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BIOREMEDIATION OF HYDROCARBON WATER POLLUTION BY BIOAUGMENTATION USING SOUTHERN AFRICAN BACTERIAL ISOLATES



by

Claire Booyjzsen

A dissertation presented to the Faculty of Science
of the University of the Witwatersrand
in fulfilment of the requirements for the degree
of Master of Science

Johannesburg 2007

"I want to know God's thoughts; the rest are details." Albert Einstein

Dedicated towards the Greater Good. . . .

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at any other university.

……

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This dissertation is submitted after examination and approval by the following supervisor:

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19th day of March, 2007.

ABSTRACT

A new, non-pathogenic bioaugmentation product was formulated specifically for underground use in South African mines, using local bacterial isolates. This was designed for the remediation of various hydrocarbons via biochemical breakdown by sub-surface microorganisms. The active microorganisms were isolated from hydrocarbon-polluted areas of a gold mine. Many commercially available bioaugmentation products are already in existence however, all, to our knowledge, have been developed and tested primarily for use in the northern hemisphere. None have been formulated and tested in Africa. Our series of bacterial isolates are the first to be isolated from mine soils for hydrocarbon biodegradation purposes. Such isolates have further, not previously been tested on sub-surface contamination. The safety associated with the use of such a product in a closed mine-environment is of paramount importance.

Initial batch-flask experiments were conducted using a readily-available commercial bioremediation product. This was tested on simple surfactant molecules and compared to the biodegradation observed under standard waste water treatment plant conditions. The bioremediation product increased biodegradation by 6% on average. Bacteria in the product were identified by 16S rDNA gene sequence analysis and found to be homologous to potentially pathogenic *Bacillus cereus*, known especially to effect immunocompromised individuals, this was of particular concern in the closed mine system.

South African isolates were sourced from various hydrocarbon-polluted sources, with six bacteria ultimately being selected from deep sub-surface mine soil and water samples. The ability of these isolates to biodegrade waterborne monograde engine oil was assessed via GC-FID. The isolate showing average percentage growth increase, homologous to *Pseudomonas pseudoalcaligenes*, was found to degrade the motor oil by 98%. The new isolates were, on average, 16% more efficient at biodegrading petroleum hydrocarbons than the commercial bioremediation product isolates. Formulation of these isolates into the first commercially-available South African developed and tested bioaugmentation product will prove a successful conclusion to this study.

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First and foremost I feel that I have to acknowledge my parents. They are the ones who have raised me and cared for me all the way through my life. Ultimately, they are responsible for me being privileged enough to pursue a degree and postgraduate research.

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DEFINITION OF TERMS

BTEX – Benzene, Toluene, Ethylbenzene and Xylene. Volatile organic hydrocarbons found in petroleum and its derivatives.

CBP – commercial bioremediation product, imported from Denmark.

CFU – colony forming units = [number of colonies on plate H $(dilution)^{-1}$] / volume plated.

Mine soil – mine soil for the purposes of this dissertation refers to samples taken from the main working area. These are generally soil, dirt, dust, cement and crushed rocks, formed from the mining process carried out in the surrounding area.

Mine water – mine water for the purposes of this dissertation refers to samples of water taken from various points in the mine water system. Some are from taps, some from dams and some from waste chutes. These will be differentiated between during the course of the dissertation.

LIST OF ABBREVIATIONS

APC – alkyl phenol carboxylate

ASTM – American Society for Testing and Materials

APE – alkyl phenol ethoxylate

Acetyl Co-A – acetyl co-enzyme A

bp. – base pairs

cfu/ml – colony forming units per millilitre

COD – chemical oxygen demand

DEC – Department of Environmental Conservation

DNR – Department of Natural Resources

DOC – dissolved organic carbon

DOE – Department of Energy

DOH – Department of Health

EIA – Energy Information Administration

EPA – Environmental Protection Agency

FID – flame ionization detector

GC – gas chromatography

LAS – linear alkylbenzene sulfonate

MBAS – methylene blue active substances

NAS – National Academy of Sciences

NA – nutrient agar

NB – nutrient broth

PAH – polyaromatic hydrocarbon

PCB – polychlorinated biphenyl

PCR – polymerase chain reaction

PT benzene – propylene tetramer benzene sulfonate

rDNA – ribosomal DNA

SDBS – sodium dodecylbenzene sulfonate

SONA – standard one nutrient agar

TCA cycle – tricarboxylic acid cycle

UCM – unresolved complex mixture

UV/Vis – ultra violet / visible

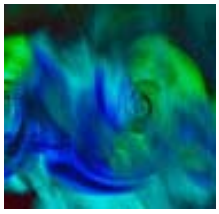
VRBG – vile red bile glucose

WRI – World Resources Institute

WWTP – waste water treatment plant

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

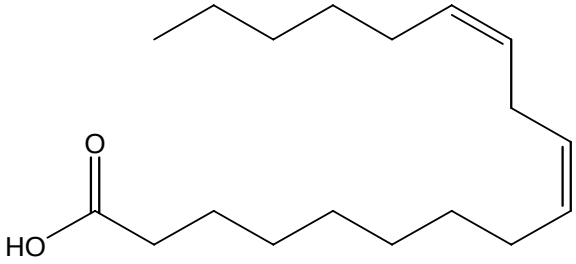


1.1 Significance of Hydrocarbon Biodegradation

Hydrocarbons are a vast class of energy rich compounds composed mainly of carbon and hydrogen. They can broadly be divided into three categories: aliphatic saturated, aliphatic unsaturated and aromatic hydrocarbons. The term is often loosely applied to derivatives containing other elements like oxygen, sulfur or the halogens, for example fatty acids, surfactants and polychlorinated biphenyls (PCB's) (Figure 1.1). Hydrocarbon compounds appear in every aspect of our daily lives from food and detergents to pesticides. Some of the most important are those that drive the world economy: fossil fuels like petroleum and coal. Rich in hydrocarbons and their derivatives, they are the world's main energy source accounting for 80% of primary energy consumption (Friedleifsson 2003). Current global crude oil utilization stands at around 80 million barrels per day. The U.S. Energy Information Administration (EIA) expects this figure to increase by 42% by the year 2030 (Figure 1.2) (DOE, 2006).

