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BIOREMEDIATION OF DRILL CUTTINGS FROM OIL BASED MUDS

by

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ABSTRACT

Analytical techniques applicable to the assay and remediation of cutting/mud matrices have been developed, utilising soxhlet extraction with dichloromethane and a drying agent followed by analysis using Gas Chromatography (FID). Calibration curves of oil content were produced for Novatec and Versaplus coated cuttings that were also sized by wet and dry sieving techniques, demonstrating their variable nature. The oil in each size fraction was assessed and showed that the finer fractions preferentially adsorbed the oil. Bacteria were isolated from the cuttings, muds and the pure oils to see if any indigenous species could, with optimum conditions, remediate the oil they contained. The resulting isolates were batch-tested in the laboratory in a minimal medium, with the drill cuttings providing the sole carbon source. Each isolate was scored for remediation performance, with reduction in oil varying from 50% to 6% within one week. Subsequently three bacteria (A,D & J) were identified using 16SrRNA sequencing; they were *Bacillus Thuringiensis* (A&D) and a novel species related to *Bacillus oleronius.* These were then tested slurry-phase in a rotating drum bioreactor designed and fabricated for the research against a known remediator, *Rhodococcus* 9737, and a non-inoculated control for four weeks. All the reactors remediated, but *Rhodococcus* 9737 reduced the oil to 35% of the original, A, D and other isolates as a consortium to 83% and J, 90%. Further tests in the bioreactors, after a modification to improve the air supply gave reductions of around 50% after four weeks. The high clay content of the cuttings was detrimental to significant levels of bioremediation in a slurry-phase bioreactor. Manures were added to the drill cuttings and tested in the bioreactors as a solid-phase system. These degraded the cuttings oil to 2% (v/v), a 96% reduction. Composting was thus more applicable for a high clay content drilling waste bioremediation system.

CHAPTER 1:
INTRODUCTION

1.1. Principal Objectives

The principal objective of this research was to develop a practical scheme for the biocleaning of drill cuttings contaminated with oil-based mud. This required cleaning the cuttings from present discharge levels of ~10 - 20% oil on cuttings to <1% v/v residual oil and was to be achieved with minimum risk to health, the environment and ecology, or of litigation.

1.2. Development of the Project

The project commenced with a thorough review of the relevant literature; this was continued episodically, as appropriate.

The hydrocarbon structure of the muds was examined to see if there was any preferential degradation of a particular chain length, and to ensure the hydrocarbons within the muds were not toxic to the bacteria.

Although commercial strains of remediating bacteria were available, the microbiology of the muds was explored to determine whether the natural mud flora would include novel remediators.

The preliminary practical work involved the isolation of several strains of bacteria from the oils and testing their ability to thrive in the mud and cuttings environment. Their remediation capabilities were then assessed, and any potential remediators were tentatively identified.

Further batch testing was carried out using bacteria isolated from the actual drill cuttings and muds; these, along with other isolates, were frozen and stored in readiness for the lab scale bioreactor experiments.

Analysis techniques were developed for the testing of the remediation capabilities of the isolated bacteria and commercial strains, using solvent extraction and gas chromatography with a flame ionising detector.

Successful remediators at the batch testing stage were taken forward and tested in the lab scale bioreactors. The reactors were designed to accommodate either a slurry material or a compost type material.

N.B. Although the UK has adopted S.I. units, the multinational oil industry still uses a mixture of metric and imperial/US units. Both systems of units have been used in this thesis and a conversion table is given below.

Table 1.1. Conversion Table

IMPERIAL/US UNITS	METRIC UNITS
1 inch	25.4 mm
1 ft (foot)	0.3048 m
bbl (42 US Gallons)	0.15899 m^3
1 psi	6.895 x 10^3 Pa
1 lb/gal (pound/gallon)	119.826 kg m^{-3}

CHAPTER 2
REVIEW OF OIL INDUSTRY

2.1. Introduction

"No other single industry affected 20th Century civilization more rapidly or more profoundly than the oil industry" (Howarth, 1997).

Oil, a single basic resource, has provided an array of products. These include fuels for heating, lighting and combustion engines; lubricants for many mechanical moving parts, and as bases for creams and lotions (human lubricants!), and more complex products, ranging from textiles and plastics to perfumes. Oil is not a new product; archaeologists have found evidence of the use of one or more of the component parts or fractions of crude oil. The Dead Sea was known as Lake Asphaltites, from which the term asphalt was derived, due to the lumps of semi-solid petroleum washed up on its shores from underwater seeps (Britannica, 1995). Examples of the use of asphalt and other hydrocarbons include, in what is now Iraq, asphalt used as an adhesive for masonry and a sealant for water-craft (Lewis, 1995), dating back to 3500 BC; bitumen, used as a waterproofing agent for baths in the Indus valley by 3000 BC, and, from 2200 BC, the Babylonians built bridges, walls, tunnels, sewers, roads, the Hanging Gardens and the Tower of Babel with asphalt as the bond (Howarth, 1997). Mesopotamian bitumen was exported to Egypt where one of its uses was the preservation of mummies (Britannica, 1995). The usage has continued from these times to the modern day mostly by the Arabs, the Greeks and the Romans, for peace and for war – bombs were made as early as 476 AD in the Byzantine Empire by mixing petroleum with calcium oxide, which would spontaneously combust when exposed to moisture ('Greek Fire'). Wars over oil are not a new thing either; they date back to the Middle Ages (Howard, 1997).

It has been suggested that fire and light-worshipping cults and religions were prompted by the eternal flames caused by the ignition of gas seeping from the earth –

Zoroastrianism, based in ancient Persia, is a religion still followed by the Parsees (Sykes, 1982).

Oil lamps were used for domestic lighting and heating in the Middle East for millennia before being introduced to Europe or North America. These times were literally the dark ages; lamps made from animal fats were at best smoky, but often smelly and inefficient.

Then, in 1859, the same year that Darwin's book *The Origin of Species* was published in London, Edwin Drake's drilling derrick bit into Pennsylvanian soil.

2.1. Drake's Derrick (tower), 1859.

Although oil had been discovered before, this was the first indication that oil could be produced commercially from drilling in the Western world. Before this, digging pits or skimming oil from natural seepages from the surfaces of streams and lakes was the main method of oil recovery in the West. China had been drilling for oil around 200 BC, with bamboo piping and brass attachments, penetrating as deep as 3,500 ft, which makes Drake's 69½ ft look trifling. Drake was filling 25 barrels daily in his first year of production. Within 6 years production had increased to 7,000 barrels daily worldwide, 6,800 from the US alone. This increased to 284,000 barrels by 1895; 7.1 million by 1945, and 1990 figures were estimated at 60 million – daily (Howard, 1997). Although still measured in barrels, which is 42 US gallons, the oil production has long since outgrown this mode of containment.

2.2. Current Status

The industry has come a long way since Drake, not only in production, but also in technical expertise, safety and accountability. Also many oil companies, with the exception of Exxon, are looking towards renewable resources of energy as an alternative to the finite resource of oil (Greenpeace, 2001). The advancing technologies concerning recovery mean that no one is sure how much oil can be recovered.

An example of technical advancement is the plan to take surplus gas via the Sullom Voe oil terminal on Shetland to the North Sea's most Northerly oil platform on the Magnus oilfield, 340 miles NE of Aberdeen, a £320 million project of BP's (Hydrocarbon online, 2001). The gas will be injected into the oil reservoir, 8,900 ft below seabed, to flush out an extra 50 million barrels of oil.

Well recovery rates have increased considerably over the last few decades, making oil fields that were uneconomical now feasible. The Enhanced Oil Recovery (EOR) technologies have increased the primary recovery life of a well from around 25% to up to 75%.

In the Gulf of Mexico half the oil comes from wells in water more than 1,000 ft deep (Schrope, 2001). Technological advances have now made it possible to drill in ultra-deep waters of more than 5,000 ft; eight wells are currently being drilled in these conditions, overcoming the immense pressures and low temperatures existing at that depth in the ocean.

Deepstar, an industry-wide collaboration, is tackling the challenges of profitably extracting oil in ocean depths up to 10,000 ft. The complications associated with this are immense – the drilling fluids will have to handle extreme pressures and changes of temperatures, from the heat within the crust to the cold of the deep ocean. Once the oil is extracted, the extreme cold can impede its pumping across a cold sea floor. The extreme cold can increase hydrate formation, which also inhibits pumping.

2.2.1. Production, Supply and Use of Crude Oil, Natural Gas Liquids (NGLs) and Feedstocks, 2001

Table **2.1.1.** illustrates that offshore development had reduced slightly from 1998 to 2000, but increased again in 2001. Exploration and appraisal has increased since 1999. Generally, development of wells is often related to crude oil prices in that prices can seriously affect the economical viability of extraction, thus E & P Rates can fluctuate quite considerably.

The UK still retains its position as a net exporter overall of oil and oil products, and even though figures are 18.7% lower than last year's (2000), they still stand at 10 million tonnes. Export of natural gas liquids increased in the same time period by 7.6% to 11.1 million tonnes.

The UK became a net importer this year (2001) of petroleum products for the first time since 1984, with net imports of 1.1 million tonnes in the second quarter. The DTI claim this is related to refinery closures for maintenance and conversions of refinery capacity to the production of Ultra Low Sulphur Petrol (DTI, 2001).

Table 2.2.1. Drilling activities on the UK Continental Shelf (DTI, 2001)

		Number of Wells Including Sidetracks					
Quarterly Figures		Offshore				Onshore	
Year	Quarter	Exploration (E)	Appraisal (A)	E&A	Development	E&A	Development
1998	1st	14	9	23	78	4	9
1998	2nd	11	5	16	61	6	9
1998	3rd	14	8	22	71	3	1
1998	4th	8	11	19	71	1	2
1998	**TOTAL**	**47**	**33**	**80**	**281**	**14**	**21**
1999	1st	7	3	10	72	-	3
1999	2nd	4	4	8	60	3	2
1999	3rd	3	6	9	62	1	5
1999	4th	2	7	9	40	4	1
1999	**TOTAL**	**16**	**20**	**36**	**234**	**8**	**11**
2000	1st	3	8	11	51	-	-
2000	2nd	10	7	17	51	8	7
2000	3rd	6	11	17	59	5	2
2000	4th	7	7	14	55	1	2
2000	**TOTAL**	**26**	**33**	**59**	**216**	**14**	**11**
2001	1st	5	8	13	54	1	2
2001	2nd	8	7	15	78	1	7
2001	3rd	6	10	16	67	1	8

However, even though the North Sea is now viewed as a mature prospect, it is thought that the UK will remain a centre for expertise and a base for major multi-nationals long after the North Sea ceases to be a major producer.

2.3. Development of Drilling Fluids

Many of the advances in recovery and availability have been enabled by the development of the drilling fluids, which have allowed for better safety practices and drilling techniques.

In the early 1900's, while rotary drilling to a depth of 300 ft for water in Wyoming, friction and cuttings removal problems occurred. It was found then that the addition of clays to the water, which was the drilling fluid at the time, gave good lubrication properties and increased the fluid viscosity, helping lift the drill cuttings. Wyoming bentonite was the only addition to the drill water for many years, but by the late 1930's crude oils were introduced. A significant problem associated with these fluids was the low flash point of the volatile fractions within the crude, and the associated safety concerns.

The 1950's and 1960's saw many advances on the original mud formulations and recipes, and as time progressed more was expected from the drilling fluid.

To understand how this progression has come about it is important to know about the drilling fluids, or 'muds', used for drilling for oil.

2.3.1. What are Drilling Fluids?

Drilling Fluids are usually non-Newtonian fluids (Moore, 1986). They were first introduced with rotary drilling methods in 1900, with their initial primary purpose being to continuously remove the rock cuttings. As time progressed, more was expected from the fluids, with additives for almost any conceivable purpose being introduced, which has led to a complicated mixture of liquids, solids and chemical conditioning agents. There are two basic sorts of drilling fluid – water-based and oil-based. Oil-based fluids can be further sub-divided into direct and invert emulsions, depending on the dominant phase. Initially mineral oils were used, e.g. diesel, but these were later replaced by synthetic drilling fluids to improve drilling, safety and environmental performance.

2.3.2. The Primary Functions and Applications Of Drilling Fluids

Most drilling fluids are engineered for specific operating requirements, and are required to perform the following actions with varying degrees of importance.

2.3.2.1. Lifting Formation Cuttings

The rock is broken up through the action of the drill bit, and obviously needs to be removed from the drill bit area or the hole will load up and the whole operation would grind to a halt. Drill solids generally have a specific gravity (SG) of 2.5 – 3.0. If this is heavier than the mud, they can slip downwards. Slip velocity is affected by thickness or shear characteristics and density (Moore, 1986), so when annular mud velocity is limited by the pump volume or enlarged hole sections, it is often necessary to thicken the mud, which can, in turn, adversely affect other drilling conditions. Thickening can be done by the addition of bentonite, drill solids, the reduction of water in oil emulsions, or the addition of polymers. If mud circulation stops for any reason, the cuttings have to be held in suspension, to prevent them settling and burying the drill bit. This is done by the addition of gelling agents to the mud; when the mud is not flowing, it sets, gel like, and prevents the descent of the drill cuttings. This 'thixotropic' property is characterised by a fluid with a time dependent viscosity. Muds are typically non-Newtonian fluids with shear rate dependent viscosities.

2.3.2.2. Control Subsurface Pressure

Subsurface pressure is controlled by the 'weight' of the mud. Minimum mud weights are desirable, as they increase drilling rates and decrease lost-circulation problems (Waller, 1997). Abnormally high formation pressures require careful measurements of pore pressure to determine mud weight requirements. If the formation pressures are higher than the annular fluid pressures, formation fluids can flow into the well-bore. This is known as a 'kick' and can have severe safety implications.

An equation for calculating drilling rates and mud weights (Moore, 1986):

$$\rho_2^{c} \log R_2 = \rho_1^{c} \log R_1$$

ρ_2 and ρ_1 = mud weights at two different times.

R_2 and R_1 = drilling rates corresponding to respective mud weights, feet per hour.

$$\text{Log } R_2 = \frac{\rho_1}{\rho_2} \log R_1$$

c = 1.5 in soft formations

c= 0.1 in hard formations.

To calculate mud weight:-

$$\rho = \rho_w (1-X) + 20.8X = \rho_w + X(20.8 - \rho_w)$$

ρ = mud weight, lb/gal

ρ_w = water weight, lb/gal

X = solids fraction

20.8 = weight of low gravity solids, lb/gal

It is worth noting that if the pressure of the mud exceeds the total pressure of the formation, the mud can be lost into the formation. Coastal formations often have higher pressures than inland formations. As mud weight rises, drilling rates decrease, mud costs increase and hole problems are more common. A reduction in circulation rate will increase the mud weight, and vice versa. Fluid loss control additives, such as shredded walnut hulls, can be added to the mud to aid loss control and give support to the hole walls by producing caking action on the sidewalls.

2.3.2.3. Lubrication and Cooling of the Drill String

Lubrication prolongs the life of the equipment and reduces hole problems such as torque, drag and differential pressure sticking (where a portion of the drill string sticks in the filter cake). Lubricant additives include bentonite, oil, detergents, graphite and other special surfactants.

2.3.2.4. Cleaning the Bottom of the Hole

This involves keeping the drill bit free from the build up of cuttings. Thin muds at high shear rates through the bit are best for this, although viscous fluids are potentially good if they have good shear thinning characteristics. Usually lower solids content are preferred.

2.3.2.5. Aid in Formation Evaluation

The mud can be utilised to evaluate the formation being drilled through. Special fluids can improve logging characteristics and formation testing. Properties that can affect evaluation include saltwater, which makes using a self-potential log difficult, thick filter cake, which makes information hard to obtain from sidewall coring and water/oil invasion, which affects resistivity. To obtain better cuttings the viscosity can be increased.

2.3.2.6. Protect Formation Productivity

There needs to be sufficient cake and pressure to prevent 'washout', i.e. the flow of mud washing away the reservoir formation, causing instability and geo-technical problems with reservoir. The ideal drilling conditions include keeping the downhole formation in a virgin state, i.e. no fluid should enter this zone. This is quite difficult to achieve, but using air with the drilling helps. Water/clay interactions can cause clays to swell, thereby significantly reducing formation permeability. Oil based muds successfully keep this zone water free, but this can cause complications in gas zones.

2.3.2.7. Aid to Formation Stability

This can be critical when drilling. Shales are inherently weak under tensile or compressional stress (MIDF, 1998). If a clay or shale contains a pressure significantly greater than the wellbore pressure, the shale will move toward the wellbore (sloughing), causing instability and hole enlargement. Clays also absorb water, swell and weaken the formation causing sidewall failure. The solution is to alter the weight of the mud to balance the pressure between the wellbore and the formation, or use oil-based muds to help prevent the clays swelling. Special hydration suppression additives have been developed which also inhibit clay swelling. Other causes of instability include natural fracturing, where the shale tends to fall into the wellbore because the shale fragments are detached. Certain additives can aid with the sealing of these fractures, but are not a panacea. Salt sections can also be unstable, but can be stabilised by drilling with either saturated saltwater of the same type found in the formation, or the use of oil-based muds.

2.3.2.8. Corrosion Protection

Some formations contain fluids of a corrosive nature, such as salt-waters or acids, which can be detrimental to the drilling hardware and can, in the case of acidic waters, cause flocculation of the fluids. The additives in the mud, for example lime for an acidic formation to raise the pH, can help counteract these effects.

2.3.2.9. Mechanical Support

This includes jet-assisted cutting, where the mud transmits hydraulic power to the bit, and drilling of down-hole motors, where the mud supports part of the weight of the drill-pipe and casings.

2.3.3. The Basic Types of Drilling Fluids

Table 2.3.2. Overview of Mud Types, Advantages and Disadvantages

MUD TYPE	ADVANTAGES	DISADVANTAGES
Fresh Water	Common, cheap, accessible, easy to control, good formation evaluation.	Swelling of clays, sloughing of shales, less cooling/lubricating ability, less wellbore stability.
Salt Water, commonly used offshore, purpose made muds.	Swelling of clays reduced, shales heave or slough less than with fresh water.	Higher mud costs, poor filter cake, less effective formation evaluation, corrosion due to electrolytes being good conductors of electricity and difficulties raising the pH.
Oil, has been used almost as long as water, initially to protect potentially productive formations.	No hydration or swelling of clays, formation damage minimised, hole problems minimised, better lubrication, temperature stable in deep wells (temps may exceed 350°C), where they are about 1/3 cheaper to maintain than comparable wt. water muds.	Generally more expensive than water, not pleasant to work with for the drilling crews, annular circulating pressures may be high, gas kicks difficult to detect due to solubility in the oil, environmental pollution problems.
Synthetic, 'Second Generation' Fluids, tailor made to suit environmental and drilling requirements.	Can be adapted to almost any drilling requirement, e.g. high temperatures, environmentally sensitive areas, shale stabilisation, water-sensitive formations. Many are water-based. Some are cheaper than the 1st generation fluids.	Some can be costly. Product inertia – for example, training and experience in dealing with a wide range of products.

2.3.4. Oil-Based Drilling Muds

2.3.4.1. Requirements for Oil-Based Drilling Mud

There is a common view that oil wells are drilled vertically downwards from surface to the oil reservoir. This could not be further from the truth, particularly in the North Sea Oil Fields, where deviations of 60° from vertical and horizontal sections within the reservoir are common. Drill strings 15,000 ft (4572 m) in length are sometimes used; the losses generated by friction can be enormous, requiring lubrication greater than could be provided by a standard water-based mud.

Drilling technology has been developed over the decades, and the drilling rates now need the cooling and lubricating properties provided by oils as well as the superior lifting abilities of oil-based muds for removal of the cuttings, attributed to the oil's flow characteristics (Philp, 1982).

Poorly consolidated shales, a feature of many wells in the North Sea, particularly the East Shetland Basin Wells, need the oil-based muds, which have had significant effects on drilling rates (Philp, 1982). Bit balling is also often reduced (shale drilling).

Well-engineered drilling fluids are a major contributor to well safety. Data brought to surface from the drill bit allows the muds to be engineered specifically for the formations being drilled through; this is key to safe practice, helping to manage potentially lethal kicks or blowouts of gas or oil.

New challenges facing the Drilling Fluids Companies include low-toxicity muds with reduced heavy metal content and easily degraded, and muds that can handle the very high pressures and extreme temperatures when drilling in sea depths of up to 10,000 ft (3048 m) (Schrope, 2001).

2.3.4.2. Water/Oil Muds

A mixture of oil and water is an oil emulsion: a 'direct emulsion', if oil is added to a continuous water phase, and an 'invert emulsion' if water is added to a continuous oil phase. Torque, drag and pipe sticking problems are reduced by the addition of oil, at rates of 4 – 10%, to water-based muds. Oil can be added in larger quantities to lower mud weight water-based muds, and water may be used in oil-based muds to reduce costs and give better control of mud viscosity.

The drilling fluids on the drill cuttings supplied for this project were invert emulsions, which are typically over 40% oil, but normally 50% is the lowest oil content used in most applications (Still, pers. comm., 1999).

2.3.5. Second Generation Synthetic Drilling Fluids (and beyond)

Included in these second generation fluids are the linear alpha olefins and isomerised olefins, as well as linear paraffins, considered pseudo-oil rather than synthetic (Friedheim *et al.*, 1996). Drilling fluid systems have been developed to cover a multitude of parameters, including chrome-free for improved environmental performance and glycols and cationic polymer water-based muds for shale stabilisation (MIDF, 2001; Baker Hughes, 2001). The organic cationic materials have provided "economical technology for inhibiting the swelling and yielding of hydratable shales" (Stamatakis *et al.*, 1995). Ingredients from previous formulas have been used in these fluids, such as lignosulfonates, bentonites and oils, as well as newly developed products; each formation and environment is assessed, with the mud tailor-made to requirements. These mud formulas are fiercely protected by the companies concerned, and are very dynamic in that products are developing continually.

2.3.6. The History of Drilling Muds in the North Sea

Initially, in the sixties, the drilling fluids were water based. These early muds gave poor drilling performance (UKOOA, 1999), especially in the shale formations common in the North Sea. By the mid to late seventies and early eighties diesel muds

were introduced; the improved lubrication reduced friction and enabled the development of advanced drilling techniques, extending the range and precision of wells and enhancing recovery rates. Further developments by the fluid companies displaced diesel by low toxicity oils. These were in turn phased out between 1992 and 1996, in line with regulations, and replaced by synthetic muds, which have a reduced environmental impact. Water-based muds have been improved for more effective drilling of shales but still fall short of the synthetic muds on performance and safety, particularly for the more challenging wells now tackled, as the more accessible reserves are used up.

2.4. Development of Drilling Techniques

Once a site has been determined for drilling, the location of the reservoir and the rock type will set the specification for the rock drilling equipment.

Cable-tool drilling has been used for drilling water wells and shallow formations, and involves repeatedly raising and dropping a heavy metal bit, pounding a hole down through the earth. Periodically the debris has to be removed from the hole by bailers (Natural Gas, 2001).

The rotary drilling method uses a sharp bit to cut through the rocks, attached to a drill pipe (drill string). The bits can be made from a variety of materials, examples include steel tooth rotary bits, polycrystalline diamond compact bits and diamond bits, plus many hybrids that combine features. Several different bits may be used when drilling deep wells, due to the different rock formations. Changing a bit involves lifting the drill string to surface, which, in deep wells, can be costly.

Casings were developed to prevent the sidewalls collapsing; the first recorded use was by David and Joseph Ruffner; they used hollow tree trunks for wall reinforcement when drilling through brine near Charleston, West Virginia in the early 20th Century (Natural Gas, 2001). Today steel pipes are used.

Slant or directional drilling has been conducted for years, particularly offshore where the cost of construction prohibits multiple platforms. Several directional wells can be drilled from a single platform, allowing one platform to give multiple intersections, reaching other oil fields and traps. Technology has moved immensely in this field; a slant well would take 2000 ft or more to bend from vertical to horizontal, whereas today's technology is capable of a 90° shift within a few feet.

The advantages of directional drilling include:

- Penetration of multiple reservoirs and multiple intersections of the same reservoir, producing up to seven times as much gas or oil as would be produced from a vertical well
- Production of formation waters is minimised
- Increase of the primary recovery life of a well by over 100%, from 25% to 50 – 75%.
- Extraction can be performed concurrently with the horizontal drilling.

Some important developments concerning the drilling process involved optimising the rate of penetration (ROP) and reducing costs (Galle & Woods, 1960).

In the late fifties and early sixties research focused on the relationships to predict drilling performance from drilling parameters and a rock strength variable (drilling strength), producing several different equations (Somerton, 1959; Galle & Woods, 1960); later, Simon (1963) and Gstalder & Raynal (1966) looked at energy balances in rock breakage, when the term "Specific Disintegration" was coined. Wilhelmi & Somerton (1967) arranged a number of drilling parameters into the term "Drillability", reflecting the energy required to drill a volume of rock, which indicated that the Specific Disintegration test caused significant over-breakage of the rock. This, with other early work, produced several ROP prediction equations that help optimise drilling.

The downturn of the oil industry in the mid eighties saw a drive to develop accurate predictions of drilling performance, with Amoco Production Co taking a leading role by the development of a drilling simulator. This enabled former theoretical data to be proved with empirical data (Rowsell & Waller, 1991). From 1987 to the present there have been seen significant amounts of research concerning logging while drilling (LWD) (Jackson & Heysse, 1994; Efnik *et al.*, 1999). Examples of its uses include:

- Geosteering the wellbore in horizontal sections, thus enabling the drilling to follow the top of the reservoir thereby increasing the available length of wellbore for reservoir drainage.
- Torque can be lost in pipe friction as opposed to requirements at the bit, particularly in deep extended reach wells, causing twist offs (Kamaruddin *et al.*, 2000), but 'at the bit' information can help prevent this.
- The determination of downhole problems and bit wear (Vos & Reiber, 2000).

Other advances included using post analysis of drilling data to optimise performance (Xu *et al.*, 1995) and *in-situ* rock strength predictions (Rampersand *et al.*, 1994).

LWD has made drilling more efficient, cost effective and considerably safer.

Automation of drilling rigs has reduced costs, time and has increased safety (Natural Gas, 2001).

2.5. Historic Perspective on Waste Materials Created from Drilling

Spent drilling fluids and drill cuttings are among the most significant waste streams from exploration and production drilling operations in the sea (Barke & Veil, 1995), and, historically, were dumped straight into it. It has been estimated by Rubin in 1996 that 90% of the hydrocarbons in the North Sea are from the dumping of drilling muds and cuttings on the sea floor near the drill-rigs. This figure is totally contradicted by other sources such as Bennett (1995) who states that the majority of the 400,000 tonnes of oil reaching the North Sea every year predominantly comes from ships

illegally washing out their tanks at sea. Knowing that drilling activities are no longer allowed to use diesel muds, and that the discharge of oily drill cuttings is no longer an approved practice, it seems logical to assume that the latter may be more accurate than the former; finding agreeing data from literature proved difficult. The drill-piles on the sea floor of the North Sea are generally from past activities, as new laws and regulations forbid their dumping (see **2.6.** Regulations and Legislation). The United Nations Report, "The State of the Marine Environment" (Schwaab, 1998) states that the main sources of man-made (global) marine oil pollution are:

- Land-based discharges and run-off (including rivers) 44%
- The atmosphere 33%
- Maritime transport 12%
- Dumping 10%
- Offshore oil and gas production 1%

Input of petroleum pollution into the global marine environment has been estimated at 6 million tonnes annually, with the majority coming from daily influxes rather than disasters (Okpokwasili & Nnubia, 1995). However, oil tanker accidents and oil well blowouts result in serious damage, due to the concentration of the contamination; the physical properties of oil lead to its coating of sea creatures, such as birds and mammals, causing death, as well as the coating of any beach it happens to be washed onto, sometimes destroying whole ecosystems (Pritchard & Costa, 1991). Although these incidents only account for a small fraction of the total amount of oil that reaches the sea, their impact can be massive. Oil-spill incidents have a powerful negative impact on public opinion.

2.5.1. Drill Cuttings in the North Sea

UKOOA reports give the sources of oil discharge into the North Sea as:-

26% Ships
21% Rivers and Runoff

20% Offshore Oil and Gas (including oil on cuttings)
7% Atmospheric
7% Other Costal Effluent
6% Coastal Sewage
4% Dredged Spoil
3% Sewage Sludge
3% Coastal Refineries
3% Others

These reports have estimated 1 – 1.5 million tonnes of cuttings accumulated in the UK sector of the North Sea over 30 years of drilling activity. To get this into some perspective, it is equal to one twentieth of household waste per annum, and only one fiftieth of that produced by mining and quarrying, which is around 74 million tonnes annually.

1970's figures from the Department of Energy state that 212 wells were drilled on the UK Continental Shelf in one year, with 76 of these drilled using oil-based muds, resulting in approximately 7000 tonnes of diesel oil being discharged, mostly attached to the drill cuttings, 91.6 tonnes per well. In 1981 223 wells were drilled, with approximately 65% using oil-based muds, resulting in 7,700 tonnes of diesel oil and 10,400 tonnes of alternative base-oils discharged into the sea (Davies *et al.*, 1984).

The drill piles left from this era of drilling are a legacy still haunting the North Sea Oil Industry. The issues surrounding drill cuttings piles have been researched in detail by the UKOOA 'Drill Cuttings Initiative Research and Development Programme' (1999, 2000), and by R. Artz, Aberdeen University (pers com, 2002). Table **2.2.1.** illustrates that the number of wells currently being developed are similar to those above, but now the cuttings are not allowed to be discharged into the sea. The regulations and legislation relating to this change of policy are discussed below.

2.6. Regulations and Legislation

The content of drilling muds and their use have long been controlled by strict regulation, as has the discharge of cuttings.

2.6.1. Responsibility for Marine Pollution

There are a number of government departments within the UK with responsibilities that cover marine pollution. These include:

- The Marine Pollution Control Unit (MPC), responsible for counter-pollution measures in the North Sea.
- The Department of Transport, whose remit includes the control of oil and chemical pollution at sea. The Secretary of State for Transport is under a duty to make an annual report to Parliament under section 26 of the Prevention of Oil Pollution Act, 1971.
- What used to be the Ministry of Agriculture, Fisheries and Food (MAFF), now the Department for Environment, Food and Rural Affairs (DEFRA) and the Centre for Fisheries, Environment and Aquaculture Science (CEFAS), control the dumping of waste at sea through a system of licences.
- The Department of Trade and Industry (DTI), responsible for the control of pollution from offshore installations, working in conjunction with the MPC.
- The Secretary of State for the Environment, responsible for the protection of wildlife in England.

Some of these have changed in 2001 due to reorganisation of Government Departments during this year.

2.6.2. Sources of Law

The main sources are International Law, Community Law and the National Law of the UK, covering matters as diverse as marine pollution and ownership of the seas.

Marine pollution recognises no boundaries, but legal frontiers are helpful in defining responsibility and regulations. International Laws assume that there can be co-operation between states, and, through international regulations, ensure the protection of the marine environment. Particular emphasis is on transfrontier pollution. International law does not have the ultimate sanction of police prosecution or court order (Read, 1988), but request that the signatory states incorporate the laws into their National Laws.

2.6.2.1. International Law

The designation of ocean waters into zones is covered under International Law. In 1982, the diverse number of treaties and conventions was codified by a global convention signed by the majority of states in the world (McEldowney, 1996). This became the United Nations Convention on Law of the Sea (UNCLOS), and provided 5 distinct categories of marine space.

- The sovereignty of the coastal state is defined to include internal waters consisting of ports, harbours and bays whose openings do not exceed 40 km.
- A coastal state may exercise its territorial sea as a sovereign zone up to 20 km; foreign shipping may have rights of passage but the sovereign state may legislate to protect its marine environment.
- A coastal state is defined as consisting of the sea and seabed to the outer limit of the continental plateau, that is to the beginning of the deep seabed.
- An exclusive economic zone consists of the maritime area that extends between the territorial sea and a line situated 360 km from the coast. This designation has existed from the 1970s and gives the coastal state rights to exploit the resources of marine life in the zone. The coastal state is under a duty to ensure that there is environmental protection of the area.
- Waters outside the designated zones outlined above are described as the high seas. These are open to exploitation by international shipping.

Problems can occur when there is a pollution event in the high seas (there is a tradition of freedom of the high seas). Coastal states can be reluctant to intervene outside their territorial waters, even when there is an imminent threat of pollution to their coasts (Read, 1988). There are powers of intervention, however, from the International Convention relating to Intervention on the High Seas in Cases of Oil Pollution Casualties (Public Law) 1969, giving states limited powers of intervention when there is "a grave and imminent danger of pollution on a large scale to their coasts or territorial water" (Read, 1988).

The International Maritime Organisation (IMO) is part of the United Nations (UN) with a membership of about 125 states. The IMO acts as a facility to extend co-operation between governments on shipping matters. The Marine Environment Protection Division of the IMO draws up conventions concerning shipping and marine pollution. The United Nations Convention on the Law of the Sea has been ratified by over 60 states since its agreement in 1982.

The UN Environment Programme, established after the Stockholm Conference of 1972, where 113 states participated, is also concerned with the protection of the marine environment.

There is an International Convention of the Prevention of Pollution from Ships (MARPOL), signed in 1973, amended by Protocol in 1978, which came in force in October 1983 which had the intention of eliminating International Pollution from the marine environment.

International Law also consists of a number of treaties and conventions with certain environmental protection afforded to the various oceans of the world. Examples include:

- The UN Environment Programme, which attempts to set out a programme for regional seas, initiated in 1974. Covers 10 areas where regional plans are under development or are operative.

- The 1983 Treaty dealing with oil-based pollution in the North Sea and the North-East Atlantic
- The 1972 Oslo Dumping Convention, amended by subsequent protocols, applies to the North Sea, the North-East Atlantic and the adjacent Arctic Seas.
- The 1974 Helsinki Convention for the Protection of the Marine Environment of the Baltic Area adopted a comprehensive approach to pollution control.
- The 1978 Barcelona Convention applies to the Mediterranean.

The aim is to extend the principles of combating pollution to other seas through a series of framework conventions. These include the Persian Gulf, the Red Sea, the Gulf of Aden, parts of the Indian Ocean, the South Pacific, the Caribbean and part of the South Atlantic.

Regional conventions include the 1984 North Sea states meeting to discuss the prevention of pollution in the North Sea. There have been conferences held in 1987, 1990 and 1995, resulting in a number of declarations and elaborations agreed in general principles.

2.6.2.2. European Community Laws

When considering environmental policy, Article 130R (3) requires that the Community should take account of available scientific and technical data. Community Directives relating to the sea include Directive 76/464/EEC, which applies to the pollution caused by certain dangerous substances discharged into the aquatic environment of the Community. There are a number of Directives on the quality of shell-fish and the Directive 76/160 on the quality of bathing waters. There are Directives prohibiting the discharge of specific chemicals into an aquatic environment.

2.6.2.3. National Laws

National Laws are important in tackling marine pollution, for example the Merchant Shipping Acts and Regulations. The Merchant Shipping (Salvage and Pollution) Act, 1994, implements the International Convention on Salvage, 1989, and various International Conventions and Protocols for oil pollution damage. Other International and European agreements are set out in National Laws.

2.6.3. Laws Specific to Petroleum Exploration and Production Operations

Until the eighties there was little in the way of legal instruments encompassing the whole field of offshore operations (Ayers *et al.*, 1982).

2.6.3.1. UNCLOS, 1982

Article 1 contains the following definition:

"Pollution of the marine environment means the introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of the quality for use of sea water and reduction of amenities".

An UNCLOS convention that came into effect on November 16th 1994 reflects the disparate economic circumstances of the International Community, in that it demands only that states use the "best practicable means at their disposal" to prevent and control marine pollution from any source. Responsibility to set any regulations and to specify limits is given to the signing states (UNCLOC, 1995). This may appear to justify poor countries polluting, but International Law cannot be policed and relies on National Laws being introduced and therefore cannot enforce a harder line. However, it reinforces the use of up to date scientific research in richer countries, technology that will filter down to the less well off, especially as many exploration and production operations conducted in these countries are by Western companies.

2.6.3.1.1. Article 81 (Drilling on the Continental Shelf)

This grants coastal states the exclusive right to authorise and regulate drilling on the continental shelf (see section 2.6.2.1. for definitions of coastal states) for all purposes.

2.6.3.1.2. Article 208 (Pollution from Seabed Activities Subject to National Jurisdiction)

This requires states to adopt laws and regulations to prevent, reduce and control pollution of the marine environment arising from or in connection with seabed activities.

2.6.3.1.3. Article 209 (Pollution from the Activities in the Area)

This deals with National Regulations.

2.6.3.2. Kuwait Convention, 1989

The Kuwait "Protocol Concerning Marine Pollution Resulting from Exploration and Exploitation of the Continental Shelf" came into force on February 17th, 1990 (Bates, 1997). It contains a general prohibition on the use of oil-based drilling fluids; however, if the use of such a fluid is considered justified because of exceptional circumstances, the coastal state can allow their use. The oil-based fluids do have to be treated to minimise their oil content before disposal and may not be discharged at sea [Article IX, 4(a) & 4(b)]. Water-based fluids being discharged from offshore installations may not contain persistent systemic toxins [Article IX, 4(c)].

2.6.3.3. The North Sea

The first formalisation of co-operation between North Sea States occurred in 1969 at the Bonn Convention, and implied that states should make equipment and information available in cases of oil spill or other pollution events.

The eighties saw a focus on the need to control oil discharges from offshore installations in the North Sea as a result of environmental damage (Read & Blackman, 1980). International Conferences ensued. The Bremen Conference gave a general

reference to pollution from offshore installations on November 1st 1984, with more specific instructions after the next one in 1987. This led to the restriction of oil-based muds and chemicals to the 'Best Available Technology', and techniques were to be implemented to reduce the impact of discharged drill cuttings.

2.6.3.3.1. The Paris Commission

Established by the Paris 'Convention for the Prevention on Marine Pollution from Land-Based Sources', 1974, the Paris Commission adopted some resolutions in respect of pollution from offshore installations concerning the North-East Atlantic. Decision 88/1 of June 1988 made the use of oil-based muds subject to prior authorisation by the coastal authority (essential for geological, safety or economic reasons), prohibited the use of diesel-based muds, set specific standards for the allowable oil content of cuttings discharged into the sea (<10%) and prohibited the dumping or discharge of oil-based muds at sea. It was proposed to outlaw drill cuttings disposal at sea altogether.

In 1992, the Convention on the Protection on the North Sea and North East Atlantic was signed in Paris, which replaced the Oslo Convention and the 1974 Paris Convention. This led to a new Decision (92/2) setting an average limit of >1% oil on dry drill cuttings. This was put into the UK Continental Shelf Regulations, 1993, 1994 and 1997 (Ferguson *et al.*, 1993; Schuh *et al.*, 1993).

2.6.3.3.2. OSPAR

The convention for the Protection of the Marine Environment of the NE Atlantic (ospar convention), Oslo, Paris, 22/9/92.

The Oslo and Paris Commissions, known as OSPAR, now formulate most of the Recommendations concerning the marine environment in the North-East Atlantic, superseding Parcom. Within OSPAR is a Working Group on Sea-Based Activities (SEBA) who give guidelines on completing Decisions, for example the Harmonised Offshore Chemical Notification Format (HOCNF), which is part of the Harmonised Mandatory Control System for the use and the reduction of the discharge of offshore

substances/preparations, as developed by OSPAR in 1995, and now the OSPAR Decision 200/2, which came into force in 2001. The Offshore Chemical Regulations 2001 will have a major impact on offshore activities; the format can be found in full on the OSPAR web site, *www.ospar.org*, but is beyond the scope of this research to cover in detail. The flow chart in figure **2.6.3.3.2.1.** sums up the processes involved in the Regulations.

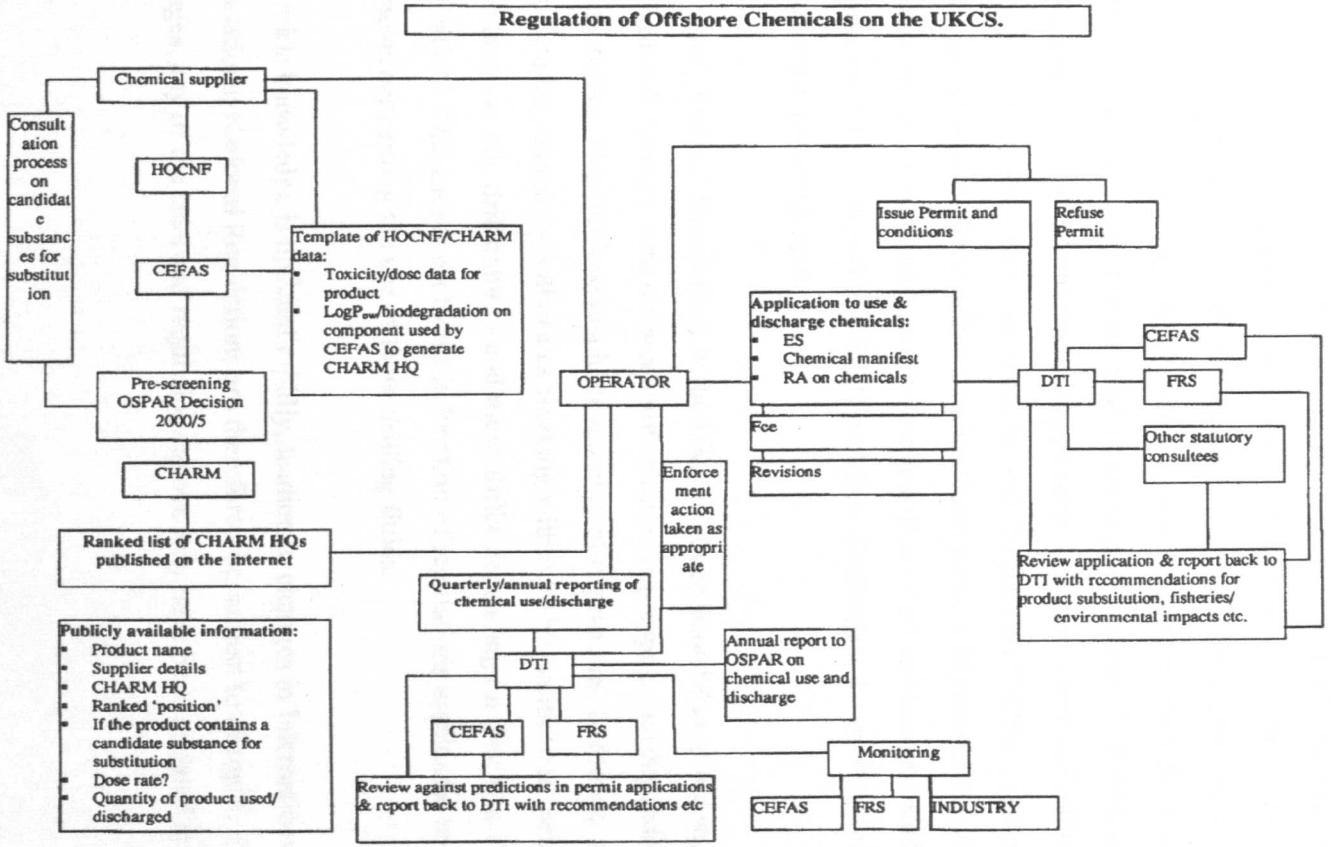


Figure 2.6.3.3.2.1. Flow Chart Summarising the Process of the Offshore Chemicals Regulations 2001 (DTI, 2001)

2.6.3.3.3. National Regulations

The DTI deals with pollution control, pipelines, installations and abandoned installations offshore. Oil exploration and production is licensed under the Petroleum (Production) Act, 1934, and is judged according to the rules set out in the Petroleum Regulations, 1982. The regulation of pipelines is provided by the Petroleum and Submarine Pipelines Act, 1975, which states that authorisation must be obtained from the Secretary of State to operate a pipeline system in controlled waters, i.e. UK territorial waters and those adjacent to the seas under the Continental Shelf Act, 1964.

There are a variety of agreements that apply to prevent oil pollution and to prescribe action to be taken when such an event occurs (McEldowney, 1996). The Merchant Shipping (Prevention of Pollution) Regulations, as part of the Prevention of Oil Pollution Act 1971, was updated in 1996 so that under Regulation 32(1) all offshore installations, when engaged in the exploration or exploitation of oil must comply with the requirements of the regulations as applicable to ordinary ships of >400 gross tonnage. This involves oil filtering equipment [Regulation 14(2)(a)] and oil content measuring equipment fitted with a 15 ppm alarm deveice [Regulation14(2)(b)]. Installations have to provide tanks for the retention of oil residues and sludges [Regulation 25(1)]. Regulation 32(2) prohibits discharges of any oil or oily mixture with an oil content>15 ppm.

In general, National Regulations in the UK have been issued due to the signing of International Treaties, with different authorities being responsible depending on the specific topic. This has lead to a huge quantity of regulations, becoming more and more detailed, issued by authorities working within their specific area, hence regulations on oily drill cuttings and muds differ depending on their specific application. This causes problems in location of regulations applicable to specific problems concerning the use of these drilling fluids.

Scientific knowledge is updated rapidly, leading to changes in International Regulations; National Regulations are therefore also subject to changes. Due to these changes, any of the laws and regulations above may have been updated in recent

times. Application of the 'precautionary principle' may lead to stricter regulations without absolute scientific basis.

2.7. Current Status of the Waste Problem

"Options for dealing with the 1.3 million cubic metres of drill cuttings which surround the North Sea oil platforms were discussed at an oil industry stakeholders' meeting", reported the February 2000 ENDS report. This doubles previous estimates, as discovered by the industry's commissioned research project (UKOOA). 60% of the piles are in the central North Sea, the remainder in the deep northern sectors.

The debate continues, with Greenpeace favouring a ship to shore policy and the UKOOA reports favouring leaving the drill cuttings on the sea floor (UKOOA, 1999, 2000). Further research conducted by R. Artz confirms this is very complex issue (2001). Moving the piles would obviously disturb the seabed and release pollution into the area. Drilling the piles to allow aerobic bacteria to reach deep into the piles will also cause the release of pollutants, as well as reducing the available oxygen to the indigenous benthic communities, which could threaten their ecosystem. Biological modification of the piles may increase the biological effects (Best *et al.*, 1985) by making the contaminant more accessible to marine flora and fauna. Currently, the piles are not showing much evidence of remediation – even after 20 years (Artz, pers. Comm., 2001), but do have sediment covering them and seem fairly stable. Anaerobic micro-organism activity by sulphur reducing bacteria (SRB), as discussed in the UKOOA report 1996, leads to the production of sulphides from metabolic respiration. Their release magnifies the toxicity of the drill cuttings and creates a corrosive, reducing environment.

Drill cuttings produced now are mostly shipped to shore where there are several options available for their disposal or treatment.

2.7.1. Disposal or Treatment Options for Drill Cuttings, Other Than Bioremediation

2.7.1.1. Re-injection

Annular re-injection is now utilised in a number of current drilling operations (Anderson *et al.*, 1996). Reinjection is dependent on the formation, as a solid cap rock is required to prevent returns to surface and contamination of other strata and aquifers. Power costs for reinjection may be considerable if cheaper power generation from produced gas is unavailable. Potential problems are re-emergence of the cuttings and a lack of data for assessing the environmental impacts. There are a number of contractors in the UK offering re-injection technology. The E & P Forum (Campbell, 1999) have some expertise in this area.

2.7.1.2. Landfill

Landfill is an option being utilised by some companies. This is not ideal; landfill is not a treatment, but is simply moving an offshore problem onshore, where there is already pressure on waste disposal. Any option that takes the cuttings onshore will have an environmental impact. While ending discharge from the rig, it increases pollution from shipping and heavy plant; the risk of spillages is higher and there is an increased risk of onshore air and groundwater pollution.

2.7.1.3. Incineration

Incineration can create atmospheric pollutants and, unless the energy is harnessed from the process, it is wasteful. Although technology has cleaned up emissions, incineration is not at all popular onshore, with the public strongly opposed to plants near any populated or environmentally sensitive areas. Fuel would have to be added to sustain the process, making it a high cost option. Offshore incineration at source or a modified installation elsewhere may be technically feasible, but is "not considered cost-effective or environmentally acceptable" (Anderson *et al.*, 1996). A licence under the Food and Environmental Protection Act would be required for this discharge. In spite of this, incineration has been utilised as a method of drill cutting

disposal (Page, pers. comm., 1999). The cuttings can also be utilised as a co-fuel in cement kilns and power stations for example.

2.7.1.4. Solvent Extraction

Solvent Extraction has been considered, but again has a contamination problem as the pollutant is moved into the solvent. The pollutant then needs to be removed from the solvent, and treated. Both extractions can prove expensive (E & P Forum Report No. 2.61/202).

2.7.1.5. Distillation/Thermal Desorption

This involves the use of heat to separate the oil from the cuttings, enabling the oil to be reclaimed. Process costs may be high. Distillation is only suitable for mineral oils, some paraffins and poly alpha olefins (PAO's) (Walker, 1995). Most of the other synthetics used, including esters and linear alpha olefins (LAO's), are unsuitable due to the high water content in the cuttings; at temperatures used in distillation this may cause the hydrocarbon chains to split, generating toxic or volatile fractions, which would make them unsuitable for re-use (Walker, 1995). Thermal desorption is a method utilised in many parts of the world and is currently being used in Aberdeen.

2.7.1.6. De-emulsification

Separating the oil and water by attacking the emulsifier, either chemically or biologically, is an attractive option. Chemical separation can prove costly, and may introduce another contaminant. Biological destruction of the oil/water bond seems a novel option, and one that could benefit from further investigation. This could lead to the oil's reuse and a clean-up that is good from all angles - the cost is low, the product has the potential to be reused, and the cuttings may be disposed of offshore, depending on legislation. Napier University, who are at the forefront of surfactant technology, are conducting research into this area.

2.7.1.7. Flotation

Oil is used as a flotation agent for coal fines, and is especially efficient in water with high chloride content. Oil is used in the coal industry for flotation, as the oil attaches

itself to the coal. If the cuttings were cleaned in this fashion, the final product could be sold as fuel, with the cuttings disposed of as a non-hazardous inert material. To date there is no evidence of research using this method.

2.7.1.8. Stabilisation

This process involves encapsulation of the cuttings using several solidifying agents, forming a dry, solidified material, creating a stable matrix suitable for land farming or landfill (Walker,1995). The risk of leachate production is therefore minimised. Stabilisation has also been used in recycling and reuse (R & R) projects in America (Sulivan *et al.*, 1998). Stabilised products from hazardous and non-hazardous materials have been used as engineered backfill or asphalt stabilised base, and the process has been utilised with clay-rich soils that can be especially difficult to remediate. In the USA the R & R process has provided construction materials for roads, car parks, dikes and work areas. This method would depend on material suitability – each formation waste would have to be assessed. Processing would be expensive, for example transportation and encapsulation.

2.7.1.9. The Cleaned Cuttings

It was hoped to find a use for the cuttings, once cleaned, for example as a construction material. However, the quantities of drill cuttings are miniscule compared to the requirements of this industry. When interviewed, civil engineering companies in the UK showed no interest whatsoever in taking the cuttings, particularly as they contain clays (Hill, Pers Comm., 2000). One use could be to make up the temporary roads on landfill sites. Further processing would be counterproductive, as stabilisation could be conducted without any prior decontamination treatment, see **2.7.1.8.** above.

2.8. Separation of Mud from Cuttings – Options at Source

All operators wish to achieve maximum recovery of mud adhered to the cuttings, as the muds are recirculated to the mud pits and consequently reused in the drilling operations. The footprint available for recovery equipment is small on offshore installations, which limits how much recovery can be achieved.

Most operations run the cuttings from the hole across 'primary shakers', which remove some of the drilling fluid. Mud losses over these shakers vary depending on the hole sections and depths drilling. For example, in a 17.5 inch hole section, there are losses of approximately 0.5 – 0.6 bbls of mud per barrel of cuttings produced (Wood, 1995); in 12.25 inch and 8.5 inch hole sections this loss rate increases to 1.5 – 1.6 bbls of mud per barrel of cuttings. These losses can be considerably reduced by secondary treatment.

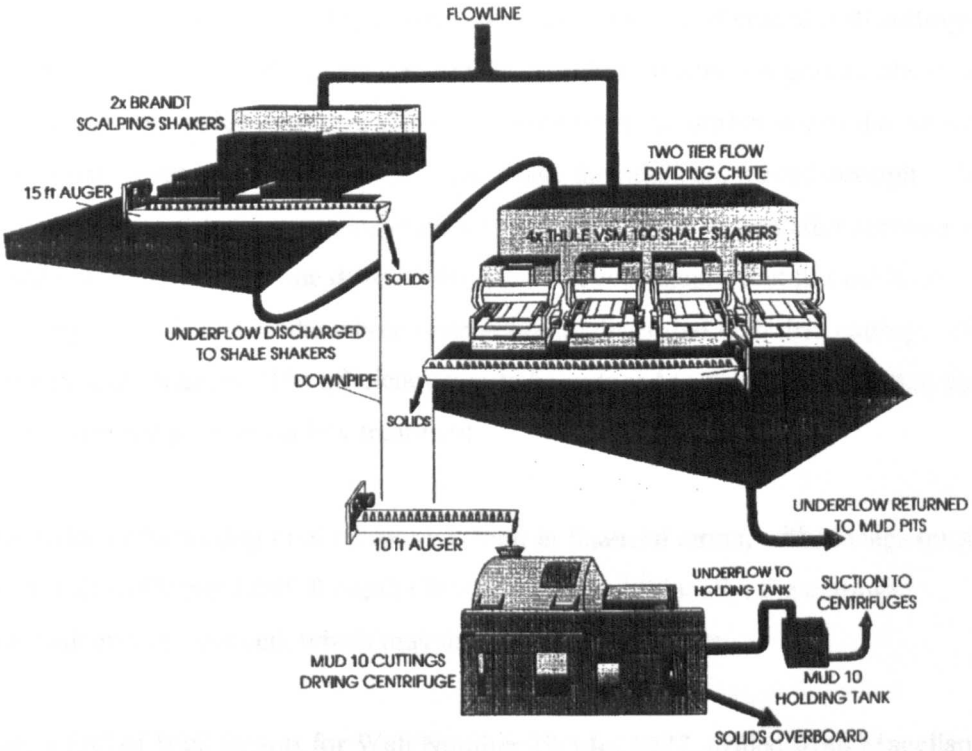


Figure 2.8.1. A Mud Separation and Solids Control System used on the Magellan Drilling Platform

Swaco have done considerable research on solids control, which include fine-mesh screen shakers, shakers in series, centrifuges and other mechanical solids removal; dilution, displacement and sedimentation (gravity). Details are available in their report, "Four Methods of Solids Control" (SW 96124 C). The reduction of solids in

the mud and the recycling of the muds reduces costs; the separation systems can also reduce the quantity of muds on the cuttings, thereby reducing the contamination content.

2.8.1. Mud Recovery Systems

There is a variety of equipment used downstream of the shakers to enhance mud recovery and solids removal. These include hydrocyclones, centrifuges and settling tanks. The MUD 10 is an example of secondary treatment of cuttings post shakers. The system "induces a centrifugal force of up to 130 G on oil coated drill cuttings" (Wood, 1995). The cuttings are fed into the distribution cone via gravity; the cone then accelerates, spreading them evenly onto the inner circumference of the conical wedge wire screen; the cuttings stay there, while the muds are forced through. The mud is then suitable for recycling back into the active mud system after secondary treatment to remove the fine drill solids. Wood (1995) claims that this achieves recovery rates of 90% of the whole mud normally discarded with drill cuttings off primary shale shakers. The efficiency would be reduced to around 75% due to the solids acquired post secondary treatment.

The value of increasing mud recovery is high in financial terms, with savings quoted as over £11,000 per 1,000 ft depth (Wood, 1995); additionally, the cuttings contamination is reduced, which may aid in future treatments.

Enaco End of Well Report for Well Number 29/14c, 1997, drilled from Magellan, an Amoco rig, confirmed that the MUD 10 and other centrifugal recovery systems reduced the average oil on cuttings, quoting figures of 32.72 g/kg, with 14 tonnes of oil recovered from the M10 and 16 tonnes from the centrifuges. The shale shakers, fitted with 200 mesh screens, discharged 114.4 g/kg of oil on cuttings, meaning the secondary treatment has reduced the contamination by 81.68 g/kg. From that well alone, 548 bbls of (expensive) synthetic oil-based mud (SOBM) were transferred back to the active system. This well already had centrifuges installed for mud weight control, which proved an advantage in minimising the reintroduction of drilled fines

into the active mud. The shaker and MUD 10 system from the Magellan can be seen in figure 2.8.1.

2.8.2. Drilling Fluid Advances

Some developments in the drilling fluid industry lead to the design of muds that reduce residual oil on cuttings. Polymeric fluids have contributed towards this (MIDF, 2001; Drill-Aid, 1990's); surfactant technology has been investigated, as conventional oil-wetting surfactants encouraged the spontaneous infiltration of the oil into the internal pores of the cuttings. This increases the oil on the cuttings and also makes it more difficult to separate.

Low oil-water ratio invert fluids with low plastic viscosities have been introduced, also reducing oil on cuttings. When these are polymer-based invert oil emulsion muds, tests showed up to a 30% reduction of oil on cuttings (Schwaab, 1998).

2.9. Summary

For the foreseeable future, oil exploration and drilling activities will continue due to the global utilisation of oil and oil products, and the lucrative nature of the industry. Drilling fluids have developed over the last few decades to deliver products that increase safety, productivity and have better environmental performance. Drill cuttings piles coated with mineral oils such as diesel are an historic legacy in the North Sea, and are being vigorously researched on several fronts; however, the issues of the cuttings being brought to surface now are scrutinised legally, by environmental bodies and by the industry itself. There is continuing research as to the best treatments for the cuttings, and for minimising their environmental impact by reducing the toxins and contaminants within the muds themselves. Methods of treatment and disposal options have to consider space availability, costs, effectiveness, materials recycling and disposal options post-treatments or without treatments as hazardous wastes.

CHAPTER 3
MUDS: MUD AND HYDROCARBON CHEMISTRY

3.1. Introduction

The main pollutant in the muds to be remediated by bacteria was the hydrocarbon. To understand the process of degradation, there had to be an understanding of hydrocarbons in general, and then of the hydrocarbons used in the constituents of the muds.

3.2. General Hydrocarbon Chemistry

The word petroleum was derived from *petra*, 'rock', and *oleum*, oil.

A hydrocarbon (HC) is a compound containing only carbon and hydrogen. The bonds are almost entirely non-polar, making the hydrocarbon molecules lacking in overall polarity (Holum, 1998). Hydrocarbons are therefore insoluble in water, but dissolve well in nonpolar solvents, such as CCl_4. Some mixtures of alkanes are in fact used as solvents, such as lighter fluid used to remove tar spots. As hydrocarbons are generally less dense than water they will float, hence oil spills floating on the sea. They are also known as non-aqueous phase liquids (NAPL).

3.2.1. Alkanes (Paraffins)

Paraffin is from the latin "*parum affinitas*" - slight affinity.

When the proportion of hydrogen is high enough for single bonds to be possible between each pair of carbon atoms, the resulting hydrocarbon molecules are saturated (Goodger, 1975). A saturated HC contains only single bonds, i.e. each carbon has the maximum number of hydrogen atoms bonded to it. These are open chain saturated hydrocarbon molecules (Brown, 1997). They do not react readily with other molecules, and are free to take up their most stable conformation in an open chain

(that is, strain free). They are known as alkanes, but are commonly referred to as aliphatic hydrocarbons because the physical properties of the higher members of this class resemble those of the long carbon-chain molecules found in animal fats and plant oils (Greek: *aleiphar*, means fat or oil). Alkanes have the general formula:

$$C_nH_{2n+2}$$

With methane as a starting point, additional hydrogen and carbon atoms can be built on, to provide an homologous series of chain-like molecules, differing progressively in physical properties but having similar chemical characteristics.

The low molecular weight alkanes – methane, ethane, propane and butane, are gases at room temperature and atmospheric pressure (Brown, 1997); higher-molecular-weight alkanes such as gasoline and kerosene are liquids, and very high-molecular-weight alkanes, such as paraffin wax, are solids.

Table 3.2.1.1. The Homologous Series of Alkanes

Name	Condensed Structural Formula	mp °C	bp °C	Density of liquid (g/ml @ 0°C)
Methane	CH_4	-182	-164	a gas
Ethane	CH_3CH_3	-183	-88	a gas
Propane	$CH_3CH_2CH_3$	-190	-42	a gas
Butane	$CH_3(CH_2)_2CH_3$	-138	0	a gas
Pentane	$CH_3(CH_2)_3CH_3$	-130	36	0.626
Hexane	$CH_3(CH_2)_4CH_3$	-95	69	0.659
Heptane	$CH_3(CH_2)_5CH_3$	-90	98	0.684
Octane	$CH_3(CH_2)_6CH_3$	-57	126	0.703
Nonane	$CH_3(CH_2)_7CH_3$	-51	151	0.718
Decane	$CH_3(CH_2)_8CH_3$	-30	174	0.730 (Water = 1g/ml at 0°C)

Molecular structure can be open-chain (alkane) or closed-chain (cyclane) forms. Alkanes can be straight chain (normal), or incorporate side chains. Alkyl groups, i.e.

alkanes deprived of one hydrogen atom, are radicals, e.g. methane (CH_4) goes to the methyl radical $CH_3^{\bullet}$. Alkyl radicals are univalent (the symbol R is used), i.e. are capable of combining with one atom of hydrogen or its equivalent, having an oxidation/co-ordination number of one (Walker, Ed, 1995).

The intermolecular forces of attraction between particles enable compounds to exist as a liquid or solid. The forces of attraction between non-polar molecules, such as those in methane, are called dispersion forces, and they are very weak (0.02 - 2kcal/mol, 0.08 – 8 kJ/mol). To convert methane from a liquid to a gas at -164°C the process of separating its molecules requires only a small amount of energy. Because interactions between alkane molecules consist of only very weak dispersion forces, boiling points of alkanes are lower than those of almost any other type of compound of the same molecular weight. As the number of atoms, and therefore the molecular weight, increases, boiling point also increases, as do melting points, but not in a direct correlation. This is because of the ability of molecules to pack into ordered patterns of solids changes as molecular size and shape change.

The average density is about 0.7g/ml; that of higher-molecular-weight alkanes is about 0.8g/ml. All liquid and solid alkanes are less dense than water.
Alkanes that are constitutional isomers of each other are different compounds and have different physical and chemical properties. The more "branches", the lower the boiling point, e.g. hexane (C_6H_{14}) b.p. = 68.7°C; isomers of C_6H_{14} (e.g. (a) 2-methylpentane and (b) 2, 2dimethylbutalne) are lower at (a) 60.3°C and (b) 49.7°C. Differences in boiling point relate to molecular shape, as the only forces of attraction are dispersion forces in alkanes. As branching increases, alkane molecules' shape becomes more compact, decreasing surface area. As surface area decreases, contact among adjacent molecules decreases, the strength of dispersion force decreases, decreasing boiling point.

Reactions that do occur with the addition of sufficient energy do so by dissociation of a carbon-hydrogen bond and the hydrogen atom replaced, i.e. substitution. This is a potential site for biodegradation.

3.2.2. Alkenes

When there is multiple bonding between the carbon atoms they are unsaturated compounds. Alkenes, or olefins (from Latin, meaning oil-forming), are hydrocarbons that have double bonds consisting of four shared electrons (Manahan, 1994). This, together with its greater exposure on either side of the molecule, make it reactive, leading readily to additional compounds with other monovalent atoms or radicals.

Alkenes have the general formula:

$$C_nH_{2n}$$

The methylene radical CH_2 is in all homologous series of alkenes. Individual members are known as homologues. A radical is a group of atoms that remain associated during a reaction but does not comprise a completely balanced molecule. Alkenes incorporating two or more C=C bonds are dienes, trienes, etc., and are, collectively, polyenes.

3.2.2.1. The C=C Double Bond

Bond energy is higher for C=C, and carbon atoms bond together more tightly due to the shorter bond length. However, the bond is weaker in reactions due to the weak link, which is usually a π bond. There are two different types of bonding, σ and π bonds. The σ bond is rotationally symmetric about a line joining the carbon nuclei, whereas the π bond has a nodal plane in the plane of the molecule, which is in fact a mirror plane (Beyer & Walter, 1991).

A variety of addition and oxidation reactions take place at the C=C double bond; it is a reactive site in a molecule. An example is ethylene, where the double bond has a tendency to add on other atoms and attain as saturated a state as a C-C bond, justifying its description as an unsaturated HC. It is also a preferred site for biological reactions, i.e. biodegradation.

3.2.3. Other Hydrocarbon Compounds

There are also triple bond compounds, known as alkynes, and the aromatics, which are normally a resonance-stable benzene ring (C_6) and can incorporate additional carbon atoms inside the chains (Goodger, 1975).

As the hydrocarbon compounds used in the Versaclean and Novatec muds are alkane and alkene, the other compounds were not focused on in this study.

3.3. Oil-Based Muds

The first oil-based muds (after the crude) were developed with diesel oil, which has a molecular size of $>C_{12}$, and a boiling point between 250 and 400°C (Holum, 1998). The industry, due to legislative pressures (see **2.6**), then moved onto low-toxicity oils; originally these were mineral oils, but these are also prohibited for use in the North Sea (Oakley *et al.*, 1993). Other low toxicity oils were then developed, such as the use of esters. An ester is the central structural feature of all of the edible fats and oils, as well as a number of constituents of body cells (Holum, 1998).

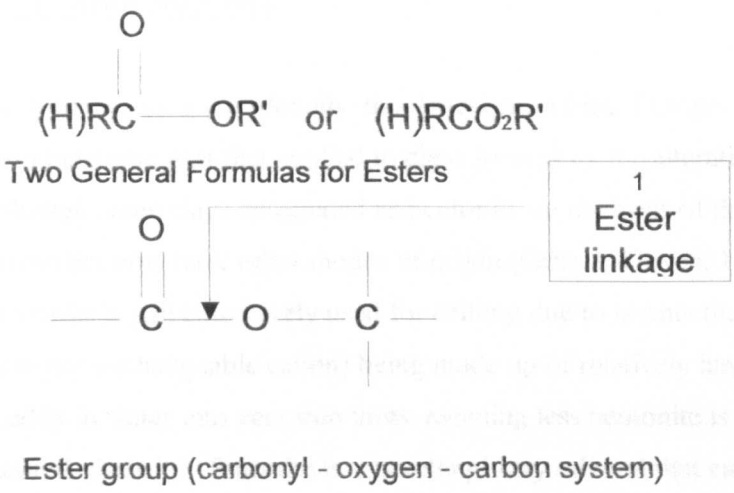


Figure 3.3.1. Ester formulas (Holum, 1998)

Esters are broken apart by water in the presence of either an acid or base (Holum, 1998), or catalysed by enzyme activity.

As there were no cuttings available containing ester based oils, it was not possible to examine which ester was used in the muds; the exact make-up and structure of the specific ester was therefore not available, and, without cuttings, was beyond the scope of this research.

By the mid nineties, the oils predominantly used were so called synthetic fluids. Examples include Poly Alpha Olefins (Walker, 1995), and, more recently, Linear Alpha Olefins (LAO), Internal Olefins (IO) and Linear Paraffins. These second-generation fluids have benefits over the older fluids, including a lower kinematic viscosity, they are less expensive (Friedheim, 1997), have less environmental impact and are less of an irritant when handling. The development of these fluid types continues to evolve, increasing performance whilst using low toxicity additives, allowing for the utilisation of biological methods of cleaning the drill cuttings, with the possibility of having a usable product after remediation.

