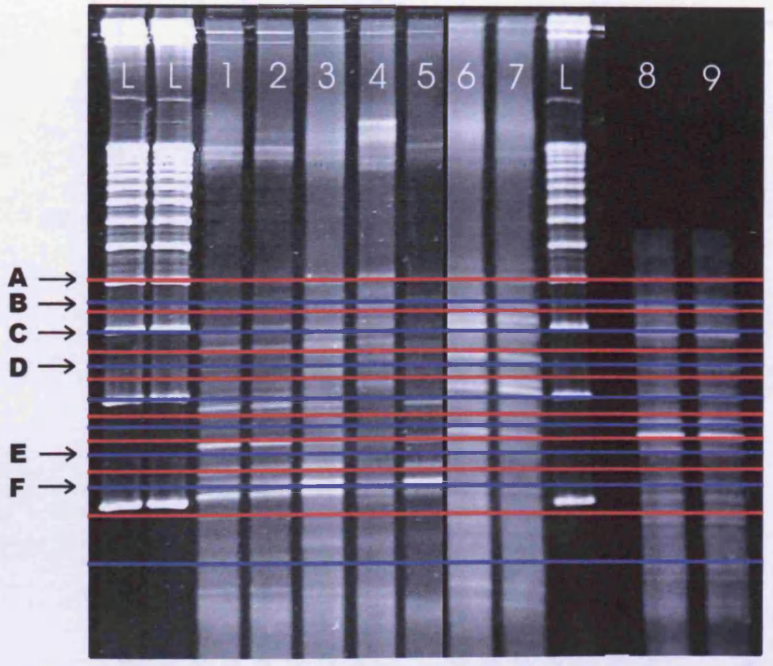
It can be seen from the data presented in Section 6.6.3 and the observations summarised above, that oil refinery sludge possesses bacterial populations potentially capable of hydrocarbon biodegradation, including *Ectothiorhodospiraceae*, *Hydrocarboniphaga effusa*, *Marinobacter*, *Pseudomonas*, and *Syntrophus*. In addition to these bacterial populations, there are many additional visible bands for the oil refinery sludge samples, which were not sequenced and therefore their identity is unknown.

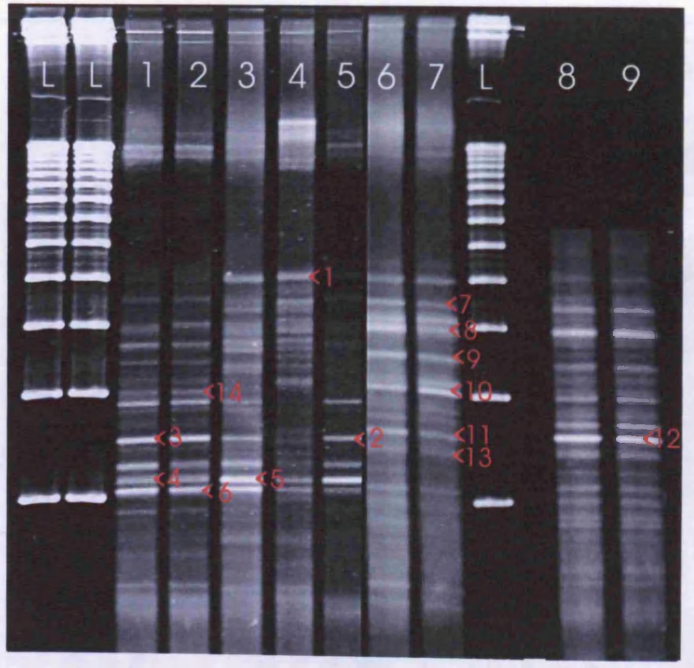
The presence of such suitable bacterial consortia is a significant factor affecting total biodegradation potential of an organic contaminant, as discussed in Section 6.6.3.1. The data presented in Section 6.6.3 indicate that the oil refinery sludge already possessed bacterial populations with potential hydrocarbon degradation ability. However it must be remembered that DGGE results are based on total genomic material extracted from an environmental sample, including that from active and non-active bacterial cells. Therefore these data cannot be used to infer the activity of these populations. Through the comparison of DGGE profiles over time and between the treatment conditions, an indication of active bacterial populations and the effect of treatment conditions on such populations can be attained. Such data is presented and discussed in Section 6.6.4.

6.6.3.3 Summary

It is evident from the baseline DGGE profiles and sequence data presented in this section that:

1. Both the chicken manure and oil refinery sludge samples possessed bacterial populations with potential hydrocarbon degradation abilities, based on sequence similarities with bacteria identified in the literature to possess hydrocarbon degradation abilities, and inferences made from the research areas recorded for the sequence matches. Such data indicate that both materials possessed bacterial consortia likely suitable for hydrocarbon degradation. The possession of such bacteria is a significant factor affecting the total biodegradation potential of an organic contaminant, as was discussed earlier. However the data presented in this section cannot be used to infer the activity of such bacterial populations. This can only be inferred through bacterial community dynamics data which are presented and discussed in Section 6.6.4.
2. A total of 16 bacterial populations were identified as common to both the chicken manure and oil refinery sludge.
3. There were several bands for both materials which were not excised for sequencing, therefore their identity are unknown. For the oil refinery sludge it is proposed by the author that such bands represent bacterial populations capable of growing in the presence of or utilisation of hydrocarbons, however this cannot be confirmed in this study. For the chicken manure it is also possible that such bands may represent potential hydrocarbon degrading bacterial populations, however it is likely that non-hydrocarbon degrading bacteria are also present. Of concern to the composting bioremediation process is the possibility that such non-hydrocarbon degrading bacteria could proliferate at the expense of hydrocarbon degrading bacteria, thus potentially having an adverse affect on TPH biodegradation. The occurrence of this can be indicated by the bacterial community dynamics data presented and discussed in Section 6.6.4.





Key:

A - G Sequenced bands common to chicken manure and oil refinery sludge

Populations common to chicken manure and oil refinery sludge

Key:

L = 100bp ladder
1 - 5 = Chicken Manure
6 - 9 = Oil refinery Sludge
1 - 11 = sequenced bands

Figure 6.12: Baseline DGGE profile for chicken manure and oil refinery sludge

Table 6.4: BLAST Sequencing Results

Band Location Number	Excised Band Reference Number	BLAST result (closest match in GenBank database (name (accession number))	Likely Function (according to GenBank research area)	% Similarity (number of compared base pairs)
1	26	Uncultured *Gammaproteobacteria* (AF154086); *Proteobacteria*; *Gammaproteobacteria*; *Ectothiorhodospiraceae* (DQ153880)	Hydrocarbon seep sediment; microbial growth on diesel fuel in saline environments.	101/102 (99%); 101/102 (99%)
2 and 3	2, 30	Uncultured *Gammaproteobacteria* (AF154086); *Proteobacteria*; *Gammaproteobacteria*; *Ectothiorhodospiraceae* (DQ153880)	Hydrocarbon seep sediment; microbial growth on diesel fuel in saline environments.	101/102 (99%); 101/102 (99%)
4 and 5	28, 29	Uncultured *Gammaproteobacteria* (AY144262; AF432282;); Uncultured bacteria (DQ123795; DQ123790)	Petroleum land treatment unit and hydrocarbon degradation; degradation of PAHs	102/114 (89%); 100/112 (89%); 102/114 (89%); 102/114 (89%)
6	31	*Actinobacteria*; *Rhodococcus* ; *Rhodococcus Gordoniae* (AY233202)	Associated with phenol contaminated soil.	104/113 (92%)
7	6, 32, 35	Uncultured *Gammaproteobacterium* (AY251203); *Proteobacteria*; *Gammaproteobacteria*; *Xanthomonadales*; *Hydrocarboniphaga* (AY363245); Uncultured bacterium (AB011569)	Associated with diesel fuel bioremediation studies; associated with alkane and aromatic hydrocarbon degradation; associated with phenol degrading bacteria.	96/99 (96%); 107/116 (92%); 107/116 (92%)
8	4, 39	*Proteobacteria*; *Gammaproteobacteria*; *Pseudomonadaceae*; *Pseuodomonas* (DQ225137); Uncultured *Gammaproteobacteria* (AJ810632)	Associated with hydrocarbon contaminated environments; Community dynamics during microcosm enrichment with various hydrocarbons	149/161 (92%); 130/114 (90%)

Table 6.4 continued...

Band Location Number	Excised Band Reference Number	BLAST result (closest match in GenBank database (name (accession number))	Likely Function (according to GenBank research area)	% Similarity (number of compared base pairs)
9	33, 36	*Proteobacteria*; *Gammaproteobacteria*; *Alteromondales*; *Marinobacter* (AF264687); Uncultured *Gammaproteobacteria* (AJ561157)	Isolation of hydrocarbon degrading bacteria from crude oil and mangrove sediments; Crude oil induced coastal bacterial community shifts	161/165 (97%); 132/140 (94%)
10	7, 34	Uncultured *Bacteroidetes* bacteria (AY437413); *Proteobacteria*; *Deltaproteobacteria*; *Syntrophobacterales*; *Syntrophus* (AJ133795); Uncultured *Deltaproteobacteria* (AF050534.	MTBE contaminated shallow aquifer; Associated with long-chain alkanes; associated with hydrocarbon contaminated aquifer.	132/140 (94%); 131/136 (96%); 128/136 (94%)
11 and 12	3, 37	*Proteobacteria*; *Deltaproteobacteria*; *Syntrophobacterales*; *Syntrophus* (AJ133795); Uncultured bacterium (DQ200731); Uncultured *Deltaproteobacteria* (AF050534 and AF351238)	Associated with long-chain alkanes; associated with degradation of petroleum hydrocarbons in the range C10 to C40; associated with hydrocarbon contaminated aquifer; associated with naphthalene contaminated aquifer waters.	130/139 (93%); 109/112 (97%); 125/139 (89%); 108/123 (87%)
13	38	Uncultured bacteria (AY351573)	Phenol degrading bacterial community dynamics.	132/161 (81%)
14	27	Uncultured *Gammaproteobacteria* (AF154086); *Proteobacteria*; *Gammaproteobacteria*; *Ectothiorhodospiraceae* (DQ153880)	Hydrocarbon seep sediment; microbial growth on diesel fuel in saline environments.	101/102 (99%); 101/102 (99%)

6.6.4 Bacterial Community Dynamics

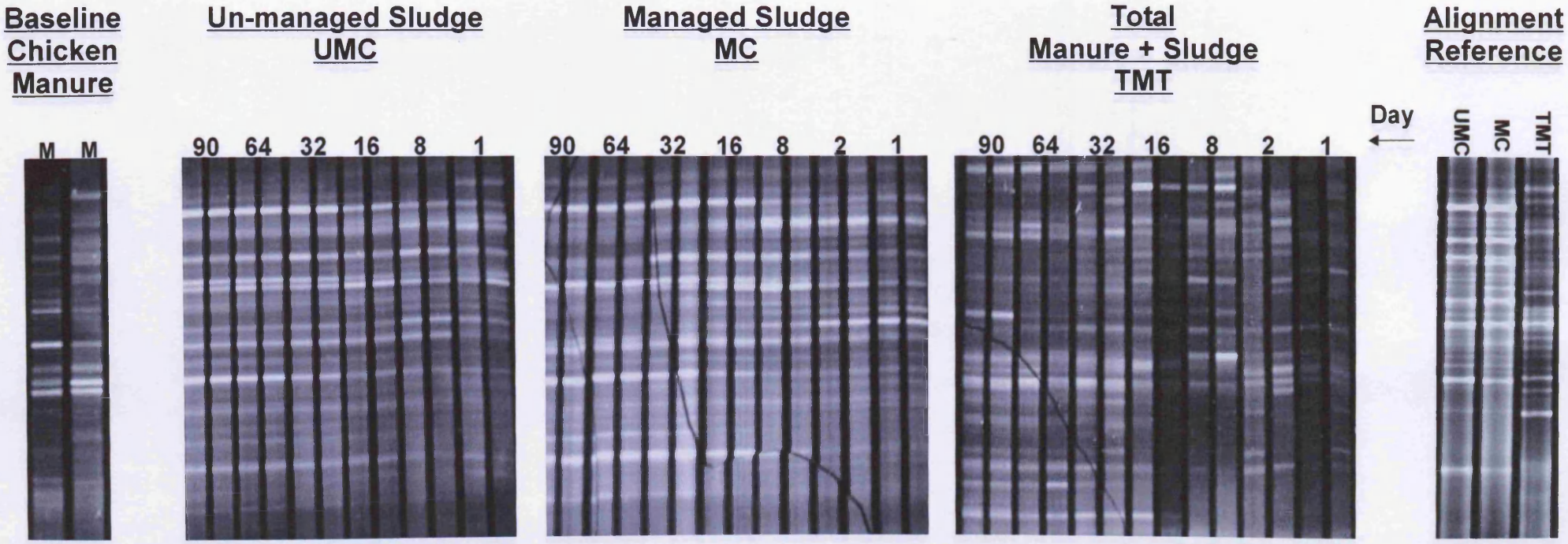
The bacterial community dynamics data (DGGE profiles) are presented and discussed in this section. These data are used to address the following aims:-

1. To determine the effects of the pile management strategy on bacterial community dynamics through comparison of data for un-managed control (UMC) condition with those for the managed control (MC) condition.
2. To determine the effects of chicken manure addition to oil refinery sludge on bacterial community dynamics through a) comparison of data for the total manure treatment (TMT) condition with those for the managed control (MC) condition, and b) comparison with baseline bacterial composition data for chicken manure and oil refinery sludge; in particular:

    a. To determine the likely survival of bacterial populations indigenous to the chicken manure.
    b. To determine the likely survival of bacterial populations indigenous to the oil refinery sludge.
    c. To indicate any predominance of non-hydrocarbon degrading bacterial populations over hydrocarbon degrading bacteria resulting from the addition of chicken manure and therefore presenting a potentially adverse effect on TPH degradation.

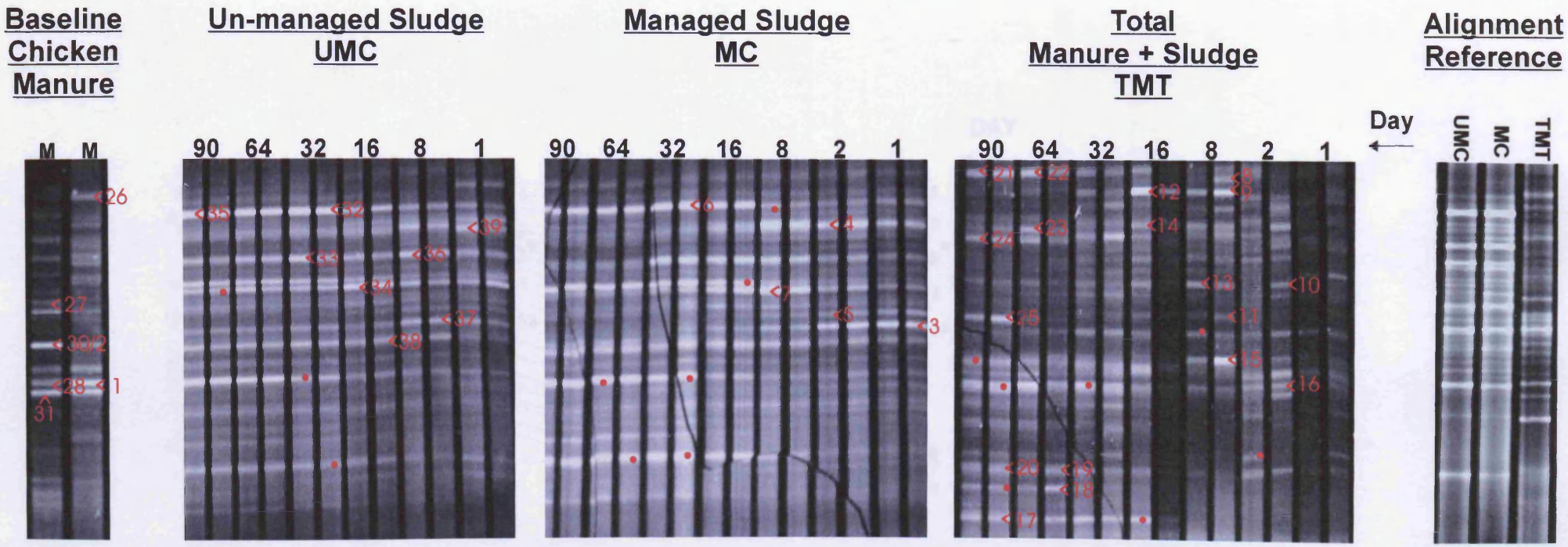
The DGGE profiles for the UMC, MC and TMT conditions were aligned in accordance with Figure 6.11 and Figure 6.12, and are presented in Figure 6.13. Several bands were excised for sequencing; their locations are shown in Figure 6.14. The BLAST results of the successfully sequenced bands are summarised in Table 6.5.

Figure 6.15 and Figure 6.16 show annotated DGGE profiles to illustrate the effects of the pile management strategy and chicken manure on bacterial community dynamics. These figures are discussed in the following sections.



Duplicate samples for each sampling event

Figure 6.13: Aligned DGGE Community Fingerprints



Duplicate samples for each sampling event

< 1, 2, 3 = **Bands successfully sequenced**

• = **Bands un-successfully sequenced**

Figure 6.14 : Location of excised DGGE bands

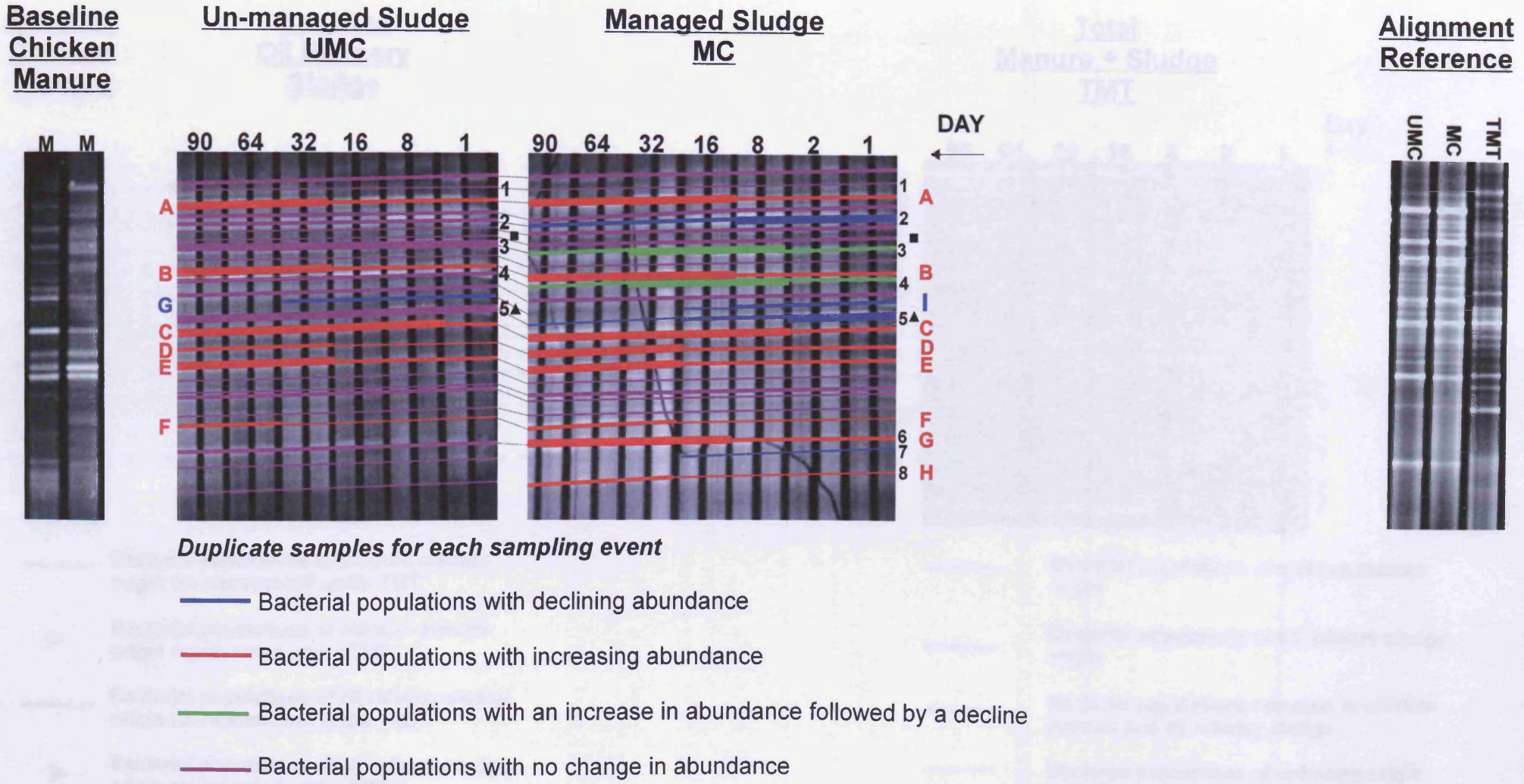


Figure 6.15: Pile Management Strategy and Community Dynamics

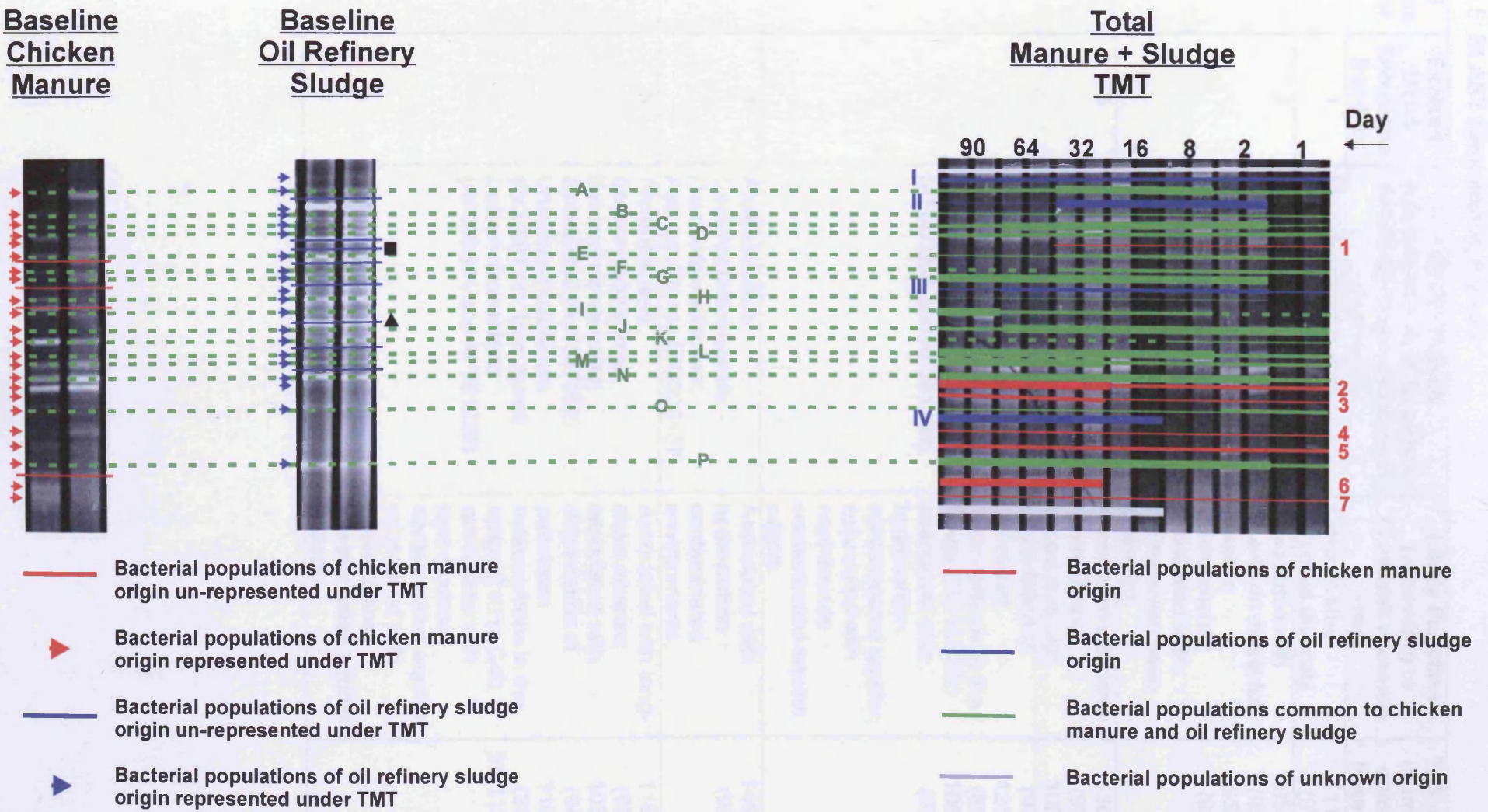


Figure 6.16: Bacterial community dynamics under TMT pile condition

Table 6.5: BLAST Sequencing Results

Band Location Number	Excised Band Reference Number	BLAST result (closest match in GenBank database (name (accession number))	Likely Function (according to GenBank research area)	% Similarity (number of compared base pairs)
1	1	Uncultured bacterium (AY795650)	Chicken litter microbial diversity	111/114 (97%)
2	2	*Proteobacteria*; *Gammaproteobacteria*; *Chromatiales*; *Ectothiorhodospiraceae* (DG153880); Uncultured hydrocarbon seep bacterium (AF154086)	Associated with growth on diesel fuel in saline environments; associated with hydrocarbon seep sediment.	95/102 (93%); 95/102 (93%)
3	3	*Proteobacteria*; *Deltaproteobacteria*; *Syntrophobacterales*; *Syntrophus* (AJ133795); Uncultured bacterium (DQ200731); Uncultured *Deltaproteobacteria* (AF050534 and AF351238)	Associated with long-chain alkanes; associated with degradation of petroleum hydrocarbons in the range C10 to C40; associated with hydrocarbon contaminated aquifer; associated with naphthalene contaminated aquifer waters.	130/139 (93%); 109/112 (97%); 125/139 (89%); 108/123 (87%)
4	4	*Proteobacteria*; *Gammaproteobacteria*; *Pseudomonadaceae*; *Pseuodomonas* (DQ225137).	Associated with hydrocarbon contaminated environments.	149/161 (92%)
5	5	*Proteobacteria*; *Deltaproteobacteria*; *Syntrophobacterales*; *Syntrophus* (AJ133795); Uncultured bacterium (DQ200731); Uncultured *Deltaproteobacteria* (AF050534 and AF351238)	Associated with long-chain alkanes; associated with degradation of petroleum hydrocarbons in the range C10 to C40; associated with hydrocarbon contaminated aquifer; associated with naphthalene contaminated aquifer waters.	115/123 (93%); 103/109 (94%); 110/123 (89%); 98/112 (87%)

Table 6.5 continued...

Band Location Number	Excised Band Reference Number	BLAST result (closest match in GenBank database (name (accession number))	Likely Function (according to GenBank research area)	% Similarity (number of compared base pairs)
6	12	Uncultured *Gammaproteobacterium* (AY251203); *Proteobacteria*; *Gammaproteobacteria*; *Xanthomonadales*; *Hydrocarboniphaga* (AY363245); Uncultured bacterium (AB011569)	Associated with diesel fuel bioremediation studies; associated with alkane and aromatic hydrocarbon degradation; associated with phenol degrading bacteria.	96/99 (96%); 107/116 (92%); 107/116 (92%)
7	13	*Proteobacteria*; *Deltaproteobacteria*; *Syntrophobacterales*; *Syntrophus* (AJ133795); Uncultured *Deltaproteobacteria* (AF050534).	Associated with long-chain alkanes; associated with hydrocarbon contaminated aquifer.	131/136 (96%); 128/136 (94%)
8	15	*Bacteria*; *Clostridia*; *Peptococcaceae*; *Desulfotomaculum* (DQ155286)	Associated with cooling water towers of petroleum refinery	115/121 (95%)
9	16	*Proteobacteria*; *Gammaproteobacteria*; *Alcanivoracaceae*; *Alcanivorax* (AY307381; AY683537; AB055207; ABO53132)	Petroleum degrading marine bacterium;	All at 151/166 (90%)
10	17	Uncultured bacterium (DQ206961); Uncultured *Bacillus* (AY862489); *Bacteria*; *Clostridia*; *Peptococcaceae*; *Desulfotomaculum* (DQ155286)	Associated with chicken litter; bacterial communities in oil; associated with cooling water towers of petroleum refinery.	160/168 (95%); 154/165 (93%); 152/162 (93%)
11	18	*Proteobacteria*; *Gammaproteobacteria*; *Chromatiales*; *Ectothiorhodospiraceae* (DG153880); Uncultured hydrocarbon seep bacterium (AF154086)	Associated with growth on diesel fuel in saline environments; associated with hydrocarbon seep sediment.	99/102 (97%); 99/102 (93%)

Table 6.5 continued...

Band Location Number	Excised Band Reference Number	BLAST result (closest match in GenBank database (name (accession number))	Likely Function (according to GenBank research area)	% Similarity (number of compared base pairs)
12	19	*Proteobacteria*; *Gammaproteobacteria*; *Alcanivoracaceae*; *Alcanivorax* (AY307381; AY683537; AB055207; ABO53132; AB053128; AF432307)	Petroleum degrading marine bacterium;	122/129 (94%); 122/129 (94%); 122/129 (94%); 151/166 (90%); 134/145 (92%); 114/119 (95%)
13	20	*Bacteria*; *Clostridia*; *Peptococcaceae*; *Desulfotomaculum* (DQ155286); Uncultured bacterium (DQ206955)	Associated with cooling water towers of petroleum refinery; associated with chicken litter.	132/134 (98%); 128/131 (97%)
14	21	Uncultured bacterium (DQ203277)	Associated with chicken litter	159/167 (95%);
15	23	Uncultured bacterium (DQ206945)	Associated with chicken litter	
16	26	Uncultured bacterium (DQ206914)	Associated with chicken litter	114/123 (92%)
17	27	*Bacteria*; *Actinobacteria*; *Actinomycetales*; *Micromonosporaceae* (AY53920)	None	86/99 (86%)
18	28	*Bacteria*; *Actinobacteria*; *Corynebacterineae*; *Dietziaceae*; *Dietzia* (AY643401; AB211032; AY822698; AY603002; AY360061; AY360062; AY205297; AF249838)	Associated with hydrocarbon degradation.	141/145 (97%); 141/145 (97%); remaining at 140/145 (96%)
19	29	Uncultured bacterium (DQ203255)	Associated with chicken litter	101/112 (90%)
20	30	Uncultured bacterium (DQ203255)	Associated with chicken litter	100/112 (89%)
21	35	Uncultured bacterium (AY907835; AF247773)	Mineralisation of BTEX and benzo(a)pyrene.	102/110 (92%); 104/113 (92%)

Table 6.5 continued...

Band Location Number	Excised Band Reference Number	BLAST result (closest match in GenBank database (name (accession number))	Likely Function (according to GenBank research area)	% Similarity (number of compared base pairs)
22	36	Uncultured bacterium (AF247773)	Mineralisation of BTEX.	107/113 (94%)
23	37	Uncultured bacterium (AY922241)	None	110/112 (98%)
24	38	Uncultured *bacteroidetes* bacterium (AF432285)	Hydrocarbon degradation and dynamics of bacterial communities.	109/114 (95%)
25	39	Uncultured bacterium (AF128781)	Oil spill bioremediation.	117/127 (92%)
26	40	Uncultured *Gammaproteobacteria* (AF154086); *Proteobacteria*; *Gammaproteobacteria*; *Ectothiorhodospiraceae* (DQ153880)	Hydrocarbon seep sediment; microbial growth on diesel fuel in saline environments.	101/102 (99%); 101/102 (99%)
27	41	Uncultured *Gammaproteobacteria* (AF154086); *Proteobacteria*; *Gammaproteobacteria*; *Ectothiorhodospiraceae* (DQ153880)	Hydrocarbon seep sediment; microbial growth on diesel fuel in saline environments.	101/102 (99%); 101/102 (99%)
28	42	Uncultured *Gammaproteobacteria* (AY144262; AF432282;); Uncultured bacteria (DQ123795; DQ123790)	Petroleum land treatment unit and hydrocarbon degradation; degradation of PAHs	102/114 (89%); 100/112 (89%); 102/114 (89%); 102/114 (89%)
29	43	Uncultured *Deltaproteobacteria* (AF211273)	Associated with hydrogen sulphide-rich black mud from marine coastal environments	106/116 (91%)
30	44	Uncultured *Gammaproteobacteria* (AF154086); *Proteobacteria*; *Gammaproteobacteria*; *Ectothiorhodospiraceae* (DQ153880)	Hydrocarbon seep sediment; microbial growth on diesel fuel in saline environments.	101/102 (99%); 101/102 (99%)
31	45	*Actinobacteria*; *Actinomycetales*; *Corynebacterineae*; *Nocardiaceae*; *Rhodococcus* ; *Rhodococcus Gordoniae* (AY233202)	Associated with phenol contaminated soil.	104/113 (92%)

Table 6.5 continued...

Band Location Number	Excised Band Reference Number	BLAST result (closest match in GenBank database (name (accession number))	Likely Function (according to GenBank research area)	% Similarity (number of compared base pairs)
32	46	Uncultured *Gammaproteobacteria* (AY251203); Uncultured bacteria (AF252601)	Diesel fuel bioremediation; microbial community composition in an oil reservoir model column.	156/164 (95%)
33	47	Uncultured *Gammaproteobacteria* (AJ561157); Proteobacteria; *Gammaproteobacteria*; *Alteromondales*; *Marinobacter* (AF264687)	Crude oil induced coastal bacterial community shifts; isolation of hydrocarbon degrading bacteria from crude oil and mangrove sediments.	132/140 (94%); 132/140 (94%)
34	48	Uncultured *Bacteroidetes* bacteria (AY437413)	MTBE contaminated shallow aquifer.	132/140 (94%)
35	49	Uncultured *Gammaproteobacteria* (AY251203); *Proteobacteria*; *Gammaproteobacteria*; *Xanthomonadaceae*; *Hydrocarboniphaga* (AY363245); Uncultured bacteria (AB011569)	Diesel fuel bioremediation; alkane and aromatic hydrocarbon degradation; phenol degrading bacteria in activated sludge.	139/143 (97%); 150/160 (93%); 150/160 (93%)
36	51	*Proteobacteria*; *Gammaproteobacteria*; *Alteromondales*; *Marinobacter* (AF264687)	Isolation of hydrocarbon degrading bacteria from crude oil and mangrove sediments.	161/165 (97%)
37	52	*Proteobacteria*; *Deltaproteobacteria*; *Syntrophobacterales*; *Syntrophus* (AJ133795); Uncultured bacteria (AF050534)	Associated with long-chain alkanes; hydrocarbon contaminated aquifer.	126/141 (89%); 122/141 (86%)
38	55	Uncultured bacteria (AY351573)	phenol degrading bacterial community dynamics.	132/161 (81%); 132/161 (81%)
39	56	Uncultured *Gammaproteobacteria* (AJ810632)	Community dynamics during microcosm enrichment with various hydrocarbons	130/114 (90%)

6.6.4.1 Pile Management Strategy and Bacterial Community Dynamics

Figure 6.15 shows the aligned community fingerprints for the UMC and MC conditions. The DGGE profiles have been annotated with coloured lines to show changes in bacterial population abundance over the duration of the treatability studies. Observations for the UMC and MC conditions are discussed individually in the following sections, and then summarised in Section 6.6.4.1.3. References are made to sequenced DGGE bands; the locations of these bands are shown in Figure 6.14, and the BLAST sequence results are recorded in Table 6.5.

6.6.4.1.1 UMC Condition

It is evident from Figure 6.13 and Figure 6.15 that the bacterial community fingerprint for the UMC treatment condition is consistent between samples (i.e. over the duration of the treatability studies. However, changes in relative abundance (indicated by changes in band intensity and therefore line thickness in Figure 6.15) of some bacterial populations are evident, as discussed later on in this section.

The majority, 72% (18 out of 25 bacterial populations) showed no change in relative abundance over the duration of the treatability studies under the UMC condition. The author therefore assumes that these populations are non-growing. However, these data cannot be used to indicate bacterial activity, or importance to TPH biodegradation. Although Alexander (1999) reports that several studies have demonstrated a positive correlation between the cell count of a population acting on a particular compound (therefore indicating that biodegradation in linked to changes in population abundance), he also states that it is likely that compound degradation in nature occasionally results from non-growing populations. Several authors have reported a lack of correlation between microbial count and hydrocarbon biodegradation, including Bento et al (2003), Venosa et al (1996), Jorgensen et al (2000), and Breedveld and Sparrevik (2000).

Some of the bacterial populations showing no change in relative abundance are represented by sequenced DGGE bands. These populations are labelled 1 to 5 on the UMC community fingerprint in Figure 6.14, and are identified as follows:-

2) Population 1 is represented by band 26 and belongs to *Ectothiorhodospiraceae* (*Gamma Proteobacteria*). A link between this population and hydrocarbon degradation can be inferred from the research area recorded for this sequence match (Table 6.4).
3) Population 2 is represented by bands 4 and 39 and belongs to the genera *Pseudomonas* (*Gamma Proteobacteria*), which have been identified by numerous authors as stated earlier (including Ijah and Antai (2003), and Kaplan and Kitts (2004)) as having the ability to degrade petroleum hydrocarbons. This ability can also be inferred from the research area recorded for these sequence matches (Table 6.4).
4) Population 3 is represented by bands 33 and 36 and belongs to *Marinobacter* (*Gamma Proteobacteria*). A link between *Marinobacter* and hydrocarbon degradation is not reported in the literature, but can be inferred from the research areas recorded for these sequence matches (Table 6.4).
5) Populations 4 and 5 are represented by bands 7 and 34, and band 5 respectively, and belong to *Syntrophus* (*Delta Proteobacteria*). A link between *Syntrophus* and hydrocarbon degradation is reported by Dojka et al (1998), and can be inferred from the research areas recorded for these sequence matches (Table 6.4).

These data indicate that populations 1 to 5 have hydrocarbon degrading abilities. For those populations showing no change in relative abundance, which are not represented by sequenced DGGE bands, their identity is unknown. Growth of these populations may be stimulated through the pile management strategy and/or addition of chicken manure, as discussed in the following sections. It may be that these populations also constitute hydrocarbon degrading bacteria, and their presence in the oil refinery sludge supports this. However, they may represent non-hydrocarbon degrading bacteria. In this latter scenario, these populations may present a risk to TPH biodegradation through adverse competition for growth factors with hydrocarbon degrading bacterial populations. This is further discussed later on.

Six bacterial populations (labelled A to F on the UMC DGGE profile in Figure 6.15) show an increase in relative abundance over the duration of the treatability study. This could reflect a) growth of these populations, and/or b) a simultaneous decline of another population. Only the increased relative abundance of population F coincides with the decline of another population (population G) thus may be, at least in part, attributable to the decline of population G. The author proposes that the increased relative abundance of the remaining populations reflects actual growth of these populations. Such growth may reflect:-

a) Environmental conditions becoming more favourable over time: - environmental conditions such as temperature, pH, water and oxygen availability affect the growth and activity of microorganisms (Alexander, 1999) as was discussed in Chapter 2. It may be that one or more environmental parameters were limiting during the earlier stages of the treatability studies, thus inhibiting bacterial growth. For example, at the start of the treatability studies, the moisture content of the oil refinery sludge was approximately 80-90%, which is higher than the 40-60% recommended for optimum microbial growth and activity (The Composting Association, 2005; Pace et al, 1995; Rynk, 2000). Therefore the growth of these populations during the later stages of the treatability studies may reflect a sufficient reduction of moisture content. *Hydrocarboniphaga effusa* is aerobic (Palleroni et al, 2004), therefore its growth between Days 16 and 32 may indicate an improvement in oxygen diffusion within the oil refinery sludge.
b) Removal of toxins:- the presence of toxins can inhibit microbial growth and activity, as was discussed in Chapter 2. In the case of complex mixtures of organic compounds such as TPH it is likely that one or more compounds within the mixture will be toxic to one or more microbial populations (Alexander, 1999). It may therefore be that populations A to F were inhibited by one or more TPH compounds during the early stages of the treatability studies. These toxins may have been removed (i.e. through biodegradation by another bacterial population, sorption, or volatilisation) thus enabling these populations to proliferate.
c) Increased availability of substrate(s):- the growth of populations A to F may indicate introduction of suitable substrate(s) (i.e. a TPH compound) which may be

the degradation product of a TPH compound broken down by another population, or increased bioavailability of substrate(s).

Population A is represented by bands 32 and 35 and belongs to *Hydrocarboniphaga effusa* (*Gamma Proteobacteria*). This bacterium has been identified by Palleroni et al (2004) as being able to utilise aliphatic hydrocarbons from C_9 to C_{19}. Population E is represented by band 28 and is an unidentified *Gamma Proteobacteria*. A link between these populations and hydrocarbon biodegradation can be inferred from the research areas recorded for these sequence matches (Table 6.5). Although the identity of populations B, C, D and F cannot be confirmed by these data, the author proposes that they are also hydrocarbon degrading bacteria as the likely sole carbon source in the oil refinery sludge was the TPH compounds. The 'growth' of these populations may coincide with increased TPH degradation rates, as discussed in Chapter 8.

One bacterial population (labelled G on the UMC DGGE profile in Figure 6.14) shows a reduction in abundance whereby it cannot be seen after Day 32. This bacterial population is represented by band 5 (Table 6.4) and belongs to *Syntrophus* (*Delta Proteobacteria*). A link between *Syntrophus* and hydrocarbon degradation has been identified by Dojka et al (1998), and can be inferred from the research area recorded for this sequence match. The reduction in the relative abundance of this population may indicate the simultaneous growth of another bacterial population (potentially at the expense of population G), or actual decline of this population. Either may be caused by changing environmental conditions, appearance of toxins, or disappearance of a particular substrate (TPH compound). It can be seen that the disappearance of population G coincides with the increase in relative abundance of population F, which may suggest that the reduction in relative abundance of population G reflects, in part at least, the increase in relative abundance of population F.

6.6.4.1.2 MC Condition

It is evident from Figure 6.13 and Figure 6.15 that the bacterial community fingerprint for the MC condition is consistent between samples (i.e. over the duration of the

treatability study) and with that of the UMC community fingerprint. However, changes in relative abundance of some bacterial populations are evident, as discussed later on in this section.

A total of 44% (11 out of 25 bacterial populations) of the bacterial populations showed no change in relative abundance over the duration of the treatability studies under the MC condition. The author therefore assumes that these populations are non-growing. However, these data cannot be used to indicate bacterial activity, or importance of these populations to TPH biodegradation. These 11 populations correspond with populations showing no growth under the UMC condition (i.e. share the same position within the DGGE gel). It can therefore be argued that the pile management strategy did not stimulate the growth of these populations.