Most forms of hydrocarbon pollutants eventually find their way into natural water systems and aquatic habitats, either actively or accidentally. It is then that their biodegradation becomes important. Biodegradation is the transformation or breakdown of substances into simpler components through the biochemical reactions of microorganisms such as bacteria, yeasts and fungi (Hemond *et al.* 2000). With levels of petroleum consumption forecast to continue rising, understanding biodegradation – the natural process for the removal of hydrocarbons from the environment – is more crucial than ever. The two specific hydrocarbon substrates of interest in this study were sodium dodecylbenzene sulfonate (SDBS) and engine oil. Further examples in this text will relate specifically to the chemical nature and biodegradation of these highlighted hydrocarbons.

a)



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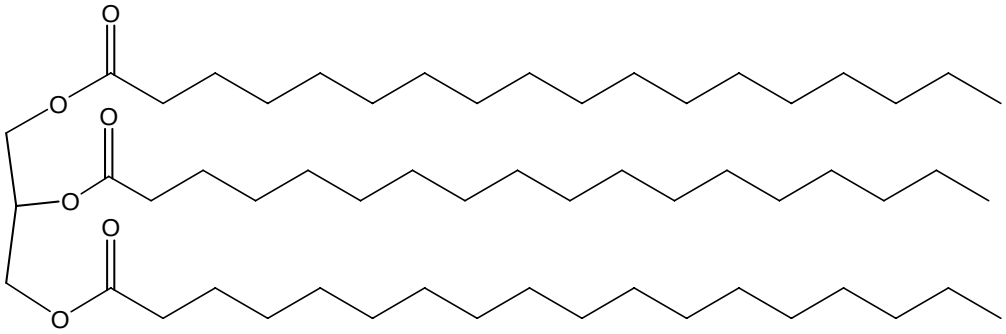

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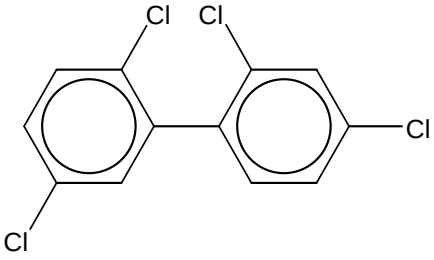


Figure 1.1 The chemical structure of some ubiquitous hydrocarbons: a) the fatty acid linoleic acid, b) glycerol tristearate and c) a polychlorinated biphenyl (found typically in pesticides).

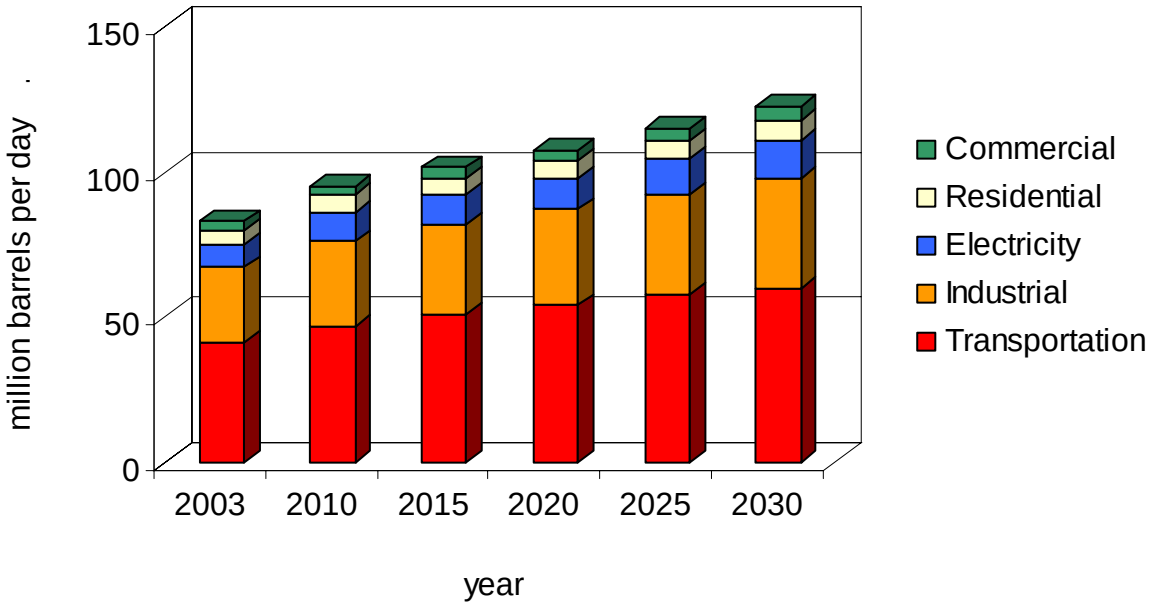


Figure 1.2 "Black Gold" or crude oil is the life blood of global economics. Consumption is shown by sector with transportation, clearly the largest consumer (adapted from DOE 2006).

There are three main paths by which surfactants find their way into the environment (Scott *et al.* 2000):

- Industrial discharge into water systems
- Effluent from wastewater treatment plants
- Sewage sludge usage on land

Surfactants were recognized as pollutants as long as six decades ago, when reports of "foaming rivers" near water treatment plants became common place. The main culprit was propylene tetramer benzene sulfonate (PT benzene). This surfactant, routinely in use at the time, was resistant to bacterial biodegradation by virtue of a branched alkyl chain. Today, most detergents are more readily biodegradable in natural environments, due mostly to the prohibition of surfactants like PT benzene.