3.3.1. Bentonite Clays

Bentonite clays occur globally: the Americas, Africa, Europe, West and East. The term bentonite was first applied to clays formed by the alteration of volcanic ash, although some clays designated as bentonite on the basis of their composition and properties now have other modes of origin (Grim & Guven, 1978). Wyoming bentonite is still extensively used for drilling due to is smectite component (with Na as major exchangeable cation) being made up of relatively large flakes which disperse readily in water into very thin units, meaning less bentonite is needed to acquire the desired viscosity. Smectite is a 'swelling' clay mineral that can take up water or organic liquids between its layers. It also has very high gel strength and low filter cake permeability.

Bentonites are also used in many industries because of their emulsifying action and their affinity for carbon particles (Grim & Guven, 1978). They are also excellent water impeders, particularly the sodium variety from Wyoming.
Bentonites are also used in microbiology as non-specific inhibitors of ribonuclease (RNase). A 2% clay solution is added as a suspension to the RNA extraction and then removed, together with the absorbed RNase (Blumberg, 1987). This illustrates the scope of bentonite utilisations and the incredible ability of this type of clay to 'hold on' to substances as diverse as proteins, oils and water.

3.3.2. M-I Drilling Fluid's Muds

M-I Drilling Fluid sent four samples of oil.

- Novatec, LAO.
- Versaplus, linear paraffin.
- Ecogreen, ester based.
- Versaclean, a lox toxicity oil.

Of these, the only contaminated cuttings available for experimentation were from drilling operations using Versaplus muds.

Versaplus is a linear paraffin; this indicates it is a straight chain alkane (see **3.2.1.**), with carbon chain lengths between C12 and C16. Experimentation was conducted on a sample of the oil using the GC to discover the chain lengths, see section **6.2.7.**, and GC/MS see appendix A.

The general structure for a C12 straight chain paraffin molecule would be:

$CH_3(CH_2)_{10}CH_3$

Novatec, the LAO, has chain lengths C14 – C16; the double bond is located between the first two carbon atoms in the chain, with the other carbons fully saturated.

The general structure for the LAO would be:

CH_3-$(CH_2)_n$-CH=CH_2

The formulation of the fluids are confidential and will therefore be omitted from this report.

3.4. Environmental Fate of the Hydrocarbons on Drill Cuttings from Oil-Based Muds.

3.4.1. Fate of the Cuttings Post Discharge

When a hydrocarbon is released in water, it would normally float (see **3.2**). However, the hydrocarbons on drill cuttings are, by the nature of the waste, 'stuck' onto and imbibed into the cuttings. Although the particle size of the cuttings and the prevailing currents can influence the spread of cuttings, oily cuttings mostly have large fall velocities due to them being aggregated particles, and settle in the immediate vicinity of the rig (Delvigne, 1996), where the major deleterious effects are recorded (Peterson *et al.*, 1987). A secondary 'plume' of lighter material will drift away with the current, but contains only 5 – 7% of the solids discharged (Ayers *et al.*, 1982). The general elliptical shape of the area more thinly covered by the deposited cuttings is dependent on the flow velocity and direction, i.e. the currents. The impacted areas can vary in size, covering a radius of several hundred metres to several kilometres (Delvigne, 1996).

Once on the seabed, the cohesive nature of the deep piles means they tend to stay in piles, and resist natural attenuation due to the cold, lack of nutrients such as N & P, lack of electron acceptor and hydrostatic pressure, except sometimes on the exposed surface of the pile where some breakdown and dispersion occurs. However, the larger area of thinly deposited material changes over time, possibly by erosion, resedimentation, leaching and degradation (Delvigne, 1996).

3.4.2. Effects of the Contaminated Drill Cuttings on the Seabed

Environmental monitoring programmes, which are conducted during the majority of North Sea oil developments when using oil-based muds, have confirmed that the zones of seabed affected by these operations are confined to the close vicinity of the installations (Peterson *et al.*, 1987).

Oil-based mud (OBM) drill cuttings can, as the E & P Forum and UKOOA agree, have adverse effects on the seabed biological community. This has been attributed initially by physical burial of the natural sediment, which is attributable when using water-based muds as well as OBM; however, the extent of biological effect is much greater when drilling has been with OBM, suggesting that these muds are more toxic (Berge, 1996).

Ecological data has been collated round a number of platforms using OBM (Peterson *et al.*, 1987), and reports that biological recovery is rapid in the area just beyond the platform. This is illustrated in the graphs in figure **3.4.1.1.** of redox potential, which increased over time even near the platform, redox reflecting oxygenation of the seabed, which can indicate biological recovery (there is a negative correlation between base oil concentration and redox potential (Berge, 1996)). Where the conditions on the seabed are more dynamic there is a more rapid regeneration; this is true for the Southern North Sea, where there were lower levels of OBM discharges per platform anyway.

Experimentation has indicated some leaching of oil and heavy metals on the sea floor (Delvigne, 1996), with coarser particles having the highest leaching rates. This is logical, as the finer particles are mostly clays that 'hold on' to organics (section **3.3.1.**). Leaching can be detrimental, making the pollutant bioavailable.

Experimentation by Berge, 1996, found that cuttings with a base oil content of 15 – 20% caused severe effects to the benthic community; cuttings with 2 – 3% base oil

content caused significant, but less severe effects. The report concluded, based on the total species matrix of a natural benthic community, that the threshold for gross effects on community structure was a sediment base oil concentration of 1000 ppm. Some individual species showed effects between 150 and 1000 ppm.

The effects from the OBM drill cuttings on the benthic communities can be a result of toxins, by a change of particle composition of the sediment (Clark & Patrick, 1987), or by anoxia caused by the heavy organic loading barrier (Barke & Veil, 1995). Experiments conducted by Plante-Cuny *et al.* (1993) confirm that diesel-based oil on drill cuttings had negative effects on macrofauna populations.

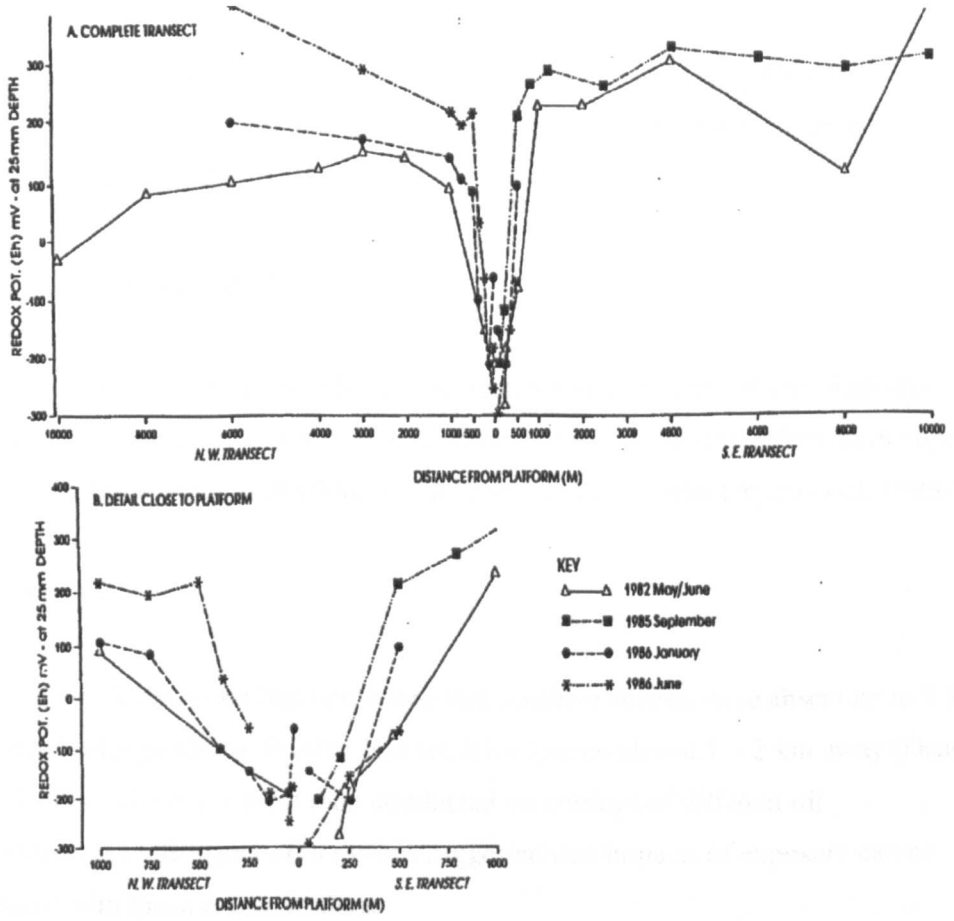


Figure 3.4.1.1. Redox Potential Graphs at Measured Distances from a Platform (Peterson *et al.*, 1987).

3.5. Toxicity Testing

The effects of the drilling fluids on the flora and fauna of the marine environment have been tested using bioassay procedures. The EPA test involves the 96-hour LC_{50} – a procedure where a group of organisms are exposed to the drilling fluid at increasing concentrations until a lethal concentration is reached with 50% of the population dying. The EPA use the possum shrimp *Mysidopsis bahia* for this test (MIDF, 1998). Other experiments have been conducted, measuring mortality, growth, bioaccumulation of hydrocarbons and metals, histopathology, reproduction and parasite infestation (Bowmer *et al,* 1996). According to the MIDF Drilling Handbook the EPA toxicity limit was 30,000 ppm – this was the minimum acceptable value for a dischargeable mud system.

There are manuals available relating to toxicity testing produced by OECD concerning the screening of chemicals for ready biodegradability in an aerobic aqueous medium (OECD, 1992).

3.5.1. Open Ocean Waters

The 96-hour LC_{50}'s observed in liquid and suspended-particulate-phase bioassays showed the discharges do not cause acute water column biological effects in the open ocean, even for the mineral OBMs, which have a higher toxicity (Ayers *et al,* 1985?).

3.5.2. Seabed

Observations in the North Sea confirmed that sensitive species were absent up to 2 km from the discharge site, with other less sensitive species absent 1 – 2 km away (Daan *et al.*, 1992). The experimentation conducted on cuttings of different oil concentrations by Bowmer *et al* (1996) suggested that impacts of exposure can be correlated with those concentrations.

- High impact from cuttings ranging from 12 – 20%

- Medium impact, 1.5 – 3%
- Low impact, 0.3%

Dab, a flat fish, was observed to assess the effects of drill cuttings by Stagg and McIntosh (1996); they were found to bioaccumulate base oil and aromatics derived from the oil in a concentration dependent manner. In both the Bowmer and Stagg experiments, the heavy metals were almost entirely non-bioavailable.

3.6. Summary

The structure of the hydrocarbons used in the muds has a bearing on its behaviour and interaction with other compounds, and therefore its degradation. Research confirms the negative impact of oil based drilling fluids and drill cuttings contaminated with the same on the natural flora and fauna of the oceans and particularly the sea-bed. Chemicals utilised toward the exploration and production of oil have to be tested for toxicity in Europe, the USA and other parts of the world. Drilling fluid development continues to evolve with increased performance and reduced toxicity.

CHAPTER 4
MICROBIOLOGY

4.1. Introduction

Bioremediation, within the remit of this project, involved the use of micro-organisms to degrade or mineralise the organic contamination of the drill cuttings. To accomplish this with any measure of success, the field of microbiology had to be understood in a general sense, and then narrowed down to the specific requirements to accomplish the objectives.

The term prokaryote is synonymous with bacteria, including archaebacteria and eubacteria (Singleton & Sainsbury, 1997). Most of the prokaryotes are single celled organisms with no nuclear membrane and a characteristic cell wall, performing all functions necessary for life for themselves. They can thrive in almost any conceivable environment, even in extreme conditions such as deep in the earth in rocks, in the heat and sulphurous conditions around the 'black smokers' under the ocean, halophilic locations such as salt lakes and within other living organisms, where they can be symbionts or pathogenic. They are essential for life, for example the Nitrogen Cycle, where dead organic proteinaceous material is recycled. Mostly their sizes are between 1 – 10 μm, and their shapes vary; rod shape, coccus (spherical or ovoid), spiral, filament and many deviations from these shapes. They are free living, and many have a flagellum, a single helical tube of protein, allowing movement. Although they are only single celled organisms they exhibit sensory behaviour, for example phototaxis, sensing light and dark. They require nutrients to sustain life; the class of bacteria appropriate for this research are heterotrophs, which require organic carbon sources to make the amino acids and sugars which build up their cell structures; they cannot fix their carbon from the atmosphere like autotrophs.

The cell wall of Gram-negative bacteria, for example *Acinetobacter* and *Pseudomonas*, contain an outer membrane composed of liposaccharide (see section **4.3.6.**), lipoprotein and other complex macromolecules (Madigan *et al.*, 1997), but

little peptidoglycan; Gram-positive bacteria, for example *Bacillus* and *Rhodococcus*, lack the outer membrane, the strength and shape of the cell wall maintained by a higher content of the polysaccaride peptidoglycan. Many of the bacteria found in environments utilising hydrocarbons as a food source are Gram-negative, for example *Pseudomonas* (Koch *et al.*, 1990; Canosa *et al.*, 2000; Nieboer *et al.*, 1993).

4.2. Health and Safety (H & S) and Laboratory Practices

There are numerous techniques specific to the field of microbiology that needed to be understood and implemented during the experimental procedures of the project.

4.2.1. Chemicals and Equipment

The H & S procedure for each chemical was laid out in Control of Substances Hazardous to Health (COSHH) amd Material Safety Data (MSDS) sheets, and was examined for each product before handling, and then handled in the manner demonstrated on those safety sheets. It was forbidden to mouth pipette any solutions, even sterile water.

Equipment was demonstrated before use, and, where appropriate, manuals were available. The safe use of the -80°C freezer procedure was discussed, i.e. the vacuum created when the temperature rises, and what to do when the alarm goes off.

Disinfectants (Trigene II) were used for decontamination of bacterial cultures in accordance with local rules and manufacturers' instructions.

4.2.2. Aseptic Technique

"The technique used in the prevention of contamination during manipulation of cultures and sterile culture media is called aseptic technique" (Madigan *et al.*, 1997, p 16). Aseptic technique is a process designed to exclude these unwanted micro-organisms, predominantly involving the Bunsen burner. The hot flame of the Bunsen

creates an updraft, so, for quick transfer work, this was the method adopted. For drying plates, a sterile cabinet was used which had sterile air drawn through it. Other techniques involved the sterilisation of equipment such as loops, blades and forceps, by dipping in ethanol and then burning off the ethanol by ignition from the Bunsen. When isolating a bacterium, a single colony was picked off with a sterile implement and re-streaked on sterile media; this is aseptic transfer. All other equipment, such as bottles, flasks, test-tubes and so on were sterilised by putting into an autoclave. The model of autoclave used was a Prior Clave Tactrol, which was programmed to heat for 20 minutes at 121°C. This was also used to sterilise the media. Some equipment was disposable, coming to the laboratory pre-sterilised in sealed packaging. This had to be handled aseptically to ensure the products stayed sterile.

4.3. Microbiology, Relating to Hydrocarbon Degradation

The samples of muds used for drilling in the North Sea were based on alkanes (Versaplus) and alkenes (Novatec). The project needed to examine past research into general hydrocarbon degradation, which could then be related to the specific oil in the mud. At the onset of the project in January 1998 the literature available was quite concise.

4.3.1. Microbiology Relating to Specific Hydrocarbons

Many alkanes and alkenes are of biogenic origin, being produced from a variety of terrestrial plants and aquatic algae (Millero and Sohn, 1991). In marine systems, good correlations have been documented between the presence of certain straight and branched-chained alkanes and blooms of primary producers (Gordon *et al,* 1978). This means that many microorganisms have evolved to use hydrocarbons as growth substrates, and have evolved specialised metabolic processes to degrade hydrocarbons, illustrating their biodiversity (Pritchard *et al.* 1995). The strategy used involves the insertion of molecular oxygen (in an aerobic organism) into these carbon-rich structures, with the process catalysed by oxygenase enzymes, the enzymes being specific to a particular hydrocarbon. Alkanes and alkenes are initially attacked,

terminally (fig. **4.3.3.1**) or subterminally (fig **4.3.3.2.**), by a hydroxylase (a mixed function oxidase) to produce the corresponding *n*-alcohol (Pritchard *et al.*, 1995). The alcohol is further oxidised to the corresponding monocarboxylic acid and metabolised by β-oxidation to provide acetate units for the cell's intermediary metabolism (fig. **4.3.3.3.**).

The susceptibility of hydrocarbons to biodegradation is determined by the structure and molecular weight of the hydrocarbon molecule (Atlas, 1993). *N*-Alkanes of intermediate chain length (C10 – C24) are degraded most rapidly; these are the length of the carbon chains in the Versaplus mud. Short chain alkanes of less that C9 are toxic to many microorganisms, but can often be evaporated off; there were little if no short chains expected in the drill cuttings as they came to the university; this was confirmed by testing by Gas Chromatography (GC), see section **6.2.1.**. As alkane chain length increases, so does resistance to biodegradation. Branching in general also reduces the rate of biodegradation because tertiary and quaternary carbon atoms interfere with the degradation mechanisms or can block degradation altogether. Aromatics are slower to degrade, and alicyclics are unable to serve as a sole carbon source except via co-metabolism (Atlas, 1984; Dean-Ross *et al.*, 2002). Co-metabolism occurs when a substrate does not support the growth of a given micro-organism, but the substrate may be modified or degraded by the organism in the presence of a second, growth-supporting substrate (Singleton and Sainsbury, 1997), which can be produced by the activity of another micro-organism. There are few complex hydrocarbons in the muds, as they are basically straight-chained hydrocarbon compounds. "It is becoming clear that co-metabolism is important in nature although this has been underestimated because of the difficulty in devising suitable enrichment and selection procedures" (Slater *et al.* 1984) – obviously enrichment and selection cannot be achieved on the basis of growth on the substrate alone.

The successful biodegradative removal of hydrocarbons from the sea depends on the enzymatic capacities of micro-organisms and various abiotic factors. Suitable growth temperatures and available supplies of fixed forms of N, P and O are required. In the oceans, temperature and nutrient concentrations often limit the rates of hydrocarbon

degradation. The low concentrations of nitrate and phosphate in seawater are particularly limiting. Reisfeld *et al.* (1972) isolated a bacterium, tentatively characterised as a member of the genus *Arthrobacter*, and found it brought about significant dispersal of crude oil, but was "absolutely dependent" on exogenous sources of nitrogen and phosphorus. Temperatures are also a limiting factor (Mulkins-Phillips and Stewart, 1974).

These factors lead to this project looking at contained bioremediation using bioreactors (see section **5.4.5.**). Sea-floor is not an option due to legislation (see section **2.6.**); however, bioreactors could perhaps be adapted for either onshore remediation, or offshore, either on barges or a designate platform which has finished production, e.g. Brent Spar, Shell's defunct platform which caused so much angst between the industry and environmentalists.

4.3.2. Aerobic Vs Anaerobic

Most muds contain halogens, with up to 2 – 3% as calcium chlorides. The method of bioremediation needed for the drill cuttings had to be appropriate for the mud and bacteria utilised.

Anaerobic processes are successfully used to slowly degrade organic compounds that are highly saturated with halogens, as the bacteria utilise the compounds as electron acceptors or reducing agents (Hoeppel and Hinchee, 1994). Anaerobic degradation produces less biomass per unit of organic waste removed, as the substrate is converted to methane. This may be harnessed as an energy source. However, most hydrocarbons are known to preferentially biodegrade under aerobic conditions, facilitated by a large, diverse group of aerobic micro-organisms (Leahy and Colwell, 1993). Madaigan *et al.* (1997) state that in the absence of oxygen, saturated hydrocarbons are virtually unaffected by micro-organisms. However, recent research has found this not to be correct, with harbour sediments contaminated with hydrocarbons degrading anaerobically (Coates *et al.*, 1996) and Zwolinski *et al.* stating that "we have only begun to reveal the diversity of organisms mediating

anaerobic hydrocarbon degradation" (2000, p 141). In slurry-phase bioremediation systems, aerobic metabolism has distinct advantages over anaerobic, as listed by Christodoulatos and Koutsospyros (1994).

- Reaction rates of the aerobic processes are much faster than those of anaerobic systems.
- Aerobic processes generally induce production of the most oxidised end products.
- Certain anaerobic microorganisms are more sensitive to heavy metal toxicity.
- Anaerobic processes are often associated with production of odorous gases.
- Costs and risks are higher, as are hazards to health from toxic by-products.

Aerobic effluent treatment of sewage is the largest controlled use of micro-organisms in the biotechnical industries (Best *et al.*, 1985). The steps involved are

- Substrate adsorption to the biological surface.
- Adsorbed solid breakdown by extracellular enzymes.
- Dissolved material absorption into cells.
- Growth and endogenous respiration at the expense of the contaminate.
- Release of excretory products.
- Ingestion of primary population by secondary grazers; these may be less abundant in a hydrocarbon environment.

This ideally results in the complete mineralisation of the waste to simple salts, gases and water.

Aerobic systems are more appropriate for hydrocarbon degradation and offer greater and more uniform process control. They are generally safer and easier to handle in both laboratory and industrial environments. After reviewing the evidence, the project proceeded by the isolation and utilisation of aerobic bacteria, and used an aerobic process for the bioreactors.

4.3.3. Electron Acceptor

The project had aimed for an aerobic system, and in aerobic respiration, bacteria utilise oxygen as the terminal acceptor of electrons removed from oxidising the hydrocarbon.

The initial mud to arrive at the university for analysis and experimentation was the Novatec, a linear alpha oleophin, which is an alkene. Alkene reduction tends to be more intricate than alkane, partly due to the double bond. The process of bacterial remediation needed to be examined, to reduce any limiting environmental factors.

Research on alkene oxidation focuses on the degradation of terminal alkenes. The products of 1-alkenes oxidation are varied since the initial attack can occur at either the methyl group or the double bond (Cookson, 1995, Britton, 1984, Atlas, 1993) to form:-

- ω-unsaturated alcohols or fatty acids
- primary or secondary alcohols or methyl ketones
- 1,2 epoxides
- 1,2 diols.

Diols are dihydric alcohols, chiefly represented by the glycols in which the hydroxyl groups are attached to adjacent carbon atoms (Walker, Ed., 1995).

It is not uncommon for a single culture to exhibit multiple modes of attack on 1-alkenes.

Methyl group oxidation is considered a major degradative pathway, with ω-unsaturated fatty acid as a metabolic intermediate; the mechanism of methyl group oxidation is similar to that of *n*-alkanes. The initial oxidation step of alkanes takes one of the O_2 atoms and incorporates it into the oxidised hydrocarbon (Madigan *et al.*, 2000). This reaction occurs through the monooxygenase enzyme which is sometimes

called hydroxylase. Enzymes are proteins that act as a specific biological catalyst, decreasing the activation energy (Singleton & Sainsbury, 1997). As monooxygenase enzymes catalyse reactions in which the main substrate becomes hydroxylated, they are also called hydroxylases. They require two substrates to serve as reductants of the two oxygen atoms (Nelson & Cox, 2000), with the main substrate accepting one and the co-substrate provides the hydrogen atom to reduce the second oxygen atom to water. For this reason, monooxygenase can also be called mixed-function oxidases or mixed function oxygenases. The general reaction equation is:

$$AH + BH_2 + O - O \longrightarrow A - OH + B + H_2O$$

This second substrate can be NADH, as shown in figure **4.3.3.5.**

Dioxygenases catalyse reactions where both the atoms of an O_2 molecule are incorporated into the organic substrate molecule (Nelson & Cox, 2000).

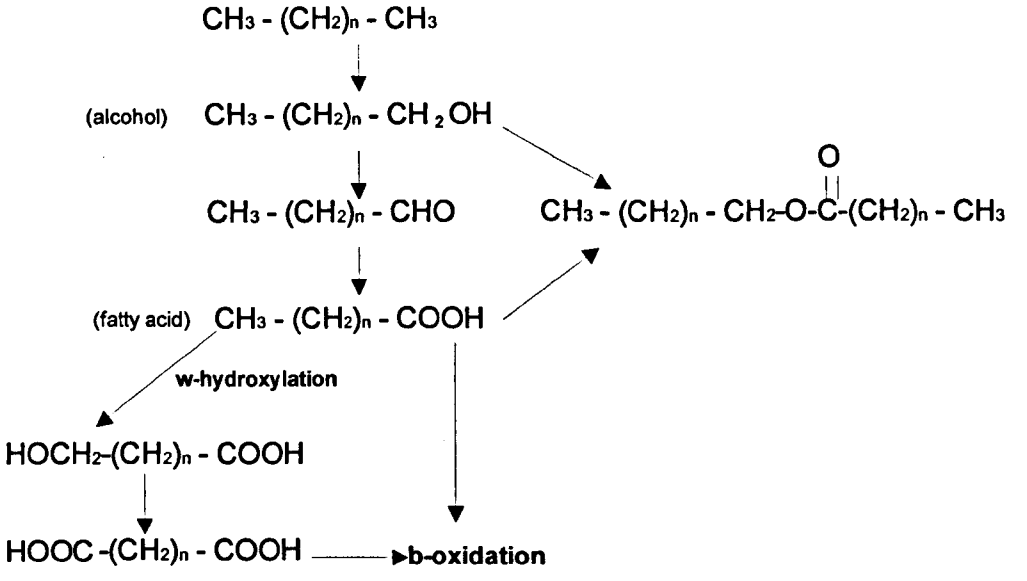


Figure 4.3.3.1. Oxidation of *n*-alkanes by attack on the terminal methyl group (Britton, 1984).

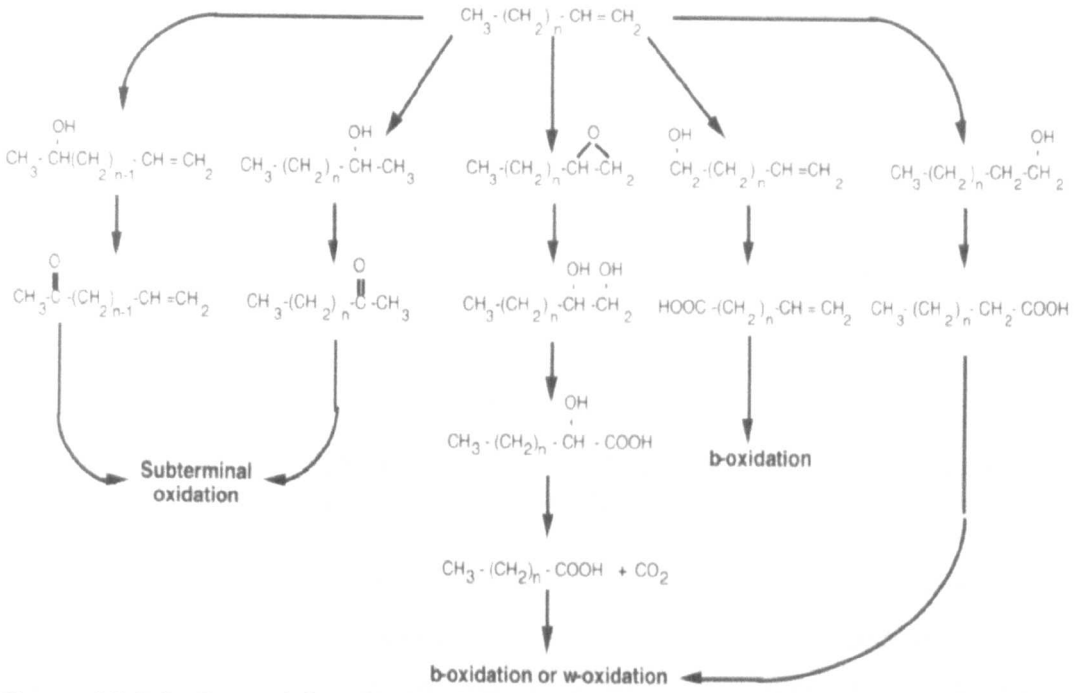


Figure 4.3.3.2. Potential pathways for 1-alkene degredation (Britton, 1984)

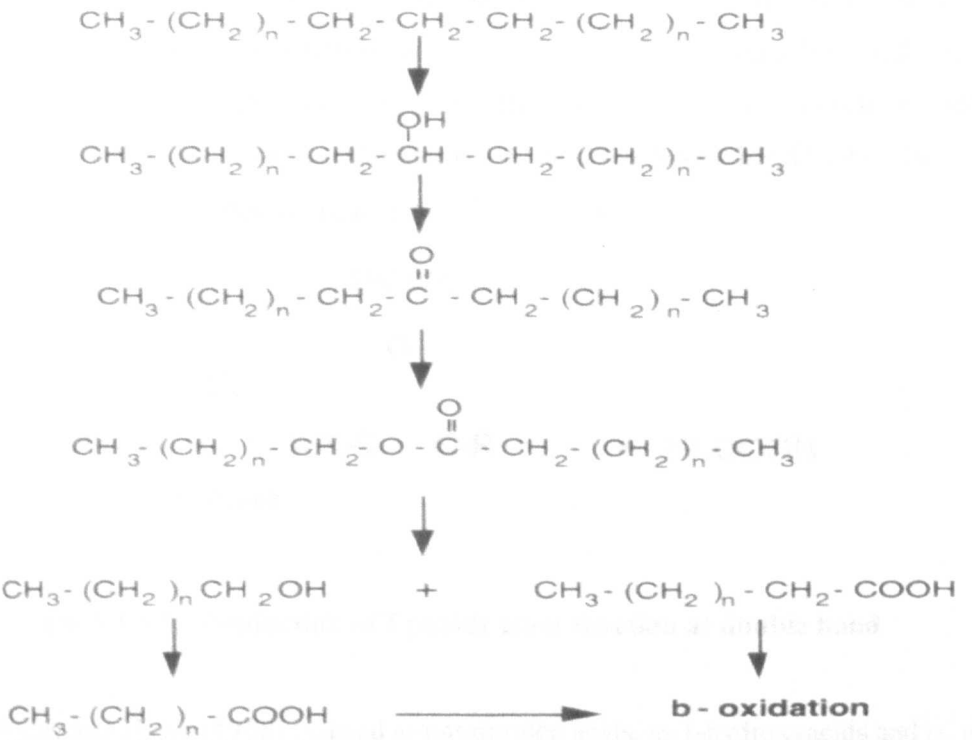


Figure 4.3.3.3. Aliphatic hydrocarbon oxidation by subterminal attack (Britton, 1984)

Pseudomonas oleovorans uses a hydrocarbon monooxygenase enzyme system which causes the epoxidation of simple, aliphatic terminal olefins (May & Katopodis, 1990). An example of benzene hydroxylation is in figure 4.3.3.4.

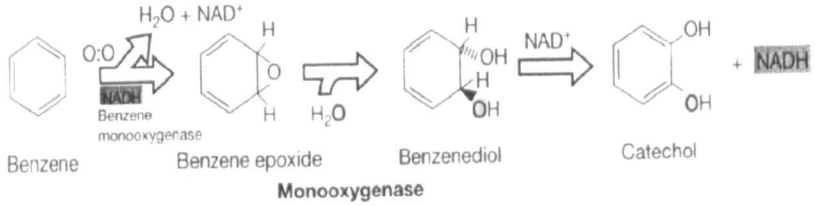


Figure 4.3.3.4. Hydroxylation of benzene to catechol by a monooxygenase in which NADH (dehydrogenase) is an electron donor (Madigan *et al.* 2000).

The isolation of products from both the methyl terminus and double-bond attack on 1-alkenes primary alkenes) indicates that many organisms can carry out diterminal oxidation of these substrates. An example is the formation of saturated fatty acids one carbon shorter than the substrate, as well as the ω-unsaturated acids in an *Acinetobacter* (Britton, 1984). It was thought that the saturated fatty acid was produced by decarboxylation of the α-hydroxy acid to CO_2 and a fatty acid one carbon shorter than the alkene substrate. Reports on *Pseudomonas* (Britton, 1984; May & Katopdis, 1990) found minor reactions at the double bond lead to the formation of epoxides, diols, and α-hydroxy acids.

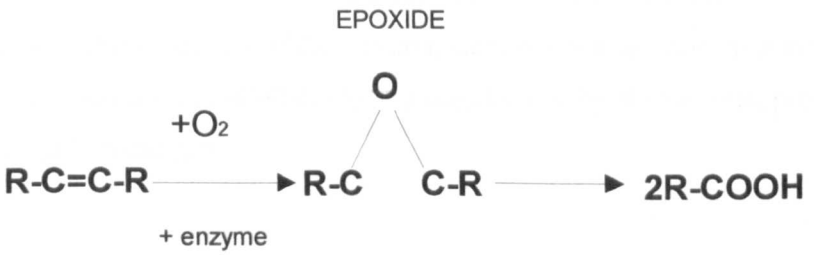


Figure 4.3.3.5. Production of Epoxide from reaction at double bond

Jones and Howe (1968) isolated ω-unsaturated acids, ω-1-hydroxyacids and α, ω-dicarboxylic acids both the same chain length and one carbon shorter than the substrate from cultures of a yeast, *Torulopsis gropengiesseri,* grown on 1-alkenes

from C14 – C18. Another possible type of diterminal attack of 1-alkenes is suggested from the finding of saturated, substrate-length fatty acids in 1-alkene grown *Mycobacterium vaccae* and *Corynebacterium simplex*, with a possible explanation being saturation of the double bond of the ω-unsaturated fatty acids.

.

Evidence has been found to suggest a carbon elongation process can occur; *Acintobacter* incorporated the growth substrate into higher cell fatty acids by an elongation mechanism (Britton, 1984), and *C. liplytica* formed fatty acids via chain elongation from C14 – C18 n-alkanes. Secondary alcohols reminiscent of subterminal oxidation can also be formed, which illustrates how diverse the products from 1-alkene/alkane oxidation can be.

May and Abbott (1973) found the ω-hydroxylase system of *Pseudomonas oleovorans* catalysed the epoxidation of alkenes as well as methyl group hydroxylation (CH_3 to CH_2OH), which illustrates reaction at the double bond. Epoxides are intermediates in the metabolism of olefins to glycols, and enzymatic hydration of epoxides to form diols have been observed in a *pseudomonad.* Methyl ketones and aldehydes could be formed directly by hydroxylation of the double bond. These compounds would then be reduced to the 1- and 2-alkanols.

Once the initial oxidation process has occurred, if the product continues to be oxidised by the enzyme activity of the bacteria, carbon atoms are removed in pairs (Acetyl with the co-enzyme, CH_3-C-O-CoA) and metabolised by the bacteria, producing biomass and carbon dioxide.

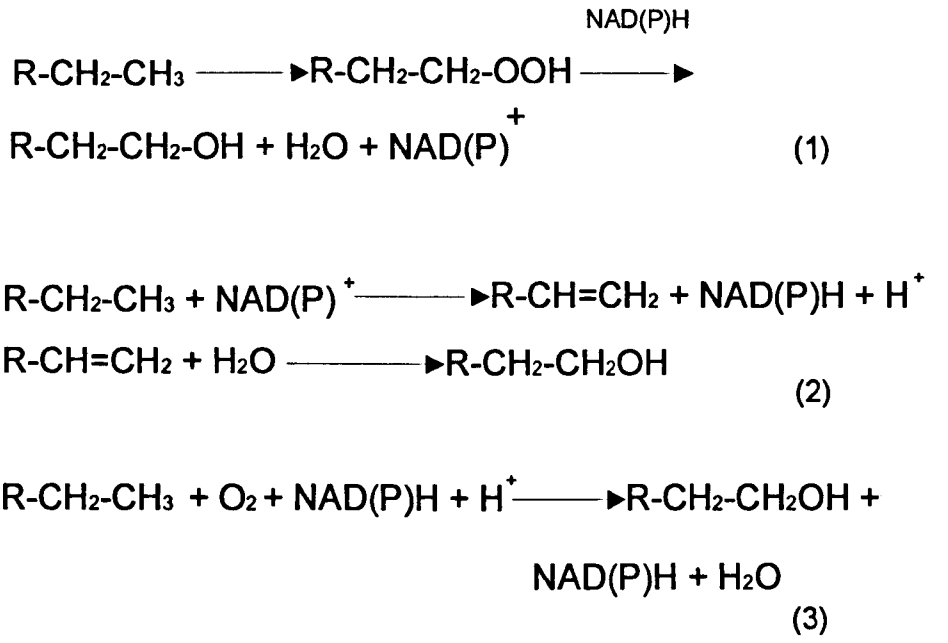


Figure 4.3.3.6. Some pathways for oxidation of *n*-alkanes (Gibson (Ed), 1984).

NAD is nicotinamide adenine dinucleotide, a coenzyme serving as an electron acceptor (and two protons) for many dehydrogenases. The reduced form, NADH, subsequently donates its electrons to the electron transport chain. NADP is a phosphorylated derivative of NAD, and serves as an electron carrier, but the electrons are primarily used for reductive biosynthesis.

Equation (1) is from *Acintobacter* sp H01-N, which did not have an alcohol dehydrogenase, with the fatty acid formed without the primary alcohol being an obligatory intermediate (Hypothesis, Britton, 1984).

Equation (2) proposes the involvement of water or molecular oxygen for hydroxylation of alkenes to primary alcohols.

Equation (3) is a widely supported hydroxylation mechanism, with direct incorporation of oxygen catalysed by a mixed-function oxidase or monooxygenase, e.g. *Pseudomonas oleovorans* (Briton, 1984)

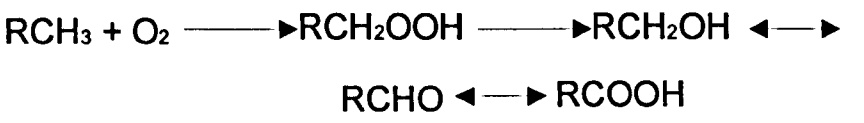


Figure 4.3.3.7. Pathway of alkane oxidation in *Acintobacter* sp H01-N (Finnerty, 1990(a))

Figure 4.3.3.8. Soluble NAD+ and NADP+ dependent primary alcohol dehydrogenase pathway (Finnerty, 1990(b))

It has been proposed that primary alcohols are formed from epoxides by a reductase that cleaved and reduced on the C-2 side of the oxygen in the epoxide group. Using this same mechanism, Klug (1971) proposed that secondary alcohols are formed by cleavage and reduction of the epoxide on the C-1 side of oxygen. Primary alcohols also could be formed directly from 1-alkenes by an hydrase analogous to fumarase, where water is added across the double bond. Fumarate reductase can, as reported by Nieboer *et al.* (1993), change fatty acid composition in *Pseudomonas oleovorans.*

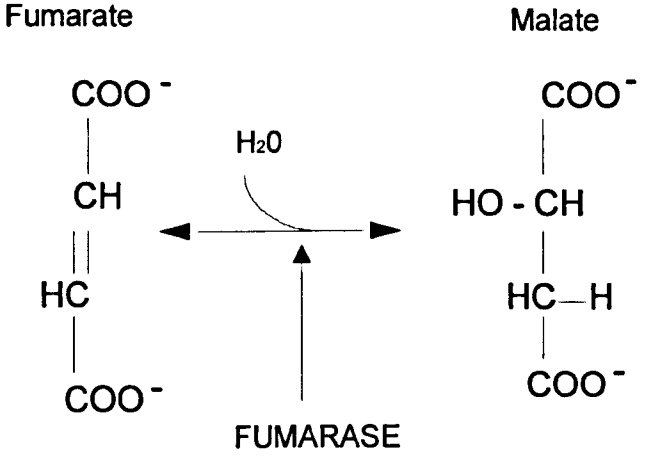


Figure 4.3.3.9. Fumarase Enzyme activity

If the oxygen is derived from water, it indicates a hydrase mechanism. Some alkenes cannot serve as sole carbon and energy sources, depending on how they are oxidised,

as the metabolites can cause depletions in the bacteria (Britton, 1984). This can be overcome by using mixed cultures, which led to the examination of co-metabolism.

The energy required by aerobic systems can be calculated based on stoichiometric equations for the theoretical mineralisation of branched alkenes in catabolic reactions – this is for complete mineralisation, skipping the intermediate steps of oxidation to an alcohol and then a fatty acid (LaGrega *et al.*, 1994). To realise this in a practical application requires further development.

$$C_{18}H_{36} + 27O_2 \longrightarrow 18CO_2 + 18H_2O$$

$$\frac{27(2 \times 16)}{(18 \times 12) \times 36} = 3.43 mgO_2/mg \text{ alkene}$$

Density of oxygen = $1492 g/m^3$, = $0.0023 m^3O_2/g$ alkene.

Figure 4.3.3.10. Oxygen requirement for mineralisation of an alkene

Not all the oxygen put in a system is available to the micro-organisms, with the concentration of oxygen that can be dissolved in water being very low, typically 0.0084g/L (Pirt, 1975). It is therefore necessary to supply oxygen continuously to a growing culture, breaking the bubbles up as much as possible to allow the maximum amount of gas to dissolve in the liquid medium.

The following equation describes the oxygen transfer rate (OTR) in a fermentation medium:

$$OTR = K_La (C^* - C)$$

K_La = mass transfer coefficient
C^* = saturation concentration of oxygen
C = actual concentration of oxygen
K_La is specific to an individual bioreactor.

4.3.4. Nutrient Amendments

Nutrients can be a limiting factor in biodegradation, particularly nitrogen and phosphorus. In all studies concerning bioremediation, nutrient amendments or enhancements encourage better growth of the bacteria, therefore improving the chances of remediation (Jackson & Pardue, 1997; Piehler & Paerl, 1996; Atlas, 1993, 1995; Mitchell *et al.* 2000; Pinelli *et al.*, 1997). Some of these have found it more beneficial to use oleophilic fertilisers (Atlas, 1993), which have a similar structure to the oils and are therefore suitable for the remediating bacteria. When used during the Exxon Valdez, it was found the pellets did not wash away, but adhered to the oil and slowly broke down, gradually releasing the nutrients. Examples include oleophilic fertilisers containing paraffinized urea and octyl phosphate. Findings by Churchill *et al.* (1995) suggest that addition of oleophilic fertiliser reduces the surface tension of the hydrocarbon, thereby increasing its availability to the micro-organism. Another example was Inipol EAP 22, developed by Elf Aquitaine, Paris, France. This fertiliser enhanced rates of oil biodegradation in field tests, even in Arctic conditions. However, Elf could not provide any of this fertiliser at the time of the project.

Other limiting nutrients include the mineral iron, particularly in open ocean areas, where iron concentrations are particularly low (Atlas, 1993).

The C:N:P ratio for optimal growth is quoted at 100:10:1 (Verheul *et al.* 1993); 20:5:1 (LaGrega *et al.*, 1994); 50:14:3 (Cookson, 1995). BOD:N:P is quoted as 100:5:1 (LaGrega *et al.*, 1994). These, as can be seen, vary considerably. The project went forward by experimentation using batch tests, and then scaling up the nutrients for the bioreactors.

The addition of particulate organic carbon with the nutrients has proved beneficial in some hydrocarbon bioremediation experiments (Phiehler & Paerl, 1996; Johnson & Logan, 1995). Piehler & Paerl used *Spartina alterniflora*, a marsh grass, for particulate organic carbon, and Johnson & Logan used Suwannee River Humic Acid

and Soil Humic Acid to represent dissolved organic matter and sediment organic matter. This additional carbon would be readily available in a compost system. However, there are significant populations of micro-organisms in soils that can use alkanes and alkenes as sole sources of carbon and energy (Cookson, 1995).

4.3.5. Solid Concentrations

Most bacteria need a liquid-based medium for cellular growth, diffusion and mixing. Exceptions include bacteria in rocks. A dry environment will cause stresses to the bacteria, aborting growth, with spore-forming bacteria resorting to this survival technique. Moisture is essential. Obviously, the higher the solids content in a reactor the better, to reduce throughput volume. Slurries up to 50% dry weight solids have been used, but the optimum is stated at around 40% (Stroo, 1989, Brown, 1999, Cookson, 1995), which was taken as the minimum for the drill cuttings to prevent degradation being inhibited and to keep the slurry in suspension (more details in section **5.4.5.3.**). Fu and Alexander, 1995, found that slurrying the contaminant was the best form of enhancement, even than the use of surfactants.

4.3.6. Surfactant

There has to be an intimate contact between the micro-organism and the substrate. The rate at which the oil desorbs from the cuttings depends on the oil and the mineralogy of cuttings. A portion of the oil is bound in a stable position along micropores within the cuttings. The (relatively) large size of micro-organisms excludes them from the micropores, preventing their direct access to the sorbed contaminant. To be accessible, the contaminant must desorb to the micropore water and then move by diffusion to the outer surface of the particle where it can be degraded by the micro-organisms that often attach to the particle. Investigations in the USA and Netherlands claim that desorption/diffusion from the sorbed phase to the solute phase is the rate limiting factor for slurry phase treatment (LaGrega *et al.* 1994, and references within), with the **rate of degradation being a function of the concentration of the contaminant in solution rather than the total sorbed mass.**

Volkering *et al.* (1998) concluded that some surfactants help, but not all, and more research is required. Some oil dispersants, which are a type of surfactant, were tested, together with drilling fluid, for their effects on marine bacteria (Okpokwasili & Nnubia, 1995), and were found to affect the ability of the bacteria to metabolise these substrates in the environment.

Cookson (1995) reported microbial cultures produce extracellular surfactants that aid in solubilising hydrocarbons. Micro-organisms within a genus produce biosurfactants with a similar structure, although there can be species-level differences in the amount produced, with the capacity for enhancement of solubilisation and biodegradation dependent on the biosurfactant structure (Miller and Zhang, 1997). The general structure of a biosurfactant includes a hydrophilic moiety composed of amino acids or peptides, anions or cations, or mono-, di- or polysaccarides. A few identified surfactants are rhamnolipids, trehalose-containing glycolipids, phospholipids and lipopolysaccharide (Cookson, 1995). Yield and composition are affected by growth conditions including carbon source, culture medium nutrients, temperature, pH and aeration. Interest has grown globally concerning biosurfactants due to their ability to meet most synthetic surfactants' requirements. Biosurfactants may be a pathway to remove the oil from the cuttings; as with surfactants in general they reduce surface tension, critical micelle concentration (CMC) and interfacial tension in both aqueous solutions and hydrocarbon mixtures (Banat, 1995). However, there are not yet many available commercially, therefore they must be produced in the laboratory. Miller and Zhang (1997) have found that "it is something of an art to maximise biosurfactant production in the laboratory".

Another alternative would be to wash the drill cuttings with a nonbiological surfactant. These have been used to promote hydrocarbon dispersion, for example Triton X-100, which is a nonionic alkylphenol ethoxylate surfactant (Cookson, 1995). If concentrations of Triton are above 0.05% there is a risk of lysing the bacteria. This product has now been phased out as the alkylphenol is toxic to marine life and has been found in rig discharges. This has led to OSPAR banning the product in the

North Sea (OSPAR, 2001). Mulkins-Phillips *et al.* (1974) tested four chemical dispersants and found that none of them were toxic to the degrading bacteria.

In situ production of biosurfactants is little recorded, yet there is evidence of large particles of oil being degraded, which means the micro-organisms can adapt to their food source. If bacteria were pre-conditioned in a medium containing the hydrocarbon to be remediated, there was a strong possibility they would develop their own surfactant or equivalent.

Once the oil is in aqueous phase, micro-organisms can cluster around a droplet of the hydrocarbon or 'accommodate' the hydrocarbons as submicron droplets (see figure **6.4.10.3.**). Below are some examples of how they prepare the oil for food (Britton, 1984).

- They can have a higher membrane lipid content, membrane 'extensions' and alkane-binding capacity (not present in glucose fed cells of the same species), known as a lipophilic edges
- A lipopolysaccharide moiety, which is responsible for alkane binding
- Use surfactants as part of the food transport system, e.g. *Pseudomonas*, by producing rhamnolipids responsible for hydrocarbon emulsification (Koch *et al.*, 1991).

Emulsification of the hydrocarbon is approached in diverse ways by differing microorganisms. *Arthrobacter paraffineus* and a number of other hydrocarbonoclastic bacteria produce trehalose-containing glycolipids (Britton, 1984).

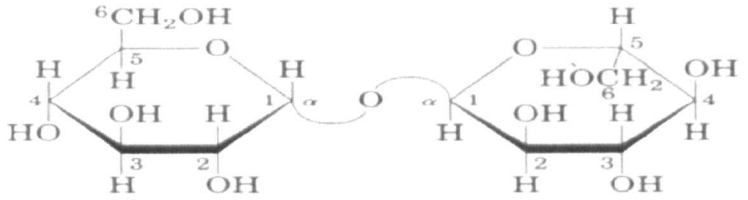


Figure 4.3.6.1. Tetrahose glycolipid (Nelson & Cox, 2000)

Corynebacterium hydrocarboclastus emulsifiers are complex mixtures of protein, lipid and carbohydrate.

It appears from this information that the micro-organisms must be acclimatised to the contaminant before addition to the reactor vessel, or these lipids may not be there, increasing the lag phase or possibly destroying the bacteria. The project used this approach to aid in the development of a bioremediation system. A small quantity of cuttings was introduced to the culture media for a minimum of 24 or 48 hours before the culture was added to the main flasks or reactor vessels.

4.3.7. pH

pH is a parameter that can be organism-specific. It can affect basic cell processes, for example cell membrane transport and the equilibrium of catalysed reactions.

The pH values of most natural environments fall between 5 and 9, resulting in the optimum pH for most organisms being between this range (Madigan *et al.,* 1997). Outside these pH ranges, biological activity can be reduced due to the oxygenase (mono/dio) enzymes' activity being limited. There are always organisms that can survive outside of the range – acidophiles such as *Thiobacillus* (Madigan *et al.*, 1997; Eweis *et al.*, 1998), and alkaliphiles, many of which are *Bacillus* species.

The pH also affects the solubility of nutrients, such as phosphorus, which is maximised at pH 6.5, and the mobility of hazardous metals, which decrease at pH levels greater than 6 (Sims *et al.*, 1990).

In batch cultures, the pH can be controlled by using buffers. For near neutral range, i.e. pH 6 – 7.5, phosphate, usually in the form of KH_2PO_4, can be used. This will keep the pH neutral even though the metabolic reactions of the micro-organisms with the substrate may produce acid or alkaline by-products.

4.3.8. Temperature

Temperature can have a major influence on growth rate, with micro-organisms having differing optimum temperatures (Atlas,1975). High temperatures can be lethal, low temperatures can lead to cells becoming dormant. Activity decreases when dropping below the optimum because of reduced enzyme activity and a loss of the fluidity of the cell membranes, restricting transport of substrate molecules (Irvine, 1988). Whatever the temperature, bacteria do not like a sudden change – this produces a much greater reduction in cell activity than a gradual one, which allows them to acclimatise. Bioreactors can be temperature-controlled, but this requires an energy input; it would be preferable for the bacteria to acclimatise to the ambient temperature. The effect of temperature on cellular activity (rate of biodegradation) can be expressed as (LaGrega *et al.*, 1994),

$$r_T / r_{20} = \theta^{(T-20)}$$

r_T = activity rate at T°C

r_{20} = activity rate at 20°C

θ = temperature-activity coefficient

T = Temperature (°C)

For hydrocarbon degradation in contaminated soils, the temperature-activity coefficient (θ) has been experimentally verified over a temperature range of 15 – 42°C (Cookson, 1995), and was found to be 1.088. θ is the ratio of the rate of progress of a reaction or process at a given temperature, to the rate at 1°C, or in the case of θ_{-10}, 10°C lower.

As temperatures increase beyond the optimum, activity decreases. Examples of growth ranges for some species of bacteria are illustrated in figure **4.3.8.1.**

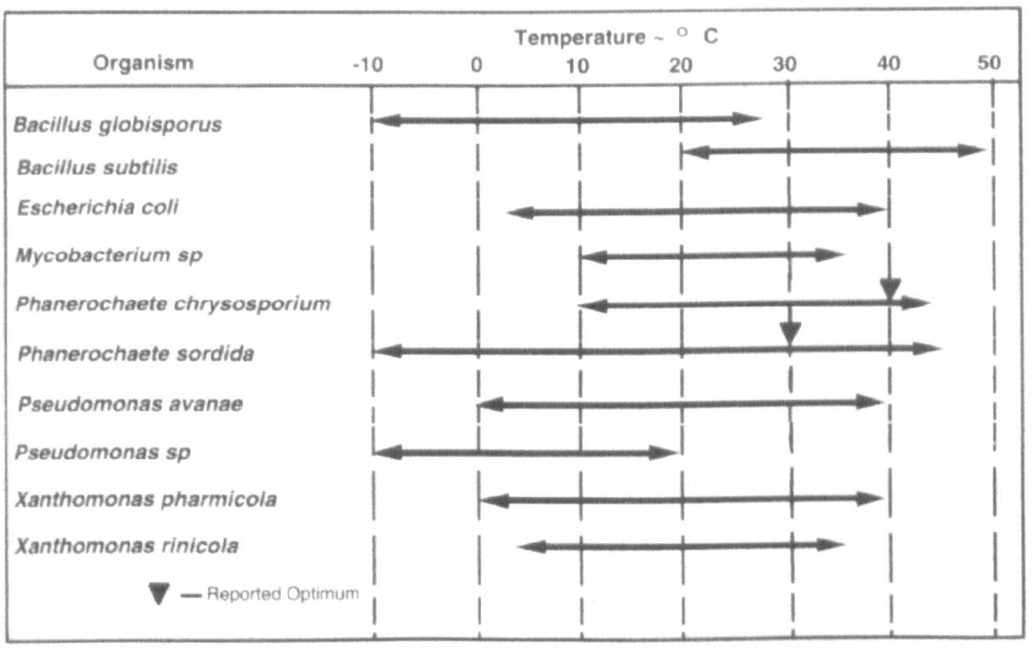


Figure 4.3.8.1. Temperature growth ranges for some remediating micro-organisms (Cookson, 1995)

4.4. Summary

Micro-organisms exhibit diverse and complex methods of utilising a growth substrate; consideration and control of their environmental conditions is essential to maximise their potential.

CHAPTER 5
BIOREMEDIATION ENGINEERING

5.1. Introduction

Industrial activities have brought about many deleterious effects on the environment. Bioremediation has been described as a biological response to environmental abuse (Sheehan, Ed, 1997), and is a process that can occur naturally, without anthropogenic interference. However, if the environment can be manipulated to improve microbial performance the process can be enhanced, leading to the bioconversion of recalcitrant pollutants to microbial biomass and stable, non-toxic end products.

Historically, bioremediation has been used for millennia; the Romans were thought to have developed the first sewage 'treatment plants', at least in the UK. There was little need for further development from that time up until the industrial revolution, when anthropogenic activities really started having detrimental effects on the environment and on the health of many workers involved in a number of industries. It was not until the last century that the backlash from these activities was felt, dealt by a concerned public to what appeared, both then and with hindsight, a financially driven and unenlightened industrial body. Now legislation has tightened up, meaning that industries have to consider the environmental impacts of their activities. Meanwhile, there is a legacy of contaminated sites throughout the country that cannot be used until some form of remediation has been carried out.

These sites have been a proving ground for many clean-up technologies, including bioremediation. Bioremediation, a technology only just past infancy, has proved to be an extremely cost effective alternative or companion technology to more conventional ones.

The following illustrates the cost; the data is not new, but it does illustrate the relationship between technologies.

TECHNOLOGY	COST RANGE (£)
Landfill UK	25 – 220
USA	100 – 200
Incineration USA (on-site)	75 – 300
USA (off-site)	100 – 500
Air Stripping	20 – 50
Soil Washing	35 – 100
Bioremediation	5 – 75

Table 5.1.1. Cost estimates for remediation technologies (after Bull, 1992)

The biological processing of waste matter draws upon a number of scientific disciplines, including biochemistry, genetics, chemistry, microbiology, chemical engineering, mechanical engineering and computing (Best *et a.*, 1985).
These disciplines have been brought together in three main areas:

- The degradation of toxic wastes, organic and inorganic.
- The recovery of resources, including materials to recycle.
- The production of valuable organic fuels.

It took one major disaster to bring bioremediation, i.e. the degradation of toxic waste via microbial activity, to the forefront.

5.2. Case Study: *Exxon Valdez* Disaster, Prince Williams Sound

In March 1989, there was a massive oil spill from the *Exxon Valdez*, which contaminated over 100 miles (160+ km) of Alaskan coastline. The clean-up task was daunting; what could be used to remove oil from such a vast area, in cold conditions, and not in itself contaminate the environment further (as chemical treatments can)? Exxon firstly physically washed the bulk of the oil from the gravel and cobbled beaches, but this still left a film of crude oil contaminant, which was ecologically available and aesthetically unpleasant. The EPA decided to conduct direct research of bioremediation on this disaster site. Pritchard, one of the principle researchers, has

written numerous papers on the work carried out (Pritchard & Costa, 1991; Pritchard, 1993); also, the word 'bioremediation' had finally come into the public and regulatory domain, and thought of as a feasible method of cleaning hydrocarbon contamination from sensitive sites. The disaster proved to be a good example of the problems and successes associated with this technology.

5.2.1. Enhancing Bioremediation *In-Situ*

Atlas had done research to stimulate biodegradation of oil slicks since the seventies (Atlas & Bartha, 1973), which focused on the use of oleophilic fertilisers; the advantages of this fertiliser and other nutrient enhancements have been reviewed in section **4.3.4.** within this report. Pritchard and his colleagues utilised this research on a grand scale in the aftermath of the spill (Pritchard, 1993). The first assumption was that there would be a significant enrichment of oil-degrading micro-organisms in the beach material following exposure to the oil (Atlas, 1981). This was authenticated by Pritchard (1993), who confirmed up to a 10,000-fold increase in the number of oil degraders within two months of the spill relative to beaches that had not been contaminated. This suggested that some nitrogen and phosphorus were available to support growth, and the low temperatures, between 10 and 16°C, were not limiting to the indigenous bacteria. The possibility of accelerating the bioremediation became feasible if the finite nitrogen and phosphorus could be provided via a fertiliser. Due to the porosity of the gravel and the high oxygen content of the seawater oxygen limitation was not considered. The team used granular fertiliser due to slower release characteristics; the tidal action helped to disperse the nutrients, but did not wash them away – granules were observed on the beach 2 – 3 weeks after application. There was a visible disappearance of oil on the cobble surface within 2 – 3 weeks of fertiliser application, in contrast to the untreated areas (Pritchard, 1991). Exxon eventually used a combination of liquid and granular in their final clean-up.

5.2.2. Relation of the *Exxon Valdez* to Drill Cuttings

The above is a fine example of *in-situ* bioremediation. The pressure was on; the oil spill had to be cleaned up within the short summer months of the North, as the severe cold of an Alaskan winter would surely stop microbial activity. 160 km of gravel and cobble beach could not feasibly be removed for *ex-situ* bioremediation. Although legislation concerning the North Sea has prevented the dumping of drill cuttings overboard, this is not necessarily the case globally. There are opportunities to enhance remediation conditions for drill piles, particularly 'new' piles, whose contaminant might be the new generation synthetic muds, by manipulation of the environment. The drill cuttings produced from operations in the North Sea are currently shipped to shore, often on barges. This leads to the view of *ex-situ* bioremediation.

5.3. Requirements for Bioremediation to Occur

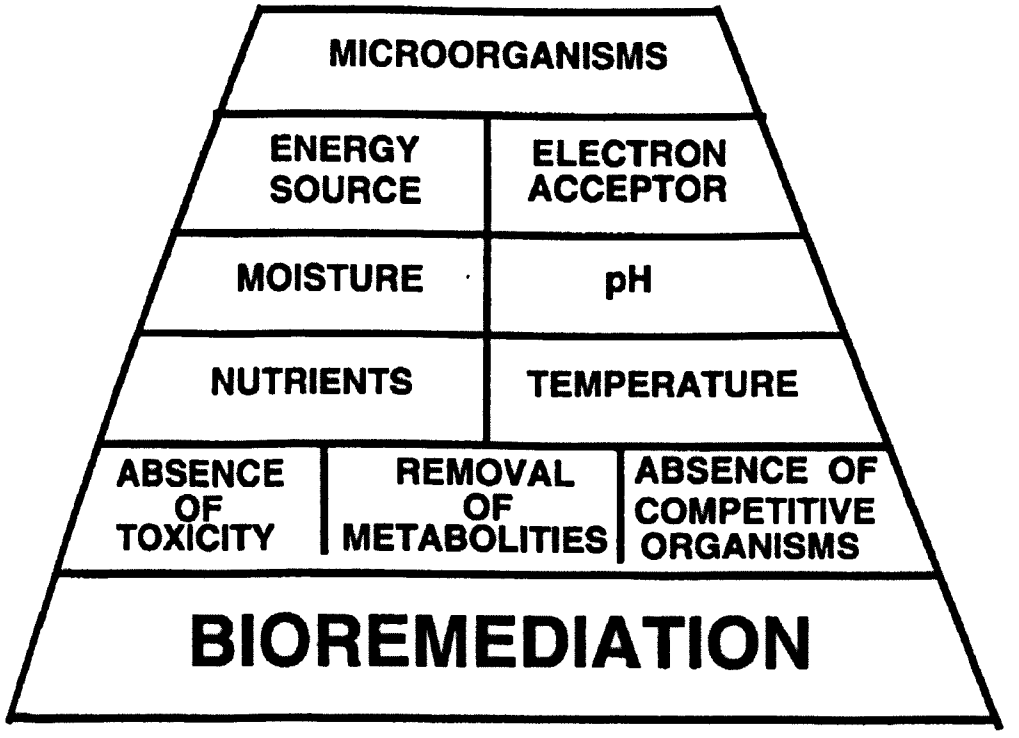


Figure 5.3.1. Requirements for Bioremediation to Occur (Cookson, 1995)

The environment influences the survival and growth of bacteria, and therefore their ability to breakdown contaminants. Bioremediation engineering considers enrichment and screening of the natural environment (in this research, the drill cuttings) for biocatalysts through environmental selection (McEldowney *et al.*, 1993), i.e. look at what the micro-organisms need to tolerate, then look for the micro-organisms in the appropriate environment. The environment can then be 'redesigned', or enhanced, to ensure that the bacteria flourish. Conditions required for the bacteria to facilitate bioremediation have been discussed in section **4.3.**

The project needed to consider what micro-organisms capable of remediation were likely to be indigenous in the drill cuttings, and thereby assess the conditions those bacteria required. As commercial strains were to be used concurrently with the indigenous isolates, conditions needed to be appropriate for them too. These conditions were assessed initially in the batch tests (see **6.4.**); conditions for the reactors used this data.

The other parameter to assess was the contaminant itself. Drill cutting waste consists predominantly of rock chippings, clays, water and oils, with other additives in smaller quantities. A bioremediation system that could deal with these products was required.

5.4. Methods of Bioremediation

There are numerous approaches to bioremediation; an *in-situ* methodology was discussed in the *Exxon Valdez* case study, **5.2.** There is another approach to *in-situ* biotreatment.

5.4.1. Gel Coating

This involves coating the cuttings after the M10 unit (or equivalent) (see **2.8.1.**) with a polymer gel containing bacteria. Response Environmental Services (RES) have developed a Bio-Gel™ which is a biological carrying medium that could be used to treat the cuttings before disposing onto the sea floor. There are many questions

arising from this product, such as the biological oxygen demand (BOD) on the sea floor - research has shown that the benthic community suffers from a lack of oxygen when there is a substance deposited which has a high BOD (Pappworth, 1997). Elf have also carried out research on gels, but are currently not following that option (Waller after pers. Comm., Elf, 1999). John Bent, Technical Director from RES, has discussed the merits of his company's gels, stating that the gels do not have a detrimental effect on the sea floor or benthic communities. However, any organic material discharged to sea will impact on oxygen (Pappworth, 1997), so more evidence to substantiate that claim is needed, such as field tests on the sea floor at depth. The bacteria used are a consortia produced by a Swiss company, which are purchased on a slope, a common method of buying bacteria. These are then inoculated using a medium containing the contaminant. The gels have proved effective on other contaminants, such as on the railways and oil spill cleanups, but the dynamics of the gels have not been fully investigated. The company does not have the time or resources to undertake detailed research. Hydrogen peroxide is not used in these gels, but would be a consideration as it does provide oxygen to the bacterial environment and is removed rapidly by the bacteria degrading the hydrocarbons (Urfer & Huck, 1997).

As the drill cuttings in the UK cannot be put overboard, the research was limited to an *ex-situ* method of remediation.

5.4.2. Landfarming

Landfarming is a form of solid-phase treatment, involving the controlled incorporation of waste into the upper soil zone and enhancing aerobic microbial activity through the addition of nutrients, lime for pH amendment and moisture (EPA, 1998), plus tilling to increase aeration. The contaminated media is usually treated in lifts up to 18 inches thick (FRTR(a), 2001). When the desired level of treatment is achieved the lift is wholly or partly removed (allowing for the old lift to inoculate the new) and a new one constructed. Liners may have to be incorporated to control leachate.

Landfarming is already popular in America and Canada for onshore disposal and treatment of drill cuttings and other heavy petroleum hydrocarbons. Generally, the higher the molecular weight of hydrocarbon, and the more rings with a PAH, the slower the degradation rate. Chlorinated or nitrated compounds can prove difficult to degrade (FRTR(a), 2001).

England does not have the space to exploit this technology efficiently, except on a small scale.

There has been research concerning radiation and heavy metal levels on land that has been contaminated from commercial oil well drilling activities in the USA (Spitz *et al.*, 1997). Spitz reported a significant rise in radium and other hazardous materials in the vicinity of where sludge ponds and waste pits were. Radium bases salts are known to be deposited as scales on pipe work in production operations. Although this refers to the production wastes rather than the drilling wastes, heavy metals are a serious consideration; they can build up in the soils of a landfarming operation (Kramer *et al.*, 1980; Berge, 1996). Concentrating the drill cuttings in one area for landfarming could lead to a build up of these toxins; this would prevent any other land use, and would eventually become a toxic environment for the micro-organisms and most flora and fauna.

A feasible solution for this would be to conduct phytoremediation. Some plants are particularly adept at removing heavy metals, chlorides and other contaminants from the soil, concentrating them in their upper growth of twigs and leaves. These can then be harvested, and, if incinerated, concentrates the contaminants further in the ash. In soil samples from the rhizosphere of poplar trees there were an increased the number of micro-organisms able to utilise hydrocarbons as food and carbon sources, thereby aiding in the remediation (Jordahl *et al.*, 1997). This may open up a new form of remediating hydrocarbon contaminants; forest remediation.

Table 5.4.2.1. Some Advantages and Disadvantages of Landfarm Bioremediation

ADVANTAGES	DISADVANTAGES
Effective on organic constituents with slow biodegradation rates (Chaineau *et al.*, 1996).	Presence of heavy metal concentrations >2,500 ppm may inhibit microbial growth.
Simple to design and implement.	Requires a large land area; may require lining to reduce leaching.
Treatment times < 2 years (Ladousse *et al.* 1996), sometimes 18 months; quicker than non-catalysed system.	Difficult to achieve reductions >95%; contaminant concentrations less than 0.1 ppm almost unachievable (EPA, 1998).
Costs very low; land rent, moving contaminant to land, occasional tilling, occasional analysis.	Dust and odour may reduce air quality and influence planning permission. Evaporation of volatile components may result in specific air pollution concerns.
Sustainable, long term; may be used for forestry after treatment.	May not be effective where total hydrocarbons exceed 50,000 ppm (EPA, 1998).
Inorganic contaminants may be degraded by further treatment using phytoremediation.	Inorganic contaminants generally not degraded by micro-organisms.
	Conditions affecting biological degradation of contaminants (temp, rainfall) uncontrolled.
	Runoff collection facilities must be constructed and monitored.