It can be seen from Figure 6.15 that not all of the populations identified under the UMC condition as showing no change in relative abundance were also identified as such under the MC condition. The populations identified as 'different' are labelled 2 to 8 on the MC community fingerprint in Figure 6.14, and are discussed as follows.

Populations 2, 5 and 7 show a reduction in relative abundance over time under the MC condition, compared with no change under the UMC condition. Population 2 is represented by band 4 and belongs to *Pseudomonas* (*Gamma Proteobacteria*). This bacterium is reported by numerous authors (including Ijah and Antai (2003) and Kaplan and Kitts (2004)) as being able to degrade hydrocarbons. Population 5 is represented by bands 3 and 37 and belongs to *Syntrophus* (*Delta Proteobacteria*). A link between this bacterium and hydrocarbon biodegradation is reported by Dojka et al (1998) and can be inferred from the research areas recorded for these sequence matches. Population 7 is not represented by sequenced DGGE bands, therefore it's identify is unknown.

The reduction in relative abundance for populations 2, 5 and 7 may reflect a) a decline of these populations (possibly due to worsening environmental conditions, reduced substrate(s), reduced bioavailability of substrate(s), or an increase in toxins), and/or b) a simultaneous growth of another population. Should the former scenario be true, this

may indicate that the pile management strategy had an adverse effect on these populations. However, even if this is the case, it may be that these populations had already carried out their function, thus it could be argued that the pile management strategy did not have an adverse effect on these populations. This cannot be confirmed by these data. There is, however, evidence for the latter scenario, as follows:-

a) The reduction in abundance of population 2 coincides with an increase in abundance of populations A (belonging to *Gamma Proteobacteria*, *Hydrocarboniphaga effusa*), B and G.
b) The reduction in abundance of population 5 coincides with an increase in abundance of population C, 3 and 4.
c) The reduction in abundance of population 7 coincides with an increase in abundance of populations D, E (unidentified *Gamma Proteobacteria*), and H.

Therefore the author proposes that the reduction in relative abundance for populations 2, 5 and 7 most likely reflects, at least in part, a simultaneous growth of other bacterial populations.

Populations 6 (G) and 8 (H) show an increase in relative abundance over time under the MC treatment condition, compared with no change under the UMC condition. For population 6, this coincides with a decline of populations I (i) and 2. For population 8, this coincides with a decline of populations 5, 7 and I. It may therefore be argued that the increase in relative abundance of populations 6 and 8 reflect, at least in part, the decline of another population rather than growth. This cannot be confirmed by these data. Should the increase reflect growth of populations 6 and 8, this indicates that the pile management strategy stimulated growth of these populations.

In addition to populations 6 and 8, populations A, B and F also show an increase in relative abundance, and that this occurred sooner than was seen for these populations under the UMC condition. The relative abundance of populations A and B increased after Day 8 under MC, versus Day 16 under UMC; population F increased

after Day 16 under MC versus Day 32 under UMC. This indicates that the pile management strategy stimulated the faster growth of populations A, B and F.

Populations 3 and 4 show an increase in relative abundance between Days 2 and 8, followed by a reduction between Days 32 and 64. The initial increase seen for these populations coincides with the decline of population 5; therefore it may be argued that it unlikely reflects the growth of these populations. However, the author argues that, on comparing relative abundance (shown by line thickness) of populations 3 and 4 with that of population 5, the decline of population 5 is most likely insufficient to account for the increase not only of populations 3 and 4, but also the simultaneous growth of population C. Therefore the author proposes that the increase in relative abundance of populations 3 and 4 between Days 2 and 8 most likely reflects, at least in part, growth of these populations. Population 3 is represented by bands 33 and 36 and belongs to *Marinobacter* (*Gamma Proteobacteria*). Population 4 is represented by bands 7 and 34 and belongs to *Syntrophus* (*Delta Proteobacteria*). A link between *Syntrophus* and hydrocarbon biodegradation is reported by Dojka et al (1998). A link between *Marinobacteria* and hydrocarbon biodegradation is not reported in the literature (to the author's knowledge) but can be inferred by the research areas recorded for these sequence matches. It may be argued that the pile management strategy stimulated the growth of these potential hydrocarbon degrading bacterial populations.

The subsequent reduction of relative abundance for populations 3 and 4 between Days 32 and 64 does not coincide with a simultaneous growth of another bacterial population. This would suggest that the reduction reflects a decline of these populations. However, the author recognises that, although this may be true, DGGE fingerprints are based on total genomic material from dead and alive bacterial cells. Therefore, once an increase in bacterial cells had occurred (in the case of populations 3 and 4, between Days 2 and 8) the band intensity would be expected (in the author's opinion) to remain the same regardless of any subsequent decline in cell numbers. Therefore the author suggests that it cannot be concluded from these data whether or not the reduction in relative abundance seen for these populations does in fact reflect a decline in cell number.

6.6.4.1.3 Summary

The effects of the pile management strategy on bacterial community dynamics of the oil refinery sludge are summarised as follows:

1. The pile management strategy did not encourage the growth of bacterial populations which were previously unrepresented in the UMC community fingerprint. This is indicated by the fact that the UMC and MC conditions shared the same bacterial community structure, which was consistent over the duration of the treatability studies.
2. The pile management strategy did encourage an increase in relative abundance of 8 bacterial populations compared with 6 under the UMC condition. Unfortunately these additional 2 populations are not represented by successfully sequenced bands, therefore their identity is unknown. It is, however, proposed by the author that these populations are able to grow in the presence of, and/or utilisation of, hydrocarbons as the TPH compounds likely presented the sole source of carbon in the oil refinery sludge.
3. The pile management strategy encouraged faster growth of 3 (labelled A, B and F in Figure 6.15) out of 6 bacterial populations (labelled A to F on the UMC community fingerprint in Figure 6.14) as compared with these populations under the UMC condition. Populations A and B showed an increase in relative abundance between Days 8 and 16, compared with an increase in relative abundance between Days 16 and 32 under the UMC condition. Population F showed an increase in relative abundance between Days 16 and 32, compared with an increase in relative abundance between Days 32 and 64 under the UMC condition. Unfortunately only Population A is represented by successfully sequenced bands. This population belongs to *Hydrocarboniphaga effusa* which was shown by Palleroni et al (2004) to degrade aliphatic hydrocarbons of C_9 to C_{19}.

6.6.4.2 Chicken Manure and Bacterial Community Dynamics

Of interest to this study is the potential negative effect of the amendment of oil refinery sludge with chicken manure on the growth and activity of hydrocarbon degrading bacterial populations. Bacterial community dynamics were therefore investigated for the TMT condition. Source identification of bacterial populations was achieved through comparison with baseline fingerprints for the chicken manure and oil refinery sludge samples. These data are presented and discussed in this section. Reference is made to Figure 6.14 and Table 6.5 for locations and BLAST results of excised DGGE bands.

The TMT community fingerprint and baseline fingerprints are presented in Figure 6.16. The TMT fingerprint has been annotated with coloured lines to indicate the likely origin of bacterial populations represented, and line thickness indicates changes in relative abundance over the duration of the treatability study. The baseline fingerprints have been annotated to identify those bacterial populations which are and those which are not represented under the TMT treatment condition.

6.6.4.2.1 Effect on Bacterial Community Diversity

The bacterial community diversity of chicken manure and oil refinery sludge was found to be 27 and 28 populations respectively (Figure 6.16), based on the total number of visible DGGE bands. A total of 16 bacterial populations were identified as being common to the chicken manure and oil refinery sludge samples (Figure 6.12).

It might therefore be expected that, upon mixing of these two materials, and therefore bacterial communities, total community diversity increases, potentially by 11-12 populations. However, it is evident from Figure 6.16 that this is not the case for the TMT condition. Over the duration of the treatability study, total community diversity for the TMT treatment condition was 23-29 populations.

It is reported by Heuer et al (2001) that for a bacterial population to be represented on a DGGE profile it must represent at least 1% of the total bacterial community. Heuer et al (2001) also reports that DGGE gels are not always this efficient.

The bacterial populations represented in the TMT community fingerprint are therefore likely those of higher abundance in each of the two materials. It is evident from the baseline fingerprints for the chicken manure and oil refinery sludge samples presented in Figure 6.16 that:-

1. All 16 bacterial populations identified in Figure 6.12 as being common to both the chicken manure and oil refinery sludge samples are represented in the TMT community fingerprint. This is discussed further in Section 6.6.4.2.2.
2. A total of 4 out of 27 bacterial populations from the chicken manure were not represented in the TMT community fingerprint. This is discussed further in Section 6.6.4.2.3.
3. A total of 8 out of 28 bacterial populations from the oil refinery sludge were not represented in the TMT community fingerprint. This is discussed further in Section 6.6.4.2.4.

6.6.4.2.2 Bacterial Populations from Chicken Manure

It is evident from Figure 6.15 that a total of 7 bacterial populations (labelled 1 to 7) of chicken manure origin only are represented in the TMT community fingerprint. Of these 7 populations (labelled 1 to 7), populations 2 and 6 are represented by sequenced DGGE bands, and are discussed as follows.

Population 2 is represented by band 31 and belongs to *Rhodococcus* (*Actinobacteria*) which is reported by Alexander (1999), Irvine et al (2000), Heiss-Blanquet et al (2005), and Behki 1994) to have hydrocarbon degrading abilities. Population 6 is represented by band 18 and belongs to *Dietzia* (*Actinobacteria*). A link between this bacterium and hydrocarbon degradation is not reported in the literature (to the author's knowledge) but can be inferred from the research areas recorded for this sequence match. These populations show an increase in relative abundance between

Days 16 and 32, which may reflect a) growth of these populations (possible due to improving environmental conditions, increased substrate(s), increased bioavailability of substrate(s), or reduced toxins), and/or b) a simultaneous decline of another population. There is no evidence for the simultaneous decline of another population, therefore the author proposes that the increased relative abundance of populations 2 and 6 largely reflects growth of these population, although this cannot be confirmed by these data. These data therefore indicate a bioaugmentation effect resulting from the amendment of oil refinery sludge with chicken manure.

In addition to populations 2 and 6, population 3 also shows an increase in relative abundance between Days 16 and 32. The author proposes that this largely reflects growth of this population (see discussion for populations 2 and 6 above). Population 3 is not represented by a sequenced DGGE band, therefore its identity is unknown. There is a possibility that this population is non-hydrocarbon degrading, and therefore may be growing at the expense of hydrocarbon degrading populations through adverse competition for growth factors. However, it can be argued that this population grows alongside populations 2 and 6, therefore may have the same metabolic requirements, which could include a particular TPH compound. It may therefore be reasonable to conclude that population 3 also possesses hydrocarbon degrading abilities. Nevertheless, should this bacterium be non-hydrocarbon degrading, the author proposes that any risk to TPH biodegradation posed is minimal as a) in the author's opinion, population 3 is of low relative abundance therefore constituting a small percentage of the total community, and b) other populations which show sequence similarity with known hydrocarbon degraders, show increases in relative abundance over this time period (for example populations 2 and 6).

Populations 1, 4, 5 and 7 are not represented by sequenced DGGE bands, therefore their identity is unknown. Should these populations be of non-hydrocarbon degrading bacteria, there is a risk to TPH biodegradation through adverse competition with hydrocarbon degrading populations. Such risk is discussed as follows:

1. Population 1 is of low relative abundance and therefore likely constitutes a small percentage of the total bacterial community. This population shows a reduction in

relative abundance between Days 32 and 64, beyond which it is not visible in the TMT community fingerprint. Such a reduction in relative abundance may reflect a) a decline of this population (possibly due to worsening environmental conditions, loss of substrate(s), lowering bioavailability of substrate(s), or increase in toxins), and/or b) a simultaneous growth of another population. There is no evidence for the latter scenario, which would indicate a decline of population 1. However, as discussed for populations 3 and 4 in Section 6.6.4.1.2, this cannot be confirmed by these data. The author proposes that any risk posed to TPH biodegradation by this population would be minimal as a) this population is of low relative abundance and therefore constitutes a minor percentage of the total community, thus utilisation of growth factors would also be low, and b) there are other 'hydrocarbon-degrading' populations represented in the TMT community fingerprint which are of high relative abundance and show signs of growth, as discussed for population 3 above.

2. Populations 4, 5 and 7 show no change in relative abundance over the duration of the treatability study and are therefore assumed by the author to be non-growing. Although these data cannot be used to indicate activity of these populations, the author proposes that any use of growth factors would be to sustain viability and not for growth. Populations 4 and 7 are of low relative abundance and therefore likely constitute a very small percentage of the total community. Therefore, the author proposes that any use of growth factors by these populations (and therefore adverse competition with hydrocarbon degrading populations) would be minimal. Population 5 is of moderate relative abundance thus may use more growth factors to sustain viability than populations 4 and 7. However, the author proposes that even population 5 presents minimal risk to TPH biodegradation as some hydrocarbon degrading populations are seen to have higher relative abundance and show signs of growth, as discussed for population 3 above.

It can be seen from the baseline fingerprint of the chicken manure samples presented in Figure 6.15 that a total of 4 bacterial populations indigenous to the chicken manure were not represented in the TMT community fingerprint. These populations are not represented by sequenced DGGE bands, therefore their identity is unknown. Their

absence from the TMT community fingerprint indicates that they constitute a minor percentage of the total community, and did not grow to a sufficient cell number to permit their detection on the TMT community fingerprint. However, their activity cannot be indicated from these data. Nevertheless, should these populations represent non-hydrocarbon degrading bacteria, the author proposes that they pose no, or very little, threat to hydrocarbon degrading bacterial populations, and therefore TPH biodegradation.

6.6.4.2.3 Bacterial Populations from Oil Refinery Sludge

It is evident from Figure 6.16 that a total of 4 bacterial populations (labelled I to IV) of oil refinery sludge origin only are represented in the TMT community fingerprint. These populations are discussed below.

Population I is represented by band 8 and belongs to *Desulfotomaculum* (*Clostridia*). A link between this bacterium and hydrocarbon biodegradation is not reported in the literature (to the author's knowledge) but can be inferred from the research area recorded for this sequence match. Population I shows no change in relative abundance over time under the TMT condition, and therefore is assumed by the author to be non-growing. However, these data cannot be used to indicate activity, or importance to hydrocarbon biodegradation. Population I was also found to be non-growing under the UMC and MC conditions. The author proposes that the addition of chicken manure to the oil refinery sludge did not stimulate the growth of this population.

Populations II and III show an increase in relative abundance between Days 1 and 2. Population II is represented by bands 6, 32 and 35 and belongs to *Hydrocarboniphaga effusa* (*Gamma Proteobacteria*). This bacterium has been identified by Palleroni et al (2004) as being able to degrade aliphatic hydrocarbons from C_9 to C_{19}. Population III is represented by bands 7 and 34 and is assumed to belong to *Syntrophus* (*Delta Proteobacteria*) based on band 7. A link between *Syntrophus* and hydrocarbon biodegradation is reported by Dojka et al (1998) and can also be inferred from the research area recorded for this sequence match. The

increase in relative abundance between Days 1 and 2 for these populations could reflect a) population growth, and/or b) a simultaneous decline of another population. There is no evidence for the simultaneous decline of another population, therefore indicating that the increase in relative abundance reflects population growth. Under the UMC and MC conditions, population II shows an increase in relative abundance between Days 16 and 32, and between Days 8 and 16 respectively. This indicates that the addition of chicken manure stimulated the earlier growth of this population. Population III showed no growth under the UMC condition, but an increase in relative abundance between Days 2 and 8 under the MC condition. This increase under the MC condition was concluded to, at least in part, reflect the simultaneous decline of another population (labelled 6 on Figure 6.14). Therefore it appears that the addition of chicken manure may have stimulated the growth of this population above that of the pile management strategy.

Populations II and III show a reduction in relative abundance between Days 32 and 64. This could reflect a) a decline of these populations, and/or b) a simultaneous growth of another population. There is no evidence for the latter scenario, however these data cannot be used to confirm a decline in cell number, as discussed in Section 6.6.4.2.2 for population 1.

Population IV is not represented by sequenced DGGE bands, therefore its identity is unknown. This population showed an increase in relative abundance between Days 8 and 16, and is of high relative abundance, therefore is assumed to constitute a high percentage of the total community. A simultaneous decline in population K is seen to occur. However, given the low relative abundance of population K in relation to that of population IV, the author proposes that the increase in relative abundance of population IV largely reflects growth. This population shows no growth under the UMC and MC conditions. These data therefore indicate that the addition of chicken manure stimulated the growth of this population. There is a chance that this population is non hydrocarbon degrading, therefore may present a risk to TPH biodegradation under the TMT condition, indicating an adverse effect of the addition of chicken manure.

It can be seen from the baseline fingerprint for the oil refinery sludge samples that a total of 8 bacterial populations indigenous to the oil refinery sludge were not represented in the TMT community fingerprint. These populations are not represented by sequenced DGGE bands, therefore their identity is unknown. All but 2 of these populations were also unrepresented in the UMC and MC community fingerprints. Their absence from the TMT community fingerprint indicates that they constitute a minor percentage of the total community and did not grow to a sufficient cell number to permit their detection on the TMT community fingerprint. However, these data cannot be used to indicate activity of these populations, or their importance to TPH biodegradation. The two populations which are represented under the UMC and MC conditions are labelled with a square symbol and a triangle symbol on Figure 6.15 and Figure 6.16. The population labelled with a square symbol showed no growth under the UMC and MC conditions and was of low relative abundance. The population labelled with a triangle symbol showed no growth under the UMC condition but was of high relative abundance. Under the MC condition, this population showed a reduction in relative abundance, which was attributed to the simultaneous growth of other populations, thus also concluded to show no growth under the MC condition. Therefore the author proposes that the 'loss' of these populations from the TMT community fingerprint unlikely represents a negative effect of the addition of chicken manure, although this cannot be confirmed by these data.

6.6.4.2.4 Common Bacterial Populations

It can be seen from Figure 6.16 that the 16 bacterial populations identified in Figure 6.12 as common to the chicken manure and oil refinery sludge samples are all represented in the TMT community fingerprint (labelled A to P). These common bacterial populations are discussed in this section. Reference is made to sequenced DGGE bands; the locations of these bands are shown in Figure 6.14, and the BLAST sequence results are presented in Table 6.5.

Populations B and D show no change in relative abundance over time under the TMT condition. The author therefore assumes that these populations are non-growing,

although their activity/importance to TPH biodegradation cannot be indicated by these data. These populations are discussed as follows:-

1. Population B is not represented by sequenced DGGE bands, therefore its identity is unknown. Under the UMC and MC conditions, this population also showed no growth. Therefore these data indicate that the addition of chicken manure did not stimulate the growth of this population.
2. Population D is represented by band 24 and belongs to *Bacteroidetes*, which is reported by Kaplan and Kitts (2004) to be associated with the degradation of petroleum hydrocarbons. This population also showed no growth under the UMC and MC conditions. These data indicate that the addition of chicken manure did not stimulate the growth of this population.

Populations G, H, N and P show an increase in relative abundance between Days 1 and 2. This does not coincide with the simultaneous decline of other populations, therefore the author proposes that the increase most likely reflects population growth. These populations are discussed as follows:-

1. Population G is represented by bands 10 and 13 and belong to *Desulfotomaculum* (*Clostridia*). A link between this bacterium and hydrocarbon biodegradation can be inferred from the research areas recorded for these sequence matches. This population showed an increase in relative abundance between Days 16 and 32 under the UMC condition, and between Days 8 and 16 under the MC condition. These data indicate that the addition of chicken manure stimulated the earlier growth of this population.
2. Population H is not represented by sequenced DGGE bands, therefore its identity is unknown. This population showed no growth under the UMC and MC conditions. These data indicate that the addition of chicken manure stimulated the growth of this population. Although the population is common to the oil refinery sludge and chicken manure samples, and may therefore be assumed to be hydrocarbon degrading, there is a possibility that this population is non hydrocarbon degrading. Therefore this population potentially presents a risk to TPH biodegradation through adverse competition for growth factors. However, the

author proposes that there is no evidence of this as a) no populations show a reduction in relative abundance between Days 1 and 2, and b) hydrocarbon degrading populations are seen to grow during this time period.

3. Population N is not represented by sequenced DGGE bands, therefore it identity is unknown. This population showed an increase in relative abundance between Days 16 and 32 under the UMC and MC conditions. These data indicate that the addition of chicken manure stimulated the earlier growth of this population. However, as with population H above, there is a chance that this population is non hydrocarbon degrading and therefore potentially presents a risk to TPH biodegradation. However, the author proposes that such risk is minimal, as for population H above.
4. Population P is represented by bands 19 and 20 but cannot be identified. This population showed no growth under the UMC condition, and an increase in relative abundance between Days 8 and 16 under the MC condition. These data indicate that the addition of chicken manure stimulated the earlier growth of this population. The same comments regarding potential risk to TPH biodegradation posed by populations H and N above also apply to population P.

Populations I (i) and L show an increase in relative abundance between Days 8 and 16. This does not coincide with the simultaneous decline of another population, therefore the author proposes that it reflects population growth. These populations are discussed as follows:-

1. Population I is represented by bands 25 and 11 and belongs to *Ectothiorhodospiraceae* (*Gamma Proteobacteria*). A link between this bacterium and hydrocarbon biodegradation is not reported in the literature (to the author's knowledge) but can be inferred from the research areas recorded for these sequence matches. This population showed a reduction in relative abundance between Days 32 and 64 under the UMC condition, and between Days 16 and 32 under the MC condition. These data indicate that the growth of this population was stimulated by the addition of chicken manure.
2. Population L is represented by band 15 but cannot be identified. Its likely presence in chicken manure is confirmed by the research area recorded for these sequence

matches. Although this population is identified as being common to the chicken manure and oil refinery sludge, it is not represented under the UMC and MC conditions. These data indicate that the addition of chicken manure enhanced the growth of this population. The same comments regarding risk to TPH biodegradation posed by populations H, N and P above apply to population L.

Population M showed an increase in relative abundance between Days 16 and 32 under the TMT condition. This does not coincide with the decline of another population, therefore the author proposes that the increase most likely reflects population growth. Population M is not represented by sequenced DGGE bands, therefore its identity is unknown. This population also showed an increase in relative abundance between Days 16 and 32 under the UMC and MC conditions. Therefore the author proposes that this population is able to utilise hydrocarbons as TPH compounds likely presented the sole source of carbon in the oil refinery sludge. These data indicate that the growth of population M was not stimulated by the addition of chicken manure.

Populations F, J, K and O show a reduction in relative abundance over time under the TMT condition. The reduction seen coincides with a simultaneous growth of populations I (i) for population J, IV for population K, and L, I and A for population O. Therefore the author proposes that the reduction reflects, at least in part, changes in abundance of other populations. For population F, however, there is no evidence of simultaneous growth of another population to account for the reduction seen. However, as explained in Section 6.6.4.2.3, these data cannot be used to confirm that such a reduction reflects a decline of this population. These populations are discussed as follows:-

1. Population F is not represented by a sequenced DGGE band, therefore its identity is unknown. This population showed no growth under the UMC and MC conditions. These data indicate that the addition of chicken manure did not stimulate the growth of this population.
2. Population J is represented by band 37 and belongs to *Syntrophus* (*Delta Proteobacteria*). A link between this bacterium and hydrocarbon degradation is

reported by Dojka et al (1998) and can also be inferred from the research area recorded for this sequence match. This population showed no growth under the UMC condition, but showed a gradual decline from Day 2 under the MC condition. It could be argued that the addition of chicken manure enabled this population to maintain its viability for longer, however the activity/importance of this population cannot be indicated by these data.

3. Population K is represented by band 38 but cannot be identified. The research area recorded for this sequence match indicates an ability to degrade hydrocarbons. This population showed an increase in relative abundance between Days 1 and 8 under the UMC and MC conditions. It could therefore be argued that the addition of chicken manure had a detrimental effect on this population. However, it can be seen in Figure 6.16 that the reduction of this population coincides with an increase in relative abundance of population IV, thus may not reflect the decline of this population.
4. Population O is not represented by sequenced DGGE bands, therefore its identity is unknown. This population showed no growth under the UMC and MC conditions. Should this population have been active under the UMC and MC conditions, it could be argued that the addition of chicken manure had a detrimental effect on this population. However, the reduction in relative abundance of population O under the TMT condition coincides with the significant increase in relative abundance of populations A, I and L. The author therefore proposes that this population most unlikely declined under the TMT condition, therefore negating any potential adverse effect of the addition of chicken manure.

Populations A, C and E showed an increase in relative abundance over time under the TMT condition. These populations are discussed as follows:-

1. Population A is represented by bands 9 and 12 and belongs to *Alcanivorax* (*Gamma Proteobacteria*). This bacterium is reported by Röling et al (2002), Syutsubo et al (2001), Heiss-Blanquet et al (2005), and Liu and Shao (2005) as being able to degrade hydrocarbons. This population showed an increase in relative abundance under the TMT condition between Days 2 and 8. This increase does not coincide with a simultaneous decline of another population therefore the

author proposes that it reflects population growth. Under the UMC and MC conditions, this population showed no growth. These data therefore indicate that the addition of chicken manure stimulated the growth of this population.

2. Population C is represented by bands 14, 23, 4 and 39, and is assumed to belong to *Pseudomonas* (*Gamma Proteobacteria*) based on band 4. *Pseudomonas* is reported by numerous authors including Ijah and Antai (2003), and Kaplan and Kitts (2004) as being able to degrade hydrocarbons. This population showed an increase in relative abundance between Days 1 and 2 under the TMT condition, which likely reflects population growth as it does not coincide with the simultaneous decline of another population. Under the UMC and MC conditions, this population showed no growth. These data indicate that the addition of chicken manure stimulated the growth of this population.
3. Population E is represented by bands 33 and 36 and belongs to *Marinobacter* (*Gamma Proteobacteria*). A link between this bacterium and hydrocarbon biodegradation can be inferred from the research area recorded for these sequence matches. This population showed an increase in relative abundance between Days 1 and 2 under the TMT condition, which likely reflects population growth as it does not coincide with the simultaneous decline of another population. Under the UMC condition, this population showed no growth, whereas under the MC condition it showed an increase in relative abundance between Days 2 and 8. This coincided with the reduction of another population thus it may be argued that population E did not show any growth under the MC condition either. These data indicate that the addition of chicken manure stimulated the earlier growth of this population.

6.6.4.2.5 Summary

The effects of the addition of chicken manure to oil refinery sludge on total bacterial community dynamics are summarised as follows:-

1. Not all bacterial populations identified in the baseline community fingerprints for the chicken manure and oil refinery sludge samples were represented in the TMT community fingerprint. It is proposed that this likely reflects the efficiency of the

DGGE gel in that only populations constituting greater than 1% of the total community are represented. The remaining bacterial populations are most likely still present in the TMT material, however their activity or importance to TPH biodegradation in unknown.

2. A total of 4 bacterial populations indigenous to the chicken manure samples were not represented in the TMT community fingerprint. The identity of these populations is unknown.
3. A total of 8 bacterial populations indigenous to the oil refinery sludge were not represented in the TMT community fingerprint. The identity of these populations is unknown.
4. Potential positive effects of the addition of chicken manure have been indicated, as follows:-
   a. Two bacterial populations, *Rhodococcus* and *Dietzia*, have been identified in the TMT community fingerprint and are of chicken manure origin only. *Rhodococcus* is reported by Alexander (1999), Irvine et al (2000), Heiss-Blanquet et al (2005), and Behki (1994) as being able to degrade hydrocarbons. A link between *Dietzia* and hydrocarbon degradation can be inferred from the research area recorded for this bacterium. These populations showed growth between Days 16 and 32 under the TMT condition, and indicate bioaugmentation effects resulting from the addition of chicken manure.
   b. The addition of chicken manure stimulated the earlier growth of several bacterial populations when compared with the UMC and MC conditions. These populations include *Desulfotomaculum*, *Ectothiorhodospiraceae*, *Alcanivorax*, *Pseudomonas*, *Marinobacter*, *Hydrocarboniphaga effusa*, and *Syntrophus*. A link between these bacteria and hydrocarbon degradation has been inferred through either reports in the literature or from the research areas recorded in Table 6.5.
5. Potential negative effects of the addition of chicken manure have also been indicated, as follows:-
   a. 5 bacterial populations of chicken manure origin only were represented in the TMT community fingerprint, and are of unknown identity. These populations could be non-hydrocarbon degrading and therefore present a risk to TPH biodegradation through adverse competition with hydrocarbon degrading

bacterial populations for growth factors. However, the author proposes that should this be the case, such risk is minimal as these populations were either a) of low relative abundance therefore likely constitute a minor percentage of the total community, or b) non growing or declining thus unlikely to be utilising vast quantities of growth factors, and c) hydrocarbon degrading populations still showed signs of growth, indicating that growth factors were still available.

b. 8 bacterial populations of oil refinery sludge origin only were not represented in the TMT community fingerprint. There is a chance that these populations are hydrocarbon degrading, therefore their lack of presence in the TMT community fingerprint may be of detriment to TPH biodegradation. However, only 2 of these populations were identified in the UMC and MC community fingerprints, and these showed either no growth or a decline, therefore their importance to TPH biodegradation is potentially low (although this cannot be confirmed by these data). The lack of presence of the remaining 6 populations under the UMC and MC conditions also indicates that these populations are of minimal importance to TPH biodegradation. Therefore the potential risk to TPH biodegradation under the TMT condition posed by the lack of these populations is proposed by the author to be minimal.
c. Population IV showed growth between Days 8 and 16 under the TMT condition, compared with no growth under the UMC and MC conditions. This population is un-identified thus there is a possibility that it is a non hydrocarbon degrading bacterium. Its growth under the TMT condition may therefore pose a risk to TPH biodegradation through adverse competition for growth factors. However, such growth did not inhibit the growth of hydrocarbon degrading populations, indicating that such risk is minimal. However, it could be that this population proliferated at the expense of the 8 populations discussed in point b above.
d. Population K is indicated to be hydrocarbon degrading, however it showed signs of population decline under the TMT condition compared with growth under the UMC and MC conditions. Therefore this could indicate that the addition of chicken manure had a detrimental effect on this population, and potentially on TPH biodegradation. However, it was concluded that the

reduction in relative abundance of this population most likely reflected the simultaneous growth of another population rather than its decline.

6.7 Conclusions

The following conclusions have been made and are based on the data presented in this chapter only.

1. The chicken manure samples had a diversity of 27 bacterial populations, and the oil refinery sludge samples had a diversity of 28 bacterial populations.
2. The chicken manure samples possessed bacterial populations with potential hydrocarbon degrading abilities, including *Gamma Proteobacteria* (*Ectothiorhodospiraceae*, *Hydrocarboniphaga effusa*, *Pseudomonas* and *Marinobacter*) and *Actinobacteria* (*Rhodococcus*). These data indicate that there is a potential for the use of chicken manure to enhance TPH biodegradation through bioaugmentation.
3. The chicken manure also possessed numerous bacterial populations of unknown identity, which were potentially non hydrocarbon degrading bacteria. These populations therefore pose a threat to TPH biodegradation through adverse competition with hydrocarbon degrading bacterial populations for growth factors.
4. The oil refinery sludge samples possessed bacterial populations with potential hydrocarbon degrading abilities. These bacteria included *Gamma Proteobacteria* (*Ectothiorhodospiraceae*, *Hydrocarboniphaga effusa*, *Pseudomonas, Syntrophus* and *Marinobacter*). These findings indicate that the oil refinery sludge already possessed bacterial consortia suitable for the biodegradation of hydrocarbons.
5. The oil refinery sludge also possessed numerous bacterial populations of unknown identity, which were potentially non hydrocarbon degrading bacteria. These populations therefore pose a threat to TPH biodegradation through adverse competition with hydrocarbon degrading bacterial populations for growth factors.
6. The bacterial community dynamics data indicated that the pile management strategy enhanced TPH biodegradation as it encouraged growth of two populations which did not show signs of growth under the UMC condition. The identity of these populations is unknown, however it is proposed that they are able

to utilise hydrocarbons as the TPH compounds likely presented the sole source of carbon in the oil refinery sludge. The pile management strategy also encouraged the earlier growth of 3 populations when compared with the UMC condition. These populations included *Hydrocarboniphaga effusa* (*Gamma Proteobacteria*) which is a known hydrocarbon degrader.

7. The bacterial community dynamics data indicated that the addition of chicken manure enhanced TPH biodegradation as a) *Rhodococcus* and *Dietzia* were introduced, and showed signs of growth under the TMT condition, and b) several bacterial populations showed earlier growth under the TMT condition than under the UMC and MC conditions, including hydrocarbon degrading *Desulfotomaculum*, *Ectothiorhodospiraceae*, *Alcanivorax*, *Pseudomonas*, *Marinobacter*, *Hydrocarboniphaga effusa*, and *Syntrophus*.
8. The bacterial community dynamics data also indicate that the addition of chicken manure was potentially detrimental to TPH biodegradation as a) bacterial populations of chicken manure origin were represented in the TMT community fingerprint, and are potentially non hydrocarbon degrading therefore present a risk of adverse competition with hydrocarbon degrading populations for growth factors, b) it encouraged the growth of populations which are potentially non hydrocarbon degrading therefore present a risk of adverse competition with hydrocarbon degrading populations for growth factors, c) it resulted in the decline of a potentially hydrocarbon degrading population, and d) 8 populations of oil refinery sludge origin and therefore potentially hydrocarbon degrading were unrepresented in the TMT community fingerprint. However, for all of these cases the author has been able to propose that the risk to TPH biodegradation posed is minimal. Therefore, in the opinion of the author, this indicates that the addition of chicken manure to oil refinery sludge likely had a stronger positive effect on TPH biodegradation than it did a negative effect.

Chapter 7

Toxicity Analyses

7.1 Introduction

Toxicology is concerned with the movement of toxic compounds in the environment and the effect of such toxicants on individuals and populations (Hodgson and Levi, 1997). As stated by Paracelsus (1493-1541), "all substances are poisons (toxicants); there is none which is not a poison. The right dose differentiates a poison and a remedy" (Timbrell, 2002). This quote recognises the well accepted 'dose-response' relationship which governs toxicity (Timbrell, 2002; Diaz-Baez and Dutka, 2005).

One of the key concerns in this study, regarding the addition of chicken manure to petroleum hydrocarbon contaminated soils (in this study, oil refinery sludge), is the potential introduction of toxic compounds (e.g. trace elements) which may have an adverse effect on hydrocarbon degrading microorganisms during the composting bioremediation process, as was discussed in Chapter 2. Toxicity analyses were therefore undertaken as a screening tool to identify the potential for this risk occurring.

Of additional interest to this study is the potential use of toxicity analyses for determining the success of bioremediation strategies in reducing the risk posed by contaminated soils to human health and the environment. It is widely accepted that chemical analyses alone are insufficient to demonstrate risk (or changes in risk during bioremediation works) associated with the toxicity of contaminants (Plaza et al, 2005; Paton, 2001; Loehr, 1996; Turner et al, 2001; Tiensing et al, 2001; Bundy et al, 2004). Risk is deemed to be associated with that portion of a contaminant (or mixture of contaminants) that is bioavailable (Loehr, 1996; Fent, 2003; Stroo et al, 2000). Measurements of the bioavailable portion of a contaminant using conventional chemical analytical methods are unattainable due to the harshness of chemical extraction steps adopted (Paton, 2001). Therefore there is increasing interest in the

use of toxicity tests for the assessment of contaminated land for supporting management decisions for remediation of such land (Plaza et al, 2005; Paton, 2001; Turner et al, 2003; Reid et al, 2000).

Toxicity tests involve the exposure of organisms to potentially toxic compounds under defined experimental conditions, and the assessment of biological end points such as mortality or changes in growth (Paton, 2001). Toxicity tests use test species ranging from bacteria to fish, rats, and plants (Loehr, 1996; Timbrell, 2002; Plaza et al, 2005; Paton, 2001). These tests are subdivided into *in vivo* (whereby target compounds are administered to test species such as rats) and *in vitro* (whereby microbial cells, cellular components such as DNA, or single celled organisms are exposed to the target compound) (Timbrell, 2002).

There has been increasing pressure to reduce the use of live animals in medical research (Timbrell, 2002), therefore the use of *in vitro* toxicity tests in the capacity of a screening tool during bioremediation investigations is deemed to be more acceptable. In this capacity, any test system will be appropriate providing it is sufficiently sensitive to the compounds present in the sample (Johnson et al, 2005). In this study, two *in vitro* toxicity tests were used, known as ROTAS (Rapid On-Site Toxicity Audit System) from Cybersense Ltd (UK), and the Plasmid Assay, to test changes in relative toxicity between samples collected during the treatability studies.

This chapter details the aims and objectives, sampling programme and methodology adopted for these analyses; the results obtained are presented and are discussed here. Further discussion of these results is presented in Chapter 8.

7.2 Aims and Objectives

As stated in Chapter 1 and 3, Objective Four, Part 3 of this study is as follows:

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 3)*

toxicological dynamics, resulting from the addition of chicken manure to TPH contaminated material.

This objective is investigated through the use of acute *in vitro* toxicity analyses to a) determine any potential adverse effect on TPH biodegradation caused by the addition of chicken manure, and therefore potential toxic compounds, and b) determine the success/failure of composting bioremediation in reducing the toxicity (and therefore risk) of the oil refinery sludge.

7.3 Sampling Programme

In order to attain a toxicological profile over the duration of the treatability studies, with consideration to existing financial and time commitments to the chemical and biological analyses, solid samples were taken on Days 1, 32, 64, and 90. These samples were of approximately 0.5kg (wet weight) taken from a 3kg composite bulk sample which was homogenised by hand using a sterile stainless steel scoop.

Toxicity was measured for total leachable compounds using the ROTAS and Plasmid Assay systems, and organic compounds extracted from the solid samples using the ROTAS system. Samples for these analyses were prepared as outlined in the following sections. The toxicity of total leachable compounds (i.e. bioavailable compounds from the oil refinery sludge and chicken manure) was measured to determine changes in toxicity over the duration of the treatability study, and potential toxic effects of the addition of chicken manure. A separate extract of organic compounds was also generated as it was anticipated by the author that the TPH compounds would likely have low solubility and therefore unlikely be leachable in water, thus sensitivity to changes in their toxicity by the leachable test suite may be low. The use of an organic solvent (in this study, methanol) enables extraction of more TPH compounds, thus provides a basis for a more sensitive assessment of reduction in toxicity owing to the bioremediation process.

7.3.1 Leachate Extraction Method

Dry matter content ratio (Equation 7.1) and moisture content ratio (Equation 7.2) were determined on test portions of each solid sample in accordance with British Standard 7755 Part 3.1 (1994b). Leachate was generated from the solid samples in accordance with British Standard 12457 Part 2 (2002). Using Equation 7.3, the wet weight of sample needed to give 95g dry residue was calculated and the corresponding sample weight placed in a 1 litre glass bottle. Using Equation 7.4 below, the volume of deionised water needed to give a 1:10 ratio of solid sample to water was calculated, and the corresponding volume of deionised water was added to the sample.

$$DR = \left(\frac{MD}{MW}\right) \times 100 \qquad \textit{Equation 7.1}$$

$$MC = \left(\frac{MW - MD}{MD}\right) \times 100 \qquad \textit{Equation 7.2}$$

$$MW = 100 \times \left(\frac{95}{DR}\right) \qquad \textit{Equation 7.3}$$

$$L = \left(10 - \left(\frac{MC}{100}\right) \times 95\right) \qquad \textit{Equation 7.4}$$

Where:

DR = dry matter content ratio of the test portion

MD = Dry mass of the test portion (kg)

MW = wet weight of sample to be tested

MC = Moisture content ratio (%)

95 = 95g dry weight of sample required for leachate test

L = volume of deionised water to be added (l) for leachate test

The 1 litre bottles containing the sample and deionised water were sealed and placed in an end-over-end tumbler for 24 hours (± 15 minutes) at room temperature (20 ± 2°C) at 8rpm (Figure 7.1). The bottles were completely filled to minimise any loss of

potentially toxic volatile compounds into headspace. The suspended solids were then allowed to settle for approximately 20 hours at room temperature (20 ±2°C), and the eluate was centrifuged at approximately 14,000xg for 45 minutes. The supernatant was then filtered over a 0.45µm cellulose nitrate membrane filter using a Sartorius filter system connected to a Charles Austen Dymax 30 Pump (Figure 7.2). The resulting leachate samples were refrigerated and used in the ROTAS leachable test, and Plasmid Assay as outlined in Sections 7.4 and 7.5.

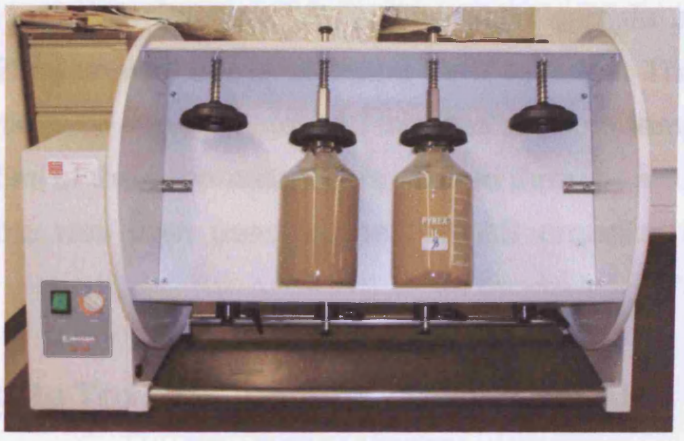


Figure 7.1: Leaching step; end-over-end tumbler.