However, around the same time that PT benzene was banned there was a switch from powdered soap-based detergents to liquid synthetic surfactant-based detergents. Use of synthetic surfactants rose by 4.5 k tons per annum during this period from 1940 – 1970. Surfactant consumption in the northern hemisphere for petroleum production and related activities exceeded 300 k tons (Scott *et al.* 2000). It was at this time that the potential risk of surfactant contamination of the environment became of greatest concern and the need for investigation into its biodegradation became obvious.

There are also three main pathways by which petroleum hydrocarbons find their way into the environment. These differ quite substantially from those for surfactants (Morgan *et al.* 1989):

- Major transportation associated spills from tankers, pipelines, refineries and storage tanks
- Seepage from natural oil reservoirs
- Domestic waste, including runoff from road surfaces

The primary difference is that accidental spillage associated mainly with the transportation of petroleum, is common. Since, as illustrated previously by Figure 1.2, transportation is the greatest consuming sector of crude oil, the impact of these spillages is amplified. Unlike surfactant contamination, which can rarely be seen, oil pollution is visible and emotive. The most famous and well-documented, although not the largest, example of this is the Exxon Valdez spill off of the coast of Alaska in 1989. It is estimated that 41 million litres (0.04 megatonnes) of oil was spilled effecting 2000 km of coastline. Clean-up efforts were among the largest and most complex in the world and included the use of skimmers and booms, burning, chemical dispersants and biostimulation fertilizers. Its significance, even by today's standards, is its claim as the largest and most ambitious bioremediation project ever undertaken. During these clean-up efforts it was noted that the addition of fertilizers –

composed mainly of nitrogen and phosphorus – accelerated removal of oil 5 fold or more (Bragg *et al.* 1994, Swannell *et al.* 1996).

It is estimated that annual global input of petroleum into the environment is between 1.7 and 8.8 million metric tons, derived mainly from anthropogenic sources (NAS 1985). Considering this and the fact that petroleum hydrocarbons are, and will most likely remain, the world's principal energy source for many decades to come (Mrayyan *et al.* 2005), biodegradation by naturally occurring microbial populations is a valuable tool that may be exploited to our advantage. Alternatively, the addition of exogenous microorganisms to enhance biodegradation can be an effective treatment for numerous forms of hydrocarbon contamination, including surfactants and petroleum hydrocarbons (Aldrett *et al.* 1997).

1.2 The Chemical Structure of Hydrocarbons

Surfactants or, surface-active agents are the most important components of any detergent or cleaning agent (Hashim *et al*. 1992). A surfactant is a large amphipathic molecule, which decreases solvent surface tension by being strongly adsorbed at any interface. It is these molecules in the detergent formulation that are largely responsible for the observed cleaning action. Figure 1.3 shows various surfactant categories (Griffiths *et al.* 1986, White *et al.* 1999) divided based on charge which is important in the microorganism's mode of interaction with the surfactant (Volkering *et al.* 1997). Clearly visible are the hydrophobic carbon chain and hydrophilic polar head group (highlighted in red) portions of the molecule. These two components provide the physical cleaning action of the surfactant. Toxicity of the surfactant must also be considered with respect to the organisms used in biodegradation, as must micelle formation (Volkering *et al.* 1997).

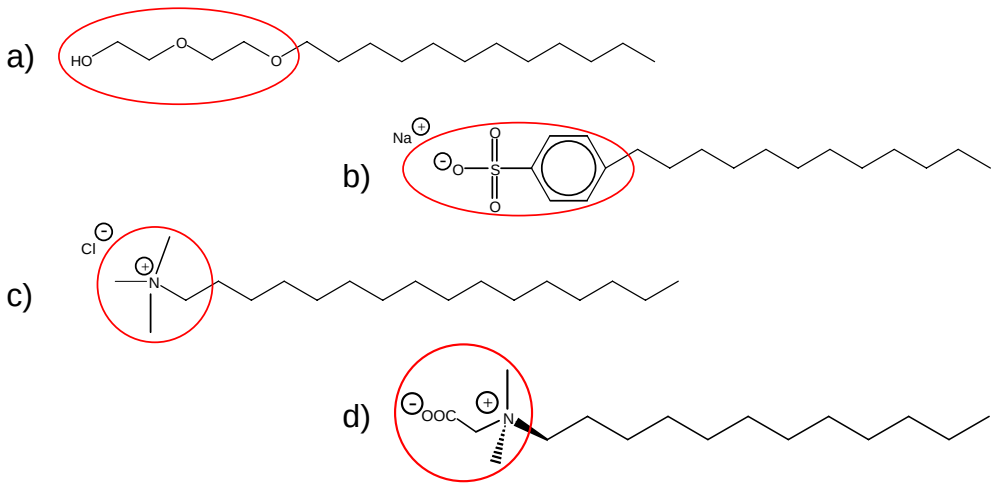


Figure 1.3 Four general types of surfactants: a) non-ionic sodium stearate, b) anionic sodium *p*-dodecylbenzene sulfonate, c) cationic hexadecyl trimethylammonium chloride and d) zwitterionic *N*-dodecyl *N*,*N*-dimethyl glycine.

There are many commercially available detergents and cleaning formulations, however, there is relatively little difference between the surfactants contained therein. Linear alkylbenzene sulfonates (LAS) are currently the major class of anionic surfactant in use, since the use of PT benzene was banned by the US detergent industry in 1965 (Hashim *et al.* 1992). With the aromatic ring randomly distributed over the length of the ten to thirteen carbon chain (Schoberl 1989), LAS are linear rather than branched molecules, making them more readily biodegradable (see section 1.3.1). Another common class of surfactant, alkyl phenol ethoxylates (APE), is used in a great variety of products: petroleum recovery chemicals, paints, textiles, cosmetics, detergents and pesticides (Scott *et al.* 2000).