5.4.3. Biopiles/Windrows

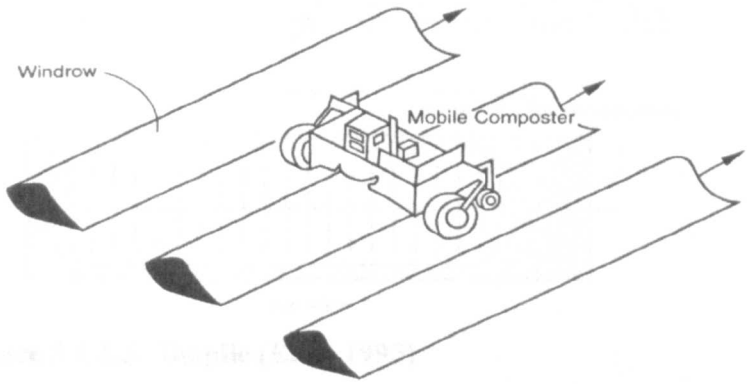


Figure 5.4.3.1. Windrows (EPA, 1993)

Biopiles, or windrows, are a more intensive form of landfarming. Contaminated material such as drill cuttings would be mixed with soil or other bulking material, and treated in aerated heaps or windrows which need turning periodically. These will require a lined base to collect the leachate produced.

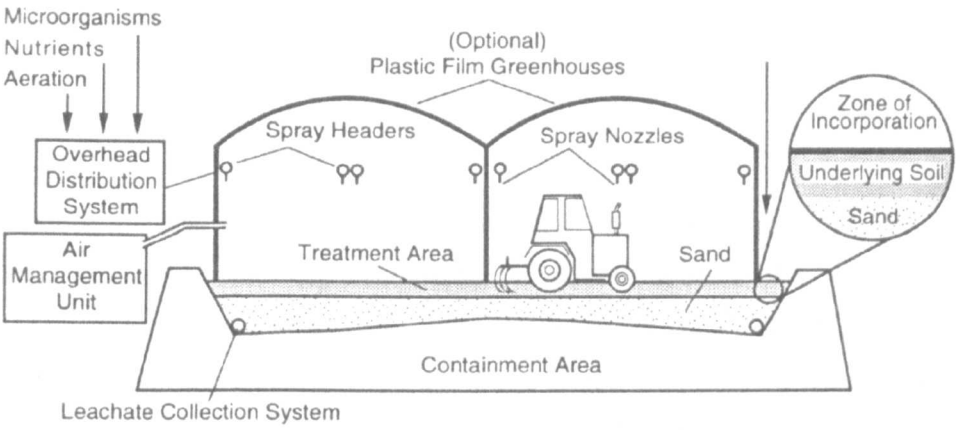


Figure 5.4.3.2. Solid-phase bioremediation system (EPA, 1993)

Organic material is added, such as waste matter created from forestry, manure on straw/shavings, hay or other organic bulking agents. These additions can help stimulate remediation by breaking down themselves, often creating heat, which may help the establishment of thermophilic and other bacteria that utilise the contaminant as a food source.

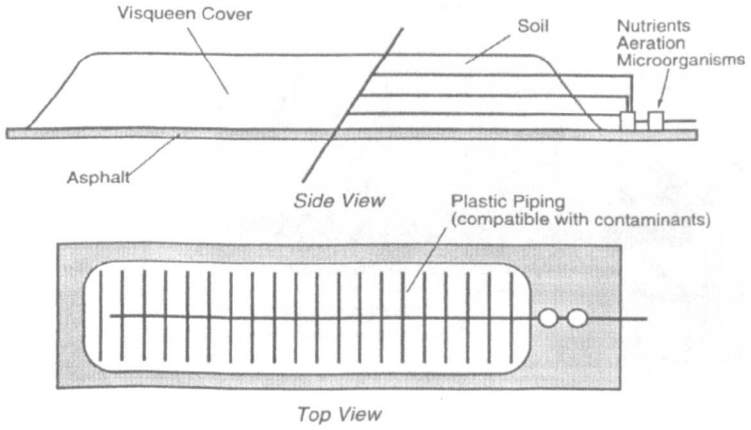


Figure 5.4.3.3. Biopile (EPA, 1993)

If the piles are covered or contained with an impermeable liner the risk of leaching is reduced as well as giving greater control of heat, moisture and other parameters. Aeration of static piles is conducted with blowers or vacuum pumps (FRTR(b), 2001). Treatability testing needs conducting to assess the appropriate oxygenation and

nutrient loading rates. Similar batch sizes require more time to degrade than slurry-phase processes.

5.4.4. Composting

Composting is an ancient and proven method of breaking down many types of waste into a reusable product, e.g. compost for the garden. When dealing with a contaminant such as drill cuttings, this could be a very good technology. The clays within the mud are a concern, as clays are so effective at 'holding' on to organic solutes. Combining the cuttings with some form of organic matter that would compost anyway should help reduce the hydrocarbon content. Ideally, with the new generation muds being developed with low heavy metal content and lower salts content, the end-product might be saleable, or at least a viable product for capping landfill sites. Composting can be conducted using biopiles or windrows, as discussed in **5.4.3.**, or in reactor vessels, see figures **5.4.4.1. – 5.4.4.3.**

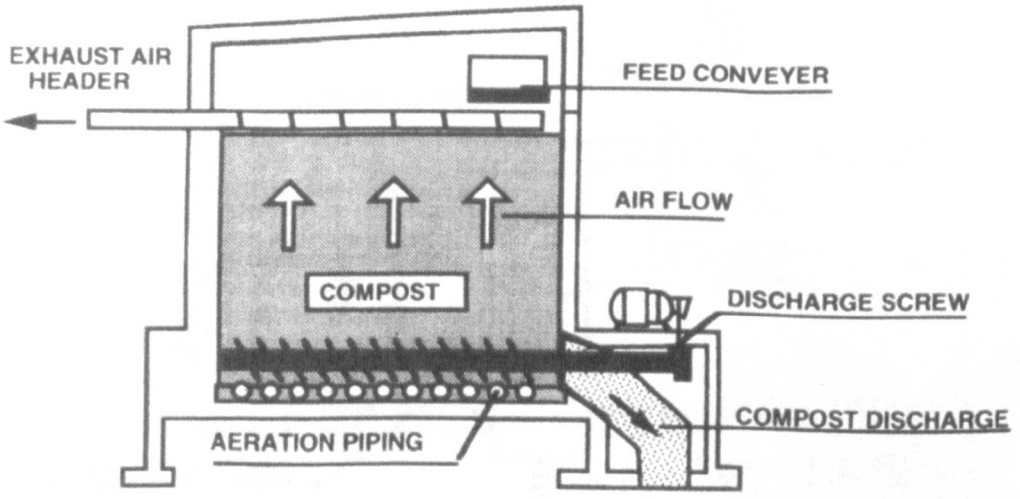


Figure 5.4.4.1. Compost reactor vessel with vertical flow (EPA, 1989)

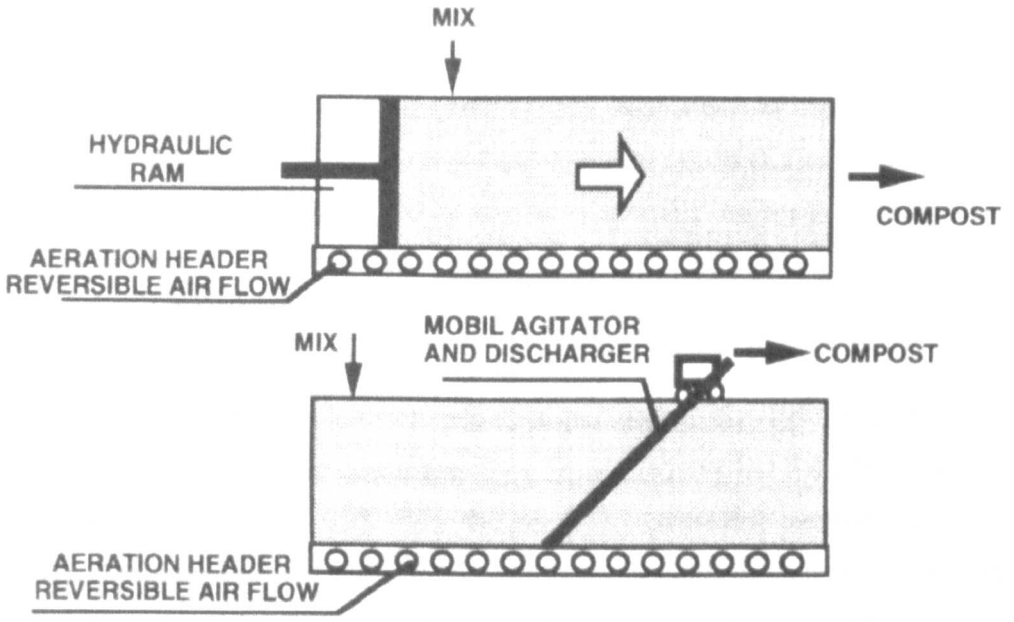


Figure 5.4.4.2. Compost reactor vessel with horizontal flow (EPA, 1989)

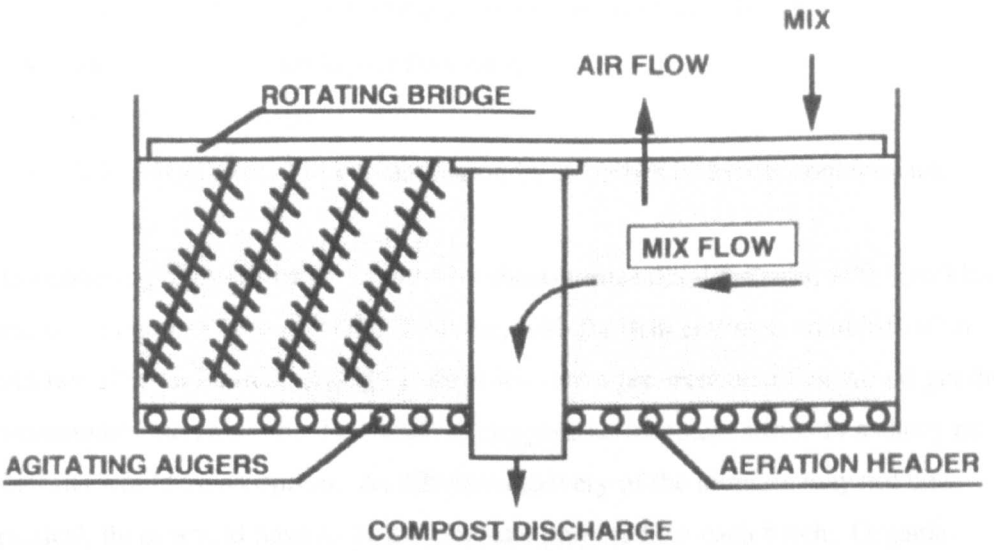


Figure 5.4.4.3. Agitated bed compost reactor vessel (EPA, 1989)

5.4.5. Bioreactors

Bioreactors are enclosed vessels in which various parameters can be controlled. They can be complex, but often achieve rapid and complete results (Karamenev & Samson, 1998). Whether the system should be aerobic or anerobic has been discussed in section **4.3.2.**

5.4.5.1 Immobilisation of Micro-organisms

Immobilisation of bacteria in macro and microparticles (Sofer, 1997, Knaebel *et al.*, 1997) or biofilms has great advantages for a liquid medium, but doesn't allow sufficient intimate contact with oils in the pore space of the drill cuttings (Fukuda 1995). There are many established and innovative methods of immobilisation from polymer beads to fixed-film reactors. Immobilisation has several advantages.

- Maximises the retention time of the biomass (Armenante, 1993),
- Higher productivity per unit bacterial biomass (Sofer, 1997),
- Systems can sustain higher flow rates
- Occupies less space
- It is more resistant to contamination by microbes or excess contaminant.

Atmospheric pollutants can be treated by sheet-immobilised bacteria, with a residence time of 2 minutes (Sofer, 1997). However, with the drill cuttings, immobilisation does not offer an intimate enough contact without a pre-treatment that would get the contaminant into solution. The research suggests an inoculate added to a slurry as free cells would be an option. As efficient recovery of the biomass may not be practical, there would have to be an inoculate prepared for each batch. Organic contaminants are well suited to a free-cell, suspended biomass system (LaGrega *et al.*, 1994).

More recent research conducted in Kuwait has discovered that the production of exopolysaccharides by hydrocarbon degrading bacteria enable the micro-organisms to adhere to a substrata, such as sawdust, Styrofoam or wheat bran (Obuekwe & Al-

Muttawa, 2001). This form of immobilisation has been utilised to store the bacteria for periods of 6 weeks – 6 months and more, even at high temperatures, indicating a potential for application as ready-to-use seeds for *in-situ* petroleum bioremediation. By using the natural ability of the bacterial to attach to surfaces, the use of extraneous chemical substances in immobilisation is avoided, and the cells are protected from environmental chemical toxicity, excessive desiccation in dry, hot conditions and from predation from other organisms. The technology may equally be utilised in cold conditions.

5.4.5.2. Continuous Vs Batch Bioreactors

Continuous flow reactors would be more cost effective to treat a waste stream that is generally continuous. With continuous flow stirred tank reactors, there is no control over whether the particles just entering the tank will exit immediately, while other particles may remain longer than necessary (LaGrega *et al.*, 1994).

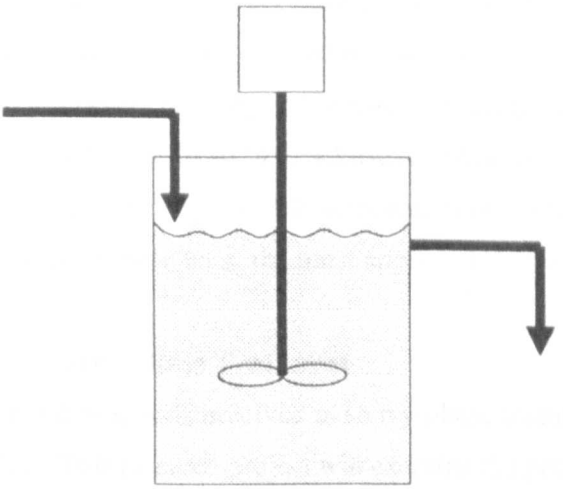


Figure 5.4.5.2.1. Continuous flow stirred tank reactor (basic principles) (Armenante, 1993).

This can lead to effluent concentrations that do not meet the required environmental standards. Plug Flow Reactors are designed to keep mixing of the influent with the entire contents to a minimum. They are often long, narrow tanks; the material moves

across the reactor to the output in a given time, as a 'slug' of material. The rate of feed will dictate the time spent in the reactor.

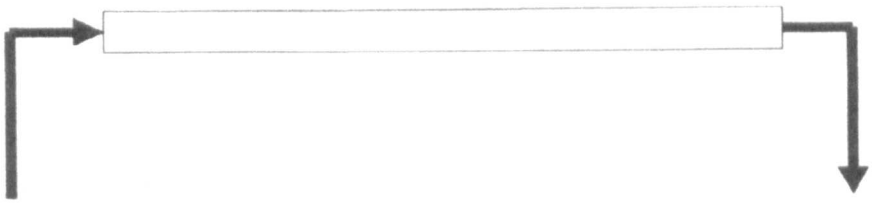


Figure 5.4.5.2.2. Plug flow continuous tank reactor (basic principles) (Armenante, 1993).

Continuous reactors are commonly used for wastewater treatment (Armenante, 1993). Batch reactors are appropriate for intermittent operations, and for biological processing, particularly slurries. Bass, the brewers, have conducted research into continuous processes for their biological reactors (brewing), but have found that the batch process is still more applicable (Harrison, 1999). Batch processes are easier to manage and control, they also reduce the danger of shock loading. The material can be analysed before entering and leaving the reactor, allowing the time allocated for each step of the treatment to be adjusted. This greater flexibility can lead to better performance. Certainly, the lab-scale and pilot studies will utilise batch reactors. However, after these tests, the most appropriate process will be reassessed.

5.4.5.3. Slurry-Phase Treatment

The general processes involved in slurry-phase treatment are illustrated in figure **5.4.5.3.1.** This research project will examine the pre-treatment of the solids before the mixing bioreactors and the bioreactors themselves. The clarifier and filter press are established technologies utilised in a number of industrial applications and are therefore not covered in this research. Recycling of the filtrate would depend on the levels of contamination contained therein – if the liquor proved high in heavy metals, for instance, it should not be recycled back into the reactor as it may be toxic to the bacteria. Advantages of recycling the filtrate include the reintroduction of the bacterial cultures into the contaminated material in the bioreactor, reducing the need

for an inoculate, and a reduction of waste material that may need further treatment before disposal.

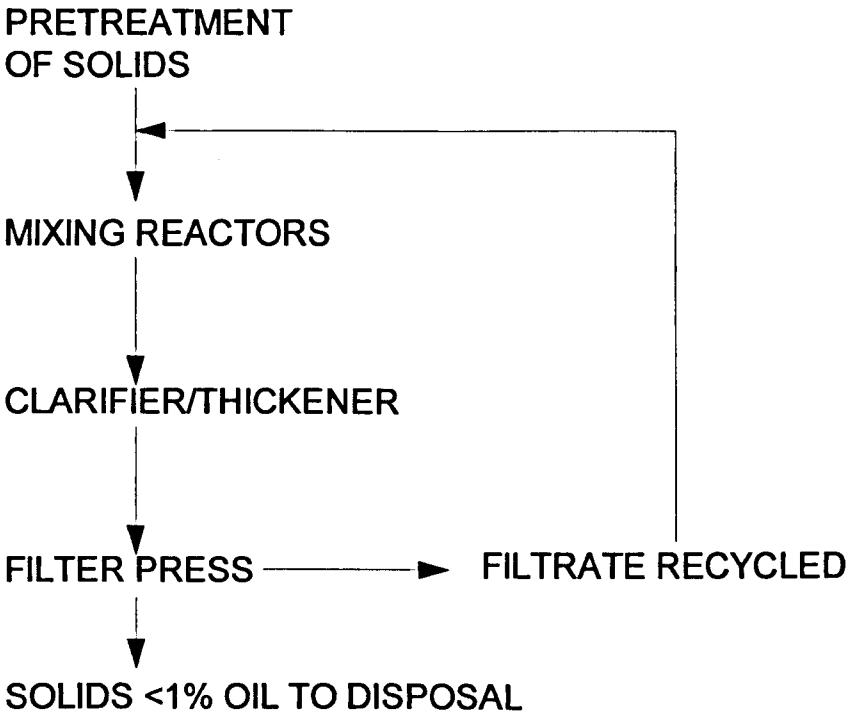


Figure 5.4.5.3.1. Process flow sequence for slurry-phase treatment

Slurry-phase treatment degrades waste at a faster rate and requires less land area than solid-phase treatment (Ross, 1991). This is illustrated by Stroo (1989), reproduced in table **5.4.5.3.1.**, which summarises the population densities of bacterial cells recovered from assorted samples of slurry treated, untreated and land treated material, illustrating the capability of slurry-phase treatment for increasing biological activity.

Table 5.4.5.3.1. Bacterial Cell Densities

Sample Source	**Contaminated Soils** (cells/gram solids)	**Waste Sludge** (cells/gram solids)
Untreated	10^6	10^6
Land Treatment	$10^7 - 10^8$	$10^7 - 10^8$
Slurry-phase Treatment	$10^8 - 10^9$	$10^8 - 10^9$

Slurry-phase bioremediation has several advantages over other methods of bioremediation (Cookson, 1995).

- There is greater and more uniform process control
- Enhanced solubilisation of the organic chemicals
- Physical breaking of particles
- Increased contact between microorganisms and contaminants
- The ability to enhance solubility of contaminants with surfactant applications
- Improved distribution of nutrients, electron acceptors and primary substrates
- Faster biodegradation rates.

There are disadvantages, as described below.

- Additional energy requirements
- Increased material handling
- Possible physical separation and breaking down processes for the large cuttings
- Liquid and solid separation processes
- Increased water supply, handling and treatment costs.

The effectiveness of slurry-phase treatment depends upon a number of theoretical factors including pretreatment, desorption, solids concentration, mixer design and retention time.

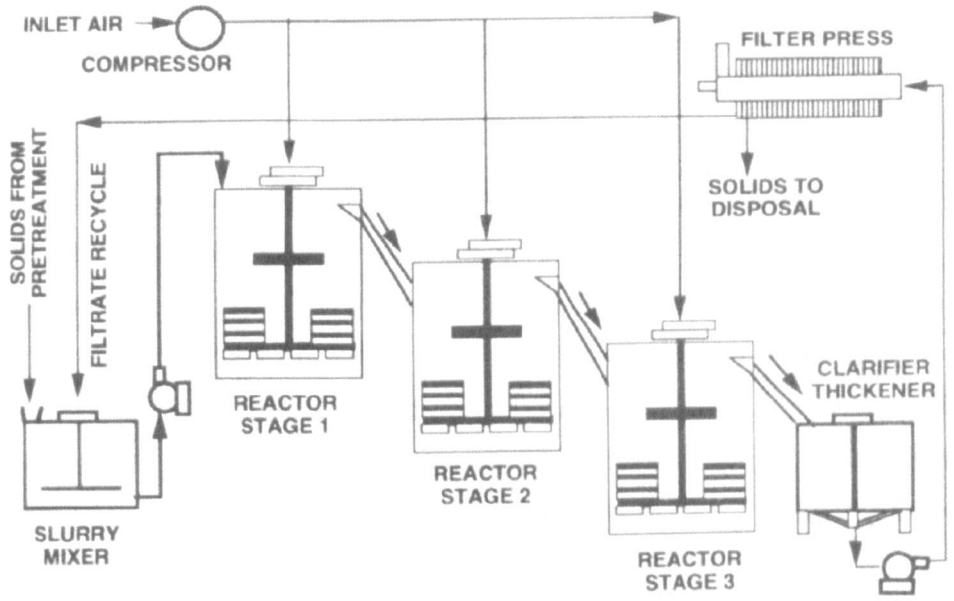


Figure 5.4.5.3.3. A typical process flow sequence for slurry-phase treatment (Cookson, 1995)

There are several options of slurry-phase treatment where the suspended solids are inert, but apposite for drill cuttings may be the inclusion of a pre-treatment to reduce the cuttings thereby increasing surface area. Post-treatment would be needed for dewatering after remediation.

Overall, slurry-phase treatment seems an appropriate technology for the contaminated drill cuttings, especially considering the clays. A personal communication with British Gas (Waller, 1999) confirmed that slurry-phase treatment was the best option for contaminated clays.

5.4.5.3.1. The Process

The wastes are suspended with water or wastewater in a mixed reactor to form a slurry. The agitation has several benefits.

- Homogenises the slurry
- Breakdown of the solid particles
- Desorption of waste from solid particles

- Contact between the organic waste and microorganisms
- Oxygenation of the slurry by aeration volatilisation of contaminants.

5.4.5.3.2. Particle Size Reduction

Material larger than 2.5 - 10 mm have been reported as too large within a slurry-type reactor, and must therefore be screened out or broken down to create an aqueous slurry (Cookson, 1995). The cuttings from the wells vary considerably in size and make-up. A small sample of cuttings from the M-10 unit, using Novatec system mud, Amoco well 30/11b-5, showed that when water was added the cuttings broke down by themselves quite considerably. However, evidence from cuttings piles indicates that cuttings can be over 1 cm (UKOOA, 1999), in which case these may have to be pre-treated. There are several types of mill that can do this such as a ball mill, roll crushers, swing hammer mill (Jackson, 1999), or other attrition mills. An option might be to wet the cuttings and screen to remove the larger particles, which could then be milled. This would reduce the amount of material needing processing. The contaminants generally preferentially adsorb to finer particles (LaGrega *et al.*, 1994), also confirmed in this study (**6.3.2.**). The advantage of breaking down the larger particles would be a reduction in energy required to keep them in suspension. If the contaminant is preferentially adsorbed onto the smaller particles, screening will concentrate the contaminants prior to degradation. In Dutch research projects (La Grega *et al.*, 1994), the mixing technique which reduced the soil particle size to 30 microns resulted in degradation rates several times greater than the technique which yielded a particle size of 60 microns.

5.4.5.3.3. Types of Reactors for Aerobic Slurry-Phase Treatment

There are several examples of slurry-phase bioreactors, as this is a common way to treat contaminated soils *ex-situ*. Reactors include Lagoons, open vessels and closed systems (Cookson, 1995). The post reactor system would need to include dewatering systems, as shown in figure **5.4.3.1.**; these are established technologies that can be adapted for the drill cuttings. Slurry reactor designs differ in the mechanics of oxygenation and mixing of the suspension. Draft tube reactors use a floating aerator, but can result in poor mixing near the reactor bottom, making them unsuitable for drill

cuttings that can fall out of suspension easily; also the air spargers were reported to have "significant clogging problems" (Cookson, 1995, p 339). Blade agitated reactors (Pinelli *et al.*, 1997), such as the turbine mixers with spargers as illustrated in figure **5.4.5.3.3.1.** (Cookson, 1995), would be appropriate to maintain suspension of the solids near the tank's bottom. There are air-lift reactors that use air to lift and stir the slurry, with rotating rakes under the air diffusers to maintain solids mixing. The slurry decontamination process touched upon by Kleijintjens *et al.*, (1995) consists of a series of compartments where the unseparated sediment is treated in a continuous process. This method has been tested in a pilot plant at 3 m^3 scale, with Petroleum Harbour Contaminated Dredged Material (Stefess, 1998). However, the level of remediation reported was below target, with the material still disposed of in a landfill. It does suggest that this method of remediation may be practical, but difficult to realise on a laboratory scale.

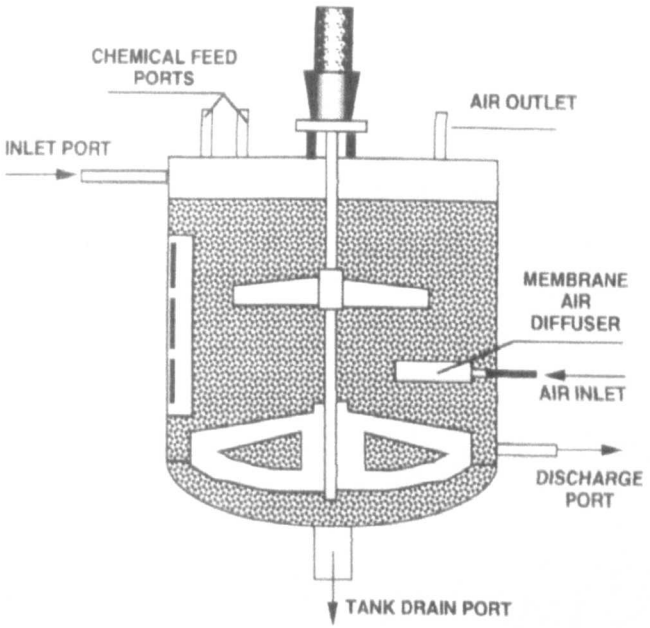


Figure 5.4.5.3.3.1. Turbine popeller slurry-phase bioreactor (Brox, 1993 in Cookson, 1995)

Trommel or rotating drum bioreactors are also commonly used for soil slurries (Cookson, 1995; Banerjee *et al.*, 1995; Kruger *et al.*, 1995; Pinelli *et al.*, 1997). The

advantages include ease of design and fabrication, and ability to handle high solids levels. Air sparging can be a problem, as can clumping of material in the reactor. The reactor design for this research is discussed in greater detail in chapter 7.

5.5. Summary

Bioremediation is a relatively young science, but has already proved itself as an efficient and cost effective technology for the clean-up of some xenobiotics. Difficulties can arise concerning optimising the environment for the micro-organisms, which also has to be suitable for the matrix binding the contaminant. There are various methods of conducting bioremediation both *in-situ* and *ex-situ*, such as composting which includes the addition of organic matter to the contaminated material and slurry-phase, which includes the addition of liquids.

CHAPTER 6
METHODS DEVELOPMENT AND RESULTS

6.1. Introduction

At the onset of this research in 1998 a succinct quantity of literature was available that dealt directly with drill cuttings. As previously discussed in sections **2.3.** and **3.3.**, muds contain a quantity of clays, specifically bentonite clay. The clay within the cuttings added a complicated dimension to the research due to their physical and chemical properties, as discussed in section **3.3.1.**. At an early stage it was recognised that a reliable method of analysis capable of resolving residual oil concentrations below 1% was required. An efficient, reliable and repeatable method of extracting the oils from the cutting and mud matrix needed development, within the constraints of the equipment available at the university. The extracted hydrocarbons from the mud and cuttings then needed analysing as accurately as possible. As a slurry system was proposed, the particle size distribution within the cuttings needed to be analysed; the concerns over particle sizing were outlined in section **5.4.5.3.2.**. It was thought that there may have been some preferential adsorption of the contaminant on a given size fraction; testing this might lead to a process reducing the quantity of cutting waste needing treatment.

Once these parameters had been explored by experimentation, the microbiology of the cuttings was assessed. If there were indigenous species within the cuttings that could be grown in a laboratory environment they could be isolated to assess their potential as remediators. These experiments did produce some isolates, which were batch tested for remediation potential within the natural flora of the cuttings, and in isolation. The knowledge acquired earlier in the research aided with optimising the environment for the bacteria (see chapters **4** and **5**), within the constraint of being able to realise the results in a practical application. The most promising of these isolates were used as inoculates in the bioreactors.

6.2. Development and Calibration of Analytical Techniques

A commonly used method of analysing organic carbons is by gas chromatography (GC). This method has been adopted by other researchers conducting bioremediation of hydrocarbon contaminated soils and of drill cuttings. Examples include Angehrn *et al.* (1998), Mulkins-Phillips and Stewart (May and Dec 1974), Chaineau *et al.* (1996) and Yeung *et al.*, (1997) all using flame ionisation detectors (FID). As the GC was an accepted and well-documented method of analysis, this was the technique researched and eventually adopted.

6.2.1. Gas Chromatography

The GC analysis was conducted on a Carlo Erba, type HRGC 5300 Mega Series Gas Chromatograph. The capillary column was J & W, 30 m by 0.541 mm, with a film thickness of 0.5 μm. The liquid phase was DB-5. DB-5 consists of 95% methylpolysiloxane and 5% phenylmethylpolysiloxane. It is aromatic in character, and can be used for diverse types of hydrocarbons (pers comm., M Cooper, 2000). Detection was by flame ionisation.

6.2.1.1. The Components of a Gas Chromatograph

The GC of the type used basically consists of the following components.

- An inlet, where the solute is injected into the apparatus.
- A surface coated capillary column, known as the stationary phase.
- A detector, which analyses the injected material as it elutes.
- A recording and data processing system.
- The column is heated in a temperature controlled oven.
- Gases are used in the system; helium as the mobile phase or carrier gas and hydrogen as the combustion gas (with air).
- The column is connected between the injection port and the detector.

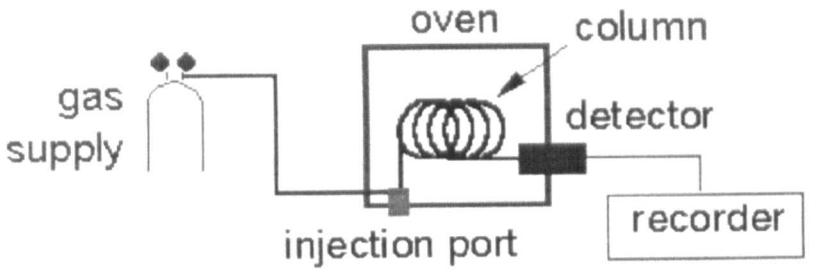


Figure 6.2.1.1. Schematic of a Gas Chromatograph

6.2.1.2. The Process

The sample (0.5 μl) was injected using a syringe via the injection port, which is a rubber septum, where it contacted a heated block at 250°C, a higher temperature than the boiling point of the least volatile component in the sample mixture. A stream of helium gas (the mobile phase) flowing at 25 ml/minute then carried it into the coiled column, coated with the stationary phase, which was confined to that column. The oven heated up; the partitioning behaviour is very temperature dependent. In the case of the base oils, it was set to start at 40°C for 2 minutes, then increased by 10°C per minute until it reached a temperature of 200°C, where it stayed for 7 minutes. The whole programme lasted for 25 minutes. The design of the programme was aided and optimised by the GC technician, K Wallgren, at the university.

The temperature of the column is critical to the analyisis (Jennings *et al.*, 1987); too low, and the material remains largely in the stationary phase, not separating or not even eluting in extreme cases. Too high, and the solutes spend most or all of their time in the mobile phase, rarely or never entering the stationary phase and eluting from the column as an unseparated mixture. A wide working temperature range was required to elute both low and high boiling components (Sigma, 2000).

The components of the mixture moved through the column at different rates, having different retention times (Atkins & Carey, 1997). The column was continuously swept with the mobile phase, carrying the solute molecules in the gas phase to the detector, an FID set at 250°C. Each solute within the mixture engaged in a highly dynamic equilibrated partitioning between the stationary phase and the mobile phase, according to its distribution constant (Jennings *et al.*, 1987).

$$Kc = Cs / Cm$$

Cs = concentration in the stationary phase
Cm= concentration in the mobile phase
Kc = distribution constant

Molecules exhibiting higher vapour pressures or boiling points partition more towards the mobile phase and are swept towards the detector more rapidly and are therefore the first solutes to elute from the column. Lower vapour pressure molecules or boiling points venture less frequently into the mobile phase, meaning the concentrations in the mobile phase are lower and require more time to reach the detector. This gives the peak separations. The detector feeds a signal to the recorder, which indicates when a substance different from the pure carrier gas leaves the column.

6.2.1.3. Quantitative Evaluation of the Chromatograms

The peaks generated during the elution are rarely proportional to the amounts of the separated solutes (Schomburg, 1990); in practice, peak-height evaluation is not usually applied. The peak height is dependent on retention, whereas the peak area is independent of retention. The total amount of an eluted solute can be determined via the peak area. The shape of the peak has no significant influence on the accuracy of the analysis providing that peak overlapping due to incomplete resolution or extremely strong leading or tailing of the peaks does not occur.

The syringe used for injecting the sample into the GC is highly accurate, but as it is not always possible to inject exactly the same size sample, time after time, the internal standard was used. This was acetophenone, as discussed in section **6.2.5.**

The FID is a recommended detector because of its high sensitivity and consistent response to most organic compounds (Sigma, 2000).

6.2.1.4. Method of Preparation of the Sample for the GC

Once the hydrocarbon had been extracted (see following sections), the solution was evaporated down to the original volume of drill cuttings added to the soxhlet. Samples of ½ ml were then measured using a 1 ml pipette into a GC vial, together with 5 µl of acetophenone, a quantity that was commensurate with the peaks produced from the sample of drilling oil, measured using a micropipette (see section **6.2.5.**). The vial is rapidly sealed to prevent further evaporation.

To inject into the GC, the syringe is filled from the vial several times and the solution discarded. The syringe is filled again, and then the plunger is gently moved up and down whilst still in the liquor to ensure the syringe is free from air. The syringe is then filled to exactly 0.5 µl, and injected into the GC.

6.2.2. Soxhlet Extraction Method

There was little agreement amongst the literature concerning the method of extracting the oil from the cuttings matrix for analysis; the semi-solid material cannot be introduced into a GC due to the nature of the apparatus (see **6.2.1.**). The oil needed to be extracted into a suitable solvent, which could then be injected and run through the GC. Investigation was conducted into methods of extraction and experimentation was conducted regarding an appropriate solvent.

Extraction methods recommended by the EPA (methods 8020 and 8240) (API, 1996) used methanol to dissolve the volatile organic constituents (VOC's). The drilling oils are not suitable for this method, as they are not VOC fractions, and the university has

no vapour trap injection systems available. Angehern *et al.*, 1998, used soxhlet extraction, which, in that research, was determined to be the most efficient extraction method when compared with cold liquid/sonication extraction and supercritical fluid extraction. The best performing solvent used in this report was tetrachloromethane. Chaineau *et al.*, 1996, also used the soxhlet for extraction. A soxhlet apparatus involves a type of reflux (see figures **6.2.2.1.** and **6.2.2.2.**).

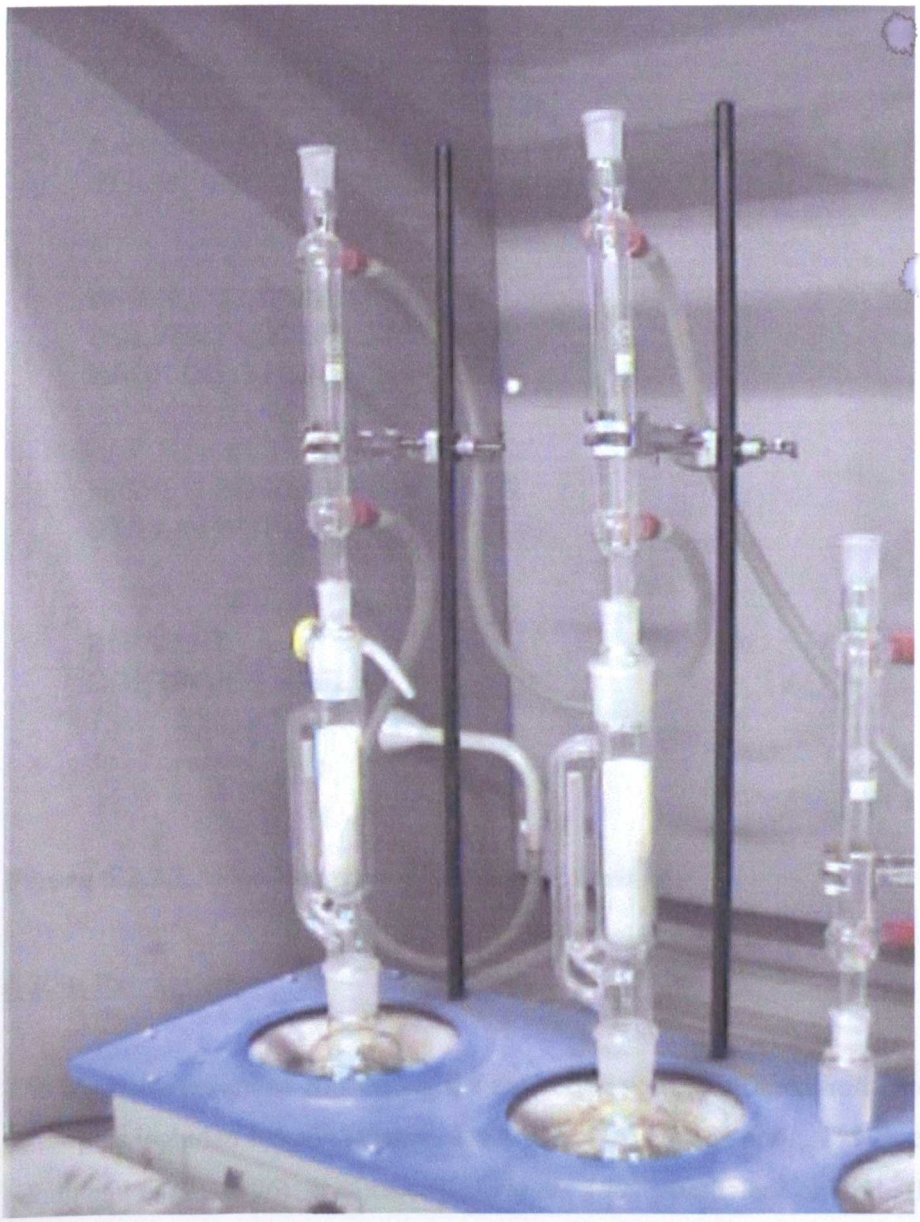


Figure 6.2.2.1. Soxhlet apparatus

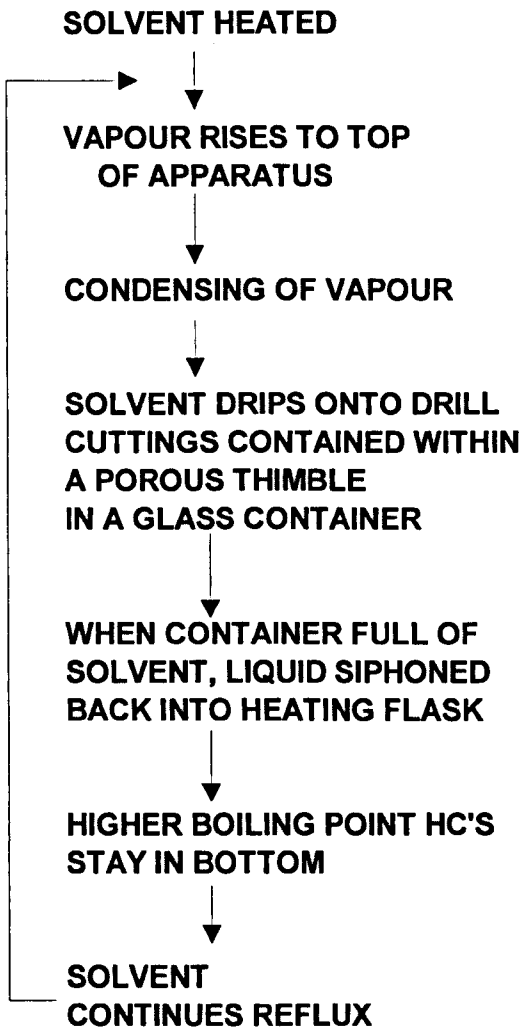


Figure 6.2.2.2. Flow diagram of soxhlet process

6.2.3. Extractions

The literature reviewed concerning the extraction solvent differed immensely. Examples included hexane (Plumb, 1984; Rojas-Avelizapa *et al.*, 1999), chloroform (Chaineau *et al.*, 1996), acetone (Pinelli *et al.*, 1997) methylene chloride (Banerjee *et al.* 1994), hexane/acetone mix (Jackson & Pardue, 1998), dichloromethane (Yeung, *et al.*, 1997) to name but a few; there appeared to be as many different solvents or

combinations as there were reports. It therefore seemed appropriate to conduct experimentation concerning the extraction solvent for the hydrocarbons contained within the drilling muds.

When carrying out these experiments the drill cuttings had not arrived, so some artificial cuttings were made up using the LAO and paraffin oils from MIDF.

Four solvents that were readily available and frequently used in analytical chemistry relating to hydrocarbons were tested. They were:

- Toluene
- Dichloromethane
- *n*-Hexane
- Acetone

As the cuttings were made up in the laboratory, the exact quantity of oil and water was known before extraction. The limestone cuttings were mixed to give a size range:-

- 80% 1 – 0.316 mm
- 10% 2.36 – 1 mm
- 10% <0.316 mm

These ratios were calculated from the small sample of drill cuttings the university had previously received. However, with hindsight, these cuttings would not have behaved in the same manner as clay/shale cuttings.

The cuttings were dried at 80°C for 24 hours.

The final volumes, in percentages, after the addition of water and oil were:

- 10.8 % oil
- 16.2% water

- 73% limestone chippings

Note that no emulsifiers were used with the mixture.
50 ml of mixture weighed 75 g, so 15 g of mixture was used for each of the experiments (10 ml), with 50 ml of the respective solvents.

The test involved the four solvents, each run through a soxhlet extraction apparatus for two hours respectively, with each test repeated. The resulting liquid was then evaporated down to 5 ml.

6.2.3.1. Results from Extractions of Four Solvents

Table 6.2.3.1. Soxhlet Extraction Using Different Solvents.

SOLVENT	OBSERVATIONS
TOLUENE	Colour change – brown tinge, evidence of water. Evaporation very long process.
DICHLOROMETHANE	Colour change. No separation. Fairly rapid evaporation.
N-HEXANE	Globules on bottom of flask after extraction. Sticky in texture, and hard to remove. However, oil and solvent are miscible when mixed together in a test tube. No colour change, rapid evaporation.
ACETONE	Oil comes out of the solvent as it evaporates, hard to transfer. Even after shaking, the oil and solvent separates in GC bottles and is therefore unreliable to test.

Dichloromethane was the solvent chosen for further experimentation for the following reasons.

- It was more efficient at extracting the oil from the sample (see graph **6.2.3.2.**).
- The oil stayed in the solvent after evaporation.
- Evaporation was done at a reasonable rate.
- With correct precautions (see safety data in appendix B), it is fairly safe to handle.

- The polarity of the solvent is the same as the hydrocarbons being extracted.

Dichloromethane proved the most efficient solvent when results were normalised using the internal standard, acetophenone (see graph **6.2.3.1.** and section **6.2.5.**).

The graph uses the different data after calculations are carried out using the internal standard in each sample (see section **6.2.5.**)

This experiment was carried out before the calibration, so the resulting areas are read as a comparison of solvents rather than exact percentages.

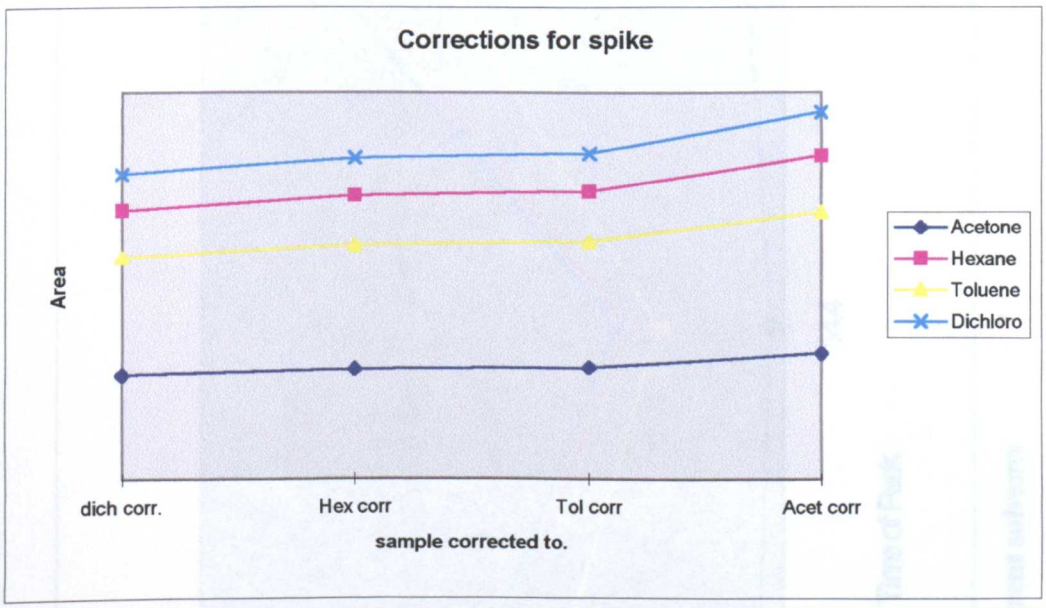


Figure 6.2.3.1. Soxhlet extraction using four different solvents – the figures after acetophenone internal standard.

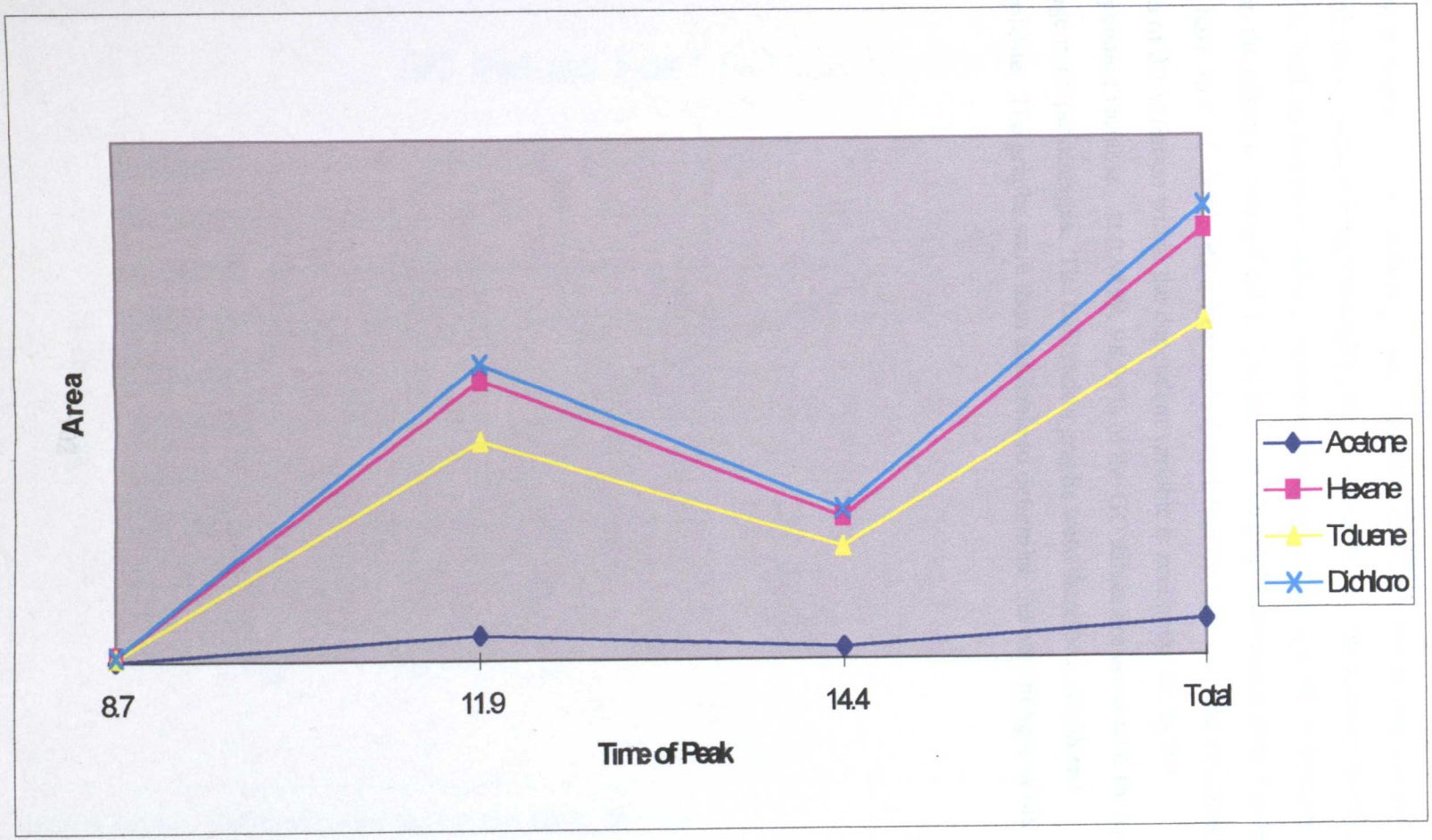


Figure 6.2.3.2. Soxhlet extractions using four different solvents

6.2.4. Calibration of the GC for the Paraffin and the LAO

The data from the GC is in the form of a relative peak area (see figures **6.2.7.1. & 2** for examples of GC traces) and needed calibration to enable the determination of future percentage results. To assess the exact oil measurement, the GC was calibrated using exact concentrations of the LAO and linear paraffin oils in dichloromethane. This was conducted by measuring exact quantities of oil into the solvent, giving a specific percentage, mixing thoroughly, then running the mixture through the GC. The GC had, by this time, had a programme designed for the specific hydrocarbons within the oils (see section **6.2.1.**). The lines on the graphs produced from the data both have an r^2 of ~0.99. R2 is Pearson's Correlation Co-efficient, and indicates how much of the variation within the dependent variable is accounted for by the independent variable. At 0.9899, 98.99% of the GC values are accounted for by the change in oil percentages. The calibration graphs were therefore considered acceptable. The graphs were then available to determine the percentages of oil.

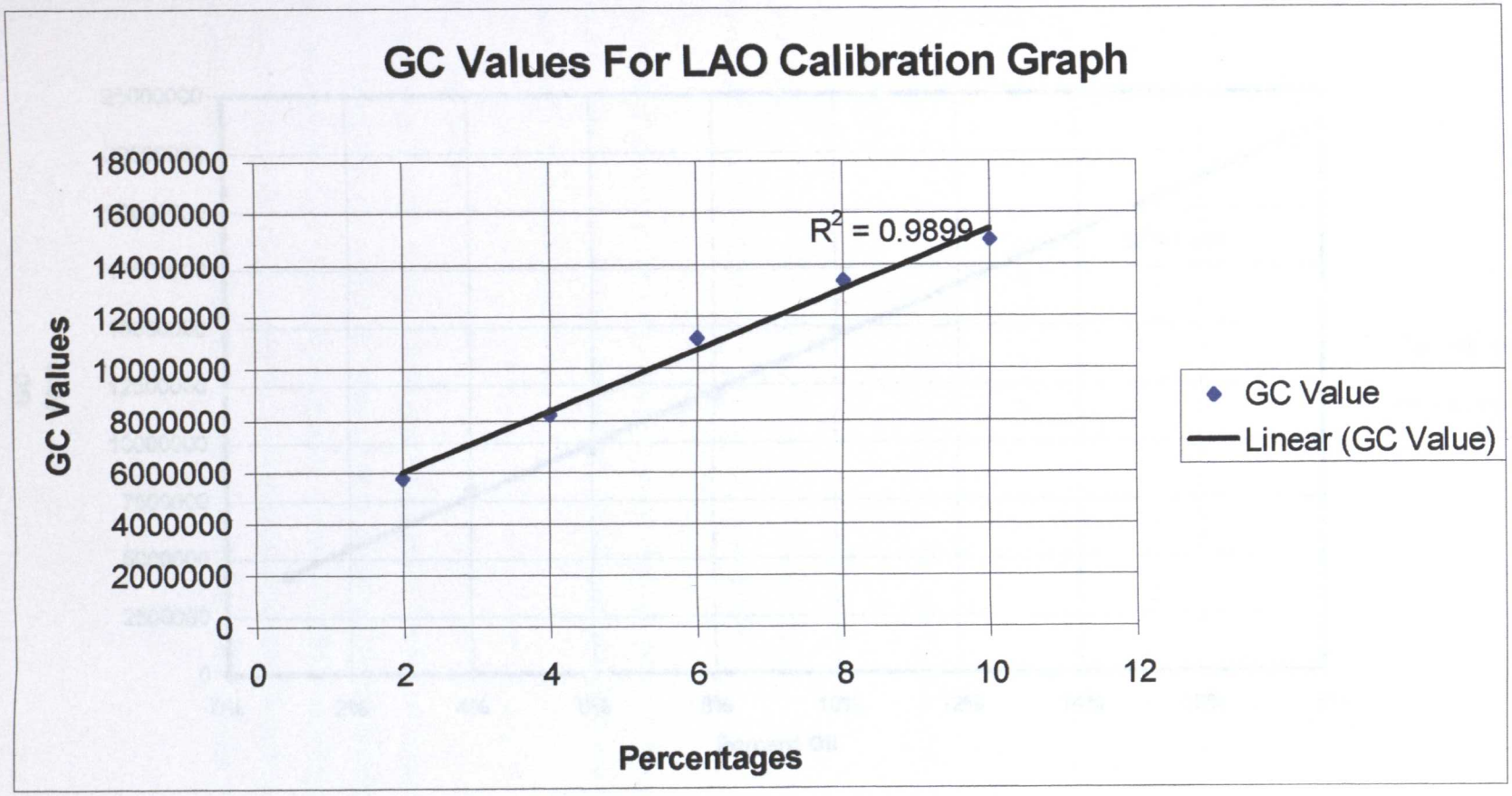


Figure 6.2.4.1. Calibration for the Linear Alpha Olephin

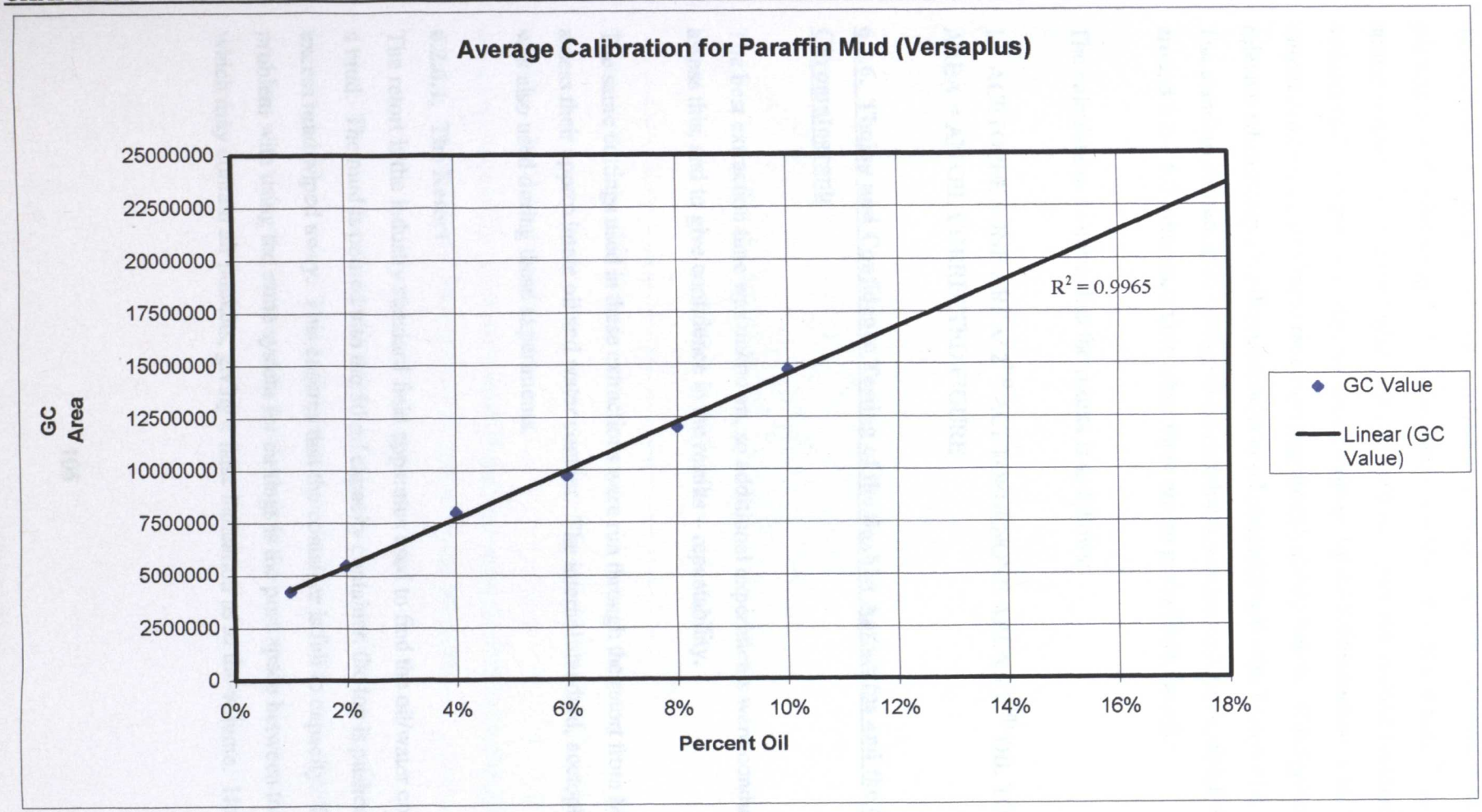


Figure 6.2.4.2. Calibration for the Linear Paraffin

6.2.5. The Use of an Internal Standard for Analysis

To normalise the results from GC analysis an internal standard of known volume (5 µl) was added to the sample. This was used to correct for drift and occasional spurious results, which are features of GC analysis. Since the internal standard volume is fixed for all samples, the area produced by the acetophenone in the GC output can be used by simple ratioing using the calculation below. This approach was relevant when comparing the reductions in oil concentration after bioremediation. The standard chosen, after testing several products, was acetophenone, which elutes at around 6.6 – 6.7 minutes, a time when there are no peaks from the oil.

The calculations to normalise the results is as follows:

1^{ST} ACETOPHENONE AREA/ 2^{ND} ACETOPHENONE AREA x 2^{ND} OIL TOTAL AREA = AN OIL CORRECTED FIGURE

6.2.6. Timing and Confidence Testing of the Soxhlet Apparatus and the Gas Chromatograph

The best extraction time was unknown, so additional experiments were conducted to assess this, and to give confidence in the results – repeatability.

The same cuttings used in these extractions were run through the retort from MIDF, to assess their approximate oil and water content. The internal standard, acetophenone, was also used during these experiments.

6.2.6.1. The Retort

The retort is the industry standard field apparatus used to find the oil/water content of a mud. The mud is poured into the 50 ml capacity container, the top is pushed on and excess mud wiped away. This ensures that the container is full to capacity. The problem with using the same system for cuttings is the pore space between the solids, which may contain air pockets, giving a false indication as to the volume. However,

the cuttings were packed in as tight as possible, and both test samples weighed in at 80.4 g.

The results from the retort were 17% and 18% oil, averaging 17.5%.

Figure 6.2.6.1.1. The Retort

6.2.6.2. The Timed Extractions

An identical quantity of the made-up drill cuttings using the linear paraffin oil were put in a soxhlet thimble and refluxed for half, one and two hours.

The results from the timed extractions, when read off the calibration graph, were between 16.5% (one hour), 17% (half hour) and 17.75% (two hours). This indicates that there may be other factors affecting these results, but that two hours gave results that were closest to the retort.

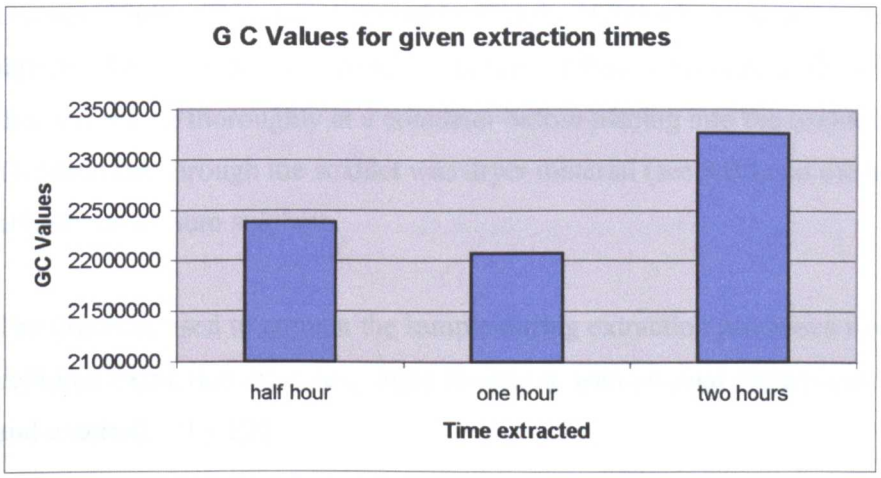


Figure 6.2.6.2.1. Extraction times using Versaplus oil.

These experiments were conducted on the made-up drill cuttings; the times did in fact change considerably once the true 'mud on cuttings' arrived at the university. The two-hour extraction was not long enough; the hydrocarbons were locked into the clay within the mud and cuttings. The reflux time, after further experimentation, was as follows:

- Run Soxhlet for 6 hours
- Leave to stand overnight
- Run for another 4 hours

This extracted all the hydrocarbons within the sample. When the sample was refluxed again, using fresh solvent, no more hydrocarbons were extracted. With hindsight, there could possibly have been some hydrocarbons that were inextricable. Using the internal standard as an addition before extraction may have given a clearer indication as to the efficiency of the extraction method.

The other parameter that caused problems with the extractions was the water within the cuttings; this became more problematic with the slurry, which had increased water content. A typical chemical traditionally used to absorb the water without affecting

the extraction of the hydrocarbon was anhydrous sodium sulphate (pers comm., Cooper, 2000). This was also used by Plumb (1984) when extracting non-aqueous liquids. The quantity of anhydrous sodium sulphate was equal to that of the sample; this was mixed thoroughly in a container before placing into the soxhlet thimble. The final run through the soxhlet was dryer material (see **8.5.**) and did not need the anhydrous sodium sulphate.

The thimbles used to contain the sample during extraction processes were Whatman cellulose extraction thimbles, single thickness, with internal dimensions 28 x 120 mm and external, 30 x 120.

6.2.7. Carbon Chain Length Compostion of the Versaplus Linear Paraffin

The Versaplus mud reportedly contained carbon chain lengths between C12 – C14. To assess the exact carbon chain length in the oil, a sample of pure paraffins containing C10, C12, C14 and C16 was run through the GC. This experiment was conducted so the relevant information on the general structure of the oil was available, plus to illustrate if any chain length is being preferentially degraded by the bacteria.

The two GC traces illustrate well the carbon chain lengths used in the linear paraffin oil; see figures **6.2.7.1.** and **6.2.7.2.** The carbon chain lengths were predominantly C12, C13, C14.

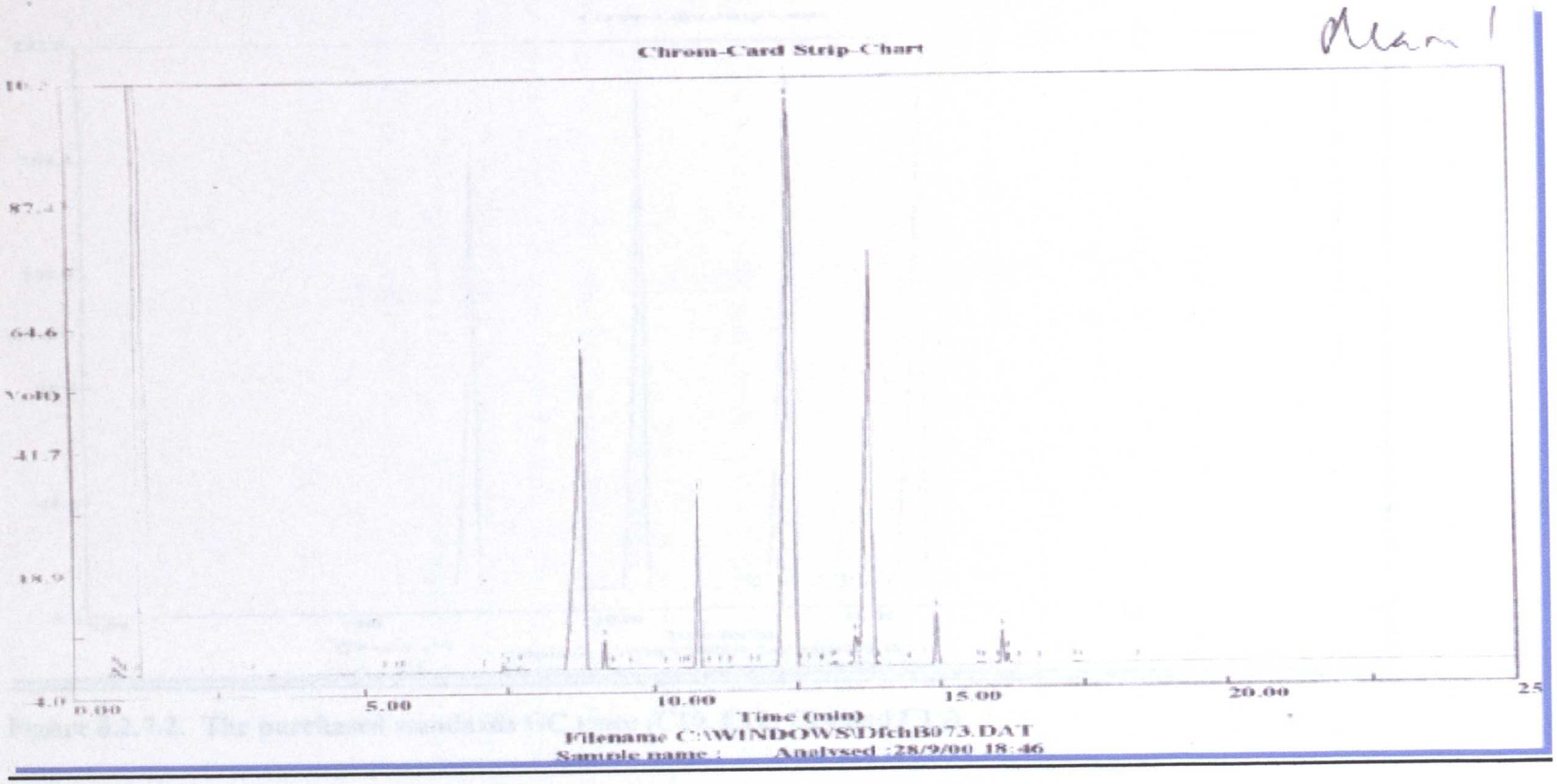


Figure 6.2.7.1. GC Trace of the muds, with acetophenone and LB broth.