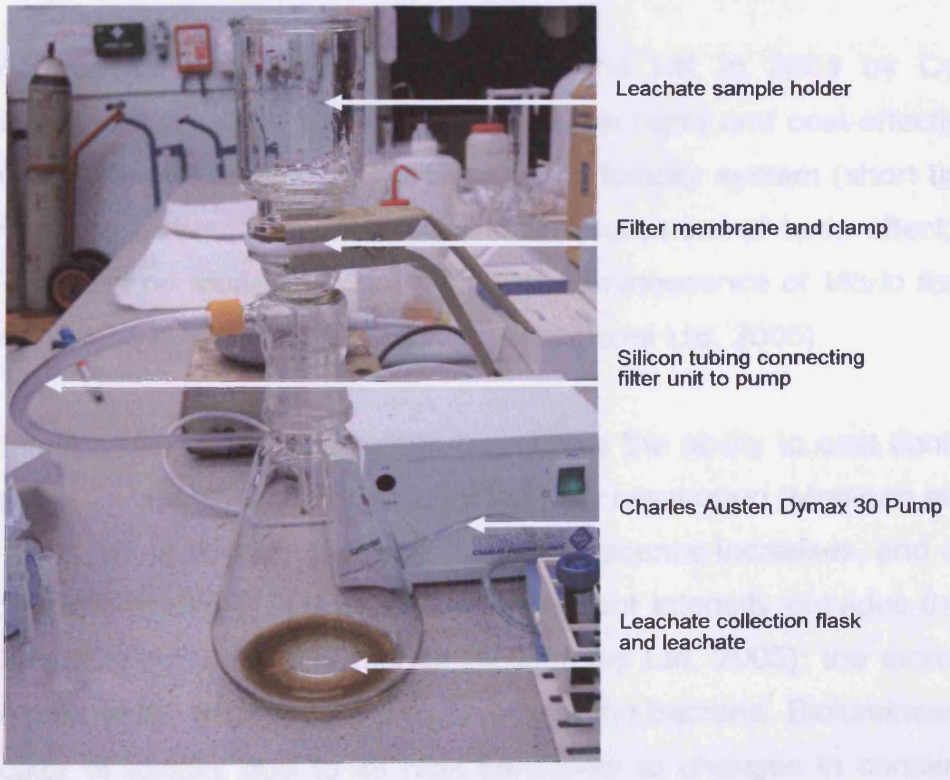


Figure 7.2: Filtering Step; Sartorius filter unit and pump.

A similar method of leachate generation from solid samples was adopted by Stroo et al (2000), whereby a 1:20 ratio of solid sample to water was applied and the slurry was shaken for 16 hours prior to filtering of the leachate.

7.3.2 Organics Extraction Method

A ratio of 1:10 (solid sample to methanol) was also adopted for the organics extraction method. The wet weight of solid sample needed to give 2g dry residue was calculated using Equations 7.1 to 7.4 (Section 7.3.1). The solid sample and methanol were placed in a sterile 25ml universal and vortexed for 2 minutes. The suspended solids were allowed to settle over approximately 10 minutes at room temperature (20 ±2°C), and approximately 5ml of the supernatant was passed through a 0.45µm syringe filter. The resulting sample was then used in the ROTAS organics test as outlined in Section 7.4.

7.4 Rapid On-Site Toxicity Assay System (ROTAS)

7.4.1 Introduction

The ROTAS toxicity system was launched in the UK in 2004 by Cybersense Biosystems Ltd, and is a field based method for the rapid and cost-effective toxicity testing of environmental samples. This is an acute toxicity system (short time period between exposure to the potential toxin and measurement of toxic effect; Timbrell, 2002) and is based on measuring changes in bioluminescence of *Vibrio fischeri* as a measure of changes in toxicity (Cybersense Biosystems Ltd, 2005).

Vibrio fischeri is a marine bacterium that possesses the ability to emit light, owing to an enzyme called luciferase, as a by-product of their respiration (Madigan et al, 2003). As the activity of *Vibrio fischeri* increases, bioluminescence increases, and vice versa. This relationship between activity and bioluminescent intensity provides the basis of the ROTAS toxicity system (Cybersense Biosystems Ltd, 2005); the more toxic the sample, the greater the loss of light emission from the bacteria. Bioluminescence is a useful indicator of toxicity due to its high sensitivity to changes in concentration of

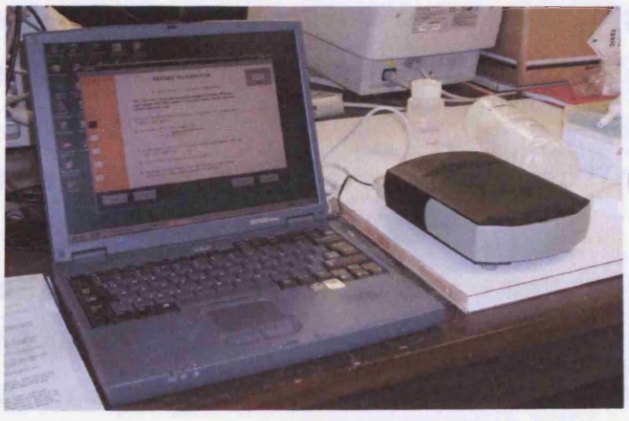
toxins, and the fact that it is a rare characteristic of bacteria thus interference from environmental samples is uncommon (Paton, 2001).

The use of bioluminescent *Vibrio fischeri* also forms the basis of Microtox®, which is another in-vitro toxicity test system (Reid et al, 2000; Turner et al, 2001). Microtox® has been used during previous bioremediation investigations such as the work of Ferguson et al (2003), Stroo et al (2000), Bundy et al (2004), and Phillips et al (2000), and showed good sensitivity to petroleum hydrocarbons. Microtox® was not chosen for this study as it was not available within Cardiff University, and its purchase was beyond the budget of this study.

The ROTAS system comprises a portable luminometer which measures light levels emitted from the *Vibrio fischeri* before and after addition of sample. The luminometer is linked to a computer (Figure 7.3) installed with proprietary software which guides the user through the ROTAS standard operating procedures, and records, analyses and reports the results (Cybersense Biosystems Ltd, 2005).

Two ROTAS test suites were used in this study, leachable and organics. The leachable test suite was used to determine the toxic effects of total leachable compounds, whereas the organics test suite was used to determine the toxic effects of total extractable organic compounds, as was discussed in Section 7.3. The methodology adopted for these test suites is outlined below.

a)



b)

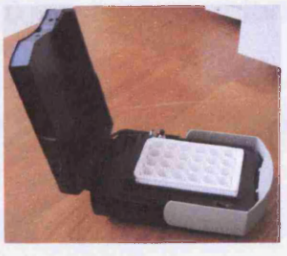


Figure 7.3: ROTAS Apparatus; *a) Luminometer attached to laptop with ROTAS software; b) Luminometer with multi-well test plate.*

7.4.2 Methodology

7.4.2.1 Sample Preparation

Although Cybersense Biosystems Ltd stipulate a protocol for sample preparation (provided in Appendix 6), in this study the samples were prepared as per Section 7.3. Sample to extractant (water for the leachable assay and methanol for the organics assay) ratio was based on the dry weight of a sample rather than wet weight as stipulated by Cybersense Biosystems Ltd. This strategy was adopted to enhance the accuracy of comparison between samples by means of accounting for moisture content and therefore any dilution effect this might have on the extracts generated. Also, a ratio of 1:10 sample to extraction was applied in this study as opposed to 1:5 stipulated by Cybersense Biosystems Ltd. This was to enable comparisons with the leachate samples tested using Plasmid Assay.

A total of 22 extract samples can be tested at any one time using the ROTAS system. For each sampling event, based on three repeats per treatment conditions, a total of 24 extract samples needed to be tested. In order to minimise costs and wastage of resources (in particular the *Vibrio fischeri*), for some treatment conditions only two repeats were undertaken.

7.4.2.2 Leachable Test Suite

The leachable test suite comprises a number of steps (protocol developed by Cybersense Biosystems Ltd; Appendix 6), as follows:

Step 1: Rehydrate freeze dried *Vibrio fischeri* with 27ml 0.9% sodium chloride, for 50 minutes.
Step 2: Add 1ml of the rehydrated bacteria solution to each well of the test plate, and calibrate for 3 minutes using the luminometer
Step 3: Add 1ml blank sample (deionised water) and 1ml control (10mg/L Copper II Sulphate) to Wells A and B of the multi-well test plate.
Step 4: Add 1ml leachate sample to remaining wells, and record locations, as prompted by the software.

Step 5: Run test for 15 minutes, and record pH using indicator paper if requested by the software. Save data file.

7.4.2.3 Organics Test Suite

The organics test suite comprises a number of steps (protocol developed by Cybersense Biosystems Ltd; Appendix 6), as follows:

Step 1: Rehydrate freeze dried *Vibrio fischeri* with 54ml 0.9% sodium chloride, for 50 minutes.
Step 2: Add 2ml of the rehydrated bacteria solution to each well of the test plate, and calibrate for 3 minutes using the luminometer.
Step 3: Add 50µl blank sample (pure methanol) and 50µl control (diesel/petrol mixture) to Wells A and B of the multi-well test plate.
Step 4: Add 50µl extract sample to remaining wells, and record locations.
Step 5: Run test for 15 minutes. Save data file.

7.4.3 Results

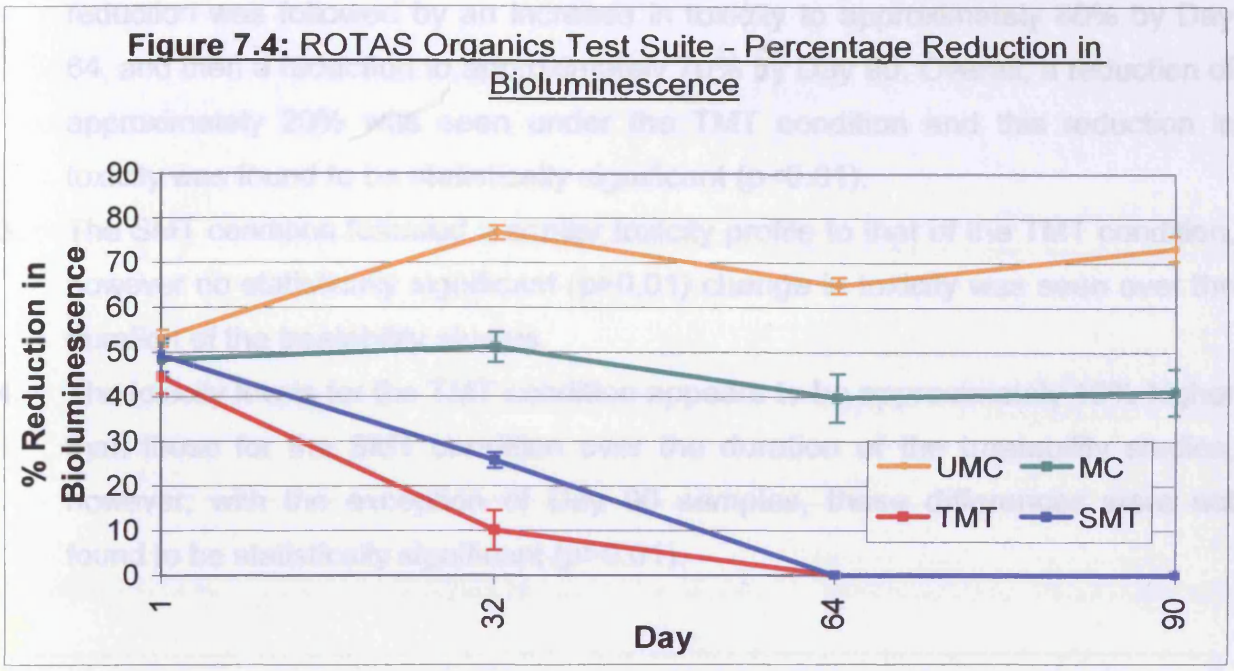
The results of the ROTAS organics and leachable test suites undertaken on samples from the four treatment conditions (un-managed sludge (UMC), managed sludge (MC), total manure treatment (TMT), and sterile manure treatment (SMT)) are presented in this section. All raw data are provided in Appendix 6. These results are discussed in Section 7.6. Statistical analyses were carried out to identify any significant differences between the conditions using two-tailed Z-test for two means in Microsoft Excel, and a significance level of 1% ($p<0.01$) in accordance with Schmuller (2005).

7.4.3.1 Organics Test Suite

The toxicity data obtained from the ROTAS organics test suite are presented in Figure 7.4. The values plotted are the mean of replicate samples (2 or 3 repeats as was outlined in Section 7.3.2.1) and the error bars show plus and minus one standard error (± 1SE). Observations are as follows:

1. Initial toxicity levels for the four conditions were approximately 40-55% reduction in bioluminescence. No statistically significant difference ($p>0.01$) in initial toxicity levels was recorded for these conditions.
2. UMC Condition - initial toxicity levels of 45-55% reduction in bioluminescence were recorded for Day 1 samples of this condition, but toxicity increased to between approximately 65-75% over the remainder of the treatability studies. This increase in toxicity between Days 1 and 90 was found to be statistically significant ($p<0.01$).
3. MC Condition – initial toxicity levels of 45-55% reduction in bioluminescence were recorded for Day 1 samples of this condition (similar to that of the UMC condition), followed by a gradual decline to approximately 40% reduction in bioluminescence by Day 90 of the treatability studies. This reduction in toxicity between Days 1 and 90 was not found to be statistically significant ($p>0.01$).
4. TMT Condition – initial toxicity levels of 40-50% reduction in bioluminescence were recorded for Day 1 samples of this condition. Toxicity levels then fell to approximately 10% by Day 32. By Day 64 no toxicity was recorded for this condition. This reduction in toxicity was found to be statistically significant ($p<0.01$).
5. SMT Condition - initial toxicity levels of 45-55% reduction in bioluminescence were recorded for Day 1 samples of this condition. Toxicity levels then fell to approximately 25% by Day 32. By Day 64 no toxicity was recorded for this condition. This reduction in toxicity was found to be statistically significant ($p<0.01$).
6. The highest toxicity levels were recorded for the UMC condition between Days 32 and 90, with statistically significant ($p<0.01$) differences in toxicity levels of approximately 25-30% higher than those recorded for the MC condition, 50-70% higher than those recorded for the SMT condition, and 65-70% higher than those recorded for the TMT condition.
7. Between Days 1 and 64, the lowest toxicity levels were recorded for the TMT condition, with statistically significant ($p<0.01$) differences in toxicity levels of up to 15% lower than those of the SMT condition. By Day 64, no toxicity was recorded for either of these conditions, with no statistically significant difference ($p>0.01$).

Figure 7.4: ROTAS Organics Test Suite - Percentage Reduction in Bioluminescence



7.4.3.2 Leachable Test Suite

A series of dilutions were made for Day 1 samples for the four pile conditions in order to determine any correlation between the ROTAS and Plasmid Assay data sets. However, as seen in Figure 7.5, the sensitivity of the ROTAS leachable assay to these samples is limited. For example samples of un-amended sludge (UMC and MC conditions) showed no variation in toxicity levels between dilutions, while the amended sludge (TMT and SMT conditions) showed a complete loss of toxic response by a dilution of 1 in 10. Therefore only neat leachate samples were tested on subsequent occasions.

The toxicity data obtained from the ROTAS leachable test suite are presented in Figure 7.6. The values plotted are the mean of replicate samples (2 or 3 repeats as was outlined in Section 7.3.2.1) and the error bars show plus and minus one standard error (± 1 SE). Observations are as follows:

1. There was no recordable toxic response for the UMC and MC conditions over the first 64 days. Day 90 samples were not run to lower costs of consumables.
2. The TMT condition showed a reduction in toxicity between Days 1 and 32 from approximately 90% (reduction in bioluminescence) to approximately 60%. This

reduction was followed by an increase in toxicity to approximately 80% by Day 64, and then a reduction to approximately 70% by Day 90. Overall, a reduction of approximately 20% was seen under the TMT condition and this reduction in toxicity was found to be statistically significant ($p<0.01$).

3. The SMT condition followed a similar toxicity profile to that of the TMT condition, however no statistically significant ($p>0.01$) change in toxicity was seen over the duration of the treatability studies.
4. The toxicity levels for the TMT condition appears to be approximately 10% higher than those for the SMT condition over the duration of the treatability studies, however, with the exception of Day 90 samples, these differences were not found to be statistically significant ($p>0.01$).

Figure 7.5 : ROTAS Leachable Sensitivity

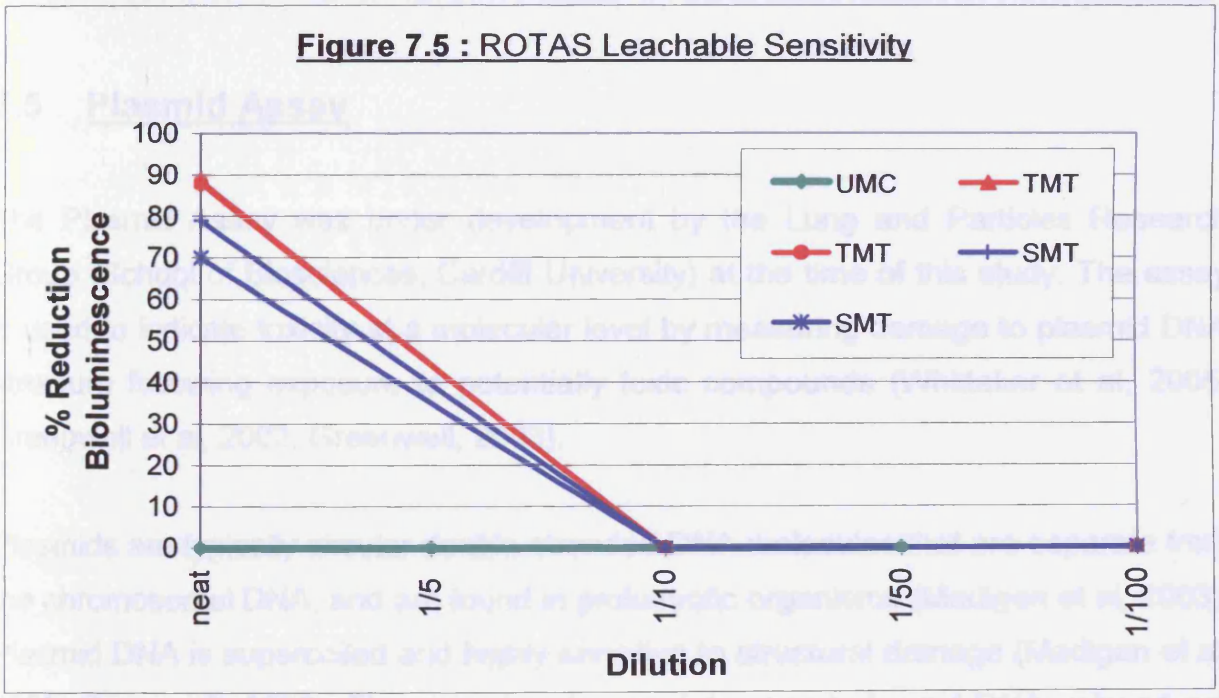
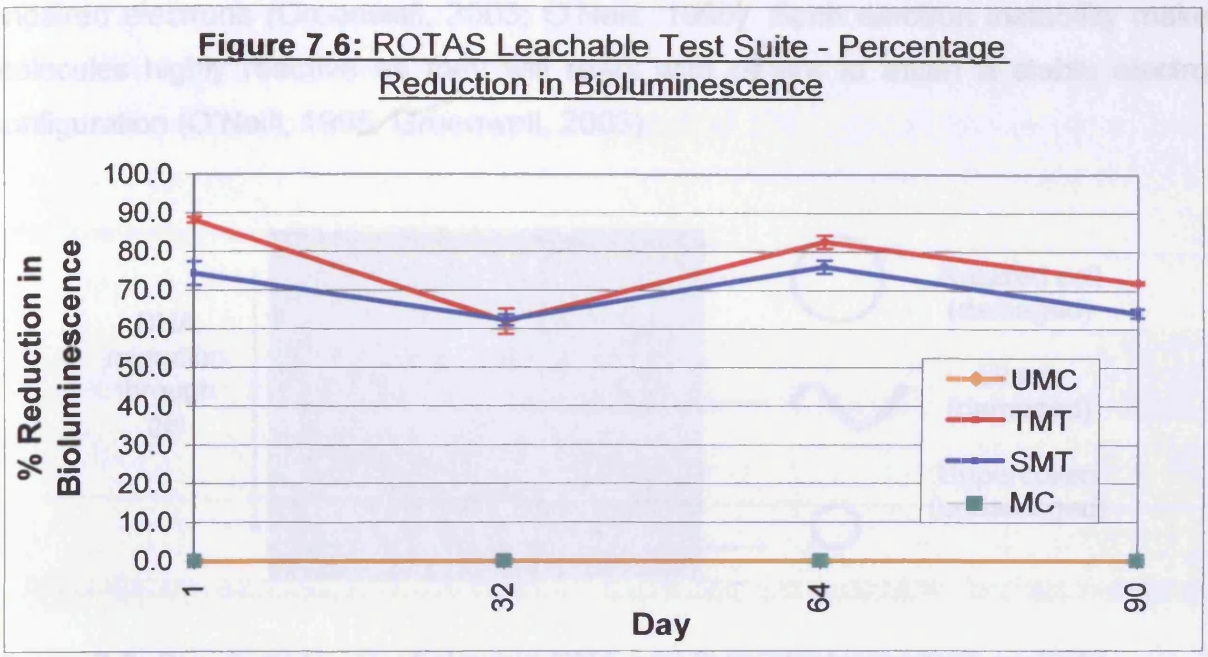


Figure 7.6: ROTAS Leachable Test Suite - Percentage Reduction in Bioluminescence



7.5 Plasmid Assay

The Plasmid Assay was under development by the Lung and Particles Research Group (School of Biosciences, Cardiff University) at the time of this study. The assay is used to indicate toxicity at a molecular level by measuring damage to plasmid DNA structure following exposure to potentially toxic compounds (Whittaker et al, 2005; Greenwell et al, 2002; Greenwell, 2003).

Plasmids are typically circular double stranded DNA molecules that are separate from the chromosomal DNA, and are found in prokaryotic organisms (Madigan et al, 2003). Plasmid DNA is supercoiled and highly sensitive to structural damage (Madigan et al, 2003; Greenwell, 2003). There are two forms of damaged plasmid DNA; relaxed coil (whereby the DNA is 'nicked' once) and linearised plasmid DNA (whereby further 'nicking' of the DNA breaks the coil) (Greenwell, 2003; Whittaker et al, 2004). The change in structure alters the electrophoretic mobility of the plasmid DNA (Figure 7.7), thus allowing separation by gel electrophoresis (as per Chapter 6) (Greenwell et al, 2002; Greenwell, 2003).

Structural damage to plasmid DNA is believed to be oxidative, and to be caused by free radicals (Greenwell, 2003). Free radicals are molecules that contain one or more

unpaired electrons (Greenwell, 2003; O'Neill, 1995). Such electron instability makes molecules highly reactive as they will react with others to attain a stable electron configuration (O'Neill, 1995; Greenwell, 2003).

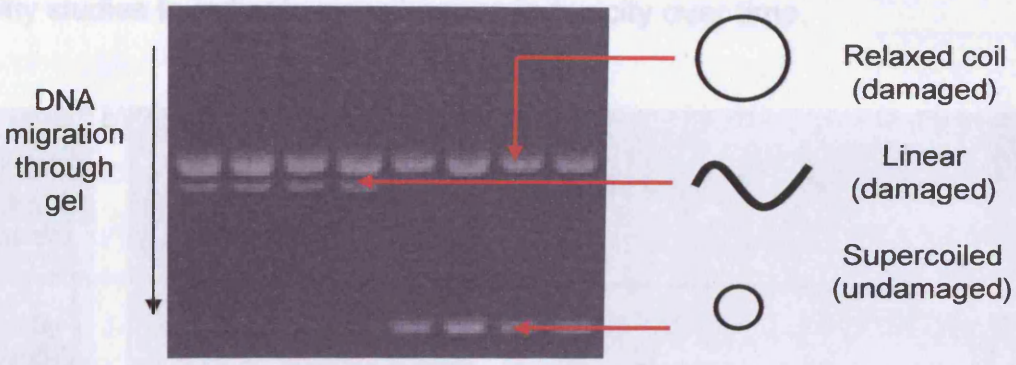


Figure 7.7: Structural forms of plasmid DNA and their electrophoretic mobility.

Oxidative damage to DNA commonly occurs at the bases (G, C, T, A), but also at the phosphate backbone, whereby bonds are broken, resulting in the process of 'nicking' (Greenwell, 2003). It is beyond the scope of this thesis to detail the processes by which free radicals attach to DNA and cause damage. The reader is referred to the work of Box et al (2001) for further detail.

7.5.1 Methodology

There are thousands of different types of plasmid known (Madigan et al, 2003). In this study, icosahedral bacteriophage ΦX174-RF (relaxed form) plasmid DNA (Promega, London, UK) was selected, based on the work of Greenwell (2003) whereby ΦX174-RF was found to be the optimum plasmid due to its size and sensitivity to oxidative damage. The Plasmid Assay was carried out as per the protocol summarised in Table 7.1, as advised by the Lung and Particles Research Group (School of Biosciences, Cardiff University) (K.BéruBé; pers.comm.). Equipment used for the Plasmid Assay is shown in Figure 7.8.

The electrophoresed gels (example shown in Figure 7.7) were imaged using Syngene Genesnap (Synoptics Ltd, Cambridge, UK), as per the molecular analyses detailed in Chapter 6. Syngene Genetools was used to perform densitometric quantification of

the bands. Percentage damage to the plasmid DNA was calculated for each sample (i.e. each lane), as per Equation 7.1 in Section 5.5.2.1. Percentage DNA damage was then plotted against leachate dilution. The dilution (*toxic dose*) necessary to cause 50% DNA damage (TD_{50}) was determined and monitored over the duration of the treatability studies to indicate any changes in toxicity over time.

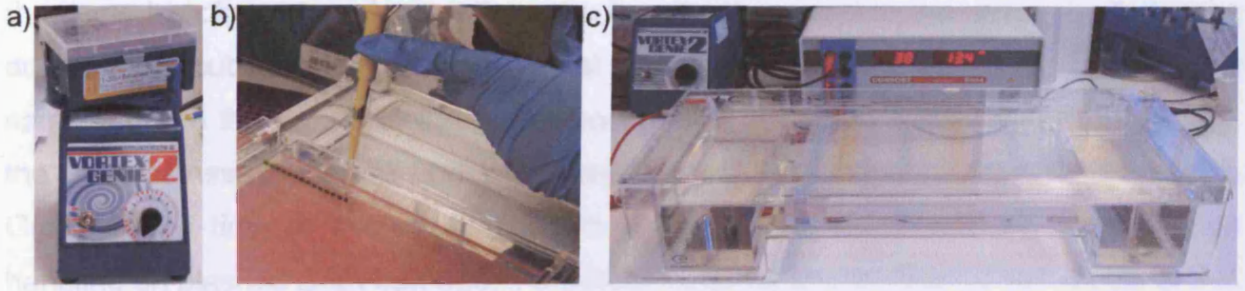


Figure 7.8: Plasmid Assay Apparatus; *a) vortex agitator; b) loading samples; c) gel electrophoresis tank with power pack and loaded gel.*

Table 7.1: Plasmid Assay Protocol (K.BéruBé; pers.comm.)

Step	Action
1	Prepare dilutions of leachate samples (i.e. neat, 1/10, 1/100, 1/1000).
2	Carefully pipette 1µl plasmid DNA (200µg ml^{-1}) into 0.2ml tubes. Store on ice.
3	Add 19µl leachate sample to the plasmid DNA (with 4 replicates of each dilution), ensuring DNA is submerged. Also make up 4 controls using HPLC grade deionised water instead of leachate sample.
4	Gently agitate the samples for 6 hours on a vortex agitator (Figure 5.5).
5	Prepare 0.6% agarose gel as per Section 6.4.6 (using 1xTBE instead of TAE buffer). Insert two 40 well combs, and allow to set for at least 2 hours at approximately 6°C. Fill electrophoresis tank with 1xTBE buffer.
6	Add 3.5µl 6 x Blue Orange Loading Dye (Promega, London, UK) to each sample and mix with a pipette before loading 20µl into the agarose gel wells (Figure 5.5). Connect electrophoresis tanks to power pack and run at 30 volts for 16 hours.
7	Remove the gel from the tank and image under UV light using Syngene Genesnap (Synoptics Ltd). Perform densitometric analysis using Syngene Genetools (Synoptics Ltd).

7.5.1.1 Assumptions

Due to the large number of leachate samples analysed using the Plasmid Assay, more than one batch of ΦX174-RF plasmid DNA was used. Comparison of toxicity data a) between samples, and b) over time therefore assumes the following:

1. Different batches of plasmid DNA have the same level of sensitivity to damage by toxic compounds.
2. Plasmid DNA damage due to handling was equal (i.e. shaking during incubation period, and even pipetting).

To minimise the effect of handling on plasmid DNA damage, all samples underwent the same handling procedure, with particular attention paid to the strength of shaking during the incubation period (the lowest possible shaking setting was used for all samples), and the length of the incubation period (i.e. 6hrs ± 5mins). Unfortunately, the Plasmid Assay was still under development by the Lung and Particles Research Group at the time of this study, thus there was no data quantifying the effect of handling on plasmid DNA damage. To collect such data was beyond the scope of this study.

The plasmid DNA was purchased in a concentrated stock and stored at -80°C. Upon first use the stock was diluted using HPLC grade water and aliquoted into 45µl aliquots and stored at -80°C until use, as per the work of Greenwell (2003). Any residual plasmid DNA was discarded after use.

7.5.2 Results

7.5.2.1 Quality of plasmid DNA and data correction

Control samples were run (4 per batch of leachate samples) whereby the plasmid DNA was incubated with HPLC grade deionised water (from the same batch as was used to dilute the plasmid DNA), to determine the quality of each batch of plasmid DNA used (i.e. whether or not the plasmid DNA is 100% undamaged). The results of these control samples are presented in Table 7.2.

It can be seen from Table 7.2 that the percentage damage to the plasmid DNA control samples was highly variable, ranging from 2.25% to 45.66%, indicating a highly variable quality of plasmid DNA. To correct for any damaged DNA prior to incubation with the leachate samples, Equation 7.5 was applied (C. Muller; pers.comm.).

Table 7.2: Percentage Damage to Plasmid DNA Control Samples

	% Plasmid DNA Damage							
Day	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
1	18.69	19.90	18.43	N/A	18.69	19.90	18.43	26.56
32	28.46	31.37	32.71	40.51	41.66	45.10	30.77	30.35
64	20.98	20.98	20.86	20.86	22.74	22.74	28.02	28.02
90	7.03	2.25	11.90	11.55	7.03	2.25	11.90	11.55

$$Damaged\ DNA(\%) = \frac{\%\ DNA\ damage\ caused\ by\ leachate}{\%\ undamaged\ DNA} \qquad \textit{Equation 7.5}$$

Where:

$\%\ DNA\ damage\ caused\ by\ leachate = total\ \%\ DNA\ damage - control$

$\%\ undamaged\ DNA = 100 - control$

7.5.2.2 TD_{50} Data

The toxicity data obtained from the Plasmid Assay are presented in Figure 7.9. It is essential that the reader understands the manipulation of the raw toxicity values undertaken to attain the data presented in Figure 7.9. Such data manipulation is summarised as follows:

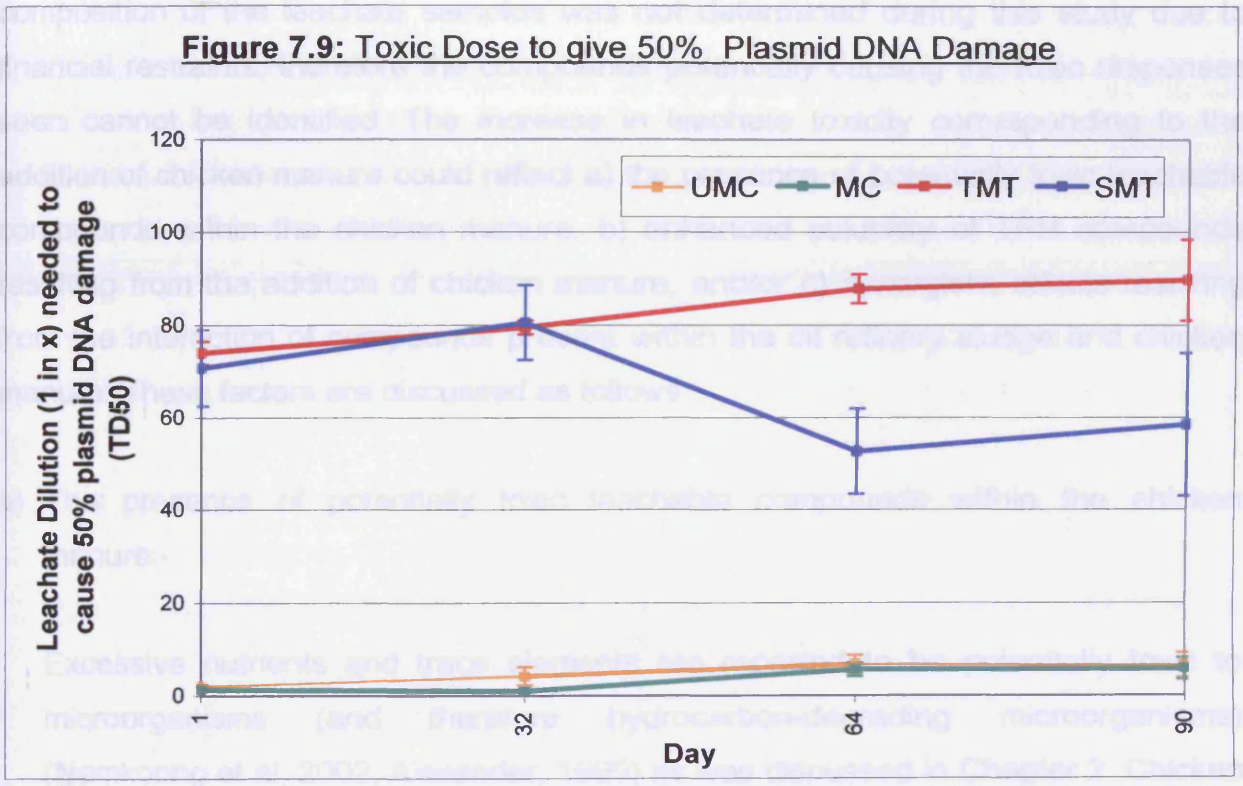
1. Percentage damage to plasmid DNA for each replicate sample was calculated in accordance with Equation 7.1.
2. Mean percentage damage (mean of 4 data points) for each sampling event (for each treatability study tray) was calculated.
3. The dilution needed to cause 50% damage to the plasmid DNA was determined for each sampling event per tray.
4. Mean dilution (mean of 2 data points) needed to cause 50% damage to the plasmid DNA, and standard error were calculated for each treatment condition (UMC, MC, TMT, SMT).

The values plotted in Figure 7.9 are the mean dilutions needed to cause 50% damage to the plasmid DNA (TD_{50}). The error bars show plus and minus one standard error (± 1 SE). An increase in dilution (i.e. reduction in toxin concentration) needed to cause TD_{50} shows an increase in toxicity). All raw data are presented in Appendix 6. Statistical analyses were carried out to identify any significant differences between the conditions using two-tailed Z-test for two means in Microsoft Excel, and a significance level of 1% ($p<0.01$) in accordance with Schmuller (2005). Observations are as follows:

1. The toxicity levels recorded for the UMC and MC conditions were statistically significantly lower ($p<0.01$) than those recorded for the TMT and SMT conditions over the duration of the treatability studies. Dilutions needed to cause TD_{50} ranged from approximately 1 in 2 to 1 in 7 for the UMC and MC conditions, compared with 1 in 70 to 1 in 90 for the TMT and SMT conditions.
2. The toxicity levels recorded for the UMC and MC conditions were not statistically significantly different ($p>0.01$) over the duration of the treatability studies.
3. The toxicity levels recorded for the UMC and MC conditions showed a gradual increase in toxicity from 1 in 2 to 1 in 7 (approx.) over the duration of the treatability studies, however this increase was not found to be statistically significant ($p>0.01$).
4. The toxicity levels recorded for the TMT condition showed a gradual increase in toxicity as shown by an increase in dilution needed to cause TD_{50} from approximately 1 in 75 to approximately 1 in 90 over the duration of the treatability studies. However, this increase in toxicity was not found to be statistically significant ($p>0.01$).
5. The toxicity levels recorded for the SMT condition showed an increase in toxicity between Days 1 and 32 as shown by an increase in dilution needed to cause TD50 from approximately 1 in 70 to approximately 1 in 80. This increase was not found to be statistically significant ($p>0.01$). Between Days 32 and 90, a general reduction in toxicity was seen, however this was not found to be statistically significant ($p>0.01$).
6. No statistically significant ($p>0.01$) difference in toxicity levels between the TMT and SMT conditions were recorded between Days 1 and 32. It appears from Figure 7.9 that between Days 32 and 90, toxicity levels recorded for the TMT

condition were higher than those recorded for the SMT condition. Between Days 32 and 64, the difference in toxicity levels recorded was found to be statistically significant (p=0.01). However, the difference in toxicity levels recorded for these conditions between Days 64 and 90 were not found to be statistically significant (p>0.01).

Figure 7.9: Toxic Dose to give 50% Plasmid DNA Damage



7.6 Discussion

The toxicity data presented in Sections 7.4 and 7.5 are discussed here to determine a) any potential increase in toxicity (and therefore potential adverse effect on TPH biodegradation) resulting from the addition of chicken manure, and b) the potential success of composting bioremediation in reducing the toxicity (and therefore environmental risk) of the oil refinery sludge. Further discussion of these data is presented in Chapter 8.

7.6.1 Chicken Manure and Potential Toxic Effects

Both the ROTAS and Plasmid Assay toxicity results of the leachate samples indicate a statistically significant ($p<0.01$) increase in leachate toxicity corresponding to the addition of chicken manure for Day 1 samples, and that this increase in leachate toxicity continued over the duration of the treatability studies. The chemical composition of the leachate samples was not determined during this study due to financial restraints, therefore the compounds potentially causing the toxic responses seen cannot be identified. The increase in leachate toxicity corresponding to the addition of chicken manure could reflect a) the presence of potentially toxic leachable compounds within the chicken manure, b) enhanced solubility of TPH compounds resulting from the addition of chicken manure, and/or c) synergistic effects resulting from the interaction of compounds present within the oil refinery sludge and chicken manure. These factors are discussed as follows:

a) The presence of potentially toxic leachable compounds within the chicken manure:-

Excessive nutrients and trace elements are reported to be potentially toxic to microorganisms (and therefore hydrocarbon-degrading microorganisms) (Namkoong et al, 2002; Alexander, 1999) as was discussed in Chapter 2. Chicken manure is reported to have high nutrient content and to contain trace elements such as cadmium, copper, and zinc (Nicholson et al, 1996; Nicholson et al 1999; Ihnat and Fernandes, 1996) as was discussed in Chapters 1 and 2. *Vibrio fischeri* is reported by Tsiridis et al (2006) to have sensitivity to trace elements such as copper, lead, zinc (as determined through the use of Microtox®). The Plasmid Assay has also been found to be sensitive to such trace elements in landfill leachate, although this finding is unpublished (E.Paris; pers.comm.).

The increase in toxicity of leachate samples seen may result solely from the presence of leachable toxins in chicken manure, and may indicate a potentially adverse effect of chicken manure additions on TPH biodegradation should these compounds be toxic to hydrocarbon degrading microorganisms.

b) Enhanced solubility of TPH compounds resulting from the addition of chicken manure:-

The addition of chicken manure may have enhanced desorption of TPH compounds and therefore bioavailability and toxicity of these compounds. Livestock manures contain humic acids (Janzen et al, 1996), and in particular, chicken manure contains uric acids (Nicholson et al, 1996). Such organic acids may enhance desorption and solubility of hydrophobic organic compounds such as hydrocarbons (Janzen et al, 1996). This was found to be true by Janzen et al (1996) who found that the addition of a compost extract (generated from livestock manure) enhanced desorption of naphthalene and α-napthol. In addition, many microorganisms are known to produce surfactants which can enhance the solubility of hydrophobic organic compounds such as hydrocarbons (Alexander, 1999; Mulligan, 2002).

However, the theory that the increased leachate toxicity seen may reflect enhanced desorption of TPH compounds (and therefore bioavailability and toxicity) due to the addition of chicken manure is not supported by the toxicity data attained from the ROTAS organics test suite (Section 7.4.3.1). These data show no increase in organics extract toxicity for Day 1 samples resulting from the addition of chicken manure, and show a complete loss of toxicity by Day 64 whereas leachate toxicity (shown by ROTAS leachable assay and Plasmid Assay) remained statistically significantly ($p<0.01$) higher than the un-amended sludge throughout the treatability studies. This would indicate that desorption of TPH compounds alone unlikely accounts for the increased leachate toxicity seen by the ROTAS leachable test suite and Plasmid Assay.

c) Synergistic effects resulting from the interaction of compounds present within the oil refinery sludge and chicken manure:-

It is recognised in literature regarding toxicology that the toxicity of mixtures of compounds is often different to that of single compounds (Timbrell, 2002; Lidman, 2005). The total toxicity of mixtures could reflect three main scenarios; additive effects (whereby the total toxicity of a mixture is the sum of the toxicities of the

individual compounds), synergistic effects (whereby the total toxicity of a mixture is greater than the sum of the toxicities of the individual compounds), and antagonistic effects (whereby the total toxicity of a mixture is less than the sum of the toxicities of the individual compounds) (Timbrell, 2002).

Therefore, the increased toxicity of the leachate samples due to the addition of chicken manure could reflect an additive or synergistic effect resulting from the interaction of compounds present in the chicken manure and oil refinery sludge.

Unfortunately the leachate toxicity of chicken manure alone was not determined during this study due to time restraints. It is apparent however, that the addition of chicken manure potentially results in an increase in leachate toxicity, and that this potentially, in part at least, reflects the chemical constituents of chicken manure. These data alone therefore suggest that further investigation is needed into the potential toxic effects of chicken manure on microbial degradation of TPH compounds.

7.6.2 Composting Bioremediation and Toxicity

7.6.2.1 Leachate Toxicity

The toxicity of total leachable compounds from the un-amended oil refinery sludge (UMC and MC conditions) was likely very low as shown by no response of *Vibrio fischeri* detected by the ROTAS leachable test suite, and very low response of plasmid DNA structure by the Plasmid Assay. Such results were anticipated due to the likely hydrophobicity and low solubility of TPH compounds (based on their physical-chemical properties which were discussed in Chapter 2). The toxicity of the total leachable compounds from the UMC and MC conditions showed no statistically significant ($p>0.01$) changes over the duration of the treatability studies. Such data could indicate that the environmental risk posed by the compounds leaching from the un-amended sludge are potentially low, or that the sensitivity of the ROTAS leachable test suite and Plasmid Assay is low. The sensitivity of the ROTAS leachable test suite to changes in concentration of leachate from the TMT and SMT conditions was found to be low, as was discussed in Section 7.4.3.2.

The toxicity of total leachable compounds from the TMT and SMT conditions was statistically significantly higher ($p<0.01$) than those from the un-amended oil refinery sludge (UMC and MC conditions), as shown by both the ROTAS leachable test suite and Plasmid Assay, as was discussed in Section 7.6.1. There were no statistically significant changes ($p>0.01$) in toxicity levels detected by the Plasmid Assay over the duration of the treatability studies, however the ROTAS leachable test suite detected a statistically significant ($p<0.01$) reduction in toxicity of 20% under the TMT condition between Days 1 and 90 of the treatability studies. These ROTAS data indicate that while the addition of chicken manure initially increased the toxicity of total leachable compounds, the composting bioremediation process led to a reduction in toxic response of *Vibrio fischeri* over the duration of the treatability studies. The reduction in toxicity seen under the TMT condition also indicates that the addition of nutrients (biostimulation) *and* microorganisms (bioaugmentation) through the addition of chicken manure contributed to the reduction in toxicity. However, despite these reductions in toxicity, total leachate toxicity levels were not reduced to the level of those from the UMC and MC conditions. This would imply that composting bioremediation failed to reduce overall leachate toxicity.