These surfactants are partially aerobically degraded in sewage treatment plants (95% MBAS; >80% DOC) where they become partially adsorbed to sewage sludge (Schoberl 1989). When this sludge is applied to land as fertilizer, surfactant contamination is spread. Further aerobic biodegradation does

occur in the soil, consequently the risk of hydrocarbon contamination of the soil is small (Scott *et al.* 2000). Thus, the naturally occurring process of surfactant biodegradation may prove useful in surfactant clean-up. Knowledge of biodegradation pathways however is vital: it was discovered in 1984 that the degradation products of APE, alkyl phenol carboxylates (APC), are ten times more toxic than the precursor surfactant.

These APC degrade ultimately to nonyl- and octyl-phenols that adsorb to suspended particles, eventually becoming incorporated into sediments. They may even adsorb to the gills of fish, causing suffocation (Scott *et al.* 2000). Nonyl-phenol is known to mimic the effects of the female sex hormone, oestrogen. Its environmental impacts may be linked to endocrine disruptors causing decreased male sperm counts, testicular and breast cancer, interference with sex determination and development, and various carcinogenic effects (Scott *et al.* 2000). Thus, APE have been banned by the E.U. From this it can readily be seen that the chemistry of the biodegradation of hydrocarbons is of great importance when considering potentially detrimental environmental implications. This is all the more true for recalcitrant petroleum hydrocarbons and their derivatives.

Petroleum, better known as crude oil, is composed of a mixture of hydrocarbons and other compounds in varying ratios. It usually contains 83 – 87% carbon; 10 – 14% hydrogen; 0.05 – 6% sulfur; 0.1 – 2% nitrogen and 0.05 – 1.5% oxygen (Speight 1991). It is generally composed of aliphatic hydrocarbons (paraffins and olefins); naphthalenes; aromatics (including polyaromatic hydrocarbons [PAH]); sulphur compounds (e.g. thiophenes, thiols and sulfides); nitrogen compounds (e.g. carbazoles and pyridine); oxygen compounds (e.g. acids, alcohols, esters, ethers, furans and ketones) and some metals (e.g. copper, iron, nickel and vanadium).

From petroleum a number of fractions are derived including oils, fuels, greases, waxes and tars. If liquid at room temperature, they are usually referred to as

oils, while those that are solid are termed greases. Liquid petroleum compounds usually have carbon chains between 5 and 18 carbons long. Increasing carbon chain length results in differing products: gasoline, kerosene, diesel fuel, engine oil or gear oil. From the different distillate fractions of crude oil, various products can be produced, for example alcohols, surfactants, explosives, rubber, paints, pesticides and fertilizers (Speight 1991). These petroleum hydrocarbons – even seemingly recalcitrant crude oil – are subject to biodegradation.

1.3 Biodegradation

Biodegradation is the transformation or breakdown of compounds, usually organic, into simpler components via the biochemical reactions of microorganisms. There are two types of biodegradation, primary biodegradation or biotransformation and ultimate biodegradation or mineralization. The former is the destruction of the molecule by metabolic activity of microorganisms such that the chemical properties of the molecule are lost or altered. Ultimate biodegradation is the complete breakdown of the compound to carbon dioxide, methane, water, mineral salts and biomass (Scott *et al.* 2000). Hydrocarbons are naturally occurring in most soils, sediments and even plant matter to some extent (Stevenson 1966, Giger *et al*. 1974). Accordingly, microorganisms capable of degrading hydrocarbons are common and widely distributed in nature (Atlas 1981, Rosenberg 1991, Van Hamme *et al.* 2003) and they do so mainly in order to produce energy and biomass but also to reduce toxicity and to perform other functions.

1.3.1 The chemistry of biodegradation (Hashim *et al.* 1992, Scott *et al.* 2000, Ellis *et al.* 2006)

LAS are highly biodegradable, with 97 – 99% aerobic biodegradation reported in some wastewater treatment plants (Wangkarn *et al.* 2005). The most thoroughly probed with respect to biodegradability (Schoberl 1989), this surfactant is worth discussing in greater detail because it represents 40% of total world surfactant

consumption and as such was the class of surfactant used in this study. There are parallels between surfactant and petroleum hydrocarbon degradation. LAS are a good model compound on which to explain the mechanisms of biodegradation as they contain a long carbon chain (like paraffins), an aromatic ring (like PAH) and a non-hydrocarbon group (i.e. sulfate).

LAS are typically broken down by microorganisms with the straight alkyl chain being degraded first, followed by the sulfonate group and finally the benzene ring (Figure 1.4) – although it is believed to differ depending on the microbe involved in the degradation (Hashim *et al.* 1992, Scott *et al.* 2000, Ellis *et al.* 2006). Biodegradation is generally considered to be initiated by the oxidation of the terminal methyl group of the alkyl chain, in a process called ω-oxidation. Molecular oxygen is essential in this step hence the process is termed "aerobic biodegradation". The methyl group is oxidised first to the corresponding alcohol, then to the aldehyde and finally the carboxylic acid. Two-carbon fragments are then successively cleaved from the paraffin-like molecule as acetyl Co-A via β-oxidation (Figure 1.4) until only four to five carbons remain. Enzymes catalysing these reactions are a membrane-bound alkane monooxygenase and two dehydrogenases. It is worth noting that although some enzymes are general for catabolic pathways, some vary depending on the species of bacterium concerned. Where general enzymes are not applicable, *Pseudomonas* enzymes have been indicated for the purposes of this discussion.

Once a carboxylic acid has been produced and undergone β-oxidation the carbon fragments produced feed into the tricarboxylic acid (TCA) cycle as Acetyl Co-A. The microorganism is then able to utilize these fragments as a carbon and energy source. At this stage in the metabolism, a problem arises with branched chain molecules such as PT benzene. β-methyl substituted side chains and *gem*-dimethyl-branched side chains are unable to undergo β-oxidation: α-oxidation is needed to degrade the carbon chain, one carbon atom at a time. The carboxyl carbon is lost whilst the second carbon is oxidized to form the new carboxyl group.