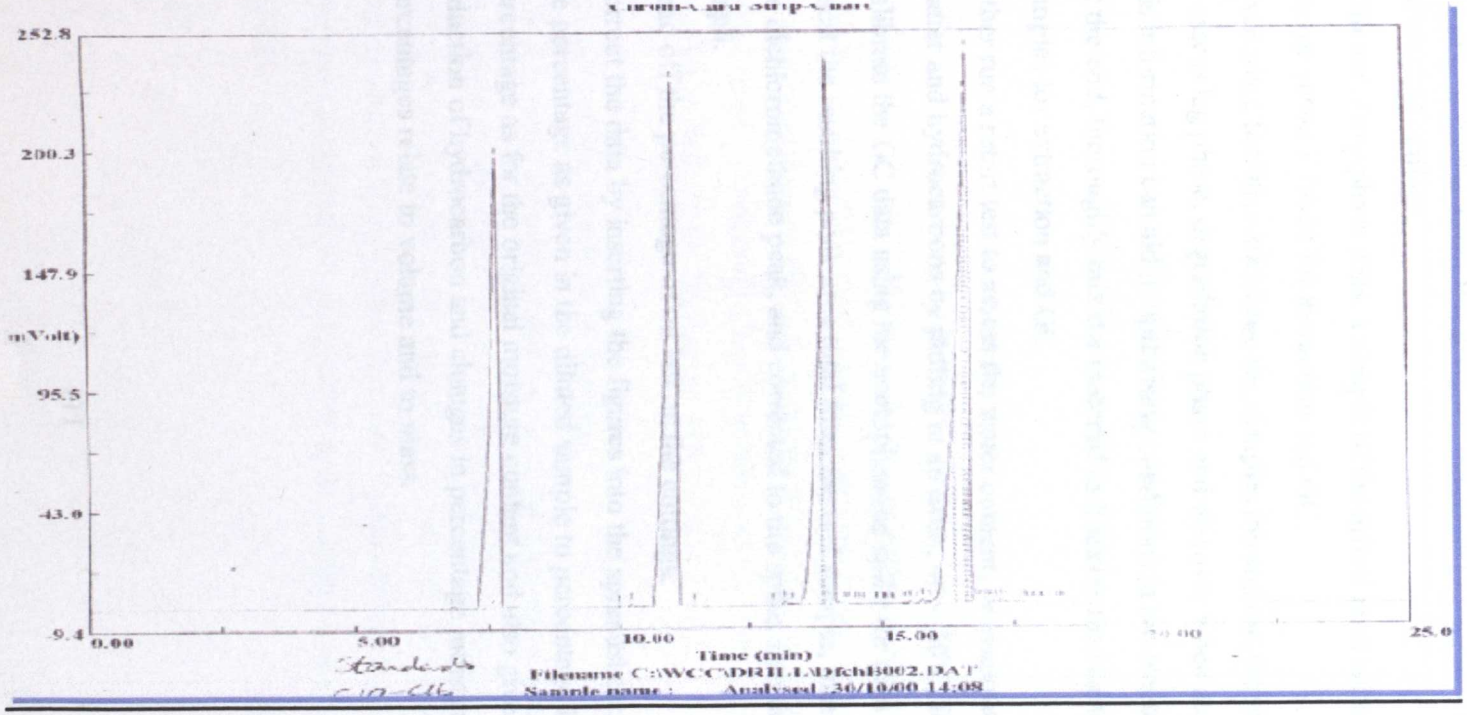


Figure 6.2.7.2. The purchased standards GC trace (C10, C12, C14 and C16)

6.2.8. Final Analysis Process as Chosen for the Research

The analysis techniques were refined throughout the research; reflection on errors and non-quantifiable results led to an analysis procedure that improved with experience. Using the information as discussed above, the final analysis process adopted is given below.

- Retort test on original material before dilution (in slurry-phase experiments and pre-screening tests); this gives an indication of the water and oil content of the original cuttings.
- With the slurry-phase tests, a sample of the mixed material as it goes into the reactor vessel is taken for extraction and GC.
- If sampling facilities are adequate, samples throughout the remediation period to assess lag phase, degradation phase and discover when activity lessens – this information can aid in optimising conditions in the reactor.
- At the end, thoroughly mix the material as it leaves the reactor, take more samples for extraction and GC.
- Either run a retort test to assess the water content, or evaporate water from dry matter and hydrocarbons by putting in an oven, 40 – 50°C, for 48 hours.
- Calibrate the GC data using the acetophenone spike for data correction.
- Insert the resulting peak area total from the end sample, minus the spike and the dichloromethane peak, and corrected to the spike, into the calibration graph.
- Read off the percentage of oil left on the cuttings.
- Correct the data by inserting the figures into the spreadsheet, which will alter the percentage as given in the diluted sample to percentage left on dry matter, percentage as for the original moisture content and also give percentage reduction of hydrocarbon and changes in percentage moisture. These percentages relate to volume and to mass.

6.3. Size Analysis and Oil on the Size Fractions

As discussed in section **5.4.5.3.2.**, particles larger than 2.5 mm are difficult to process in a slurry-phase system. Some of the rock fragments within the cuttings were larger than this; the project needed to define whether these larger cuttings needed some form of pre-treatment. Reports indicated that contaminants preferentially adsorb onto the finer particles (LaGrega, *et al.*, 1994); with clays being the finer fraction, this was almost guaranteed; the ability of clays to adsorb the oil are well documented and outlined in this report in section **3.3.1.**. There was a possibility that the larger cuttings contained relatively small amounts of oil, which could lead to a reduction of the quantity of material needing remediation. This hypothesis was tested.

6.3.1. Particle Size Distribution of a Sample of Drill Cuttings

The Versaplus cuttings were separated into size fractions using both dry sieving, via a stack system and by wet sieving, using a cascade of sieves (British Standards Institute, 1989).

Sieving is an established method of assessing size distribution within the mineral processing industry. The sieve sizes are British Standard (British Standards Institute, 1989). Both wet and dry methods of sieving were used to see if the washing process of wet sieving altered the size distribution within the cuttings; a previous sample of cuttings broke down considerably with the addition of water and agitation. The dry sieving samples were dried for 24 hours at 50°C.

6.3.1.1. Results of Size Analysis by Sieving

After dry sieving some tenacious clays remained on the surface of the cuttings; this was left as it came off the shaker/sieve to mimic a genuine process. The wet sieving left oil residue on all the equipment, especially the vacuum filter. This will have affected the results, but also suggests that washing the cuttings (particularly with warm water) may remove some of the oil from the cuttings as they come from the drilling system.

As can be seen from the graph (figure **6.3.1.1.**), washing influences the particle size distribution of the cuttings considerably. This could be due to the rock being in an oil-wetted condition, protected by the OBM when it comes to surface. When the cuttings are washed, some of the invert emulsion that protected them from hydration would have been removed, which may have destabilised the cuttings leading to hydration, causing the rock-chippings to break-up. Another possibility might have been mechanical attrition.

These results cannot be extrapolated to all cuttings. Differing geologies, muds and mud removal processes could have a massive impact on particle size and the effects of washing. Each drill hole and mud system would have to be judged individually.

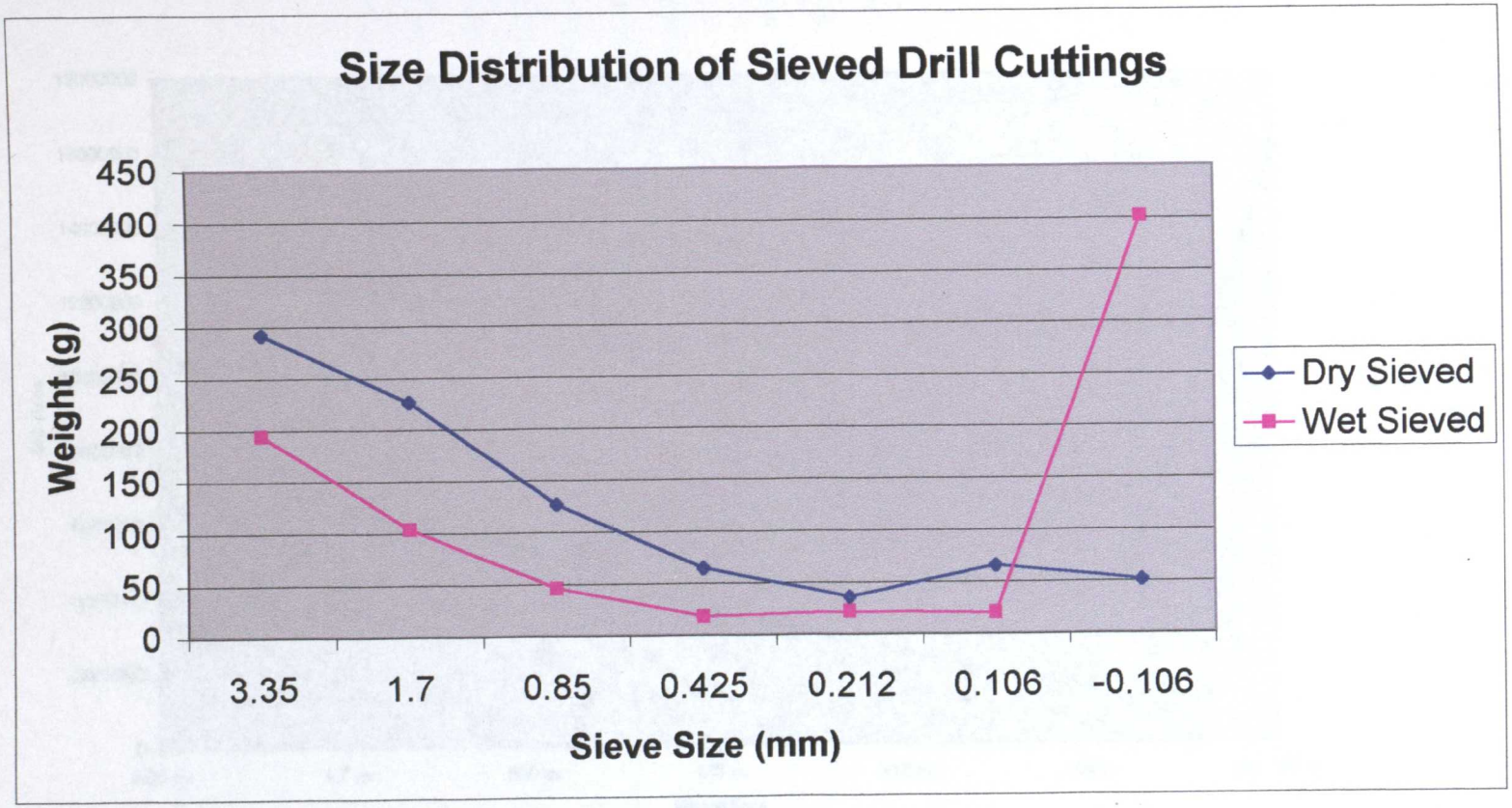


Figure 6.3.1.1. Particle size distribution using BS sieve sizes

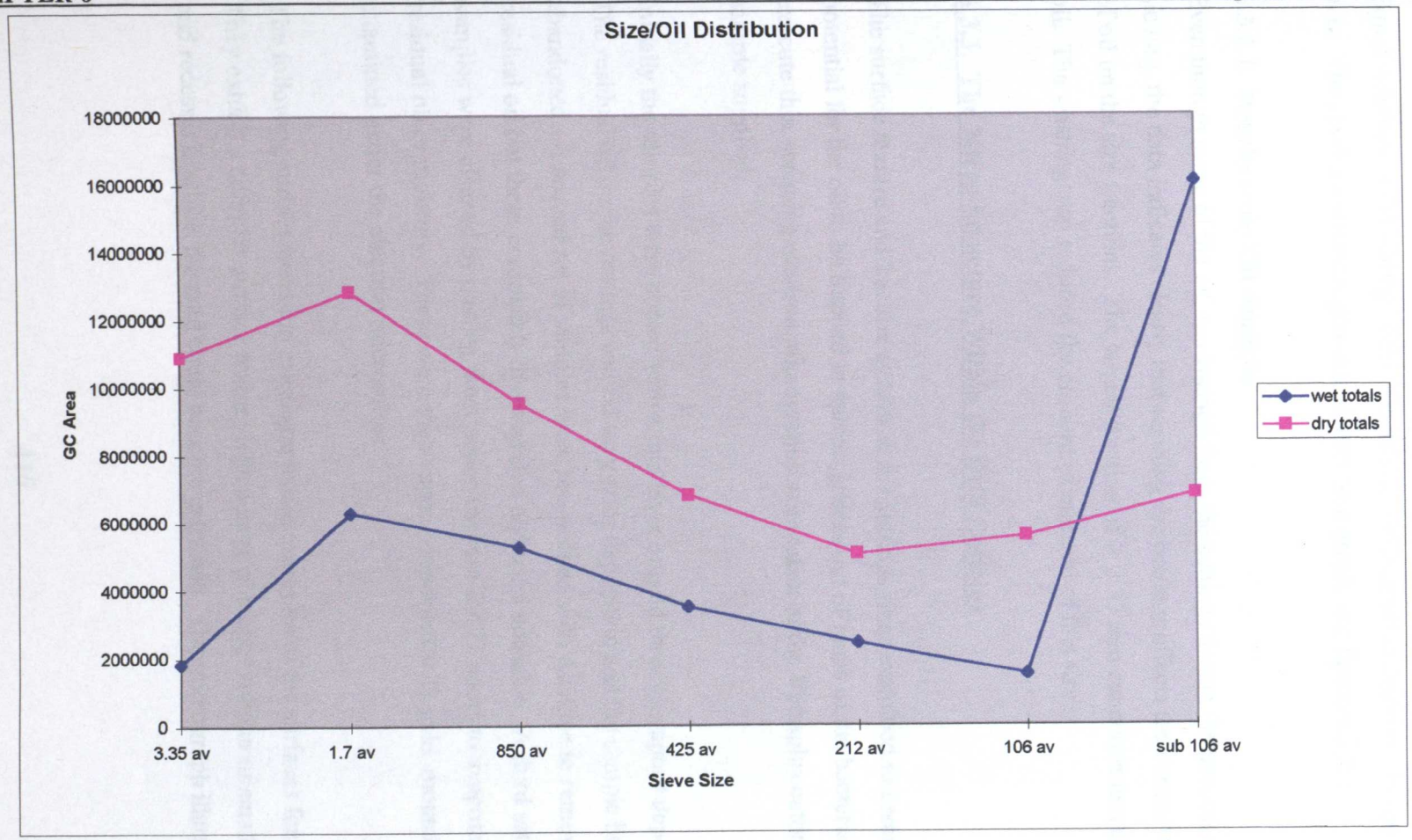


Figure 6.3.2.1. Oil on the drill cuttings within BS sieve size fractions

6.3.2. Oil on the Size Fractions

The wet cuttings were dried for 48 hours in a 50°C oven after sieving to remove the water; the dry cuttings were also dried, to ensure comparable conditions.

Equal volumes of the cuttings were weighed and extracted as described in section **6.1.**. The results were averaged and plotted on a graph, see figure **6.3.2.1.**

6.3.2.1. Results from Oil Analysis

Even though some of the oil was washed from the cuttings during the process of wet sieving, the data indicates clearly that washing the cuttings affects the concentrations of oil on the size fractions. The largest fraction of > 3.35 mm contained less than 1% oil. The washing also reduced the amount of material of this size.

6.3.3. The Micro-Structure Within the Drill Cuttings

The surface texture and fracture system in the cuttings were examined to assess the potential for the oil to be trapped in narrow spaces out of reach of the bacteria. To execute this, scanning electron micrographs were taken of the Versaplus cuttings sample supplied.

Initially the samples were coated with a thin layer of gold through vapour deposition. The residual oil on the cuttings caused very poor deposition and the sample had to be abandoned. A second set of samples were pre-washed with Acetone to remove the residual oil but these completely disintegrated and were unusable. A third set of samples were cleaned by placing them under vacuum for 72 hours to evaporate any residual oil or moisture. These were then vapour deposited with gold, mounted and examined under the electron microscope.

The following surface emission micrographs are examples of the surfaces found. They exhibit a complex surface texture with a large potential volume of small voids and recesses in which the mud could become entrained. The micrograph illustrated in

figure **6.3.2.1.** is a freshly broken sample; figure **6.3.2.2.** is an unbroken sample. Notice the deep fracture system in figure **6.3.3.1.** with an opening ranging from ~20μm down to 5μm.

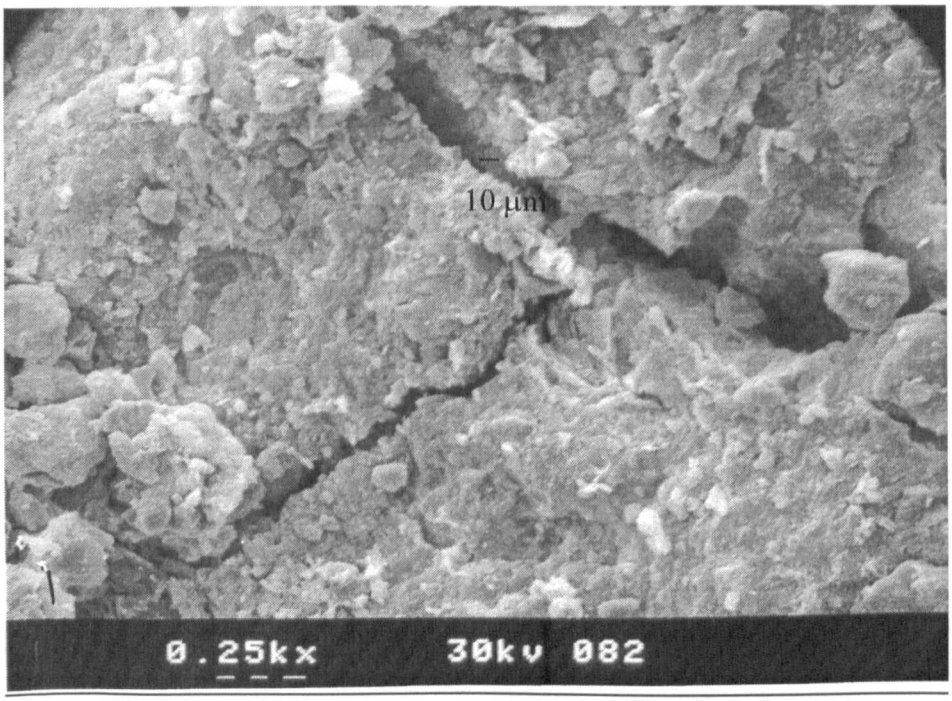


Figure 6.3.3.1. EM photograph of a sample of drill cuttings

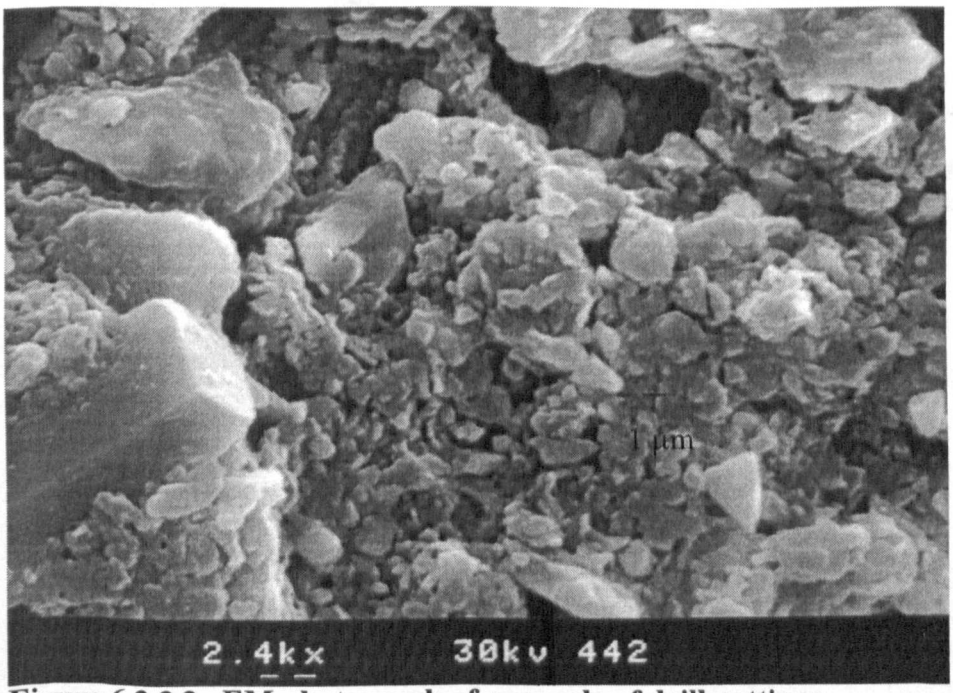


Figure 6.3.3.2. EM photograph of a sample of drill cuttings

6.4. MICROBIOLOGY

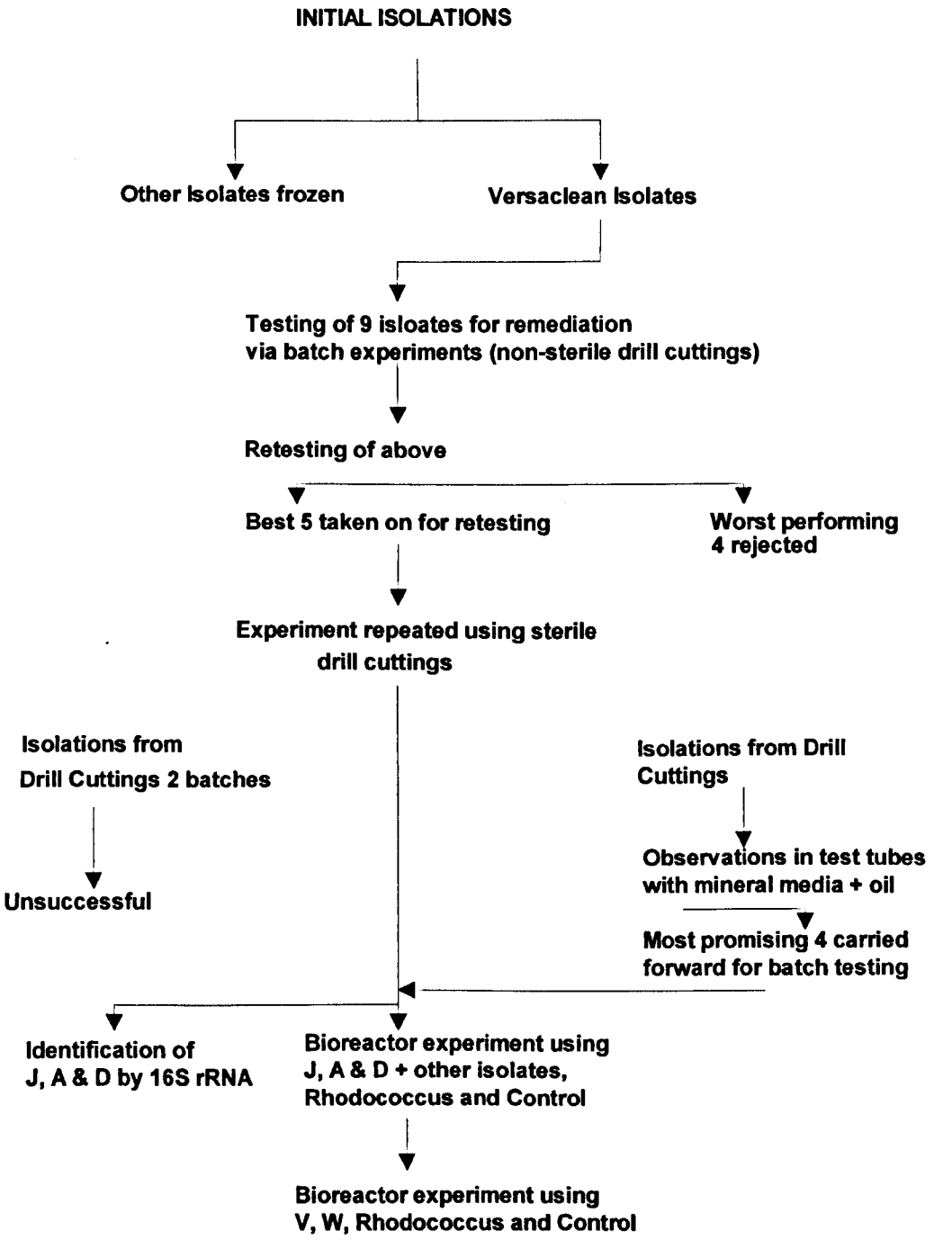


Figure 6.4.1. Flow Chart of the Experimentation Conducted

6.4.1. Initial Isolation

It was assumed that any remediating bacteria would be Gram-negative, as, with a few exceptions, the literature reviewed suggested these were the most common remediators (Bossert & Compeau, 1995). There are at least twenty-eight hydrocarbon utilising bacteria that are gram negative and non fermentative, with coryneforms seen to be the most frequently isolated hydrocarbon degrading bacteria (Bossert & Compeau, 1995). Examples include *Acinetobacter* (Hanson *et al*,1997), *Pseudomonas* (Staijen and Witholt, 1998; May and Katopodis, 1990)and *Arthrobacter* (Britton, 1984). Plasmids containing genes are well known to confer bioremedation properties on several Gram-negative bacteria.

The drilling fluids are often saline, and the majority of drilling in the UK takes place offshore, hence the consideration of salinity of the medium. The water phase salinity of the muds are typically 200,000 mg/l (MIDF, 1999).

Bacteria were isolated using enrichment procedures with Versaplus, Novatec, Ecogreen and Versaclean muds. Enrichment increases the number of a given micro-organism in a mixed culture (Singleton & Sainsbury, 1997) and have been widely used in studies to isolate potential remediators (Lal & Khanna, 1996; Mercade *et al*, 1996). By the addition of oil or drilling mud, it was hoped that micro-organisms able to tolerate the high levels of hydrocarbon in the medium would have some potential as remediators.

The enrichment was achieved by preparing a sterile nutrient broth, then adding the hydrocarbon aseptically at the rate of 4 ml per 400 ml broth (Ball, 1998), followed by an incubation period of 7 days on a shaker at 28°C, or until the flasks showed evidence of growth. Four flasks were sterile nutrient broth alone, four had the addition of 2% NaCl. The salt concentrations were lower than that of the muds, as it was not the pure muds being remediated, but the drill cuttings coated with mud, which would reduce the overall percentage of salt; also, if adopting a slurry system, the mud and cuttings would additionally be diluted with water.

The concentration of mud was increased to 10% as the broths became turbid to ensure that the 'right sort' of bacteria (possible remediators) outgrew other organisms.

After 7 days, all the cultures were turbid. A sample from Novatec + 2% salt and Ecogreen + 2% salt were examined under the light microscope. There was evidence of growth, so the concentration of NaCl was increased to ~3%, nearer that of seawater.

Once the broths became turbid, the solution was serial diluted 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, then streaked out onto a solid nutrient agar media, the same salinity as the original broths. Single colonies were then picked off and streaked out again individually. The problem at this stage was the fact that a method to keep the oil contained within the solid media had not been found. This was resolved later, as described in section **6.4.2.**. However, some isolates were found, and these were then prepared for storage in the freezer so as to retain their original characteristics and be tested later for remediation potential.

These were revived at a later date by inoculating the defrosted bacterial broth in Luria Bertani (LB) broth (see **6.4.2.1.**), on a shaker at 180 rpm at 29°C for 48 hours. The resulting turbid broths were then streaked onto LB agar plates, which were incubated at 29°C for 48 hours. Observations were taken, then colonies were picked off and re-incubated in LB broth for 24 hours; observations were taken. The samples were then prepared for the freezer, as described in section **6.4.7.**

6.4.1.1. Results from Initial Isolation

These results were from cultures grown on LB plates.

Table 6.4.1.1. Growth Descriptions of Revived Bacteria from Initial Isolations on LB plates.

CODE	DESCRIPTION
A1	Creamy white, round colonies; slight sheen.
J	Orange colonies; round; slight sheen.
B1 & B2	Off white, good individual colonies, very round, bright and shiny.
C1 & C2	Off white, mucoid, clumpy colonies, matt.
D	Mucoid, off white, large colonies, clumpy; slight sheen.
E1 & E2	Off white, tight growth pattern. Only grows where streaked, tiny shiny colonies.
F	Well spread, irregular colonies, off white, covers most of the plate, slight sheen.
G	Covers nearly all the plate. Very fuzzy colonies, well spread, roundish but appears blurred due to hazy appearance. Matt.
H	Shiny off white colonies, but different to E. Small, slightly spread over the plate but mostly concentrated where streaked.

The samples were put in a broth and left for 48 hours on a shaker at 180 rpm, in LB broth. The observations are in table **6.4.1.2.**

Table 6.4.1.2. Descriptions of Broths of Initial Isolates

CODE	DESCRIPTION
A1 & A1b	Turbid, good growth
B1 & B1b	Settled out, turbid at the bottom.
B2 & B2b	Settled out, turbid at the bottom.
J	No growth. Slight presence at the bottom of the tube.
C1 & C2	Turbid, good growth.
D & Db	Turbid, good growth.
E1, E1b, E2	Very minimal growth, just at the bottom of the tube.
E2b	Particles at the bottom of the tube, looks like it has perished.
F & Fb	Good growth throughout the tube. Sediment at the bottom.
G	Good growth throughout the tube. Sediment at the bottom.
H	Good growth but settled out

E and J were put back into the incubator to see if they were a slow growing species. After 24 hours there was still no evidence of growth from J but a little from E.

E and J were re-incubated, with sterile oil to see if the addition of oil influenced their growth; 2 at 1%; 2 at 2%. The results from this were quite surprising, with vigorous growth from both percentages using the 'J' bacteria, and fairly good growth from E, particularly at 1%. J was put into another broth with 4% oil, which grew vigorously too.

The samples were frozen at –80°C ready for future experimentation.

6.4.2. Oil-Based Growth Media Development

A project objective was to utilise remediating bacteria from the microflora of the muds and cuttings. The consideration when developing a medium was; would it demonstrate bacterial growth on oil, not just survival? It is possible for some bacteria to survive on plates with very little nutrition in a microbiology laboratory, utilising any nutritional aerosols present in the atmosphere. There would possibly be some

resting spores within the cuttings that would grow well using LB or Lab-Lemco, but could they demonstrate strong growth using a minimal medium with the addition of an oil as used in the manufacture of the MIDF muds?

The muds used in the drilling industry are basically an emulsion of water and oil, with other ingredients added to give the mud various properties (see **2.3.**). Mixing the oil into the agar mix using a sterile flea proved totally ineffective, as the oil and medium separated rapidly once the stirring stopped, unless the mixture was stirred up to the point of setting. This made a lumpy medium, which was unusable.

The initial formulation was as follows (adapted from the MIDF drilling fluid formulation & a standard microbiological nutrient/agar medium).

Agar	200ml (4g select agar, 2.6 g nutrient broth, 200ml water)
Novatec P	6.9 ml
Novatec S	4.9 ml
Novatec F	4.0 ml
Novatec BF	132.85 ml (Oil)

The Novatec P, S and F are all types of emulsifier.

The recipe was then changed, leaving the select agar (setting agent) out until the mixture was emulsified, having been mixed using a food mixer/liquidiser. It was not possible to add the emulsifiers aseptically. The mixture was left for over two hours at room temperature, and seemed to emulsify, remaining stable. However, when the agar was added to the emulsion (in solution), the medium would not set properly, and was therefore unsuitable for plates. There may not have been enough shear to create a stable emulsion.

Further attempts at making a suitable medium were conducted using an emulsifier, as seen in fig **6.4.2.1.**. The oil, emulsifiers and water, as per the MIDF formulation, with the addition of agar, were added to one jar, on one side of the pump, the apparatus

was switched on and the mixture pushed through the shearing action pump to emulsify the mix. However, even after 20 minutes running the mixture through the emulsifier, the two elements within the mix separated out within hours, plus the agar lost its setting properties. Another run was carried out using the recipe described below, along with the oils and emulsifiers above, minus the select agar; 500 ml of an agar-based medium was added to the 500 ml after running through the emulsifier. This was then sterilised by pressure cooker. Unfortunately, the mixture separated out after the sterilisation process.

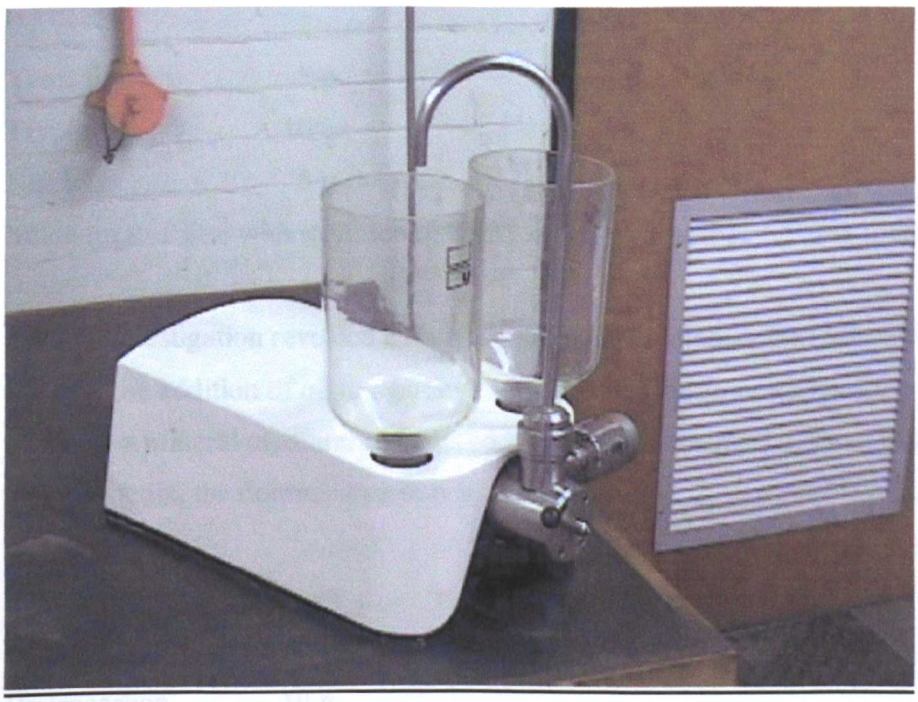


Figure 6.4.2.1. The emulsifier

The recipe used in the emulsifier was taken from NCIMB and described as an inorganic liquid medium:

	g/l
K_2HPO_4	1
$MgSO_4$	0.2
NaCl	0.1
$FeCl_3$	0.02
$(NH_4)SO_4$	1
Trace Mineral	1 ml

After these set backs, the previously used Lab-lemco (off the shelf product containing amino acids and vitamins) was used for the plating out of the initial Versaplus isolates, plus Luria Bertani (LB), a commonly used media in both liquid and solid form. However, this was non-selective; it was just to get the isolates growing until a formula was created. All the growth media are pre-sterilised by autoclaving at 121°C for 20 minutes.

6.4.2.1. Luria Bertani (LB)

Yeast Extract	5 g
Tryptone	10 g
NaCl	5 g

Made up to 1 litre with sterilised distilled water, with agar at 1.5% in solid media.

Further investigation revealed a recipe for making an oil powder (Atlas, 1993). This enabled the addition of oil to agar, and kept it in suspension. The powder could be added to a mineral medium, autoclaved, then used in the petri dishes. When in this type of media, the dominant carbon source is the oil.

6.4.2.2. Oil Powder

per 10 g

Hydrocarbon	10 g
Silica Gel	10 g
Diethyl ether	30 ml

Prepared weighing 10 g of hydrocarbon, which was added to 30 ml of diethyl ether (see appendix B for safety information) in the fume cupboard, and mixed thoroughly. 10 g silica gel was then added, and the ether allowed to evaporate. This process was carried out at room temperature.

6.4.2.3. Mineral Medium

Another medium utilized was from Atlas's Media Handbook (Atlas, 1993), which was described as being used for "the cultivation and enumeration of hydrocarbon-utilising bacteria by direct plating of estuarine water and sediment samples", and is as follows:

Agar, purified	20 g
NaCl	10 g
Oil Powder	10 g
NH_4NO_3	1 g
$MgSO_4$	0.5 g
*(Amphotericin B solution	10 ml)
K_2HPO_4 solution	7 ml
KH_2PO_4 solution	3 ml
$FeCl_3$	0.1 ml

Made up to 1 l with distilled water.

*Amphotericin B solution is a hazardous material. It is used as a fungal and protozoal inhibitor (Singleton & Sainsbury, 1997), and as it is not an essential ingredient it was decided to omit it. Vitamin stock was also added to the recipe, at the rate of 1 ml per litre.

For 100 ml of vitamin solution:
1 g Nicotinic Acid
0.5 g Thiamine-HCl
0.01 g Biotin

The iron chloride was described as a solution, but the lab only had this as powder. There was no indication as to the concentration or method, so, after discussion (R E Sockett, pers comm., 1999) 0.2 g of powder was mixed with 20 ml of distilled water and added as described in the recipe. With hindsight this was not prepared correctly. It should have been autoclaved separately and added as a sterile solution. The

K_2HPO_4 solution and the KH_2PO_4 solution were made using the same method, as follows (per 100 ml):

10 g of powder was added to deionised/distilled water, bringing the volume up to 100 ml, and mixed thoroughly. This was then autoclaved for 15 minutes at15 psi pressure, 121°C, then cooled to room temperature.

All the components were combined except the vitamin solution, K_2HPO_4 solution and KH_2PO_4 solution - with distilled/deionised water to bring the volume to 980 ml. It was mixed thoroughly and heated gently up to boiling point, then autoclaved for 15 mins at 15 psi pressure, 121°C then cooled to 45 - 50°C. 1 ml of sterile vitamin solution, 7 ml of sterile K_2HPO_4 solution and 3 ml of sterile KH_2PO_4 solution were then added aseptically, and mixed in thoroughly. This was then ready to use in sterile petri dishes or sterile tubes.

However, when used to grow-on some of the Versaplus isolates, the above recipe failed to support any growth. One known problem was the preparation of the $FeCl_3$, which may not have been correctly prepared (see above) and therefore may have proved toxic to the bacteria.

A further examination of the literature provided another recipe (Livingston & Islam, 1999), which described the concentration of the $FeCl_3$ solution.

0.2 g $MgSO_4.7H_2O$
0.02 g $CaCl_2$
1 g KH_2PO_4
1 g NH_4NO_3
1 g K_2HPO_4
2 drops of $FeCL_3$ solution (1.5 g / 25 ml aqueous solution)
1 l distilled water

The pH needed to be 6.5 – 7.0; in this case it was, so no buffer was needed.

The $FeCl_3$ was made up at the above concentration and autoclaved.

This recipe was used as a mineral base, with the addition of the oil powder and agar as appropriate. It seemed the most appropriate medium as the bacteria grew readily on plates and in the flask, with colonies up and visible in 48 – 72 hours. It was also used in the batch and reactor experiments to supply appropriate nutrients for the bacteria during remediation.

6.4.2.4. Oil Plates

The oil powder was tested to ensure that it was not toxic to the bacteria. Nine plates were made up containing LB agar and oil powder (see subsection **6.4.2.2.**), nine with LB agar and silica gel powder. Also, all the initial isolates were inoculated into a broth with the oil powder, 15 ml per sterile tube, and put onto a shaker. Both plates and broths were incubated at 29°C.

The oil plates were prepared in two ways, both of which were efficient at growing the oleophillic bacteria.

6.4.2.4.1. Oil Powder Plates

Using the mineral media, 15 g Agar and 10 g oil powder were mixed per litre, and autoclaved. This was then heated up in a microwave when required and poured into the plates, which were left to set aseptically.

6.4.2.4.2. Oil Film Plates

Using the mineral media, 15 g Agar was added per litre, then autoclaved. This was then heated and plated as above, but the oil was added as a liquid medium to the surface of the set plate. The oil was filter sterilised using membranes of pore size 0.2μm.

Further plates were produced using the muds by streaking the mud onto the oil powder plates, incubating them at 27°C, then picking off single colonies (where

possible). These single colonies were then streaked onto some additional oil powder and oil film plates.

6.4.2.4.3. Sterilisation of Oil

The oil was sterilised to ensure that it was not contaminated when testing the isolates. This was achieved by filtration using a Millex filter with a pore size of 0.2 μm, and conducted as follows.

The filter container was opened aseptically, as was a 10 ml syringe. The plunger was removed from the syringe, aseptically, and the syringe was attached to the filter. The oil was then poured into the syringe and the plunger inserted, over a sterile container. The oil was then gently passed through the filter into the container, which was then sealed and ready for use.

6.4.2.5. Results from Oil Powder

The broths were turbid within 24 hours, demonstrating that the oil powder is non-toxic to the bacteria. The plates all showed signs of growth within 48 hours, meaning the silica is non toxic in an agar environment.

6.4.3. Identification of Isolates

It was important to identify the isolated bacteria that were to be used for future experimentation, for several reasons.

- To know the health and safety aspects of handling and utilising them in a university laboratory environment, i.e. the ACDP classification. The laboratory was licensed for groups 1 and 2, but any scaled up process might require 1 only.
- To see if any of them were traditional remediators
- To see if there were any novel species
- To find out if they posed an environmental threat.

The initial isolates, having had individual colonies picked from the plates and streaked out, were left for 3 days incubation and Gram stained. As initial identification was to involve API 20 NE, suited to Gram negative bacteria (see 6.4.3.1), only species that appeared to be Gram-negative strains were taken for further analysis. Any results were noted; the bacteria were then prepared for freezing and frozen at –80°C.

Later during the project, the isolates were revived from –80°C ready for identification. Firstly, it had to be ensured that there was no contamination of the individual vials of bacteria. This was done using a visual method, by plating out the subsequent broths and examining the colonies for uniformity. Individual colonies were taken off the plates and restreaked onto new ones. There appeared to be no contamination, with the individual plates containing identical morphology.

6.4.3.1. API 20 NE Identification Tests

As many of the remediating bacteria are Gram-negative, an *in vitro* diagnostic test known as the API 20 NE was used to give an indication as to the genus or species of the isolated bacteria.

The API 20 NE is a strip consisting of 20 microtubes, each containing various dehydrated substrates and media, developed by BioMerieux. The test combines eight conventional and twelve assimilation tests for the identification of non-fastidious, Gram-negative rods not belonging to the enterobacteriacae. These tubes are "inoculated with a saline based bacterial suspension which reconstitutes the media" (BioMerieux, 2000) and then incubated in its closed incubation box for 24 hours at 30°C. During the incubation period, in the conventional tests the metabolism of the bacteria produces either spontaneous colour changes or ones that are revealed by the addition of reagents. In the assimilation tests, the bacteria grow if they are capable of utilising the corresponding substrate. The data is compared to the "Reading Table", using a points system, which is then compared to the Analytical Profile Index, or, as at the University of Nottingham, the identification software. For more details of the API 20 NE Identification System, please refer to Appendix C.

The isolates A, D and J were run through the system as outlined above, after first testing for Gram-stain; this was the first test, as the API can only give a positive identification with Gram-negative rods. As they all tested Gram-negative, the rest of the test was executed, with incubation periods of 24 and 48 hours. Section **6.4.3.2.1.** will disclose that the Gram-negative results were correct but gave a false answer. This is likely due to poor up-take of the first stain in the Gram reaction, possibly due to secreted surface products such as polysaccharides or to wall properties – the lattice of the peptidoglycan and peptides which form the mechanical strength of the cell wall may not have been porous enough for the first stain to take up.

6.4.3.1.1. Results from API 20 NE

Table 6.4.3.1.1. API 20 NE results

BACTERIA	Closest Match	%	TESTS AGAINST
A	*Aeromonas salmonicida*	99.9	2
D	*Aeromonas salmonicida*	98.9	1
J	*Aeromonas salmonicida*	70	2

These test proved inconclusive due to the tests against the predicted identities and the percentage confidence, particularly for J. Further confirmation was required, which lead to examining other methods of identification. The literature reviewed suggested that 16S rRNA was a more reliable method of identification.

6.4.3.2. 16S rRNA

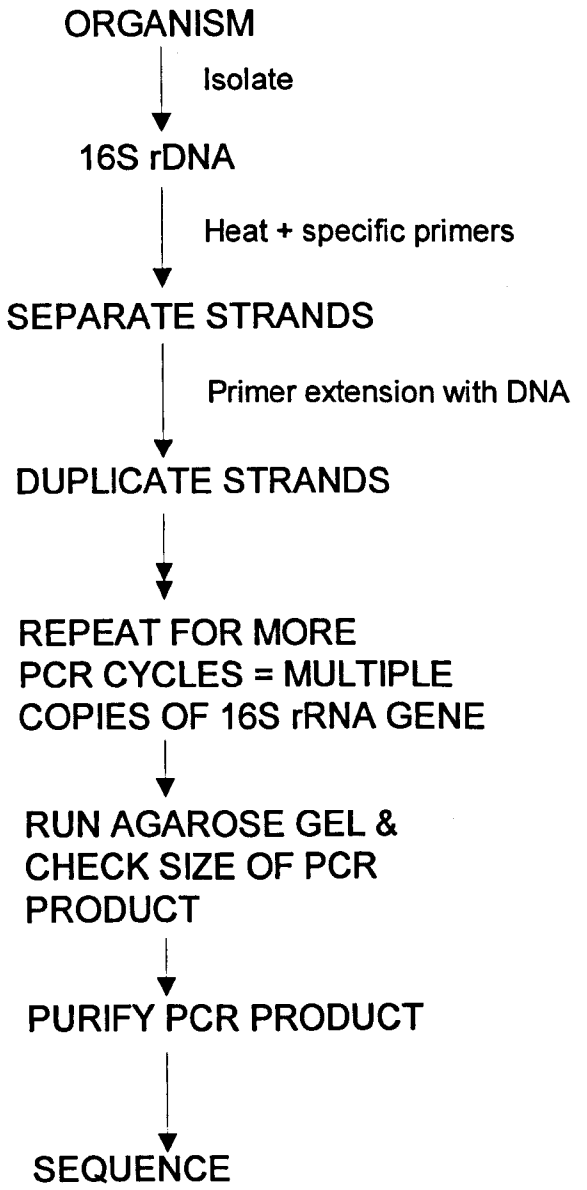


Figure 6.4.3.2.1. Ribosomal RNA amplification process for sequencing using DNA

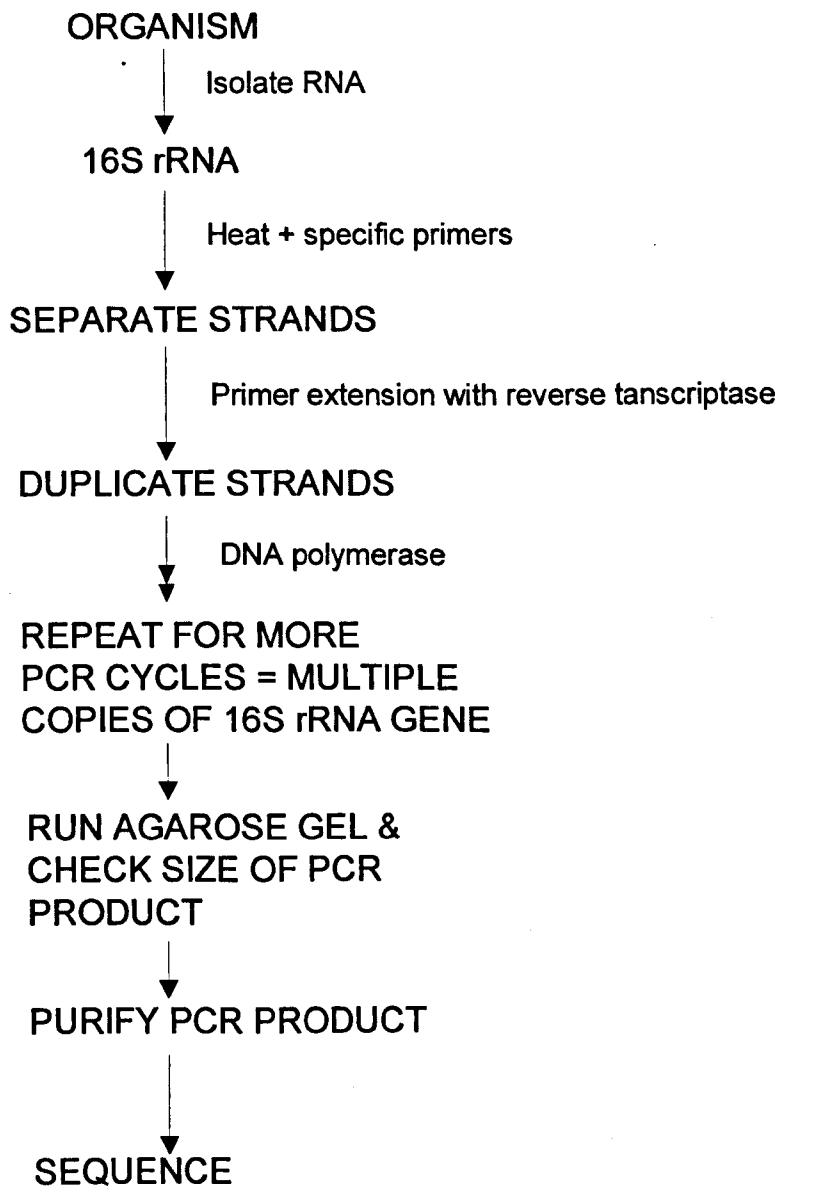


Figure 6.4.3.2.2. Ribosomal RNA amplification process for sequencing using RNA

The process of identification using this method can be very time consuming when undertaken in a standard microbiology laboratory. Suitable specific PCR (polymerase chain reaction) primers have to be designed, and used for amplification of 16SrDNA or 16SrRNA directly. The complication for a non-specific laboratory is in the design of the primer; it is preferable to have some prior knowledge of the genus of the bacterium, as without this the process can be very time consuming, requiring a large

bank of primers to test specific for different bacterial groups. There is a company in the USA called Midilabs who have set up an identification service using a wide range of primers for all genera.

The 16S rRNA is a test based on gene sequence similarity. Ribosomal RNA is employed for identification and evolutionary chronometers for several reasons (Madigan *et al.*, 2000); they are ancient molecules, being protein synthesizers, are functionally constant, universally distributed and fairly well "conserved in sequence across broad phylogenetic distances" (Madigan *et al.*, 2000, p 434). There are a large number of possible sequences in such a large molecule that some similarity between sequences indicates a phylogenetic relationship; how related they are depends on the degree of similarity. To calculate this degree, phylogenetic trees are constructed. Of the three ribosome RNA molecules of prokaryotes (5S, 16S and 23S), 16S is used as it is large enough to obtain proper sequence alignments but is more experimentally manageable than the 23S RNA molecule.

Once the PCR product has been produced (see figures **6.4.3.2.1.** and **6.4.3.2.2.**), it is ready for sequencing. The exact process that Midi Labs use can be found in appendix C. The new raw data is aligned with previously known sequences and then imported into a treeing programme, which compares the data. At Midilabs the sample sequences were compared using the PE Applied Biosystem's MicroSeq™ database and sequence analysis software. The top ten alignment matches were presented in a percent genetic distance format, which is basically the percent difference between two aligned sequences.

The isolates were prepared for the process by being streak plated several times until a plate with single colonies was produced. These plates were then sent to America for 16SrRNA identification.

6.4.3.2.1. Results from 16S rRNA

Table 6.4.3.2.1.1. 16S rRNA results with confidence levels

Sample	Closest Match	% Difference	Confidence Level
A	*Bacillus thuringiensis*	0.37	Species
D	*Bacillus thuringiensis*	0.09	Species
J	*Bacillus oleronius*	3.92	Genus

A 16SrRNA sequence homology of greater than 99% indicated a 'species level match', as with A and D.

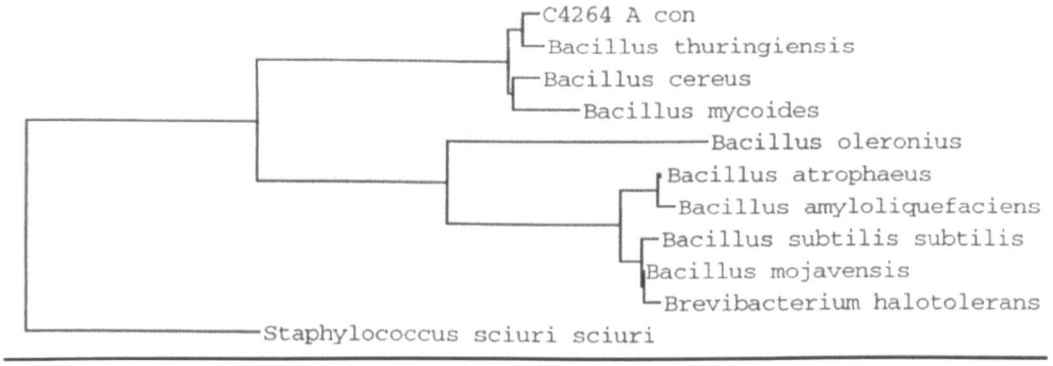


Figure 6.4.3.2.1.1. Phylogenetic Tree including 'A'

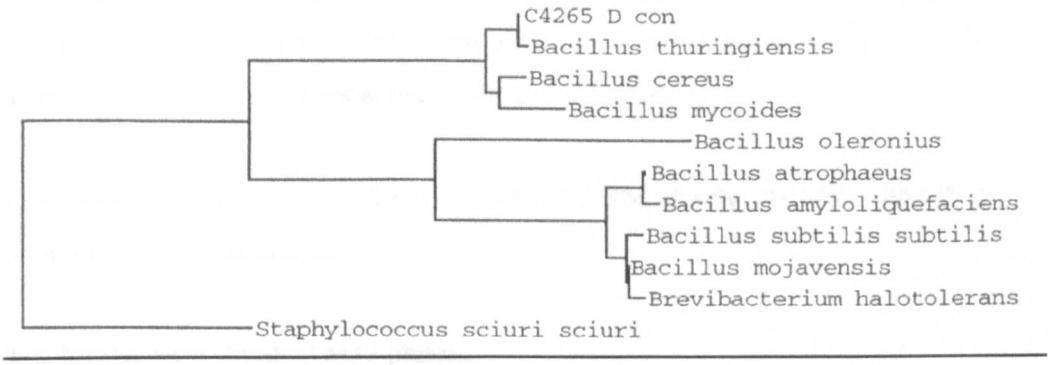


Figure 6.4.3.2.1.2. Phylogenetic Tree including 'D'

A 'genus level' match indicated that the sample appears to group within a particular genus, as with J, but the alignment did not produces a species match, meaning it is not in the database.

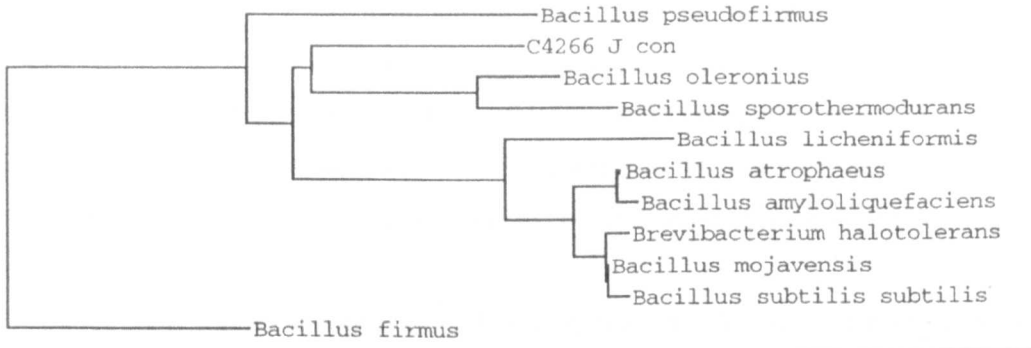


Figure 6.4.3.2.1.3. Phylogenetic Tree including 'J'

No match would indicate that the sample did not group well within any particular genus found in that database, or the GenBank database. None of the isolates fell into this category.

6.4.4. Second Isolations

The samples had arrived from MIDF. One had come from Getliff (M), and the other from Aberdeen, provided by Still (I). There were obviously two different rock types. The density of the samples was determined, which lead to 59.3 g and 47 g (equal volumes) of I and M respectively being added to flasks with 100 ml of LB broth. These were left for 48 hours in the incubator on a shaker.

This proved a little too thick; the broths were diluted at a rate of 10:1, and left for another 24 hours as above.

The broths were streaked onto plates.

6.4.4.1. Results from Second Isolations

The broths were extremely pungent, even though they had a foil cap and were enclosed in an incubation unit, with the whole lab smelling. All the cuttings had broken down, with no whole cuttings left, just small gritty pieces and sludge. The M's were grey, and the I's brown. This reflected the differing mineralogy. They proved difficult to extract, with lots of debris going into the solvent, which is totally unacceptable for injecting into the GC due to contamination of the equipment.

The plates produced some interesting micro-organism morphology; the I's were very typical of the type described in table **6.4.1.1.**, i.e. white irregular / spotted colonies. However, the M's were very different, with white hairy strands; a Medusa type head, typical of *Bacillus cereus.*

6.4.5. Third Isolation

The drill cuttings had arrived from MIDF; 4 'wet' batches and two dryer batches from one source, plus another drum from a second source whose mineralogy appeared to be different. The four wet drums and part of the two dry drums were thoroughly mixed in the mineral resources laboratory to ensure an homogenous batch. These were put back into the buckets, and frozen at between $-5^{\circ}C$ and $-10^{\circ}C$, in a small chest freezer. To ensure the constant temperature, a tube of frozen water was left inverted in the freezer – if this migrated to the opposite end, then the freezer must have defrosted at some point. This did not happen. The rest of the samples were kept at ambient temperature. This enabled the utilisation of these samples for further isolation experiments, as freezing bacteria without the correct preparation can burst the cell walls (see **6.4.7.**).

6.4.5.1. Method of Third Isolations

2 g of the unfrozen drilling mud with cuttings were added to flasks containing 25 ml of mineral broth, and left on the shaker for one week; the flasks were coded V, W, Y and Z. The material from the flasks was then streaked onto plates of mineral media

with sterile oil, both as powder and as a film, and incubated. The plates did not grow. The flasks had another 2 g of drill cuttings and some LB broth, and left for another 48 hours; the material was streaked out again.

There was no growth within the first 48 hours; however, after another 48 hours, the oil powder plates exhibited vigorous growth with the material from flasks V and W. Individual colonies were picked from these plates, and put into LB and mineral broths, both with oil, as W, W1, W2, W3, WW, WO; V, V1, V2.

The process was repeated for flasks Y and Z, which were 48 hours later going into their respective flasks; the growth was not as vigorous as for V and W, and were also slow to establish, but were put into the same broths as Y1, Y2, Y3, Y4 and Z. A, D and J were revived from the freezer and put into broths to grow alongside the other isolates to compare growth rates.

The isolates that appeared the most vigorous in the broths were plated out onto LB with sterile oil film plates.

Some of the broths had 1 ml taken from each to start new broths towards preservation in the freezer with final concentration of 15% sterile glycerol. The rest of the broths from these were added to flasks for batch testing.

6.4.5.2. Results from Third Isolations

The table **6.4.5.2.1.** illustrates the progress of the bacteria grown in the broths. It should be noted that V had to have another half millilitre of sterile oil added to it, as the oil had either been metabolised or disappeared as an emulsion.

The broths were repeated several times, and plated out several times. After plating onto the oil powder/mineral agar plates, the pigment in the bacteria seemed to fade. V, W2 and Z grew more vigorously on the oil/mineral solid media over time.

Table 6.4.5.2.1. Progress and descriptions of broths from third isolations.

Broth ID	LB + oil, progress and descriptions	Mineral media + oil, progress and descriptions
A	Turbid within 24 hours	Slight, after 72 hours
D	Turbid within 24 hours	Moderate after 72 hours
J	Minimal, but turbid after 72 hours	Slight to moderate after 72 hours
W	Turbid within 24 hours	Slight, moderate after 72 hours, bottom of flask
W1	Turbid, yellow, within 24 hours	Yellow at bottom of test-tube
W2	Turbid, no obvious pigment	Better than A and D after 24 hours, risen to interface of oil/broth by 72 hours
W3	Slight	Slight, but on bottom of test tube
WW	Turbid, rises to interface post shaking	Minimal growth, on bottom
WO	Slight after 24 hours; turbid after 72 hours	Slight, after 72 hours
V	Turbid after 24 hours	Slight after 24 hours, but very turbid after 72 hours, plus vigorous growth at broth/oil interface
V1	No Growth	Slight, even after 72 hours
V2	None until 72 hours, then stringy growth within the broth	Slight, even after 72 hours
Y1	Turbid within 24 hours	Slight, even after 72 hours, on the bottom
Y2	Turbid within 24 hours	Slight, even after 72 hours, on the bottom
Y3	Turbid within 24 hours	Slight, even after 72 hours, on the bottom
Y4	Turbid within 24 hours	Slight, even after 72 hours, on the bottom
Z	Turbid within 24 hours, clumpy.	Slight, even after 72 hours, on the bottom

Table 6.4.5.2.2. Plates from some of the broths.

Isolate Code	Description, 72 hours
V	No growth.
WW	Creamy white, slight sheen.
W1	Bright yellow, glossy, small colonies.
W2	Vigorous, whole plate covered, creamy colour, with sheen
Y1	Creamy to yellowish, blotchy, glossy.
Y2	Blotchy, 'splatter' like appearance, creamy colour, slight sheen
Z	Creamy white, slight sheen

V would not grow on the plate, yet exhibited promising activity within the broths.

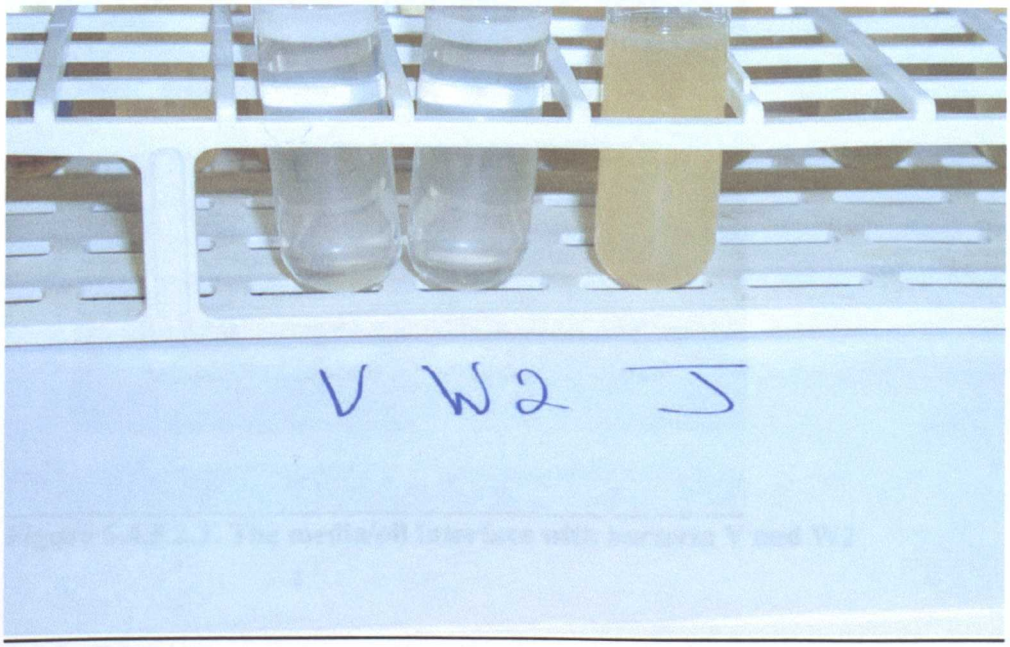


Figure 6.4.5.2.1. Test tubes containing cultures V, W2 and J, showing the bacteria on the interface between the mineral medium and the oil, and in the oil itself. Notice the breaking up of the oil globules in the tube containing J.

V displayed an unexpected reaction to the oil; in one of the test-tubes, the oil on the surface changed colour to a dark grey/black. This oil was run through the GC/MS, but no indication of the change could be interpreted from the trace; the peaks eluted without separation, possibly illustrating a complex mix of hydrocarbon chain lengths.