7.6.2.2 ROTAS Organics Test Suite

The ROTAS organics test suite results show that the toxicity of total extractable organic compounds showed no statistically significant ($p>0.01$) changes under the MC condition over the duration of the treatability studies. This may indicate that the pile management strategy alone did not succeed in reducing toxicity levels. However, toxicity levels under the UMC condition showed a statistically significant ($p<0.01$) increase over the duration of the treatability studies, and between Days 32 and 90 were statistically significantly ($p<0.01$) higher than those of the MC condition. It may therefore be argued that the pile management strategy potentially restricted increases in toxicity levels. However, the author recommends caution when interpreting these data as the increase in toxicity levels seen under the UMC condition may reflect the potential heterogeneous nature of the oil refinery sludge given that this condition was undisturbed (through tilling and mixing) over the duration of the treatability studies.

The addition of chicken manure under the TMT and SMT conditions showed a statistically significant ($p<0.01$) reduction in toxicity of total extractable organics between Days 1 and 64, and beyond Day 64 no toxic response was seen at all. The differences in toxicity levels recorded between the manure amended oil refinery sludge (TMT and SMT conditions) and un-amended oil refinery sludge (UMC and MC conditions) were found to be statistically significant ($p<0.01$). These data suggest that the addition of chicken manure and the composting bioremediation process likely caused a reduction in toxicity of total extractable organic compounds.

The reduction in toxicity seen under the TMT condition was statistically significantly ($p<0.01$) greater than that seen under the SMT condition between Days 1 and 32. No statistically significant difference was seen by Day 64. These data therefore indicate that the addition of nutrients (biostimulation) *and* microorganisms (bioaugmentation) through the addition of chicken manure potentially contributed to the reduction in toxicity.

The reduction in toxicity of the total extractable organic compounds under the TMT and SMT conditions indicates that the compounds within chicken manure deemed to be potentially toxic to microorganisms under the ROTAS leachable test suite and Plasmid Assay, unlikely had a significant adverse effect on hydrocarbon degrading microorganisms, and therefore TPH biodegradation. This assumes that the reduction in toxicity seen reflects biotic processes. This is discussed further in Chapter 8.

7.6.3 General Discussion

The use of the ROTAS and Plasmid Assay toxicity test systems have enabled the identification of potentially adverse effects resulting from the addition of chicken manure on TPH biodegradation, and the potential success of composting bioremediation to reduce the toxicity of the oil refinery sludge.

The author recognises, however, that the ROTAS system is based on a single bacterium and therefore these data cannot be used to determine the potential toxic effects of chicken manure and oil refinery sludge on all microorganisms. Therefore the

author recommends further investigation using a battery of toxicity tests to confirm the relationships seen in this study.

Also, the author recognises that the reduction in toxicity of total extractable organic compounds resulting from composting bioremediation and the addition of chicken manure shown by the ROTAS test system may not represent a sufficient reduction in risk posed to the environment and human health. The author recognises that further investigation is needed to fully quantify such reduction in risk, and that additional toxicity tests should be undertaken.

The author also recognises that toxicity tests, although potentially useful in tracking biodegradation processes, should not completely replace chemical analyses but should be undertaken as a complementary tool, or as a screening tool to focus the need of chemical analyses (and therefore potentially reduce analytical costs). However, even as a screening tool, the author recommends that laboratory trials should be undertaken to identify the optimum toxicity assay based on sensitivity to the contaminants present at a site.

7.7 Conclusions

The following conclusions have been made based on the toxicity data presented in this chapter only:-

1. The addition of chicken manure caused a statistically significant ($p<0.01$) increase in toxicity of total leachable compounds as shown by the ROTAS leachable test suite and Plasmid Assay results presented in this chapter. This may indicate a potentially adverse effect on hydrocarbon degrading microorganisms and therefore TPH biodegradation resulting from the addition of chicken manure as part of the composting bioremediation strategy. The chemical composition of the leachate samples is unknown therefore the extent to which compounds within chicken manure contributed to this total leachate toxicity cannot be determined from these data. Further investigation is recommended to determine the potential adverse effect on TPH biodegradation caused by the addition of chicken manure.

2. The pile management strategy did not result in a statistically significant ($p>0.01$) change in toxicity levels of total extractable organic compounds (using methanol), indicating that the pile management strategy alone was not successful in reducing toxicity of the oil refinery sludge, as shown by the ROTAS organics test suite results.
3. The addition of chicken manure caused a reduction in toxicity of total extractable organic compounds (using methanol), indicating that the addition of chicken manure was successful in reducing toxicity of the oil refinery sludge, as shown by the ROTAS organics test suite results.
4. The reduction in toxicity of total extractable organic compounds caused by the addition of chicken manure likely reflects both biostimulation (addition of nutrients) and bioaugmentation (addition of microorganisms) effects resulting from the addition of chicken manure, as shown by comparison of the ROTAS organics test suite results for the total manure and sterile manure treatment conditions.
5. The reduction in toxicity of total extractable organic compounds caused by the addition of chicken manure indicates that the compounds within chicken manure identified as potentially toxic to microorganisms unlikely had a significant adverse effect on potential hydrocarbon degrading microorganisms within the chicken manure and oil refinery sludge, or at least on those microorganisms acting on the hydrocarbons present in the organic extracts.
6. The author recommends the use of a battery of toxicity assays to strengthen the conclusions made based on the ROTAS and Plasmid Assay toxicity tests, and the use of chemical analyses to determine the likely source and significance of the observed toxicity.

Chapter 8

Discussion and Conclusions

8.1 Introduction

Treatability studies combined with an extensive suite of laboratory analyses have been designed and undertaken in order to investigate the following:

Aim: ***To investigate the use of chicken manure as a co-composting amendment to enhance the biodegradation of petroleum hydrocarbons and to help develop an improved understanding of the chemical, biological and toxicological processes involved.***

Objective One: - *To determine the potential for using chicken manure to enhance the biodegradation of Total Petroleum Hydrocarbons (TPH).*

Objective Two: - *To determine the potential for using chicken manure to enhance the biodegradation of the more resistant TPH compounds.*

Objective Three: - *To determine the potential enhancement of environmental conditions for microbial growth and activity through the amendment of oil refinery sludge with chicken manure.*

Objective Four: - *To help develop an improved understanding of the composting bioremediation process on TPH degradation with particular attention to: (Part 1) bioaugmentation versus biostimulation, (Part 2) microbial population dynamics, and (Part 3) toxicological dynamics, resulting from the addition of chicken manure to TPH contaminated material.*

The results of the laboratory analyses have been presented and discussed in Chapters 4 to 7. In this chapter, the conclusions drawn from these data are summarised and further discussed, final conclusions are stated, and recommendations for further research are presented.

8.2 Discussion

8.2.1 Objectives One and Two – Total and Fractionated TPH Degradation

Oil refinery sludge was chosen as the test material for this study as such sludge is reported to contain a variety of hydrocarbon compounds ranging from readily biodegradable to less biodegradable, reflecting variations in chemical properties and structure. The oil refinery sludge was collected from a former oil refinery site in the UK and is believed to be in excess of 10 years in age. The baseline chemical characterisation of the oil refinery sludge showed a predominance of high molecular weight hydrocarbons (constituting approximately 64% of total TPH) therefore indicating a low potential for biodegradation.

It is evident from the data and discussion presented in Chapter 5 that there was no statistically significant ($p>0.01$) change in total TPH concentration over the duration of the treatability studies under any of the four pile conditions (un-managed control (UMC), managed control (MC), total manure treatment (TMT) and sterile manure treatment (SMT)) investigated. It is therefore concluded that the addition of chicken manure did not enhance the degradation of total TPH compounds.

However, it is evident from the data and discussion presented in Chapter 5 that the addition of chicken manure enhanced the degradation of C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbons. It is also evident that volatilisation potentially contributed to the degradation of these hydrocarbons fractions between Days 1 and 2 under the TMT condition, but that biodegradation was likely the dominant degradation process over the remaining duration of the treatability studies.

The C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbon fractions have the highest biodegradation potential out of the aliphatic and aromatic hydrocarbons (C_9-C_{36}) quantified in this study, owing to their lower molecular weight and higher solubility, therefore likely higher bioavailability. However, these fractions formed a small percentage of total TPH compounds detected (approximately 7.25%). Therefore their degradation did not have a statistically significant effect on total TPH degradation. The addition of chicken manure did not enhance the degradation of the C_{12}-C_{36} aliphatic hydrocarbon fractions or the C_9-C_{36} aromatic hydrocarbon fractions. This likely reflects the lower biodegradability and bioavailability of such higher molecular weight hydrocarbons.

The lack of enhanced TPH degradation seen under the TMT condition in this study may reflect adverse competition between non-hydrocarbon and hydrocarbon degrading microorganisms resulting from the addition of chicken manure, and/or adverse toxic effects on potential hydrocarbon degrading microorganisms resulting from the addition of chicken manure. These factors are discussed in sections 8.2.4 and 8.2.5.

8.2.2 Objective Three – Pile Viability

It is evident from the data and discussion presented in Chapter 4 that the addition of chicken manure enhanced the ability of the materials to support microbial growth and/or activity, as shown by significantly increased pile temperatures (by approximately 25-30°C on Days 2 and 3, and by approximately 1-3°C for the remainder of the treatability studies) and microbial respiration (by approximately 10-20ppm CO_2 min^{-1} g^{-1} soil dry weight).

Although these data indicate enhanced environmental conditions for microbial growth and/or activity resulting from the addition of chicken manure, this may reflect the growth and/or activity of microorganisms indigenous to the chicken manure only. Therefore this did not necessarily have a positive effect on TPH biodegradation, hence the lack of TPH degradation recorded. This is discussed further below.

8.2.3 Objective Four Part 1 – Biostimulation versus Bioaugmentation

It is evident from the data and discussion presented in Chapters 4 to 7 that the addition of chicken manure resulted in potential biostimulation and bioaugmentation effects, as discussed below.

It is evident from the data and discussion presented in Chapter 4 that the enhanced pile viability resulting from the addition of chicken manure likely reflected biostimulation *and* bioaugmentation effects (i.e. the activity of both the microorganisms indigenous to the chicken manure and those indigenous to the oil refinery sludge was enhanced) as shown through comparison between the TMT and SMT conditions. However, whereas the pile temperature data indicate a greater effect on microorganisms indigenous to the oil refinery sludge, the microbial respiration data indicated a greater effect on microorganisms indigenous to the chicken manure. Therefore, the enhanced growth and/or activity of microorganisms indigenous to the chicken manure may not necessarily have had a positive effect on TPH biodegradation.

It is evident from the data and discussion presented in Chapter 5 that the enhanced degradation of C_9-C_{12} aliphatic hydrocarbons resulting from the addition of chicken manure mostly reflects biostimulation. However, the data presented indicate that bioaugmentation effects potentially caused the enhanced degradation of C_9-C_{10} aliphatic hydrocarbons between Days 1 and 2, and of C_{10}-C_{12} aliphatic hydrocarbons between Days 1 and 16, through comparison between the TMT and SMT conditions. Such bioaugmentation effects likely resulted in the increased pile temperatures recorded and therefore potentially higher contribution of volatilisation to TPH degradation over this time period under the TMT condition compared to the remaining duration of the treatability studies.

It is evident from the data and discussion presented in Chapter 6 that the addition of chicken manure resulted in the introduction of two potential hydrocarbon-degrading bacterial populations (*Rhodococcus* and *Dietzia*). These two populations showed signs of population growth over the duration of the treatability studies, and therefore indicate bioaugmentation effects resulting from the addition of chicken manure. In

addition to this, the addition of chicken manure was seen to result in the enhanced growth of seven potential hydrocarbon-degrading bacterial populations, including *Desulfotomaculum*, *Ectothiorhodospiraceae*, *Alcanivorax*, *Pseudomonas*, *Marinobacter*, *Hydrocarboniphaga effusa*, and *Syntrophus*. Of these bacterial populations, *Desulfotomaculum, Ectothiorhodospiraceae, Alcanivorax*, *Pseudomonas* and *Marinobacter* were identified as being common to both the chicken manure and oil refinery sludge. Therefore the enhanced growth of these populations under the TMT condition may reflect biostimulation and/or bioaugmentation resulting from the addition of chicken manure. These populations are further discussed in Section 8.2.4.

It is evident from the data and discussion presented in Chapter 7 that the addition of chicken manure resulted in a reduction in toxicity of total extractable organic compounds, and that this likely reflects both biostimulation and bioaugmentation.

8.2.4 Objective Four Part 2 – Bacterial Community Dynamics

It is evident from the data and discussion presented in Chapter 6 that the addition of chicken manure had potentially positive effects on bacterial community dynamics. The addition of chicken manure introduced two potential hydrocarbon-degrading populations (*Rhodococcus* and *Dietzia*), and enhanced the growth of several bacterial populations above that of the pile management strategy, including *Desulfotomaculum*, *Ectothiorhodospiraceae*, *Alcanivorax*, *Pseudomonas*, *Marinobacter*, *Hydrocarboniphaga effusa*, and *Syntrophus*. These populations are discussed as follows:

1. Although the introduction of *Rhodococcus* and *Dietzia* through the addition of chicken manure may represent a potentially positive effect on TPH biodegradation, over the duration of their 'growth' period under the TMT condition (between Days 16 and 32) there was no recorded difference in degradation rate of C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbons between the TMT and MC condition. Therefore it is considered that the introduction of these bacterial populations unlikely had a significant effect on the degradation of C_9-C_{10} and C_{10}-C_{12} aliphatic hydrocarbons. It is also interesting to note that over this time period, degradation of these hydrocarbon fractions was faster under the SMT condition. This could indicate that

the introduction of *Rhodococcus* and *Dietzia*, although potentially capable of hydrocarbon degradation, had an adverse effect on degradation that could otherwise have been enhanced through biostimulation only. However, it may be that these bacterial populations had a significant effect on the biodegradation on $<C_9$ hydrocarbons, which were not quantified during this study.

2. The growth of *Desulfotomaculum*, *Ectothiorhodospiraceae*, *Alcanivorax*, *Pseudomonas*, *Marinobacter*, *Hydrocarboniphaga effusa*, and *Syntrophus* was seen to be enhanced through the addition of chicken manure (above that of the pile management strategy). Of these, only the growth of *Ectothiorhodospiraceae* did not coincide with an enhanced degradation rate of C_9-C_{12} aliphatic hydrocarbons. This bacterial population, however, may have been effective on the $<C_9$ hydrocarbons, which were not quantified in this study. The growth of the other bacterial populations coincided with enhanced degradation of C_9-C_{10} aliphatic hydrocarbons when comparisons are made between the TMT and UMC/MC conditions. Such growth appeared to reflect bioaugmentation rather than biostimulation effects resulting from the addition of chicken manure, as determined through comparisons between the TMT and SMT conditions. In contrast, when the TMT and UMC conditions are compared, the growth of these populations did not coincide with a statistically significant enhanced degradation of C_{10}-C_{12} aliphatic hydrocarbons, but did so when the TMT and MC conditions are compared.

However, the data presented in Chapter 6 also indicate that the addition of chicken manure a) introduced potentially non-hydrocarbon-degrading bacterial populations which therefore present a risk of adverse competition with hydrocarbon degrading populations for growth factors, b) encouraged the growth of populations which are potentially non hydrocarbon degrading therefore present a risk of adverse competition with hydrocarbon degrading populations for growth factors, c) resulted in the decline of a potentially hydrocarbon degrading population, and d) resulted in 8 bacterial populations of oil refinery sludge origin, that were potentially hydrocarbon degrading, being unrepresented in the TMT community fingerprint. However, for all of these cases the author has been able to propose that the risk to TPH biodegradation posed is minimal. Therefore, in the opinion of the author, this indicates that the addition of

chicken manure to oil refinery sludge likely had a stronger positive effect on TPH biodegradation than it did a negative effect.

8.2.5 Objective Four Part 3 – Toxicity

It is evident from the data and discussion presented in Chapter 7 that:-

1. The addition of chicken manure resulted in an increase in toxicity of total leachable compounds which may have a detrimental effect on potential hydrocarbon degrading bacterial populations and therefore on TPH biodegradation. It was hypothesised that such toxicity was unlikely to have had a detrimental effect on those bacterial populations acting on the hydrocarbons represented in the ROTAS organics test suite as the addition of chicken manure resulted in a reduction in toxicity of such extractable organic compounds. This is further discussed below.

2. The addition of chicken manure resulted in a decrease in toxicity of total extractable organic compounds above that of the pile management strategy. Through comparison with the TPH and headspace analyses results, it is likely that the total extractable organic compounds represented those hydrocarbons less than C_9. This is evident from the lack of decline in toxicity for the UMC and MC conditions, despite a loss of 90-100% of C_9-C_{12} aliphatic hydrocarbons over the duration of the treatability studies. This potentially indicates that the ROTAS organics test suite was only sensitive to $<C_9$ hydrocarbons, and therefore, unlikely suitable as a screening tool for use in bioremediation studies of petroleum mixtures containing higher molecular weight hydrocarbons. However, it could also indicate that those hydrocarbons $>C_9$ were less bioavailable, which could explain the lack of degradation of these hydrocarbon fractions seen in this study. This can only be confirmed through chemical analysis of the organics extracts, to determine the range of hydrocarbons extracted.

The possibility that the addition of chicken manure resulted in increased toxicity of leachable compounds and that such compounds could have a detrimental effect on hydrocarbon ($>C_9$) degrading microorganisms therefore remains. The author therefore recommends further toxicity testing (using a suite of toxicity tests) and chemical

analysis of the leachate samples to confirm this. Despite this, it is evident from Section 8.2.4 that several bacterial populations showed growth under the TMT condition, and that such growth coincided with the enhanced degradation of C_9-C_{12} aliphatic hydrocarbons.

8.2.6 General Discussion

The addition of chicken manure to the oil refinery sludge was seen to have potentially positive effects on TPH degradation through:-

- Enhanced pile viability.
- Addition of potential hydrocarbon degrading bacterial populations (bioaugmentation effect).
- Enhanced growth of potential hydrocarbon degrading bacterial populations (biostimulation and bioaugmentation effects).
- Reduction in toxicity of total extractable organic compounds (likely $<C_9$ hydrocarbons) which may have been toxic to potential hydrocarbon degrading bacterial populations.
- Enhanced degradation of C_9-C_{12} aliphatic hydrocarbons.

However, despite these potentially positive effects, the addition of chicken manure failed to statistically significantly enhance the degradation of total TPH compounds in the oil refinery sludge. The C_9-C_{12} aliphatic hydrocarbons constituted only approximately 7% of the total TPH (C_9-C_{36}) compounds measured in this study, therefore the degradation of these hydrocarbon fractions did not significantly affect total TPH concentrations.

In contrast, previous authors, such as Ijah and Antai (2003), Ibekwe et al (2006), Atagana (2003), and Atagana (2004b) found that the addition of poultry manure statistically significantly enhanced total TPH degradation, as was discussed in Chapter 2.

The author recognised in Chapter 2 that the addition of chicken manure could have a potentially adverse effect on TPH biodegradation through a) adverse competition for growth factors between hydrocarbon- and non-hydrocarbon-degrading microorganisms, and b) toxic effects on potential hydrocarbon degrading microorganisms potentially due to the addition of trace elements. These potentially adverse effects are discussed below.

It is seen from the discussion in Section 8.2.4 that the addition of chicken manure unlikely resulted in the adverse proliferation of non-hydrocarbon degrading bacterial populations at the expense of hydrocarbon degrading bacterial populations.

It is seen from the discussion in Section 8.2.5 that the addition of chicken manure resulted in a significant increase in toxicity of total leachable compounds. These data indicate a potentially adverse toxic effect on hydrocarbon degrading microorganisms resulting from the addition of chicken manure. However, it is unlikely that such toxic effects had a significantly adverse impact on those bacterial populations degrading C_9-C_{12} aliphatic hydrocarbons, as 100% reduction of these hydrocarbon fractions was recorded over the duration of the treatability study, and there is evidence of enhanced growth of potential hydrocarbon degrading bacterial populations resulting from the addition of chicken manure. The author recommends further toxicity testing using a suite of toxicity tests, and chemical analysis of the leachate, to confirm the potential toxic effect of chicken manure on hydrocarbon degrading bacterial populations, as such toxic effects could render the use of chicken manure in composting bioremediation of TPH compounds unviable.

Alternative explanations for the lack of TPH degradation recorded may include:-

1) Lack of bioavailable and biodegradable substrate:

   It is evident from the data and discussion presented in Chapter 5 that the biodegradability of the TPH compounds in the oil refinery sludge was seen to be high based on the ratio of aliphatic to aromatic hydrocarbons, but low based on the dominance of high molecular weight hydrocarbons over low molecular weight hydrocarbons. Such high molecular weight hydrocarbons are likely more resistant

to biodegradation and less bioavailable owing to their physical-chemical properties, as was discussed in Chapter 2. The ROTAS organics test suite shows a reduction in toxicity of total extractable organic compounds, however based on comparison of these toxicity profiles with the fractionated TPH degradation profiles, it is considered by the author that the extractable organic compounds likely only comprised $<C_9$ hydrocarbons. This may reflect a low sensitivity of the ROTAS organics test suite to higher molecular weight compounds, or a lack of bioavailability of such compounds.

In contrast to this study, the work of Ijah and Antai (2003) and Ibekwe et al (2006) was based on soils spiked with petroleum hydrocarbons. It is reported by Loehr and Webster (1996) that hydrocarbons freshly applied to soils are more biodegradable than those which have undergone the ageing process. This could explain the higher biodegradation recorded by these authors as opposed to that recorded in this study. In addition, Ijah and Antai (2003) and Ibekwe et al (2006) monitored the degradation of total crude oil. The distribution of hydrocarbon fractions is not recorded by these authors. Therefore it may be that the biodegradation potential was much higher for these soils than for the oil refinery sludge used in this study.

2) Inappropriate contaminated soil to organic amendment ratio:

It was recognised in Chapter 2 that the addition of chicken manure could present a preferential source of carbon and energy, and therefore be detrimental to the degradation of TPH compounds. Also, the contaminated soil to organic amendment could significantly affect the biodegradation process as excessive nutrients and/or trace elements could have toxic effects on hydrocarbon degrading microorganisms (Ladislao et al, 2005). Previous authors, Ijah and Antai (2003), Ibekwe et al (2006), Atagana (2003) and Atagana (2004b), between them used varying contaminated soil to poultry manure amendment ratios of 10:1 (Ijah and Antai, 2003), 10:9 (Ibekwe et al, 2006), 9:1 (Atagana, 2003) and 4:1 (Atagana, 2004b). It is difficult to make comparisons between the results of these investigations owing to inconsistent experimental design adopted in these investigations.

The contaminated soil to chicken manure amendment ratio used in this study was based on the balance of carbon to nitrogen (C:N). Based on published literature, a C:N ratio of 25:1 was deemed to be optimum, as was discussed in Chapter 3. However, this ratio is based on the assumption that all carbon and nitrogen is bioavailable. It may be that this ratio is not optimum for the bioremediation of the oil refinery sludge using chicken manure given the likely low bioavailability of carbon due to the predominance of high molecular weight hydrocarbons. Therefore the author recommends further investigation into the effectiveness of varying C:N ratios.

3) Inappropriate pile management strategy:

A pile management strategy was adopted in an attempt to optimise environmental conditions to encourage the growth and/or activity of hydrocarbon degrading microorganisms. Such microbial growth/activity can be affected by various environmental factors, including moisture content, oxygen availability, temperature and pH. While the addition of chicken manure was seen to enhance pile viability, as indicated by increased carbon dioxide evolution rates and pile temperature which are considered to reflect increased microbial growth and/or activity, it is possible that its full potential was not realised given the limited scope of this study to ensure that optimal environmental conditions were achieved. It may therefore be that the increase in microbial growth and/or activity recorded was insufficient to cause a significant effect on TPH biodegradation, and that this may reflect environmental conditions. Further investigation into the optimisation of environmental conditions is therefore recommended.

It is therefore evident from this study that although the addition of chicken manure did not enhance the degradation of total TPH compounds in the oil refinery sludge there is a potential for the use of chicken manure in composting bioremediation to enhance the biodegradation of low molecular weight TPH compounds. However, there is also a potential for the addition of chicken manure to result in an adverse toxic effect on hydrocarbon degrading microorganisms. Further investigation is recommended to confirm the results of this study. Such recommendations are presented in Section 8.4.

8.3 Conclusion

Based on the data and discussions presented in this thesis, the following conclusions can be made:

1. The addition of chicken manure can have a positive effect on the biodegradation of total petroleum hydrocarbons. This is evident from:

    a. Enhanced ability of the pile to support and encourage the growth and/or activity of microorganisms (pile viability).

    b. Addition of potential hydrocarbon degrading bacterial populations (bioaugmentation).

    c. Enhanced growth of potential hydrocarbon degrading bacterial populations (biostimulation and bioaugmentation).

    d. Enhanced reduction in toxicity of methanol extractable organic compounds (potentially $<C_9$ hydrocarbons) which may elicit toxic effects on hydrocarbon degrading microorganisms.

    e. Enhanced degradation of C_9-C_{12} aliphatic hydrocarbons.

    f. In the case of the oil refinery sludge investigated in this study, there was no significant evidence of potentially adverse competition between hydrocarbon and non-hydrocarbon degrading microorganisms resulting from the addition of chicken manure. The author, however, recognises that this is dependent on the baseline microbial composition of the material to be treated through the addition of chicken manure.

2. However, the addition of chicken manure was seen to result in an increase in toxicity of total leachable compounds. This could render the use of chicken manure in the composting bioremediation of petroleum hydrocarbons unviable should this toxicity be found to have an adverse effect on hydrocarbon degrading microorganisms.

3. Although it has been seen that the addition of chicken manure can have a positive effect on TPH degradation, it is evident from the data and discussions presented in this thesis that the potential for the use of chicken manure to enhance the degradation of total petroleum hydrocarbon is restricted to hydrocarbons of low molecular weight rather than of high molecular weight. Therefore the potential alternative end use for surplus chicken manure could be restricted to soils contaminated with low molecular weight petroleum hydrocarbons.

8.4 Recommendations for Further Research

The author recommends that further investigation into the potential use of chicken manure to enhance the biodegradation of petroleum hydrocarbons is needed, with particular attention to:

1. The use of a battery of toxicity tests and chemical analysis of leachate samples to confirm the potential toxic effect of chicken manure on hydrocarbon degrading microorganisms.

2. The investigation of varying carbon to nitrogen (C:N) ratios to determine the optimum contaminated soil to chicken manure ratio for the biodegradation of Total Petroleum Hydrocarbons.

3. The investigation of an optimum pile management strategy to enhance conditions for growth and/or activity of hydrocarbon degrading microorganisms, with particular attention to oxygen and moisture availability.

4. Confirmation of the potential use of chicken manure to enhance the degradation of low molecular weight hydrocarbons through the investigation of materials contaminated with lighter petroleum products such as jet fuel/ diesel.

5. Assessment of the potential long term use of materials bioremediated using chicken manure during site re-development works, with particular attention to

geotechnical properties and long term stability in terms of potential leachate and land gas production.

Chapter 9

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APPENDIX 1

Questionnaire Survey of Poultry Farming in Wales (UK) – Summary of Results

SUSTAINABLE MANAGEMENT OF FARM WASTE IN WALES

QUESTIONNAIRE

The objective of this questionnaire is simply to attain information to aid research being undertaken by the Geoenvironmental Research Centre (GRC) at Cardiff University.

Any information you provide will be dealt with in a strictly confidential manner, and will *only* be used by the GRC.

1) Which type of poultry farm do you run? Please tick one of the following.

a) Organic ☐

b) free range ☐

c) large scale/ battery hen ☐

2) How many chickens do you keep? .....

.....

3) Approximately how much manure is produced each year? .....

.....

4) What do you do with the manure? Please tick the relevant answers.

a) Store it for use on the farm. (please specify use) ☐

b) Spread it on the farm as a waste disposal method ☐

c) Composting for use on the farm ☐

d) Composting for sale to other users. ☐

e) Sell to other users in an untreated state. ☐

f) Other, please state

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5) If you store the manure, how do you store it? Please tick one of the following. *Details are not necessary.*

a) Outside with no protection (i.e. directly on land with no barrier such as concrete) ☐

b) Outside on a concrete base ☐

c) In a barn / shed with no concrete flooring ☐

d) In a purpose built container ☐

e) Other, please state

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6) Have you ever received any complaints from nearby land users about odours or other problems associated with the poultry manure? If so, did you take any actions to reduce/rectify the problem? Please state the complaints made and actions taken. *This information will only be seen by the GRC and will not be passed on to any authorities.*

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7) Have you identified any problems associated with the manure? Such problems may include the following, please tick the relevent box/boxes.

a) Over-application of nutrients to crop/grass land. ☐

b) Too much manure to store. ☐

c) Not enough land to utilise the manure. ☐

d) Other, please state. ☐

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8) If you have any additional comments/information please write it below.

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Questionnaire Survey of Poultry Farming in Wales (UK) 2003
Summary of Results

Farm Type	Flock Size	Quantity of Manure Produced per Year	Manure Use	Manure Storage	Any Complaints Received?	Storage and Handling Problems	Additional Comments
Free Range	4000	200 tons	Compost on farm	Storage outside, no base or covers	No	No	None
Free Range	3000	110.5m^3	Land application as waste disposal method	Storage outside, no base or covers	No	Over-application	Have found that is they apply to grassland more often than 3 years, sheep will not eat the grass. This effect tends to last for 2 years.
Free Range	12750	100 tons	Compost on farm for use on farm	Storage outside, no base or covers	No	No	None
Free Range	12	unknown	Spread on farm as waste disposal	Storage outside on concrete base with no covers.	No	No	None
Free Range	Unknown	Unknown	Spread on farm as waste disposal	Storage outside, no base or covers	No	No	None
Free Range	1000	Unknown	Composting on farm for use on farm	Storage outside, no base or covers	No	Too much manure to store and not enough land beneficially spread it on.	None
Free Range	8000	Unknown	Give to others in untreated state		No	Too much manure to store and not enough land beneficially spread it on.	None
Free Range	10500	400 tons	Spread on farm as waste disposal method		No	No	None
Free Range	1200	50 tons	Store for use on farm	Storage outside, no base or covers	No	No	None
Free Range	500	25	Compost on farm for use on farm	Storage outside, no base or covers	No	No	None
Free Range	7500	Unknown	Give to others in untreated state	None	No	No	None
Free Range	15000	40 tons	Give to other in untreated state	None	Fly problems in past	None	None

Appendix 1 – Questionnaire Survey

Battery/ caged	1000	Unknown	Compost on farm	Storage outside, no base or covers	No	No	None
Battery/ caged	800	Unknown	Land application as waste disposal method	Unspecified	No	No	None
Battery/ caged	18000	>500 tons	Give to neighbour in untreated state	Storage outside, no base or covers.	Complaints from EA about contamination of water courses.	No	
Battery/ caged	12000	800tons	Spread on farm as waste disposal method	Store in slurry pit	No	No	None
Battery/ caged	20000	600-800 tons	Spread on farm as waste disposal method	Storage outside on concrete base, no covers	Flies are a big problem	No	None
Battery/ caged	7500	500 TONS	Spread on farm	Storage outside on concrete base, no covers	Complaints regarding odours	No	None
Battery/ caged	6000	Unknown	Spread on grassland	Open lagoon	No	No	None
Battery/ caged	50000	8500 tons	Give to local farmers in untreated state	Storage outside with no base or covers	Complaints regarding odours	Too much manure, not enough land	None
Battery/ caged	7060	Unknown	Spread on farm as waste disposal method	Storage outside on concrete base, no covers	No	No	None
Battery/ caged	200000	1950 tons	Give to others in untreated state		No	Over application	None
Battery/ caged	160000	Unknown	Give to others in untreated state		No	None	None
Battery/ caged	20000	Unknown	Spread on farm as waste disposal method	Storage outside with no base or covers	Complaints regarding odours	None	None
Battery/ caged	7000	Unknown	Give to others in untreated state		No	None	None
Organic	500	30 tons	Composting on farm for use on farm	Indoor storage, no concrete base.	No	No	None
Organic	30000	Unknown	Compost on farm for use on farm and for sale to others	Storage outside, no base or cover	No	No	None
Organic	50000	400	Spread on farm as waste disposal method	Storage outside, no base or cover	No	No	none

Appendix 1 – Questionnaire Survey

APPENDIX 2

Material Characterisation

Material Characterisation

ALcontrol Geochem Analytical Services
Sample Descriptions

Job Number: 04/14814/02/01
Client: Cardiff University
Client Ref :

Grain sizes

<0.063mm	Very Fine
0.1mm - 0.063mm	Fine
0.1mm - 2mm	Medium
2mm - 10mm	Coarse
>10mm	Very Coarse

Sample Identity	Depth (m)	Colour	Grain Size	Description	Batch
SLUDGE 1		Black	<0.063mm	Silt with some Tar	1
SLUDGE2		Black	<0.063mm	Silt with some Tar	1
SLUDGE3		Black	<0.063mm	Silt with some Tar	1

lidated ☑
reliminary ☐

ALcontrol Geochem Analytical Services
Table Of Results

ISO 17025 accredited
M MCERTS accredited
* Subcontracted test
» Shown on prev. report

ob Number: 04/14814/02/01 **Matrix:** SOLID
lient: Cardiff University **Location:** Not Specified
lient Ref. No.: **Client Contact:** K Brice

Sample Identity	SLUDGE 1	SLUDGE2	SLUDGE3							Method Code	LoD/Units
Depth (m)											
Sample Type	SLUDGE	SLUDGE	SLUDGE								
Sampled Date	23.09.04	23.09.04	23.09.04								
ample Received Date	24.09.04	24.09.04	24.09.04								
Batch	1	1	1								
Sample Number(s)	1-3	4-6	7-9								
)(C4-C10)	1434399	1362083	866259							TM089#	10 ug kg
TBE	181282	152529	93616							TM089#	10 ug kg
zene	19828	25905	5944							TM089#	10 ug kg
ene	11145	8912	4327							TM089#	10 ug kg
rl benzene	13335	15224	8415							TM089#	10 ug kg
t p Xylene	43865	45898	18902							TM089#	10 ug kg
ylene	28299	22344	12603							TM089#	10 ug kg
phatics C5-C6	90054	79781	44347							TM089#	10 ug kg
phatics C6-C8	656803	619992	426479							TM089#	10 ug kg
phatics C8-C10	95845	96580	59892							TM089#	10 ug kg
phatics C10-C12	60070	60019	40759							TM089#	10 ug kg
phatics C12-C16	7159686	1568638	1619562							TM061#M	100 ug kg
phatics C16-C21	18376202	4252786	3767376							TM061#M	100 ug kg
phatics C21-C35	19852032	4232104	3778782							TM061#M	100 ug kg
l Aliphatics C5-C35	46290692	10909900	9737197							TM61 89#	100 ug kg
matics C6-C7	19828	25905	5944							TM089#	10 ug kg
matics C7-C8	11145	8912	4327							TM089#	10 ug kg
matics EC8-EC10	229267	228337	129757							TM089#	10 ug kg
matics EC10-EC12	90105	90029	61138							TM089#	10 ug kg
matics EC12-EC16	560779	218476	202055							TM061#M	100 ug kg
matics EC16-EC21	1597038	622740	591942							TM061#M	100 ug kg
matics EC21-EC35	2226834	829004	867508							TM061#M	100 ug kg
l Aromatics C6-C35	4734996	2023403	1862671							TM61 89#	100 ug kg
Aliphatics and Aromatics C5-C35)	51025688	12933303	11599868							TM61 89#	100 ug kg

results expressed on a dry weight basis.

Date 07.10.2004

Validated ☑
Preliminary ☐

ALcontrol Geochem Analytical Services
Table Of Results

ISO 17025 accredited
M MCERTS accredited
* Subcontracted test
» Shown on prev. report

Job Number: 04/14814/02/01 **Matrix:** SOLID
Client: Cardiff University **Location:** Not Specified
Client Ref. No.: **Client Contact:** K Brice

Sample Identity	SLUDGE 1	SLUDGE2	SLUDGE3							Method Code	LoD/Units
Depth (m)											
Sample Type	SLUDGE	SLUDGE	SLUDGE								
Sampled Date	23.09.04	23.09.04	23.09.04								
Sample Received Date	24.09.04	24.09.04	24.09.04								
Batch	1	1	1								
Sample Number(s)	1-3	4-6	7-9								
PAH by GCMS											
phthalene	17641	6398	3032							TM074#M	10 ug kg
enaphthylene	303	282	344							TM074#M	5 ug kg
enaphthene	8085	7002	6499							TM074#M	14 ug kg
orene	15013	11833	8982							TM074#M	12 ug kg
enanthrene	19299	20806	10599							TM074#	21 ug kg
thracene	1103	1023	392							TM074#M	9 ug kg
uoranthene	4180	4053	3091							TM074#M	25 ug kg
rene	4431	4427	3873							TM074#M	22 ug kg
zo(a)anthracene	947	1155	929							TM074#M	12 ug kg
rysene	1978	2357	2152							TM074#M	10 ug kg
zo(b)fluoranthene	420	522	435							TM074=M	16 ug kg
zo(k)fluoranthene	250	250	250							TM074=M	25 ug kg
zo(a)pyrene	268	377	311							TM074=	12 ug kg
deno(123cd)pyrene	110	110	214							TM074=M	11 ug kg
benzo(ah)anthracene	80	80	122							TM074=M	8 ug kg
zo(ghi)perylene	100	100	1072							TM074=	10 ug kg
16 Total	73668	60235	42047							TM074=	25 ug kg

results expressed on a dry weight basis.

Date 07.10.2004

alidated ☑
reliminary ☐

ALcontrol Geochem Analytical Services
Table Of Results

\# ISO 17025 accredited
M MCERTS accredited
* Subcontracted test
» Shown on prev. report

ob Number: 04/14814/02/01 **Matrix:** SOLID
lient: Cardiff University **Location:** Not Specified
lient Ref. No.: **Client Contact:** K Brice

Sample Identity	SLUDGE 1	SLUDGE2	SLUDGE3							Method Code	LoD/Units
Depth (m)											
Sample Type	SLUDGE	SLUDGE	SLUDGE								
Sampled Date	23.09.04	23.09.04	23.09.04								
ample Received Date	24.09.04	24.09.04	24.09.04								
Batch	1	1	1								
Sample Number(s)	1-3	4-6	7-9								
OC											
zene	28686	42975	20953							TM116#M	1 ug kg
iene	<10	<10	<10							TM116#M	1 ug kg
ylbenzene	41552	55658	27560							TM116#	1 ug kg
p-Xylene	214329	200899	115116							TM116#	1 ug kg
ylene	90914	12996	53653							TM116#	1 ug kg

sults expressed on a dry weight basis.

Date 07.10.2004

ALcontrol Geochem Analytical Services
Table Of Results - Appendix

Job Number: 04/14814/02/01
Client: Cardiff University
Client Ref. No.:

Report Key :

NDP	No Determination Possible	*	Subcontracted test
NFD	No Fibres Detected	»	Result previously reported (Incremental reports only)
#	ISO 17025 accredited	M	MCERTS Accredited
		EC	Equivalent Carbon (Aromatics C8-C35)

Note: Method detection limits are not always achievable due to various circumstances beyond our control.

Summary of Method Codes contained within report :

Method No.	Reference	Description	ISO 17025 Accredited	MCERTS Accredited	Wet/Dry Sample [1]
TM061	Method for the Determination of EPH,Massachusetts Dept.of EP, 1998	Determination of Extractable Petroleum Hydrocarbons by GC-FID (C10-C40)	Y	Y	DRY
TM074	Modified: US EPA Method 8100	Determination of Polynuclear Aromatic Hydrocarbons (PAH) by GC-MS	Y	N	DRY
TM074	Modified: US EPA Method 8100	Determination of Polynuclear Aromatic Hydrocarbons (PAH) by GC-MS	Y	Y	DRY
TM089	Modified: US EPA Methods 8020 & 602	Determination of Gasoline Range Hydrocarbons (GRO) and BTEX (MTBE) compounds by Headspace GC-FID (C4-C10)	Y	N	WET
TM116	Modified: US EPA Method 8260, 8120, 8020, 624, 610 & 602	Determination of Volatile Organic Compounds by Headspace / GC-MS	Y	N	WET
TM116	Modified: US EPA Method 8260, 8120, 8020, 624, 610 & 602	Determination of Volatile Organic Compounds by Headspace / GC-MS	Y	Y	WET
TM61/89		see TM061 and TM089 for details	Y	N	WET

[1] Applies to Solid samples only. **DRY** indicates samples have been dried at 35°C. **NA** = not applicable.

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CARDIFF

CF24 0YF

Woodthorne
Wergs Road
Wolverhampton
WV6 8TQ

Tel: 01902 743222
Fax: 01902 746183
www.directlabs.co.uk

Laboratory Report

Batch number : **02229927**
Date received : **17 January 2005**

Work code : **Not supplied**
Sample date : **14 January 2005**

Lab sample no. / Sample type Your reference	Determination [1]	Result	Method Ref
25100818 / Contaminated Soil **SCN3**			
	* Oven Dry Matter, % m/m	59.1	SAMP/015
	* Total Nitrogen (Kjeldahl), g/kg 100% DM	1.4	Z/002
	* Organic Carbon, % m/m air dried	1.64	PHYS/002
	* C:N Ratio	12	PHYS/002
25100819 / Sewage Sludge **SLCN1**			
	* Oven Dry Matter, % m/m	58.1	SAMP/015
	* Total Nitrogen (Kjeldahl), g/kg 100% DM	3.5	Z/002
	* Organic Carbon, % m/m air dried	28.3	PHYS/002
	* C:N Ratio	81	PHYS/002

* indicates that this determination is not included in the UKAS accreditation schedule for our laboratory.
[1] unless otherwise stated the results are expressed on an "as received" basis.
[2] this determination was sub-contracted.

For further information please contact your consultant Mr A Scott. Telephone 01902 743222.