The occurrence of both the α- and β-systems in the same microorganism is rare thus explaining the observed recalcitrance of compounds such as PT-benzene and branched alkylbenzene sulfonates.

If a sulfonate group is present this is degraded next, with three possible mechanisms for this step proposed:

1) Hydroxyative desulfonation

$$R\text{-}SO_3H + H_2O \rightarrow R\text{-}OH + 2H^+ + SO_3^{2-}$$

2) Monooxygenase catalysis under acidic conditions

$$R\text{-}SO_3H + O_2 + 2NADH \rightarrow R\text{-}OH + H_2O + SO_3^{2-} + 2NAD^+$$

3) Reductive desulfonation

$$R\text{-}SO_3H + NADH + H^+ \rightarrow R\text{-}H + NAD^+ + H_2SO_3$$

Irrespective of which mechanism is used, the breakdown product is a sulfite, which is oxidised to sulfate in the environment, alternatively it may be incorporated into biomass in the reduced form.

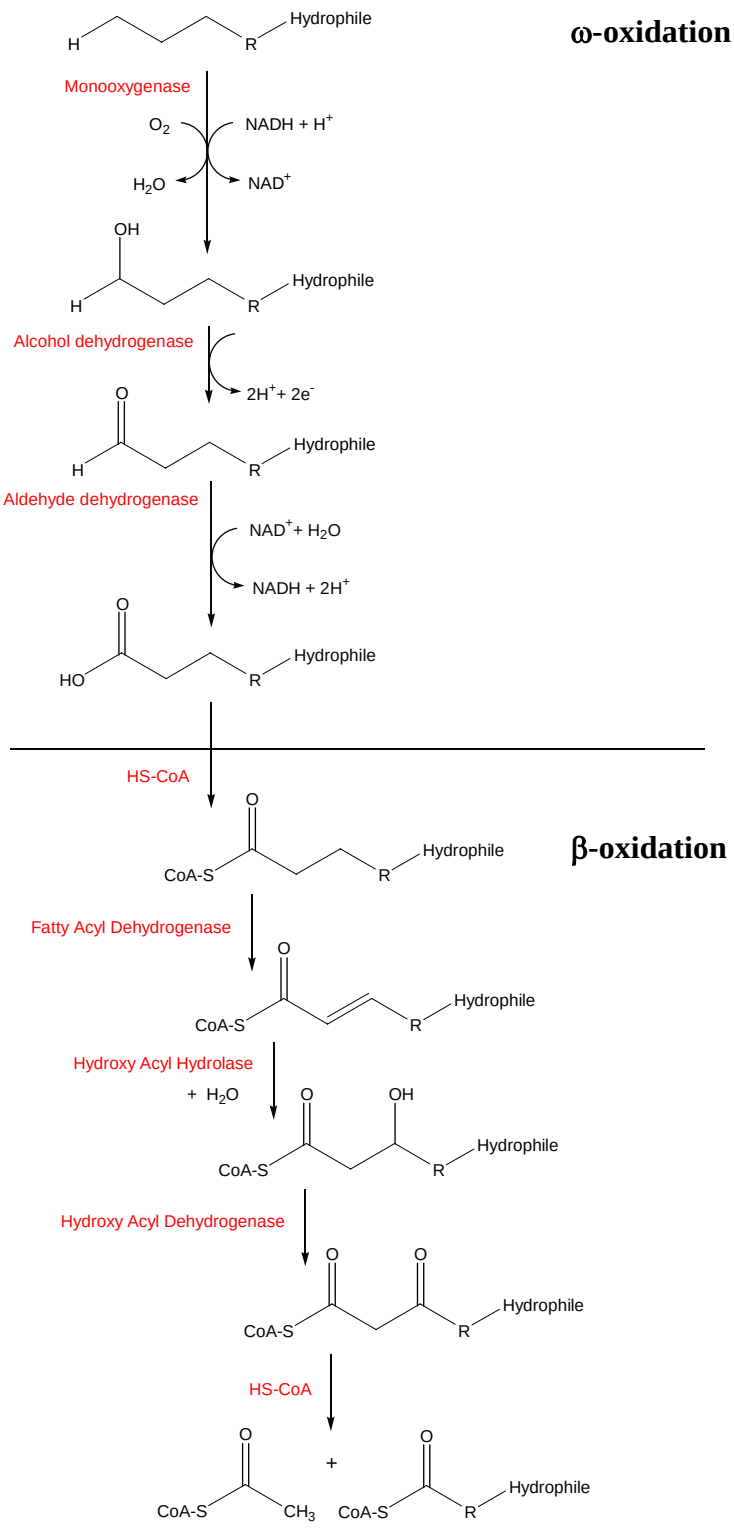


Figure 1.4 General biodegradation scheme showing first ω-oxidation of the alkyl chain, followed by 2-carbon β-oxidation (adapted from Scott *et al.* 2000 and Ellis *et al.* 2006).

All that remains to be degraded at this point are the resultant phenylacetic, benzoic or other similar acids. These aromatic substrates (Figure 1.5) are then hydroxylated forming *cis*-dihydrodiols (Cerniglia 1984) which are usually degraded to catechol. Analogous pathways exist for more complex aromatics such as PAH. Up to 80% biodegradation of the aromatic ring has been observed for LAS (Schoberl 1989). These aromatic acids and any PAH diols, are oxidatively cleaved and degraded via *ortho*- or *meta*- ring-cleavage, resulting in fumaric and acetoacetic acids. *Ortho*-cleavage usually only takes place when no side chain is present. *Meta*-cleavage is favoured in the case of LAS (Schoberl 1989). Further degradation to the simplest metabolites (such as pyruvate; acetaldehyde; formate; acetate; succinate and fumarate) yields short-chain carbon compounds able to feed into anabolic pathways of microorganisms such as the Krebs (TCA) cycle or glyoxylate pathway. Fatty acids, often formed as intermediates of oxidation, are degraded to acetate and proprionate.