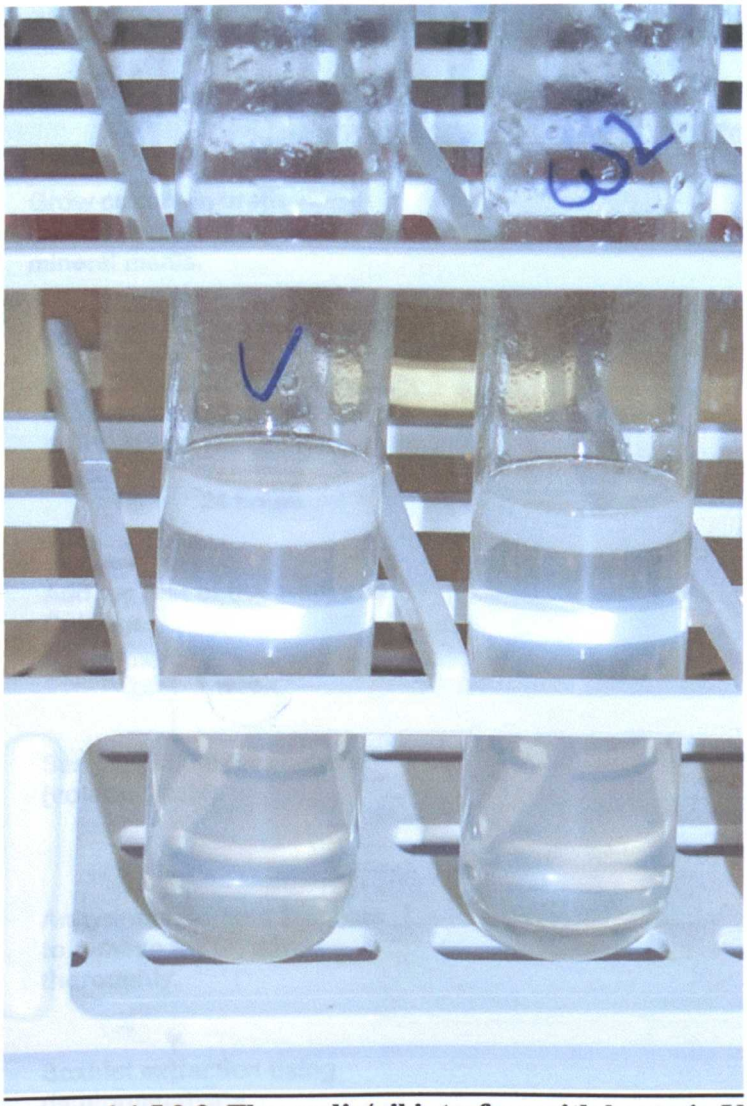


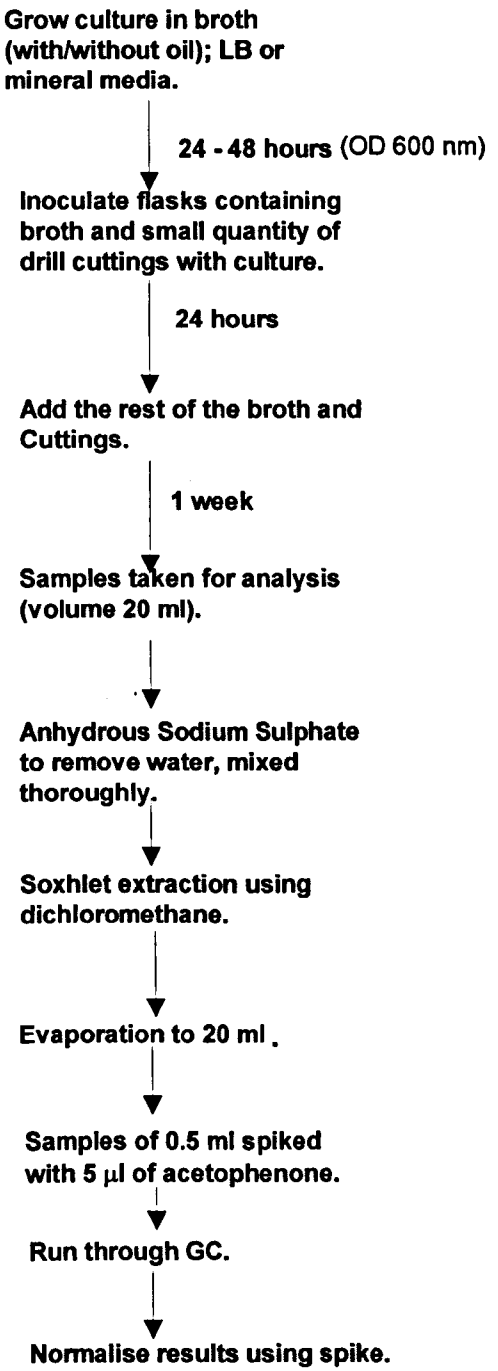
Figure 6.4.5.2.2. The media/oil interface with bacteria V and W2

6.4.6. Freezing

Once bacteria have been isolated, it is important that they are preserved so they can be reused at a later date and to prevent adaptation to laboratory, non-oil media and loss of remediation genes if subcultured. For this project, the bacteria were frozen at –80°C. To prevent cell damage, the bacterial broth to be frozen has to be combined with glycerol at 15 – 20%. The glycerol in the laboratory was 80%, which meant that for every 4 ml of broth there needed to be 1 ml of glycerol.

6.4.7. Pre-Screening of the Nine Bacteria from the Initial Isolation

Screen Tests – General Overview



6.4.7.1. OD 600 nm

A rapid method of obtaining estimates of cell growth is by measuring the turbidity (Maligan *et al.* 2000). When a broth looks cloudy, or turbid, to the eye it is because the cells scatter light passing through the suspension; the more cells, the more turbid. This turbidity can be measured with a spectrophotometer, which pass light through a cell suspension. The amount of unscattered light that emerges is measured. A spectrophotometer will measure the optical density in OD units, which, if a standard curve is prepared with known cell numbers, can indicate cell number. It is a fixed value for a fixed number of one cell type.

The apparatus used for measuring the optical density was a spectrophotometer (Biorad).

The optical densities were measured so that there was a standard reproducible amount of bacteria to add to the cuttings. It was conducted by picking off a colony from a plate using a loop, and put into 15 ml of broth, which was incubated for 24 hours at 29°C. All the broths were then left to settle on the bench for half an hour – this allowed the oil to rise and separate from the bacterial broth to prevent micelles of oil affecting the OD readings. It was then diluted at a rate of 100 µl of bacterial broth to 900 µl of LB broth. During the OD600 tests, each sample was blanked against the identical media alone.

6.4.7.2. Results from OD600 nm Turbidity Tests

Table 6.4.7.2.1. Results from OD600 nm

Bacteria	1st Reading	2nd Reading	Average
A	1.382	1.377	1.3795
B	0.534	0.535	0.5345
C	1.536	1.536	1.536
D	1.409	1.393	1.401
E	0.111	0.098	0.1045
F	1.574	1.607	1.5905
G	1.625	1.625	1.625
H	0.568	0.574	0.571
J	0.020	0.017	0.0185

The broths were then used to inoculate the batch tests.

6.4.7.3. First Pre-Screening

To give an initial indication of the remediating potential of the bacteria before they went into the bioreactors, pre-screening tests were undertaken with these initial isolates. Bioreactors generally are dealing with much larger quantities, the bacterial inoculate is a very small portion of the whole, so the process takes much longer than flask tests. The results were for comparative evaluation of the inoculates and therefore simple area evaluation was valid.

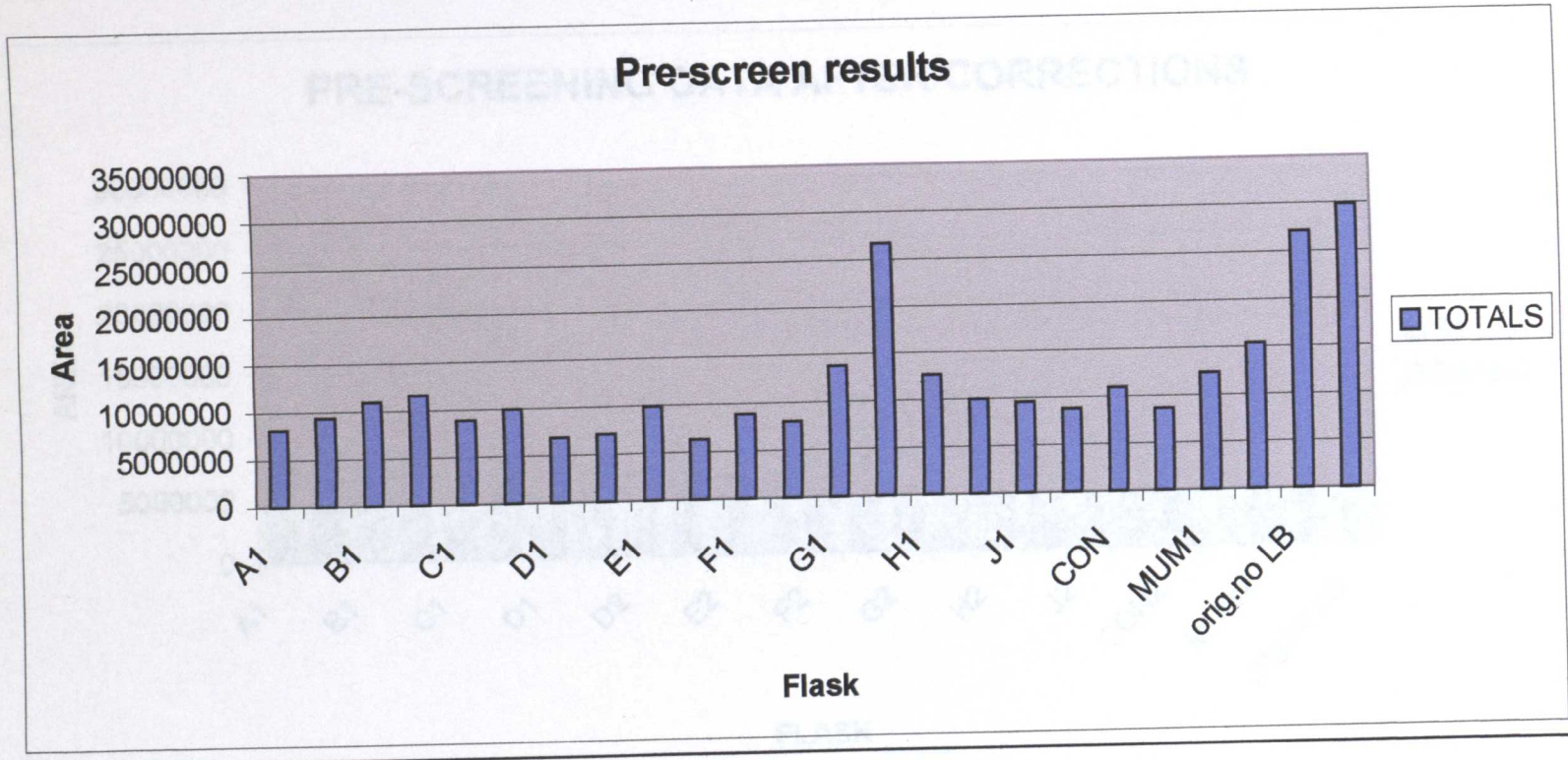
250 ml flasks were put onto a shaker, at ambient temperature, with 33 ml of LB broth and the inoculate in each flask. These were left for 24 hours, until they were turbid. At this point, 66 ml of cuttings were added to the flasks; they were reinstalled onto the shaker table and left for one week. A control was also made up with 33 ml of LB and 66 ml of drill cuttings, unsterilised. Two further samples were made up prior to analysis, to get some measure of possible remediation in the control flask with unsterilised cuttings (made up mix, labelled MUM on the graph); do any of the

natural flora within the cuttings perform as remediators when supplied with nutrients, warmth, moisture and oxygen, even without enrichment and inoculation?

6.4.7.3.1. Results of First Pre-Screening

The data showed that there had been some remediation within the flasks in one week. There were flasks that performed worse than the control, which would have contained the natural flora within the drill cuttings. For examples of data, including remediation rates (%) and peak areas (% of totals) see appendix D.

The percentage peak areas showed no particular trend in preferential HC chain-length degradation. The results from these experiments were (from best to worse)
D, E, F, A, J, C, Control, B, H, G.



Graph

Figure 6.4.7.3.1. First Pre-screen results using initial isolates

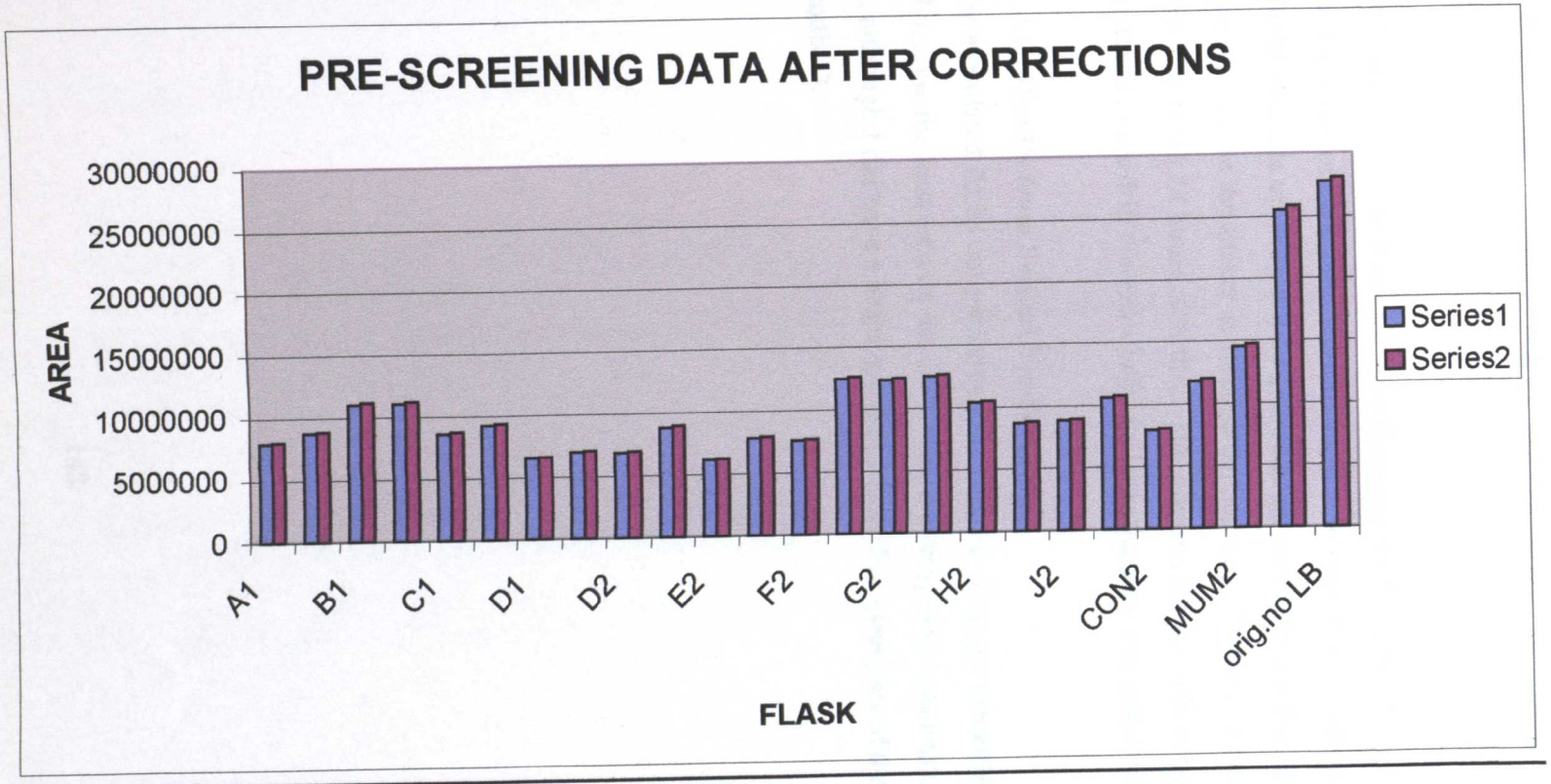


Figure 6.4.7.3.2. Pre-screening results after using acetophenone internal standard

Further experimentation was justified on the best 5; E had a very poor SD, i.e. the spread of data was large, one result very good, the other very poor.

6.4.7.4. Second Pre-Screening

The five best performers in the above tests were carried forward for further experimentation. The method above was refined to try to improve conditions for the bacteria, particularly by introducing the cuttings slowly. The quantities were kept the same.

The refinement of the technique involved putting the bacteria into test tubes containing 10 ml of LB broth for 24 hours. These were then transferred to the flasks and the broth made up to 33 ml; 20 g of cuttings were added at this time and the mixture was left on the shaker at ambient temperature. The rest of the cuttings were added to the flasks 24 hours after that. This allowed the bacteria to become acclimatised to the drill materials before a large quantity was added (total 116 g).

6.4.7.4.1. Results from Second Pre-Screening

All the inoculated flasks out-performed the control. The best remediators were A, D and J, having the least area on the GC traces, meaning there was less oil left in the flask, although J did have a larger spread of data. For examples of the data, see appendix D.

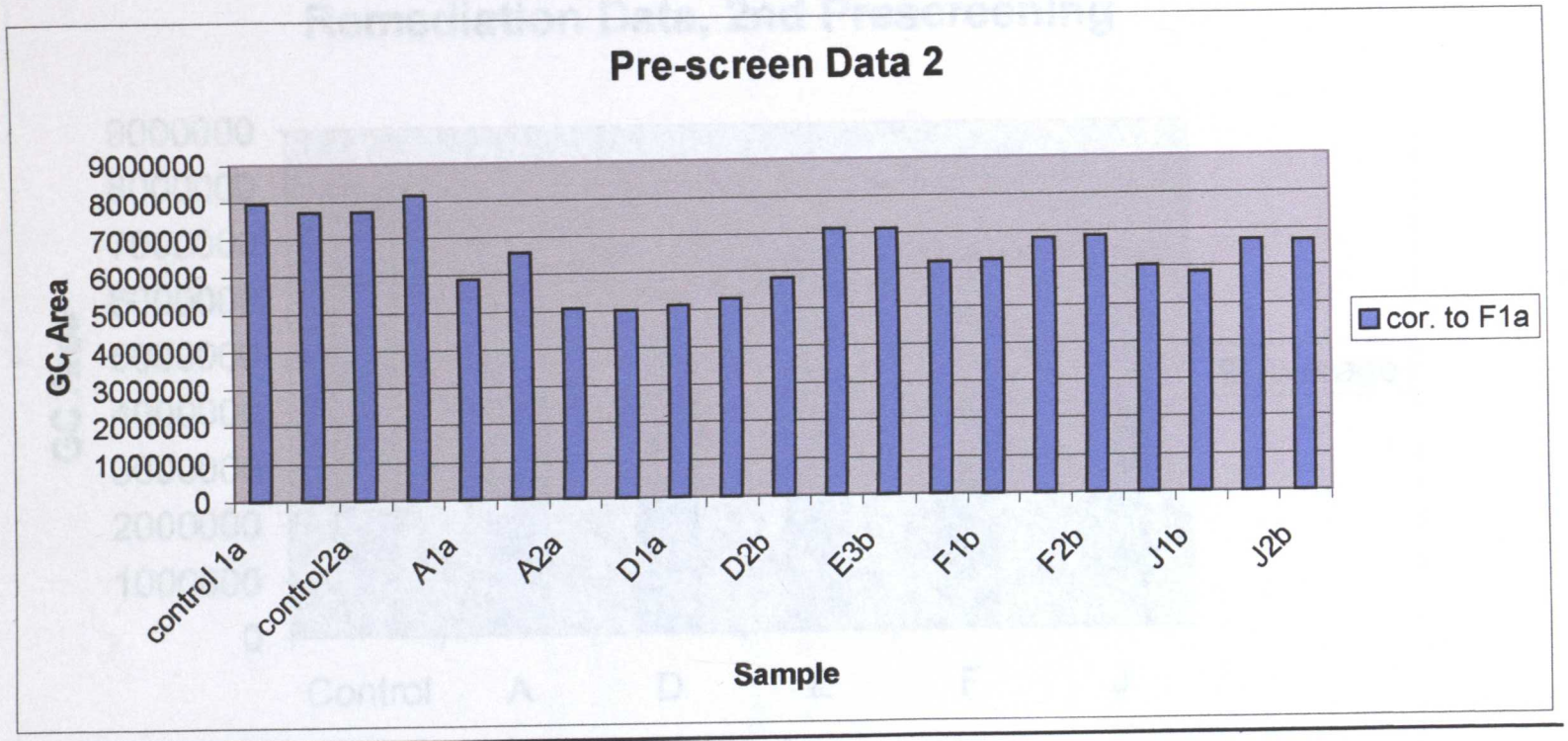


Figure 6.4.7.4.1. Results from second pre-screen batch experiment

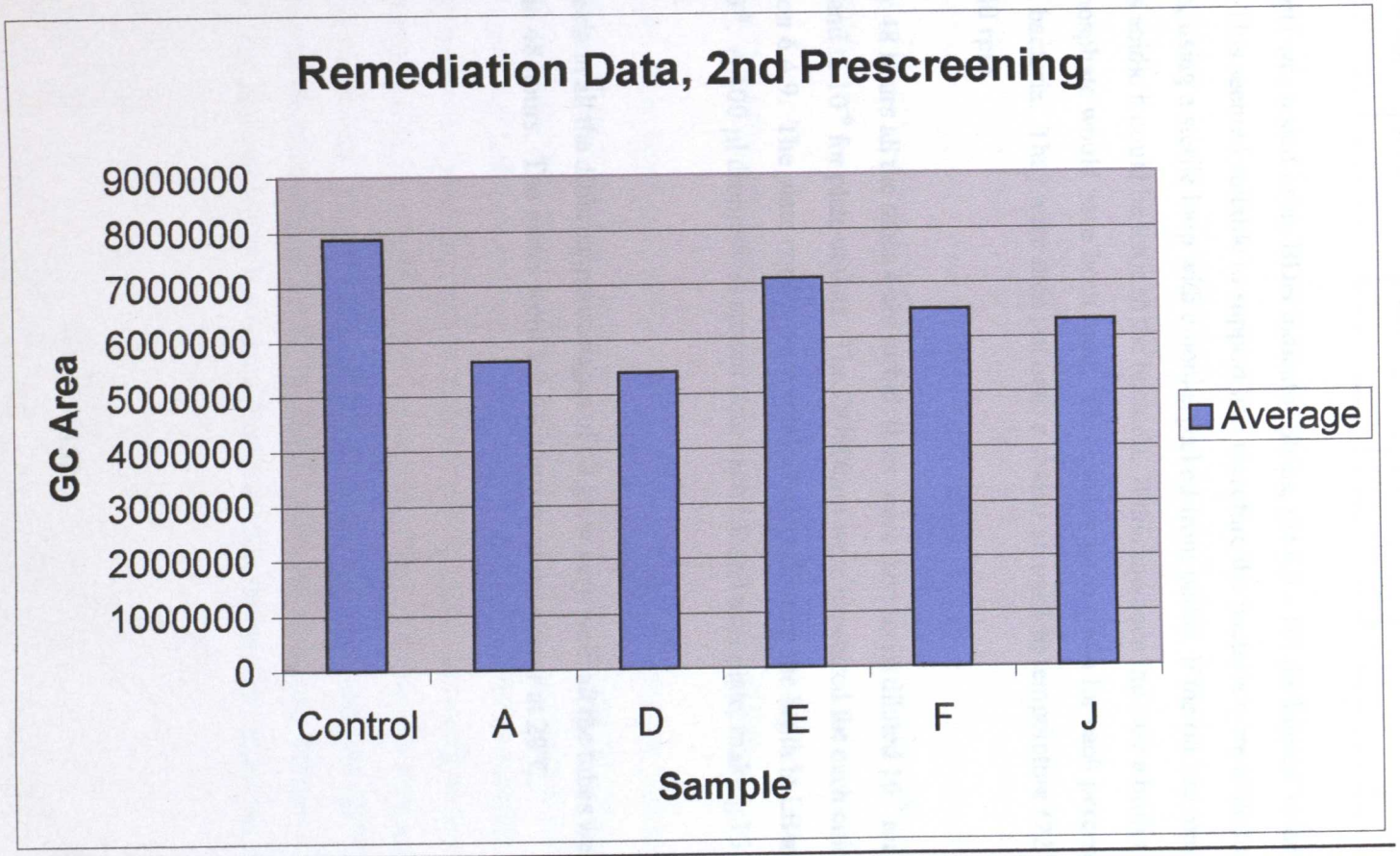


Figure 6.4.7.4.2. Results from second pre-screening, averages

6.4.8. Oil Enrichments of A, D & J

Test tubes were set up with different concentrations of sterile oil in LB. The percentages were as follows.

Table 6.4.8.1. Oil Enrichment test tubes

% OIL	1/2	1	2	4	6
LB ml	9.95	9.9	9.8	9.6	9.4
Sterile Oil ml	0.05	0.1	0.2	0.4	0.6

The pH was tested using BDH Indicator Strips, pH 6.5 - 10; the highest value was at 7.5. This seemed suitable to support life, therefore the bacteria were added to the tubes, using a sterile loop with colonies picked from plates. If the oil had made the tubes acidic it could be toxic to the bacteria. If this had been the case a buffer solution of phosphate would have been used. Three tubes were made for each percentage of each bacteria. They were then put onto a shaker at ambient temperature (22 – 23°C) at 180 rpm.

After 48 hours all the tubes were turbid; these were then serial diluted 10^{-4} for OD 600 nm, and to 10^{-6} for plate counts. The OD600nm were measured for each culture as in section 6.4.9. The plate counts were conducted by diluting the broth in LB at the rate of 10^{-6}. A 100 µl droplet was spread onto each LB and agar plate, making 15 plates in total.

Bacteria in all the differing percentages of oil grew very well; all the tubes were turbid within 48 hours. The plates were left 48 hours in an incubator at 29°C.

Table 6.4.8.2. Plate Counts from Enrichment Plates

% OIL	J	D	A
6	32	ALL	ALL
4	1	PLATES	PLATES
2	1	TOTALLY	TOTALLY
1	24	COVERED	COVERED
1/2	0		

The plate counts were inconclusive.

All the tubes were left for a further week to observe the growth.

The interface between the oil and the water was starting to bubble, suggesting emulsification.

All the J strain bacteria (J's) were very vigorous after the extra time; all the test tubes had turned very orange. This confirmed that J was a slow growing species. A & D's had started to drop out of suspension, with cells building up at the bottom of the test tubes. This can cause a cessation of growth by anoxia and the release of toxins from the dead cells.

6.4.9. Third Pre-Screening – The Use of Sterilised Drill Cuttings

To date, all the batch tests had used the cuttings in their raw state, i.e. with indigenous bacteria within the cuttings. This was to enable the measure of remediation with these as a natural consortium; not all bacteria within a sample can be grown in a laboratory environment (McEldowney *et al.*, 1993), implying that some of the indigenous bacteria have not been observed in broths or plates. Also, this would be more representative of the conditions that would prevail in a 'real' reactor treating field-produced cuttings. By providing nutrients, a fairly warm environment (22°C), increasing the moisture and gentle swirling on a shaker at around 180 rpm it was hoped that there would be some remediation even in the control. However, the

isolates needed testing in isolation to see how they could perform totally alone. The flasks were set up as described in section **6.4.7.4.**, with the exception of the drill cuttings, which were autoclaved to sterilise them, ensuring they were free from micro-organisms. The flasks were left on the shaker for 1 week, and samples extracted and prepared for GC as described in the previous sections.

6.4.9.1. Results from Pre-Screening using Sterilised Drill Cuttings

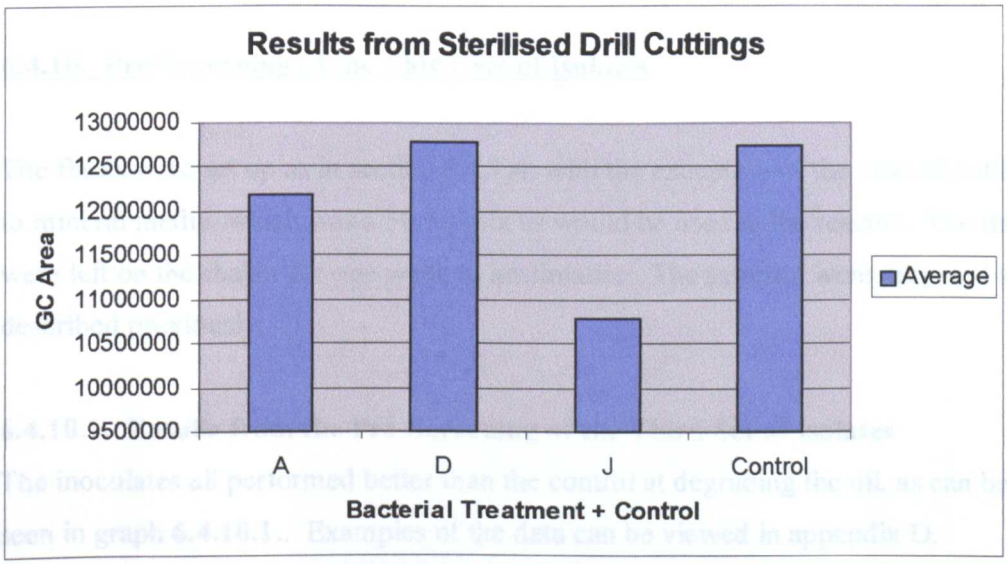


Figure 6.4.9.1. Results from Pre-Screening in flasks using sterile drill cuttings

Remediations were J, 15%; A, 4.5%; D, 0, as reduction of oil compared to the control.

Reading the data from the calibration graph gave the following approximate percentage concentrations left in the flasks:

Control	9%
D	9%
A	7.5%
J	6%

It was interesting that the worst remediator of the three when using non-sterile cuttings became the best performer when the cuttings were sterilised. However, all the remediation percentages were lower than when used with non-sterilised drill cuttings. This lead to further investigations concerning J as well as A and D, including the identification using 16S rRNA as described in section **6.4.3.2.** and testing its remediation abilities in the bioreactor experiments. J had proved to be a species that was slow growing; the extra time in the reactor might have helped to release its potential as a remediator.

6.4.10. Pre-Screening of the Third Set of Isolates

The flasks were set up as in section **6.4.7.4.** with the exception of the ratio of cuttings to mineral media, which was a 50/50 mix as would be used in the reactor. The flasks were left on the shaker for one week to acclimatise. The samples were prepared as described previously.

6.4.10.1. Results from the Pre-Screening of the Third Set of Isolates

The inoculates all performed better than the control at degrading the oil, as can be seen in graph **6.4.10.1.**. Examples of the data can be viewed in appendix D.

V, W and Y had reduced the oil content more than Z. However, all looked promising for further testing. It was decided to examine these, as well as J, using an electron microscope and take some slides, some of which were developed into photographs.

All these pre-screened isolates had potential for testing their remediation capabilities using the bioreactor; initially V and W were tested. Time ran out before Y and Z could be run in the reactors.

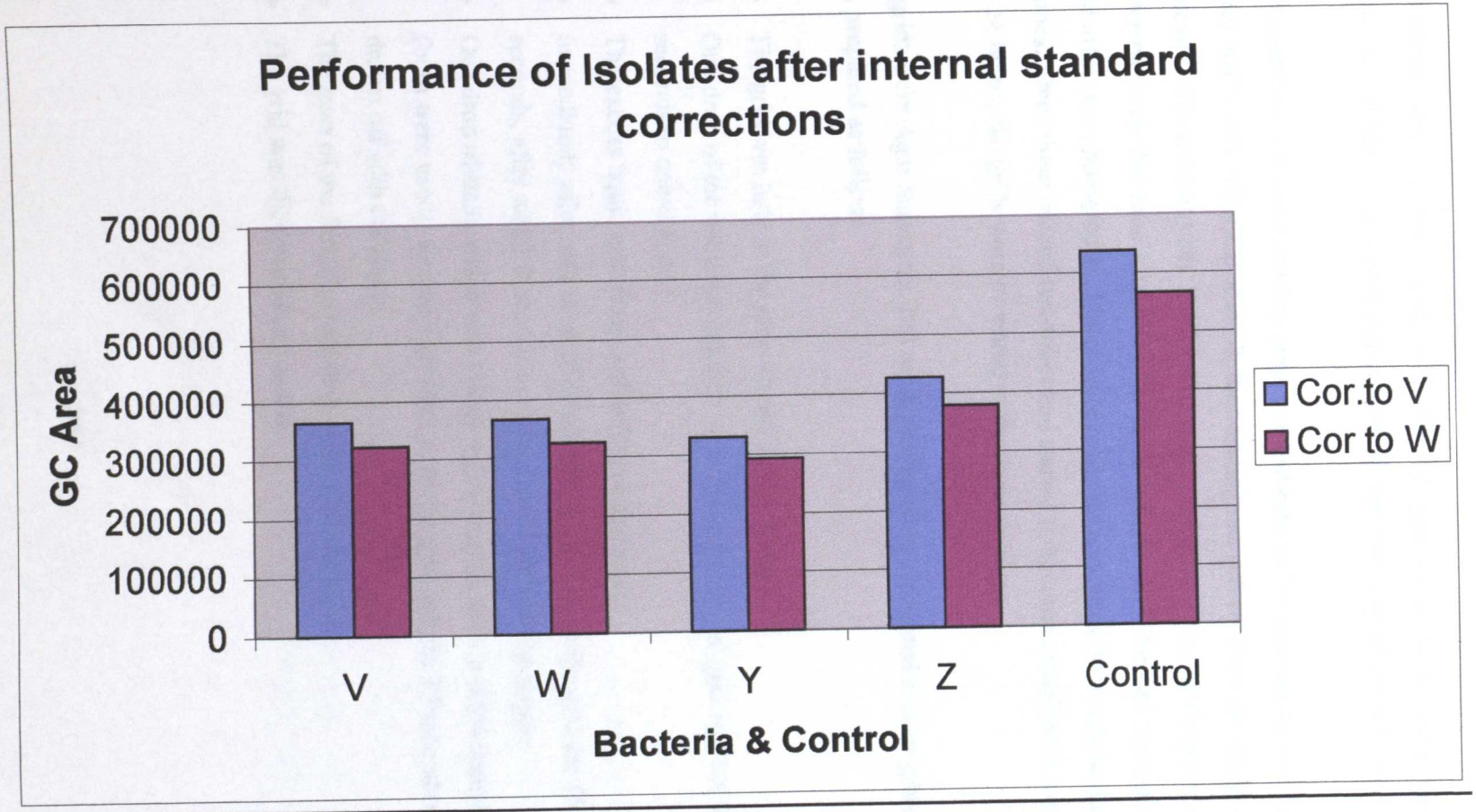


Figure 6.4.10.1. Performance of Pre-screening 3rd Isolates

6.4.11. Transmission Electron Micrographs of Negatively Stained Bacteria

A Jeol 100S Transmission Electron Microscope was used to take the EM photographs, at 80 KiloVolts operational voltage and magnifications of 7 – 21,000 times.

The bacteria were required in a liquid medium at approximately the mid phase of the culture, i.e. not heavily concentrated and turbid, but with enough growth to be viable.

The preparation was conducted by preparing test-tubes of an appropriate medium, i.e. mineral media with oil or LB with oil, then adding one colony from the chosen plate aseptically. These tubes were left for 48 hours. One drop from each broth was transferred to another tube containing LB broth with no oil. This was because the oil would affect the photographs. Plates were made up concurrently to ensure purity of the tubes. Once these second test-tubes had started to become established, which was after 24 hours, the grids were prepared using these cultures.

The grids were Agar Scientific, 200 mesh carbon-formvar coated copper grids, and were prepared as follows.

- The grid was held in the jaws of dry, sterile forceps.
- One drop of the bacterial medium was pipetted onto the grid and left for 30 seconds to one minute.
- The excess liquid was drawn off with blotting paper.
- Immediately after, one drop of sterile water was left on the grid for 15 seconds, after which it was once again drawn off with the paper.
- One drop of stain, which was 1% phospho-tungstic acid or 0.5% uranyl acetate (both were used), was then pipetted onto the grid, left for 15 seconds and drawn off with the paper.
- The jaws of the forceps were then dried with the paper.
- The grid was then stored and noted.

The grids were inserted into the electron microscope and the view moved until bacteria could be seen. The magnification was then set to allow a good photograph; the magnifications used were 5.6K, 7K, 10K and 14K, depending on the size of the bacteria. The photos were then scaled to enable the bacteria sizes to be assessed. This was conducted by measuring the flagella on figure **6.4.11.1.**; all flagella are the same size, 15 – 25 nm wide (a nano is 10^{-9}, or a thousandth of a micro) and 1 – 3 μm long (Sockett, pers comm., 2001) . The bacterium in that photograph was then measured; a multiplying factor was used to find the scale. Knowing the magnification used for the photograph allowed for extrapolation for the other magnifications. There is a 40 mm bar on each photograph; the micron measurement is scaled to this bar. This bar may change size, depending on the shrinkage of the photo for the report; the scale of bar size to microns stays constant.

Table 6.4.11.1 Scales for the Electron Microscope

Magnification	Bar Scale (micron)
14	1
10	1.4
7	2
5.6	2.5

6.4.11.1. Results of Electron Microscope

The bacteria displayed hydrophobic tendencies, and were therefore not easy to capture on film. This propensity could be interpreted as an indication that the bacteria were hydrocarbon-utilising species. This is emphasised by figure **6.4.11.3.**, which shows the bacterium surrounded by micro-droplets of oil. The different isolates were definitely separate species, with appearance and size varying. This can be noticed particularly in figure **6.4.11.8.** of the Y2 isolate, which is bigger than any of the others. W2, a rounder rod like J, had only one flagellum, as can be seen in figure **6.4.11.4.** V2 (figure **6.4.11.7.**) was rod-like, as was Y2 (figure **6.4.11.8.**), but much

smaller. Not all the photographs were of an adequate quality to develop; hence none of the Z isolates were available.

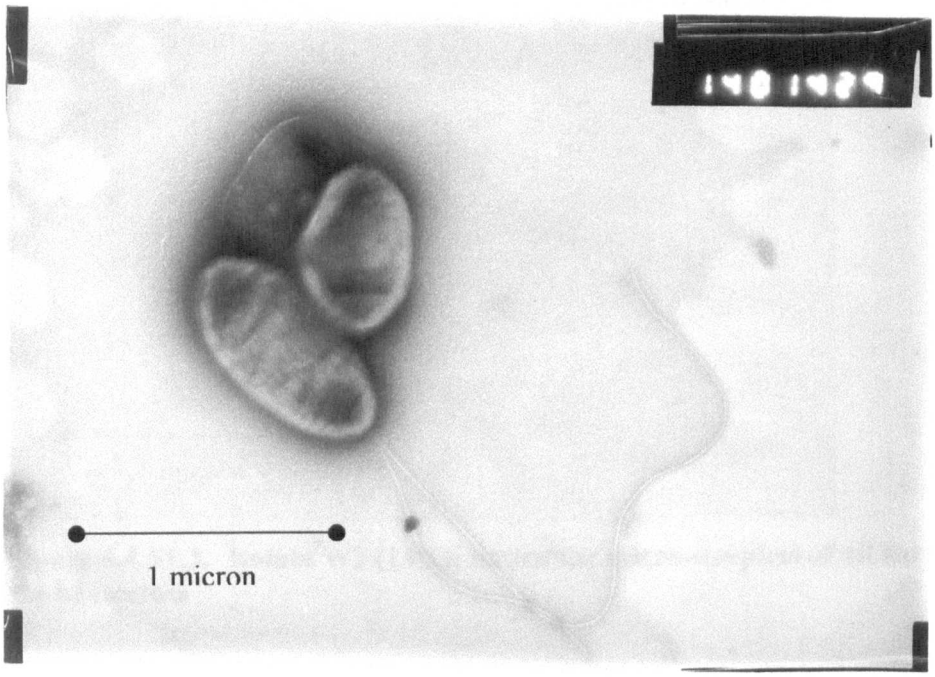


Figure 6.4.11.1. Bacteria J (14K Magnification). Notice the two flagella.

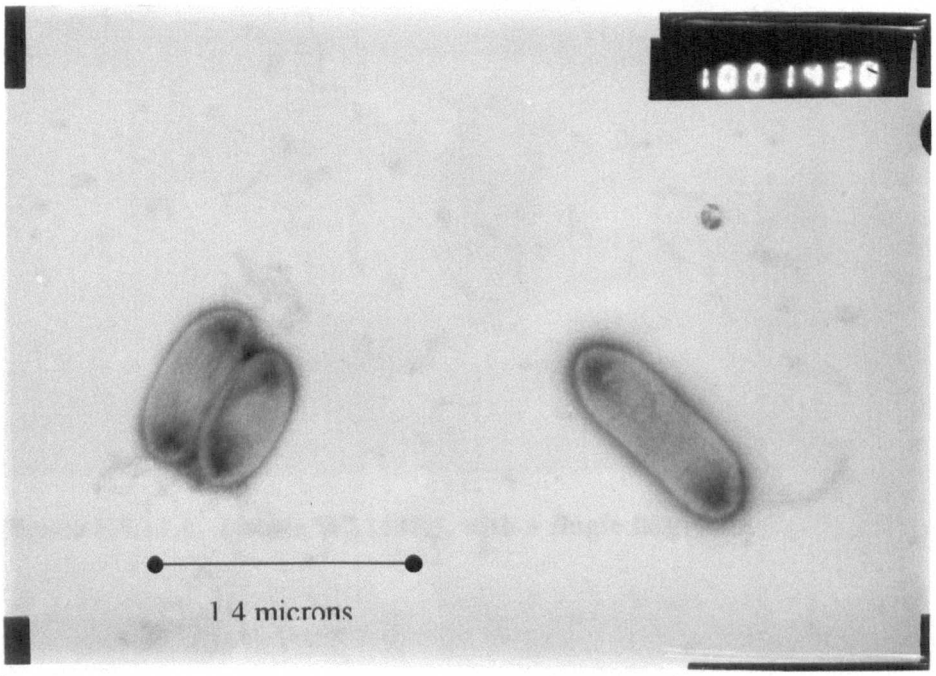


Figure 6.4.11.2. Bacteria J (10K).

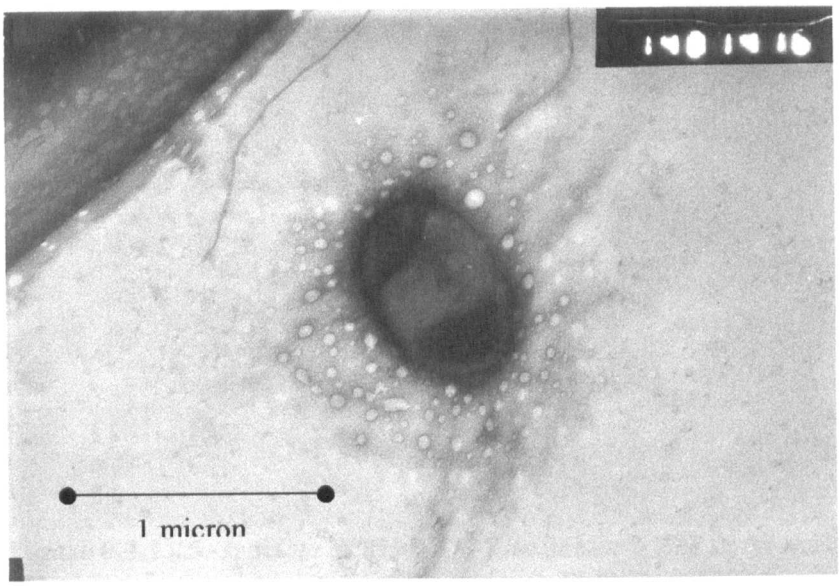


Figure 6.4.11.3. Isolate W2 (14K); notice the micro-droplets of oil surrounding the bacterium

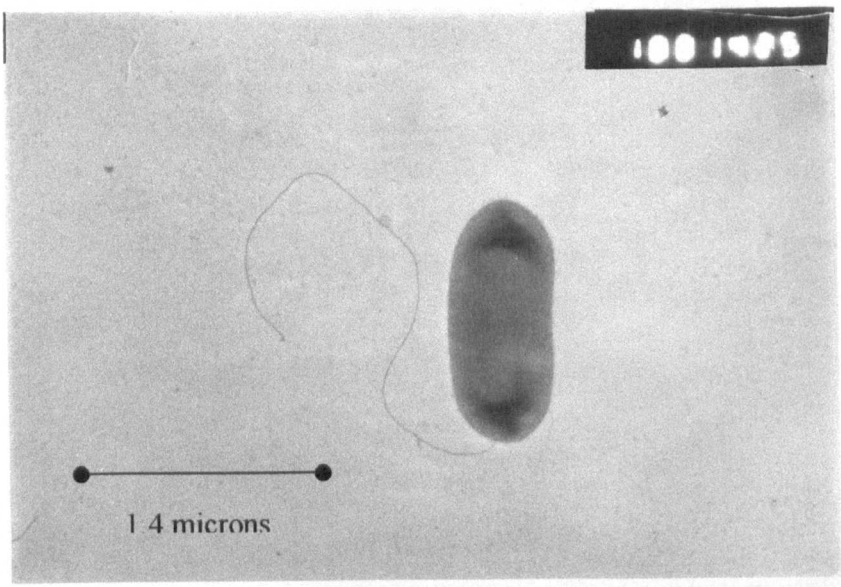


Figure 6.4.11.4. Isolate W2 (10K), with a single flagellum

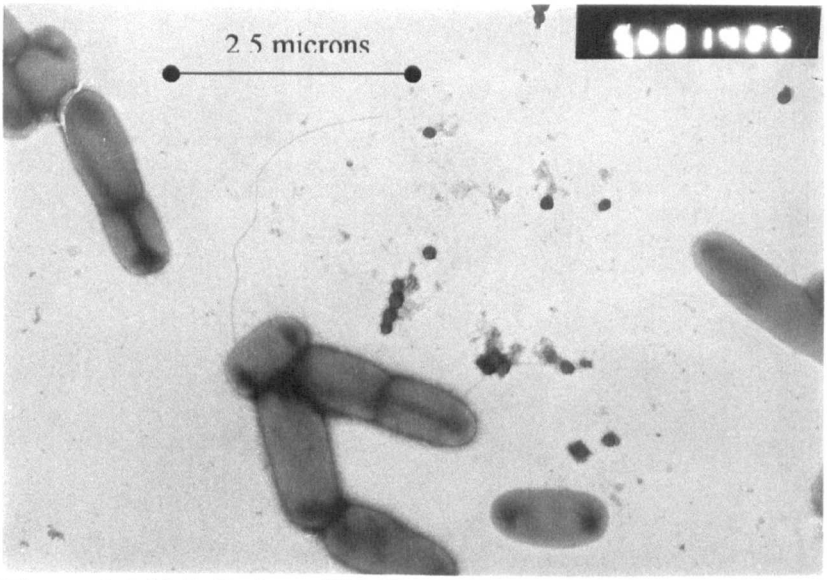


Figure 6.4.11.5. Isolates W2 (5.6K). The bacteria like to form chains or associate as pairs.

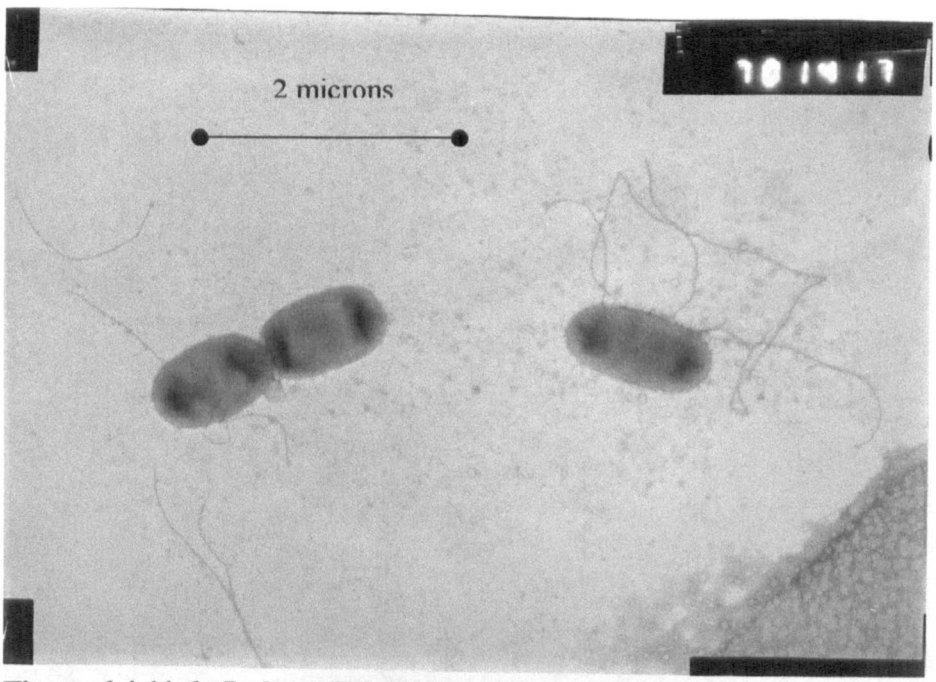


Figure 6.4.11.6. Isolates W2 (7K)

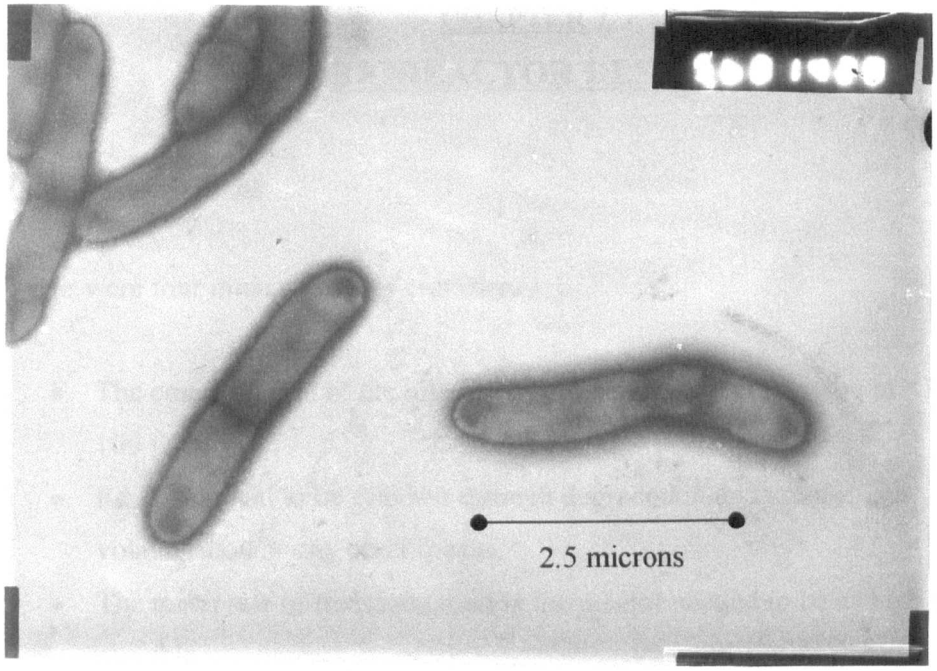


Figure 6.4.11.7. Isolate V2 (5.6K)

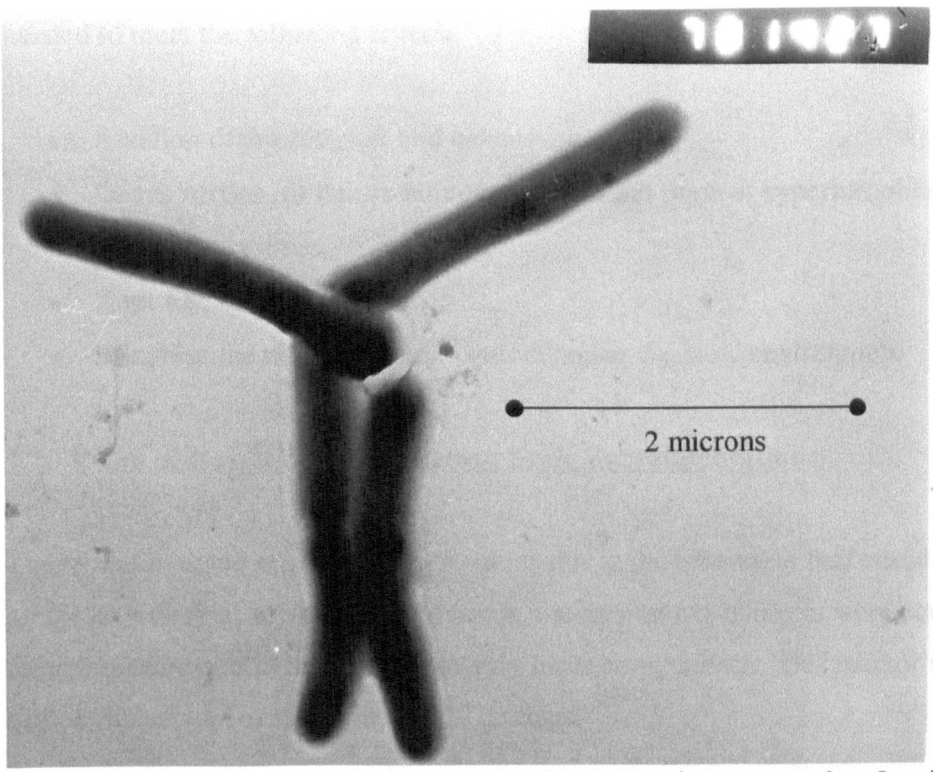


Figure 6.4.11.8. Isolate Y2 (7K). A very large species, over twice the size of J

CHAPTER 7
BIOREACTOR DESIGN

7.1. Considerations

There were four main criteria to consider.

- The concentration of the oil on the cuttings needed a reduction to <1% v/v or 100 ppm.
- Reduction had to be effected through degradation and not through volatilisation or any other means.
- The mean rate of transformation in the reactor needed to be as high as possible.
- The ability to suspend and aerate high solids concentrations.

Based on prior experimentation and the literature the design of the bench scale reactor needed to meet the following criteria.

- Aeration of the cuttings, and exhausting of air.
- Gentle mixing, to ensure homogenisation, but prevent hyperturbobiosis.
- Sampling facilities.
- Kept free of contamination.
- Minimise the risk of bacteria contaminating the local environment

7.2. Types of Reactor for Laboratory Scale Experimentation

The research aimed at utilising a type of reactor in the laboratory that could be scaled up for an industrial application. However, the earliest experiments were conducted using a reactor system made up from very basic components. This reactor was used prior to the arrival of the genuine drill cuttings.

7.2.1. First Basic Reactors

Continuously stirred aerobic bioreactors were designed using four 5 l plastic buckets with lids. These were chosen because they pose no toxicological hazard to the bacteria, were readily available, and the indentation in the bottom of the bucket served to hold the aeration system. The lids kept them fairly free of contamination, although they were not perfect in this respect.

The mixing system consisted of a stainless steel impeller and a motor/gearbox, designed and manufactured in-house by utilising a power screwdriver, modified to hold the impeller, adapted for mains power and suspended via a clamp. The rotational speed was 10 rpm.

Air was delivered into the reactor using perforated tubes placed at the bottom of the reactor vessel, just below the impeller. Two variable speed aquarium pumps provided 60 l/minute each into the four reactors.

Figure 7.2.1.1. The first basic reactor test set-up

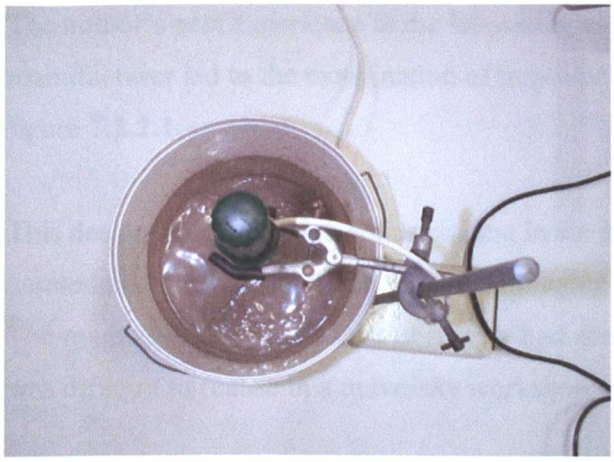


Figure 7.2.1.2. The mud and simulated drill cuttings being stirred in the reactor

7.2.2. The Reactor Design for Testing of the Isolates and *Rhodococcus*

Looking at all the parameters required for the bioreactor, the problems associated with the first design and the condition of the contaminant, i.e. the hydrocarbon locked into a rock and clay matrix, a change of design was required.

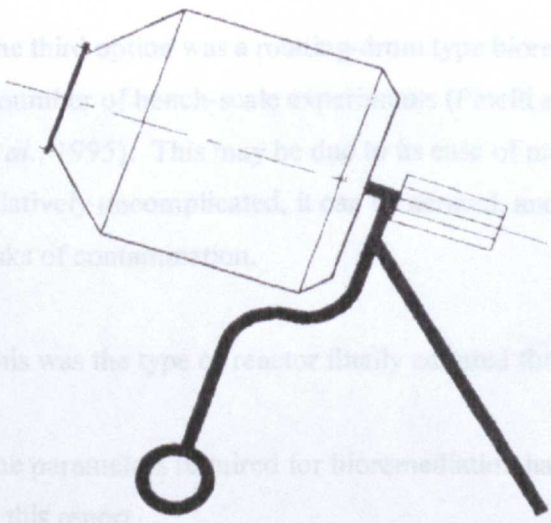


Figure 7.2.2.1. Bench-scale semisolid-phase bioreactor

The author's past experience in the laboratory of a construction materials manufacturer led to the examination of bench-scale mixers of the type illustrated in figure **7.2.2.1.**

This design allows for high solids content in the mixture whilst keeping the cuttings aerated and thoroughly mixed without damaging the bacteria by hyperturbobiosis. The manufacturers of this type of reactor had gone into liquidation, and the design was difficult to realise in a university workshop.

The second option was a domed bottom tank with a stirrer modelled on a marine propeller. This shape of propeller can rotate at slow speeds, induce better mixing than a flat blade and eliminate the shearing problems that can lead to hyperturbobiosis. The propeller would have gone into the reactor at an angle for improved mixing, and the design should have managed solids content of ~40%.

This design also proved difficult to realise practically, with problems encountered in purchasing the tank and the challenges of keeping the system free from contamination and from contaminating the laboratory environment.

The third option was a rotating-drum type bioreactor. This design has been adopted in a number of bench-scale experiments (Pinelli *et al.*, 1997; Kruger *et al.*, 1995; Truax *et al.*, 1995). This may be due to its ease of manufacture; furthermore, scaling up is relatively uncomplicated, it can be aerated, and measures can be taken to reduce the risks of contamination.

This was the type of reactor finally adopted for this research.

The parameters required for bioremediation have been discussed in chapters 4 and 5 in this report.

7.2.3. Rotating Drum Reactor

The reactor consisted of a bed with five drive rollers capable of housing four self-contained reactor vessels (see figures **7.2.3.1.** and **7.2.3.2.**).

Figure 7.2.3.1. The reactor vessels on the rolling bed.

Figure 7.2.3.2. The reactor vessels on the rolling bed

The design is illustrated in figures **7.2.3.3., 7.2.3.4., 7.2.3.5., 7.2.3.6.** and **7.2.3.7.**.

The reactors were housed in a laboratory with restricted access due to the potential for biological contamination. There was no external light source for the laboratory, and the reactors were designed to be predominantly dark, with only the end pieces clear to allow some observations. This was because biodegradation can be inhibited by high doses of solar UV-B radiation (Santas *et al.*, 1999).

Note that the air sparge protection system was added after the first drum reactor experiment. There were problems encountered with the material in the reactor being carried up the air pipes, blocking them. The end caps were adapted to help minimise this, by the addition of a Perspex cylinder to keep the mixing material away from the air extraction pipes.

The system was designed for hazard group 2 organisms. It had to ensure bacteria within the reactors could not escape into the external environment and that rogue bacteria could not contaminate the reactors running with single species, i.e. to keep them as isolates. These considerations were taken into account during fabrication.