Signed: Andrew Scott
Laboratory Manager

Reported on 25 January 2005 at 11:43 MSS

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CARDIFF

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Laboratory Report

Batch number : **02229927**
Date received : **17 January 2005**

Work code : **Not supplied**
Sample date : **14 January 2005**

Lab sample no. / Sample type Your reference	Determination [1]	Result	Method Ref
25100820 / Sewage Sludge **SLCN2**			
	* Oven Dry Matter, % m/m	61.4	SAMP/015
	* Total Nitrogen (Kjeldahl), g/kg 100% DM	3.0	Z/002
	* Organic Carbon, % m/m air dried	24.4	PHYS/002
	* C:N Ratio	81	PHYS/002
25100821 / Sewage Sludge **SLCN3**			
	* Oven Dry Matter, % m/m	60.5	SAMP/015
	* Total Nitrogen (Kjeldahl), g/kg 100% DM	3.5	Z/002
	* Organic Carbon, % m/m air dried	31.5	PHYS/002
	* C:N Ratio	90	PHYS/002

* indicates that this determination is not included in the UKAS accreditation schedule for our laboratory.
[1] unless otherwise stated the results are expressed on an "as received" basis.
[2] this determination was sub-contracted.

For further information please contact your consultant Mr A Scott. Telephone 01902 743222.

Signed: Andrew Scott
Laboratory Manager

Reported on 25 January 2005 at 11:43 MSS

Geo Enviro Research Centre
Cardiff School of Engineering
Queens Buildings, The Parade
Newport Road
CARDIFF

Laboratory Report

Batch number : **02267715**

Order number. : **Not supplied**

Date received : **4 August 2005**

Quotation : **Not Supplied**

Determination	C:N Ratio	Total Potassium	Ammonium-N	Total Nitrogen (Kjeldahl)
Units		mg/kg	mg/kg	g/kg
Basis [1]		100% DM	100% DM	100% DM
Method ref.	PHYS/002	ICP/003/016	H/047	Z/002
Lab sample no / Your reference / Sample date				
25601738 AG1 1 / 3 Aug 05	7	20800	3790	35.5
25601739 AG1 2 / 3 Aug 05	7	25800	3800	29.1
25601740 AG1 3 / 3 Aug 05	7	24200	4810	32.2
25601741 A1 / 3 Aug 05	6	21900	5070	30.4
25601742 A2 / 3 Aug 05	6	23100	5200	33.4
25601743 A3 / 3 Aug 05	6	26100	6540	36.6
25601744 S1 / 3 Aug 05		5630	78.0	3.8
25601745 S2 / 3 Aug 05		5580	50.0	3.6
25601746 S3 / 3 Aug 05		5740	48.0	2.4

[1] unless otherwise stated the results are expressed on an 'as received' basis.
[2] This determination was sub-contracted

For further information please contact your consultant Mr A Scott. Telephone 01902 743222

Authorised by: **Andrew Scott**
Laboratory Manager

Reported on 18 August 2005 at 07:33

Geo Enviro Research Centre
Cardiff School of Engineering
Queens Buildings, The Parade
Newport Road
CARDIFF

Laboratory Report

Batch number : **02267715**

Order number. : **Not supplied**

Date received : **4 August 2005**

Quotation : **Not Supplied**

Determination	Organic Carbon	Total Phosphorus
Units	% m/m	mg/kg
Basis [1]	air dried	100% DM
Method ref.	PHYS/002	ICP/003/016
Lab sample no / Your reference / Sample date		
25601738 AG1 1 / 3 Aug 05	24.9	10900
25601739 AG1 2 / 3 Aug 05	20.8	13200
25601740 AG1 3 / 3 Aug 05	22.6	12500
25601741 A1 / 3 Aug 05	17.0	12300
25601742 A2 / 3 Aug 05	20.2	11500
25601743 A3 / 3 Aug 05	23.7	12800
25601744 S1 / 3 Aug 05		930
25601745 S2 / 3 Aug 05		962
25601746 S3 / 3 Aug 05		1450

[1] unless otherwise stated the results are expressed on an "as received" basis.
[2] this determination was sub-contracted

For further information please contact your consultant Mr A Scott. Telephone 01902 743222.

Authorised by: **Andrew Scott**
Laboratory Manager

PRODUCT VALIDATION

Customer: **Geoenvironmental Research Centre**

Product Description: **Chicken Manure**

The attached report summarises data generated to confirm the distribution of absorbed dose across customer's product and to establish parameters to ensure that Isotron has irradiated goods within an agreed processing specification.

Relevant data is obtained by placing the appropriate type of perspex dosimeters in a pre-determined pattern across an irradiation container loaded with the customer's product. Dosimeter positions are chosen to ensure that the positions can be related to the absorbed dose throughout the product.

Loaded containers will be processed through the irradiation plant amongst products of approximate equal density. Key parameters of both the product being validated and the plant cycle used are recorded. After irradiation, dosimeters are recovered and the absorbed dose at each position on the product calculated. A Product Validation cannot be used to release Routine Processing, it can only be used to release the product it was validated on.

This data along with a three-dimensional illustration of dosimeter placement and absorbed doses calculated, are recorded in the attached report.

PRODUCT VALIDATION

Site:	Swindon	Date:	22.07.05
		A/C No:	GEO 002
Plant:	Auto	Ref No:	
Customer Name	Geoenvironmental Research Centre		
Type of Package:	Drum		
Product Description	Chicken Manure		
Dose Range Specifications:	Min: 25.0 kGy	Max:	40.0 kGy
Dimensions of Package:	200 x 230 (d)		mm
Weight of Package:	2.4 kg	Density:	0.29 g per cc
No of Packages/Irradiation Container:	22		
Plant Batch No:	S205 07 A569		
Dwell Time:	249 Secs		
Current Cobalt Loading:	1.697112	Mega Curies	
Standard Plant dwell Time:	235 Secs		

Dosimetry Results

Minimum Dose reading:	25.4 kGy	R to Min	Ratio 1: 0.78
Maximum Dose reading:	33.0 kGy	R to Max	Ratio 1: 1.02
Routine Dosimeter Reading:	32.4 kGy		

Comments
Insufficient product to validate a full tote

Signatures

Plant Manager/
Regional Manager

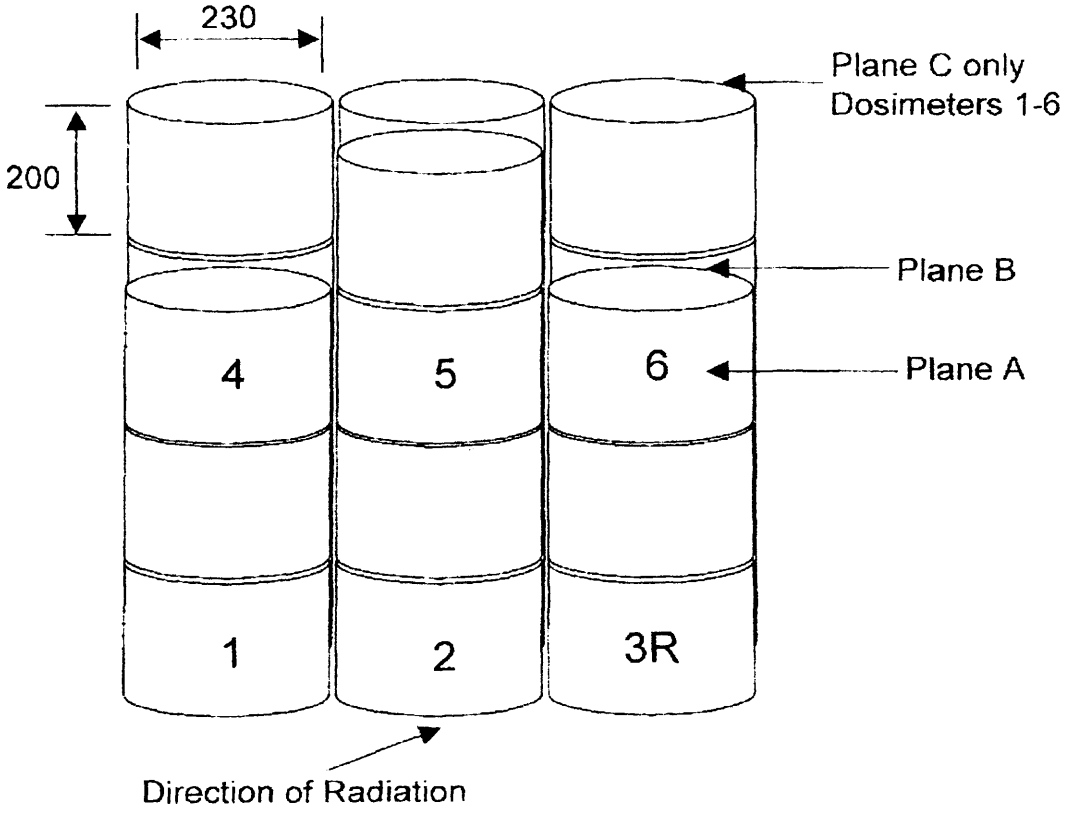
Date:

Approved:
QA Officer/
Quality Manager/Quality Engineer

LOADING DIAGRAM

Site	Swindon	Date:	22.07.05
		A/C No:	GEO 002
Plant	Auto	Ref No:	0
Customer Name	Geoenvironmental Research Centre		
Type of Package	Drum		
Product Description	Chicken Manure		
Plant Batch No:	S205 07 A569		
Fit per Tub	22		

R = Routine Dosimeter Position



Authorised By: Date:

DOSIMETER READINGS

Site Swindon

Date: 22.07.05
A/C No: GEO 002
Ref No: 0

Plant Auto

Customer Name Geoenvironmental Research Centre

Type of Package Drum

Product Description Chicken Manure

Plant Batch No: S205 07 A569

Fit per Tub 22

R = Routine Dosimeter Position

Number	Dose (kGy)	
3A	32.4	**R**
1B	25.4	
2B	25.4	
3B	27.8	
1C	31.5	
2C	33.0	
3C	31.5	
4C	30.4	
5C	30.3	
6C	31.1	

eurofins

GRC Cardiff University
FAO Kathryn Brice
5 The Parade
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GLAMORGAN
CF24 3AA

MICROBIOLOGY LABORATORY
CERTIFICATE OF ANALYSIS

Sample Date:01.06.06 Date Analysed:01.06.06
Batch No: 01789457 Date Completed:04.06.06
Order number

Lab Number:	Sample Type:	Sample Description:
26012797	Manure	Sample A

Determination	Result	Units	Method Ref.
Escherichia coli 44°C	< 10	cfu/g	Micro/027
Enterobacteriaceae 37°C	< 10	cfu/g	Micro/007
Aerobic Colony Count 30°C	< 10	cfu/g	Micro/001

26012798	Manure	Sample B

Determination	Result	Units	Method Ref.
Escherichia coli 44°C	< 10	cfu/g	Micro/027
Enterobacteriaceae 37°C	< 10	cfu/g	Micro/007
Aerobic Colony Count 30°C	< 10	cfu/g	Micro/001

26012799	Manure	Sample C

Determination	Result	Units	Method Ref.
Escherichia coli 44°C	< 10	cfu/g	Micro/027
Enterobacteriaceae 37°C	< 10	cfu/g	Micro/007
Aerobic Colony Count 30°C	< 10	cfu/g	Micro/001

P S Anderson

Signed: P.S.ANDERSON
Manager Microbiology Services

KEY:
< denotes less than
> denotes greater than
cfu = colony forming unit

copy to : P S Anderson

Date:05.06.06 Time:08:08:47

Page: 1 Last

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INVESTOR IN PEOPLE

APPENDIX 3

Pile Viability
Raw Data

pH data

Pile Temperature

Ambient Greenhouse Temperature

Carbon Dioxide Evolution
(example GC-TCD chromatograms on CD)

Treatability Study Raw Data

Pile Viability

Ph

	Tray Number															
Day	**1**	**1**	**2**	**2**	**3**	**3**	**4**	**4**	**5**	**5**	**6**	**6**	**7**	**7**	**8**	**8**
1	7.9	7.8	8	8.1	7.9	8	8.1	8.1	8.2	8.1	8.3	8.3	8.1	8.2	8	8
4	7.9	7.8	7.6	7.6	7.9	7.9	8	8	8	8	8	8	8	8	8.1	8.1
8	7.6	7.6	7.4	7.4	7.7	7.7	7.8	7.8	7.9	7.9	8.1	8.1	8.1	8.1	8.1	8.2
16	7.3	7.3	7.3	7.4	7.4	7.4	7.4	7.4	8	8	7.9	7.9	7.9	7.9	7.9	7.8
32	7.2	7.3	7.3	7.3	7.2	7.3	7.3	7.3	7.6	7.6	7.5	7.5	7.5	7.5	7.5	7.5
64	7.2	7.2	7.3	7.3	7.2	7.2	7.2	7.2	7.6	7.6	7.5	7.5	7.8	7.9	7.8	7.8
90	7.1	7.1	7.2	7.3	6.6	6.6	6.9	7	7.8	7.8	7.7	7.8	7.8	7.9	7.8	7.8

Treatability Study Raw Data
Pile Viability
Pile Temperature (degrees C)

Day →	1	2	3	4	7	8	9	10	12	16	19	20	23	24	27	28	30	31	32
Tray 1	22.3	21.6	21.7	21	22	20.6	22.8	21	21.3	23.7		24.8		23.2		23.2		22.4	
	21.9	21.3	21.5	20.9	21.6	20.7	22	20.6	21.4	23.7		24.2		22.6		23.4		22.6	
	22.5	21.3	21.7	21	21.6	20.8	22	20.9	21.7	23.6		24.3		22.3		23		22.4	
	22.7	20.9	22	20.8	21.6	20.7	22.1	20.6	21.2	23		24.5		22.5		23		22.3	
	22.6	21.1	21.7	21	21.6	20.9	21.8	20.5	21.2	23.1		24.5		22.6		23.1		22.5	
Tray 2	22.4	23	22.2	22	22.3	21.9	23	21.5	21.8	24.1		25		24.1		24		23.8	
	22.2	21.8	22.4	21.5	22.2	21.8	22.2	21.3	21.8	24		25		23.7		24.5		23.9	
	22.9	21.9	22.1	21.6	22	21.9	22.6	21.9	22.3	24.2		25.4		23.7		24.4		23.2	
	22.3	22.2	22.3	21.4	22	21.5	22.6	21.3	21.8	23.8		24.5		23.2		24		23.1	
	22.8	22.3	22	21.7	22.2	21.7	22.6	21.4	21.7	23.7		24.8		23.8		23.9		23.3	
Tray 3	21.5	20.9		20.9		22.7			23.1	22.1	23.2		22.3		23.5		23.6		23.4
	21.8	21.5		21.4		22.9			23.7	22.4	23.6		22.9		23.3		23.9		23.7
	21.8	22.2		21.9		23.2			23.8	22.2	23.6		22.5		23.6		24.2		24.4
	22.3	22.1		21.7		23			23.5	21.9	23.1		22.1		23.5		24		24
	21.8	21.1		21.3		22.9			23.2	22	23.5		22.7		23.5		23.8		23.5
Tray 4	22.2	21.3		21		22.6			23	22	23.2		22.4		23.2		23.8		24.1
	21.6	21.1		21		22.9			23.5	22.7	23.7		22.8		23.5		24.1		24.1
	21.8	21.6		21.7		22.9			23.6	22.4	24.1		22.9		23.3		24.1		24.6
	21.7	20.9		21.2		22.9			23.5	22.5	23.5		23.4		23.3		24		24.1
	21.8	21.1		21		22.7			23.7	22.3	23.5		22.2		23.2		23.9		24.2
Tray 5	28.1	50	44.6		27	25.8	26	24.8	24.3	26.8		26		24.2		26.6		24.3	
	28.1	49.9	49.8		27.7	26.6	26	24.8	24.3	26.4		26.2		24.3		25.3		24.2	
	26.3	48.9	39.7		27.5	27.3	26	25.7	24.8	26		25.7		24.3		25.3		24	
	27	50.2	29.2		26.8	26	25.8	24.9	24	25.6		25.6		24		25.1		24.3	
	27.3	51.2	38.5		26.1	25.5	25	24.3	24.4	25.1		25.9		24.2		25		24.1	
Tray 6	29	48.7	52.3	33.5	27.1	25.3	24.7	27.1	23.1	25.2		25.6		23.4		23.7		23	
	30	50	49.2	34	27.4	26.3	25.4	25.7	23.6	25.6		25.4		24		24		23.4	
	29.3	49.3	43.2	31.1	27	26.5	25.5	25	24.2	25.8		25.5		23.6		24.3		23.4	
	28.4	50.1	49.5	30.2	26.3	25.4	25.3	24.6	23.9	25.8		24.9		23.7		24		23.4	
	28.5	49.7	45.3	30.1	26.2	26.1	25.5	24.4	23.9	25.5		25.1		23.8		23.9		23.5	

Treatability Study Raw Data
Pile Viability
Pile Temperature (degrees C)

Day →	35	36	38	40	44	52	54	56	62	64	65	70	73	78	82	86	90
Tray 1	23.7		23.7	23.7	21.8	20.8			18.7	18.6			20.5	19.1		16.2	13.6
	23.7		23.6	23.7	22.1	20.4			18.5	18.4			20.8	18.9		16.7	13.9
	24.2		23.6	23.5	22.3	19.8			18	18.9			20.1	19.4		16.3	13.4
	23.7		23.4	23.7	21.9	19.9			18.1	18.2			19.8	18.7		16.4	13.9
	23.5		23.5	23.8	21.7	20.4			18.4	18.3			20.1	19.5		17	14.1
Tray 2	24.1		24.2	23.7	22.1	20.3			18.9	19.2			20.2	19.4		17.2	13.7
	24		24.2	24	22	20.2			18.6	19			20.2	19.2		16.6	13.9
	24.2		24.1	24	21.9	20			18.1	18.6			19.7	18.9		16.5	13.6
	24.1		24.1	24	22.1	19.7			18.2	18.4			19.9	18.7		16.4	14
	24.1		23.9	23.8	22.1	19.6			18.7	18.6			19.8	19.4		16.7	13.9
Tray 3		22.5			20.8		19.6	19.8			22.2	20		17.2	14.8		11.8
		22.6			20.5		19.6	19.5			22.1	20.1		17.1	15.1		12
		22.3			20.4		19	19			20.9	19.1		16.8	13.9		11.8
		22.1			20.8		19.5	18.9			22	19.7		17	14.4		11.7
		22.4			20.6		19.5	19.6			21.6	19.9		17	14.5		11.7
Tray 4		21.8			20.9		19.6	19.5			22.2	20.3		17.2	15.1		12.1
		22.1			20.9		19.2	19.9			22.3	20		17.1	14.7		12
		23.1			20.5		19.7	19			21.4	19.8		16.7	14.7		11.2
		22.6			20.9		19.4	19.9			21.8	19.9		17.2	15		11.6
		22.3			21		19.8	19.9			22	19.7		17.1	14.9		11.8
Tray 5	24.9		24.7	24.1	22.5	21.1			20.2	19.5			22	19.4		16.6	14.1
	24.8		24.9	24.3	22.9	21.2			20	19.8			21.1	18.8		16.4	13.8
	24.7		25.1	25	23.9	21.3			19.8	19.4			20.6	18.8		15.7	13.3
	25.5		25.1	24.4	23.2	21.4			19.8	19.7			21.1	18.8		16.2	13.5
	24.8		25.2	24.8	23.1	21.1			19.9	19.6			21	18.7		16.3	13.6
Tray 6	24.4		24.1	24.2	22.7	20.8			19.8	19.3			21.5	19		16.5	14.2
	24.7		24.4	24	22.7	20.6			19.8	19			21.6	19		16.3	13.8
	24.4		24.5	24	23.2	20.7			19.9	19.4			21.2	18.8		16	13.9
	24.4		24.4	23.8	22.6	21			19.5	19.5			21.4	19.1		16.1	13.7
	24.8		24.5	23.8	22.8	21.1			19.9	19.5			21.4	19		16.1	14

Treatability Study Raw Data

Pile Viability

Pile Temperature (degrees C)

Day →	1	2	3	4	7	8	9	10	12	16	19	20	23	24	27	28	30	31	32
Tray 7	24.8	40.5		28.7		26.2			25.9	23.4	24.6		23.3		24.3		25.1		24.6
	25.8	44.3		35.2		26.9			26.9	24.1	25.1		23.4		24.8		25.5		25.1
	25.6	42.4		33.3		26.9			26.9	23.9	25.9		23.6		24.9		25.6		25.8
	25.5	40.2		30.8		26			26.9	24.3	25.6		23.6		24.8		25.3		25.4
	25.4	41.8		33.9		25.8			26.3	24.5	25.5		23.5		24.3		25.4		25.3
Tray 8	25.1	41.2		33		24.7			26	24.5	23.7		23		23.9		24		24.2
	25.6	42.8		35.7		25.5			26.3	24.4	25.1		23.3		24.4		25		24.8
	25.6	42.3		33.3		26.8			26.2	24	25.5		23.6		24.2		25.2		25.1
	25.3	40.5		34.7		26.4			26	23.9	25.2		23.3		24.6		25.2		24.5
	25.2	42		34.7		26			26	23.8	24.8		23.3		24.5		25.1		24.7

Treatability Study Raw Data

Pile Viability
Pile Temperature (degrees C)

Day →	35	36	38	40	44	52	54	56	62	64	65	70	73	78	82	86	90
Tray 7		23.2			22.1		20.2	20.5			21.9	20.6		18.2	15.5		13.4
		23.4			21.7		20.1	20.4			22.8	20.5		18	15.3		13
		24.9			21		20.1	20			23	20.1		17.7	15		13.3
		23.8			21.6		20.3	20.4			22.9	20.6		17.9	15.1		13.6
		23.8			21.6		20.1	20.1			22.9	20.2		17.8	15.2		13.8
Tray 8		22.8			21.6		20.3	20.4			23.1	20		18.1	15.5		13.8
		23.4			21.6		20.2	20.6			21.9	20.1		18.1	15.2		13.9
		24.3			21.2		20	20.1			22.8	19.9		17.4	15.3		13.4
		24			21.7		19.9	20.6			23.1	20.1		17.8	15.2		13.6
		23.7			21.6		19.8	20.6			23	20		17.7	15.5		13.3

Treatability Study Raw Data
Pile Viability
Greenhouse Ambient Temperature

UMC, TMT Conditions		MC, SMT Conditions	
Day	Temperature	Day	Temperature
1	29	1	23.75
2	26	2	21.5
3	28.5	3	
4	25.75	4	
6	25.5	6	
7	26.5	7	
8	27.25	8	24.75
9	23.75	9	
10	21.5	10	
12		12	24.5
16	24.75	16	24
20	24.5	20	23.75
23		23	23.5
24	24	24	
27		27	23.75
28	23.75	28	
30		30	23.75
31	23.5	31	
32		32	23.25
35	23.75	35	
36		36	21.5
38	23.75	38	
40	23.25	40	
44	21.5	44	23
52	23	52	
54		54	20
58		58	15
59		59	16.5
62	20	62	
65		65	23.5
66	15	66	
67	16.5	67	
70		70	23
73	23.5	73	
78	23	78	20.5
82		82	19
86	20.5	86	15.5
90	19	90	13.5

Treatability Study Raw Data
Pile Viability
Microbial Respiration by Carbon Dioxide Evolution

Peak integration and evolution rate calculation

Day	Tray	Avg DW	Time Start	Time Finish	hrs	Mins	Total Mins	CO_2 Start ppm	CO_2 Finish ppm	Total CO_2 ppm	CO_2 ppm/min/g
1	T1	6.20	14.3	14.15	23	45	1425	11885.34	66089.95	54204.61	6.13
	T2	6.00	14.5	14.39	23	49	1429	14683.03	68744.90	54061.87	6.30
	T3	3.13	9.38	14.5	5	12	312	350.00	7471.53	7121.53	7.30
	T4	3.04	9.52	15.07	5	15	315	350.00	8228.75	7878.75	8.22
	T5	5.42	15.15	14.55	23	40	1420	40975.81	150180.00	109204.19	14.19
	T6	5.86	15.37	15.1	23	33	1413	54560.95	169150.00	114589.05	13.83
	T7	3.07	10.13	15.28	5	15	315	350.00	12099.64	11749.64	12.14
	T8	3.00	10.28	15.43	5	25	325	350.00	10877.34	10527.34	10.81
8	T1	3.40	12.2	17.2	5	0	300	350.00	11176.44	10826.44	10.61
	T2	3.42	12.34	17.34	5	0	300	350.00	11257.42	10907.42	10.62
	T3	3.55	11.51	17.04	5	13	313	350.00	10133.81	9783.81	8.81
	T4	3.74	12.07	17.19	5	12	312	350.00	11592.28	11242.28	9.63
	T5	3.28	12.48	17.48	5	0	300	350.00	37803.60	37453.60	38.06
	T6	3.51	13.01	18.01	5	0	300	350.00	24943.45	24593.45	23.36
	T7	3.58	12.23	17.47	5	24	324	350.00	16077.31	15727.31	13.57
	T8	3.39	12.37	18.15	5	38	338	350.00	16838.36	16488.36	14.41
16	T3	3.62	9.26	15.37	6	11	371	350.00	26110.11	25760.11	19.18
	T4	3.43	9.28	15.52	6	24	384	350.00	24543.54	24193.54	18.38
	T7	3.43	9.31	16.08	6	37	397	350.00	46949.68	46599.68	34.19
	T8	3.74	9.34	16.24	6	50	410	350.00	57489.56	57139.56	37.22
24	T3	3.39	8.45	14.17	5	32	332	350.00	21979.75	21629.75	19.24
	T4	3.61	8.48	14.31	5	43	343	350.00	21983.17	21633.17	17.46
	T7	3.29	8.5	14.46	5	56	356	350.00	79779.80	79429.80	67.84
	T8	3.29	8.53	15	6	7	367	350.00	78951.36	78601.36	65.12
32	T1	4.24	8.32	13.1	4	38	278	350.00	18533.17	18183.17	15.42
	T2	4.10	8.35	13.25	4	50	290	350.00	17641.67	17291.67	14.54
	T5	3.23	8.39	13.44	5	5	305	350.00	39545.89	39195.89	39.73
	T6	3.42	8.42	14	5	18	318	350.00	34568.66	34218.66	31.48

Peak integration and evolution rate calculation - continued

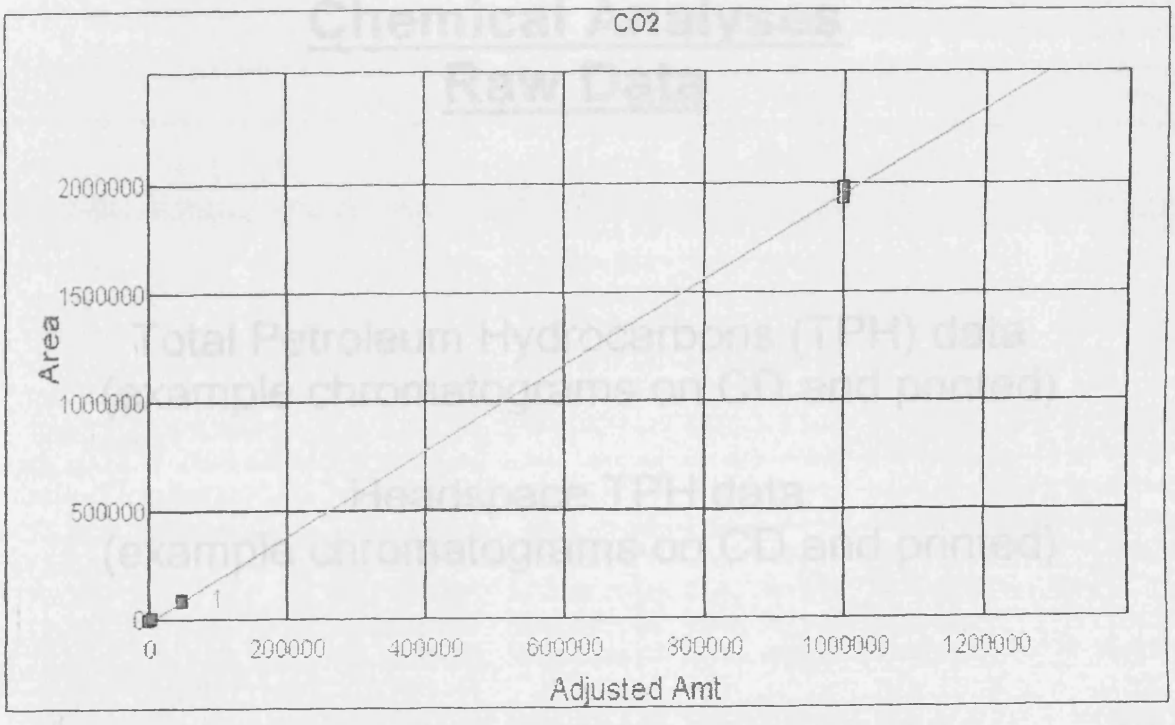
Day	Tray	Avg DW	Time Start	Time Finish	hrs	Mins	Total Mins	CO2 Start	CO2 Finish	Total CO2	CO_2 ppm/min/g
43	T3	4.14	8.43	13.53	5	10	310	350.00	7263.20	6913.20	5.38
	T4	4.26	8.46	14.07	5	21	321	350.00	7222.48	6872.48	5.02
	T7	3.47	8.58	14.51	5	53	353	350.00	17956.50	17606.50	14.37
	T8	3.42	9	15.04	6	4	364	350.00	18022.61	17672.61	14.18
58	T3	4.08	9.44	14.51	5	7	307	350.00	9876.24	9526.24	7.60
	T4	3.83	9.46	15.06	5	20	320	350.00	10547.30	10197.30	8.32
	T7	3.38	9.55	15.48	5	53	353	350.00	10386.21	10036.21	8.40
	T8	3.41	9.59	16.05	6	6	366	350.00	10671.73	10321.73	8.28
64	T1	4.38	9.38	14.24	4	46	286	350.00	3334.93	2984.93	2.38
	T2	4.48	9.41	14.37	4	56	296	350.00	2909.38	2559.38	1.93
	T5	3.55	9.49	15.22	5	33	333	350.00	23729.17	23379.17	19.79
	T6	3.60	9.53	15.36	5	43	343	350.00	28552.57	28202.57	22.83
79	T3	3.73	8.4	13.29	4	49	289	350.00	10885.84	10535.84	9.78
	T4	3.82	8.42	13.42	5	0	300	350.00	9420.63	9070.63	7.91
	T7	3.39	8.51	14.25	5	34	334	350.00	9975.55	9625.55	8.51
	T8	3.29	8.53	14.39	5	46	346	350.00			0.00
87	T1	4.79	8.34	13.01	4	27	267	350.00	1445.95	1095.95	0.86
	T2	4.63	8.37	13.15	4	38	278	350.00	1213.87	863.87	0.67
	T5	3.44	8.44	14.11	5	27	327	350.00	10883.31	10533.31	9.37
	T6	3.20	8.51	14.25	5	34	334	350.00	7561.31	7211.31	6.74

Treatability Study Raw Data
Pile Viability
Microbial Respiration by Carbon Dioxide Evolution
Carbon Dioxide Evolution Rates (ppm min^{-1} g^{-1} soil dry weight)

Day	T1		T2	T3	T4	T5	T6	T7	T8
1		6.13	6.30	6.43	7.05	14.19	13.83	9.56	8.52
8		10.27	10.24	8.70	9.20	34.49	20.64	12.08	12.91
16				19.18	18.38			34.19	37.22
24				19.24	17.46			67.84	65.12
32		15.42	14.54			39.73	31.48		
43				5.38	5.02			14.37	14.18
58				7.60	8.32			8.40	8.28
64		2.38	1.93			19.79	22.83		
79				9.78	7.91			8.51	
87		0.86	0.67			9.37	6.74		

CO2 Calibration Curve for Microbial Respiration Measurements

CO2 Standard	CO2 Measurement 1	CO2 Measurement 2
500	846	665
5000	8541	9170
50000	85491	94220



$y = (-1095.9) + (1.945)\ xR2=0.999783$

APPENDIX 4

Chemical Analyses
Raw Data

Total Petroleum Hydrocarbons (TPH) data
(example chromatograms on CD and printed)

Headspace TPH data
(example chromatograms on CD and printed)

Treatability Study Raw Data
Chemical Analyses - TPH Analyses
Fractionated Aromatic Hydrocarbons - Raw Data

Sample	C9-10 Total in 6.5cm3	C10-12 Total in 6.5cm3	C12-16 Total in 6.5cm3	C16-22 Total in 6.5cm3	C22-36 Total in 6.5cm3	Dry Weight g soil
T1:1	55.51224554	425.8785607	3856.250657	7145.422375	4029.050314	6.002122034
T1:1B	74.87036194	487.6537262	4053.817315	7997.179496	4696.335973	6.311948052
T1:2	17.08069094	204.683613	3109.255107	5927.284433	3337.567009	6.491386382
T1:2B	0	177.3545076	2569.505273	5067.556322	2761.663046	6.740464826
T1:4	0	0	223.1876949	1021.425318	945.1315651	6.623317203
T1:4B	22.77425458	150.3100802	2317.84976	4427.030412	2965.777303	6.76632803
T1:8	21.63554185	293.4462703	2925.23913	4729.017028	2198.569602	6.977102
T1:8B	0	82.55667286	1857.809817	3485.884342	1376.988368	6.655454466
T1:16	0	81.98731649	2253.797169	4563.391262	1731.128026	6.849240765
T1:16B	0	135.5068148	2390.442696	4837.251673	3114.663992	7.652774068
T1:32	3.700816369	75.15504012	3343.829929	6556.138538	2097.224169	8.128649463
T1:32B	0	75.4397183	3358.917873	6387.039697	1971.111734	8.257777423
T1:64	0	36.01179006	2932.754634	7876.760625	9803.320224	8.788806818
T1:64B	0	30.88758278	2708.001209	7489.882975	9059.883151	8.763247513
T1:90	0	0	2112.169773	5647.161102	5168.047721	7.66332167
T1:90B	0	0	2847.635857	7158.802249	6309.607232	8.419617284
T2:1	10.24841456	109.6011002	2036.01836	4183.915245	2239.278582	6.23163521
T2:1B	0	79.42521285	1784.932203	3790.774675	2007.265863	6.130193147
T2:2	23.91296731	225.4651204	2644.944991	4878.245331	2777.605024	6.37249706
T2:2B	37.00816369	296.0653096	2747.144459	4901.019586	2784.437301	6.648198154
T2:4	19.92747276	292.9338495	3734.977751	7383.982692	5536.421289	6.731231685
T2:4B	26.19039277	318.8395641	3923.15003	7526.891139	5259.714096	6.463525653
T2:8	0	244.8232367	2681.668477	4692.919835	2084.413651	6.751803304
T2:8B	0	295.7806314	3516.629586	6142.501139	2662.879717	6.974121884
T2:16	0	315.9927823	3639.61056	7052.047931	2638.682071	7.17245443
T2:16B	67.4687292	583.0209173	5090.330577	8898.470621	5660.540976	7.20440273
T2:32	4.270172734	162.2665639	4727.935251	8657.063523	2961.791808	8.635248722
T2:32B	0	102.1994674	4136.943345	7832.920185	2577.76094	8.42522706
T2:64	0	78.00182194	3774.548019	9469.962072	10722.54607	9.011921605
T2:64B	0	54.23119372	3591.642287	9411.176027	10232.61492	8.927378891
T2:90	1.565730002	49.53400371	3617.832679	9516.933972	8660.622	9.063338023
T2:90B	0	53.80417645	3542.677639	8938.610245	8074.469623	9.00173886
T3:1	44.12511825	280.6926877	2450.225115	4129.82639	1640.031008	5.984231868
T3:1B	42.41704916	282.5146281	2001.743106	3048.163169	1406.367156	5.971090591
T3:2	0	249.3780877	2740.027504	4813.338706	1925.563225	6.338640408
T3:2B	44.9791528	273.291055	2960.368417	5302.131145	2039.719176	6.262266667
T3:4	31.02992187	324.5331278	3683.735678	5701.534634	2616.192495	6.73390223
T3:4B	30.4605655	409.3672261	3880.732981	5224.414001	3397.918784	6.718396603
	0	208.6691076	3171.599629	5822.80754	2122.845205	7.105392882
	0	228.027224	2984.281385	4020.22529	1801.158859	6.595547214
T3:16	0	85.97281104	2802.087348	5031.686872	2260.344767	7.205836465
T3:16B	3.700816369	98.49865106	2308.170702	5033.110262	1029.965663	7.044648285
T3:32A	0	44.40979643	2545.022949	5101.290687	1622.665639	8.053236486
T3:32B	0	31.02992187	2527.088224	4828.568988	1563.452577	7.695478123
T3:64	0	0	2027.335675	0	5054.176448	7.405076568
T3:64B	0	0	1646.578606	4736.760275	4960.802004	7.473311824
T3:90	0	0	0	0	0	7.843755976
T3:90B	3.273799096	0	1797.173365	4736.760275	10971.07013	7.801676444

Fractionated Aromatic Hydrocarbons - Raw Data

continued...

Sample	C9-10 Total in 6.5cm3	C10-12 Total in 6.5cm3	C12-16 Total in 6.5cm3	C16-22 Total in 6.5cm3	C22-36 Total in 6.5cm3	Dry Weight g soil
T4:1	32.45331278	163.6899548	1882.292141	3731.846291	2251.804422	6.618576483
T4:1B	54.54433972	322.2557023	2256.700886	3997.337164	3074.809047	5.985650605
T4:2	21.63554185	253.078904	2822.299499	4953.400371	1947.198767	6.360282553
T4:2B	9.109701832	187.0335657	2857.314915	5064.994219	1870.905014	6.377970395
T4:4	0	284.6781823	2962.930521	5331.452997	2150.743667	6.40529703
T4:4B	0	260.4805368	2790.415543	5117.659683	1952.607652	6.372714797
T4:8	0	196.9973021	3207.184401	5893.407729	2314.433622	6.585033557
T4:8B	25.6210364	208.9537858	2929.053817	5419.133878	2117.43632	6.64807239
T4:16	4.839529098	195.0045549	3152.52619	5355.650643	2759.385621	7.014465798
T4:16B	0	131.5213202	2659.178901	4592.997793	1286.176027	6.964357802
T4:32	0	37.29284188	2700.741915	5474.646123	1759.026488	7.449380064
T4:32B	0	57.50499282	3243.907887	6372.236432	2088.968501	7.533001722
T4:64	0	0	2029.328422	5764.590852	5844.585421	7.702604284
T4:64B	0	0	1732.836095	5151.394047	5018.164658	7.506662352
T4:90	3.416138187	0	1426.380032	4280.563488	4035.597912	7.336440353
T4:90B	4.127833643	0	1692.411794	4900.023212	4636.695894	6.783483376
T5:1	0	29.17951368	572.4878245	1296.282103	824.0009986	5.248225962
T5:1B	10.10607547	104.1922147	1163.62207	2143.342034	1224.68554	5.189083395
T5:2	0	0	369.0852633	786.1388003	437.408027	5.854507859
T5:2B	0	23.05893276	550.9946218	1141.132494	628.7117655	5.878612556
T5:4	0	46.2317368	905.9029116	1753.845345	1049.039102	5.773024214
T5:4B	0	14.63245857	606.0229144	1278.375845	877.6628359	6.533864542
T5:8	0	36.09719351	885.2352756	1626.024841	739.6508532	6.543312754
T5:8B	0	27.44297677	750.1839459	1398.794716	642.5186574	6.623267851
T5:16	0	19.30118076	1039.53085	2091.98609	972.1190568	7.355981091
T5:16B	0	0	843.6437931	1939.085438	715.1115938	7.255120013
T5:32	2.989120914	0	589.8531937	1241.196875	367.3771942	6.74404199
T5:32B	0	0	799.5186749	1629.355576	511.1396763	6.500283395
T5:64	0	0	589.3977086	1666.961564	1820.516976	7.247888452
T5:64B	0	0	518.7405837	1483.344137	1667.473985	7.002039244
T5:90	0.313146	0	402.335675	1262.319996	1263.629515	7.328499299
T5:90B	0.740163274	0	289.9731965	903.5685505	853.4082548	6.559133042
T6:1	2.419764549	20.06981185	390.2937879	928.1932133	499.4678708	5.896790757
T6:1B	2.846781823	22.06255913	423.1741179	932.463386	514.4134754	4.283826495
T6:2	0	0	110.0281174	388.4433797	209.5231421	6.692581559
T6:2B	3.53000946	37.23590624	479.3411233	929.1895869	467.7831891	6.083203484
T6:4	0	69.11986265	954.7536877	1539.084125	656.7525665	6.921531725
T6:4B	0	106.1280263	1265.451456	2088.626888	986.5807085	9.656037307
T6:8	1.081777093	56.87870082	1077.620791	1956.991696	921.6740829	6.732654099
T6:8B	0	67.29792229	1092.025507	1919.926597	893.2632003	7.371074948
T6:16	0	31.82702078	1023.588872	1886.391507	831.2602922	6.891323851
T6:16B	0	18.78876003	1001.213167	1926.559598	612.0580919	7.470640375
T6:32	0	0	747.8495848	1414.565888	422.7471007	3.518499973
T6:32B	0	0	1108.109824	2026.339301	652.1977156	7.094018758
T6:64	0	0	806.9772433	2037.527154	2148.836323	7.232799499
T6:64B	0	8.170263831	876.239445	2203.950019	2404.135717	7.19965187
T6:90	0	0	705.6318104	2013.898865	2179.41076	6.80784121
T6:90B	0	0	832.6552153	2439.635086	2464.003539	6.797672094

Fractionated Aromatic Hydrocarbons - Raw Data

continued...