The hindrance of substituents to biodegradation has already been mentioned. Specifically with regard to LAS there is a further factor, known as the distance principle: a steric consideration in which either of the methyl groups to be oxidized should be as far from the sulfophenyl group as possible. The first enzymatic conversion in the oxidation, the hydroxylation, is sterically hindered and accordingly substrate specific in certain species. This indicates that the active site must reside in the centre core of the protein and be of a finite, substrate-matched size (approximately 5 × 8 Å). Although all ring isomers can be biodegraded to carbon dioxide, water and sulfate, the rate of biodegradation is thus limited (Schoberl 1989).

The necessity for a range of enzymes to accomplish ultimate degradation is thus clearly evident. For example, the second major class of surfactants, APE, is far less biodegradable with only 0 – 20% biodegradation occurring at maximum. Pseudomonads are the only Gram negative bacteria able to biodegrade APE with nine or ten ethoxy groups. They degrade this down to four or five ethoxy groups, at which point other bacteria degrade the products further (Anderson *et al.* 1990).

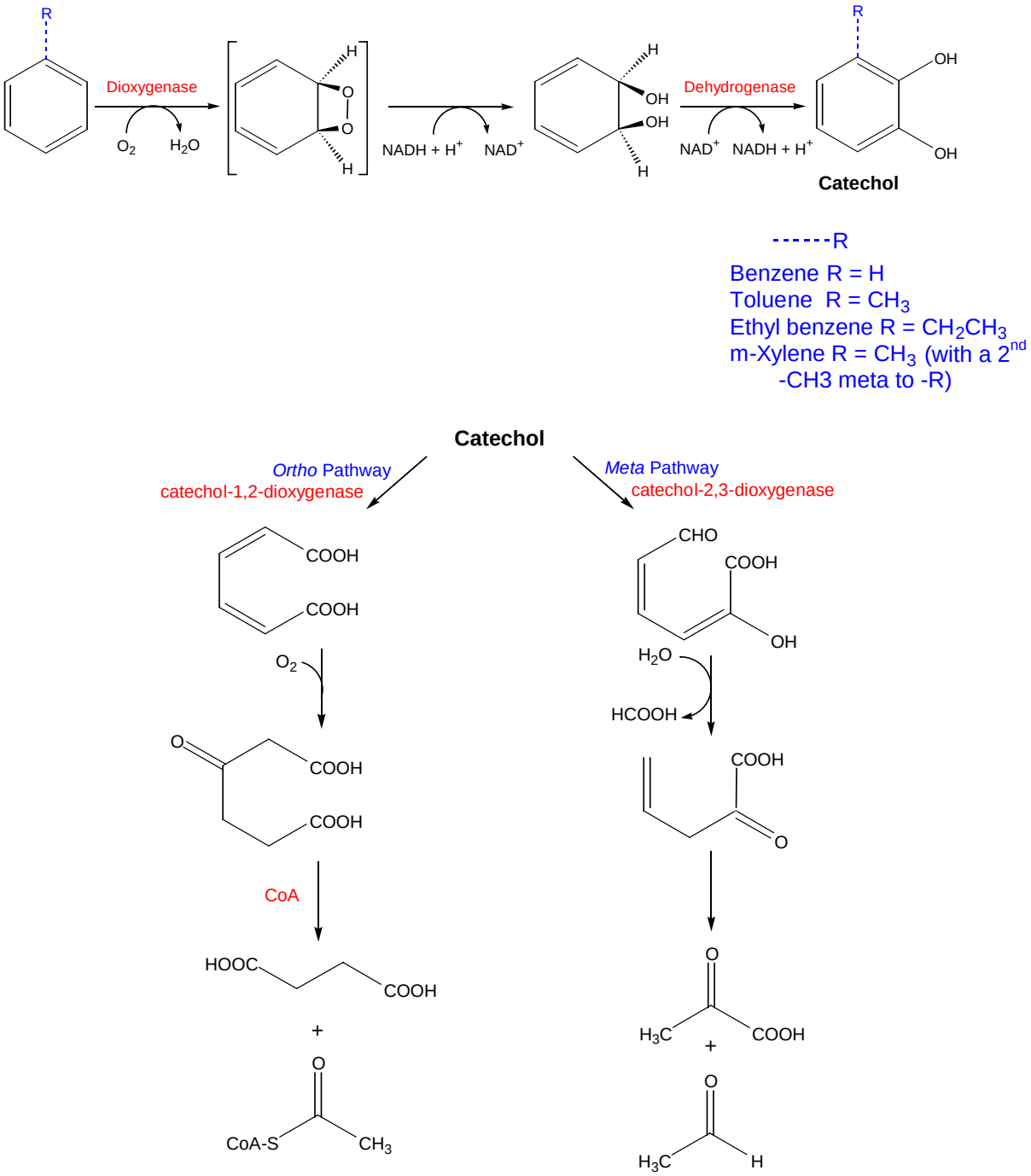


Figure 1.5 Generalised biodegradation of the BTEX compounds (benzene, toluene, ethylbenzene and *m*-xylene) (adapted from Schoberl 1989, Juhasz *et al.* 2000 and Ellis *et al.* 2006).

1.3.2 The role of microbial flora

Complete biodegradation of hydrocarbon mixtures requires a bacterial consortium (Scott *et al.* 2000, Van Hamme *et al.* 2001). This is because each microorganism has its own specific metabolic capabilities, and consequently deficiencies, when presented with a range of structurally unique substrates. Microorganisms require the necessary metabolic machinery to deal with carbon chains of varying lengths, cleavage of carbon-sulfur bonds and cleavage of aromatic rings, present in varying positions (Schoberl 1989). The more complex the hydrocarbon mixture, the more this applies. Degradation of petroleum requires an intricate consortium, especially if complete mineralization to CO_2 and H_2O is desired (Ghazali *et al.* 2004).