Figure 7.2.3.3. ***Reactor*** - Plan View
Scale 1:3

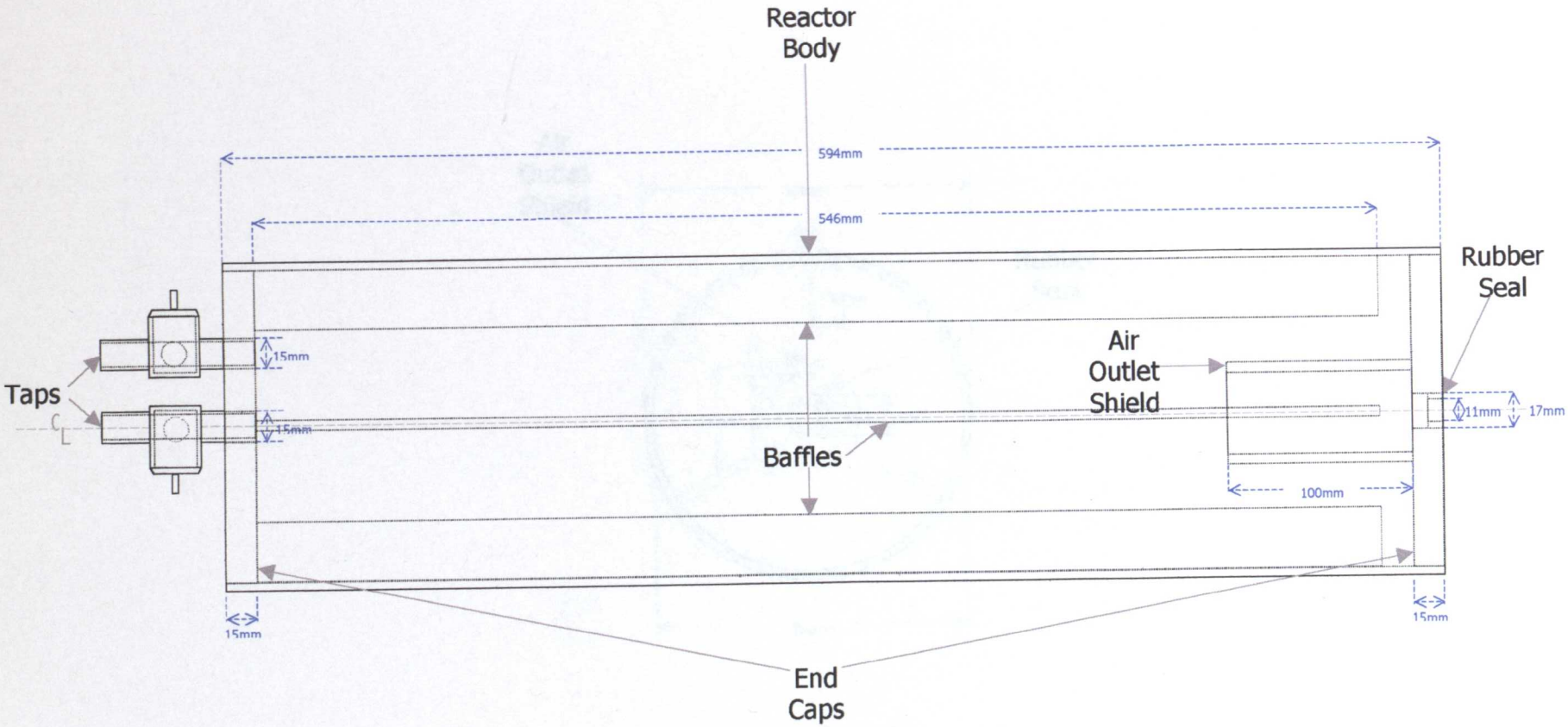


Figure 7.2.3.4. ***Reactor*** – End Elevation (Air Output)
Scale 1:3

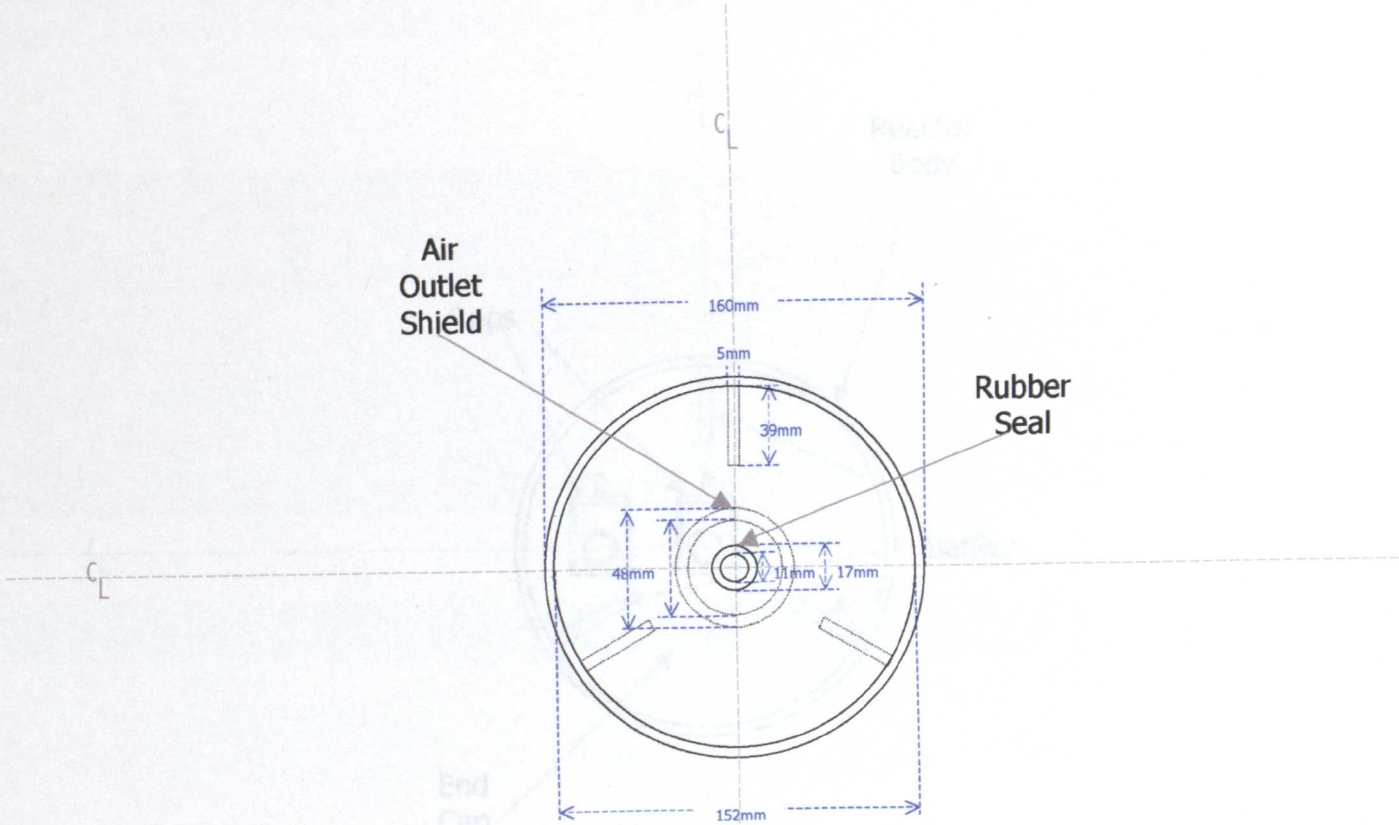


Figure 7.2.3.5. *Reactor* – **End Elevation (Air Intake & Sampling Port)**

Scale 1:3

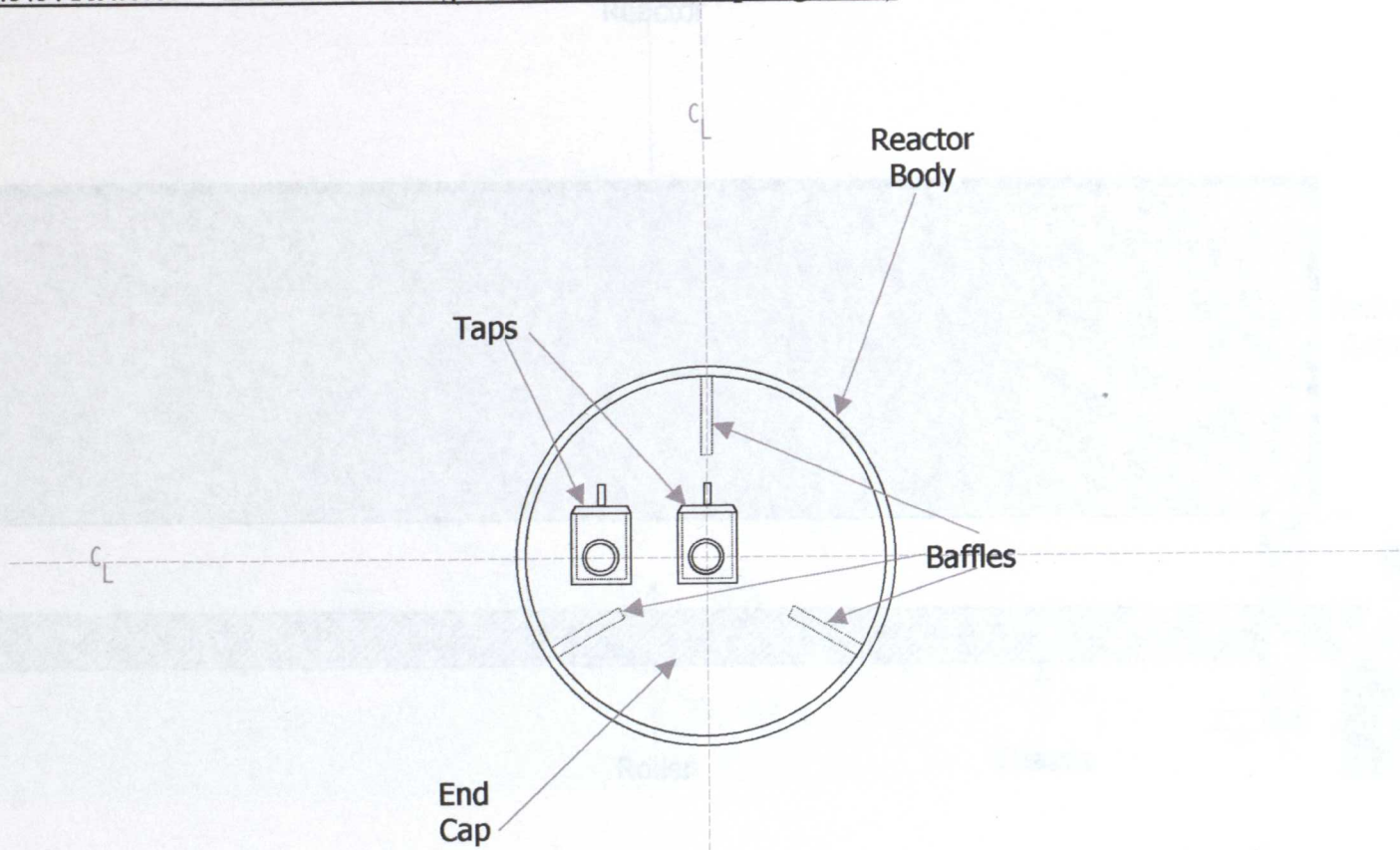


Figure 7.2.3.6. ***Apparatus*** – Diagram Showing Drive Mechanism (Side Elevation)

Not Drawn To Scale

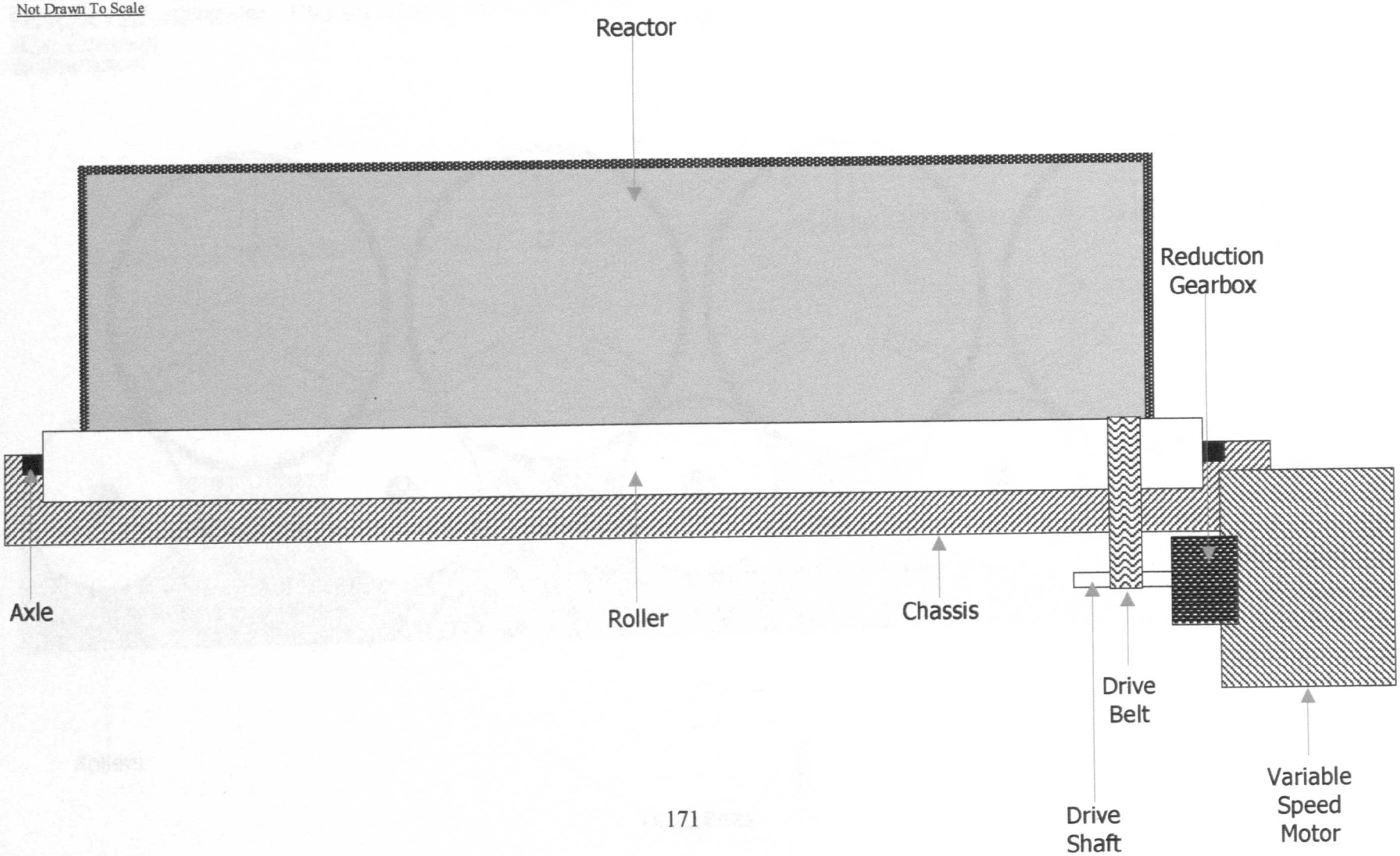
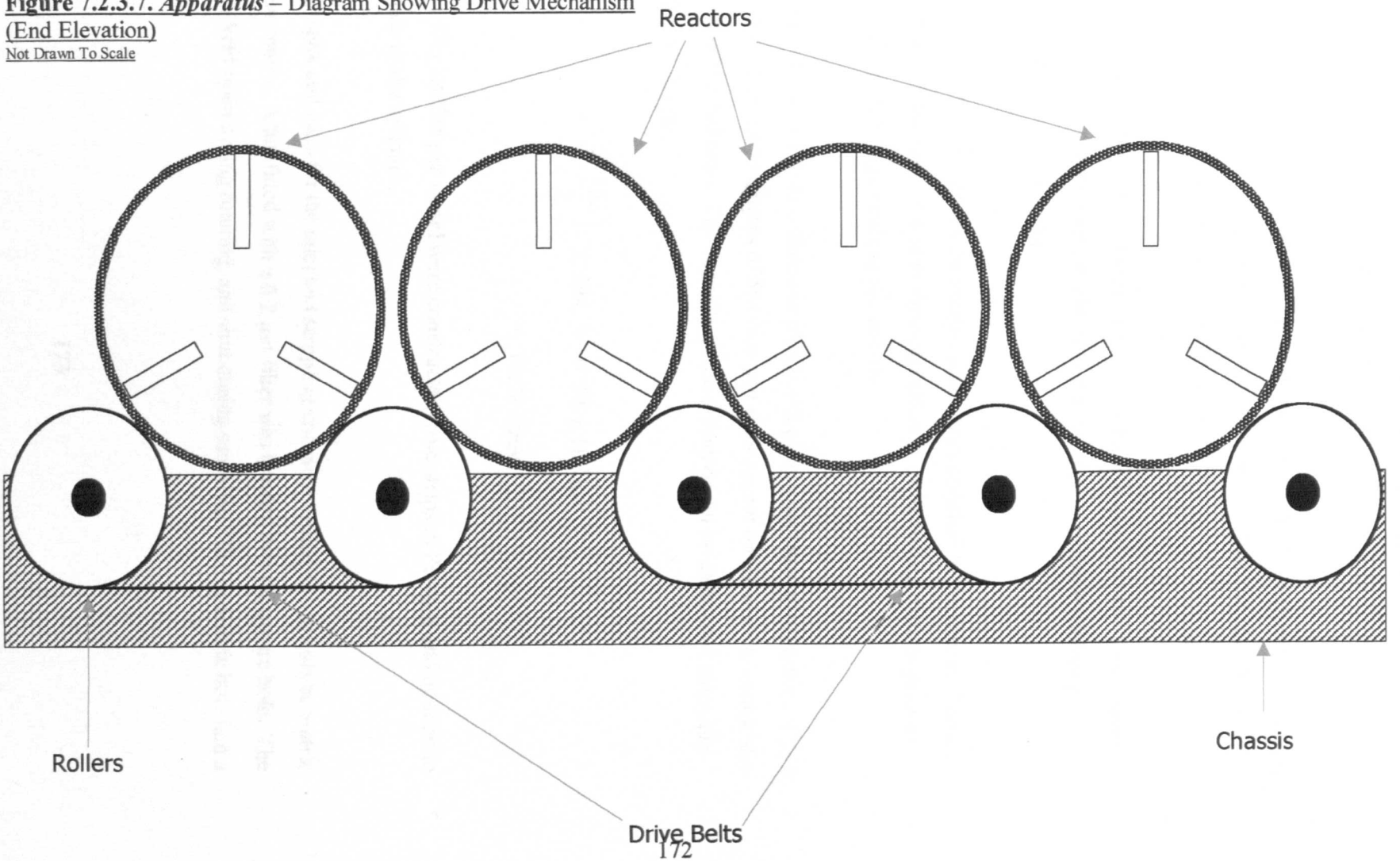


Figure 7.2.3.7. ***Apparatus*** – Diagram Showing Drive Mechanism (End Elevation)
Not Drawn To Scale



7.2.3.1. Fabrication of the Reactor

The chassis was constructed from 25 mm angle and box steel, with a bracket welded on to house a 30W AC motor (mains powered) directly coupled to a reduction gearbox giving a final drive of approximately 4 rpm.

The rollers were stock items consisting of spindle roller bearings supporting a rotating outer cylinder. There were grooves in the outer cylinder to take the drive belts, and roller ends which slotted into the top of the frame.

The drive belts were a vacuum cleaner type, but these proved to have a very short lifespan. Consequently custom-made belts that were constructed by splicing elasticated cord replaced them.

Perspex brackets to clamp the air suction pipes were attached to the frame. The air pipes went into the reactor vessels through rubber seals in the end caps to prevent bacteria either entering or exiting the vessels.

The cylinders were 160 mm diameter plastic soil pipes with internal diameters of 152 mm. They were cut to lengths of 594 mm and chamfer cut on the ends to enable the insertion of the end caps. The capacity of the cylinders can be calculated using the equation πr^2.length.

$$\pi \times \left(\frac{152}{2}\right)^2 \times 594 = 10.78 \times 10^{-3}\ m^3$$

$$= 10.78 \text{ litres}$$

Three lifting baffles per vessel were constructed from strips of Perspex and bonded to the inside of the cylinder.

The Perspex end caps for the inlet and sampling end were drilled as one hole eccentric and one centre. A tap fitted with a 0.2 μm filter was bonded into the centre hole. The tap was kept open during running, and shut during sampling. The eccentric hole had a

tap bonded into it that served as the sampling port. The air outlet end cap had one hole drilled in the centre, and was fitted with a rubber seal. The air pipes entered the vessel here. Both end caps press-fitted tightly into the ends of the cylinders and were secure.

Once constructed the reactors were tested for leakage by filling with water. There appeared to be none.

CHAPTER 8
BIOREACTOR EXPERIMENTS

8.1. 1st Remediation Experiment – Continuously Stirred 5 l Vessels

The system was set up as described in **7.2.1.** and illustrated in figure **7.2.1.1.**.

Drill cuttings were simulated by combining limestone chippings with Novatec drilling fluid; the oil:water ratio in the mud was 83:17, and the cuttings were set to ~7.5% v/v oil on cuttings (for data see appendix E).

The bacteria used were *Rhodococcus* sp 11273 (R1 and R2) and an unidentified strain from the first set of isolations, henceforth known as X (X1 and X2).

The bacteria were revived from the freezer as described in chapter 6. They were streaked on a plated and left at ambient temperature for 48 hours for examination. Further plates with an oil film and mud film were made concurrently.

Three of the reactor vessels were inoculated; R1; X1; R2 and X2 together; the fourth was kept as a control.

Sampling was conducted on days 1, 2, 3, 4 and 12, and frozen, with additional samples for retort analysis on day 12.

150 ml of distilled water was added to each reactor vessel on day 4 to prevent excessive dehydration.

Extraction was by soxhlet with dichloromethane for 2 hours; water was separated after extraction using a separatory funnel and purified with anhydrous sodium sulphate, then evaporated down from 100 ml to 2 ml.

The samples were run through the GC (see section 6.2.1.2). The analysis methodology was still being developed at this stage, so the readings were interpreted in two ways, as recommended by K Wallgren (Pers. Comm., 1999).

8.1.2. GC Methods of Evaluation

1. The compounds in each sample were divided into groups with the following retention times:

0 – 5	(>Carbon 10, short chain length)
5 – 10	(~ C_{10})
10 – 12.5	(~ C_{12})
12.5 – 15	(~ C_{14})
+15	(> C_{16})

The total percentage areas in each group was calculated and tabulated, see tables in appendix E.

2. Four areas with pertinent peaks common to all the analyses eluted within the 5^{th}, 11^{th}, 14^{th} and 15^{th} minute of the run. Total area of peaks within each minute were tabulated against the dates of sampling, see tables in appendix E.

8.1.2.1. Assumption

The assumption was made that the longer chain hydrocarbons would remain fairly constant, with the shorter ones being degraded first. This provided a constant value to relate the rest of the data to.

All the values in the table, including the total areas, were divided by these constants and the results recorded. The internal standard had not been used at this stage in the experimentation.

8.1.3. Results

8.1.3.1. Plates

The *Rhodococcus* was slow to adapt to the ambient temperature, the X was much quicker. The plates with oil and drilling muds showed no evidence of growth.

8.1.3.2. GC and Retort Results

The results illustrate that the reactor containing *Rhodococcus* sp 11273 reduced the oil better than the other reactors. The tables in appendix E show some evidence of an increase in short chain length hydrocarbons, but is inconclusive. The production of these shorter-chained hydrocarbons imply that some microbial activity was taking place degrading some longer-chained hydrocarbons within the 10 – 12.5 minute elution time (C_{12}). All the vessels showed an increase in the 12.5 – 15 minute elution time (C_{14}). The R1 reactor was placed next to the control; there is no guarantee, with the set up of the system, that bacteria did not contaminate the control reactor.

The magnitude of the reduction is not given by using this method of evaluation. For example, if the reduction in total hydrocarbons was from 6 ppm to 2 ppm, the percentage change would be 66.66%; this percentage would be identical for a reduction from 60 ppm to 20 ppm. This means a small change in concentrations could be interpreted as a large reduction, and vice versa.

Due to some erroneous data on the first day of sampling for R1, it is not possible to get an accurate percentage reduction, but, even discounting this day's data the reduction was 26.71% for the *Rhodococcus*.

X1 appeared to degrade shorter-chain hydrocarbons, with 71.36% reduction in the five-minute elution band, with 36.91% degrading at the rate of 12.3% per day, according to the data. 43.21% at 11 minutes elution and 7.8% at 14 minutes. Overall, it possibly degraded 21.66% of the hydrocarbons. Given more time these results might be enhanced.

The combination of *Rhodococcus* and X did not perform well, but there appeared to be an increase in short-chain hydrocarbons. This might have been due to the cleaving of long-chain molecules.

The control remediated too, by 16.55%.

The assumption for the second set of tables was that the longer-chained hydrocarbons would be degraded first. Although this was true for R2 +X2, it was not proved as an hypothesis.

Although percentages are to two decimal points, they are only indications of reductions due to some experimental errors.

- Adding water to the cuttings, but not knowing evaporation rates in the reactors.
- Evaporation of the extracted solution was not always accurate; it was performed on a hot plate, both inaccurate and risky!
- The oil may have preferentially stuck to a particular size fraction, making the samples unrepresentative.
- The limestone cuttings, only just introduced to the mud before experimentation, may not represent real drill cuttings at all.

The retort did corroborate the trend that all the samples, including the control, underwent degradation. The table in appendix E illustrates reductions from the 7.5% starting value. R1 was the best performer. There could have been some competing in the reactor containing both the bacterial species.

8.1.4. Summary

The reactor vessels were inadequate. There was poor mixing due to the shape, and an extremely high risk of cross contamination due to the positive pressure sparge and no mist extraction system. The mixture was not homogenous, making sampling difficult.

At this stage the GC method of analysis had not been developed for this project; this experiment illustrated some of the pitfalls in sample preparation and data interpretation. There were definitely problems caused by the lack of control over the evaporations, as volumetric reduction would have had a significant influence over the concentrations in the residues and therefore the GC results.

8.2. 1st Rotating Drum Bioreactor Experiment: Method

8.2.1. Preparation of Bacteria for Remediation Testing in the Bioreactor

The bacteria chosen for the initial bioreactor experiment were:

- J (*Bacillus oleronius*).
- A, D (*Bacillus thuringiensis*) and other species isolated from the V, W, Y and Z flasks, as a consortium.
- *Rhodococcus* sp. 9737.
- Control, with natural micro-flora.

The *Rhodococcus* was purchased freeze-dried from the National Collections of Industrial, Food and Marine Bacteria (NCIMB) Ltd, and had to be resuscitated; details of this procedure are in appendix C. The species was classified for Laboratory Containment Level 1 (see appendix F). *Rhodococcus* sp. 9737 was described in the UK National Culture Collections database as being able to utilise various paraffins, which is why it was chosen. A known remediator would allow comparison of the isolate, consortium and control, and would give an indication as to the effectiveness of the bioreactor. The other species were treated as Laboratory Containment Level 1 / 2 (see appendix F).

The *Rhodococcus* was resuscitated in LB broth and incubated at 29°C on a shaker; it exhibited a lengthened lag period, but the broth turned turbid after 48 – 72 hours. Once it was established it was rebrothed and plated out to ensure a pure culture before

enrichment procedures. A sample was prepared for preservation by freezing (see section **6.4.6.**). The second broth appeared well established within 24 hours, and was prepared for the bioreactor experiments in the same manner as the other species.

The bacteria were prepared as for the batch cultures for the pre-screen tests. This involved introducing the broths of culture, which were first grown in mineral media with oil, into a 250 ml flask containing 2 g of drill cuttings and 25 ml of mineral media. After 48 hours, further mineral media and drill cuttings were added, to make the totals in each flask half media to cuttings (66ml cuttings: 33 ml mineral media). These were left at ambient temperature (22 – 24°C) for one week. The contents were then added to the drill cuttings and sterile nutrient water in the bioreactors.

8.2.2. Preparation of Material for the Reactor Vessels

The drill cuttings were from Stirling Pegasus and were dated 14/7/99 and 15/7/99. They had already been thoroughly mixed to ensure homogenisation and frozen, as mentioned in section **6.4.5.**, for storage. After defrosting the cuttings specific gravity (SG) was tested.

With highly particulate drill cuttings the density was measured by displacement in water, reading off the volume displaced by a mass of cuttings introduced into measuring cylinder. The wet cuttings from the Sterling Pegasus could have their density measured quite directly, i.e. by filling a measuring cylinder to an exact volume and weighing. If 50 ml of the mud weighed 68 g, that would mean 136 g would equal 100 ml. Scaled up, the mud SG would be 1.36. All tests were repeated.

Another way was to fill a container with exactly 50 ml of drill cuttings, then top up with water, to 50 ml. The water fills in any air gaps in the container. The SG of water is 1; the difference between 50 g and the weight of cuttings with water indicates the approximate SG of the drill cuttings. The process has to be repeated and an average taken.

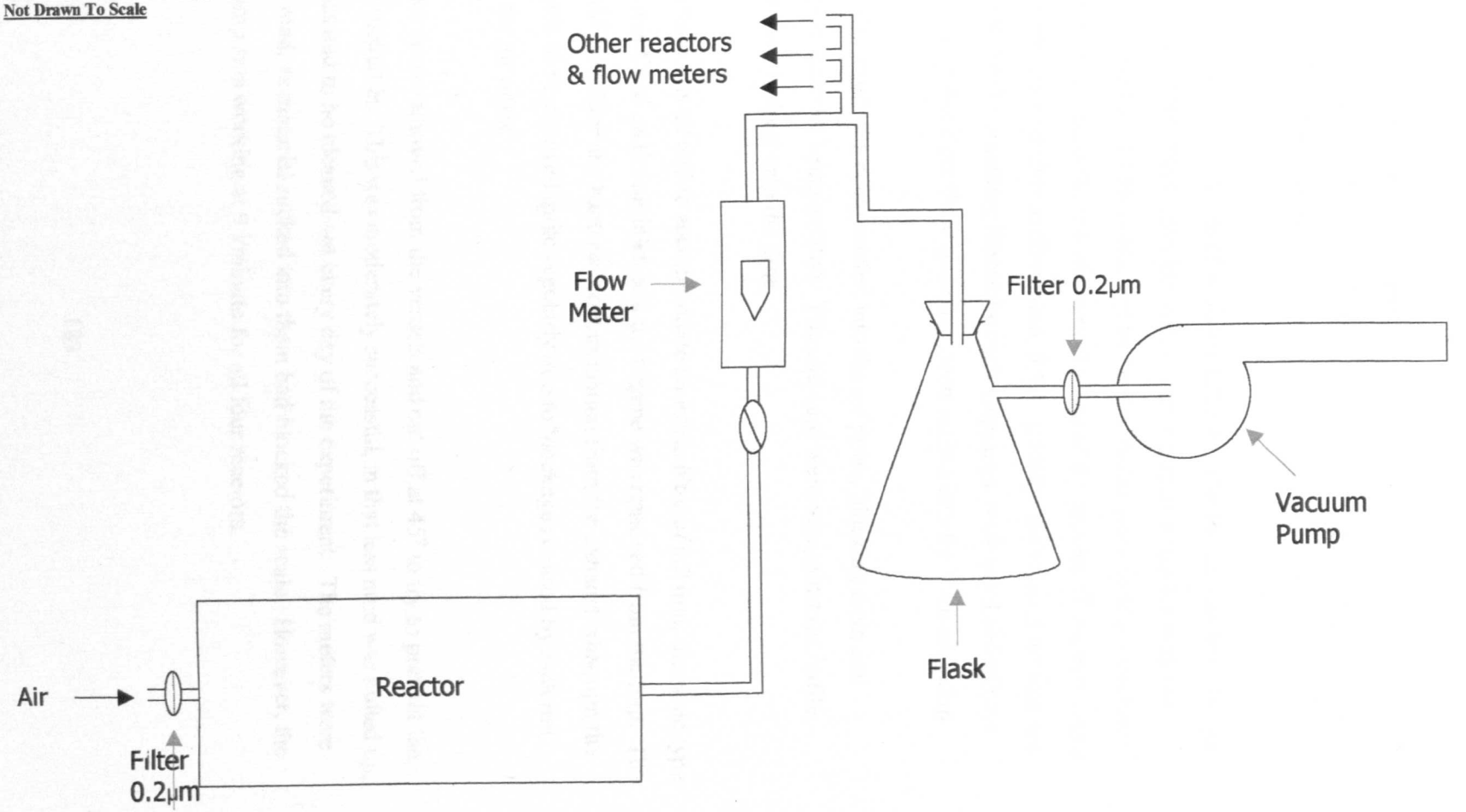
Assessing the SG allowed the material to be assessed as a volume for percentage oil on cuttings, but be weighed for adding to the reactors, which was easier than trying to measure the cuttings as volume.

The cuttings had a SG of 1.734; this lead to 1734 g of cuttings going into each reactor vessel, which equalled a litre of cuttings.

The liquid addition to the vessels was sterile nutrient water. The mineral media, as described in section **6.4.2.3.**, was made up in bulk and added to the vessels. This media worked well for the pre-screen experiments, having a good balance of nitrates and phosphates with additional minerals, so was used for the reactor experiments. 500 ml was added to the vessels, which gave a 2:1 cuttings to fluid ratio.

Within 24 hours, the material had gone extremely viscous and very sticky; this could possibly have been due to clay hydration or alternatively to the mud reaching a critical moisture level which lead to the plastic-like behaviour. It was decided at this point to add another 500 ml of sterile water to increase the flow properties and stir-ability of the material.

Figure 8.2.3.1. ***Apparatus*** – System Diagram
Not Drawn To Scale



8.2.3. The Reactor System

There was 1 l of cuttings and, after the additional nutrient water, 1 l of water, making 2 l in the 10 l vessel. The proportions of water/solids/hydrocarbons at the outset was:

1300 ml water	65%
520 ml solids	26%
180 ml hydrocarbons	9%

The system was set up as illustrated in figure **8.2.3.1.**, with the exception of the air cleaning system. An exhaust cleaning system was set up as a trap for material accidentally sucked up the air tubes, and to sterilise the exhaust air to prevent any contamination of the laboratory environment – one of the species of bacteria was a known bioinsecticide (details in discussion, **9.1.**). Initially, the exhaust air was run into a Buchner flask containing limestone rock-chippings, rock-wool and trigene. Within 24 hours foam from the trigene had been sucked into the vacuum pump.

Also, too much mud was being sucked into the air pipes, blocking them and preventing the material being aerated. The end caps were up against the baffle, causing the mud to drop onto the pipe.

The trap system was altered to accommodate an inline filter of 0.2 mm, the same type of filter as was fitted on the air inlet, and the trigene was removed from the trap. The system could then filter any bacterial contamination from the exhaust, although the filter did have to be changed quite regularly due to blockages caused by material sucked up the air pipes.

The air pipes were removed from the vessels and cut off at 45° to try to prevent the mud being sucked in. This was moderately successful, in that less mud was pulled up, but the pipes had to be cleaned out every day of the experiment. The meters were difficult to read, as material sucked into them had blocked the scale. However, the vacuum pump was working at 9 l/minute for all four reactors.

The calculations for oxygen were evaluated using the largest hydrocarbon molecule in abundance in the linear paraffin, C_{14}. There were traces of longer chain carbon molecules, but only in small quantities, see GC trace **6.2.7.1.**.

8.2.3.1. Air Requirements

1 l drill cuttings = 1.7 kg (as m = ρ.V)

Contamination 18% Oil on cuttings

(18/100) x 1.7 = 0.306 kg oil

$$C_{14}H_{30} + \frac{43}{2} O_2 \longrightarrow 14CO_2 + 15H_2O$$

Relative molecular mass of $C_{14}H_{30}$ = 14(12) + 30 = 198

No of moles of $C_{14}H_{30} = \frac{306}{198}$

The molar ratio:

$$C_{14}H_{30} + \frac{43}{2} O_2 \longrightarrow 14CO_2 + 15H_2O$$

$$\frac{306}{198} : \frac{43}{2} \times \frac{306}{198} : 14 \times \frac{306}{198} : 15 \times \frac{306}{198}$$

Volume of oxygen required:

$$\text{Mass of oxygen} = \left[\frac{43}{2} \times \frac{306}{198}\right] \times 32 = 1063.27 \text{ g}$$

$\rho_{oxygen} = 1492 \text{ g/m}^3$

$$1063.27 \times \frac{1}{1492} = 0.71 \text{ m}^3$$

Air is 21% oxygen = 3.38 m^3 of air per reactor.

Reactors will run for a maximum of 28 days, minimum of 21 days

$3.38 \times \frac{1}{28}.\frac{1}{24}.\frac{1}{60} = 83.83 \times 10^{-6}$ m^3 air per minute for mineralisation.

Worst-case scenario might be only 3% of the air used for degradation:

$(83.83 \times 10^{-6}$ m^3 / 3) x 100 = 2.79×10^{-3} m^3 air per reactor per minute for 28 days at 3% uptake.

Convert to litres = 2.79 litres per minute per vessel.

If 5% of the air is used for degradation:

$(83.83 \times 10^{-6}$ m^3 / 5) x 100 = 1.68×10^{-3} m^3 air per reactor per minute.

Convert to litres = 1.68 litres per minute per vessel.

This equation illustrates that at 3% utilisation there might not be quite enough air for complete mineralisation in 28 days, but enough for a considerable reduction. Considering the problems with the air pipes blocking, the lack of electron acceptor may have been a limiting factor to biodegradation.

8.2.3.2. Running Conditions

The reactors were set to turn at 4 rpm.

The temperature was kept at ambient, which was mostly around 20°C. This temperature is warm enough for good microbial activity with both the *Bacillus* species and the *Rhodococcus*.

The pH was tested, and found to be 7.5 ± 1.

Figure 8.2.3.2.1. The running reactors

The experiment ran for 28 days, with sampling via the tap conducted weekly and the sample frozen at -5°C.

At the end of the 28 days there were no large drill cuttings whatsoever. The material was smooth enough to measure its density directly; 50 ml weighed 68.6 g. As 20 ml was required for the soxhlet, 27.5 g of the material was weighed and mixed with a similar quantity of anhydrous sodium sulphate.

These, and some of the frozen samples, were prepared and solvent extracted as described in section **6.2.2.**, and prepared for GC as described in section **6.2.1.** at the end of the experiment.

8.2.3.3. Problems Encountered with the Reactor System Set-Up

The problems with air and exhaust gas have been discussed at the beginning of this section. There were additional problems with the system.

Sampling was difficult using the tap due to the viscous nature of the mud. However, samples were extracted and the tap was cleaned to keep contamination down to a minimum.

Testing viscosity was a major problem, as the H&S regulations at the university forbade the reactors being opened in the laboratory and the material being bench tested out in the open. It would also have allowed external micro-organisms into the reactor vessel material, or, alternatively, reduced the amount of material in the reactor to a ridiculously low level if the test substrate was not reintroduced to the vessel. The decision was taken not to add extra water; this would reduce changes of the parameters within the system.

As time went on the material started to build up on the reactor walls. This could have been due to a lack of suspension of the material; a sedimentation effect.

At the end of the 28-day cycle, all the material was emptied out of the vessels into a mixing container. The mixture was not homogenous. There was some wet and some cake material. This was mixed up before sampling, but the end of the run was the only opportunity to do this. The material looked as if it was trying to separate all the time, having a watery phase and a more solid phase in the mixing container. The mud was thoroughly mixed and samples taken for analysis. However, when trigene was added, the material changed appearance rapidly, with the tendency to separate gone, becoming an homogenous material. Trigene contains NP9 detergent and quaternary ammonium salts; these salts make the mixture homogenous, ensuring that the biocide contacts the bacteria (www.medichem.co.uk/environmental_products.html). The addition of trigene instantly kills all the bacteria in the material. This may also have some bearing on the change of behaviour of the material.

The residual material was disposed of in accordance with the university H&S procedures.

The original hydrocarbon and water content of the cuttings for this experiment was assessed using the retort, and was ~18% oil, ~30% water.

8.2.4. 1st Rotating Drum Bioreactor Experiment: Results

The vessels were coded as:

1. *Rhodococcus*
2. Control
3. Consortium
4. J

The vessels containing inoculums of bacteria performed better than the control containing natural flora.

The results were calculated by assessing the oil content at the start of the experiment, using the GC, and again at the end of the experiment. The start/end data were then compared in each reactor individually.

The best remediator was the *Rhodococcus*, which reduced the oil content by 65.6% in 28 days; at 18 % original content, there was 6.2% oil left on the cuttings. That does not include the dilution factor of the water, as that would be removed by post-reactor treatments.

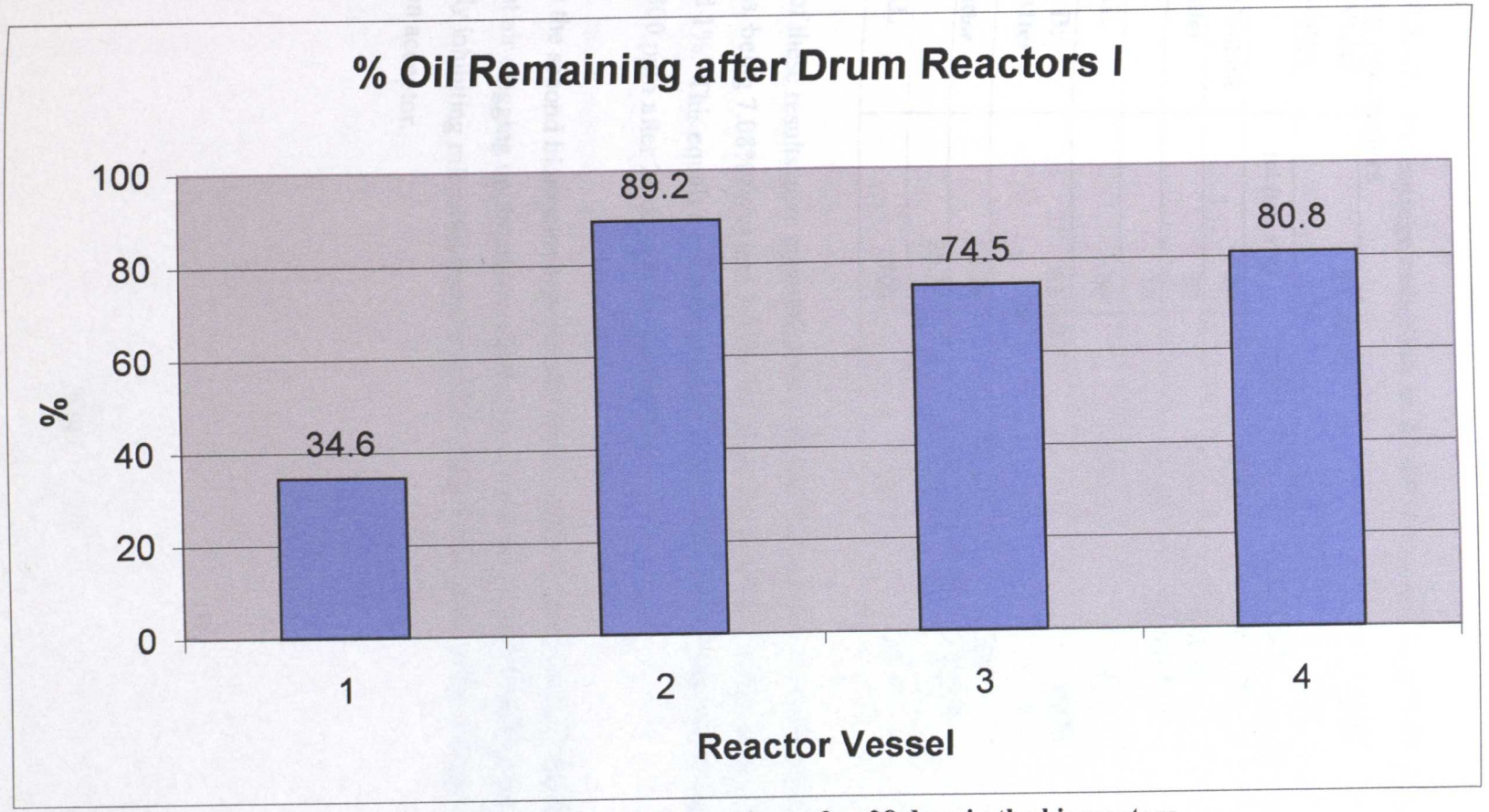


Figure 8.2.4.1. Graph of percentage oil remaining on the cuttings after 28 days in the bioreactors

The novel isolate, J, did not perform as well as hoped; the oil content was only reduced by 19.2%, leaving the oil on cuttings at 14.5%. The consortium was slightly better, reducing the oil by 25.5%, leaving oil on cuttings at 13.4%.

Data for the *Rhodococcus* bioremediation was processed, and can be seen in appendix G, giving the following results.

Table 8.2.4.1. Percentage reduction in the *Rhodococcus* bioreactor using differing parameters

As Whole Cuttings	START %		END %	
	ml (i.e. v/v)	g (i.e. w/w)	ml (i.e. v/v)	g (i.e. w/w)
Water Content	30	17.20	33.99433	18.18
Dry Matter	52	74.54	58.92351	78.79
HC	18	8.26	7.082153	3.03
TOTAL	100	100.00	100	100.00
As Dry Matter	START %		END %	
Dry Matter	74.29	90.02	89.27	96.30
HC	25.71	9.98	10.73	3.70
TOTAL	100	100	100.00	100.00

None of these results gave encouragement for the prospect of an industrially viable process, being 7.08% (v/v) and 3.03% (w/w) on the original cuttings; both over the desired 1%. This equals a reduction from 180,000 ppm on the original cuttings down to 70,800 ppm after 28 days in the bioreactor.

Before the second bioreactor experiment, the air sparge system was redesigned to prevent air clogging up the pipes, which was a major problem during the first tests, possibly inhibiting microbial transformation of the hydrocarbons due to lack of electron acceptor.

8.3. 2nd Rotating Drum Bioreactor Experiment: Method

The first modification to the design was to make a cover for the air pipes to prevent the mud material landing on the pipe and being sucked up, blocking the air flow; it can be seen in the diagrams of the reactor design. This alteration solved the restriction of air, resulting in a more adequate airflow, which should have reduced the problems associated with lack of oxygen.

Bacteria were prepared as in section **8.2.** The control and *Rhodococcus* were as before although the *Rhodococcus* was revived from the freezer rather than from freeze-dried. Two different isolates were tried for the other reactors: V and W, both of which had performed fairly well in the batch tests. Y performed well too, but it was hoped to utilise this in a third reactor experiment, if it responded to revival after freezing. The enrichment procedure was altered slightly to accommodate the change in drill cuttings to fluid ratio in the reactors, and was 50/50 drill cuttings to mineral media.

The materials were the same, and prepared as described in 8.1.2. The running procedure was as described in 8.1.3.2. The enrichment flasks, drill cuttings and sterile mineral water were added to each vessel, and left to run for 24 hours. A sample was then extracted from the sampling tap, with difficulty. Attempts were made to take samples weekly; the difficulty in extracting these got worse over time, until it was almost impossible to guarantee a representative sample. When the reactors were switched off, the material was extracted and mixed as described for the 1st drum reactor experiment and samples taken for analysis.

The samples were prepared for extraction and GC as described in the first drum reactor experiment.

8.3.1. 2nd Rotating Drum Bioreactor Experiment: Results

The caking was considerably worse in this second test. This may be due to the drying effect of the air sparge.

The results were calculated by assessing the oil content at the start of the experiment, using the GC, and again at the end of the experiment. The start/end data were then compared in each reactor individually.

Due to the sampling problems discussed in **8.3.**, the only data examined are the start and end figures. At the beginning the problem was not so acute and at the end the material was mixed in a container before the final sampling. It was very difficult to extract a sample from the sampling tap, and H & S regulations prevented any dismantling of the reactors before the end of the experiment.

The percentage of oil remaining on the cuttings can be examined in figure **8.4.1.** The *Rhodococcus* did not perform as well as in the first reactor experiments, however, the control performed much better than in the first drum reactor experiment. It is difficult to perceive why this should be so. The conditions were identical to the first experiments with the exception of the air system which appeared to be much more efficient, having cured the problem of mud blocking the pipes. This could account for improvements, but not for deterioration in the performance. The worsening result may be a reflection on the physical state of the material, which coated the sidewalls therefore reducing the opportunity for the bacteria to be in intimate contact with the oxygen or even the contaminant. . If the bacteria are trapped in the clays they can only remediate the hydrocarbons they can contact. If the hydrocarbons are absorbed onto the clays, and the bacteria is not a species that produces biosurfactants in sufficient amounts then it would be difficult to get that hydrocarbon into solution. Rate of degradation is a function of the concentration of the contaminant in solution rather than the total sorbed mass.

V and W both performed quite well considering the conditions of the material in the reactor.

Data for the bioremediation performance of V, as the highest reducer of hydrocarbons, was processed as in experiment I for the *Rhodococcus*.

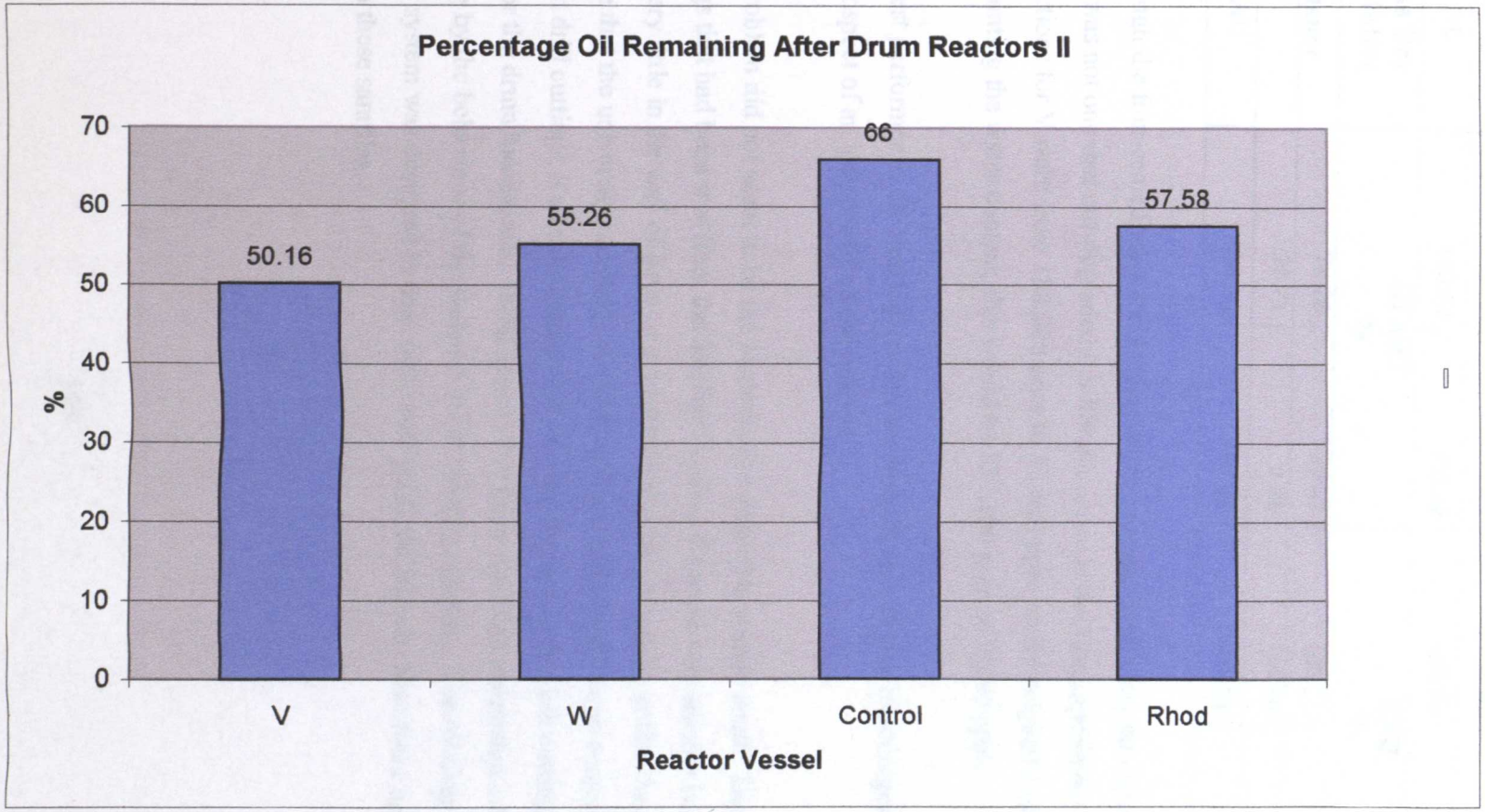


Figure 8.3.1.1. Graph of percentage oil remaining on the cuttings after 28 days in the bioreactors

Table 8.3.1.1. Percentage reduction in the 'V' bioreactor using differing parameters

As Whole Cuttings	START %		END %	
	ml (i.e. v/v)	g (i.e. w/w)	ml (i.e. v/v)	g (i.e. w/w)
Water Content	30.00	17.20	32.96	17.94
Dry Matter	52.00	74.54	57.12	77.74
HC	18.00	8.26	9.92	4.32
TOTAL	100.00	100.00	100.00	100.00
As Dry Matter	START %		END %	
Dry Matter	74.29	90.02	85.21	94.74
HC	25.71	9.98	14.79	5.26
TOTAL	100.00	100.00	100.00	100.00

Although the four-remediation levels were more grouped in this second experiment, there was not one that out-degraded the *Rhodococcus* in the first experiment. Reductions for V were from 180,000 ppm to 82,600 ppm on the original cuttings. Discounting the water content, this would be 257,100 ppm to 9,980 ppm.

The best performer in the slurry-phase bioreactor still did not give encouragement for the prospect of an industrially viable process.

The problem did not seem to be the bacteria, but rather the system itself. The drill cuttings that had been sent from the Sterling Pegasus did seem very smooth in texture, with very little in the way of large or even medium rock chippings, unlike the first sample that the university received. As size fractions had already been conducted on the first drill cuttings, it was decided to test the size fraction in the drill cuttings being used for the drum bioreactors. It did appear that there was high percentage of clay, judging by the behaviour of the material in the rotating reactors. The rotating drum reactor system was designed to deal with more rock and less clay than there appeared to be in these samples.

8.4. Size Fractions in the Cuttings Used in the Rotating Drum Bioreactor Experiments

A sample of drill cuttings as used in the experiments discussed in **8.2** and **8.3** was dried for 24 hours at 50°C. The sample was weighed, then dry sieved as described in section **6.3.**.

The results can be seen in figure **8.4.1.**; the graph illustrates the considerable differences in size fraction percentages. The high percentage of clays in the Sterling Pegasus drill cuttings samples could be due to:-

- Different rock formations, e.g. drilling through clays
- Different solids removal systems
- Equipment breakdown on the platform, leading to a higher percentage of fine solids.
-

Whatever the cause, the change in mineralogy had a detrimental effect on the rotating drum bioreactor system as run with slurry. Another approach was required.

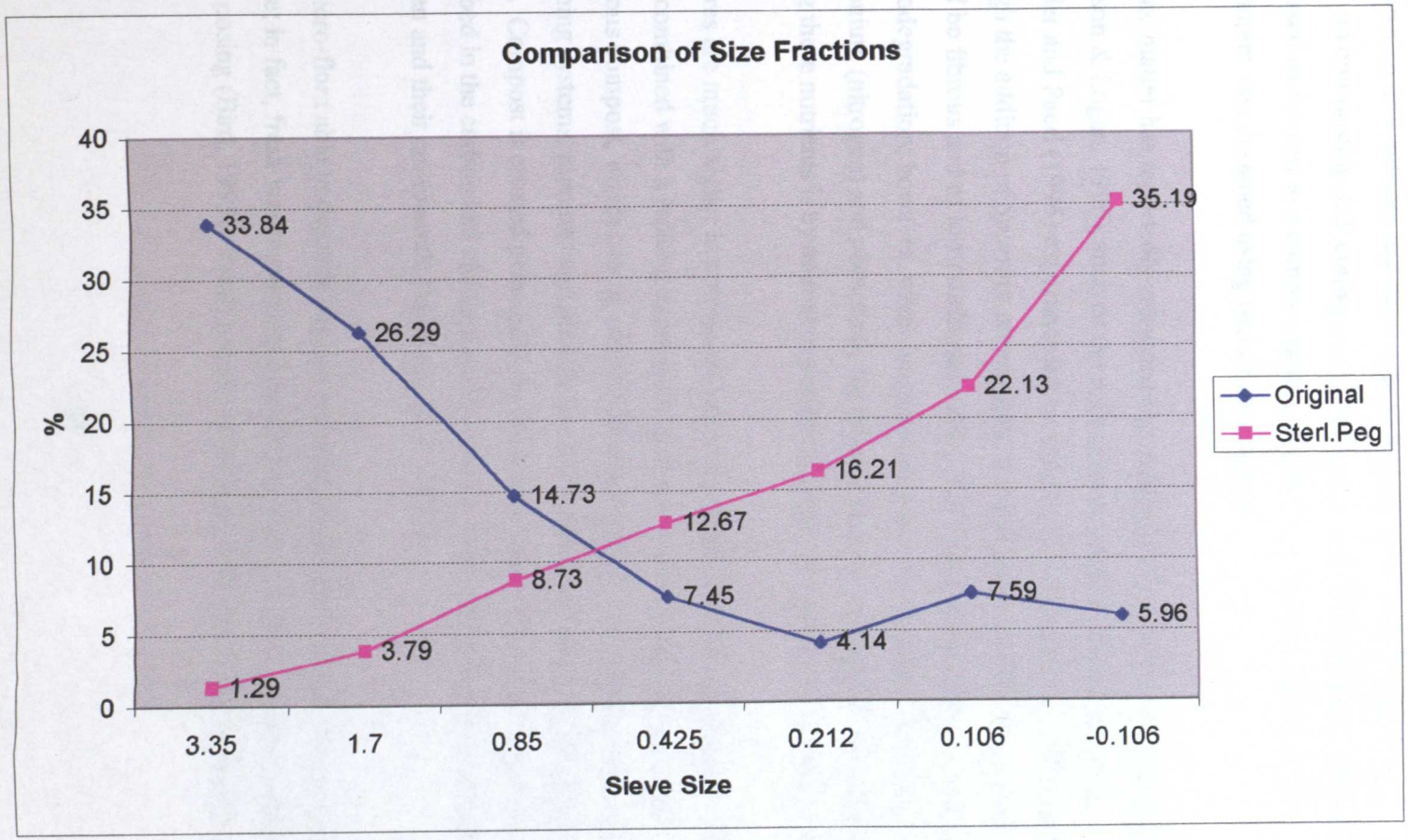


Figure 8.4.1. Comparison of the drill cuttings from the Sterling Pegasus with the original drill cuttings

8.5. 3rd Experiment: Composting

As discussed in sections **8.3** and **8.4.**, major problems had been experienced with processing the high clay content slurries in the small drum reactors. As time was a very finite resource by this stage of the research, it was not possible to redesign and manufacture a different bioreactor. Another approach was to redesign the material within the reactor.

Composting drill cuttings had been utilised in other studies, but there were no reports found on composting drill cuttings in a trommel or drum style reactor. MIDF also expressed an interest in a compost system (Getliff, pers comm., 2001), so an experiment was designed using two of the reactors.

Organic matter has reportedly enhanced the transport of bacteria in porous media (Johnson & Logan, 1996), such as the rock content of the drill cuttings waste. Phiehler and Paerl (1996) experimented on enhanced biodegradation of diesel fuels through the addition of *Spartina alterniflora*, a marsh grass, which, by its nature, would be fibrous and of low nutritional value. The marsh grass alone did not aid in the biodegradation; however, when inorganic nutrients were added, specifically ammonium (nitrogen) and phosphate, the degradation was enhanced. Another way of adding these nutrients is by animal or poultry manures (Singleton & Sainsbury, 1997).

Manures are much higher in nutritional value than would be the marsh grass, and when combined with a bulking agent such as straw or wood-shavings produce nutritious compost, which can be utilised for plant growth. In organic farming and gardening systems, manures can provide the total nutritional needs of the crops and plants. Compost is created principally by the action of aerobic micro-organisms, as described in the carbon and nitrogen cycles – the cyclical interconversion of carbon or nitrogen and their compounds (Madigan *et al.*, 1997).

The micro-flora able to degrade organic material are normally found within fresh manure; in fact, fresh is recommended rather than material that has already started decomposing (Bird, 1993). Fresh poultry manure in particular is recognised as a

powerful activator of microbial activity in compost heaps (Caplan, 1992). The drill cuttings need to be introduced at the beginning of the composting, to allow adequate mixing and to expose the possible degrading species of micro-organism to the contaminant, encouraging them to 'prepare' for that nature of food source, such as functional plasmids and enzymes (more details in chapter 4). The wide and diverse microbial populations within a composting system might tolerate and even degrade the hydrocarbon within the drill cuttings.

The addition of this organic material might also reduce the problems associated with the clays, i.e. the sedimentation causing a build up of material on the vessel walls.

8.5.1. Method

There were two types of manure used for the compost experiments; fresh horse manure (dung and urine) on wood-shavings, and fresh poultry manure on wheat straw.

The mass of material into each reactor was:

1. 2 kg Versaplus Drill Cuttings
   1 kg Horse Manure (as described above).

2. 2 kg Versaplus Drill Cuttings
   1 kg Poultry Manure (as described above).

The dry matter within the manure was assessed to determine whether any additional moisture was required.

1 kg of each type of manure was dried in an oven at 50°C for 72 hours, and reweighed. The retort was used to assess the water and oil content of the drill cuttings. The total moisture content in the reactor containing the horse manure was 55.86% and in the reactor containing the poultry manure was 48.6% (both including the drill cuttings); this was judged to be adequate to support microbial growth.

The system was set up as in the previous experiments, with the exception of the air system. This was altered slightly in that air was pulled through directly to the vacuum pump rather than through the flow meters, and was measured at 9 l/minute for both reactors. A flask to catch particulate material was located before an in-line filter set before the vacuum pump to ensure that the pump was particulate free and bacteria were filtered out of the exhaust air. A Y shaped pipe junction was used to split the air supply to the two reactors.

The reactors were run for 24 days. Any material building up on the sidewalls of the reactor vessels was removed weekly. Temperatures were checked weekly, but this was possibly not frequent enough as a rise in temperature could easily be missed in the interim period.

At the end of the run the reactors were emptied, the balls (see figure **8.5.2.2.1.**) were broken up as much as possible and samples of the mixture were taken for analysis. The dry matter of the material was reassessed to see how much drying out had occurred due to the air system, which ideally might have had some form of water mister. Two 100 g samples from each vessel were dried in an oven at 50°C for 48 hours and reweighed. These were smaller samples and appeared quite dry, hence the shorter drying time.

The material was assessed for density and was found to be ~1; hence 2 x 20 g of material from each vessel was solvent extracted using the soxhlet and dichloromethane. The material was so dry it did not need the addition of anhydrous sodium sulphate. Samples of the dried mud were also extracted, 24.63 g per thimble. Samples were prepared and run through the GC as described in chapter 6.

8.5.2. Results of Composting Reactors

Table 8.5.2.1. Dry matter and moisture content

	Horse		Poultry		Drill Cuttings	
	DM %	Moisture %	DM %	Moisture %	DM %	Moisture %
Beginning	12.42	87.58	34.20	65.80	60	40
End (1, including drill cuttings)	93.60	6.40	82.83	17.17	-	-
End (2, including drill cuttings)	94.45	5.55	78.67	21.33	-	-
Average End	94.00	6.00	80.75	19.25	-	-

The reactor moisture content appeared to reduce considerably in the last 4 days of the experiment; at this high dry matter content, the material would no longer support the majority of microbial growth and activities.

8.5.2.1. Observations

During the whole process the temperature in the reactors never rose more than 2 degrees over ambient, which was 21°C – 23°C; this rise was in the poultry manure reactor. It appeared that there was no thermophilic composting, but as previously mentioned, the temperatures were only taken weekly and this heating activity may have occurred unobserved.

After 96 hours, the contents of the reactor containing the horse manure and drill cuttings had physically changed; some of the material had formed balls, which rattled round as the drum rotated. This indicated the clays had accreted.

Figure 8.5.2.1.1. Horse manure balls

The material in the poultry manure reactor was loose and looked well aerated. Some balls did form in this reactor, but not so many and only after 2 weeks running. The caking of the clays on the sidewalls of the reactors was still occurring but to a much lesser degree than with the slurry system, and was initially worse in the poultry reactor. However, by day 24, there was little caking in this reactor, except where the baffle met the drum wall. This build up could have been reduced by the addition of a fillet.

Figure 8.5.2.1.2. The horse manure and drill cuttings as they came out of the bioreactor.

Figure 8.5.2.1.3. The poultry manure and drill cuttings as they came out of the bioreactor.

8.5.2.2. Retort Analysis

The drill cuttings that were used in the compost experiments were tested for oil and water content. The 50 ml cylinder was filled as described in section **6.2.6.1.**, and weighed 80 g. The retort showed that there was 40% water and 20% oil on cuttings. Assuming the water SG was 1 and the oil SG was ~0.8, the SG of the dry cuttings was 2.5.

8.5.2.3. Bioreactor Remediation Results

To calculate the remediation of the compost reactors, some assumptions were made.

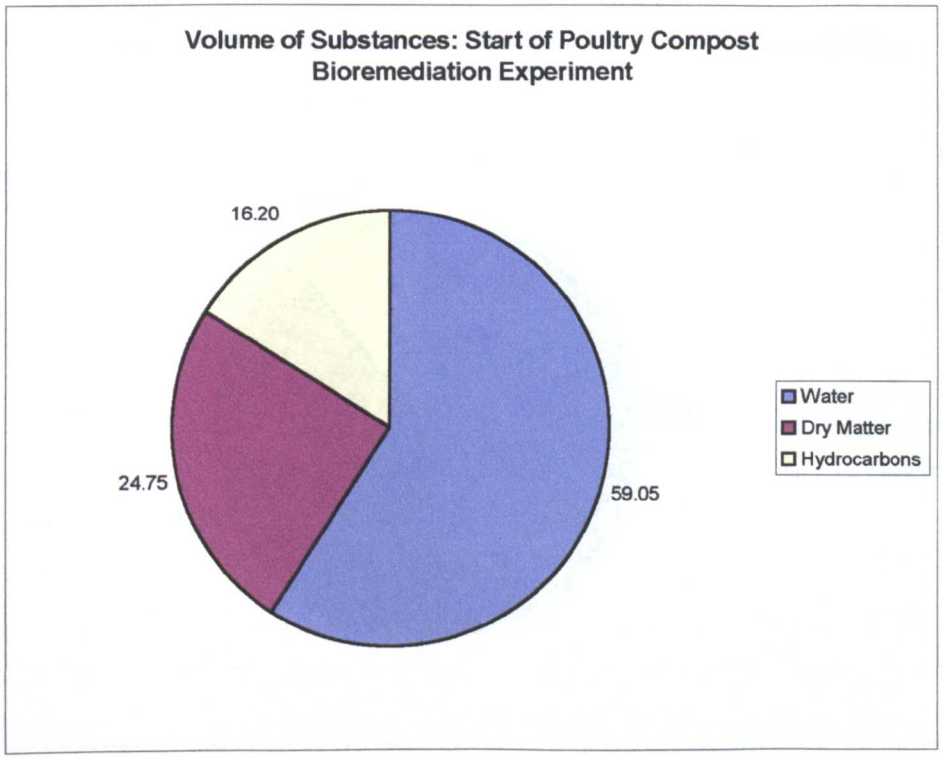
- The dry matter within the mix stays constant, i.e. no dry matter is converted to water or carbon dioxide by the bacteria or other micro-organisms.
- The SG of the drill cuttings combined with the manures was ~2.0 and the oil ~0.8.
- The ratio of drill cuttings to manure was 2:1.
- The retort result of 20% oil and 40% water on cuttings was correct.
- The GC calibration graph was correct.

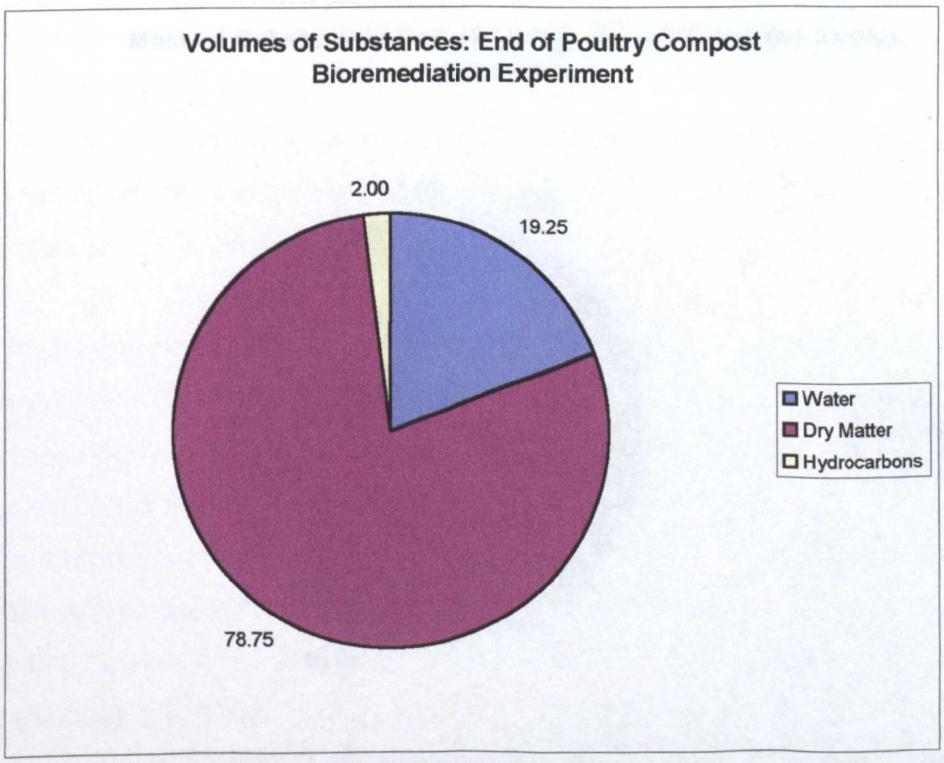
Table 8.5.2.3.1. Percentage Composition of the Poultry Compost

Content	START % Volume	END % Volume	START % Mass	END % Mass
Water	59.05	19.25	48.60	10.79
Dry matter (SG 2.0)	24.75	78.75	40.73	88.31
Hydrocarbon (20%, SG 0.8)	16.20	2.00	10.67	0.90
TOTAL	100.00	100.00	100.00	100.00

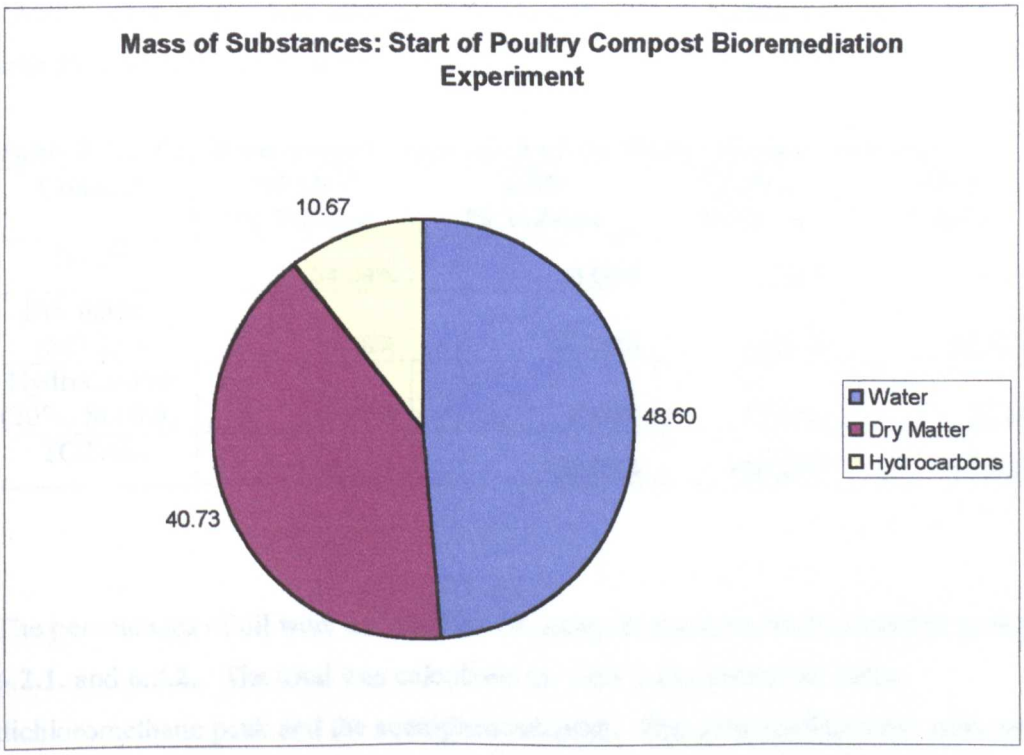
This data is illustrated graphically in figures **8.5.2.3.1. – 8.5.2.3.4.** The spreadsheet calculations can be found in appendix G.

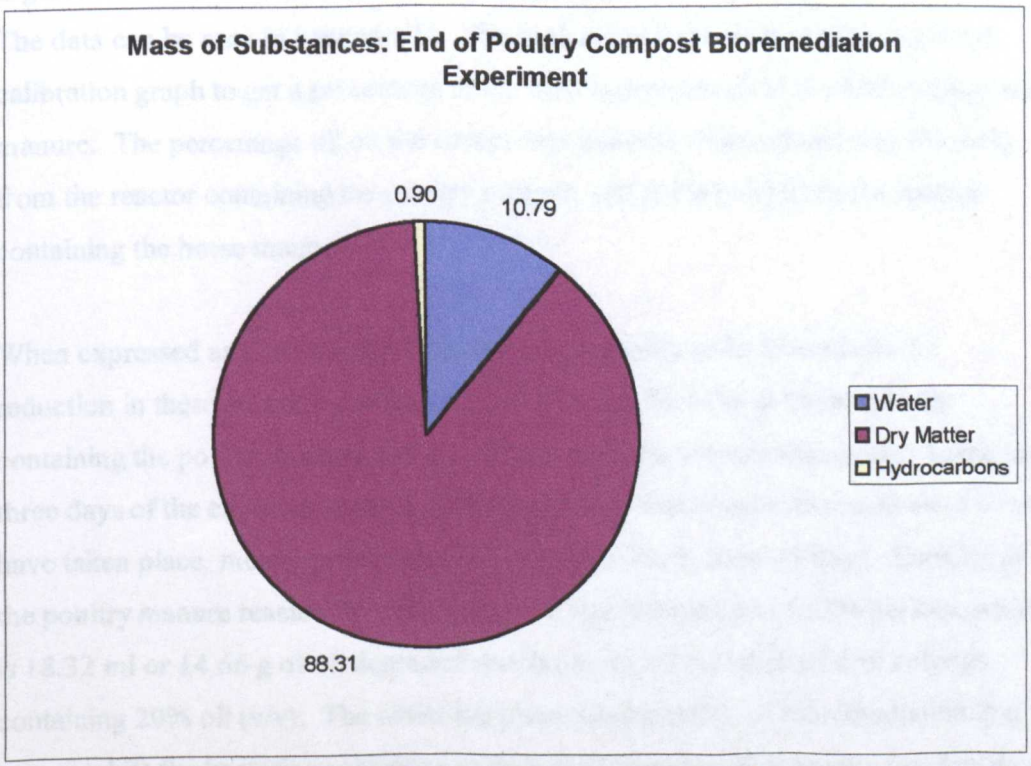
When looked at as ppm, including compostable material, the hydrocarbon content went from 162,000 ppm to 20,000 ppm.





Figures 8.5.2.3.1. & 2.: Volumes of water, dry matter and hydrocarbons at the start and end of the poultry compost bioremediation experiment





Figures 8.5.2.3.3. & 4.: Mass of water, dry matter and hydrocarbons at the start and end of the poultry compost bioremediation experiment

These figures were gained after GC analysis, followed by reading the resulting peak area from the calibration graph.

Table 8.5.2.3.2. Percentage Composition of the Horse Manure Compost

Content	START % Volume	END % Volume	START % Mass	END % Mass
Water	64.99%	6.00%	55.83%	3.22%
Dry matter (SG 2.0)	19.50%	87.60%	33.50%	94.03%
Hydrocarbon (20%, SG 0.8)	15.52%	6.40%	10.67%	2.75%
TOTAL	100.00%	100.00%	100.00%	100.00%

The percentages of oil were assessed by GC analysis; methods as discussed in section **6.2.1.** and **6.2.2.**. The total was calculated on total peaks minus the initial dichloromethane peak and the acetophenone peak. This assumes that there were no organics in the manure and that all the organics were sourced from the drill cuttings. The data can be seen in appendix G. The peak area total was read off against the calibration graph to get a percentage of the total hydrocarbons in the drill cuttings and manure. The percentage oil on the composted material with cuttings was 2% (v/v) from the reactor containing the poultry manure, and 6.4% (v/v) from the reactor containing the horse manure.