Sample	C9-10 Total in 6.5cm3	C10-12 Total in 6.5cm3	C12-16 Total in 6.5cm3	C16-22 Total in 6.5cm3	C22-36 Total in 6.5cm3	Dry Weight g soil
T7:1	4.042430188	47.4843208	530.9248099	916.0374549	363.5909744	6.143442721
T7:1B	4.782593462	39.45639606	429.1238919	752.5752426	309.7867979	6.157688794
T7:2	5.864370555	58.98531937	753.6000841	1493.706422	1065.038015	6.63474042
T7:2B	6.889212011	63.48323464	739.8785957	1421.227357	1002.124137	6.674898702
T7:4	0	64.76428647	1024.699117	2110.177026	1308.523265	6.878773469
T7:4B	0	48.67996917	910.9701832	1828.772643	1108.394503	7.042030249
T7:8	1.850408185	49.39166462	1007.903104	1885.138923	655.1868365	6.577116695
T7:8B	1.992747276	52.38078554	1134.442556	2076.585001	743.1523948	7.165565647
T7:16	0	42.55938825	1048.612084	1800.020146	552.5603518	7.395833333
T7:16B	1.992747276	62.77153919	1376.276672	2252.516117	710.1297257	6.851110568
T7:32	0	19.07343821	955.3799797	2216.902877	1708.923128	7.048236536
T7:32B	0	0	715.9086928	1655.745244	1696.340352	6.839147433
T7:64	0.341613819	14.94560457	719.7518482	1746.187502	1791.109719	6.887353096
T7:64b	0	0	865.2508672	2124.069321	1866.435566	5.92800216
T7:90	0.654759819	0	662.8731474	1712.567009	1660.584773	7.091261209
T7:90B	0.48395291	0	716.2503066	1850.778266	1808.418153	7.384487388
T8:1	8.881959287	82.44280158	851.9848639	1444.115483	622.5911846	6.062817824
T8:1B	7.401632739	64.22339792	653.848849	1146.228233	477.4053117	6.559648345
T8:2	14.23390911	127.4219544	1174.525244	2009.144739	911.2548614	6.600731558
T8:2B	7.23082583	68.37969938	767.3215725	1344.136505	560.9298903	6.190068822
T8:4	6.205984373	76.12294594	855.7995515	1463.188921	653.5641708	7.082368827
T8:4B	9.963736379	112.7894958	1119.240741	1907.628499	896.4515959	6.835549806
T8:8	3.302266914	78.11569321	1092.822606	1883.886339	852.0987352	6.910481061
T8:8B	0	54.37353281	781.498546	1352.676851	582.8501104	6.713719116
T8:16	0	77.0054483	1871.047353	3010.756456	1009.611173	7.580633694
T8:16B	4.270172734	124.6890438	2642.382888	4221.208087	1438.051838	7.708231139
T8:32	0	18.44714621	865.592481	2045.583546	2033.740934	7.021372845
T8:32B	0	16.51133457	930.897656	2185.92989	2247.192635	7.199319767
T8:64	0.654759819	4.697190007	548.7171963	1404.915297	1443.944676	7.096210904
T8:64b	0.512420728	0	505.1614344	1278.632056	1285.834414	6.78139778
T8:90	0.597824183	0	634.0352475	1636.785677	1474.376774	6.652919911
T8:90B	0	3.21686346	728.6622753	1933.733489	1675.558845	6.958980155

Treatability Study Raw Data
Chemical Analyses - TPH Analyses
Fractionated Aliphatic Hydrocarbons - Raw Data

Sample	C9-10 Total in 6.5cm3	C10-12 Total in 6.5cm3	C12-16 Total in 6.5cm3	C16-22 Total in 6.5cm3	C22-36 Total in 6.5cm3	DW
T1:1	802.2231176	2518.832557	10800.40556	14923.11499	8133.540345	6.002122034
T1:1B	865.9910304	2645.514348	11290.33671	16553.46694	9357.656529	6.311948052
T1:2	571.3491118	1952.89233	10562.98395	14875.00438	8060.378053	6.491386382
T1:2B	474.2738517	1511.925826	7778.261974	10508.8951	5501.69055	6.740464826
T1:4	769.2004485	3111.247854	16485.42886	22955.02523	17598.52055	6.623317203
T1:4B						6.76632803
T1:8	432.5969658	1437.681756	7360.012789	9238.604113	4965.926211	6.977102
T1:8B	325.6718405	918.6564942	5517.34785	6989.703409	2684.799937	6.655454466
T1:16	264.1813531	1072.667391	7084.501244	9727.16881	3546.805473	6.849240765
T1:16B	177.0698294	797.6682667	6084.142111	8404.269297	2873.541572	7.652774068
T1:32	152.0181493	802.5077958	9841.894117	13642.63253	4293.801023	8.128649463
T1:32B	175.3617603	1092.025507	9982.240461	13812.01605	4494.78382	8.257777423
T1:64	28.75249641	629.423461	8427.043551	15530.90291	16312.91388	8.788806818
T1:64B	10.39075365	554.2684209	8218.089766	15531.75695	15805.048	8.763247513
T1:90	0	339.7634105	6165.133054	11663.97682	9183.148803	7.66332167
T1:90B	0	431.4297852	7447.323587	13348.98698	10528.82257	8.419617284
T2:1	375.7752006	1217.283907	6469.881048	8451.241197	4280.421149	6.23163521
T2:1B	325.6718405	1064.696402	5702.958025	7878.468694	3972.114677	6.130193147
T2:2	560.8160191	1921.008374	9123.935742	12877.70225	6870.992607	6.37249706
T2:2B	856.5966504	2847.920535	12365.5662	17074.99737	9244.639291	6.648198154
T2:4	714.8269157	2683.945902	12393.46466	17259.46883	12131.27606	6.731231685
T2:4B	821.8659122	3208.607792	14652.67072	20167.17179	14484.14124	6.463525653
T2:8	537.1877299	1828.203287	8365.268386	10269.76543	4003.713955	6.751803304
T2:8B	701.4470411	2214.226902	10934.2043	13609.89454	6071.616271	6.974121884
T2:16	793.1134158	2378.486213	10704.75369	14214.83567	5531.58176	7.17245443
T2:16B	858.0200413	2770.772748	12684.97512	16781.20949	7371.456852	7.20440273
T2:32	158.8504257	1274.219544	13390.69234	18426.93406	6229.612662	8.635248722
T2:32B	140.0616657	1308.096248	12806.24803	17833.66473	5931.839284	8.42522706
T2:64	15.51496093	717.5313584	10937.33576	19830.11282	21921.64343	9.011921605
T2:64B	8.540345468	618.7480292	9886.87327	18232.92588	17447.07176	8.927378891
T2:90	0	674.1179356	9891.855138	18469.35111	15121.25101	9.063338023
T2:90B	0	750.1270103	9934.556866	18241.7509	14717.15033	9.00173886
T3:1	702.870432	1609.855121	6939.315371	8558.564872	3373.151782	5.984231868
T3:1B	415.2315967	1175.948635	4995.361935	5848.997933	2911.40377	5.971090591
T3:2	698.6002593	1864.357416	8417.649171	10740.33846	4346.751165	6.338640408
T3:2B	710.8414211	1934.388248	9063.583967	11126.93143	4553.142847	6.262266667
T3:4	703.1551102	2207.679303	10973.77457	13665.40678	6118.018815	6.73390223
T3:4B	858.5893977	2778.174381	13470.40223	17098.62566	7298.863915	6.718396603
T3:8	666.7163029	2086.691076	10025.51154	12943.46291	4962.794751	7.105392882
T3:8B	594.4080446	1823.079079	8735.065345	11381.14905	4407.387618	6.595547214
T3:16	236.2828913	1274.219544	9397.796153	13045.66238	5074.957955	7.205836465
T3:16B	0	1171.45072	7023.010756	8411.101573	2230.453558	7.044648285
T3:32	183.3327494	932.0363687	8512.162328	11707.9596	3990.618759	8.053236486
T3:32B	112.447882	876.8088014	8555.148733	11333.03844	3332.72748	7.695478123
T3:64	6.547598192	297.4887005	5338.996969	9746.669265	8803.530447	7.405076568
T3:64B	6.405259101	277.8459059	4903.581689	9443.487001	8611.515013	7.473311824
T3:90	0	0	0	0	0	7.843755976
T3:90B	9.109701832	274.0027504	5740.108528	10666.32213	10542.62946	7.801676444

Fractionated Aliphatic Hydrocarbons - Raw Data

continued...

Sample	C9-10 Total in 6.5cm3	C10-12 Total in 6.5cm3	C12-16 Total in 6.5cm3	C16-22 Total in 6.5cm3	C22-36 Total in 6.5cm3	DW
T4:1	662.7308083	2168.963071	8773.496899	11364.92239	8142.650047	6.618576483
T4:1B	595.0343366	1949.590063	7610.017168	9982.411268	7450.540451	5.985650605
T4:2	691.4833047	1900.226867	8616.069864	11018.75372	4274.727585	6.360282553
T4:2B	615.4742301	1693.835184	7869.074314	10145.36106	3824.082022	6.377970395
T4:4	698.0309029	2036.303038	9218.16422	12131.27606	4556.274307	6.40529703
T4:4B	690.9139484	2046.83613	9390.963877	12389.47917	4765.228093	6.372714797
T4:8	612.6274482	1931.826145	9632.086297	12687.25255	4906.71315	6.585033557
T4:8B	546.0127536	1728.281245	8835.272065	11800.19533	4564.245296	6.64807239
T4:16	242.8304895	1269.664693	8677.84503	10624.18976	2918.236046	7.014465798
T4:16B	166.5367366	919.7952069	4633.422095	5193.099401	1036.228583	6.964357802
T4:32	71.45422375	730.7688939	8548.031779	12179.95603	3615.697593	7.449380064
T4:32B	175.0770821	984.7018325	9074.971094	12595.01682	4360.700396	7.533001722
T4:64	7.828650012	385.0272415	6234.73687	11615.01218	10254.53514	7.702604284
T4:64B	8.113328195	317.7008514	5248.184629	9989.072737	8847.228548	7.506662352
T4:90	0	206.107004	4016.809152	7567.45778	7892.417925	7.336440353
T4:90B	0	251.0861568	4472.8636	8357.866753	8115.890298	6.783483376
T5:1	100.7760765	387.0199888	2145.61946	3132.883396	1691.842437	5.248225962
T5:1B	246.6736449	980.4316597	4585.026804	6437.712414	3568.013997	5.189083395
T5:2	16.65367366	196.854963	1849.981167	2893.611384	1381.827897	5.854507859
T5:2B	48.25295189	311.8649487	2405.53064	3656.83359	1865.638467	5.878612556
T5:4	18.73182439	277.5612277	2630.426404	3919.733892	2673.697488	5.773024214
T5:4B	8.938894923	183.1050068	1771.381521	2771.342104	1873.69486	6.533864542
T5:8	8.369538559	188.4569567	2242.808591	3360.91062	1563.28177	6.543312754
T5:8B	13.66455275	157.4270348	1915.827231	2929.907852	1415.932343	6.623267851
T5:16	3.074524368	136.8163344	2395.851582	4158.40808	1945.262955	7.355981091
T5:16B	3.416138187	103.3381802	2283.546039	4197.722137	1578.255842	7.255120013
T5:32	0	75.29737921	1199.064504	2467.163467	838.661925	6.74404199
T5:32B	33.7343646	47.39891735	1555.623927	3039.224274	1062.988333	6.500283395
T5:64	0	0	762.1973652	2297.75148	2645.48588	7.247888452
T5:64B	3.302266914	14.5185873	797.0989103	2235.264619	2617.30274	7.002039244
T5:90	0	0	588.6290775	1680.882327	1833.156687	7.328499299
T5:90B	0	0	433.6787429	1221.013192	1223.888441	6.559133042
T6:1	46.82956098	216.9247749	1482.888651	2208.106321	1019.57491	5.896790757
T6:1B	69.0344592	277.5612277	1634.622123	2417.487124	1083.200484	4.283826495
T6:2	20.21215094	77.57480467	796.9565712	1370.583109	1542.67107	6.692581559
T6:2B	36.32493606	235.1441786	1498.033531	2059.76052	1152.377282	6.083203484
T6:4	27.9553975	354.9367576	2482.621492	3292.872534	1339.695526	6.921531725
T6:4B	54.48740409	508.4352335	3485.314985	4631.827897	2084.015101	9.656037307
T6:8	13.77842402	269.533303	2906.45037	4255.824954	2009.714096	6.732654099
T6:8B	10.24841456	197.3958516	2405.587576	3692.560702	1785.444624	7.371074948
T6:16	0	172.8565923	2128.709576	3539.80239	1544.777688	6.891323851
T6:16B	11.10244911	160.1314775	2546.873358	4218.645983	1601.741793	7.470640375
T6:32	37.00816369	65.33364283	1578.825199	2797.390158	890.4733541	3.518499973
T6:32B	0	114.2982902	2355.000263	4100.504537	1396.061806	7.094018758
T6:64	0	0	994.637101	2801.318717	2823.836761	7.232799499
T6:64B	18.07706457	81.93038086	1507.541782	3658.086174	4449.320714	7.19965187
T6:90	2.789846186	13.86382748	1185.172208	3102.052749	3267.507708	6.80784121
T6:90B	0	15.51496093	1134.869574	3098.266529	3164.14106	6.797672094

Fractionated Aliphatic Hydrocarbons - Raw Data

continued...

Sample	C9-10 Total in 6.5cm3	C10-12 Total in 6.5cm3	C12-16 Total in 6.5cm3	C16-22 Total in 6.5cm3	C22-36 Total in 6.5cm3	DW
T7:1	89.61669178	347.5920605	1746.102099	2160.536596	925.0332854	6.143442721
T7:1B	68.15195683	275.9670299	1447.987106	1799.906275	682.2597316	6.157688794
T7:2	93.60218633	506.0439368	2566.601556	3509.227953	2375.52556	6.63474042
T7:2B	77.60327249	475.9249851	2428.304895	3279.49266	2208.476402	6.674898702
T7:4	191.5884167	1092.594864	5634.635262	7791.499509	5130.470201	6.878773469
T7:4B	200.5557794	1047.188693	5245.622525	7248.760555	5136.306104	7.042030249
T7:8	47.68359553	442.1052171	2982.857994	4269.745717	1415.846939	6.577116695
T7:8B	85.83047195	481.9601626	3103.134526	4338.21082	1487.158824	7.165565647
T7:16	83.12602922	438.1197225	3087.050208	4337.926141	1318.202323	7.395833333
T7:16B	108.8894047	446.8024071	3159.073789	4350.451981	1401.755369	6.851110568
T7:32	1.821940366	179.9735468	2588.407904	5291.142567	4258.443993	7.048236536
T7:32B	4.611786553	48.79384044	1203.334676	2761.036754	2560.224764	6.839147433
T7:64	0.711695456	55.62611681	1126.898584	2709.851617	2499.360569	6.887353096
T7:64B	0	75.46818612	1502.816124	3411.839547	3175.528188	5.92800216
T7:90	0	17.22303003	638.6470341	1642.450773	1482.205424	7.091261209
T7:90B	0	8.255667286	1046.334659	2641.898935	2590.42912	7.384487388
T8:1	154.7510599	543.279843	2573.092218	3146.718755	1354.38492	6.062817824
T8:1B	134.0264882	472.4519113	2236.659542	2826.512736	1189.214639	6.559648345
T8:2	182.0232297	740.2202095	3589.336393	4590.435689	2040.744017	6.600731558
T8:2B	107.8930311	461.0078484	2441.172349	3162.31912	1372.547388	6.190068822
T8:4	83.80925686	461.5772047	2670.452157	3589.108651	1486.646403	7.082368827
T8:4B	128.6176027	650.2049683	3415.511895	4530.083914	1837.142181	6.835549806
T8:8	64.56501174	493.005676	3050.554466	4182.207176	1806.795487	6.910481061
T8:8B	61.14887355	374.1810028	2245.370695	3063.820469	1977.773203	6.713719116
T8:16	83.83772468	569.4987036	4823.729459	6483.68794	2235.150748	7.580633694
T8:16B	0	0	0	0	0	7.708231139
T8:32	0	96.16428997	1793.814162	3906.012403	3719.434323	7.021372845
T8:32B	4.668722189	75.72439648	1643.447146	3674.853719	3438.456957	7.199319767
T8:64	1.76500473	20.41142567	832.9683613	2126.602957	1887.587155	7.096210904
T8:64b	2.163554185	27.6422515	807.2049858	1976.77683	1891.060229	6.78139778
T8:90	1.793472548	25.36482604	992.7297572	2371.255387	2279.959094	6.652919911
T8:90B	4.241704916	29.86274132	1175.8917	2748.112365	2650.638555	6.958980155

Treatability Study Raw Data
Chemical Analyses - TPH Analyses
Fractionated Aromatic Hydrocarbons - Manipulated Data - mg kg^{-1}

	C9-10	C10-12	C12-16	C16-22	C22-36
T1:1	138.7315483	1064.319981	9637.218225	17857.24033	10069.06463
T1:1B	177.9253282	1158.882461	9633.675568	19004.86054	11160.58608
T1:2	39.46928267	472.9735707	7184.725089	13696.49891	7712.297834
T1:2B	0	394.6786582	5718.089196	11277.16661	6145.710535
T1:4	0	0	505.4590201	2313.248679	2140.464218
T1:4B	50.48732742	333.216361	5138.347748	9814.105359	6574.712214
T1:8	46.51402943	630.8771255	6288.94159	10166.86519	4726.682228
T1:8B	0	186.0654444	4187.114104	7856.452988	3103.443292
T1:16	0	179.554171	4935.869346	9993.935281	3791.211506
T1:16B	0	265.6033228	4685.443491	9481.369037	6104.970494
T1:32	6.829208935	138.6854738	6170.452934	12098.20629	3870.060171
T1:32B	0	137.0339398	6101.371532	11601.86217	3580.464149
T1:64	0	61.46190968	5005.380186	13443.39588	16731.48658
T1:64B	0	52.8700965	4635.269981	12820.3893	15507.7495
T1:90	0	0	4134.309894	11053.6162	10115.81128
T1:90B	0	0	5073.216088	12753.79036	11240.90387
T2:1	24.66868057	263.8178339	4900.84454	10070.98884	5390.106704
T2:1B	0	194.345947	4367.559455	9275.665343	4911.588791
T2:2	56.28790508	530.7145336	6225.844359	11482.73264	6538.108213
T2:2B	83.49968556	667.9974845	6198.245889	11057.92759	6282.38788
T2:4	44.40674536	652.7791568	8323.092844	16454.6023	12337.46262
T2:4B	60.78043356	739.9357129	9104.512553	17467.76808	12206.29663
T2:8	0	543.9063293	5957.671653	10425.92539	4630.793189
T2:8B	0	636.1674695	7563.596488	13211.34311	5727.34409
T2:16	0	660.8465458	7611.642422	14748.18976	5518.366336
T2:16B	140.473954	1213.884632	10598.37457	18527.15129	11785.58693
T2:32	7.417573375	281.8677883	8212.737241	15037.89376	5144.828893
T2:32B	0	181.952605	7365.279265	13945.47612	4589.361667
T2:64	0	129.8310594	6282.591301	15762.3909	17847.26922
T2:64B	0	91.1205759	6034.76507	15812.88776	17193.08945
T2:90	2.591313485	81.9797357	5987.583168	15750.71008	14333.49718
T2:90B	0	89.65630522	5903.322171	14894.80597	13454.8498
T3:1	110.603464	703.580745	6141.703318	10351.77066	4110.881006
T3:1B	106.5560349	709.7061009	5028.586678	7657.302604	3532.940427
T3:2	0	590.1378016	6484.105411	11390.46798	4556.72613
T3:2B	107.7385119	654.6137434	7090.966997	12700.18851	4885.736949
T3:4	69.12022363	722.9087609	8205.648566	12700.36549	5827.659221
T3:4B	68.0085606	913.9842069	8664.417739	11664.42153	7586.450274
	0	440.5156289	6695.476974	12292.36927	4481.480279
	0	518.5935676	6787.036666	9143.044147	4096.306495
T3:16	0	178.9649504	5832.953666	10474.19039	4705.237438
T3:16B	7.880059201	209.7308064	4914.732308	10716.88051	2193.081092
T3:32A	0	82.7179169	4740.37293	9501.690461	3022.385425
T3:32B	0	60.48341904	4925.791842	9411.830385	3047.476489
T3:64	0	0	4106.646953	0	10237.92881
T3:64B	0	0	3304.917509	9507.351733	9957.035357
T3:90	0	0	0	0	0
T3:90B	6.294414642	0	3455.359968	9107.196976	21093.67815

late GCFID result - may be erroneous

Fractionated Aromatic Hydrocarbons - Manipulated Data - mg kg^{-1}

continued

	C9-10	C10-12	C12-16	C16-22	C22-36
T4:1	73.5505124	370.9784617	4265.929719	8457.663746	5103.373273
T4:1B	136.687747	807.5706141	5655.277184	10017.29994	7705.450709
T4:2	51.02495449	596.8576913	6656.071036	11682.02905	4592.245904
T4:2B	21.42460987	439.8740214	6719.96279	11912.08309	4400.079252
T4:4	0	666.662719	6938.62558	12485.2594	5036.636842
T4:4B	0	613.1151599	6568.038029	12045.8702	4596.01845
T4:8	0	448.7387204	7305.621999	13424.55056	5272.031498
T4:8B	57.80856819	471.4609894	6608.803979	12227.15449	4777.557002
T4:16	10.34903278	417.0051444	6741.481706	11452.72669	5900.774984
T4:16B	0	283.2737575	5727.402963	9892.508232	2770.196615
T4:32	0	75.09250747	5438.187927	11023.69474	3541.958807
T4:32B	0	114.5061324	6459.392962	12688.63993	4159.63366
T4:64	0	0	3951.90058	11225.92562	11381.70651
T4:64B	0	0	3462.596319	10293.64411	10027.42182
T4:90	6.984596117	0	2916.359904	8751.98996	8251.136213
T4:90B	9.127685764	0	3742.351163	10835.1925	10252.90909
T5:1	0	83.39822036	1636.232401	3704.915087	2355.084379
T5:1B	29.21346999	301.186761	3363.663622	6195.724382	3540.178814
T5:2	0	0	945.6437813	2014.188432	1120.695465
T5:2B	0	58.83769141	1405.930268	2911.739333	1604.23508
T5:4	0	120.1235308	2353.799875	4557.001531	2725.709427
T5:4B	0	33.59219909	1391.26602	2934.807962	2014.878401
T5:8	0	82.74981237	2029.328206	3727.526643	1695.588032
T5:8B	0	62.1512916	1698.973896	3167.910647	1455.13968
T5:16	0	39.35813697	2119.766563	4265.887985	1982.303335
T5:16B	0	0	1744.238121	4009.069667	1478.497101
T5:32	6.648359214	0	1311.942885	2760.652017	817.1150063
T5:32B	0	0	1844.962657	3759.887401	1179.501674
T5:64	0	0	1219.798799	3449.890768	3767.684176
T5:64B	0	0	1111.263231	3177.668858	3572.117907
T5:90	0.640948415	0	823.5021768	2583.721328	2586.401657
T5:90B	1.692670211	0	663.1361064	2066.359711	1951.648753
T6:1	6.155291875	51.05271496	992.8123719	2361.097548	1270.524658
T6:1B	9.968127186	77.25298569	1481.762106	3265.06006	1801.240582
T6:2	0	0	246.6046543	870.6133267	469.6016185
T6:2B	8.704318709	91.81652316	1181.962245	2291.201312	1153.462621
T6:4	0	149.7931355	2069.094802	3335.426722	1423.281564
T6:4B	0	164.8627014	1965.793134	3244.540417	1532.586314
T6:8	2.41014259	126.7227604	2400.882569	4360.074795	2053.441487
T6:8B	0	136.9500163	2222.251533	3907.014805	1817.773947
T6:16	0	69.27628449	2227.99471	4106.014057	1809.362708
T6:16B	0	37.72519975	2010.295872	3868.26196	1228.926962
T6:32	0	0	3188.217666	6030.549517	1802.247139
T6:32B	0	0	2343.050947	4284.607999	1379.044244
T6:64	0	0	1673.578626	4225.598583	4456.441085
T6:64B	0	17.02220603	1825.587114	4591.78456	5008.858263
T6:90	0	0	1554.747949	4437.307223	4801.986473
T6:90B	0	0	1837.368449	5383.39093	5437.163277

late GCFID result - may be erroneous

Fractionated Aromatic Hydrocarbons - Manipulated Data - mg kg^{-1}

continued

	C9-10	C10-12	C12-16	C16-22	C22-36
T7:1	9.870109575	115.9390336	1296.320729	2236.622436	887.7537992
T7:1B	11.65029678	96.11494844	1045.336748	1833.257415	754.6341046
T7:2	13.25832704	133.3556001	1703.759385	3377.011747	2407.866656
T7:2B	15.48161025	142.6611192	1662.673762	3193.81781	2251.998529
T7:4	0	141.2263831	2234.480729	4601.496987	2853.393713
T7:4B	0	103.6916218	1940.428011	3895.409232	2360.955144
T7:8	4.220104957	112.64434	2298.658708	4299.313081	1494.241778
T7:8B	4.171507263	109.6510481	2374.779492	4347.008533	1555.674244
T7:16	0	86.3176325	2126.762537	3650.745086	1120.685784
T7:16B	4.362972812	137.4336436	3013.256008	4931.717482	1554.776526
T7:32	0	40.59193697	2033.231947	4717.994775	3636.916382
T7:32B	0	0	1570.170916	3631.472914	3720.508373
T7:64	0.744002407	32.5501053	1567.551071	3803.030303	3900.86662
T7:64b	0	0	2189.39917	5374.667377	4722.76034
T7:90	1.385000072	0	1402.16203	3622.558015	3512.601053
T7:90B	0.983046387	0	1454.908653	3759.458516	3673.413044
T8:1	21.97482972	203.9714964	2107.893281	3572.881929	1540.351045
T8:1B	16.9253724	146.8601544	1495.161359	2621.08921	1091.68652
T8:2	32.34620812	289.5632551	2669.079709	4565.731968	2070.804244
T8:2B	17.52200025	165.7001756	1859.401554	3257.160488	1359.26572
T8:4	13.1438743	161.2234857	1812.528207	3098.939684	1384.206725
T8:4B	21.86452442	247.5064165	2456.073264	4186.119376	1967.182498
T8:8	7.167953037	169.559165	2372.098114	4089.193622	1849.579054
T8:8B	0	121.4830377	1746.048351	3022.192679	1302.221839
T8:16	0	152.3727133	3702.290788	5957.463275	1997.74428
T8:16B	8.309635486	242.6413562	5142.002439	8214.351666	2798.408244
T8:32	0	39.40927213	1849.194951	4370.050398	4344.750618
T8:32B	0	34.40186387	1939.55336	4554.450895	4682.093673
T8:64	1.384034018	9.928939692	1159.880682	2969.715774	3052.216237
T8:64b	1.133440504	0	1117.383431	2828.248903	2844.180039
T8:90	1.347883766	0	1429.527011	3690.37738	3324.20229
T8:90B	0	6.93391141	1570.622977	4168.139826	3611.647414

late GCFID result - may be erroneous

Treatability Study Raw Data
Chemical Analyses - TPH Analyses
Fractionated Aliphatic Hydrocarbons - Manipulated Data - mg kg^{-1}

	C9-10	C10-12	C12-16	C16-22	C22-36
T1:1	2004.848735	6294.855076	26991.46776	37294.59741	20326.66189
T1:1B	2057.980413	6286.920439	26830.86889	39338.4106	22237.95994
T1:2	1320.247505	4512.654652	24408.46222	34372.48263	18625.55449
T1:2B	1055.432816	3364.588048	17309.4783	23386.1359	12243.27408
T1:4					
T1:4B					
T1:8	930.0357779	3090.857256	15823.21598	19861.98019	10676.19381
T1:8B	733.9960979	2070.459273	12434.94613	15753.32709	6050.976573
T1:16	578.5634398	2349.16707	15515.22604	21302.73079	7767.588251
T1:16B	347.0698882	1563.488468	11925.366	16472.98592	5632.352817
T1:32	280.5228901	1480.887691	18161.49318	25175.09075	7923.458336
T1:32B	318.5392715	1983.630918	18132.43434	25089.1045	8164.637268
T1:64	49.07235476	1074.247291	14382.57273	26506.84541	27841.51629
T1:64B	17.78579283	948.7380449	14066.85664	26585.61838	27053.4091
T1:90	0	665.0446605	12067.48193	22830.78538	17974.87278
T1:90B	0	768.6153135	13267.8066	23781.93664	18757.6624
T2:1	904.5182875	2930.091059	15573.47509	20342.75333	10303.28558
T2:1B	796.8880409	2605.210903	13954.59626	19277.8641	9719.387093
T2:2	1320.085393	4521.795042	21476.51617	30312.37708	16173.39139
T2:2B	1932.696568	6425.621956	27899.81417	38525.47031	20858.22145
T2:4	1592.933394	5980.954218	27617.8237	38461.31654	27033.55781
T2:4B	1907.316431	7446.263768	34004.6706	46802.25515	33613.56173
T2:8	1193.431678	4061.588891	18584.52033	22815.60561	8894.765831
T2:8B	1508.678195	4762.377842	23517.37857	29272.27563	13058.88334
T2:16	1658.665294	4974.209811	22387.21861	29727.97349	11568.3867
T2:16B	1786.449356	5768.915589	26410.88151	34939.48794	15347.81673
T2:32	275.9337296	2213.403895	23260.5211	32008.80713	10821.25054
T2:32B	249.3612303	2328.891978	22799.82712	31750.47616	10560.85357
T2:64	25.82406108	1194.303596	18204.77847	33006.46691	36487.73988
T2:64B	14.34969699	1039.635547	16612.16588	30635.40727	29314.99598
T2:90	0	1115.678242	16371.21188	30567.13387	25025.96334
T2:90B	0	1249.970181	16554.39636	30397.04526	24523.84571
T3:1	1761.806145	4035.242508	17394.00024	21452.79058	8455.099642
T3:1B	1043.104916	2954.105161	12548.86823	14693.29056	7313.748785
T3:2	1653.194252	4411.886373	19919.8455	25416.34586	10286.31746
T3:2B	1702.677622	4633.437902	21709.99204	26652.32581	10906.13772
T3:4	1566.302315	4917.681965	24444.4622	30440.16601	13628.0984
T3:4B	1916.951577	6202.762083	30075.03804	38175.68388	16295.99519
T3:8	1407.486498	4405.156289	21164.58241	27324.58949	10476.81986
T3:8B	1351.839412	4146.15881	19865.82401	25883.71066	10023.55258
T3:16	491.8573141	2652.473901	19562.88392	27156.44973	10564.26547
T3:16B	0	2494.341816	14953.92773	17909.55609	4749.251065
T3:32	341.4765288	1736.015769	15854.79765	21807.30621	7432.947174
T3:32B	219.1830323	1709.072758	16675.66705	22090.32029	6496.141162
T3:64	13.26305974	602.6042359	10814.87191	19743.21773	17832.76047
T3:64B	12.85626624	557.6762601	9842.186045	18954.42186	17284.53572
T3:90	0	0	0	0	0
T3:90B	17.51489292	526.8151385	11036.29823	20507.75024	20269.93084

Fractionated Aliphatic Hydrocarbons - Manipulated Data - mg kg^{-1}

continued...

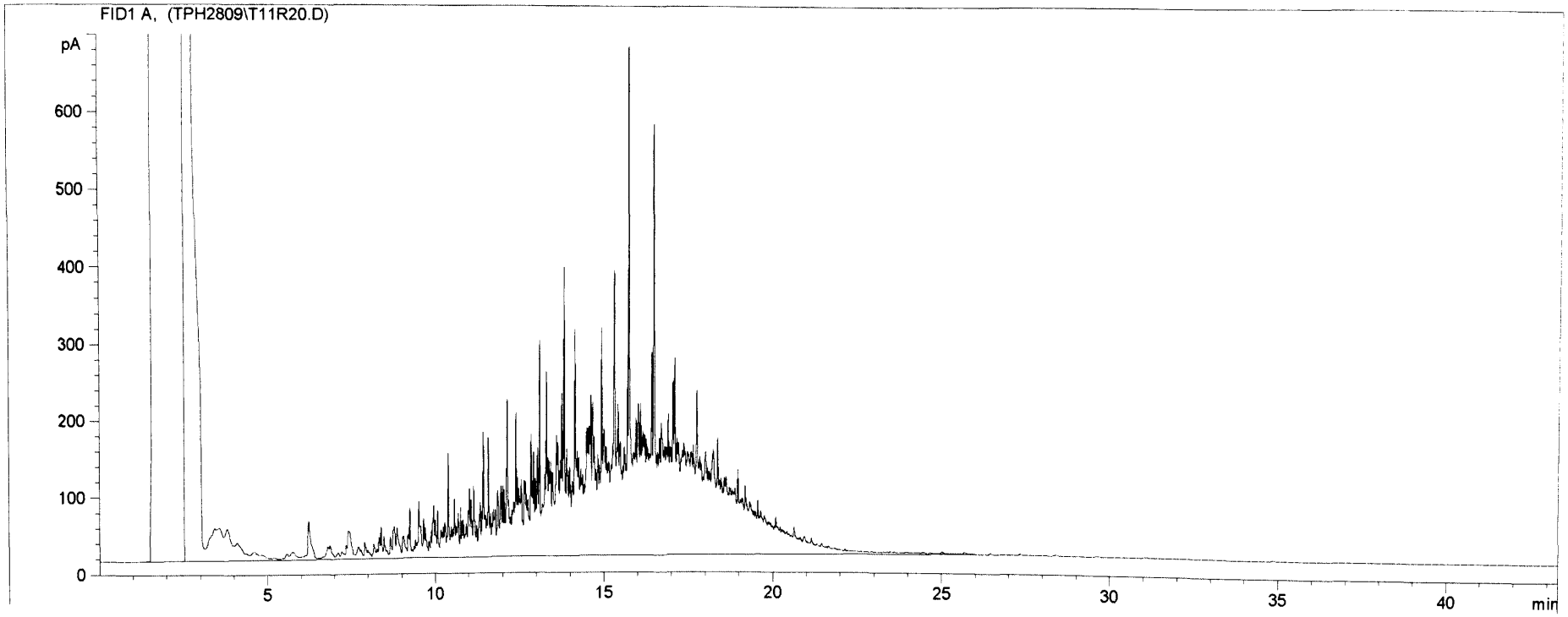
	C9-10	C10-12	C12-16	C16-22	C22-36
T4:1	1501.978885	4915.625912	19883.80036	25756.8733	18454.08163
T4:1B	1491.15203	4885.659535	19070.65164	25015.85523	18671.00406
T4:2	1630.784401	4481.468042	20320.01674	25986.47221	10081.4568
T4:2B	1447.500204	3983.638398	18506.84581	23860.32021	8993.649512
T4:4	1634.656987	4768.638429	21587.2055	28409.16511	10669.93682
T4:4B	1626.262834	4817.812021	22104.30917	29162.16926	11216.32203
T4:8	1395.499604	4400.492711	21940.85925	28900.20022	11176.96616
T4:8B	1231.96482	3899.509083	19934.96358	26624.69955	10298.27526
T4:16	519.2779389	2715.099188	18557.03331	22719.17079	6240.466766
T4:16B	358.6907968	1981.076862	9979.575058	11185.02139	2231.853847
T4:32	143.8795372	1471.469211	17212.23452	24525.44223	7280.533873
T4:32B	348.6201555	1960.775801	18070.42816	25079.6773	8683.19275
T4:64	15.24546061	749.7994717	12141.48483	22618.99693	19969.61306
T4:64B	16.21225482	634.8377678	10487.05347	19960.41437	17678.75282
T4:90	0	421.4039657	8212.720935	15472.33552	16136.74523
T4:90B	0	555.2150927	9890.634394	18481.36044	17946.2892
T5:1	0.893488137	1106.145176	6132.41353	8954.121122	4835.469498
T5:1B	1.037020784	2834.118047	13253.86332	18609.39184	10314.00074
T5:2	0.180778739	504.3676628	4739.889019	7413.803483	3540.420297
T5:2B	0.387960632	795.7616165	6138.006079	9330.858827	4760.40507
T5:4	0.105089438	721.184991	6834.614684	10184.61143	6947.045575
T5:4B	0.071560971	420.35997	4066.616724	6362.257941	4301.5007
T5:8	0.08030739	432.0218911	5141.452065	7704.607925	3583.693373
T5:8B	0.144758535	356.5317869	4338.856454	6635.488518	3206.722968
T5:16	0.02370778	278.9899798	4885.517415	8479.646756	3966.696483
T5:16B	0.032467532	213.6522483	4721.243828	8678.813298	3263.052519
T5:32	0	167.4753345	2666.941811	5487.42906	1865.339642
T5:32B	0.476031066	109.3773482	3589.74486	7013.288704	2452.943052
T5:64	0	0	1577.419486	4755.353567	5475.013648
T5:64B	0.018925592	31.10219778	1707.571643	4788.457779	5606.872474
T5:90	0	0	1204.808215	3440.436286	3752.112019
T5:90B	0	0	991.7745381	2792.319924	2798.895296
T6:1	119.1230016	551.8038128	3772.107692	5616.884874	2593.550335
T6:1B	241.7270843	971.8924006	5723.69863	8464.933606	3792.872394
T6:2	45.30124309	173.8674471	1786.208874	3071.870913	3457.569525
T6:2B	89.57024736	579.8199398	3693.860154	5078.969967	2841.538882
T6:4	60.58354988	769.2013237	5380.214071	7136.149913	2903.321647
T6:4B	84.64249208	789.8197014	5414.200786	7195.230946	3237.376319
T6:8	30.69760562	600.5060538	6475.418892	9481.754649	4477.537534
T6:8B	20.85533243	401.6968752	4895.325836	7514.292138	3633.346498
T6:16	0	376.2483	4633.455679	7704.91084	3362.440341
T6:16B	22.29216349	321.5215888	5113.765145	8470.450533	3216.073279
T6:32	157.7724768	278.5291033	6730.816587	11925.77879	3796.248519
T6:32B	0	241.6788581	4979.54758	8670.341897	2951.913126
T6:64	0	0	2062.763736	5809.615041	5856.314892
T6:64B	37.66237223	170.6965469	3140.863911	7621.381368	9269.866366
T6:90	6.146984265	30.54674834	2611.339275	6834.881983	7199.435785
T6:90B	0	34.23589881	2504.246067	6836.751948	6982.113177

Fractionated Aliphatic Hydrocarbons - Manipulated Data - mg kg^{-1}

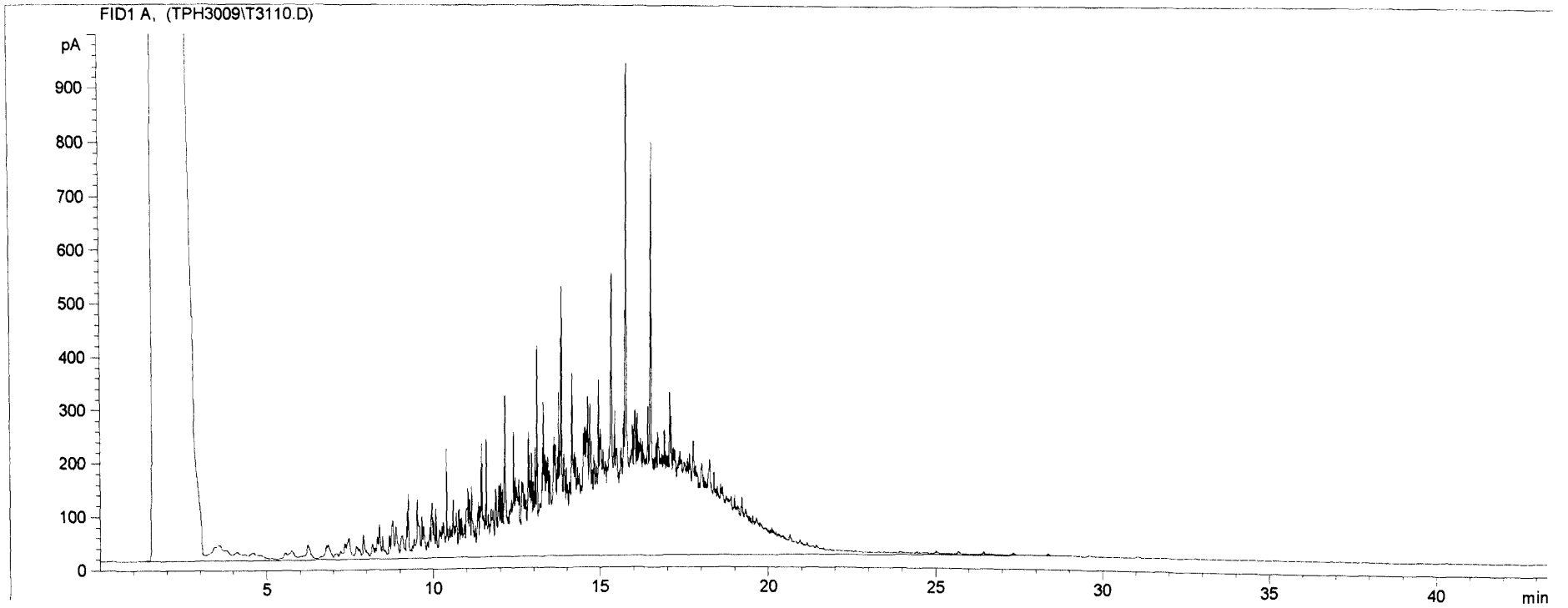
continued...