Bacterial assemblies may provide a range of metabolic capabilities that cover the full spectrum of reactions required to completely degrade hydrocarbon mixtures and then utilise all of the breakdown products. Therefore, the bacteria benefit from living in association due to synergistic and commensalistic relationships. Faster and more complete biodegradation is possible than by individual species alone (Gazhali *et al.* 2004). This is especially pertinent when microorganisms are added to a contaminated site to facilitate pollution clean-up, known as bioaugmentation.

In the case of LAS, for example, an estuarine consortium of four members was found to carry out biodegradation in a study by van Ginkel (1996). Three members oxidised the alkyl chain, while the synergistic action of *all four* was essential to mineralize the aromatic ring. It is preferable if the members of the consortium are from different genera (Gazhali *et al.* 2004), especially for petroleum transformation where each organism is believed to play a distinct role. Over the course of the biodegradation, as oil composition changes, so does the bacterial profile. Numerous instances of this have been reported (Horowits *et al.* 1975, Sorkhoh *et al.* 1995, Venkateswaren *et al.* 1995).

Rambeloarisoa and colleagues (1984) described a crude oil degrading consortium of eight strains from six different genera. The association was able to successfully degrade the crude oil but only when all members of the consortium were inoculated. Degradation decreased markedly when three of the species were removed. The synergistic interactions of microbial consortia are not completely understood but the benefit may lie in one species removing metabolic wastes toxic to another degrader, or in the ability to totally degrade those compounds only partially degraded by others (Bouchez *et al.* 1995, Kanaly *et al.* 2000, Settings 2006, Vandermeer *et al.* 2007). It is clear however that mixed populations with broad enzymatic capacities are especially necessary when complex carbons are the sole energy source (Gazhali *et al.* 2004). Greater understanding of the individual roles played by each member is therefore essential in influencing the effectiveness of microbial consortia and their exploitation in bioaugmentation.

1.4 Bioremediation

Bioremediation is the process of utilizing biological organisms to remove hazardous substances from the environment. Once the pollutant has been consumed by the microorganisms, they should die out, therefore posing little or no risk of environmental contamination (EPA 1996, Blumenroth 1998, Leung 2004). The process can be accelerated by adding additives, such as oxygen or nutrients, thus providing optimal conditions for bacterial growth and thereby increasing the speed of biodegradation and site recovery.

Bioremediation can take place *in* or *ex situ* (i.e. on site or off site) and while some compounds, like hydrocarbons, are susceptible to bioremediation, others, for example certain pesticides, are more difficult to biodegrade. *In situ* methods have the following advantages: there is less likely to be contaminant mobilization, they are usually less expensive, create less dust and the treatment of large volumes is possible.

This works best for permeable soils (e.g. sandy soil) but the process is slow (can require years) and may often be difficult to manage and monitor. *Ex situ* methods are faster, easier to monitor and control, prevent the spread of contaminants and can be used to treat a wide range of soil and pollutant types. However, excavation is required which increases costs considerably. Bioremediation is possible for contaminated soil, water and air. It is just one of the technologies available for remediation, including chemical and physical means.

1.4.1 Factors effecting bioremediation

The greatest limitation of most hydrocarbon biodegrading microorganisms is a deficit, typically in oxygen, nitrogen and phosphorus. Concentrations of these in hydrocarbons are usually minimal hence the biodegradation process is stunted. Biodegradation and its useful application in bioremediation are contingent upon several endemic factors.

The ultimate biodegradation of LAS, for example, is effected by: the concentration of dissolved oxygen; complexation competition by cationic surfactants; formation of insoluble calcium and magnesium salts; the presence of other organic contaminants and pH, to name but a few. Environmental determinants along with the type of compounds present and their exact nature, in a hydrocarbon mixture, greatly effect the extent of microbial activity (Atlas 1981).

It is well-documented that improved levels of dissolved atmospheric oxygen in solution increase the rate of biodegradation. Biodegradation of LAS occurs aerobically and slowly (if at all) under anaerobic conditions. This is because the ω-oxidation of the alkyl chain and the cleavage of the benzene ring, require molecular oxygen as mentioned previously (section 1.3.1).

By comparison, research into surfactant pollution since the 1940's has shown that ordinary soap is 92 – 97% biodegradable in 28 days. Sodium-based soaps show especially high levels of biodegradation. Furthermore, since the main bacterial biodegradation pathway is *β-oxidation*, oxygen is not needed for biodegradation to occur. This means that soap is highly biodegradable under aerobic *and* anaerobic conditions. From an environmental point of view, it may be suggested that a return to traditional soap-based surfactants may prove prudent (Scott *et al.* 2000).

1.4.2 Current methods of remediation (Crawford 1996 and DEC 2004)

Currently, mechanical methods of soil and water rehabilitation are the most commonly used because of their relative cost-effectiveness and the need for mainly unskilled labour in their implementation. Which technology will work best is dependant on the type and concentration of contaminant, climate, groundwater flow, site location, miscellaneous variables (e.g. cost, availability, time) and how all these factors interact. Therefore remediation technologies (Figure 1.6) are not only site specific, but usually country specific as well.

One of the least expensive technologies is natural attenuation: a technique whereby natural exposure to the elements allows soil rehabilitation. But this is extremely slow. What's more, contamination may not be contained during the time of treatment. Natural attenuation generally consists of dilution, volatilisation, biodegradation and adsorption. This kind of treatment is usually only allowed in situations where the spread of contamination and further harm to the environment are unlikely. This method, applicable for both soil and water, requires careful and consistent monitoring.