When expressed as a percentage of the original quantity of hydrocarbons the reduction in these compost reactor experiments was **96.12%** in the bioreactor containing the poultry manure and **90.82%** in the horse manure bioreactor. In the last three days of the experiment the material was so dry that little or no remediation could have taken place, meaning these results were more likely from 21 days. Looking at the poultry manure reactor, the resulting mean transformation is 4.58% per day, which is 18.32 ml or 14.66 g of oil degraded per day from a 2 kg batch of drill cuttings containing 20% oil (v/v). The initial lag phase characteristic of bioremediation that occurs while the bacteria acclimatise to their environment will mean the first few days would be less than this, but rates midway through the experiment would be higher.

The figures are erring on the cautious side, as there may be organic material within the manures that would be picked up on the GC. Ideally, the manures should have been extracted in isolation and the graphs compared to the manure/drill cuttings graphs. This would have enabled peaks solely from the manure to be removed from the data. These tests were not conducted due to time constraints.

The composting was a more efficient degrading system than the slurry-phase system when using high percentage clay drill cuttings in a rotating drum bioreactor. Although this experiment had not reduced the oil cuttings (dry) to below 1%, it was considerably closer than previously attained using the slurry system. If the moisture content had been kept up then there might have been more degradation. There were problems encountered during the experiment, such as material build up between the baffles and the reactor wall, and some caking of the reactor sidewalls. Solving these problems would improve the results.

CHAPTER 9

DISCUSSION, CONCLUSIONS AND POTENTIAL FOR FUTURE RESEARCH

9.1. Discussion Concerning the Microbiology

Three isolates were sent away for identification using 16S rRNA as discussed in section **6.4.3.2.**. The results identified A and D as *Bacillus thuringiensis*, and J as within the genus of *Bacillus oleronius*, but a novel species.

There are different strains of *Bacillus thuringiensis*, possibly illustrated by the slight difference in alignment of A and D. *Bacillus* are typical endospore-forming bacteria; vegetative growth ceases when a key nutrient, such as carbon or nitrogen, becomes limited. Adjacent to the spore is a parasporal crystal which is toxic to insects; the gene for this has been used in genetically modified crops. Spores of *Bacillus thuringiensis* have been reported to survive in both sterile and mixed culture clay soils (Vilas-Boas *et al.*, 2000). This may account for its presence within the drilling mud, which contains significant amounts of clay. These spores, being characteristically resistant to adverse environmental conditions, would stay dormant until the environment could support growth, when they would rapidly convert to vegetative cells (Madigan *et al,*, 1997).

There is conflicting research concerning the safety of exposure to *Bacillus thuringiensis* (Bt), a known bioinsecticide. The toxin gene has been engineered into genetically modified (GM) maize (corn) grown in North America; there has been some concern as to its effect on the monarch butterfly (*Danaus plexippus*) populations. However, the Bt expression in the pollen of most of the commercial hybrids of the GM maize is low (Sears *et al.*, 2001), and field and laboratory studies indicated no acute toxic effects at the pollen densities encountered in the field of these particular hybrids. It is toxic to the butterfly larvae, but as populations are widespread and only a portion of the monarch population utilizes milkweed stands in and near

cornfields, the impact is said to be negligible. The risk of gene transfer and soil and plant contamination was not discussed in the report.

Bacillus thuringiensis is considered non-pathogenic for humans, and is widely sprayed in urban areas as an insecticide. However, bacterial super-infections are the main cause of complication and mortality after an influenza virus infection. Research into combined infections, which typically occur during influenza outbreaks, suggest that there is a possible risk to the workers spraying the Bt-based bioinsectide (Hernandez *et al.*, 2000) at concentrations of 10^{11}. This puts some doubt as to the safety of the bacteria as used in the bioremediation experiments. For this reason, the *Bacillus thuringiensis* was treated as a hazard group 2 organism, restricting the methods of handling and sampling of the biologically active material.

Isolate J is most related to *Bacillus oleronius*, but is free-living. *Bacillus oleronius* is known as a member of the hindgut flora of the termite *Reticulitermes santonensis* (Kuhnigk *et al.*, 1995), a species of termite found in, amongst other places, France. The termites were collected and fed on pine or beech wood; the micro-organisms within the termite gut were then cultured aerobically with a mixture of different aromatic compounds. It can utilise lignin as a food source, which is a tough recalcitrant material, as well as the pine-oils, which are complex aromatic molecules. J, as isolated from the drill cuttings, had slightly different morphology to *B. oleronius*, which is described as rod shaped. The EM pictures showed J to be rounder rods, with two flagella. However, its presence in the drilling mud might be explained by the fact that lignosulfonates, which are derivatives of lignin, are used within the drilling muds. Lignosulfonates are obtained as by-products of the wood-pulp, paper-manufacturing industry (Davis). Research concerning degradation of lignin monomers by the hindgut flora of Xylophagous termites (Kuhnigk *et al.*, 1994) conclude that the flora could degrade lignin and aromatic compounds aerobically. This could mean that the isolate J may be able to degrade more complex hydrocarbon rings; however, there was no time to assess this.

The isolate J did utilise the hydrocarbons within the drilling fluid, thereby degrading them. The growth rate was low, however. There would still be potential for this novel isolate, as the genes for oil remediation could be cloned out and introduced into a *Bacillus* species that could grow more rapidly.

The *Bacillus oleronius* Gram-stained negative, just as J did, and both are therefore Gram-variable.

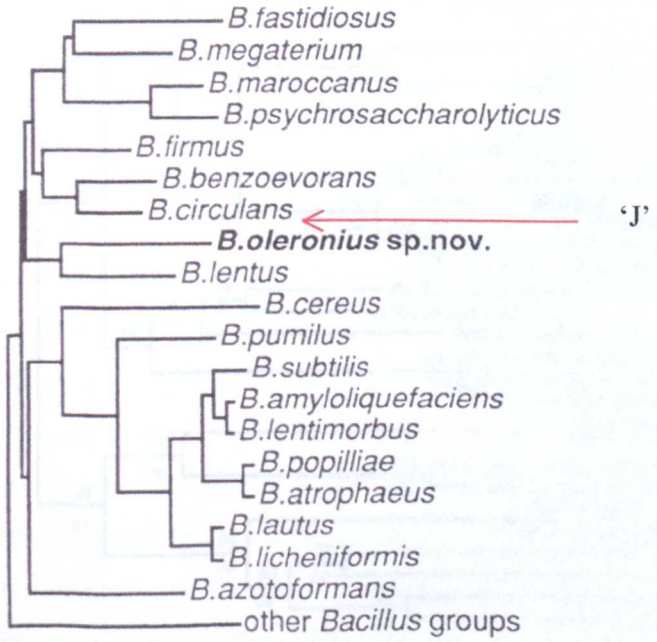
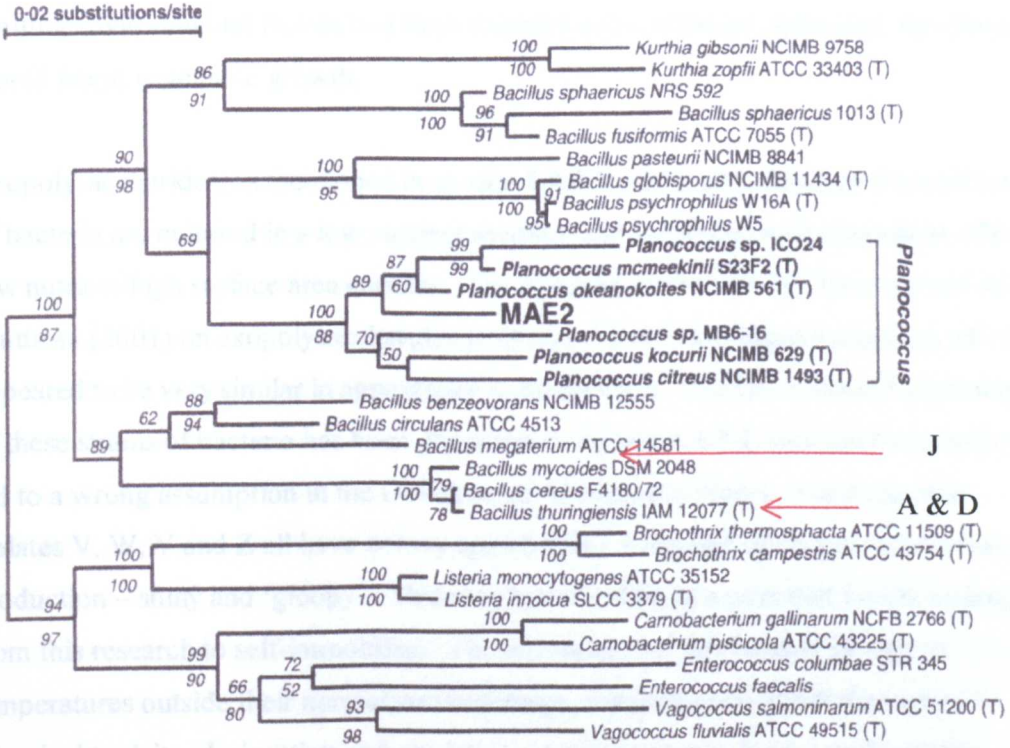


Figure 9.1.1. Phylogenetic Tree containing *Bacillus oleronius* from termites (Kuhnigk *et al.*, 1995)

There has been increasing evidence of Gram-positive hydrocarbon degrading bacteria within the vicinity of the J species on a phylogenetic distance tree. Gram-positive bacteria are useful in environmental applications as many form dry resistant spores which are easy to store and have very long shelf lives, and can therefore be used as an alternative to freeze dried organisms. *Bacillus lichenifomis*, as can be seen near the bottom of the phylogenetic tree in figure **9.1.1.**, has been described as a candidate for microbial enhanced oil recovery (Sarkar *et al.*, 1994). A novel *Planococcus*, isolated from beach sediment, was capable of extensive degradation of the alkanes within crude oil (Engelhardt *et al.*, 2000); its position on the phylogenetic tree can be seen in

figure **9.1.2.**. The novel species had an obligate requirement for NaCl but could not tolerate high saline concentrations. All known species of *Planococcus* have been isolated from saline environments, particularly marine, including some in sensitive Antarctic ecosystems; as a hydrocarbon degrader, it may have a role to play in future contamination incidents in sensitive marine environments.



Phylogenetic distance tree of selected Gram-positive bacteria. Bootstrap values for distance analysis are given above the nodes and bootstrap values for parsimony analysis are given below the nodes. Bootstrap values below 50% have been omitted

Figure 9.1.2. Phylogenetic tree containing the *Planococcus* and *Bacillus*.

There is also evidence of a reduction of biological activity due to substrate diminution, accumulation of recalcitrant or toxic residues or a possible switch to alternate substrates (Obuekwe & Al-Muttawa, 2001). At this point in the remediation there is the option of introducing additional micro-organisms to augment the bacteria in the contaminated substrate. To conduct this successfully, an inoculate would need to be stable and resistant to adverse environmental conditions, tolerate periods of storage and be able to survive and flourish in the hydrocarbon environment once introduced. The *Bacillus* species isolated during this research can do all these things,

being endospore-forming bacteria. Transportation and storage is simplified, plus there are no complex preservation processes to undertake such as freeze drying or keeping frozen at -80°C. The spores form when nutrition is limited; this starvation can be conducted in a laboratory, and are then recalcitrant. This means that the *Bacillus* could be used for *in-situ* hydrocarbon degradation; the spores could be spread onto the contaminated area, or injected via a solution sub-surface. Providing the limiting environmental factors had been assessed and conditions optimised, the spores would begin vegetative growth.

Exopolysaccharides, as mentioned in section **5.4.5.1.**, are produced when some strains of bacteria are cultured in a low nutrient medium and incubated with sawdust or other low nutrient/high surface area carriers. The research conducted by Obuekwe and Al-Muttawa (2001) on exopolysaccharides worked with a Gram-negative *Bacillus*, which appeared to be very similar in appearance to the isolate J. The Gram-negative staining of these strains of bacteria has been discussed in section **6.4.3.1.** and this has possibly led to a wrong assumption in the Obuekwe & Al-Muttawa report. J and the other isolates V, W, Y and Z all have colony appearances indicative of exopolysaccharide production – shiny and 'gloopy'. This may be indicative of a potential for the isolates from this research to self-immobilise. The advantages of this include storage at temperatures outside their normal survival range, reduced costs and resistance to chemical toxicity, desiccation and predation. Additional uses for the drill cuttings would be providing a low nutrient carbon-bulking agent that would be introducing a highly concentrated and metabolically active culture into the cuttings, with the beneficial effect of bulking up the clay material that may aid bacterial contact with the hydrocarbon contaminant.

Drill cuttings can contain varying quantities of heavy metals, being heterogeneous mixtures of differing strata; some drilling muds also contain heavy metals. *Bacillus* have biodegraded hydrocarbons in the presence of heavy metals (Amor *et al.*, 2001), although at a slower rate. Hassen *et al.* (1998) looked at the effects of heavy metals on *Bacillus thuringiensis* and found zinc concentrations of between 0.05 and 0.2 M (zinc is highly toxic to many bacteria) and Cd concentrations below 1.5 mM had no

substantial effects on its growth. Nickel was found to be the least toxic heavy metal (Amor *et al.*, 2001).

Bacillus megaterium can be seen on both the phylogenetic trees in figures **9.1.1.** and **9.1.2.**, indicating it is closely related to the isolates from the drill cuttings. Research has found this species a potential bioremediation and biocontrol agent (Lopez *et al.*, 1998). It is spore-forming and very persistent in soils, where it plays an important role in the degradation of herbicides and insecticides; it is also a fungal inhibitor. The accumulation of reserve polymers, such as poly-3-hydroxybutyrate (PHB), have increased the survival of the bacteria in the research conducted by Lopez. The results of this research were interesting, in that when the soil was sterile there was no difference between the PHB-wild-type strain and the PHB-negative strain (a mutant), but in non-sterilised soil the survival of the PHB-wild-type strain was higher, and numbers were greater even during the first part of the experiment. There was also a higher tendency to sporulation in the sterile soil. The same results were noticed in sterile and non-sterile river water microcosms. In a non-sterile environment there are biological interactions and possible changes in substrate conditions, which may help to stimulate the production of reserve polymers. The *Bacillus thuringiensis*, i.e. A and D isolates, were particularly affected by a change in sterility of the drill cuttings. This may suggest that there might be some transformation of the oil to PHB within the cells of the bacteria.

The *Bacillus megaterium* enzyme CYP102 has been engineered into a mutant strain for the degradation of polycyclic aromatic hydrocarbons (PAHs) (Carmichael & Wong, 2001). Bacilli in general are well characterised for cyclic organics.

Bacillus subtilis, as can be seen on the phylogenetic tree in figure **9.1.1.**, has produced a biosurfactant preparation which was obtained from a 24 hour culture, that increased biodegradation rates of aliphatic and aromatic hydrocarbons (Moran *et al.*, 2000). The enhancement was more noticeable in the complex molecules, the longer chain alkanes, and when high biosurfactant concentrations were added to cultures of indigenous microbial communities. The drill cutting degradation would probably

have been enhanced by the addition of a biosurfactant; with hindsight, a biosurfactant might have been generated from the isolates themselves and reintroduced to the contaminated material in high concentrations. Considering the nature of the clays, and the fact that the clay content was particularly high in the drill cutting material sent to the university to work with, a surfactant of some description would have been advantageous.

When producing a suitable media for the bacteria (see **6.4.2.3.**), vitamins were omitted from the recipe finally adopted. Recent research suggests the number of oil-utilising bacteria grown on vitamin-containing media were several-fold higher than the numbers counted on vitamin-free media (Radwan & Al-Muteirie, 2001). The organisms grown on the vitamin media were tested for growth on the same medium lacking any vitamins, and 90% failed to grow. The 10% that did had their growth enhanced when vitamins were added. In view of this it might have been appropriate to add vitamins to the mineral media recipe (Livingston & Islam, 1999).

Enrichment procedures were fairly basic for this research. The bacteria were cultured in a mineral broth with the oil, added to flasks containing the mud/cuttings mix with additional nutrients and left to acclimatise and multiply. Other researchers have used more sophisticated methods of enrichment, for example a Biological Activated Carbon system (BAC). The BAC includes an activated carbon column inoculated with bacteria from the contaminant's environment, with the hydrocarbon contaminant provided as their sole carbon source, and a nutrient supplying apparatus. This system produces an effluent containing an abundance of the indigenous bacteria that have the ability to decompose the target hydrocarbons (Li *et al.*, 2000).

This system has several advantages over the simple method utilised for the drill cuttings research.

- Selection and retention of micro-organisms capable of degrading the target compounds in the reactor. This would enable the researcher to examine the

effluent and possibly identify species; it also would be a massive advantage in gaining knowledge of bacteria in consortia.

- The activated carbon offers a large interface area allowing for thorough mixing of bacteria, water and target hydrocarbons (Weber & Corseuil, 1994).
- The system can work continuously to produce bacteria to be utilised for bioremediation.

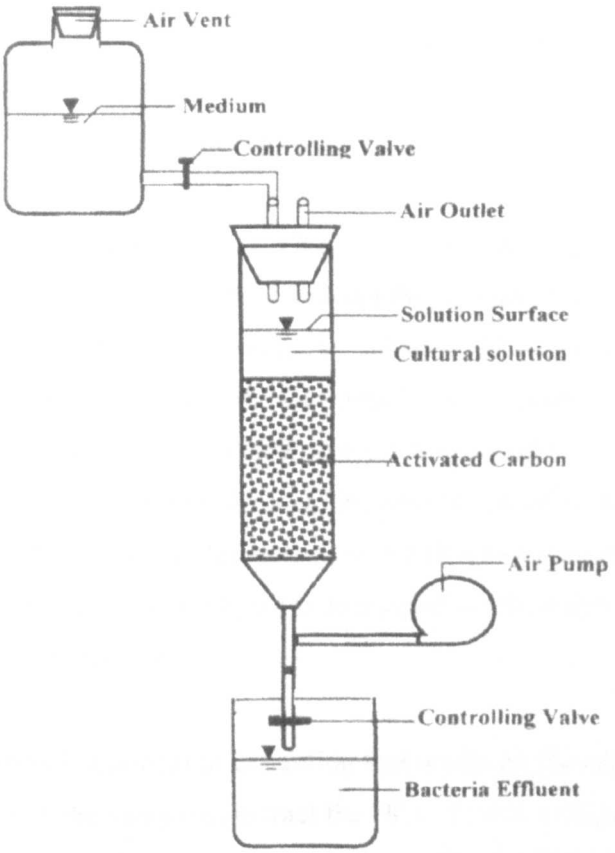


Figure 9.1.3. BAC system (Li *et al.*, 2000)

9.1.1. Conclusion

It appears that Bacilli able to utilise hydrocarbons are being discovered in diverse environments, and are being utilised for bioremediation clean-ups. The *Bacillus* species isolated from the drill cuttings appear, according to very recent research, extremely useful and versatile hydrocarbon degraders. There were parameters that

could have improved their chances of bioremediation; these include the addition of a surfactant, a more efficient enrichment technique and a media containing vitamins. The late identification of the remediating species as *Bacillus*, just before the bioremediation experiments were conducted, did not allow for specific conditions to be optimised for this species. The main reason the pre-screen data did not scale up to the bioreactor experiments was the condition of the drill cuttings; being of very high clay content, the cuttings did not physically behave in a manner congruent with bioremediation in the drum reactors as used for these experiments. Some modification to enable the processing of clay-rich cuttings would be necessary.

9.2. Discussion Concerning the Analysis

Gas Chromatography (GC) is a recognised and frequently used method to quantify the hydrocarbon (HC) content in a sample of material, once that HC has been extracted into an appropriate solvent. A method specific to the HCs being analysed had to be developed. However, the GC, which is an extremely sensitive apparatus, can occasionally produce illogical or spurious results. There needed to be a reference point. For this research, acetophenone was used as the internal standard as it eluted from the GC at a different time to the HCs being assessed. To ease interpretation of data, calibration graphs were developed to allow direct reference as to the percentage HC in the sample.

Soxhlet is a system of extraction that works on the principle of refluxing a solvent through the sample to extract the HCs. This is a documented and reliable method of extraction. However, the water content in the cuttings can complicate this. The research dealt with this complication by the use of a compound that holds onto the water within the cuttings – anhydrous sodium sulphate (Plumb, 1984; Cooper, 1999). However, there is the doubt that the addition of another compound may effect the extraction. Another approach would be to dry the sample first in a low temperature oven to remove the water, then to extract the hydrocarbons. This is the method as adopted by Chaineau *et al.* (1996) during their research on contaminated drill cuttings.

Initial remediation experiments used the data to assess the percentage reduction from the start of the experiment to the end of the experiment. The problem with doing this is that evaporation from the vessel is not taken into account, meaning that the data looks worse than it might. To overcome this, a spreadsheet was designed to allow for changes in moisture content. The moisture content has to be assessed in the material at the end of the experiment as a percentage; the dry matter stays constant, the HC content is expressed as a percentage as read off the calibration graph. The spreadsheet computes an assortment of result data, for example percentage reduction of HCs and percentage HCs left on the cuttings, as a volume or a mass figure; also the exact quantity of HC, in millilitres or grams. The specific gravity (SG) of each fraction needs to be known. This system is appropriate for the slurry-phase or for the composting bioremediation experiments.

9.2.1. Conclusion

The methods developed and adopted for analysing the hydrocarbon content appeared to be repeatable and reliable. The only doubts are the use of anhydrous sodium sulphate, even though this compound is commonly utilised in other research and analysis. More experimentation concerning this might be appropriate. The results are reliant on the calibration graph being correct and the SG of the different fractions within the cuttings/water matrix assessed.

9.3. Discussion Concerning the Design of the Bioreactor

Drum bioreactors have been utilised in past bioremediation research and for practical bioremediation applications (Cookson, 1995; Pinelli *et al.*, 1997; Kruger *et al.*, 1995; Truax *et al.*, 1995). Truax *et al.* and Kruger *et al.* (1995) studied fuel contaminated soils of a sandy nature, with very low levels of clay. The study by Pinelli *et al.* (1997) was conducted on a silt-clay soil, but there was no discussion concerning the behaviour of the material in the reactors.

The reactors were quite simple to construct with readily available materials. The motor/gearbox allowed for the speed to be varied, depending on requirements. The frame, rollers and belts meant multiple reactors could run concurrently. The belts initially used were not substantial enough for the work load; these were changed to elasticated cords, which proved adequate.

The reactor was designed to keep ultraviolet radiation to a minimum, as this can reduce microbial activity (Santas *et al.*, 1999). They were of a practical size for laboratory scale experimentation, and were of a design that could be scaled up if the experiments successfully remediated the contaminant in the drill cuttings. Initial problems with the air pipes blocking were cured by the installation of a shield to deflect material from the pipe ends.

The sampling facilities were appropriate for hazard group 2 organisms; however, the mud-like substance lacked fluidity, which meant that sampling was not always possible. Sampling through these ports was impossible during the compost reactor experiments. There were difficulties in loading and unloading the material into and out of the reactors via the end caps. The vessels were difficult to clean, and it was difficult to ensure they were sterile.

The reactor design was appropriate for keeping contamination of the environment and cross-contamination within the reactors to a minimum. They were constructed so as to seal effectively, and the air system designed to ensure clean air into and out of the reactor. A vacuum pump was used to 'pull' the air through the reactor rather than a pump 'pushing' air in, as was used with the bucket and impeller reactors, which would be difficult for contamination control. The vacuum pump model was inappropriate, as it was not a low vacuum pump of continuous rating; this resulted in some breakdowns. Each breakdown resulted in oxygen starvation in the reactors.

The baffle design was inadequate; material quickly accumulated in the area where the baffle connected to the sidewall of the vessel, and the vessels were difficult to sterilise here. This problem could easily be solved by the addition of a fillet, which would

round off the acute angle. These would need careful sealing, as bacteria could survive between experiments in any crevices, contaminating the next experiment. The use of the baffle for mixing the slurry-phase experiments was insufficient due to the caking effect of the material. This might be solved in a drum reactor by removing the baffles entirely and installing a plough in the vessel, which could scrap material from the reactor sidewalls constantly. This would prevent cake build up and allow better mixing of the material, facilitating improved aeration.

A more adaptable design would have been the scaled-down concrete-mixer type reactor as mentioned in section **7.2.2.** A reactor of a similar design to this was used by Woo & Park (1999), with a stainless steel drum body and a two-part lid consisting of a stationary centre part, which housed the air inlet and outlet ports, and a rotating outer part. The reactor was housed at an angle of ~16° and equipped with a screwed baffle 3 cm wide inside the reactor wall. The temperature was controlled via a ceramic heating jacket. This style of reactor would have made loading and unloading the vessel simple, but may not have withstood the rigors of the university's H & S officer if using hazard group 2 organisms.

9.3.1. Conclusion

The reactor was simple to construct, and some initial problems were cured after the first experiments. However, further problems ensued; the mixing of the material in the reactors was inadequate for slurry-phase bioremediation of the drill cuttings when they are predominantly clays. The baffles were traps for material to lodge in and did not achieve their purpose of stirring the material in the reactors. Operating conditions were not fully optimised to ensure good mixing and suspension of the material. Making the end caps the only entrance/exit into the reactors made materials amendments during the experiments difficult. The length of the vessels and the baffles did not allow for ease of cleaning. The reactor design was more appropriate for solid or semi-solid-phase material, as used in the compost bioremediation experiment.

9.4. Discussion Concerning the Bioremediation Experiments

The drill cuttings are diverse by nature, making them a difficult material to design a specific process for. Each well might drill through many differing rock formations; the drill cuttings will reflect this e.g. mud-making or hydratable shales will produce totally different cuttings to limestone formations. The differences in mineralogy were illustrated well during this research, in that each sample received for testing was different from the last. Some rocks break up rapidly in contact with water, others may not, and may require pre-treatment to allow the release of hydrocarbons within the micropores of the rock. Some drill cuttings, as discovered during the reactor experiments, will be predominantly clays, which are renowned for absorbing organics, making them less available for the bacteria to degrade. Having some prior knowledge as to the mineralogy of the cuttings would have been useful, in that the behaviour of the material might have been more predictable. Also, if experiments were conducted on various types of cuttings, the data could be utilised to predict the best remediating technology to adopt.

Ortega-Calvo *et al.*, (1997) claimed in their paper "clays represent an important hindrance for bioremediation technologies, as they may cause a retardation in the biological removal of hydrophobic pollutants". This appeared to be the case during the slurry-phase bioreactor vessel experiments. However, if a pollutant is too toxic for the bacteria, as many xenobiotic organic contaminants can be, this property of the clays can have advantages. 'Clay hutches' can be organised and occupied by bacteria (Timmis, 2001). The high adsorptive capacity of the clays for hydrophobic organics is utilised by the bacteria – the clay hutch protects them from direct contact with droplets of the toxic pollutant, with the clays acting as nutrient shuttles – the bacteria get the substrate from the clay rather than directly from the droplets.

Another implication of clays adsorbing contaminants is that the hydrocarbons adsorbed into the clays are not bioavailable and therefore have a reduced environmental impact.

As already discussed in section **9.1.**, the conditions for the bioreactor bioremediation experiments might have been enhanced with prior knowledge of the species *Bacillus*, which would have enabled the conditions to be specifically enhanced. However, the isolates were not identified until just before the first reactor experiment. All the bacteria, as discussed in section **9.1.**, were potential degraders of hydrocarbons. The purchased *Rhodococcus* was a confirmed degrader of paraffins, as stated in the NCIMB catalogue. However, none of the species remediated to an adequate level in these experiments. This could have been due to a number of factors, but the very high clay content, which consisted of over 57% within the fine fraction <212 μm may have been a major contributor. However, this is contradicted in other reports on remediation which state that particle sizes of <30 microns gave higher degradation rates than particles larger than that (La Grega *et al.*, 1994) as well as being easier to maintain in suspension. The muds are designed to coat sidewalls, as well as having other properties (see section **2.3.2.**), and contain additives other than oil and clay to carry out this function. The combination of these additives with other factors, such as the operating speed and water content in the mud slurry, led to the sedimentation of the material, which then was deposited on the sidewalls of the bioreactors. The swelling of the clays as the oil was remediated may have enhanced this tendency; it was not observed until 7 – 14 days after its introduction into the reactors. The material did not display this characteristic during the pre-screening experiments. Also, the muds can reach a point of critical moisture, when they become extremely thixotropic. The impact of this could perhaps have been reduced by the addition of more water to make the slurry more aqueous. Although the experimental process did not want to alter the parameters within the reactor, with hindsight this was a mistake. With more time to conduct a series of experiments, the question of whether extra fluid would solve the problem would have been answered. Humidifying the air might have been a solution. The data can be calculated back to dry matter by assessment of the water content at the end of the remediation experiment using the retort or by evaporation of the moisture by drying a sample in a low temperature oven. The addition of water would not affect the results as the data could be programmed into the designed spreadsheet. However, adding more and more water to the system serves

to increase the amount of material to process, and increase the amount of filtrate that would need secondary treatment, meaning it is not an ideal solution.

Another approach would be to add another material into the reactor with the drill cuttings. The addition of grinding beads would be one solution. The grinding beads would help prevent the clays sticking to the sidewalls as they help prevent agglomeration of soils (Scholz *et al.*, 1998) providing the moisture content is kept slightly above the plastic limit, but would also break up any lager size fraction rock chippings. By stopping the sticking of the material in the reactors, aeration would be more efficient, reducing one of the limiting factors in degradation – lack of electron acceptors. The mechanical action of the grinding beads might also help release the hydrocarbon from the mud/cuttings matrix, making it available for the bacteria. This could increase the rate of degradation. Conducting more trials using grinding beads would have been beneficial, if time had allowed. However, this system may not easily scale up for an industrial process, and would mean even more post-treatment. The beads would either have to be reclaimed, if possible, from the other solids waste, or disposed of with the solids waste. More processing, or more waste.

The research took a different approach; as the slurry-phase system had proved to be inappropriate for the type of drill cuttings sent for remediation, a solid-state bioreactor bioremediation experiment was conducted. Clay surfaces are said to be a significant contributor to hydrophobic sorption when other organic matter (such as straw, manure, or humus in a soil) is below 6 – 8 % (Ortega-Calvo *et al.*, 1997). Humic acids within a soil can also contribute to the binding of organic compounds when in combination with clays, which discouraged the use of a soil amendment to the drill cuttings. The research then looked towards a compost type system, which utilised an organic bulking agent and a biologically active material, in the form of poultry and horse manure on straw and wood-shavings respectively (for conditions, refer to **8.5.**). The mixture in the reactors was kept solid. The problem of caking the sidewalls was considerably reduced, but not eliminated. The area where there was significant build-up of material was where the baffles met the reactor wall, as discussed in section **9.2.**

The compost system results were superior to the slurry-phase reactor experiments, particularly the reactor containing the poultry manure, which is a powerful activator of degradation (Caplan, 1992). When the mixed material was added to the reactor, it was full; within 72 hours, the material had reduced size considerably due to the action of the drum reactor and baffles. As it was only possible to conduct one experiment using these materials, it was essential to set some parameters, and keeping the material in its original state was one of them. However, if there had been time for more experimentation on this system, then additional material (drill cuttings and manure) could have been added to the vessel; water should also have been added, but only enough to maintain the moisture levels. The restriction of airflow had been solved by this stage of experimentation. It was not possible to assess the air requirement per reactor for one experiment as there was a multitude of organics to degrade beside the hydrocarbons, i.e. in the manures themselves. There needed to be a high air input; this is why only two reactors were run concurrently instead of four from the single vacuum pump. Considering that bacteria nearly always display a lag phase when introduced to a new environment, and that the material at the end of the three weeks was too dry to support microbial growth and metabolism, the degradation was achieved in a reasonable time.

There is potential for a marketable material from bioremediation via composting. The material would first have to be assessed for heavy metal content and comply with statutory regulations.

9.4.1. Conclusion

The bacteria used for the experiments had the potential to remediate the hydrocarbons in the slurry-phase bioremediation experiments; they did achieve a reduction in hydrocarbons, but not enough to fulfil the prerequisite given at the onset of the research, i.e. to below 1% (v/v). This was due to the reactor type being inappropriate for the high clay content material used in the experiments. Clays can be particularly difficult to remediate as slurries in drum reactors, as discovered during this research. The caking of the sidewalls would have reduced oxygen and substrate availability to

the micro-organisms trapped in the mud matrix. Considering the variable nature of drill cuttings, the system needs to be able to remediate diverse substances. This also includes the varying hydrocarbon types used in the drilling muds themselves.

However, changing the physical nature of the substance to be remediated in the bioreactor to semi-solid/solid, via the addition of compostable matter, increased the rate of degradation and reduced the final content of oil on drill cuttings to a figure that was very close to the target of <1%. Further investigation into this type of system is therefore justified.

9.5. Potential for Future Research

Some points related to the microbiology have already been discussed in section **9.1.**, and are include with the following ideas for future research.

- Conducting isolation experiment on a basic mineral media containing vitamins as well.
- Analysis of plasmids to assess where the remediation gene is.
- Cloning oil remediation gene from the slower growing species J into a faster growing species
- Identification of V and W isolates
- Testing of Y and Z isolates
- Testing of J to assess whether it can degrade more complex HCs, e.g. rings
- Analysis of bacteria to assess whether they produce exopolysaccharides
- Conducting bioremediation experiments using better enrichment techniques
- Looking at the microbiology of the reactors at the end of the experiment – if the *Bacillus* had formed spores it would indicate that the environmental conditions had limited its growth.
- Development of a consortia
- Analyse rates of degradation throughout the experiment, to assess optimum conditions for bioremediation
- Conducting experiments with the addition of surfactants

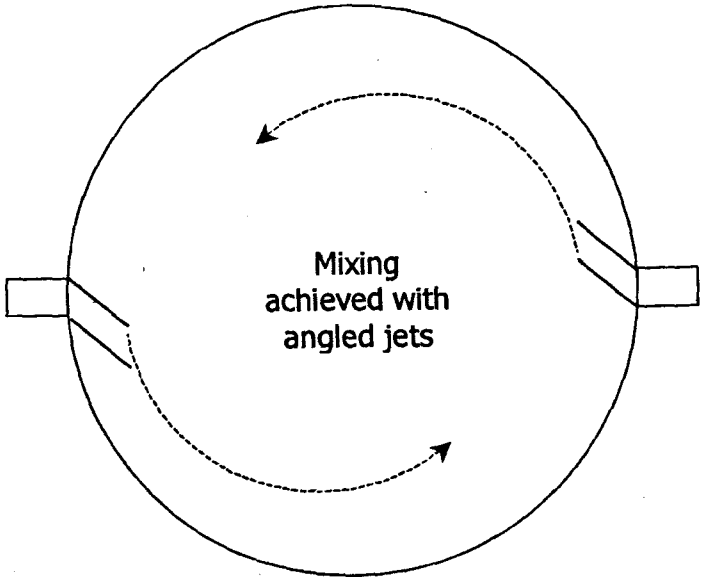
- Producing biosurfactants in the laboratory using the isolates
- Modification of the bioreactor, or using a different style of reactor altogether for slurry-phase remediation
- Modification of the material in the reactor, e.g. by the addition of grinding beads
- Tests to assess whether anhydrous sodium sulphate affects the extraction and therefore the results of the experiments

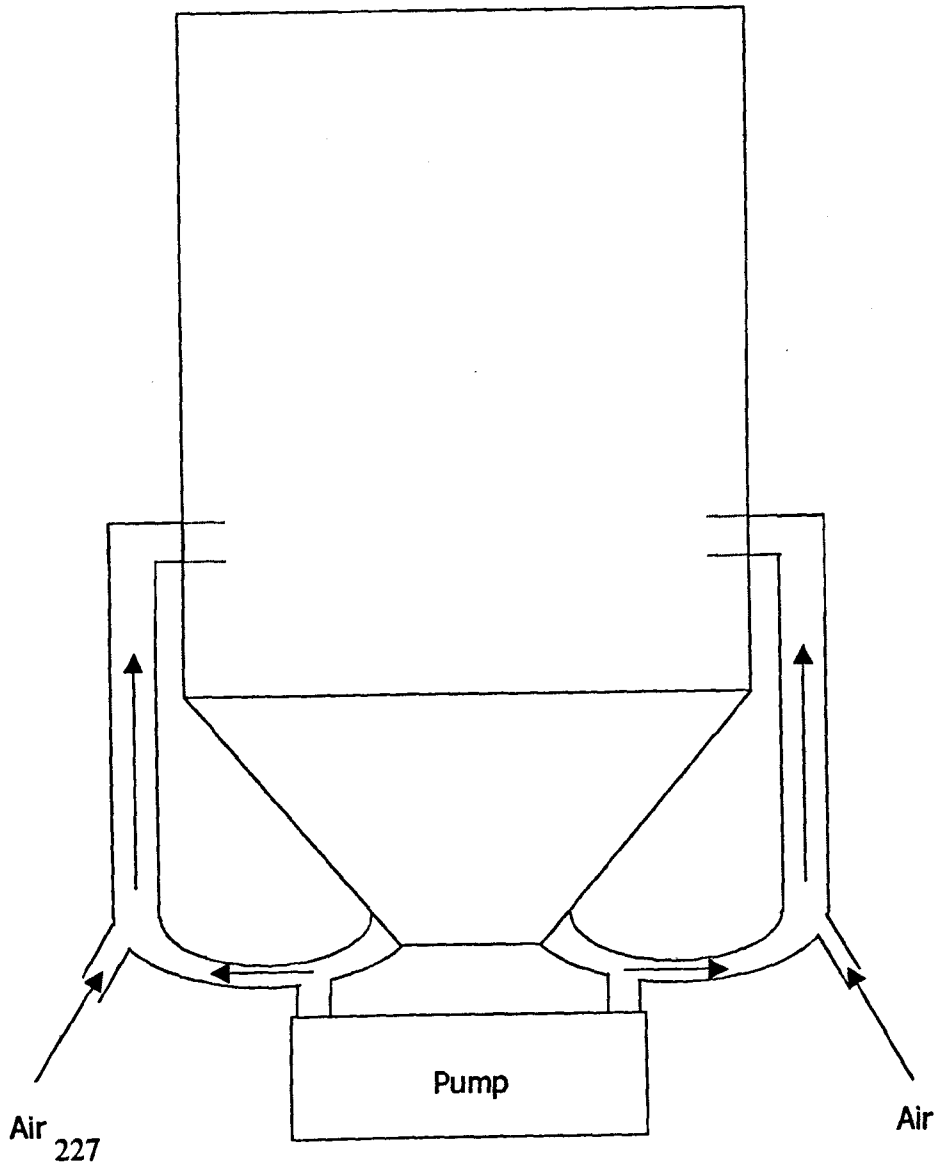
It would be advisable to analyse each batch of drill cuttings before any decision as to method of remediation is taken to assess their mineralogy and heavy metal content.

Although not a biological method of remediation, flotation using coal fines may prove efficient and is certainly worth considering for future research (see **2.7.1.7.**).

An example of a slurry system that might deal with a substrate predominantly clay in nature can be seen in Figure **9.5.1.**. The problem of caking encountered with the drum reactor might be eased, as the material is continuously pumped from the bottom of the reactor, where solids often settle out. Air is introduced into the slurry as it travels through the pipe, with the material pumped back into the tank about halfway up the sidewall. Mixing within the vessel is achieved with angled jets. This would be appropriate for a slurry-phase material; the clays, which are prone to settling out, would be redistributed into the slurry constantly. Stirrage would be constant, and air would be diffusing throughout the material, enabling a more intimate contact with the micro-organisms. The size of the vessel would be smaller per unit input than for a rotating drum reactor. The design would be difficult to realise on a laboratory scale. However, during a meeting with the sponsors BP/Amoco and MIDF, there was some discussion concerning some vessels of this shape that might have been available for larger scale testing.

Figure 9.5.1. ***Batch Slurry Tank*** – Plan View & Elevation
Not Drawn To Scale



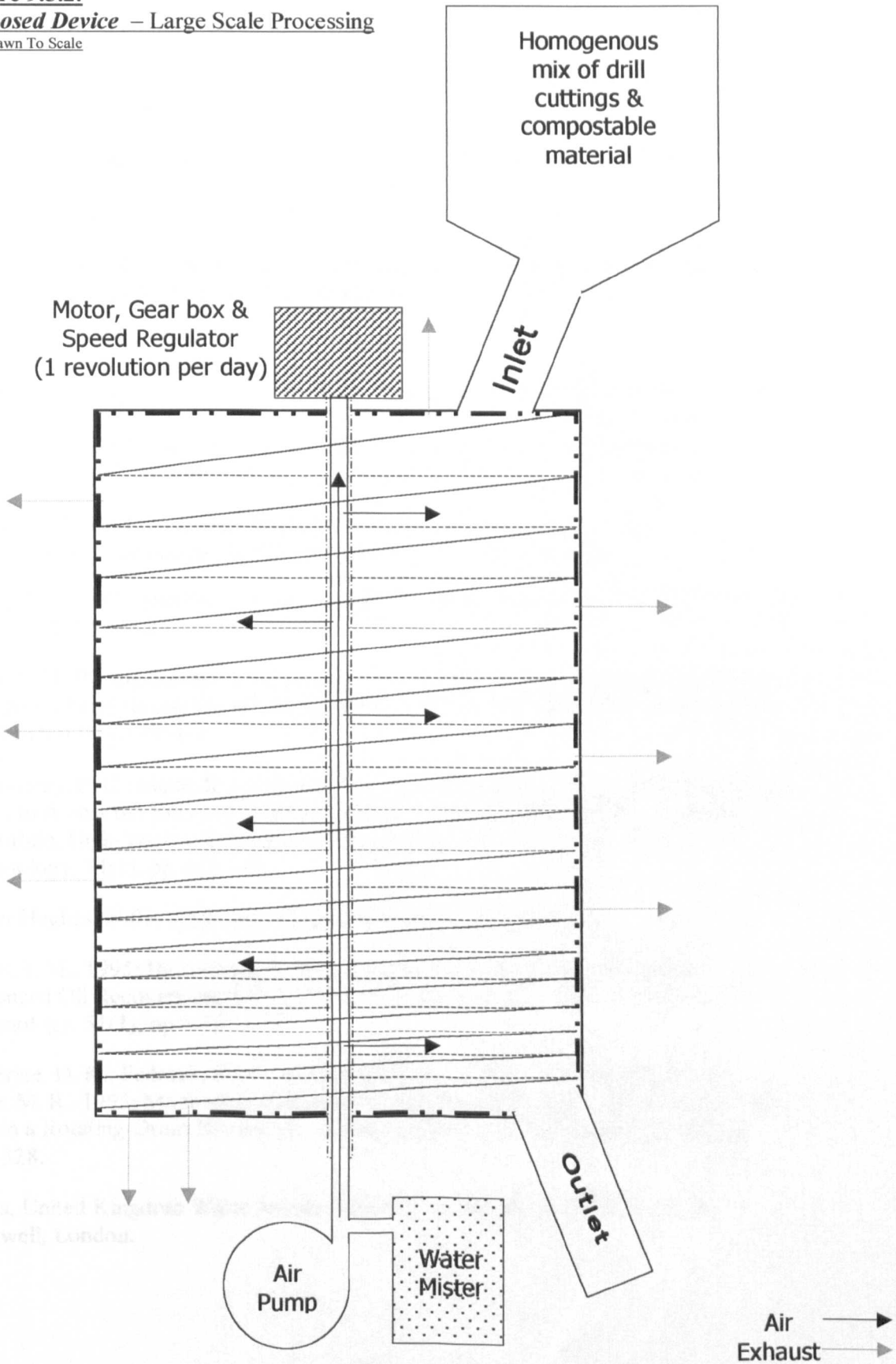


Future experiments conducted on composting drill cuttings might take regular samples and observations of the conditions in the reactor and discover when the peak rate of transformation occurs. The conditions in the reactor during this period could then be used to optimise the running conditions throughout; this would include moisture content but would not necessarily apply to the initial lag phase. Most compost systems become thermophilic, but did not in this experiment. There has to be quite a bulk of material to create these conditions; in a larger scale reactor, this could occur. The heat could increase the rate of degradation, but may cause the complication of evaporation of some of the hydrocarbons in the drill cuttings, leading to the necessity of air pollution clean-up equipment. However, most of the hydrocarbons are above C12 and are therefore not VOCs, so unless the microbial activity lessens the chain lengths considerably this should not occur. The compost system lends itself to a continuous process using bioreactors, or a low-tech process such as biopiles, as discussed in section **5.4.3.**.

The research has included a possible design of compost bioreactor; this is illustrated in figure **9.5.2.**. This reactor has a hopper with a mixing blade, where the manure and drill cuttings are mixed before introduction into the reactor. This would reduce the size as well as ensuring a more homogenous mix, which proved to be difficult to achieve with large particles such as straw. The mix is then fed into the reactor, which has an auger running down throughout the vessel. The auger is larger at the top to accommodate the bulkier material, and becomes tighter as it feeds down the vessel; this reflects the reduction in size of the mix as it degrades and mixes. An air pump keeps the system aerobic by pumping air into the shaft, which has outlets into the reactor itself. A water mister could be added to the air system to ensure the moisture levels remained adequate for degradation. The shaft is attached to a motor/gearbox, which could be run at a speed relative to the required retention time in relation to the number of auger sections. The example in the diagram is a 20-day cycle, with 20 turns of the auger turning at one revolution per day. Air can exhaust through the perforated sidewalls of the reactor, providing that caking did not become an issue; this would have to be assessed by experimentation. The material, at the end of its cycle, is fed through an outlet pipe at the bottom of the reactor. Having the vessel upright allows

gravity to help feed the material down, meaning less power would be needed than using a similar but horizontal design. This also reduces the 'footprint' required by the reactor. The material is continuously fed in and out of the bioreactor vessel. Another modification might be a solar heating jacket that would heat up the material and may increase the rates of degradation. A modification such as this would have to be designed around the exhausting of the air, and would only be needed for the top section of the vessel. Heat from the thermophilic activity in the vessel might be circulated upward, encouraging the thermophiles in the upper part of the reactor.

Figure 9.5.2.
Proposed Device – Large Scale Processing
Not Drawn To Scale



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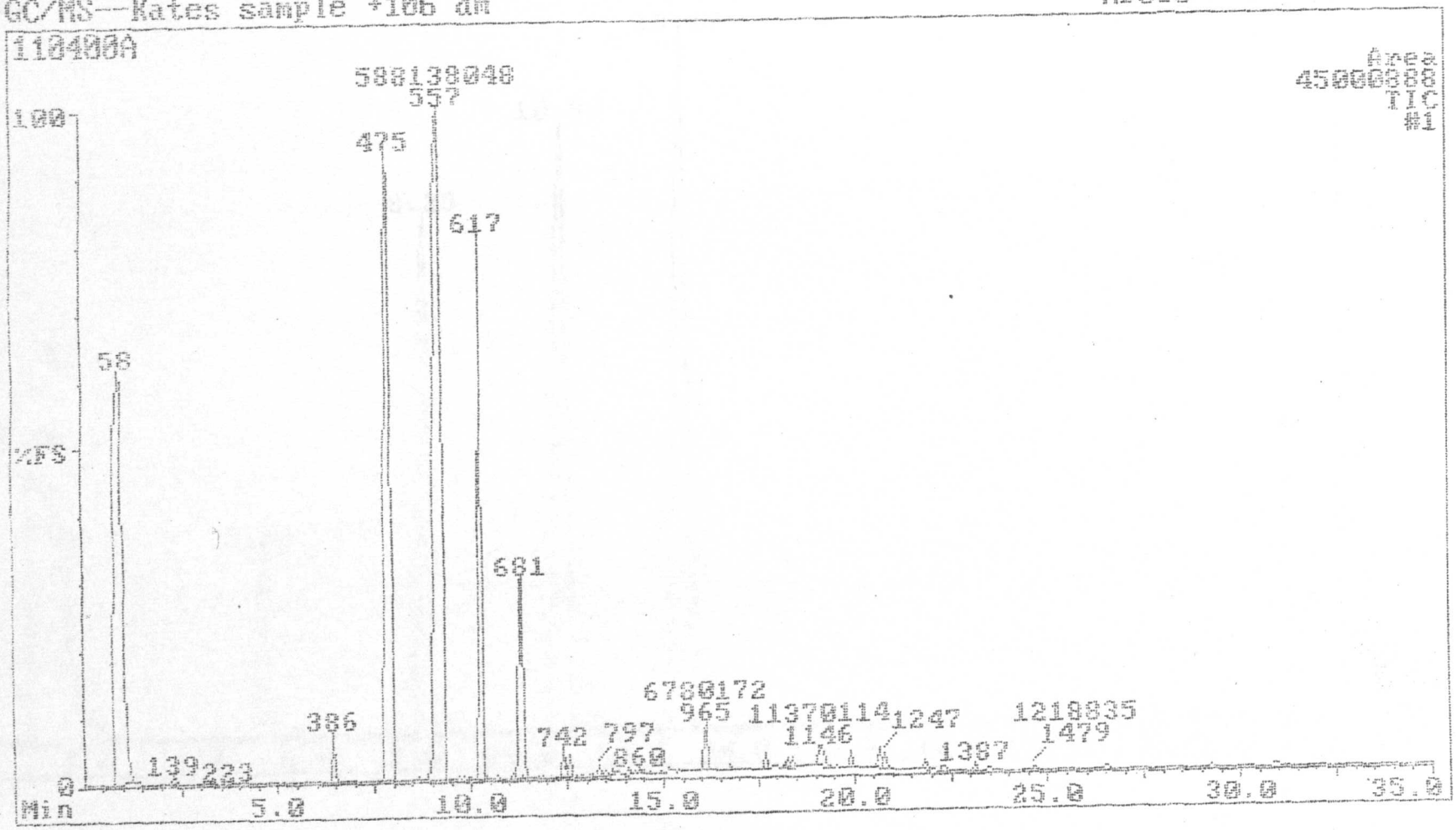
Appendix

A

U of Nottm School of Chem,Env & Min Eng

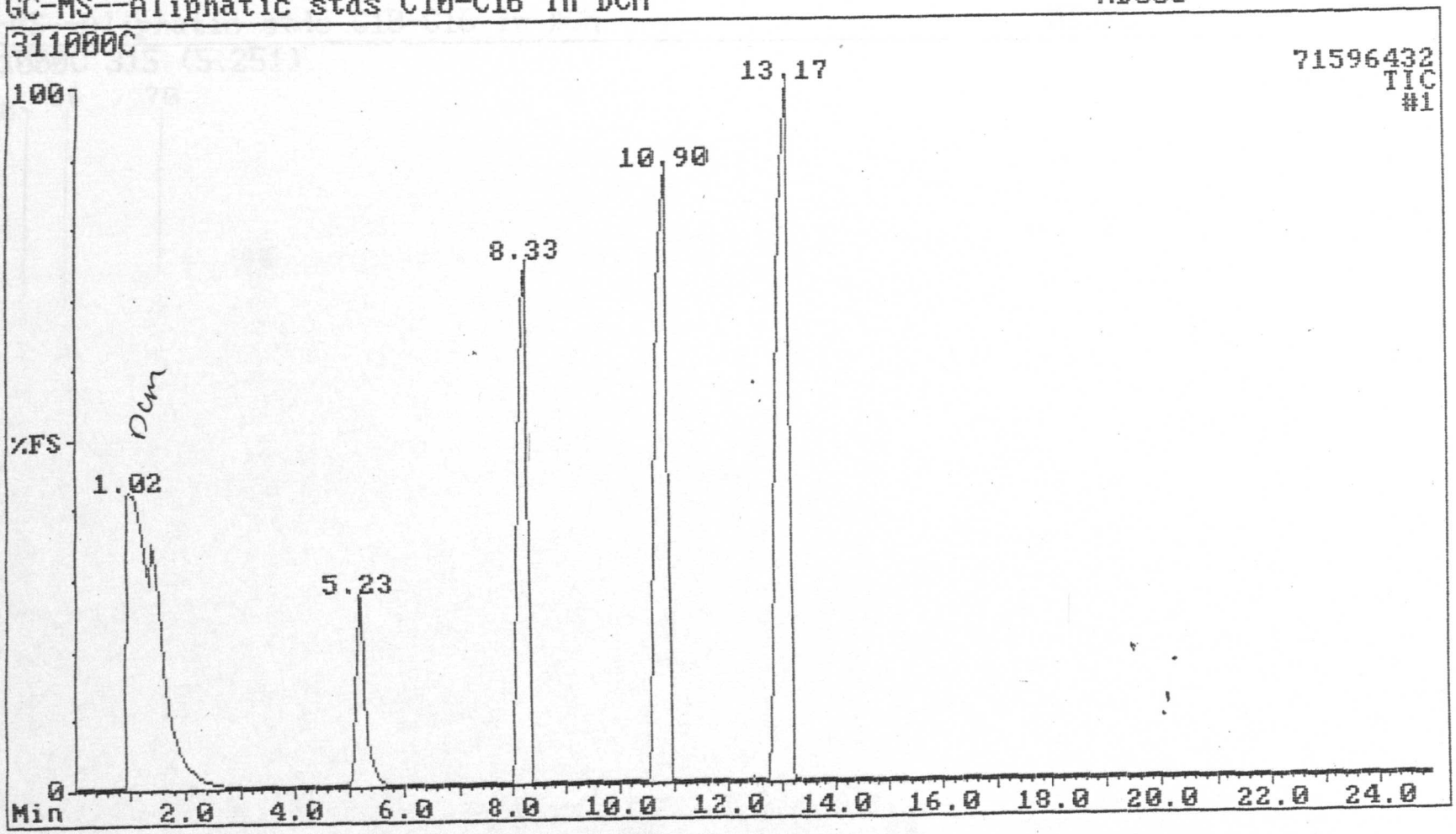
MD800

GC/MS--Kates sample +106 dn



SChEME The University of Nottingham

GC-MS--Aliphatic stds C10-C16 in DCM MD800



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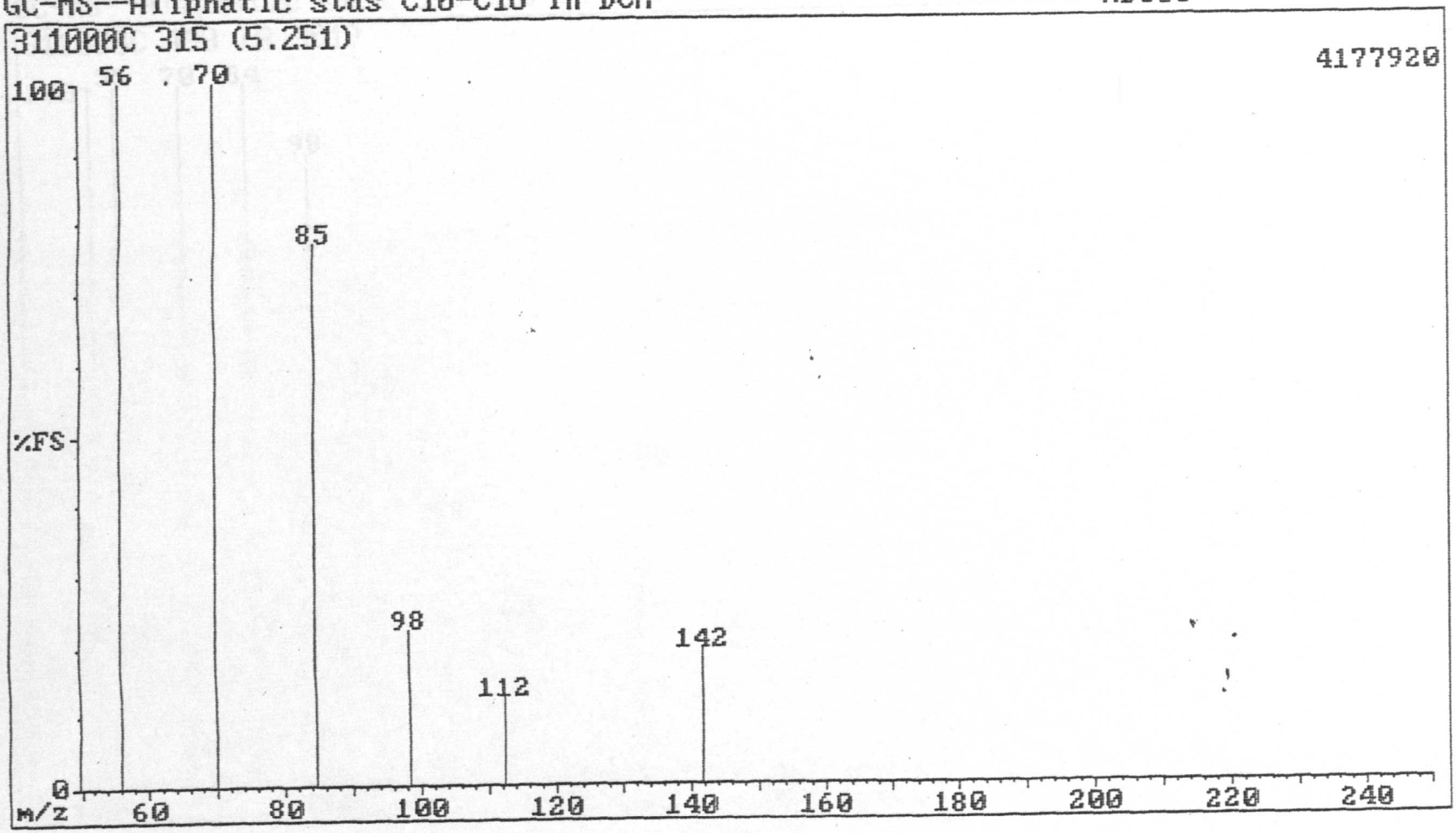

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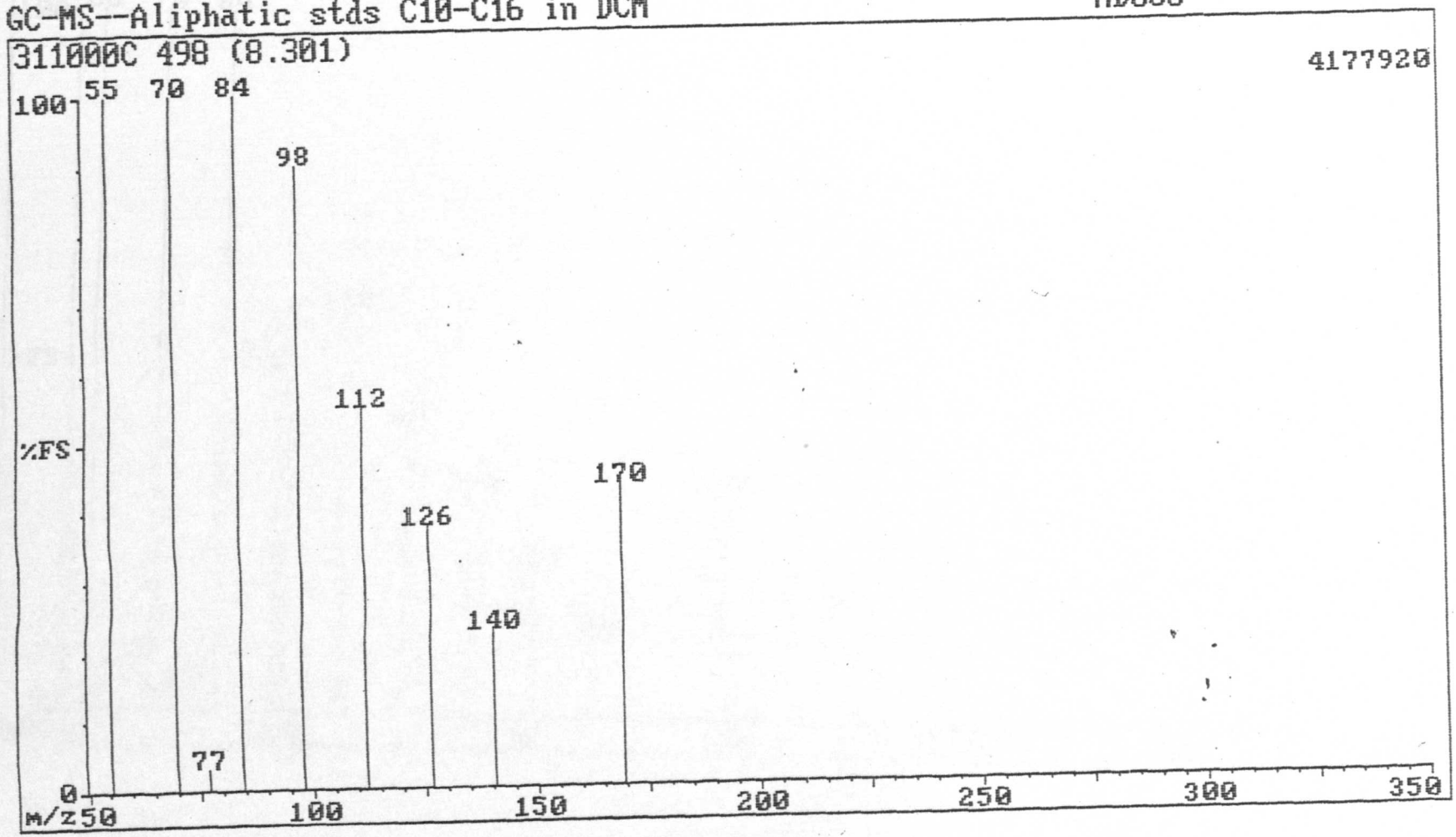

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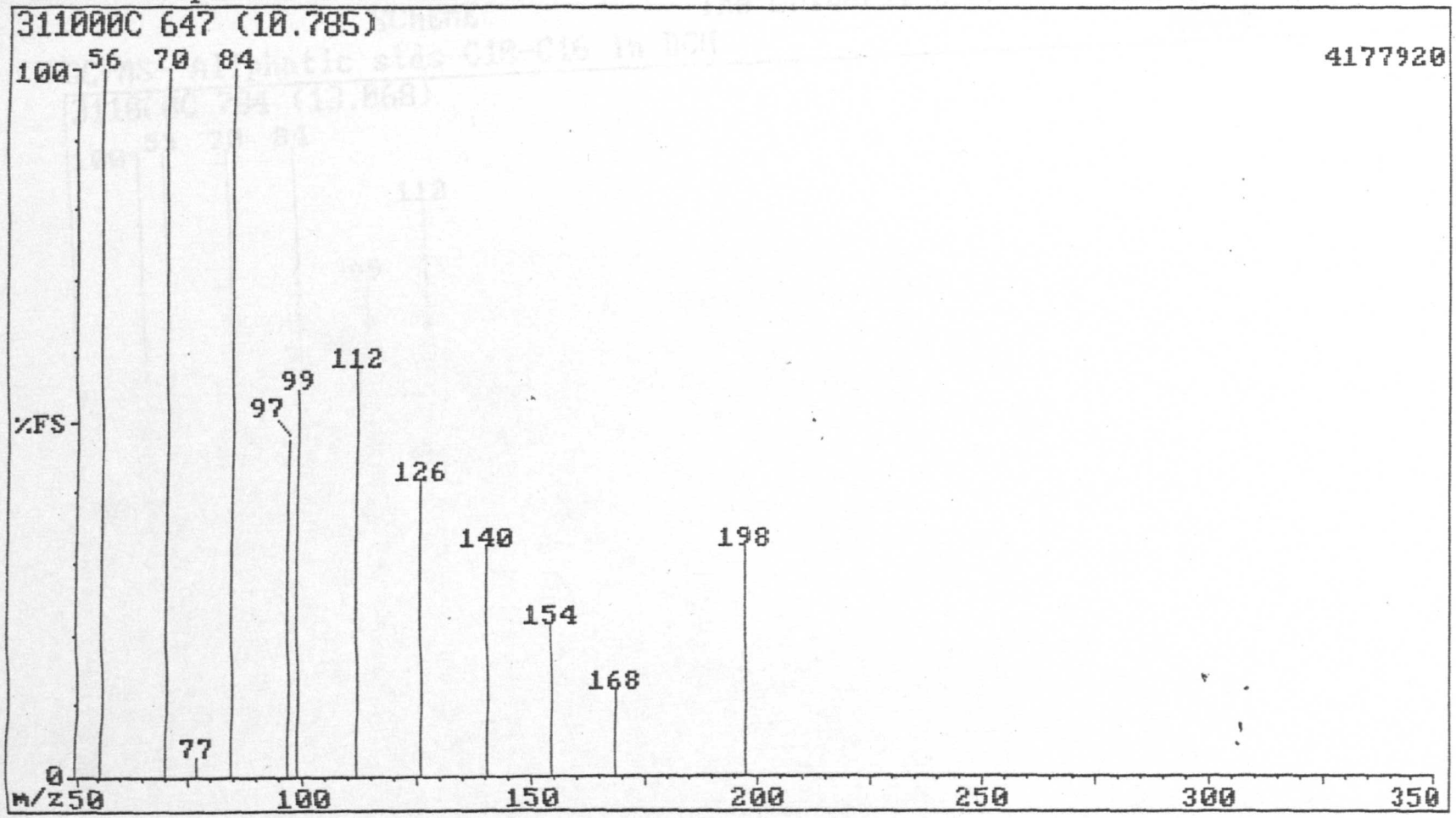

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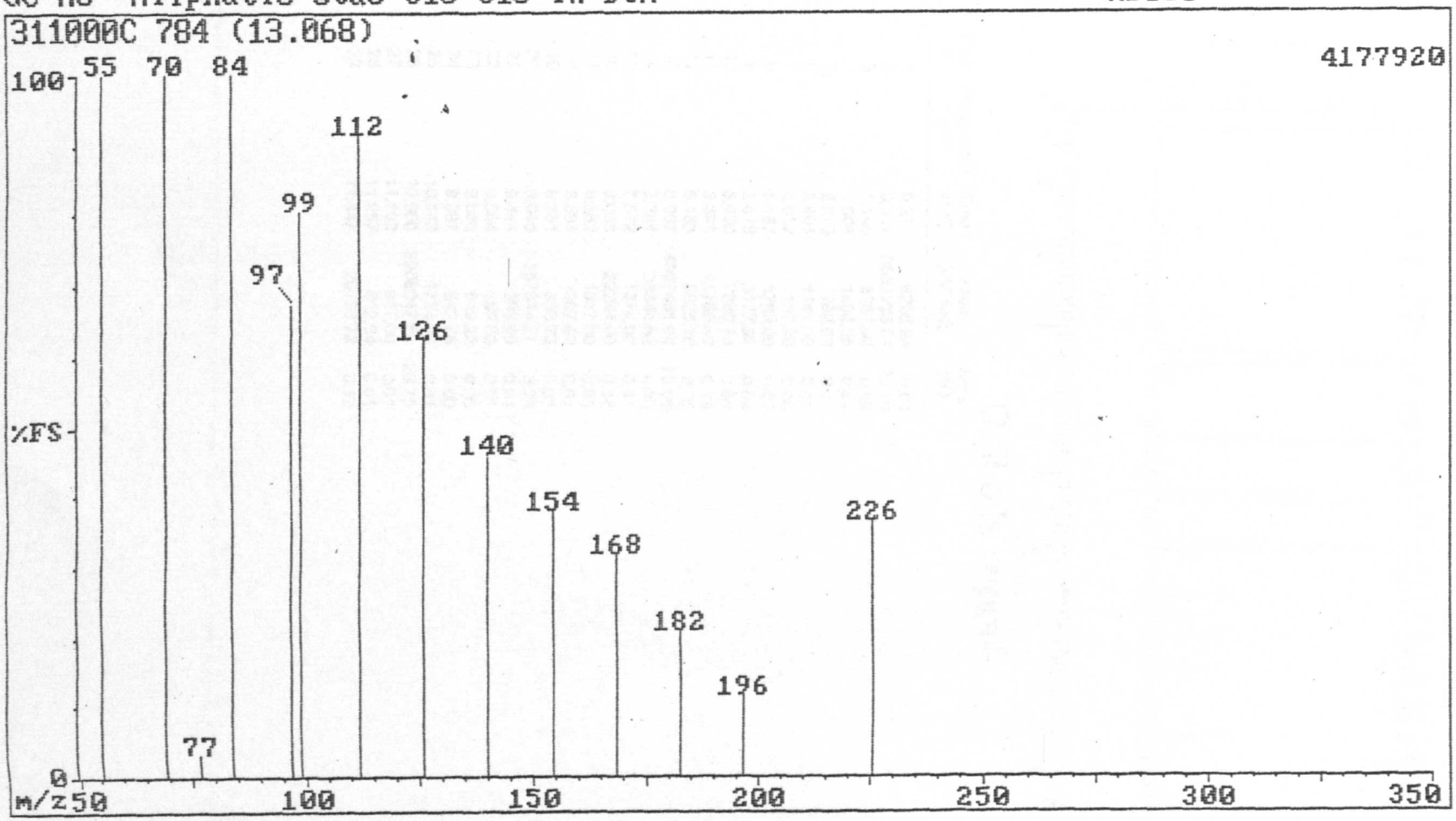