	C9-10	C10-12	C12-16	C16-22	C22-36
T7:1	218.8105982	848.6904078	4263.331274	5275.226029	2258.586905
T7:1B	166.0167291	672.249863	3527.266044	4384.533716	1661.970313
T7:2	211.6183462	1144.077774	5802.641987	7933.757157	5370.652224
T7:2B	174.3920229	1069.510579	5456.947745	7369.758267	4962.943636
T7:4	417.7817838	2382.535641	12287.00571	16990.30985	11187.61264
T7:4B	427.1973543	2230.582637	11173.53023	15440.34951	10940.67887
T7:8	108.7488585	1008.280461	6802.809191	9737.729877	3229.029539
T7:8B	179.6727771	1008.908828	6495.930703	9081.371311	3113.136277
T7:16	168.5936367	888.5808456	6261.059578	8798.047385	2673.537106
T7:16B	238.4053001	978.2408326	6916.55847	9524.99293	3069.039732
T7:32	3.877438755	383.018247	5508.628771	11260.56682	9062.78607
T7:32B	10.11482776	107.0173752	2639.220798	6055.659966	5615.227898
T7:64	1.550005014	121.1483919	2454.27794	5901.799093	5443.36961
T7:64B	0	190.9619398	3802.670994	8633.194088	8035.240462
T7:90	0	36.43152364	1350.917027	3474.242573	3135.27886
T7:90B	0	16.76961484	2125.404115	5366.450228	5261.900353
T8:1	382.8691485	1344.127084	6366.079996	7785.287749	3350.879803
T8:1B	306.4794357	1080.35954	5114.58715	6463.409136	2719.386565
T8:2	413.6433094	1682.132207	8156.678609	10431.65212	4637.54055
T8:2B	261.4503187	1117.130992	5915.537659	7663.046755	3326.006772
T8:4	177.502596	977.5907242	5655.845286	7601.500441	3148.621118
T8:4B	282.2397753	1426.816394	7495.034033	9940.862205	4031.443484
T8:8	140.1458404	1070.125954	6621.581996	9077.965351	3921.858995
T8:8B	136.6207146	836.0068309	5016.677022	6845.282956	4418.802387
T8:16	165.8919189	1126.882118	9544.840816	12829.44448	4422.751788
T8:16B	0	0	0	0	0
T8:32	0	205.439076	3832.186814	8344.548472	7945.955309
T8:32B	9.727423577	157.7740653	3424.171726	7656.668626	7164.128837
T8:64	3.730874309	43.14575612	1760.731972	4495.222139	3989.989547
T8:64b	4.785637685	61.14281832	1785.483639	4372.498622	4182.899213
T8:90	4.043651297	57.18878262	2238.25727	5346.348863	5140.507757
T8:90B	9.142945133	64.36878822	2534.620749	5923.523929	5713.420277

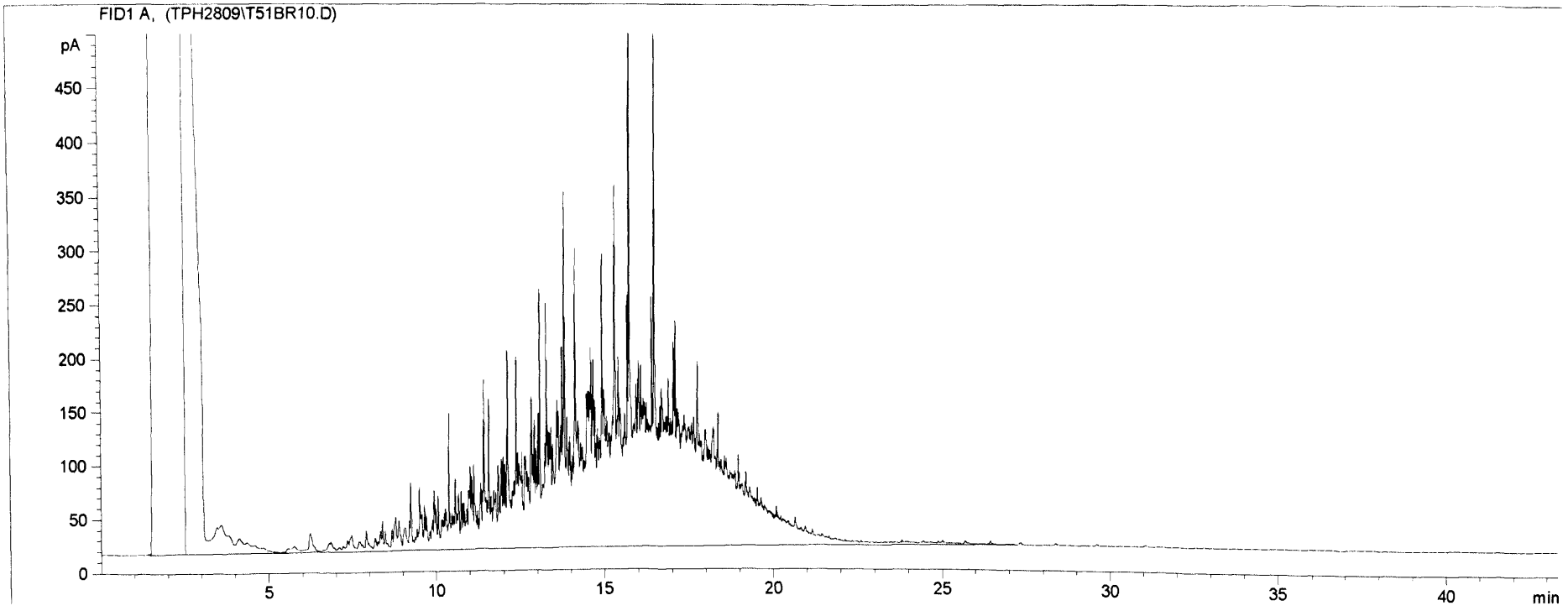
Un-managed Control (UMC) Day 1 GC-FID Chromatogram



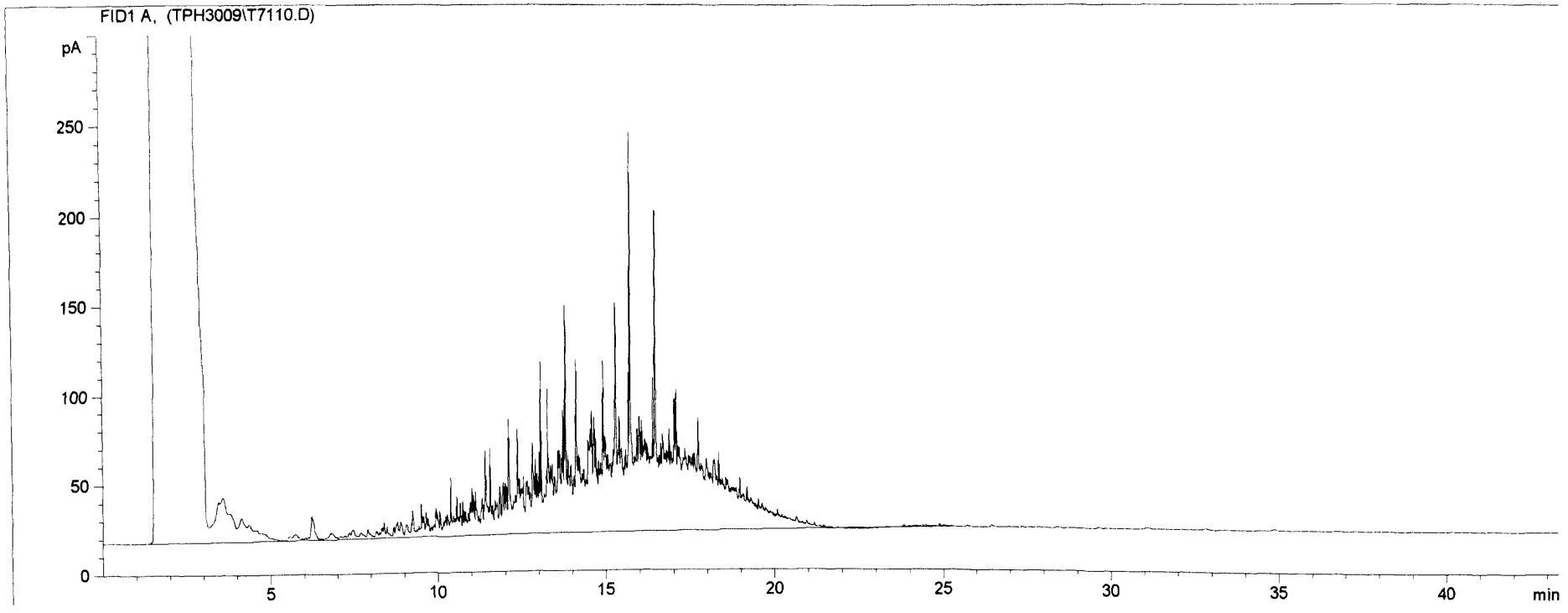
Managed Control (MC) Day 1 GC-FID Chromatogram



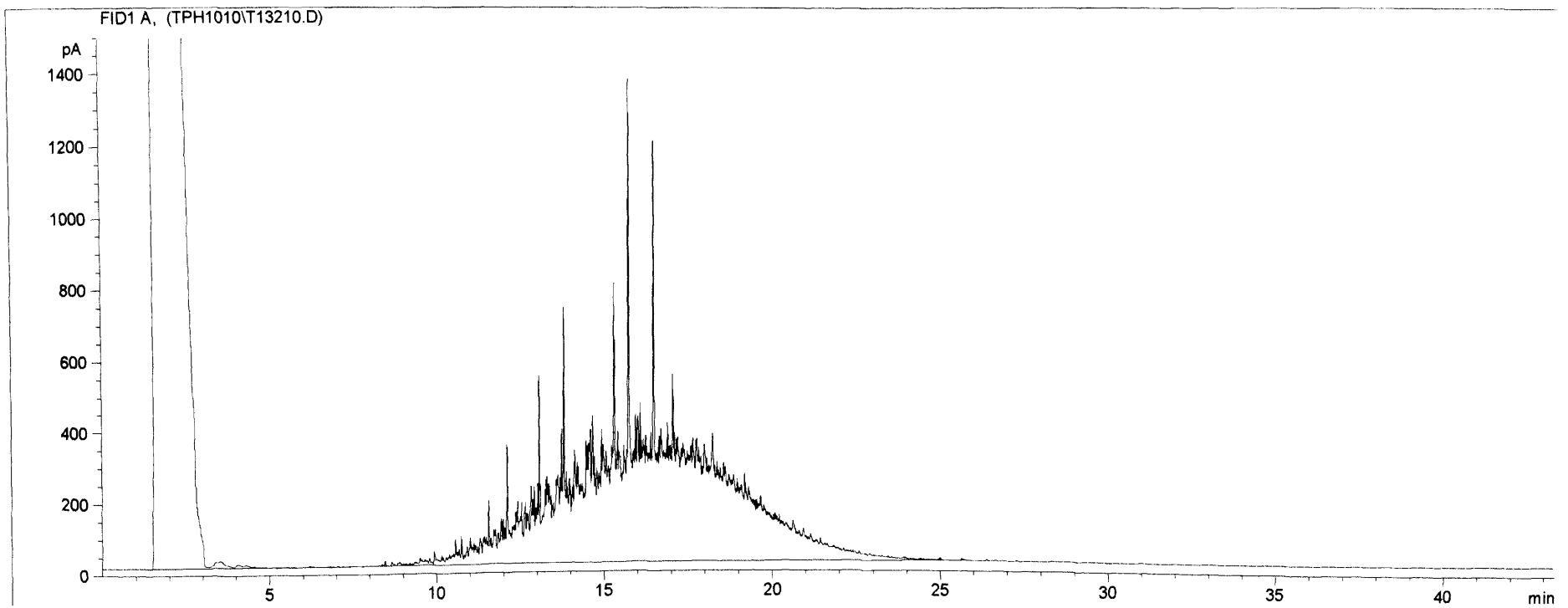
Total Manure Treatment (TMT) Day 1 GC-FID Chromatogram



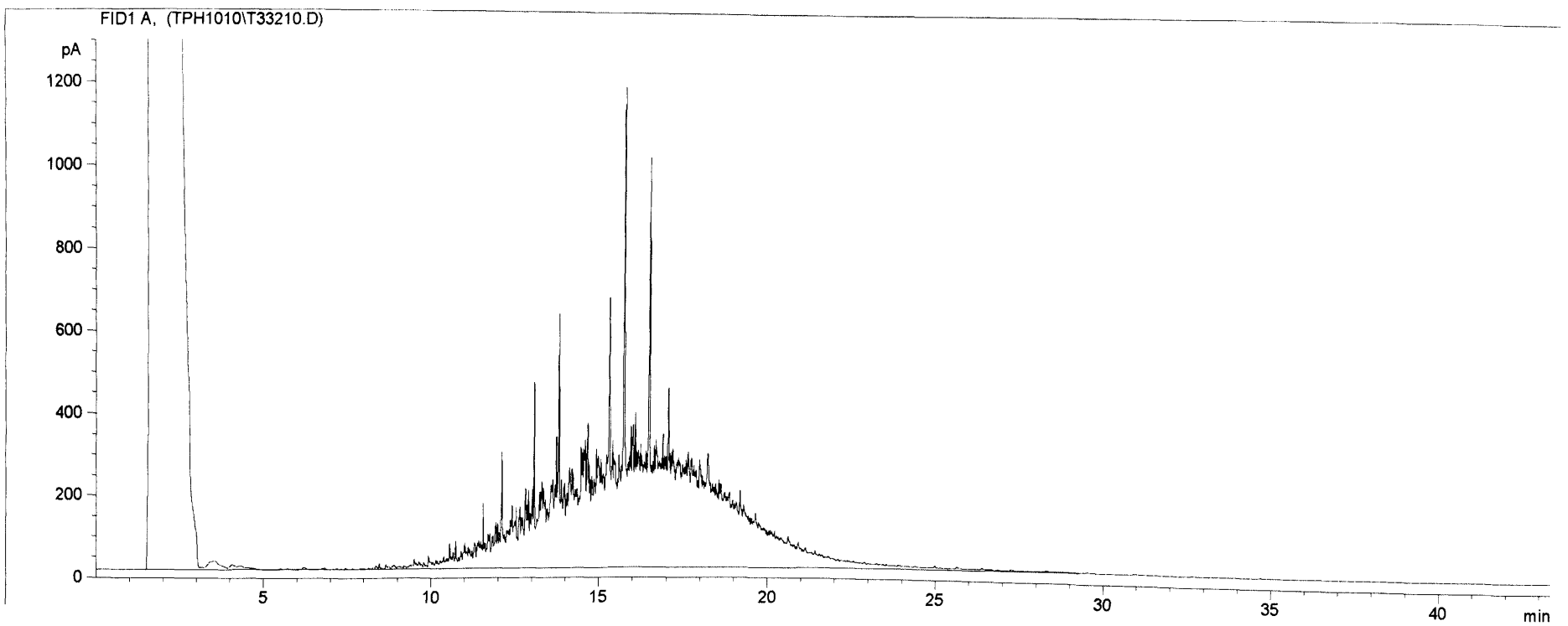
Sterile Manure Treatment (SMT) Day 1 GC-FID Chromatogram



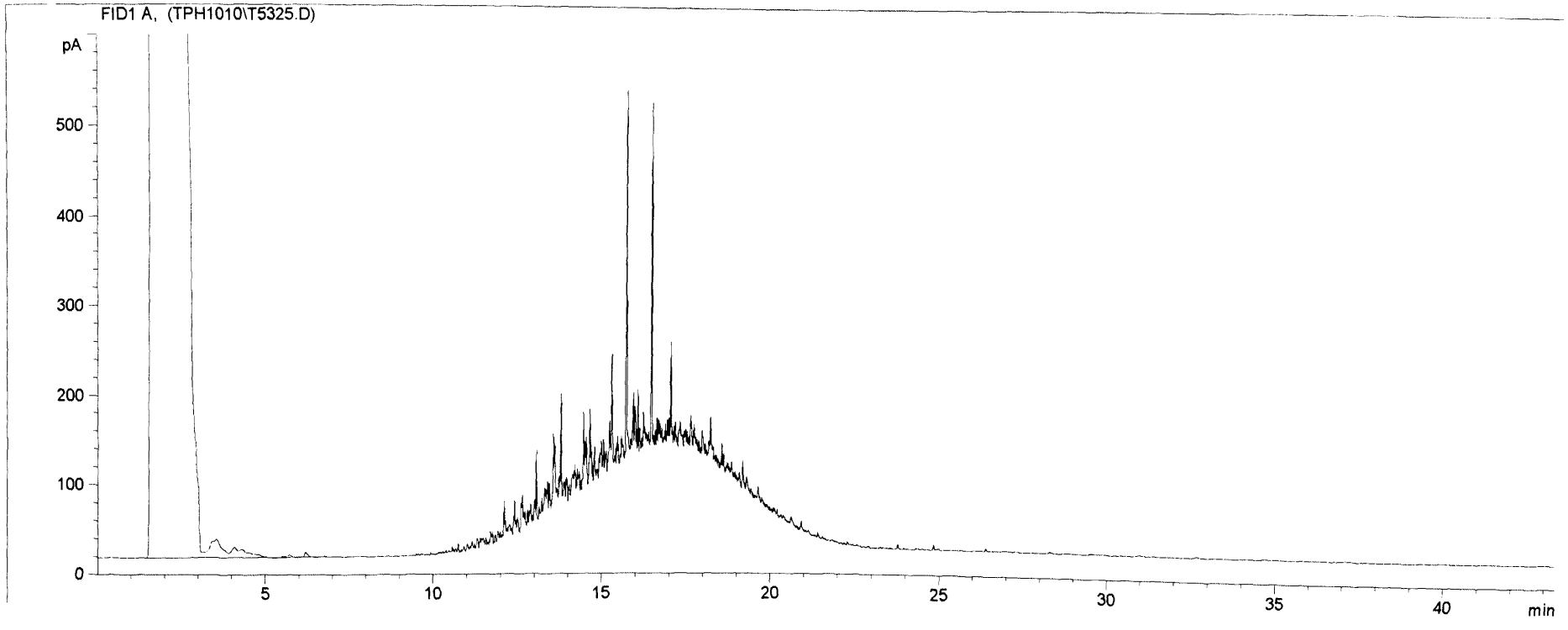
Un-managed Control (UMC) Day 32 GC-FID Chromatogram



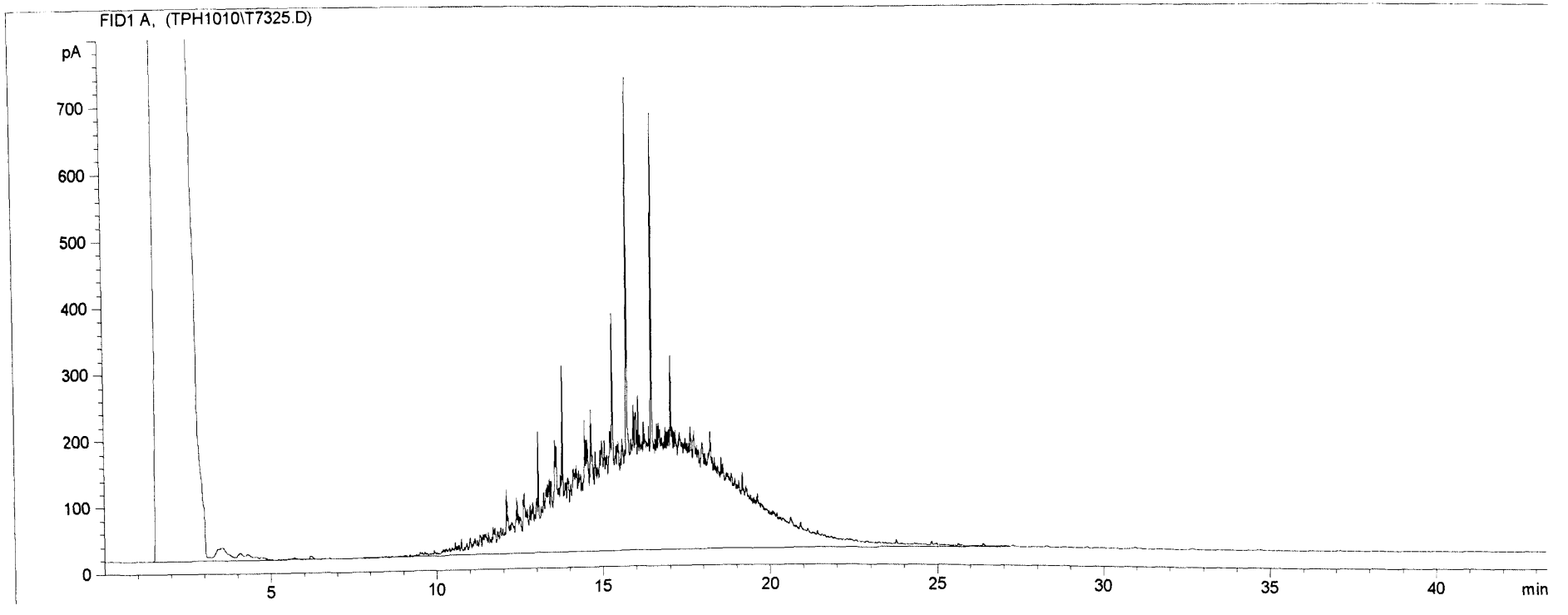
Managed Control (MC) Day 32 GC-FID Chromatogram



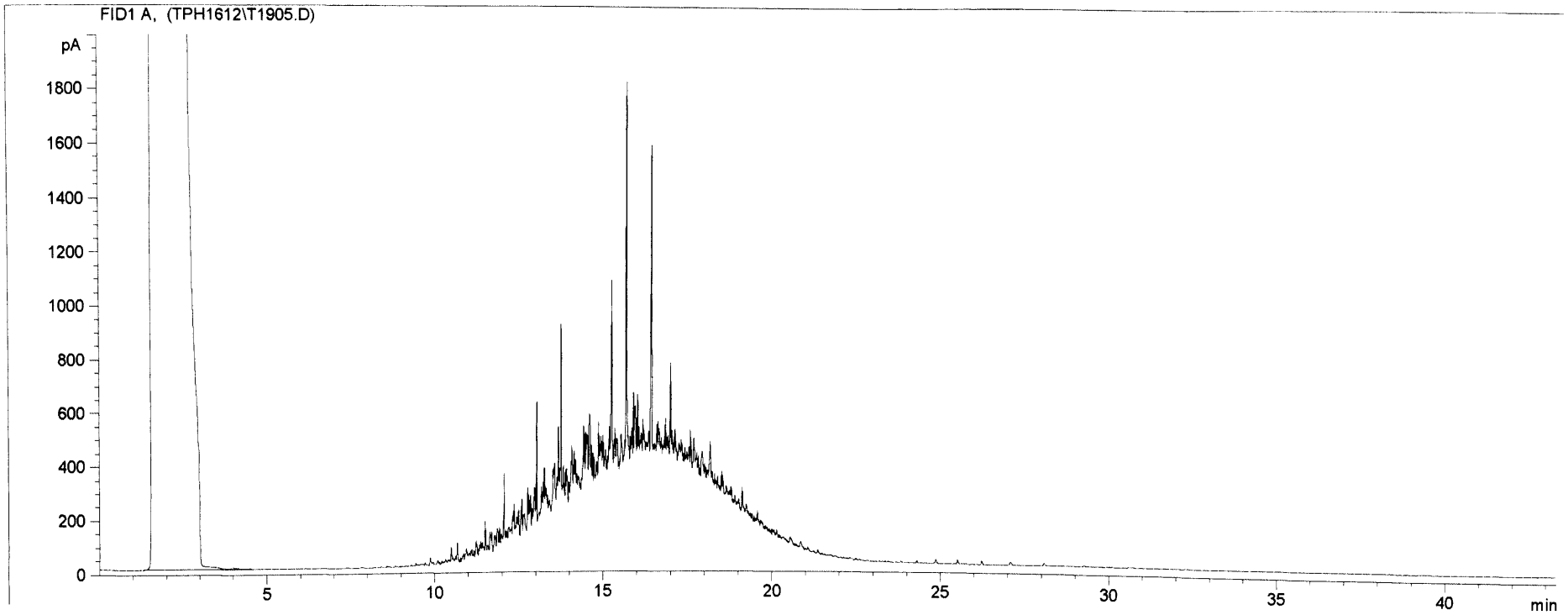
Total Manure Treatment (TMT) Day 32 GC-FID Chromatogram



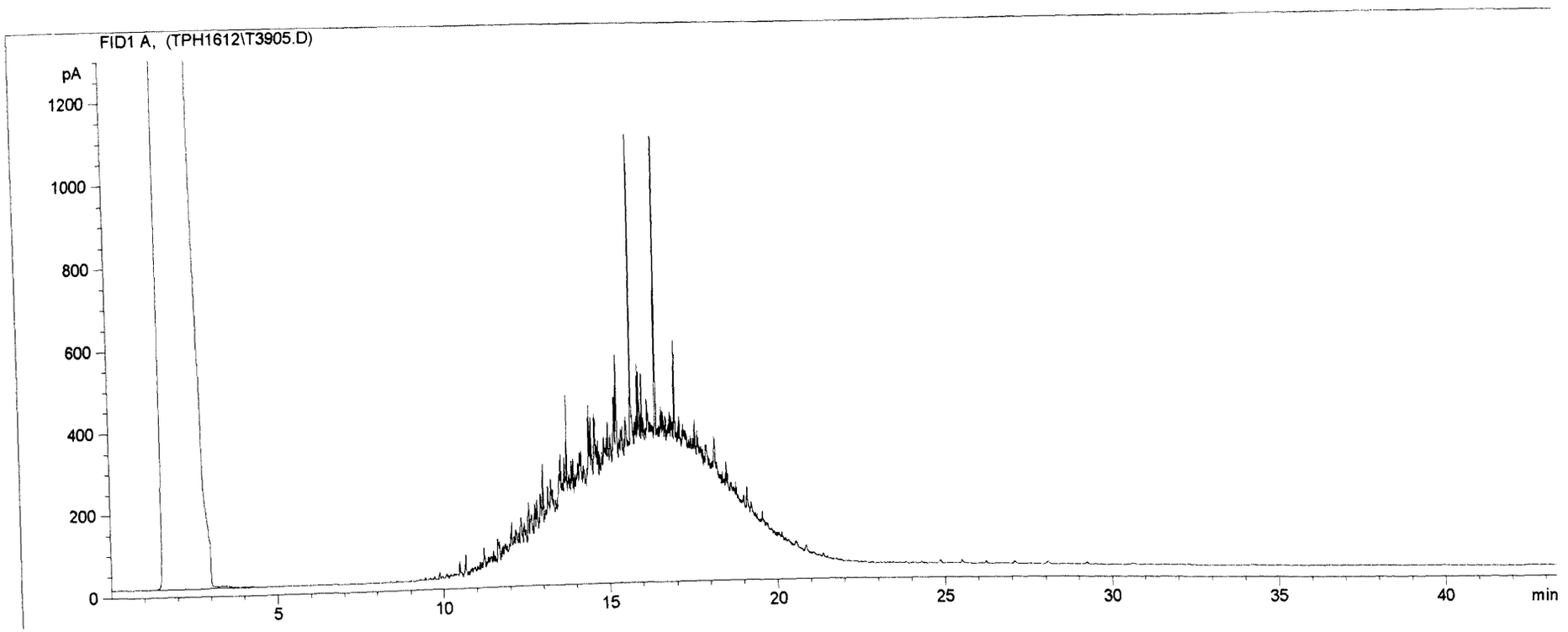
Sterile Manure Treatment (Day 32) GC-FID Chromatogram



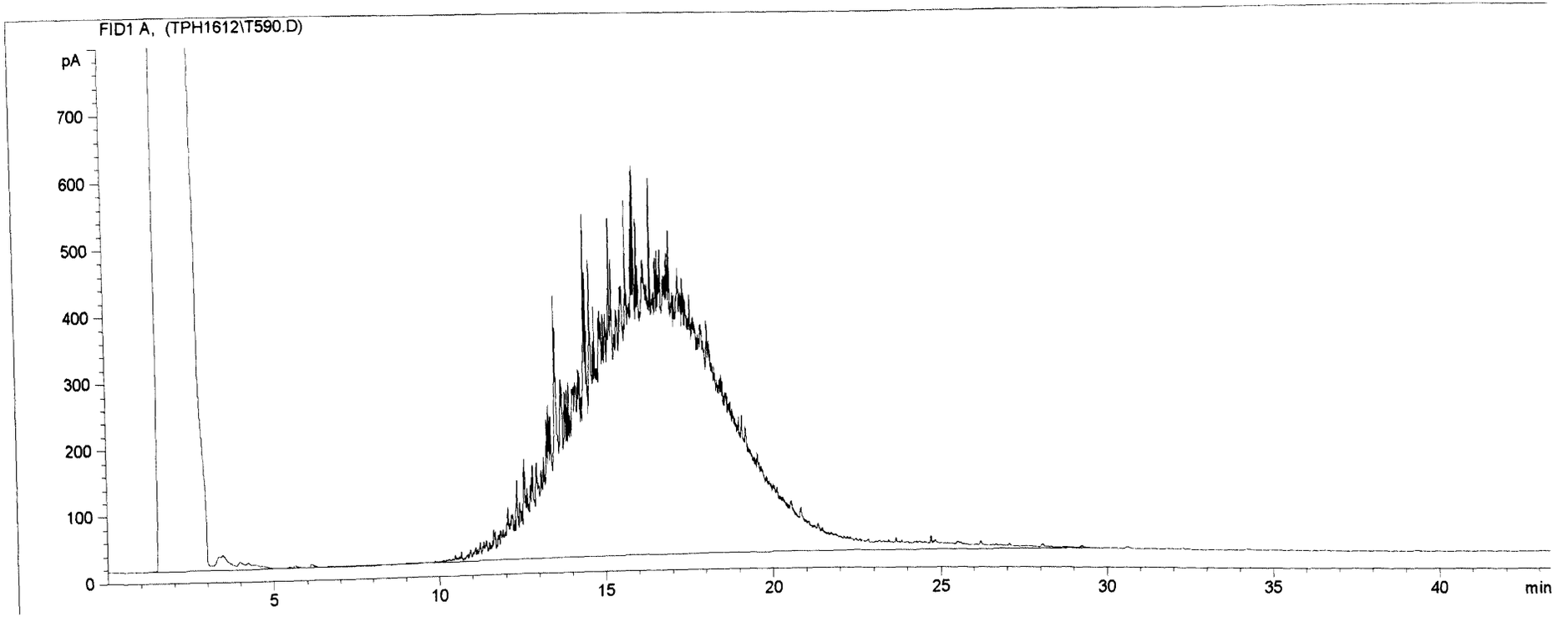
Un-managed Control (UMC) Day 90 GC-FID Chromatogram



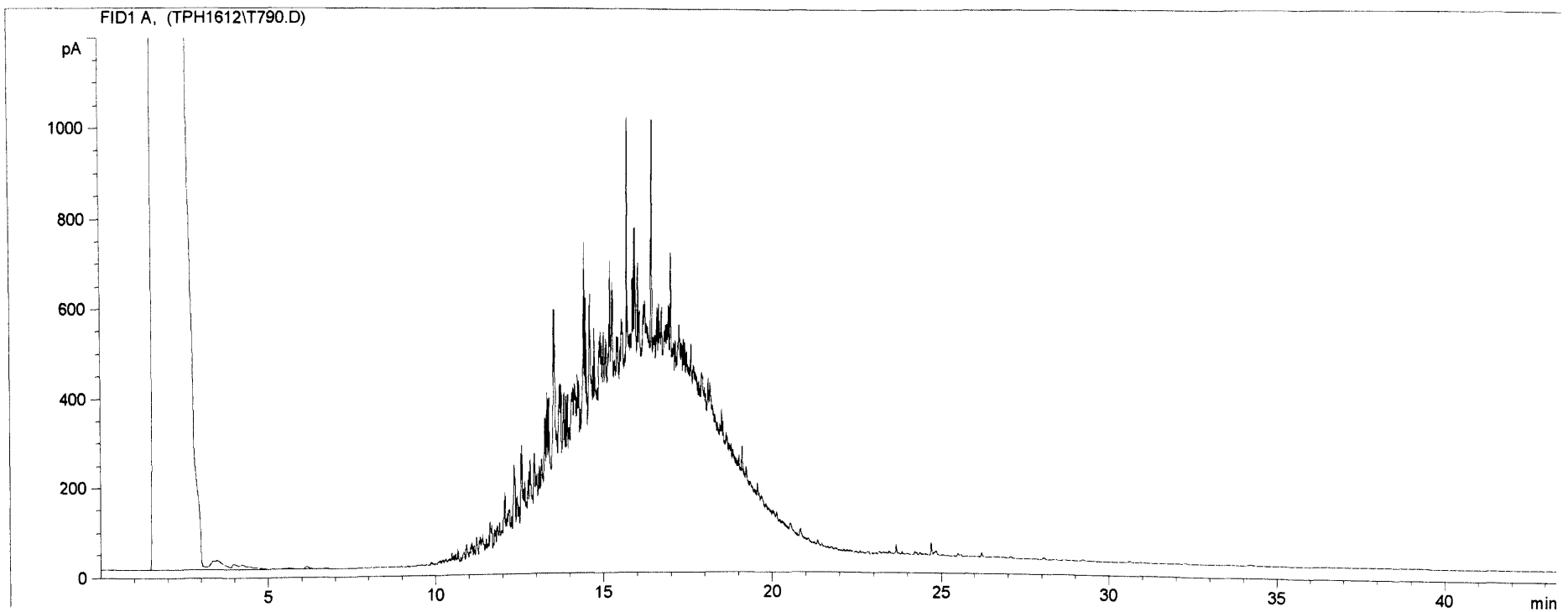
Managed Control (MC) Day 90 GC-FID Chromatogram



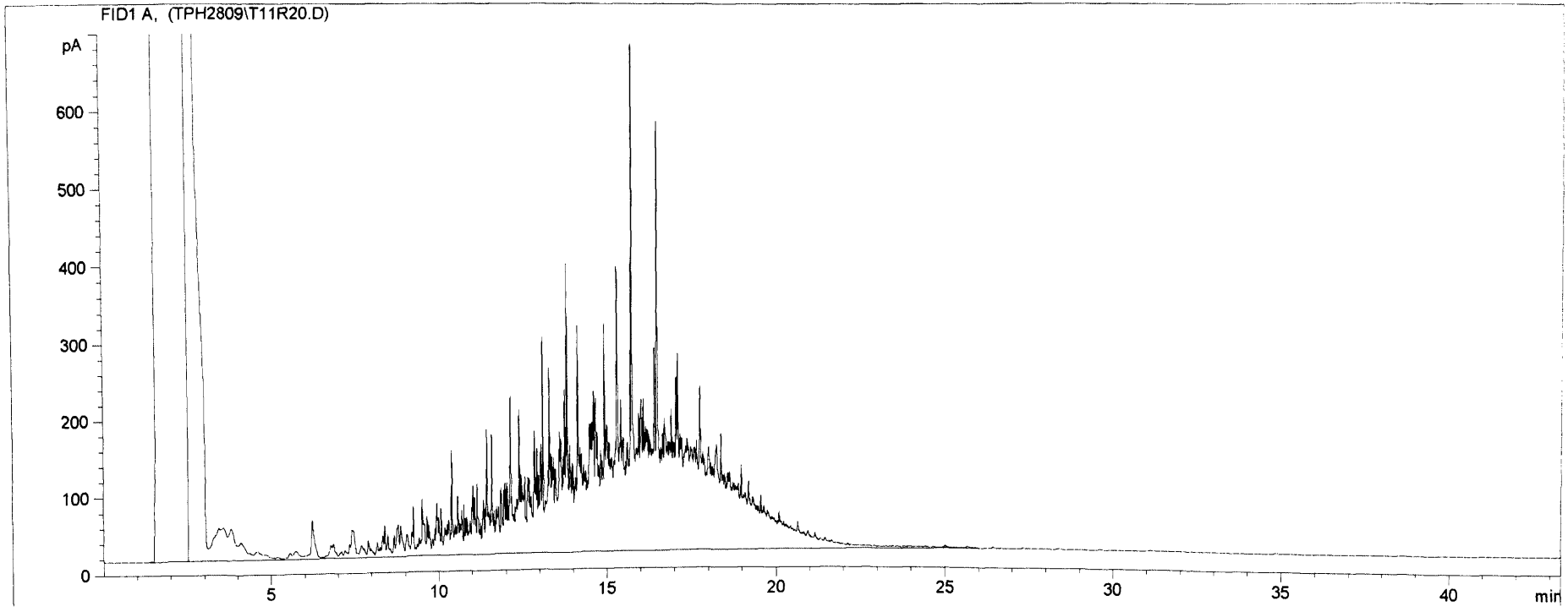
Total Manure Treatment (TMT) Day 90 GC-FID Chromatogram



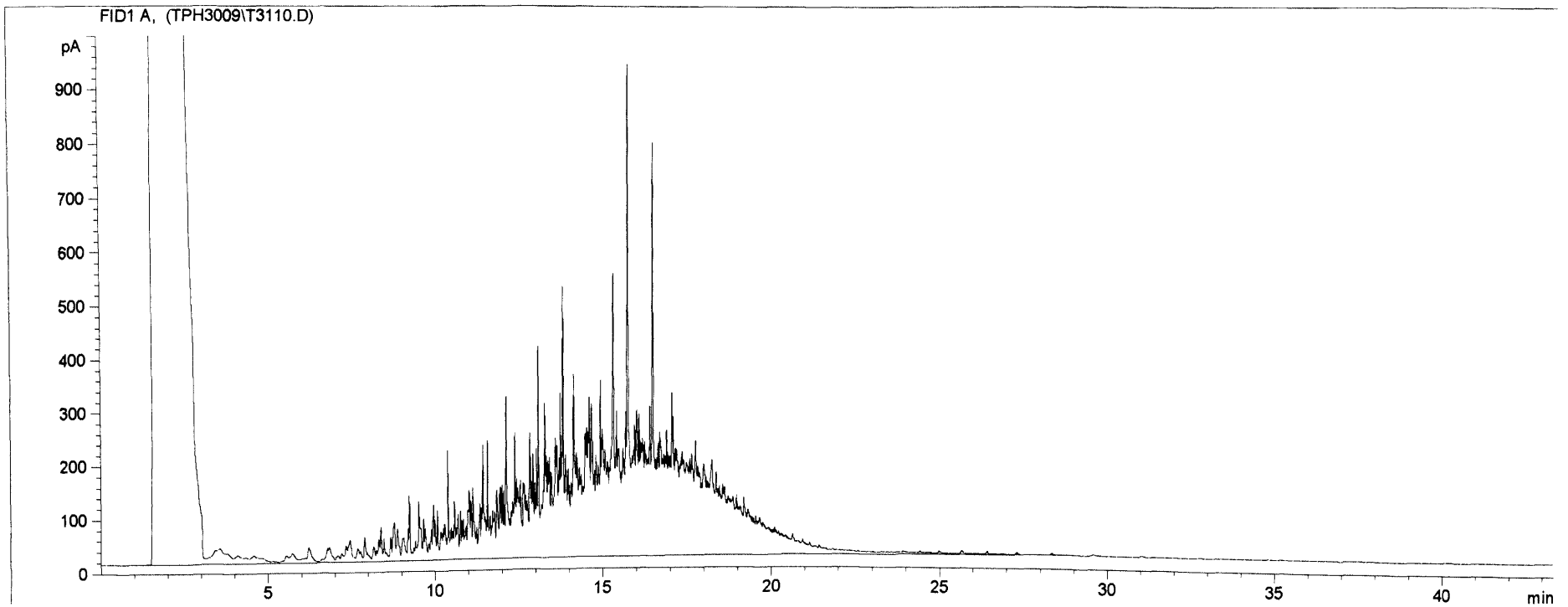
Sterile Manure Treatment (SMT) Day 90 GC-FID Chromatogram



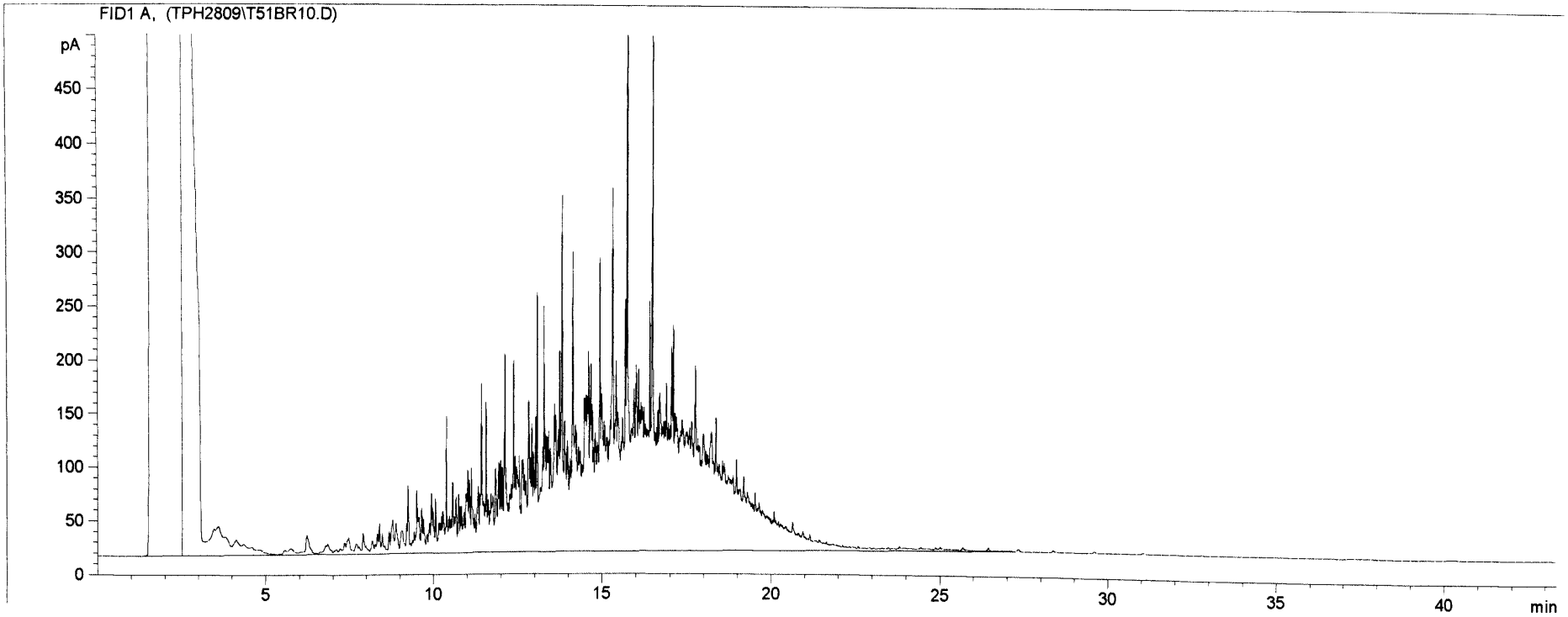
Un-managed Control (UMC) Day 1 GC-FID Chromatogram



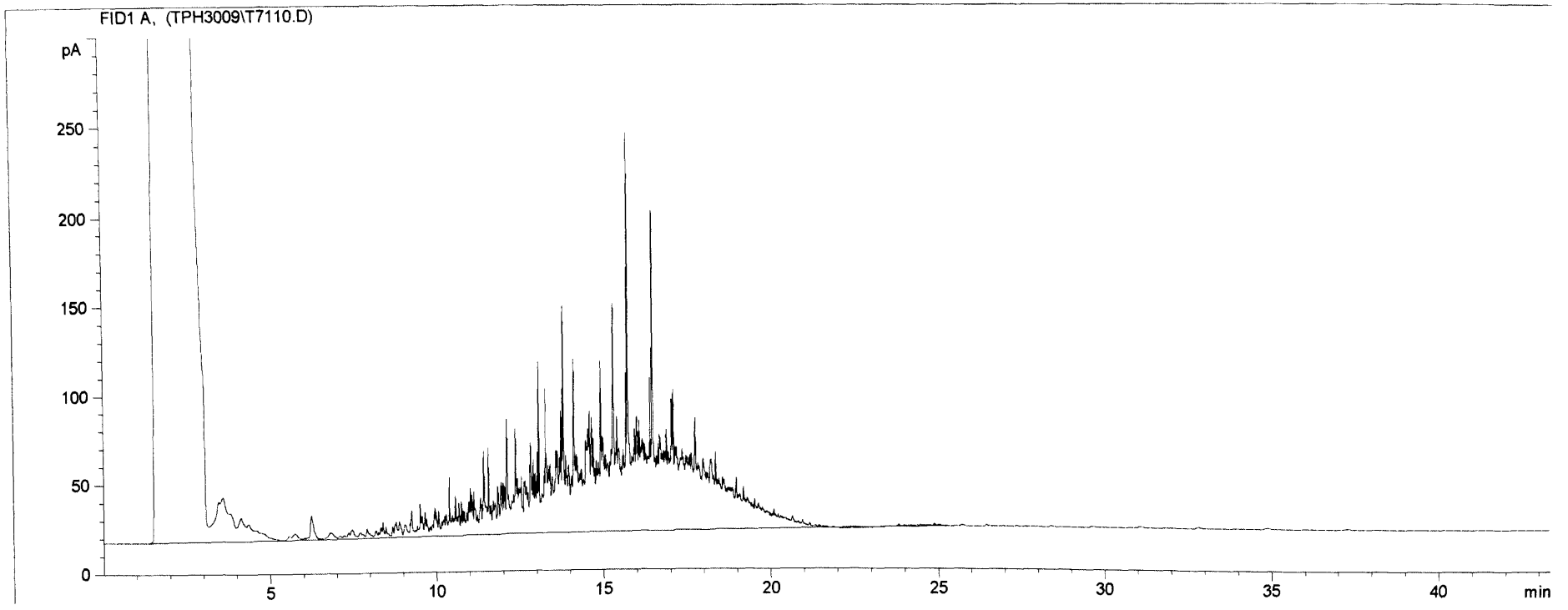
Managed Control (MC) Day 1 GC-FID Chromatogram



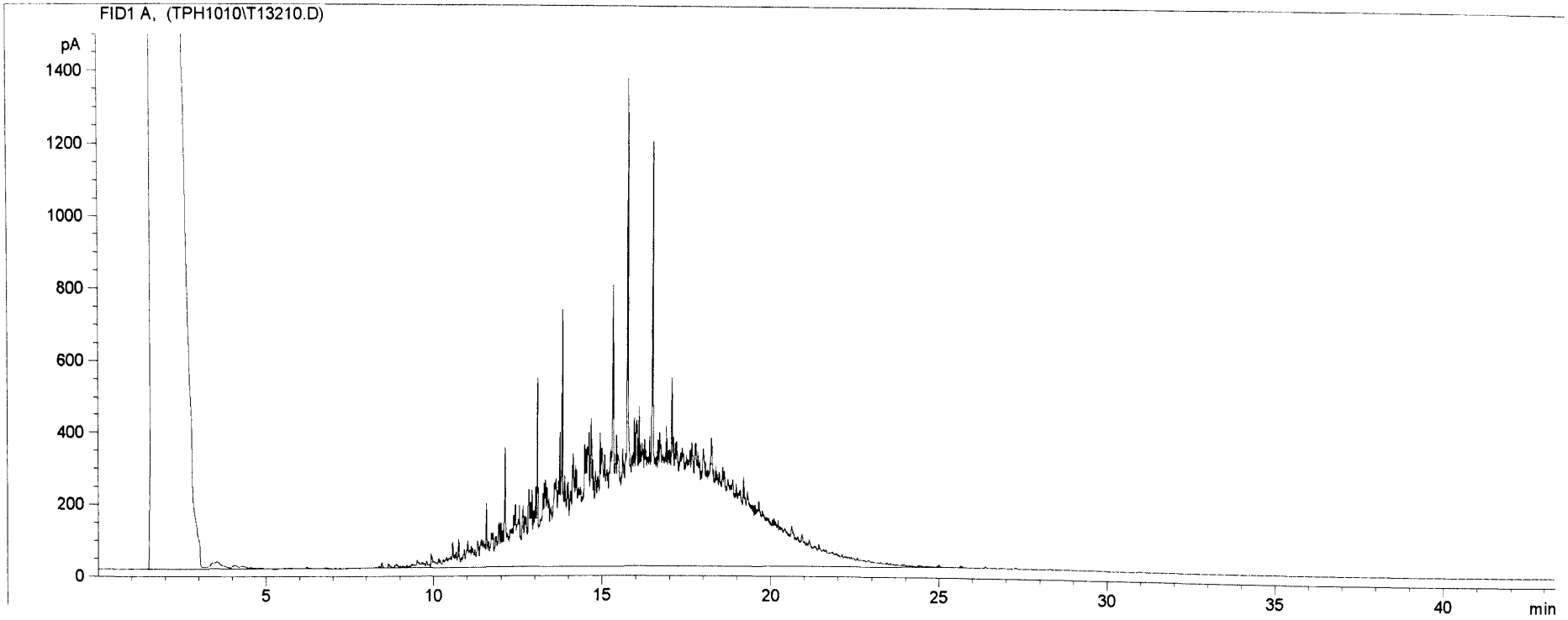
Total Manure Treatment (TMT) Day 1 GC-FID Chromatogram



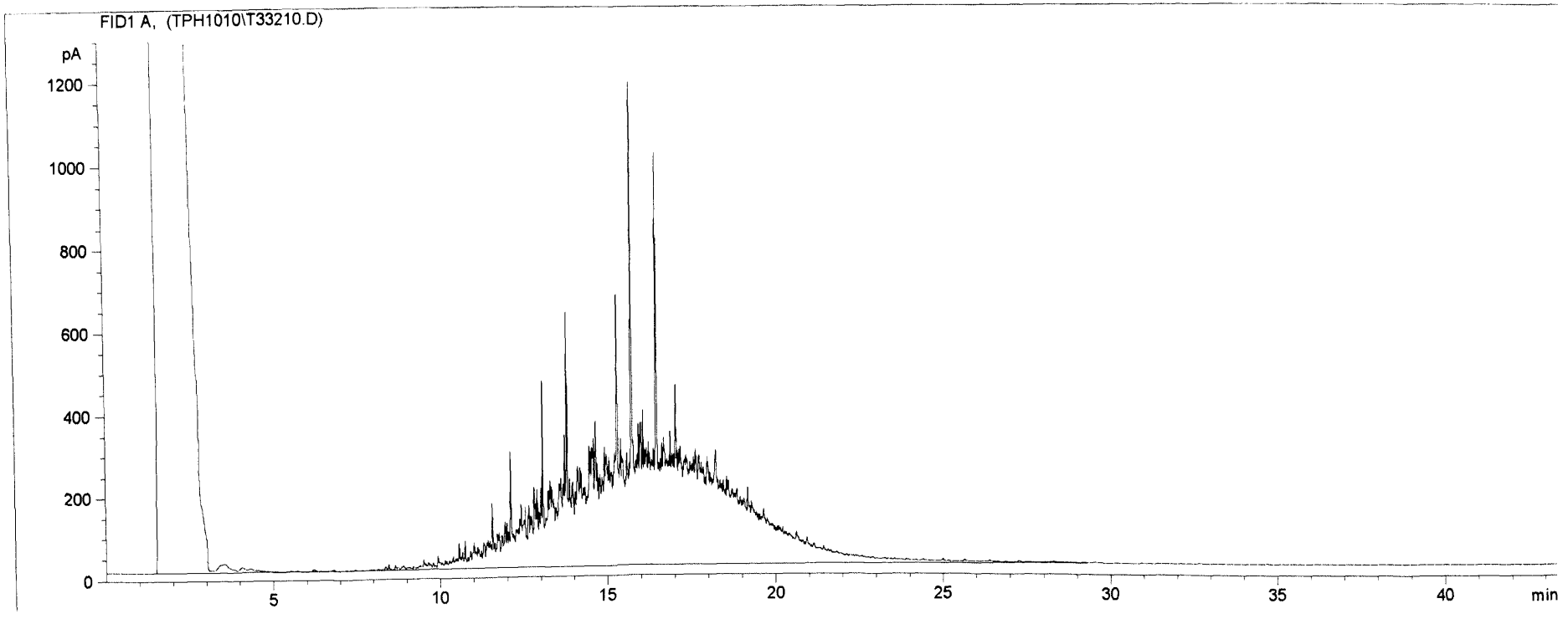
Sterile Manure Treatment (SMT) Day 1 GC-FID Chromatogram



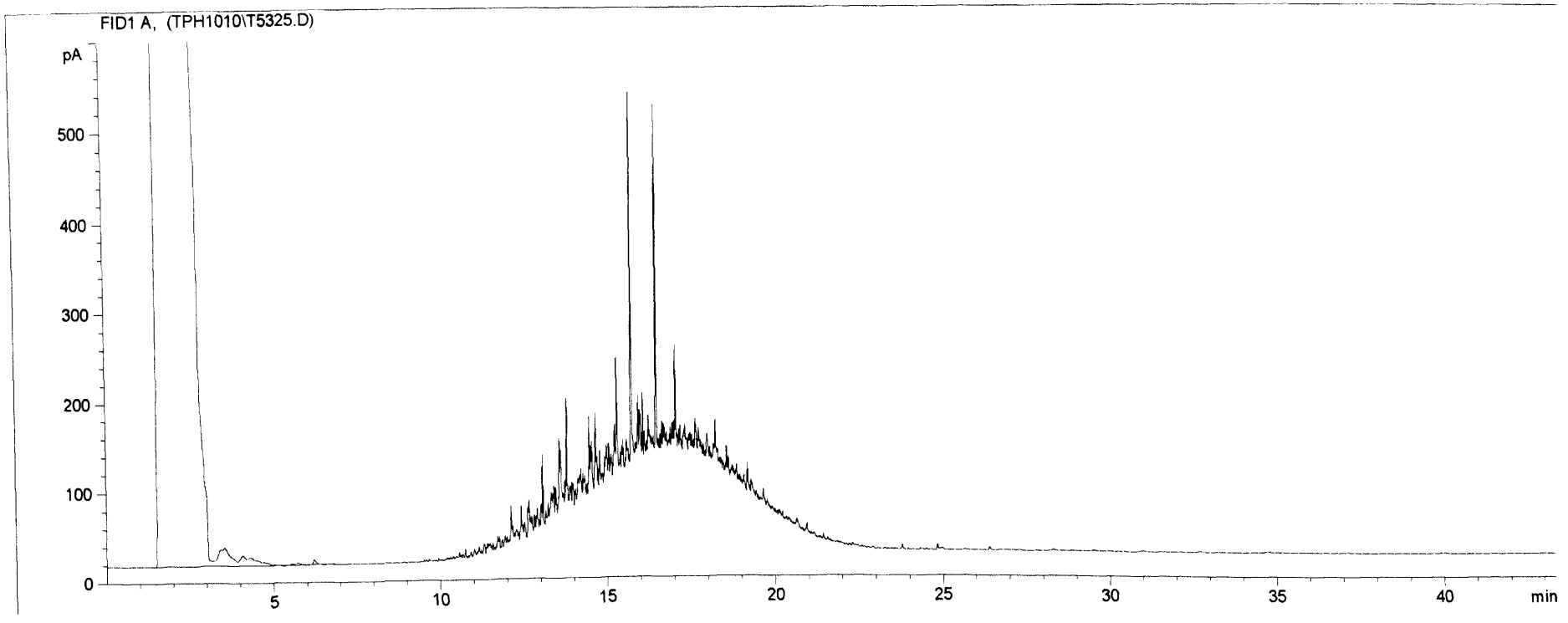
Un-managed Control (UMC) Day 32 GC-FID Chromatogram



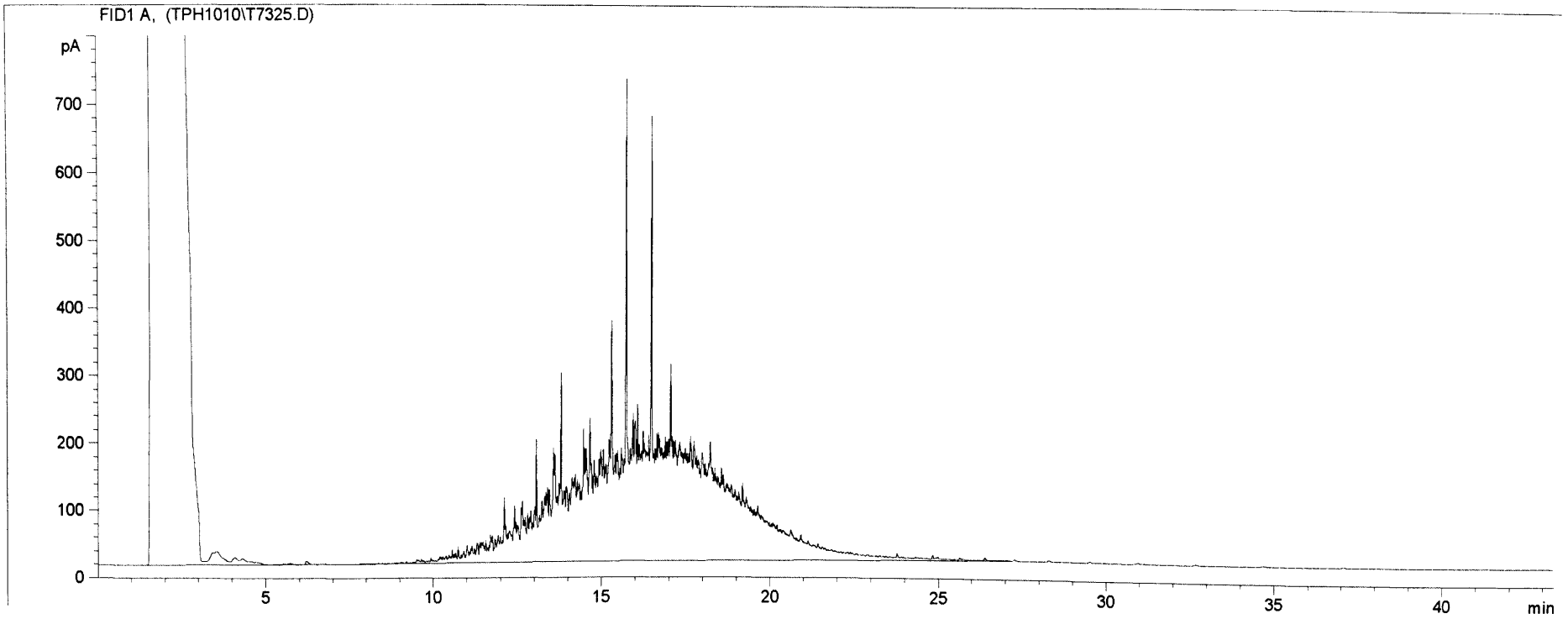
Managed Control (MC) Day 32 GC-FID Chromatogram



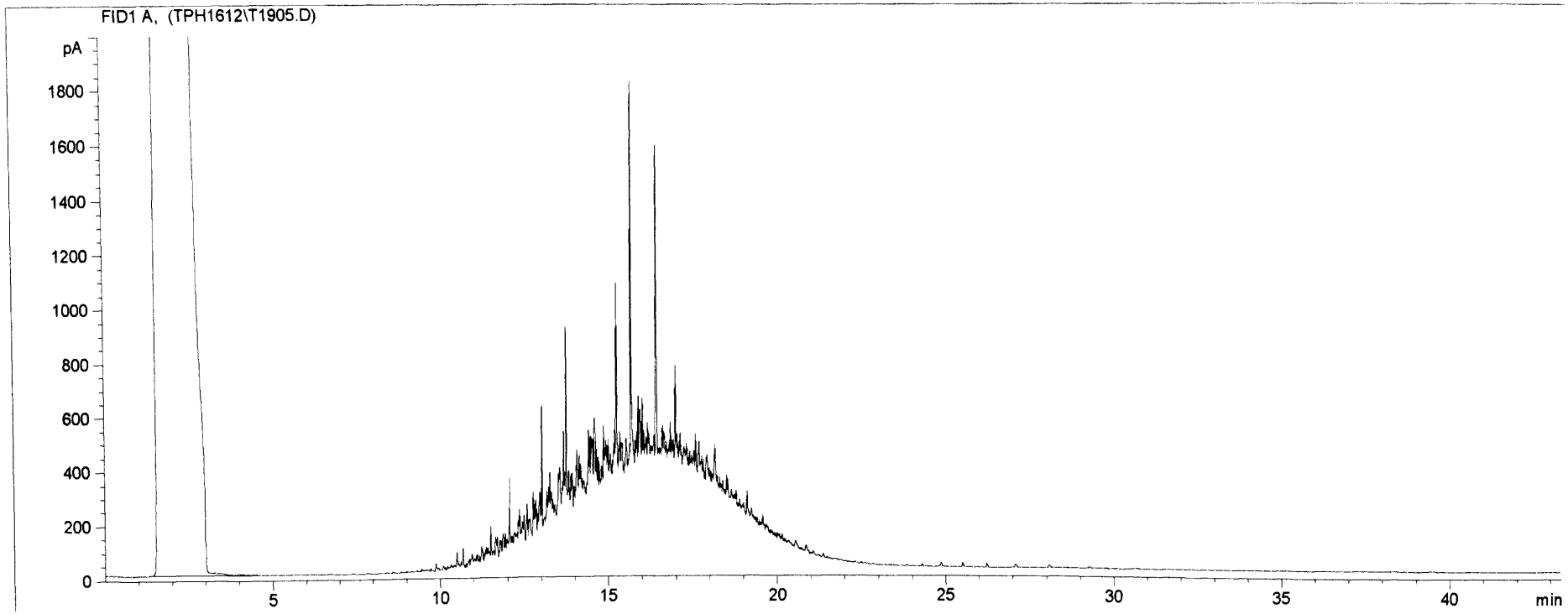
Total Manure Treatment (TMT) Day 32 GC-FID Chromatogram



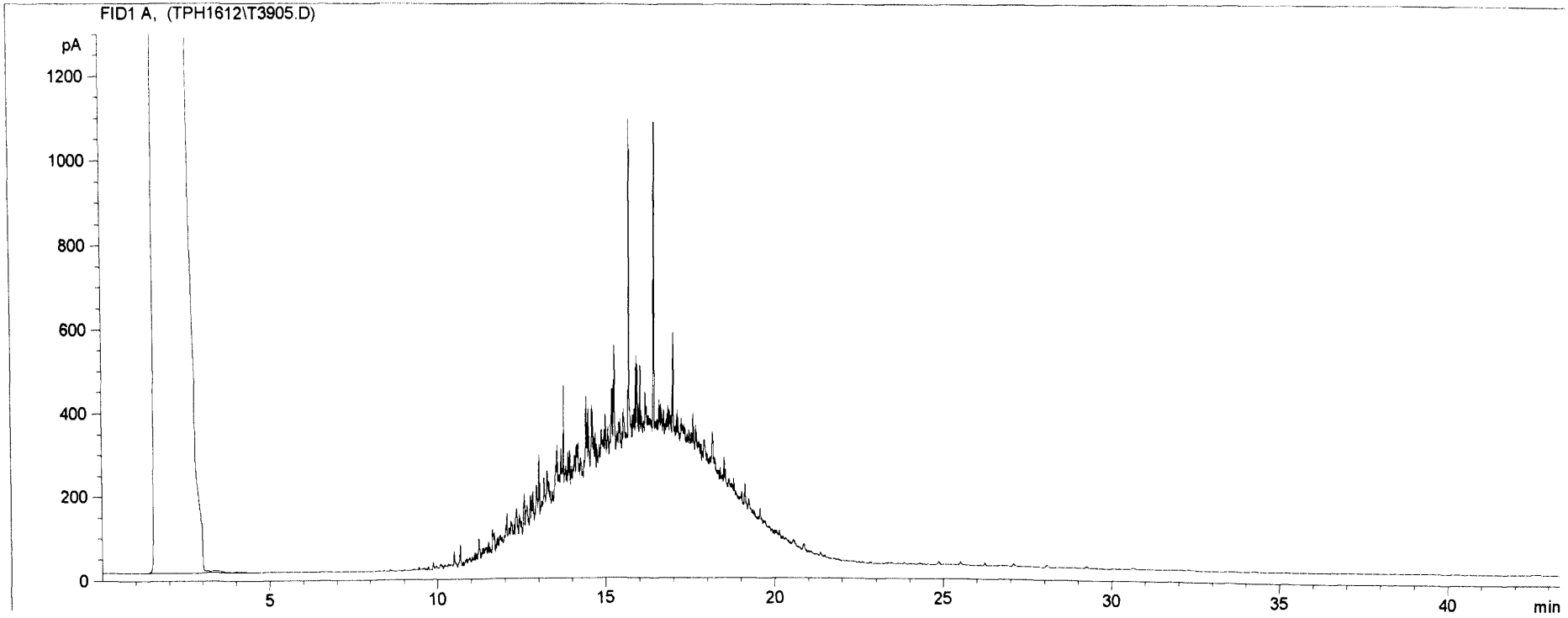
Sterile Manure Treatment (Day 32) GC-FID Chromatogram



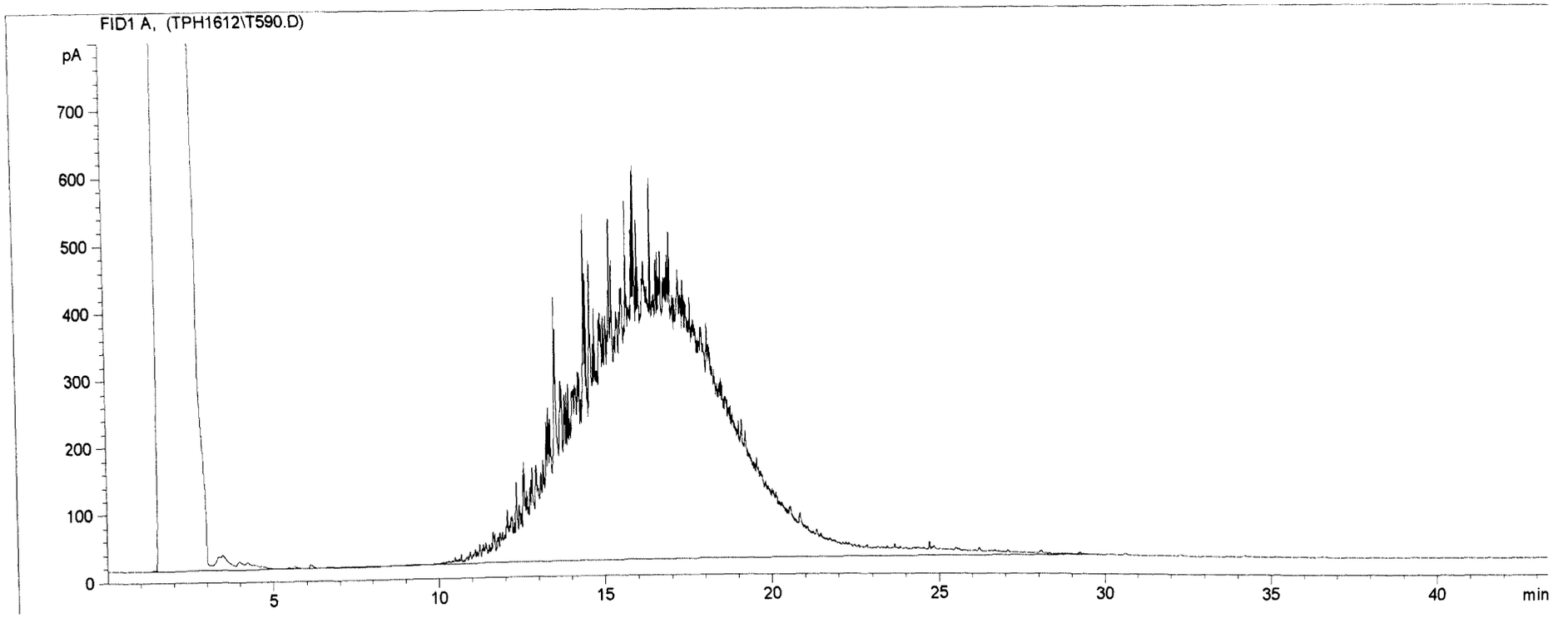
Un-managed Control (UMC) Day 90 GC-FID Chromatogram



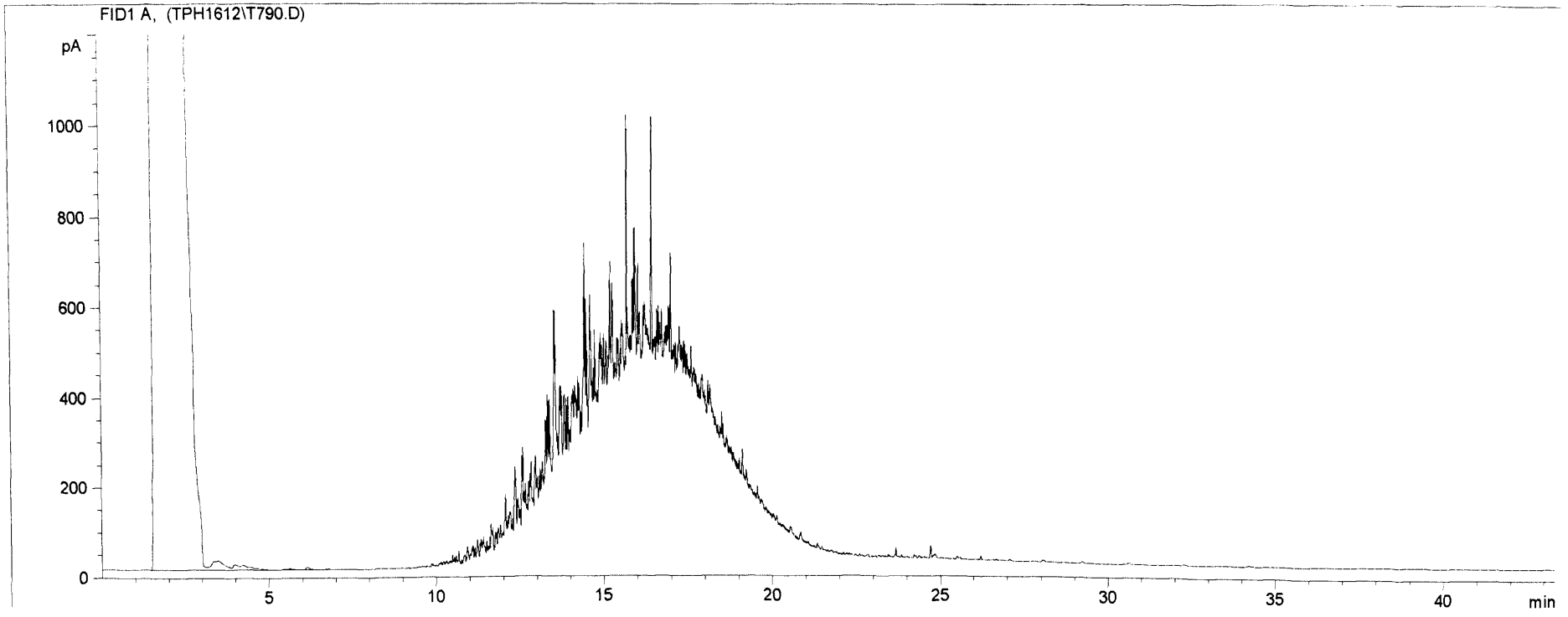
Managed Control (MC) Day 90 GC-FID Chromatogram



Total Manure Treatment (TMT) Day 90 GC-FID Chromatogram



Sterile Manure Treatment (SMT) Day 90 GC-FID Chromatogram



Treatability Study Raw Data
Chemical Analyses - Headspace Analyses

SPME total peak areas (uncorrected)

Day	Tray 1	2	3	4	5	6	7	8
1	258367663	19927364439	1250214984	945860945	28657677091	26882814459	533695635	699105641
	20909901330	28657831541	1131105479		32927269097		668413882	
2	1095719858	999915595	875770575	863592831	625681738	730533355	553284626	598467050
	1000531911	984767585	976327520	841003605	811708142	711422921	475938663	708367673
4	985942586	1032585693	1075994162	948468714	228626675	386231206	527985202	687839703
	1041556684	1063622237	935461347	1005198129	160686094	401577773	623968710	626021038
8	708868734	749255668	1000097641	817274863	130463982	177897919	338446594	204845302
	607028814	386754776	740934279	674578010	80411677	108785703	333438677	198007415
16	898165110	1089755517	608694944	331008544	57421491	162314475	131171912	171979228
	669133038	900760747	341517874	283916100	58329055	168894552	123746051	142618028
32	45909893	43941451	80141811	93439424	3291157692	2335535017	645147822	578452389
	38386420	60725085	115683066	41960193	1124172077	2353549459	468846963	701934099
64		497003555	6192895979	1459850362	909203692	557249153	1355531117	526686225
	399961511	459732238	1371553081	1177382690	582389447	570323716	511899914	593528160
90	3644478672	664091371	1407742449	1599908595	326222293	266631283	198789428	290589706
	1626635279	915760195	665954013	920029468	278112274	257261224	236405235	254913654

Hexadecane Standard - peak area and correction factors

Hexadecane batch	contents	area	correction factor
1	T1,2,5,6 DAY 1, 2, 4	13836635	1
2	T1,2,5,6 DAY 8	23144442	0.597838349
3	T3,4,7,8 DAY 1	25285092	0.547225021
	T3,4,7,8 DAY 2		
4	T3,4,7,8 DAY 4	37946860	0.364631883
	T1,2,5,6 DAY 16		
5	T3,4,7,8 DAY 8	29475660	0.469425791
	T3,4,7,8 DAY 16		

Hexadecane batch	contents	area	correction factor
6	T1,2,5,6 DAY	13469432	1.027261951
7	T3,4,7,8 DAY	27516732	0.502844415
8	T1,2,5,6 DAY	27385048	0.505262397
9	T3,4,7,8 DAY	32806102	0.421770163
10	T1,2,5,6 DAY	17986296	0.769287629
11	T3,4,7,8 DAY	386389472	0.035810072
baseline =		13836635	

SPME Total Area corrected for hexadecane correction factors and split/splitless injection

Day	Tray 1	2	3	4	5	6	7	8
1	258367663	19927364439	684148920.8	565471946	28657677091	26882814459	292051605	382568099.1
	20909901330	28657831541	618969219.5		32927269097		365772801	
2	1095719858	999915595	479243571.3	516288913	625681738	730533355	302771191	327496144
	1000531911	984767585	534270847.6	502784207	811708142	711422921	260445545	387636514.7
4	985942586	1032585693	392341776.9	345841933	228626675	386231206	192520238	250808285.8
	1041556684	1063622237	341099032	366527286	160686094	401577773	227518885	228267229.6
8	423788913.8	447933771.7	469471625.8	383649899	77996371.64	106354198.2	158875560	96159667.85
	362905104.1	231216836.9	347813659.7	316664316	48073184.24	65036265.11	156524715	92949787.34
16	327499634.9	397359605.7	285737105.3	155383947	20937706.36	59185032.59	61575478.5	80731485.08
	243987239.3	328446086.9	160317298	133277540	21268633.13	61584338.45	58089587.8	66948580.55
32	47161486.27	45139380.7	40298862.05	46985492.5	33808810.73	23992062.59	3244089.79	2908715.53
	39432908.71	62380569.31	58170583.63	21099448.7	11548192.01	24177118.1	2357570.77	3529636.412
64		2511172.076	26119787.49	6157213.26	4593864.37	2815570.429	5717225.81	2221405.352
	2020855.118	2322854.126	5784801.67	4965848.9	2942594.881	2881631.279	2159041.1	2503324.69
90	28036523.56	5108772.761	504113.5914	572928.428	2509587.742	2051161.475	71186.6383	104060.3844
	12513503.97	7044829.889	238478.6148	329463.219	2139483.318	1979078.77	84656.886	91284.76427

APPENDIX 5

Microbial Analyses

Protocols
(on CD)

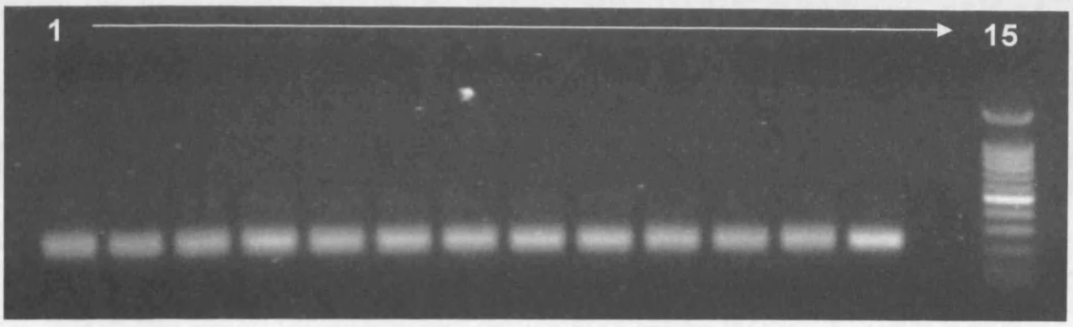
Polymerase Chain Reaction (PCR) Images

Denaturing Gradient Gel Electrophoresis (DGGE) Images
(on CD)

DGGE Band Sequencing Raw Data Files
(on CD)

Polymerase Chain Reaction (PCR) Images

Un-managed Control (UMC)



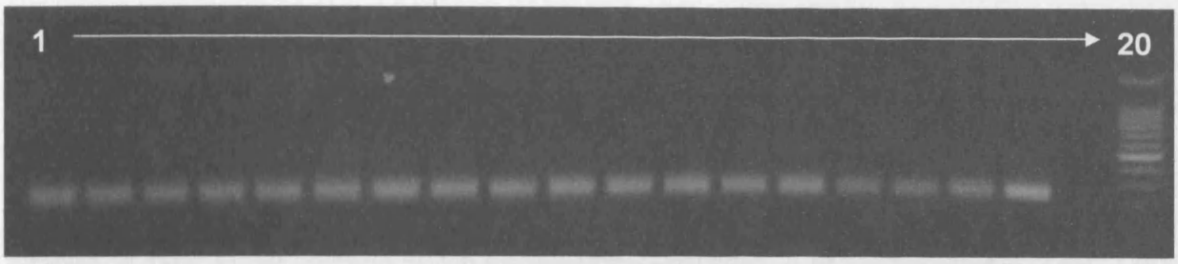
Lanes 1 to 15 – contents

1	Day 1A	1/10
2	Day 1B	1/10
3	Day 8A	1/10
4	Day 8B	1/10
5	Day 16A	1/10
6	Day 16B	1/10
7	Day 32A	1/10
8	Day 32B	1/10
9	Day 64A	1/10
10	Day 64B	1/10
11	Day 90A	1/10
12	Day 90B	1/10
13	Positive control	
14	Negative control	
15	100bp ladder	

Comments

All samples run at 1 in 10 dilutions where indicated, as this dilution was identified as being optimum based on previous PCR results.
No contamination in negative control lane therefore proceeded with DGGE.

Managed Control (MC)



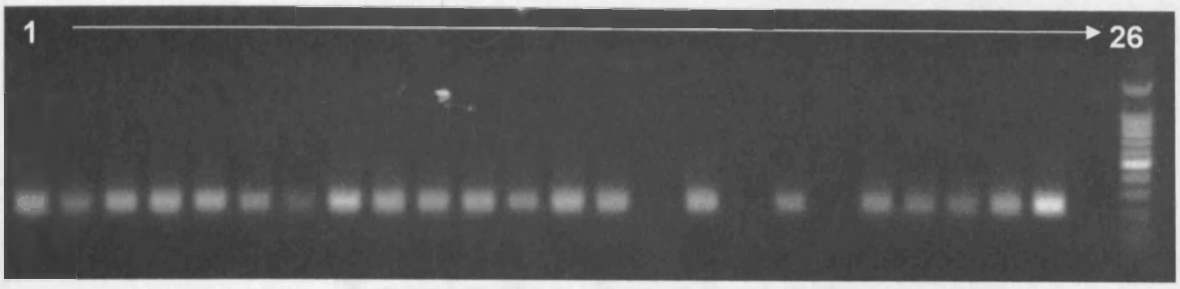
Lanes 1 to 20 – contents

1	Day 1A
2	Day 1B
3	Day 2A
4	Day 2B
5	Day 8A
6	Day 8B
7	Day 16A
8	Day 16B
9	Day 32A
10	Day 32B
11	Day 64A
12	Day 64B
13	Day 90A
14	Day 90B
15	Manure A
16	Manure B
17	Manure C
18	Positive control
19	Negative control
20	100bp ladder

Comments:

All samples were run as neat.
No contamination in negative control lane therefore proceeded with DGGE.

Total Manure Treatment (TMT)



Lanes 1 to 26 – contents

1	Day 1A	1/10
2	Day 1B	1/10
3	Day 2A	1/10
4	Day 2B	1/10
5	Day 8A	1/10
6	Day 8B	1/10
7	Day 16A	1/10
8	Day 16B	1/10
9	Day 32A	1/10
10	Day 32B	1/10
11	Day 64A	1/10
12	Day 64B	1/10
13	Day 90A	1/10
14	Day 90B	1/10
15	Sterile Manure A	neat
16	Sterile Manure A	1/10
17	Sterile Manure B	neat
18	Sterile Manure B	1/10
19	Sterile Manure C	neat
20	Sterile Manure C	1/10
21	Manure A	1/10
22	Manure B	1/10
23	Manure C	1/10
24	Positive control	
25	Negative control	
26	100bp ladder	

Comments

All samples run at 1 in 10 dilutions where indicated, as this dilution was identified as being optimum based on previous PCR results.
No contamination in negative control lane therefore proceeded with DGGE.

APPENDIX 6

Toxicity Analyses

Rapid On-Site Toxicity Audit System

Protocols (on CD)
Leachable Test SUITE Raw Data Files (on CD)
Organics Test Suite Raw Data Files (on CD)
Leachable Test Suite Raw Data Summary
Organics Test Suite Raw Data Summary

Plasmid Assay

Gel Images and Densitometry Raw Data Files (on CD)
Raw Data Summary

Treatability Study Raw Data
Toxicity Analyses - ROTAS Leachable Assay
Summary of Raw Data

% light emittence

Un-managed control

Day	Tray 1			Tray 2		
1	100	100	100	100	100	100
32	100	100	100	100	100	100
64	100	100	100	100	100	100
90	100	100	100	100	100	100

Managed Control

Day	Tray 3			Tray 4		
1	100	100	100	100	100	100
32	100	100	100	100	100	100
64	100	100	100	100	100	100
90	100	100	100	100	100	100

Total manure treatment

Day	Tray 5			Tray 6		
1	12	10	12	15	10	11
32	35	35	29	46	45	
64	20	20	23	13	14	14
90	29	27	28	28	27	29

Sterile manure treatment

Day	Tray 7			Tray 8		
1	23	22	18	25	25	40
32	42	39	41	34	35	35
64	28	28	27	20	20	20
90	36	39	39	35	32	34

Treatability Study Raw Data
Toxicity Analyses - ROTAS Organics Assay
Summary of Raw Data

% light emittence

Un-managed Control (UMC)

	Tray					
Day	1			2		
1	39	46	45	49	51	51
32	26	24	27	19	20	
64	28	38	37	36	36	
90	27	22		30	19	35

Managed Control (MC)

	Tray					
Day	3			4		
1	45	58	52			
32	38	57	54	45	50	
64	42	59	58	68	74	
90	61	78		54	48	53

Total Manure Treatment (TMT)

	Tray					
Day	5			6		
1	55	59	72	53	47	47
32	97	97	97	77	81	80
64	100	100	100	100	100	100
90	100	100	100	100	100	100

Sterile Manure Treatment (SMT)

	Tray					
Day	7			8		
1	62	37	57	44	50	54
32	66	77	74	77	76	75
64	100	100	100	100	100	100
90	100	100	100	100	100	100

Treatability Study Raw Data
Toxicity Analyses - Plasmid Assay
Summary of Raw Data

% Plasmid DNA Damage

T1:1		T2:1		T3:1			
1	72.44272	1	53.75446	1	50.92066		
5	15.21795	2	47.01915	2	30.34212		
10	14.81617	5	26.38452	5	10.54505		
50	4.200785	10	16.18294	10	6.384626		
100	5.890348	50	10.61452	50	3.625385		
		100	6.538761	100	3.319635		

T1:32		T2:32		T3:32		T4:32	
1	100.0559	1	61.08252	1	33.17995	1	41.5908
5	57.06562	5	25.33315	5	25.74486	5	16.29572
10	27.87946	10	19.69039	10	24.67683	10	10.53731
100	16.00137	100	20.83441	100	18.31633	100	11.15055
1000	10.62906	1000	18.80389	1000	11.09807	1000	6.154043

T1:64		T2:64		T3:64		T4:64	
1	100.0253	1	100	1	100	1	93.25436
2	100.0253	2	97.63339	2	100	2	88.39724
5	84.96169	5	45.37853	5	67.17481	5	35.17382
10	38.49384	10	22.25655	10	27.12366	10	22.25169

T1:90		T2:90		T3:90		T4:90	
1	99.96499	1	93.323	1	89.89681	1	100
2	94.18526	2	71.57184	2	69.84554	2	96.87757
5	90.4652	5	36.74517	5	29.28458	5	70.0092
10	42.67835	10	33.49232	10	12.21753	10	37.9285
100	22.32927	100	33.2117	100	21.34514	100	25.90366

T5:1		T6:1		T7:1		T8:1	
1	100	1	100	1	100	1	100
5	100	5	100	5	100	5	100
10	100	10	72.38368	10	100	10	100
50	85.36732	50	100	100	14.20769	50	100
100	3.362586	100	5.682092			100	13.8586

T5:32		T6:32		T7:32		T8:32	
1	100	1	100	1	100	1	100
50	100	50	88.1167	50	71.94892	50	100
100	19.90119	100	20.02069	100	23.27002	100	35.46095
1000	-4.934393	1000	-11.50784	1000	14.98624	1000	31.63469

T5:64		T6:64		T7:64		T8:64	
10	84.41371	10	100	10	100	10	100
25	100	25	100	25	100	25	95.70544
50	97.27611	50	94.4072	50	59.8346	50	33.84296
100	29.17326	100	40.39327	100	18.58737	100	15.46847

T5:90		T6:90		T7:90		T8:90	
10	100	10	100	10	100	10	100
25	93.55197	25	100	25	76.15835	25	98.81329
50	87.74735	50	97.62149	50	39.70746	50	64.14174
100	43.01071	100	48.69328	100	37.07568	100	34.54302

NB. T1:1 = Tray 1, Day 1

Treatability Study Raw Data
Toxicity Analyses - Plasmid Assay
Summary of Raw Data

Dilution (1 in x) needed to cause 50% plasmid DNA damage (TD_{50})

Un-managed Control (UMC)

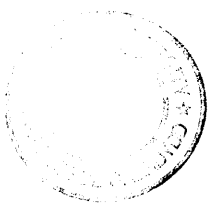
Day	1	Day	2
1	2.56	1	1.56
32	6.2	32	2.24
64	8.76	64	4.83
90	9.25	90	3.86

Managed Control (MC)

Day	3	Day	4
1	1.05	1	1.723333
32	1	32	1
64	7.1	64	4.16
90	3.45	90	8.14

Total Manure Treatment (TMT)

Day	5	Day	6
1	71.5	1	76.5
32	81	32	78
64	84.7	64	91
90	81	90	98.5

Sterile Manure Treatment (SMT)

Day	7	Day	8
1	62.5	1	79
32	72.5	32	88.6
64	62	64	43.5
90	43	90	74