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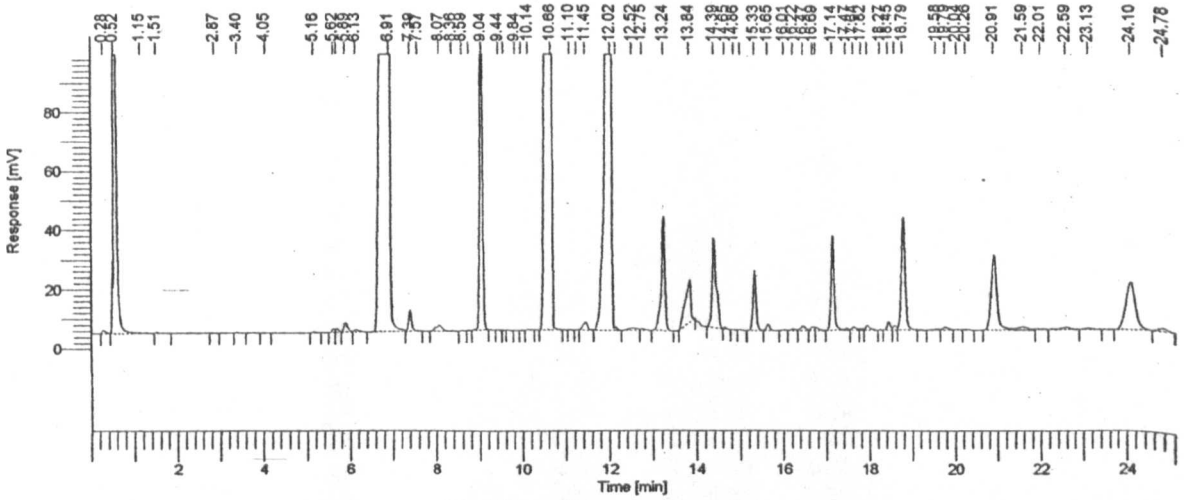

Drill oils report

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		0.277	6226.94	0.04
2		0.517	3353225.01	20.39
3		1.145	3257.00	0.02
4		1.505	1494.55	0.01
5		2.870	282.00	0.00
6		3.403	1141.00	0.01
7		4.052	167.00	0.00
8		5.157	1789.00	0.01
9		5.618	6177.37	0.04
10		5.696	7280.13	0.04
11		5.893	21769.40	0.13
12		6.133	8525.10	0.05
13		6.907	4367431.98	26.56
14		7.394	33344.52	0.20
15		7.572	1374.00	0.01
16		8.068	22264.00	0.14
17		8.362	1017.00	0.01
18		8.594	1397.00	0.01
19		8.751	853.73	0.01
20		9.045	557614.27	3.39
21		9.444	988.50	0.01
22		9.564	189.00	0.00
23		9.836	549.22	0.00
24		9.987	642.78	0.00
25		10.143	1724.00	0.01
26		10.660	3906017.50	23.75
27		11.103	947.00	0.01
28		11.257	852.54	0.01
29		11.447	20188.72	0.12

10/12/01 11:49:13 Result: \\penkw3\Drill\Data\compost001.rst

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
30		12.025	2384727.74	14.50
31		12.143	4894.00	0.03
32		12.520	10752.84	0.07
33		12.746	4601.66	0.03
34		13.243	217117.50	1.32
35		13.841	124824.50	0.76
36		14.390	199596.87	1.21
37		14.654	2895.13	0.02
38		14.857	675.00	0.00
39		15.002	913.00	0.01
40		15.330	108433.34	0.66
41		15.649	10874.66	0.07
42		16.013	1577.20	0.01
43		16.223	3854.10	0.02
44		16.471	11183.10	0.07
45		16.685	6875.80	0.04
46		16.757	6173.57	0.04
47		17.139	172417.00	1.05
48		17.467	5681.00	0.03
49		17.636	8351.92	0.05
50		17.817	3084.52	0.02
51		17.951	12046.29	0.07
52		18.271	607.95	0.00
53		18.454	14079.01	0.09
54		18.597	7712.78	0.05
55		18.793	234906.27	1.43
56		19.579	2779.25	0.02
57		19.786	8581.11	0.05
58		20.043	2508.73	0.02
59		20.264	1260.90	0.01
60		20.914	246077.11	1.50
61		21.593	20290.00	0.12
62		22.015	4155.60	0.03
63		22.592	14714.45	0.09
64		23.128	5692.34	0.03
65		24.097	239314.64	1.46
66		24.785	13536.36	0.08
			16446497.50	100.00

Warning -- Signal level out-of-range in peak

Horse 2

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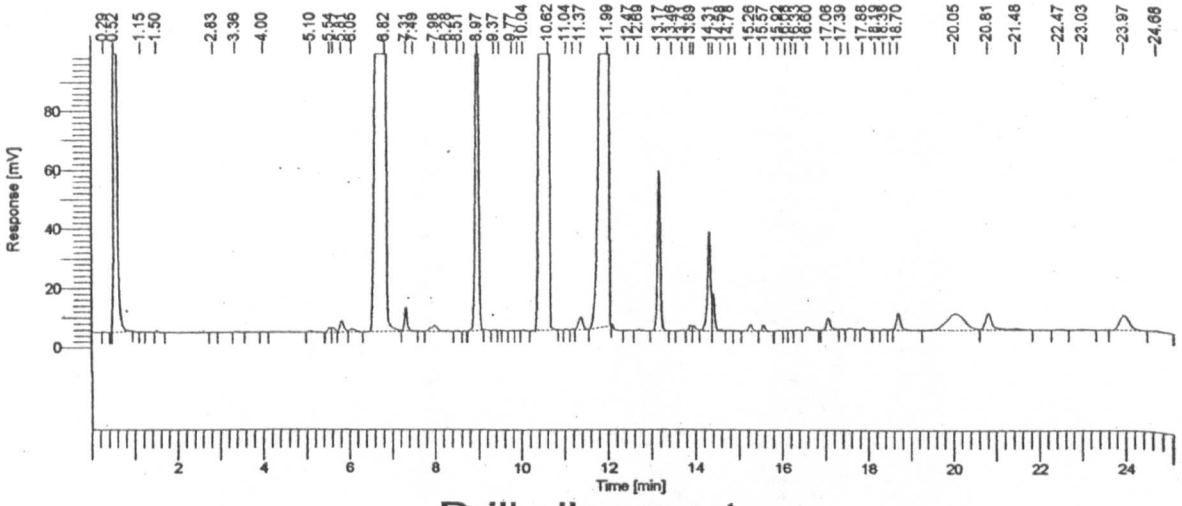
Instrument Name	: Carlo Mega	Channel	: B
Rack/Vial	: 0/0	Operator	: Kate
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	:		

Result File : \\penkw3\Drill\Data\compost002.rst
Sequence File : \\penkw3\Drill\Sequences\compost.seq



Drill oils report

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		0.288	1593.00	0.01
2		0.517	4252720.00	20.21
3		1.145	301.00	0.00
4		1.500	977.00	0.00
5		2.835	269.00	0.00
6		3.362	1129.00	0.01
7		4.000	159.00	0.00
8		5.096	1868.50	0.01
9		5.540	7039.86	0.03
10		5.617	8041.30	0.04
11		5.814	24429.47	0.12
12		6.052	9922.48	0.05
13		6.823	4366671.52	20.75
14		7.313	37067.38	0.18
15		7.487	1356.00	0.01
16		7.983	22549.50	0.11
17		8.280	1180.00	0.01
18		8.512	1539.00	0.01
19		8.668	683.00	0.00
20		8.966	672082.00	3.19
21		9.365	1005.00	0.00
22		9.477	180.50	0.00
23		9.767	1049.82	0.00
24		9.913	1736.67	0.01
25		10.045	2443.51	0.01
26		10.622	6330887.50	30.08
27		11.035	1611.00	0.01
28		11.187	1360.81	0.01
29		11.374	30587.69	0.15

10/12/01 12:32:07 Result: \\penkw3\Drill\Data\compost002.rst

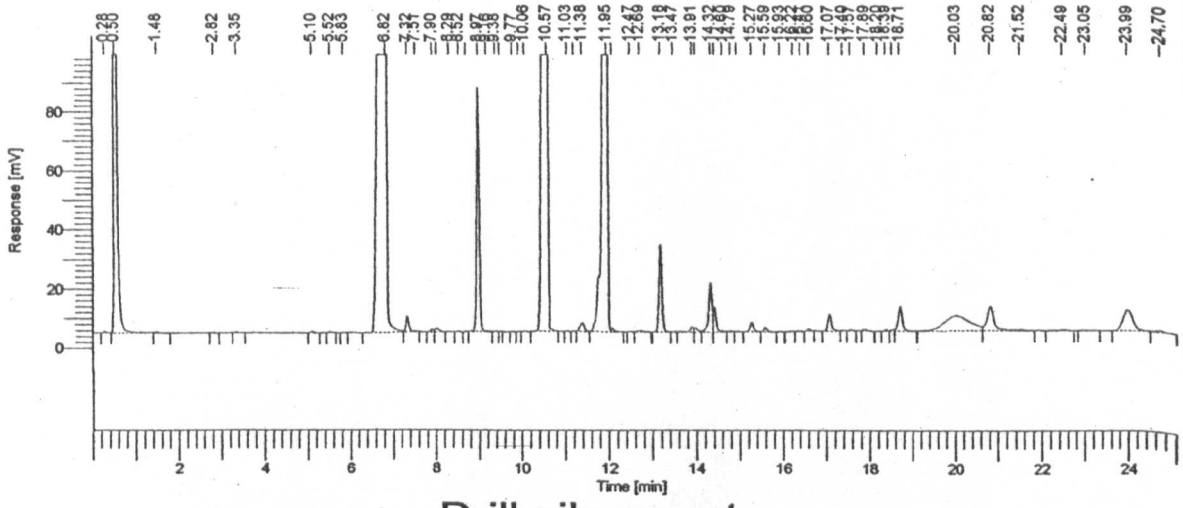
Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
30		11.992	4321405.00	20.53
31		12.469	1282.68	0.01
32		12.689	4671.82	0.02
33		13.170	249067.64	1.18
34		13.456	869.70	0.00
35		13.713	1375.23	0.01
36		13.893	9130.85	0.04
37		13.960	9935.90	0.05
38		14.311	168620.91	0.80
39		14.393	51104.71	0.24
40		14.585	2923.00	0.01
41		14.783	2050.27	0.01
42		14.916	1439.01	0.01
43		15.262	14922.10	0.07
44		15.573	10655.18	0.05
45		15.918	1438.00	0.01
46		16.084	454.87	0.00
47		16.217	828.99	0.00
48		16.326	2176.63	0.01
49		16.596	9427.50	0.04
50		17.058	27684.47	0.13
51		17.389	1344.74	0.01
52		17.553	2482.29	0.01
53		17.879	4529.00	0.02
54		18.193	583.00	0.00
55		18.380	2594.95	0.01
56		18.538	2538.35	0.01
57		18.703	41606.70	0.20
58		20.047	161891.52	0.77
59		20.808	62732.48	0.30
60		21.478	7314.00	0.03
61		22.467	1610.65	0.01
62		23.027	2264.85	0.01
63		23.967	77792.57	0.37
64		24.680	3241.43	0.02
			21046431.50	100.00

Warning – Signal level out-of-range in peak

Poultry ①

Software Version	: 6.1.2.0.1:D19	Date	: 10/12/01 13:29:41
Sample Name	:	Data Acquisition Time	: 10/12/01 13:04:18
Instrument Name	: Carlo Mega	Channel	: B
Rack/Vial	: 0/0	Operator	: Kate
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	:		

Result File : \\penkw3\Drill\Data\compost003.rst
Sequence File : \\penkw3\Drill\Sequences\compost.seq



Drill oils report

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		0.278	2438.81	0.02
2		0.500	4206223.74	29.66
3		1.480	1400.45	0.01
4		2.821	217.00	0.00
5		3.350	974.50	0.01
6		5.096	1503.50	0.01
7		5.519	1550.00	0.01
8		5.829	130.50	0.00
9		6.820	3700791.10	26.10
10		7.322	24257.90	0.17
11		7.505	1237.00	0.01
12		7.899	4854.99	0.03
13		8.006	8914.13	0.06
14		8.295	926.88	0.01
15		8.522	1459.13	0.01
16		8.681	996.59	0.01
17		8.966	360317.50	2.54
18		9.159	2481.00	0.02
19		9.377	1017.55	0.01
20		9.485	245.24	0.00
21		9.772	678.43	0.00
22		9.921	946.20	0.01
23		10.059	2209.22	0.02
24		10.574	2972718.15	20.96
25		11.034	1018.00	0.01
26		11.187	1172.68	0.01
27		11.380	20618.58	0.15
28		11.949	2004272.24	14.13
29		12.068	5506.00	0.04

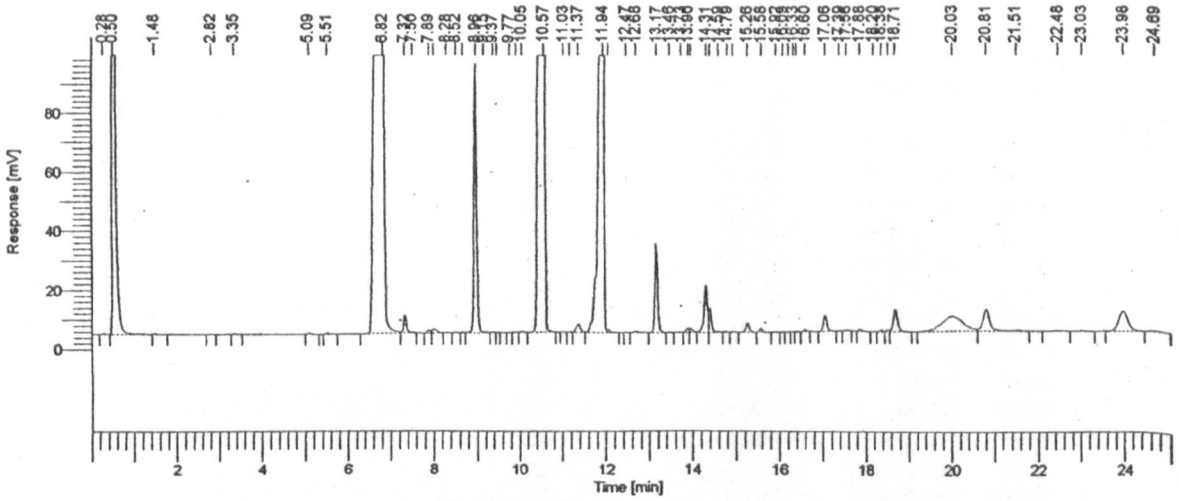
10/12/01 13:29:41 Result: \\penkw3\Drill\Data\compost003.rst

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
30		12.472	662.23	0.00
31		12.695	4410.27	0.03
32		13.176	134387.53	0.95
33		13.466	430.47	0.00
34		13.905	8772.31	0.06
35		13.973	8107.50	0.06
36		14.317	82341.13	0.58
37		14.397	35929.61	0.25
38		14.596	2561.00	0.02
39		14.794	1951.44	0.01
40		14.930	1454.77	0.01
41		15.270	19297.50	0.14
42		15.586	6992.73	0.05
43		15.929	1045.00	0.01
44		16.223	970.33	0.01
45		16.408	1549.67	0.01
46		16.604	3300.00	0.02
47		17.069	33638.03	0.24
48		17.400	1028.35	0.01
49		17.566	1726.62	0.01
50		17.888	3628.50	0.03
51		18.203	339.00	0.00
52		18.389	3212.08	0.02
53		18.546	3299.45	0.02
54		18.715	55817.76	0.39
55		20.031	212514.36	1.50
56		20.822	94245.72	0.66
57		21.518	5088.00	0.04
58		22.492	4359.46	0.03
59		23.046	2598.00	0.02
60		23.991	108753.93	0.77
61		24.696	5505.07	0.04
			14180994.83	100.00

Warning – Signal level out-of-range in peak

Software Version	: 6.1.2.0.1:D19	Date	: 10/12/01 14:30:56
Sample Name	:	Data Acquisition Time	: 10/12/01 14:05:33
Instrument Name	: Carlo Mega	Channel	: B
Rack/Vial	: 0/0	Operator	: Kate
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	:		

Result File : \\penkw3\Drill\Data\compost004.rst
Sequence File : \\penkw3\Drill\Sequences\compost.seq



Drill oils report

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		0.278	1977.69	0.01
2		0.500	4288431.21	29.28
3		1.482	1293.10	0.01
4		2.819	231.50	0.00
5		3.348	1049.50	0.01
6		5.088	1720.00	0.01
7		5.514	2103.50	0.01
8		6.825	4154032.54	28.37
9		7.317	27323.46	0.19
10		7.501	1396.00	0.01
11		7.891	5217.51	0.04
12		8.001	10151.27	0.07
13		8.285	1315.22	0.01
14		8.515	1569.00	0.01
15		8.672	995.93	0.01
16		8.962	397163.05	2.71
17		9.151	2990.00	0.02
18		9.369	1134.15	0.01
19		9.476	264.37	0.00
20		9.769	671.67	0.00
21		9.918	1669.14	0.01
22		10.048	3059.89	0.02
23		10.569	3009338.30	20.55
24		11.026	1059.00	0.01
25		11.180	1109.52	0.01
26		11.370	19687.21	0.13
27		11.939	1918645.27	13.10
28		12.061	5734.00	0.04
29		12.466	582.64	0.00

10/12/01 14:30:56 Result: \\penkw3\Drill\Data\compost004.rs

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
30		12.683	4307.36	0.03
31		13.168	133025.77	0.91
32		13.463	435.23	0.00
33		13.730	808.01	0.01
34		13.901	6497.48	0.04
35		13.963	7273.75	0.05
36		14.312	81515.82	0.56
37		14.394	34971.17	0.24
38		14.588	2133.00	0.01
39		14.786	1624.49	0.01
40		14.922	1087.27	0.01
41		15.264	18071.56	0.12
42		15.581	6554.45	0.04
43		15.924	1077.50	0.01
44		16.090	342.86	0.00
45		16.217	616.89	0.00
46		16.326	590.45	0.00
47		16.402	769.79	0.01
48		16.599	3498.00	0.02
49		17.063	32030.41	0.22
50		17.395	1039.31	0.01
51		17.560	1753.28	0.01
52		17.883	3808.00	0.03
53		18.196	376.00	0.00
54		18.383	3158.67	0.02
55		18.541	3472.78	0.02
56		18.706	51536.32	0.35
57		20.032	178590.78	1.22
58		20.813	81668.31	0.56
59		21.509	4680.00	0.03
60		22.482	3837.91	0.03
61		23.032	2285.09	0.02
62		23.977	104040.39	0.71
63		24.687	5065.61	0.03
			14644459.35	100.00

Warning -- Signal level out-of-range in peak

Appendix

B

Reviewed: 2. September 1997 Reviewer: K. Wallgren	COSHH Form: S18

Chemical Engineering Department

Substance	Sodium sulphate	
Synonyms	Sulphuric acid disodium salt	
Hazards	Harmful if ingested in quantity. Irritating to eyes, skin and respiratory system.	
Exposure Control	Safety glasses, lab coat and chemical-resistant gloves.	
End Storage	Chemical store.	
Disposal	Mix with a combustible material and incinerate. Small amounts can be run to drain with excess water.	
Fire fighting measures	Noncombustible. May emit toxic fumes in fire	
First Aid	Eye contact - Immediately flush with copious amounts of water at least 15 min. Inhalation - Remove to fresh air. Skin contact - Wash with soap and copious amounts of water. Ingestion - Wash mouth with water and give plenty of water to drink. In severe cases obtain medical attention.	
Exposure limit	Long term	Short term

Reviewed: 15. August 1997 Reviewer: K. Wallgren	COSHH Form: D7

Chemical Engineering Department

Substance	Dichloromethane	
Synonyms	Methylene chloride	
Hazards	Carcinogen, irritant. May cause mutagenic or teratogenic effects. May be fatal if swallowed or inhaled. Harmful if absorbed through skin.	
Exposure Control	Safety glasses, lab coat and nitrile gloves. Use only in fume cupboard.	
End Storage	Solvent store.	
Disposal	Mix or dissolve with combustible solvent and incinerate.	
Fire fighting measures	Noncombustible. May emit toxic fumes in fire.	
First Aid	Eye contact - Immediately flush with copious amounts of water at least 15 min. Inhalation - Remove to fresh air, rest and keep warm. In severe cases obtain medical attention. Skin contact - Wash with soap and copious amounts of water. Ingestion - Wash mouth with water and give plenty of water to drink. **Do not induce vomiting.** Obtain medical attention.	
Exposure limit	Long term TWA (8 hr): 350 mg/m^3 (100 ppm)	Short term STEL (10 min): 1740 mg/m^3 (500 ppm)

Reviewed: 30. September 1999 Reviewer: K. Wallgren	COSHH Form: D16

Chemical Engineering Department

Substance	Diethyl ether
Synonyms	Ether
Hazards	Extremely flammable, possible mutagen. May be harmful by inhalation or ingestion. Irritating to eyes, respiratory system and skin.
Exposure Control	Safety glasses, lab coat and chemical-resistant gloves. Use only in fume cupboard.
End Storage	Solvent store. Keep tightly closed, away from heat and open flame.
Disposal	Contact chemical waste disposal company. At no circumstances allow to enter drains.
Fire fighting measures	Carbon dioxide, dry powder or vaporising liquids. Forms explosive mixtures in air.
First Aid	Eye contact – Immediately flush with copious amounts of water at least 15 min. If irritation persists, obtain medical attention. Inhalation - Remove from exposure, rest and keep warm. In severe cases obtain medical attention. Skin contact – Wash with soap and copious amounts of water. In severe cases obtain medical attention. Ingestion - Wash mouth with water provided person is conscious and give plenty of water to drink. Obtain medical attention.

	Long term	Short term
Exposure limit	TWA (8 hrs): 1200 mg/m^3	

Appendix

C

NO_3 test :

- Add 1 drop of NIT 1 and 1 drop of NIT 2 reagents to the NO_3 cupule.
- After 5 minutes, a red color indicates a positive reaction to be recorded on the result sheet.
- A negative reaction may be due to the production of nitrogen : add 2-3 mg of Zn reagent to the NO_3 cupule.
- After 5 minutes, a cupule remaining colorless indicates a positive reaction to be recorded on the result sheet. If the cupule turns pink-red, the reaction is negative as nitrates were present in the tube and were reduced to nitrite by the zinc.

The reaction used for the identification of the bacterium is the reduction of nitrates. It is positive when either of the above reactions (production of NO_2 or N_2) is positive.

The production of N_2 may, however, be useful alone as an additional test (refer to the Analytical Profile Index).

TRP test :

Add 1 drop of JAMES reagent. The reaction takes place immediately : a pink color which develops in the whole cupule indicates a positive reaction to be recorded on the result sheet.

Assimilation tests :

Observe the bacterial growth. An opaque cupule indicates a positive reaction.

Occasionally, a cupule may show weak growth. In this case, the results should be recorded as ∓ ou ± by comparing the intensity to that of the other tests on the strip.

Once these readings have been made, identification should be possible as indicated in the paragraph "Identification". However, in the following cases, the strip must be reincubated :

- if the profile cannot be found in the Analytical Profile Index
- if the following note is indicated for the profile obtained :

IDENTIFICATION NOT VALID
BEFORE 48-HR INCUBATION

Remove the NIT 1, NIT 2 and JAMES reagents by suction and immediately cover tests NO_3 and TRP with mineral oil so that a convex meniscus is formed. Reincubate the strip at 30°C for a further 24 hours and read the all the tests again, except the first 3 (NO_3, TRP and GLU) which should only be read once at 24 hours.

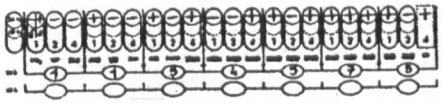
Identification

Identification can be obtained :

- using the Analytical Profile Index : the pattern of the reactions obtained must be coded into a numerical profile.

  On the result sheet, the tests are separated into groups of 3 and a number 1, 2 or 4 is indicated for each. By adding the values corresponding to positive reactions within each group, a 7-digit number is obtained which constitutes the numerical profile. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

- using the identification software by manually entering the 7-digit numerical profile via the keyboard.



1 154 575 *Pseudomonas aeruginosa*

DISPOSAL OF USED MATERIAL

After use, all ampoules, pipettes, strips and incubation boxes should be autoclaved, incinerated, or immersed in a disinfectant for decontamination prior to disposal.

LIMITATIONS

The API 20 NE system is intended uniquely for the identification of those non-fastidious, non-enteric Gram-negative rods included in the database (see Identification Table at the end of this package insert). It cannot be used to identify any other microorganisms or to exclude their presence.

QUALITY CONTROL

The media, strips, and reagents are systematically quality controlled at various stages of their manufacture. For those who wish to perform their own quality control tests with the strip, it is recommended that the following stock cultures be used, to obtain the results below :

	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
1.	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	–	+	–*	–	+
2.	+	–	–	V	V	–	+	–	+	–	–	+	+	–	+	+	+	+	+	–	+
3.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	+
4.	–	–	–	–	+	+	–	+	+	+	+	–	+	+	–	–	–	–	–	–	+

Profiles for tests ADH to PAC obtained after 48 hours of incubation after culture on Trypcase Soy agar.

* Weak reactions may occur.

1. *Aeromonas hydrophila* ATCC 35654
2. *Pseudomonas aeruginosa* ATCC 27853
3. *Alcaligenes faecalis* ATCC 35655
4. *Sphingobacterium multivorum* ATCC 35656

ATCC : American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA.

READING TABLE

TESTS	SUBSTRATES	REACTIONS/ENZYMES	RESULTS	
			NEGATIVE	POSITIVE
NO_3	potassium nitrate	reduction of nitrates to nitrites	NIT 1 + NIT 2 / 5 min colorless	pink-red
		reduction of nitrates to nitrogen	Zn / 5 min pink	colorless
TRP	tryptophane	indole production	JAMES / Immediate colorless pale green / yellow	pink
GLU	glucose	acidification	blue to green	yellow
ADH	arginine	arginine dihydrolase	yellow	orange / pink / red
URE	urea	urease	yellow	orange / pink / red
ESC	esculin	hydrolysis (β-glucosidase)	yellow	grey / brown / black
GEL	gelatine (with India ink)	hydrolysis (protease)	no pigment diffusion	diffusion of black pigment
PNPG	p-nitrophenyl-β-D-galactopyranoside	β-galactosidase	colorless	yellow
GLU	glucose	assimilation	transparent	opaque
ARA	arabinose	assimilation	transparent	opaque
MNE	mannose	assimilation	transparent	opaque
MAN	mannitol	assimilation	transparent	opaque
NAG	N-acetyl-glucosamine	assimilation	transparent	opaque
MAL	maltose	assimilation	transparent	opaque
GNT	gluconate	assimilation	transparent	opaque
CAP	caprate	assimilation	transparent	opaque
ADI	adipate	assimilation	transparent	opaque
MLT	malate	assimilation	transparent	opaque
CIT	citrate	assimilation	transparent	opaque
PAC	phenyl-acetate	assimilation	transparent	opaque
OX	tetramethyl-p-phenylene diamine	cytochrome oxidase	OX / 1-2 min colorless	violet

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Printed in France

api 20 NE

For in vitro diagnostic use

Identification system for non-enteric Gram-negative rods

The API 20 NE system is a standardized micromethod combining 8 conventional tests and 12 assimilation tests for the identification of non-fastidious Gram-negative rods not belonging to the *Enterobacteriaceae* (e.g. *Pseudomonas, Acinetobacter, Flavobacterium, Moraxella, Vibrio, Aeromonas*, etc.). Fastidious organisms having demanding nutritional requirements and requiring appropriate handling precautions (i.e. *Brucella* and *Francisella*) are not included in the API 20 NE database. Alternative procedures must be used to exclude or confirm their presence. The complete list of those bacteria that it is possible to identify with this system is given in the identification Table at the end of this package insert.

PRINCIPLE

The API 20 NE strip consists of 20 microtubes containing dehydrated media and substrates.
The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents.
The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate.
The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

REAGENTS

Kit contents (25 tests) :

- 25 API 20 NE strips
- 25 incubation boxes
- 25 ampoules of AUX Medium
- 25 result sheets
- 1 package insert

Additional products (not included in the kit) :

- NaCl 0.85 % Medium, 2 ml (ref. 20 070)
- Reagents : JAMES (ref. 70 540)
  NIT 1 (ref. 70 440)
  NIT 2 (ref. 70 450)
  OX (ref. 70 460)
  Zn (ref. 70 380)
- Mineral oil (ref. 70 100)
- Pipettes or PSIpettes (ref. 70 250)
- McFarland Standard (ref. 70 900) No. 0.5
- API 20 NE Analytical Profile Index (ref. 20 090) or identification software (consult bioMérieux)
- Ampoule rack (ref. 70 200)
- Wooden applicator sticks

Required laboratory equipment :

- 30°C incubator
- Refrigerator
- Bunsen burner
- Marker pen

COMPOSITION OF MEDIA AND REAGENTS

NaCl 0.85 % Medium 2 ml	Sodium chloride Demineralized water	8.5 g 1000 ml
AUX Medium 7 ml	Ammonium sulphate Agar Mineral base Amino acids Vitamins and nutritional substances Phosphate buffer 0.04 M pH 7.1 Final pH : 7.0-7.2	2 g 1.5 g 82.8 mg 250 mg 35.9 mg qsp 1000 ml
JAMES reagent 5 ml	Compound J 2183 (confidential) HCl 1N S24/25 : Avoid contact with skin and eyes. In case of contact with eyes, rinse immediately with plenty of water for at least 10 minutes. In case of contact with skin, wash with soap and plenty of water.	0.5 g qsp 100 ml
NIT 1 reagent 5 ml	Sulfanilic acid Acetic acid H_2O CORROSIVE R34 : Causes burns. S2 : Keep out of reach of children. S23 : Do not breathe vapours. S26 : In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.	0.4 g 30 g 70 ml
NIT 2 reagent 5 ml	N,N-dimethyl-1-naphthylamine Acetic acid H_2O CORROSIVE R34 : Causes burns. S2 : Keep out of reach of children. S23 : Do not breathe vapours. S26 : In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.	0.6 g 30 g 70 ml
OX reagent 5 ml	Tetramethyl-p-phenylenediamine Isoamyl alcohol HARMFUL R10 : Flammable. R20 : Harmful by inhalation. S24/25 : Avoid contact with skin and eyes.	1 g 100 ml
Zn reagent 10 g	Zinc dust FLAMMABLE R15 : Contact with water liberates highly flammable gases R17 : Spontaneously flammable in air. S7/8 : Keep container tightly closed and dry. S43 : In case of fire use od by metals, use powder extinguishers. "Never use water"	

STORAGE OF THE STRIPS AND MEDIA

The strips and media should be stored at 2-8°C until the expiration date indicated on the packaging.

STORAGE OF THE REAGENTS

The reagents should be stored in the dark at 2-8°C (except NIT 1 which can be stored at 2-30°C and Zn at 8-30°C) until the expiration date indicated on the packaging.
The reagents may be kept for up to 1 month after the ampoules have been opened and the reagents transferred into the dropper-bottles, (or until the expiration date if this comes first) : record the date opened on the bottle label.
The OX and JAMES reagents are very sensitive to light : wrap the bottles in aluminum foil and only leave them out of the refrigerator while being used. Do not leave them on the bench for prolonged periods of time.

USE OF THE REAGENTS

Allow reagents to come to room temperature (20-30°C) before using.

1. NIT 1, NIT 2 and OX reagents :

- Open the ampoule of reagent as indicated in the paragraph "Warnings and Precautions" (ampoule with dropper-cap).
- Dispense one drop of reagent.
- Carefully close the bottle after use and store it as indicated in the paragraph "Storage of the reagents".

2. JAMES reagent :

- Open the ampoule of solvent associated with the JAMES reagent as indicated in the paragraph "Warnings and Precautions" (ampoule with no dropper-cap).
- Take up the contents of the ampoule using a completely dry pipette and transfer this solvent into the dropper-bottle (contains the dehydrated active ingredient).
- Fit the dropper to the bottle.
- Carefully close the bottle.
- Shake.
- Wait 5-10 minutes until the active ingredient is completely dissolved.
- Use the reagent thus reconstituted, carefully close the bottle and store it as indicated in the paragraph "Storage of the reagents".

3. Zn reagent :

- Open the bottle.
- Take up an aliquot of powder (approximately 2-3 mg) using the spatula fixed to the cap and deposit this quantity in the reaction cupule.
- Carefully close the bottle after use and store it as indicated in the paragraph "Storage of the reagents".

- For *in vitro* diagnostic use only.
- Qualified laboratory personnel shoul technique and established precaution agents.
- Do not pipette specimens or reagents by
- Do not use reagents past the expiration
- Upon removal from refrigerator, allow re to room temperature (20-30°C) before us
- Open ampoules carefully as follows :

  - Hold the ampoule in vertical position (whil uppermost).
  - Press the cap down as fa
  - Cover the flattened part the upper part of the thun

  - Apply thumb pressure motion to the flattened p snap off the top of the an cap.
  - *For ampoule with no dropp*
    - Carefully remove the cap
  - *For ampoule with dropper-*
    - Turn the ampoule up maintain it in a vertical po
    - Squeeze on the cap to reagent into the dropper-
- All inoculated products should be cons and handled appropriately.
- All patient specimens and microbi potentially infectious and should be tre the recommended precautions (I *Protection of Laboratory Workers Biohazards and Infectious Disea by Blood, Body Fluids, and TI Guideline* - December 1997).
- After completing test, reading and specimens, spills and inoculated pr autoclaved, incinerated or immersed in to disposal.
- Interpretation of the test results shou competent microbiologist who shoul consideration the patient history, th specimen, colonial and microscopic n necessary, the results of any other particularly the antimicrobial susceptib

api 20 NE

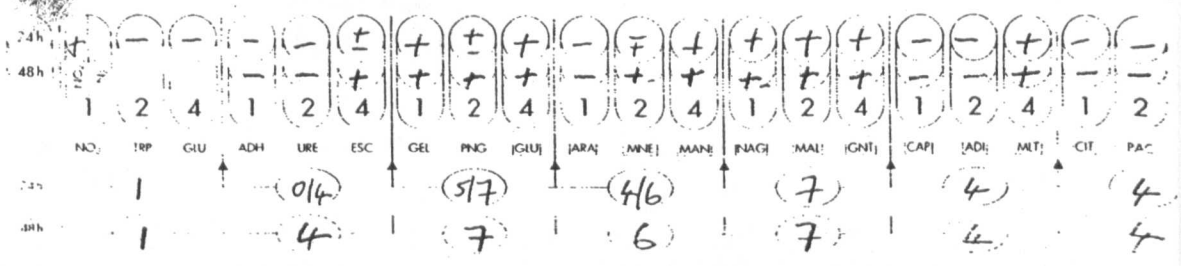
07224 A

REF. : J

24h le 25/10
48h le 26/10

Origine / Source / Herkunft / Origen / Prelievo :

bioMérieux



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Ident. :

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24h le 25/10/00
48h le 26/10.

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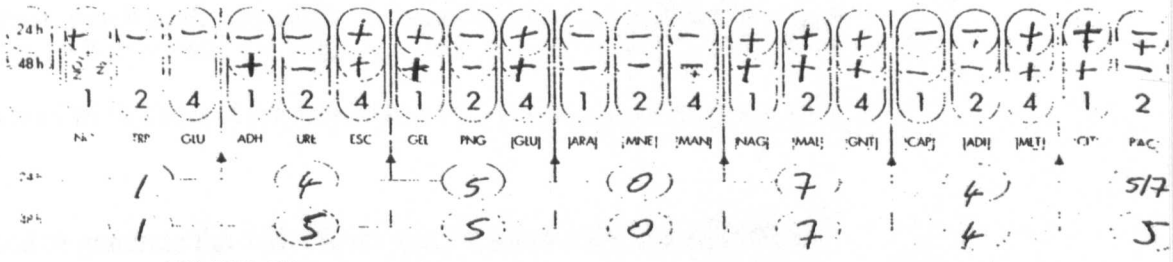

Autres tests Other tests / Weitere Tests / Altri tests / Otros tests :

Ident. :

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REF. : D

24h le 25/10
48h le 26/10

Origine / Source / Herkunft / Origen / Prelievo :

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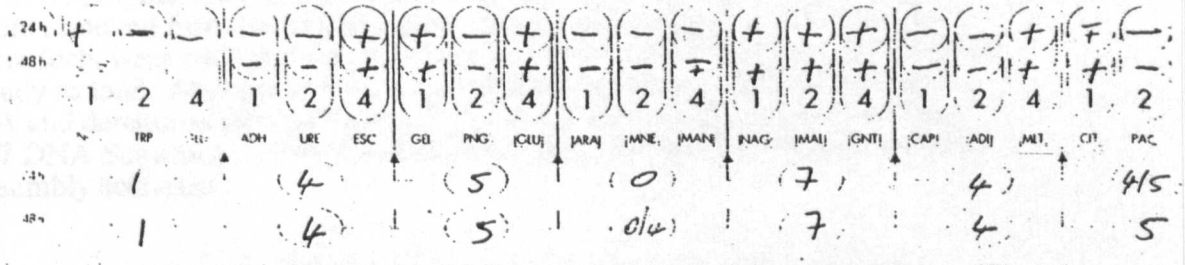


Autres tests Other tests / Weitere Tests / Altri tests / Otros tests :

Ident. :

MIDI LABS

Enclosed are your sample results, including 16S rRNA gene alignment profiles and phylogenetic tree displays.

The bacterial identifications assigned in this report are based on 16S rRNA gene sequence similarity. Sequences analysis was preformed using PE Applied Biosystem's MicroSeq™ microbial analysis software and database. The top ten alignment matches are presented in a percent genetic distance format. In this format a low percent indicates a close match.

Also provided with the report are neighbor joining (Saitou and Nei, **Mol. Biol. Evol.** 4(4):406-425, 1987) phylogenetic trees. The trees are generated using the top ten alignment matches.

Concise alignments are also included. These illustrate positions that differ between your sample and the first match in the database. The position of the mismatch is read vertically from top to bottom and the sequences are read horizontally from left to right.

The results provided in this report are intended for research use only and will be kept confidential.

The protocol used to generate the 16S rRNA gene sequence data is as follows:

The 16S rRNA gene was PCR amplified from genomic DNA isolated from bacterial colonies. Primers used for the amplification correspond to E. coli positions 005 and 1540 (full length packages) and 005 and 531 (500 bp packages). Amplification products were purified from excess primers and dNTPs using Microcon 100 (Amicon) molecular weight cut-off membranes and checked for quality and quantity by running a portion of the products on an agarose gel.

Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labeled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under vacuum and frozen at -20 °C until ready to load. Samples were resuspended in a solution of formamide/ blue dextran/ EDTA and denatured prior to loading. The samples were electrophoresed on a ABI Prism 377 DNA Sequencer. Data was analyzed using PE/Applied Biosystems DNA editing and assembly software.

Thank you very much for choosing MIDI Labs for your bacterial identification needs. Do not hesitate to contact MIDI Labs should you have any questions or comments concerning the data reports.

Please keep us in mind for your future identification or sequencing needs.

MIDI LABS 125 SANDY DRIVE NEWARK DE 19713 USA PH 800 276 8068 FX 302 737 7781 info@midilabs.com http://www.midilabs.com

NCIMB LTD

REVIVAL OF CULTURES

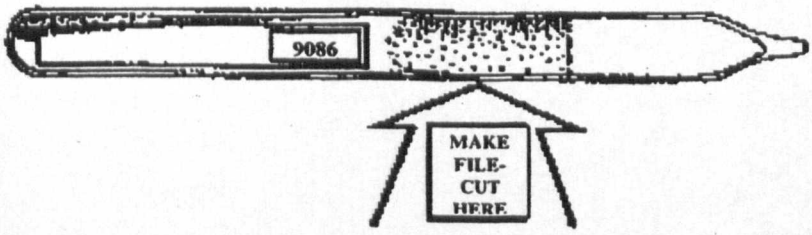
Care should be taken when opening ampoules of freeze-dried cultures as the contents are in a vacuum.

Make a file cut on the ampoule at the mid-point of the cotton wool plug and crack the glass by applying a red hot wire or glass rod to the file cut. Allow air to enter slowly before gently removing the pointed part. If the ampoule proves difficult to crack, use the ampoule snappers provided (place one at either side of the file cut) and physically snap the ampoule using thick wadding or gloves to protect the operator. Discard the upper part of the ampoule and the cotton plug into a disinfectant solution.

After flaming the open end of the ampoule, up to 0.5 ml of a suitable liquid medium should be added to the ampoule and the contents mixed, avoiding frothing. The suspension should be sub-cultured into suitable solid and liquid media. The numbered filter paper strip should be transferred on the tip of a Pasteur pipette or with a loop to the surface of the solid medium with the number upwards. Ready recognition of the culture is thus ensured and confusion is avoided if several ampoules are opened at the same time. If a liquid medium only is used for resuscitation, then the paper strip should be placed into an inoculated tube of medium. It is advisable to prepare more than one subculture from the ampoule contents as a precaution against accidents. Sub-cultures should be incubated at the optimum temperature for the organism under appropriate gaseous conditions.

Resuscitated freeze-dried cultures tend to exhibit a lengthened lag period, but if after prolonged incubation the culture appears to be non-viable, the Collections should be informed. The number and date on the paper strip should be quoted, together with details of the growth medium and its pH, the incubation temperature and time, and any gas mixture employed.

Ampoules not opened soon after receipt should be stored in a cool, dark place (e.g. a refrigerator). They should not be stored exposed to light, particularly direct sunlight. N.B. Organisms should be sub-cultured at least twice before they can be optimally used in experiments.



The Catalogue number of the culture is read from the rounded end of the ampoule: thus the above number is 9086 not 9806. The date the culture was freeze-dried is stamped on the reverse side of the paper slip.

Appendix

D

PRE-SCREENING DATA

Sample	correc.to F	total	spike
control 1a	7944728	7748430	5273614
control 1b	7708163	7345245	5152631
control2a	7714481	8064014	5652209
control2b	8158636	8142793	5396715
A1a	5883768	5822665	5351061
A1b	6578102	6051950	4974717
A2a	5071311	4885935	5209560
A2b	5000419	5025197	5434009
D1a	5118861	5073041	5358814
D1b	5269327	5315047	5454131
D2a	7507210	5790554	4170760
D2b	5810359	5952999	5539958
E1	11949502	11351964	5136826
E2	14255995	13618213	5165308
E3a	7116344	7072737	5374081
E3b	7091317	7083691	5401400
F1a	6174765	6174765	5407215
F1b	6229418	5754157	4994682
F2a	6792917	6615201	5265752
F2b	6854353	6837566	5393972
J1a	6028159	5916152	5306745
J1b	5852459	7460811	6893206
J2a	6694654	6972042	5631259
J2b	6657495	6918123	5618897
MuM1a	15653276	17847459	6165166
MuM1b	16818487	16943910	5447539
MuM2a	7731475	8779425	6140127

Sample	Average
Control	7881502
A	5633400
D	5399516
E	7103831
F	6512863
J	6308192

Table of Results

Sample	correction to F1a	Average	S/D	total	spike
control 1a	7944728	7881502	186241.2	7748430	5273614
control 1b	7708163			7345245	5152631
control2a	7714481			8064014	5652209
control2b	8158636			8142793	5396715
A1a	5883768	5633400	646481.8	5822665	5351061
A1b	6578102			6051950	4974717
A2a	5071311			4885935	5209560
A2b	5000419			5025197	5434009
D1a	5118861			5073041	5358814
D1b	5269327	5399516	296933.4	5315047	5454131
D2a	7507210			5790554	4170760
D2b	5810359			5952999	5539958
E3a	7116344	7103831	12513.5	7072737	5374081
E3b	7091317			7083691	5401400
F1a	6174765	6512863	312128.6	6174765	5407215
F1b	6229418			5754157	4994682
F2a	6792917			6615201	5265752
F2b	6854353			6837566	5393972
J1a	6028159	6308192	373321.7	5916152	5306745
J1b	5852459			7460811	6893206
J2a	6694654			6972042	5631259
J2b	6657495			6918123	5618897

D2a not used in averages – maybe an air bubble in the spike measurement, as it is considerably less than all the other figures.

Pre-screening of V, W, Y, Z

Area	V	W	Y	Z	Control
Total	843787	750487	744501	775398	1029665
Spike	479668	425335	440896	410248	441939
Total-spike	364119	325152	303605	365150	587726

Area	V	W	Y	Z	Control
Total-spike	364119	325152	303605	365150	587726

	V	W	Y	Z	Control
Cor.to V	364119	366687	330304	426939	637901
Cor to W	322874	325152	292890	378579	565645

Pre-screens 2

Sample	cor. to F1a	Average	S/D	total	spike
control 1a	7944728	7881502	186241.2	7748430	5273614
control 1b	7708163			7345245	5152631
control2a	7714481			8064014	5652209
control2b	8158636			8142793	5396715
A1a	5883768	5633400	646481.8	5822665	5351061
A1b	6578102			6051950	4974717
A2a	5071311			4885935	5209560
A2b	5000419			5025197	5434009
D1a	5118861	5926439		5073041	5358814
D1b	5269327	5399516	296933.4	5315047	5454131
D2a	7507210			5790554	4170760
D2b	5810359			5952999	5539958
E1	11949502			11351964	5136826
E2	14255995			13618213	5165308
E3a	7116344	7103831	12513.5	7072737	5374081
E3b	7091317			7083691	5401400
F1a	6174765	6512863	312128.6	6174765	5407215
F1b	6229418			5754157	4994682
F2a	6792917			6615201	5265752
F2b	6854353			6837566	5393972
J1a	6028159	6308192	373321.7	5916152	5306745
J1b	5852459			7460811	6893206
J2a	6694654			6972042	5631259
J2b	6657495			6918123	5618897
MuM1a	15653276	16235882	582605.5	17847459	6165166
MuM1b	16818487			16943910	5447539
MuM2a	7731475	7731475		8779425	6140127

Bioremediation Data, Screening Tests (1)

Sample	Total Peak Area	Mean + SD	% Remediation + rating (1-10)	Peak Heights 1 (%of total)	PH 2 (%)
A1	8049625	8722095;	34	621823(7.7)	4253154(52
A2	9394565	672470	(11)	725947(7.7)	5021030(53
			4		
A1+	7944889	8349359;	37	592061(7.5)	4050335(51
A2+	8753829	404470	(10)	675464(7.7)	4639132(53
B1	10921065	11273620;	14		
B2	11626175	352555	(+15) 8		
B1+	11005928	11048577;	16		
B2+	11091225	42648	(+20)		
C1	8946055	9445541;	28		
C2	9945026	499485	(3) 6		
C1+	8587125	8904493;	32		
C2+	9221861	317368	(4)		
D1	6844726	6978035;	47	535189(7.8)	3608009(52
D2	7111343	133309	(28)	559307(7.8)	3788041(53
			1		
D1+	6524743	6780098;	48	530733(8.1)	3591250(55
D2+	6971052	187809	(27)	619562(8.9)	4246733(60
D2+	6844500			524242(7.7)	3552126(51
E1	9920948	8121804;	38	773247(7.8)	5258669(53
E2	6322660	1799144	(17) 2	495584(7.8)	3347301(52
E1+	8766136	7451324;	43 2	700839(8)	4811662(54
E2+	6136512	1314812	(19)	467588(7.6)	3138872(51
F1	8921951	8465340;	36	696388(7.8)	4778092(53
F2	8008729	456611	(13) 3	627537(7.8)	4274254(53
F1+	7807339	7698628;	42	633517(8.1)	4318672(55
F2+	7589917	108711	(17)	596427(7.9)	4066432(53
G1	13790152	20209765;	+53		
G2	26629378	6419613	(+107%)		
			10		
G1+	12421539	12351093;	6		
G2+	12280647	70446	(+34)		
H1	12649911	11307881;	14		

H2	9965851	1342030	(+16)	9		
H1+	12523029	11432135;	13			
H2+	10341241	1090894	(+24)			
J1	9586784	9143122;	31		746515(7.8)	5134873(53.
J2	8699460	443662	(6)	5	678580(7.8)	4657082(53.
J1+	8614897	8677492;	34		663031(7.7)	4535975(52.
J2+	8740087	62595	(6)		671685(7.7)	4621375(52.
Control 1	10916218	9749090;	26	7		
Control 2	8581962	1167128	(0)			
Control 1+	10597733	9242667;	30	7		
Control 2+	7887601	1355066	(0)			
Made-up-mix 1	12231460	13770465;	0		931775(7.6)	6602005(54
Md-up-mx 2	15309470	1539005	(+41)		1153017(7.5)	8289727(54
Md-up-mx 1+	11799410	13164072;	0		892680(7.6)	6288413(53
Md-up-mx 2+	14528734	1364662	(+42)		1098486(7.6)	7841835(54

Appendix

E

Drill Cutting Preparation for Simulated Drill Cuttings

Limestone	(>1 mm, kg)	=	1.421
	(<1 mm, kg)	=	2.476
Drilling Fines	(kg)	=	2.355

0.982 kg of cuttings was 600 ml as a crushed rock density with drilling fluid.

$$\text{Density} = \frac{0.982}{.0006} = 1636.7 \text{ kg/m}^3$$

Volume of simulated cuttings

$$\frac{1.421+2.476+2.355}{1636.7} = 3.82 \times 10^{-3} \text{ m}^3$$

$= 3.82$ l

Need 7.5% of oil on cuttings

$$\therefore \quad 0.075 = \frac{\alpha}{\alpha + 3.82}$$

$\alpha = 0.309$ l

Novatec 83:17

$$\text{So } \frac{0.309}{0.83} = 0.373 \text{ l} = 373 \text{ ml oil}$$

GC Analysis Results for 1st Reactor Experiments

The following tables illustrate the ratio of short/medium/long chain hydrocarbons.

Retention Times	Day 1 % Area	Day 2 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
0 – 5	0.045	2.221	0.062	0.66	0.104
5 – 10	0.81	19.686	1.072	3.727	0.146
10 – 12.5	59.277	44.223	57.357	55.564	56.17
12.5 – 15	33.411	26.738	34.647	33.998	36.196
15+	6.456	7.133	6.862	6.05	7.381

Table 1: R1

Retention Times	Day 1 % Area	Day 2 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
0 – 5	0.291	0.032	0.016	0.871	2.864
5 – 10	3.694	0.59	2.585	7.519	1.351
10 – 12.5	58.441	59.853	57.814	53.718	51.722
12.5 – 15	32.1	33.53	33.967	32.223	37.767
15+	5.472	5.993	5.473	5.668	6.297

Table 2: X1

Retention Times	Day 1 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
0 – 5	0.020	0.221	0.353	5.420
5 – 10	0.345	3.500	6.038	2.080
10 – 12.5	60.309	57.297	54.442	51.890
12.5 – 15	32.857	33.089	33.281	34.610
15+	1.489	5.892	5.886	5.996

Table 3: R2 + X2

Retention Times	Day 1 % Area	Day 2 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
0 – 5	0.032	0.579	0.525	0.617	0.406
5 – 10	2.105	10.253	6.975	8.328	0.359
10 – 12.5	58.931	51.16	54.203	52.029	56.301
12.5 – 15	33.234	31.68	32.435	33.357	36.107
15+	5.698	6.328	5.861	5.67	6.828

Table 4: Control

Time Eluted	Day 1 % Area	Day 2 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
5	173312	113323	78745	271905	17575
11	2722157	17397645	1748322	1929863	673071
14	1490730	9475086	1021931	1124665	486665
16	231400	1509942	166648	204973	**81938**
Total	4691346	29270800	3044756	3616030	1301316
Constant	2.82	18.43	2.03	2.5	1
5	61369	6150	38718	108694	17575
11	963907	944095	859620	771463	673071
14	527863	514172	502466	449585	486665
16	81938	81938	81938	81938	81938
Total	1661191	1588399	1497055	1445509	1301316

Table 5: X1

Time Eluted	Day 1 % Area	Day 2 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
5	63606	281844	666802	126942	22807
11	5226979	633162	40081147	1881198	8732516
14	2844059	382822	23521382	1144026	5513833
16	502091	**65574**	3903889	206014	990069
Total	12086720 8878488	1431735	90627300 70326710	3405453	15632080
Constant	7.66	1	59.53	3.14	15.1
5	8307	281844	11200	126942	1511
11	682653	633162	673247	1881198	578370
14	371439	382822	395091	1144026	365191
16	65574	65574	65574	206014	65574
Total	1159547	1431735	1181285	1083951	1035340

Table 6: R1

Time Eluted	Day 1 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
5	61408	93560	118876	27040
11	39389209	1521734	1071710	1333327
14	20947714	874864	649158	889610
16	3476683	157495	**115860**	154996
Total	66057140	2673215	1968548	2585092
Constant	30.01	1.36	1	1.34
5	2046	68827	118876	20212
11	1312640	1119452	1071710	996666
14	698080	643587	649158	664986
16	115860	115860	115860	115860
Total	2201345	1966530	1968548	1932364

Table 7: X2 + R2

Time Eluted	Day 1 % Area	Day 2 % Area	Day 3 % Area	Day 4 % Area	Day 12 % Area
5	58620	196339	203988	110023	8075
11	1628797	971768	1573997	687355	1266232
14	914377	600903	936962	440667	812065
16	158652	101128	171371	74902	153563
Total	2784484	1914904	2924168	1321073	2249057
Constant	2.12	1.35	2.29	1	2.05
5	27675	145421	89158	110023	3939
11	768980	719755	687955	687355	617618
14	431691	445068	409523	440667	396093
16	74902	74902	74902	74902	74902
Total	1314597	1418303	1278081	1321073	1097002

Table 8: Control

Code	Water Volume(ml)	Oil Volume (ml)	% Oil
Blank	18	3.0	6
R1	18.5	1.5	3
X1	16	2.5	5
R2 + X2	18	2.0	4

Table 6: Retort Analysis, day 12

Appendix

F

LABORATORY CONTAINMENT LEVEL 1 - CUSTOMER INFORMATION SHEET

Laboratory Containment Level 1 is suitable for work with agents in Group 1[1]. Although defined as unlikely to cause disease by infection, some agents in this group are nevertheless hazardous in other ways (i.e. are allergenic or may be toxigenic) and due precautions must be taken. Guidance on respiratory sensitisation is available[2]. Laboratory personnel must receive suitable and sufficient information, instruction and training in the procedures to be conducted in the laboratory.

1. The laboratory should be easy to clean. Bench surfaces should be impervious to water and resistant to acids, alkalis, solvents and disinfectants.

2. Effective disinfectants should be available for immediate use in the event of spillage.

3. If the laboratory is mechanically ventilated, it is preferable to maintain an inward airflow while work is in progress by extracting room air to atmosphere.

4. All procedures should be performed so as to minimise the production of aerosols.

5. The laboratory door should be closed when work is in progress.

6. Laboratory coats or gowns should be worn in the laboratory and removed when leaving the laboratory suite.

7. Personal protective equipment, including protective clothing, must be:

   (a) stored in a well-defined place;
   (b) checked and cleaned at suitable intervals;
   (c) when discovered to be defective, repaired or replaced before further use.

8. Personal protective equipment which may be contaminated by biological agents must be:

   (a) removed on leaving the working area;
   (b) kept apart from uncontaminated clothing;
   (c) decontaminated and cleaned or, if necessary, destroyed.

9. Eating chewing, drinking, taking medication, smoking, storing food and applying cosmetics should be forbidden.

10. Mouth pipetting should be forbidden.

11. The laboratory should contain a basin or sink that can be used for hand washing.

12. Hands should be decontaminated immediately when contamination is suspected and before leaving the laboratory.

13. Bench tops should be cleaned after use.

14. Used glassware and other materials awaiting disinfection should be stored in a safe manner. Pipettes, for example, if placed in disinfectant, should be totally immersed.

15. Contaminated materials whether for recycling or disposal, should be stored and transported in robust and leakproof containers without spillage.

16. All waste material, if not to be incinerated, should be disposed of safely by other appropriate means.

17. Accidents and incidents should be immediately reported to and recorded by the person responsible for the work or other delegated person.

Whilst NCIMB believes the information contained herein to be correct, it is the responsibility of the recipient to ensure it is accurate, up to date and compliant with current Health & Safety legislations.

[1] Some agents that would qualify for inclusion in this group may be pathogens of animals or plants (see Appendix 20)* of categorisation of pathogens according to hazard and containment categories (HMSO 1995). Certain additional control measures specified by Agriculture Departments may be necessary to prevent their release to the environment.

[2] See Preventing asthma at work - how to control respiratory sensitisers 1994 HSE Books ISBN 0 7176 0661 9

*Pages 31 & 32 Advisory Committee on Dangerous Pathogens (Ref)

LABORATORY CONTAINMENT LEVEL 2

Laboratory Containment level 2 must be used for work with biological agents in Hazard Group 2[1]. Laboratory personnel must receive suitable and sufficient information, instruction and training in working safely with agents in Group 2. A high standard of supervision of the work should be maintained.

1 Access to the laboratory is to be restricted to authorised persons.

2 There must be specified disinfection procedures.

3 If the laboratory is mechanically ventilated, it must be maintained at an air pressure negative to atmosphere while work is in progress (see paragraph 17 below).

4 Bench surfaces must be impervious to water, easy to clean and resistant to acids, alkalis, solvents and disinfectants.

5 There must be safe storage of biological agents.

6 Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet, isolator or be otherwise suitably contained.

7 There must be access to an incinerator for the disposal of infected animal carcasses (see paragraph 24).

8 Personal protective equipment, including protective clothing, must be:

(a) stored in a well-defined place;
(b) checked and cleaned at suitable intervals:
(c) when discovered to be defective, repaired or replaced before further use.

9 Personal protective equipment which may be contaminated by biological agents must be:

(a) removed on leaving the working area:
(b) kept apart from uncontaminated clothing;
(c) decontaminated and cleaned or, if necessary, destroyed.

10 There should be adequate space (24 m^3) in the laboratory for each worker.

11 The laboratory door should be closed when work is in progress.

[1] Some agents in this group may be pathogens of animals (see Appendix 20). Certain additional control measures specified by Agriculture Departments may be necessary to prevent their release to the environment.

Appendix

G

Rotating Drum Reactor 1

	1Rhod/star	1Rhod/end	2 start	2 end	3 start	3 end	3 end/tap	4 start
Total	1190610	384508.4	1049453	931548.3	1053886	665768.7	967562.6	914821.4
Spike	446169.6	416692.6	450399.4	448300.8	439673.6	373003.3	427271	419217.6
Cor.total to 3 start	1173275	405714.4	1024461	913621.4	1053886	784767.6	995648.5	959460.7

	% left		4 end	
1	34.6	Rhod	802653.6	
2	89.2	control	454937.5	380080.9
3	74.5	Consotia		
4	80.8	J	775723.3	

Rotating drum reactor 2

	SPIKE	TOTAL		cor to each other	corr to 1,1/6
1, 1/6	462719.4	954688	22-Aug	954688	
1, 26/6	461914.4	478035	22-Aug	478868	
2, 1/6	464543	1171383	22-Aug	1171383	1166783
2, 26/6	439571.2	612548	22-Aug	647347	644805
3, 1/6	517939.4	1496882	09-Aug	1496882	1337292
3, 26/6	483737.8	922447	09-Aug	987665	882365
4, 1/6	619188.5	1071525	10-Aug	910758	
4, 26/6	526288.3	765394	10-Aug	526288	

	%reduction	%
Reactor 1	49.84	50.16
Reactor 2	44.74	55.26
Reactor 3	34	66
Reactor 4	42.22	57.78

	%reduction
V	49.84
W	44.74
Control	34
Rhod	42.22

Reactor Experiment 1

END ml	SG	g		Rhodococcus percentage ml	percentage g	% reduction ml	% reduction g
300.00	1.00	300.00		33.99%	18.18%	0.00%	0.00%
520.00	2.50	1300.00		58.92%	78.79%	0.00%	0.00%
62.50	0.80	50.00		7.08%	3.03%	65.28%	65.28%
882.50		1650.00		100.00%	100.00%	11.75%	5.39%
520.00	2.50	1300.00		89.27%	96.30%	0.00%	0.00%
62.50	0.80	50.00		10.73%	3.70%	65.28%	65.28%
582.50		1350.00		100.00%	100.00%	16.79%	6.51%

Reactor Experiment 2

END ml	SG	g		V' percentage ml	percentage g	% reduction ml	% reduction g
300.00	1.00	300.00		32.96%	17.94%	0.00%	0.00%
520.00	2.50	1300.00		57.12%	77.74%	0.00%	0.00%
90.29	0.80	72.23		9.92%	4.32%	49.84%	49.84%
910.29		1672.23		100.00%	100.00%	8.97%	4.12%
520.00	2.50	1300.00		85.21%	94.74%	0.00%	0.00%
90.29	0.80	72.23		14.79%	5.26%	49.84%	49.84%
610.29		1372.23		100.00%	100.00%	12.82%	4.97%

Reactor Experiment 3 including manure (Poultry)

END ml	SG	g		percentage ml	percentage g	% reduction ml	% reduction g
149.36	**1.00**	149.36		**19.25%**	10.79%	89.76%	89.76%
611.00	**2.00**	1222.00		78.75%	88.31%	0.00%	0.00%
15.52	**0.80**	12.42		**2.00%**	0.90%	96.12%	96.12%
775.88		1383.78		100.00%	100.00%	68.58%	53.87%
611.00	2.00	1222.00		97.52%	98.99%	0.00%	0.00%
15.52	0.80	12.42		2.48%	1.01%	96.12%	96.12%
626.52		1234.42		100.00%	100.00%	38.03%	19.95%

END ml	SG	g		percentage ml	percentage g	% reduction ml	% reduction g
34.42	**1.00**	34.42		**6.00%**	3.22%	97.95%	97.95%
502.50	**2.00**	**1005.00**		87.60%	94.03%	0.00%	0.00%
36.71	**0.80**	29.36		**6.40%**	2.75%	90.82%	90.83%
573.63		1068.78		100.00%	100.00%	77.74%	64.37%

Reactor Experiment 1 Rhodococcus

	START ml	SG	g	ml	percentage	g	percentage
WC	300.00	1.00	300.00	30.00%		17.20%	
DM	520.00	2.50	1300.00	52.00%		74.54%	
HC	180.00	0.80	144.00	18.00%		8.26%	
TOTAL	1000.00		1744.00	100.00%		100.00%	
Remove WC							
DM	520.00	2.50	1300.00	74.29%		90.03%	
HC	180.00	0.80	144.00	25.71%		9.97%	
total	700.00		1444.00	100.00%		100.00%	

Reactor Experiment 2 V'

	START ml	SG	g	ml		g	
WC	300.00	1.00	300.00	30.00%		17.20%	
DM	520.00	2.50	1300.00	52.00%		74.54%	
HC	180.00	0.80	144.00	18.00%		8.26%	
TOTAL	1000.00		1744.00	100.00%		100.00%	
Remove WC							
DM	520.00	2.50	1300.00	74.29%		90.03%	
HC	180.00	0.80	144.00	25.71%		9.97%	
total	700.00		1444.00	100.00%		100.00%	

Reactor Experiment 3 including manure (Poultry)

	START ml	SG	g	ml	percentage	g	percentage
WC	**1458.00**	**1.00**	1458.00	59.05%		48.60%	
DM	611.00	**2.00**	1222.00	24.75%		40.73%	
HC	**400.00**	**0.80**	320.00	16.20%		10.67%	
TOTAL	2469.00		3000.00	100.00%		100.00%	
Remove WC							
DM	611.00	**2.00**	1222.00	60.44%		79.25%	
HC	400.00	0.80	320.00	39.56%		20.75%	
total	1011.00		1542.00	100.00%		100.00%	

Reactor Experiment 3 including manure (Horse)

	START ml	SG	g	ml	percentage	g	percentage
WC	**1675.00**	**1.00**	**1675.00**	64.99%		55.83%	
DM	502.50	**2.00**	1005.00	19.50%		33.50%	
HC	400.00	**0.80**	320.00	15.52%		10.67%	
TOTAL	2577.50		3000.00	100.00%		100.00%	

Appendix

H

Input Sheet

Start		
Units g/ml	ml	
	%	SG
WC	59.05%	1.00
DM	24.75%	2.00
HC	16.20%	0.80
	Vol / Mass	
TOTAL	2469	

End		
Units g/ml	ml	
	%	SG
WC	19.25%	1.00
DM	78.75%	2.00
HC	2.00%	0.80
	Vol / Mass	
TOTAL	775.88	

Title Compost

Strain Poultry

Output Sheet

	START ml	SG	g	% ml	% g	END ml	SG	g	% ml	% g	% reduction ml	% reduction g
WC	1457.94	1.00	1457.94	59.05%	48.60%	149.36	1.00	149.36	19.25%	10.79%	89.76%	89.76%
DM	611.08	2.00	1222.16	24.75%	40.74%	611.01	2.00	1222.01	78.75%	88.31%	0.01%	0.01%
HC	399.98	0.80	319.98	16.20%	10.67%	15.52	0.80	12.41	2.00%	0.90%	96.12%	96.12%
TOTAL	2469.00		3000.08	100.00%	100.00%	775.88		1383.78	100.00%	100.00%	68.58%	53.88%
Remove WC												
DM	611.08	2.00	1222.16	60.44%	79.25%	611.01	2.00	1222.01	97.52%	98.99%	0.01%	0.01%
HC	399.98	0.80	319.98	39.56%	20.75%	15.52	0.80	12.41	2.48%	1.01%	96.12%	96.12%
total	1011.06		1542.14	100.00%	100.00%	626.52		1234.43	100.00%	100.00%	38.03%	19.95%

Title Compost

Strain Poultry