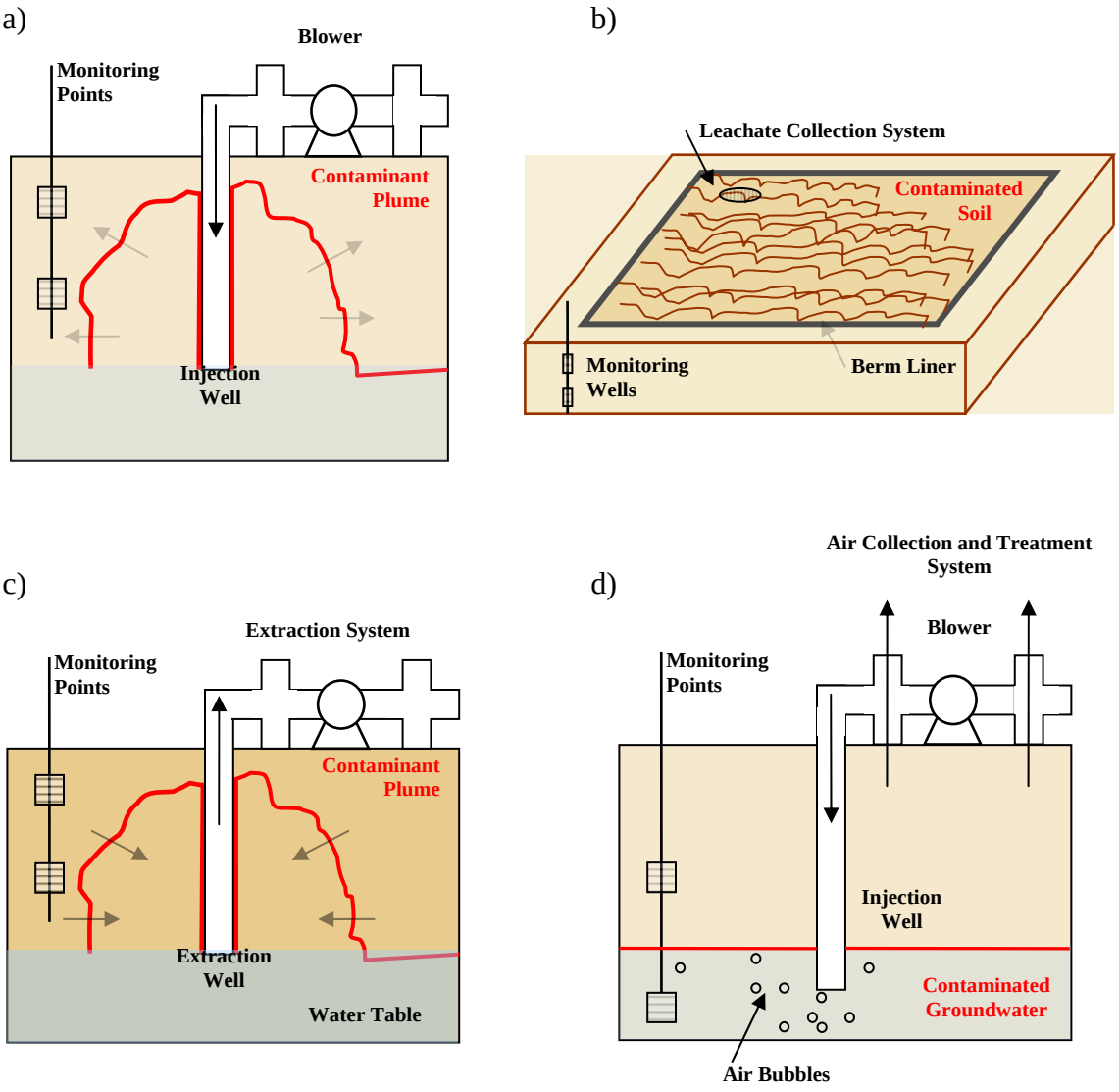


Figure 1.6 Some remediation technologies: a) a generalized bioventing system used in the bioremediation of soil and groundwater; b) a biocell used in landfarming (Note that leachate collection as well as monitoring points are in place.); c) a generalized soil vapour extraction system. Air is sucked through a soil pile and out through a decontamination system, causing volatile contaminants to evaporate and the soil to be rehabilitated and d) volatilization and containment of soil and groundwater pollutants through a stream of high pressure bubbles in the process of air sparging (adapted from DEC 2004).

Many soils have the correct indigenous microorganisms present to affect bioremediation without the necessity for bioaugmentation. Instead the conditions need only be altered to allow the bacteria to function optimally. Oxygen is commonly added to speed the process along via bioventing. Air can be blown or sucked into the soil, above the water table, at a rate dependant on the bacteria's oxygen demand requirements. Alternatively, oxygen can be supplied by the addition of liquid hydrogen peroxide. As the risk of water table contamination is high, this method is seldom employed, unless the water table is already polluted. These mechanisms work well for substances such as gasoline. The soil may be isolated in a biocell to confine contamination while biodegradation proceeds. This is referred to as landfarming. Soil is contained by a liner or a berm, fertilized and tilled to aid biodegradation. A leachate collection system is used to prevent contamination of the surrounding groundwater. A more passive variation of this is landspreading in which soil is not confined but merely tilled to oxygenate the soil allowing naturally occurring microorganisms and the elements to assist with the clean-up. In all the aforementioned soil treatments, regular monitoring is needed to follow the breakdown of pollutants. These regimes are time and labour intensive but are relatively inexpensive and can easily be maintained by the person or company responsible for the initial contamination.

The next class of remediation technology uses a mechanical or engineered process, for example soil vapour extraction. In this method, air is pulled or pumped through contaminated soil. Volatile compounds like gasoline evaporate easily and the extracted air is treated before being released into the environment. The soil can be treated in place or excavated and stacked in a pile.

Alternatively, soil can be washed with water or a solvent to dissolve and remove the pollution from the particulate surface. The disadvantages of this type of remediation are that the soil must be removed, cleaned several times (which uses a lot of water), and then replaced. Furthermore, the contaminated water used in the

washing process still needs to be rehabilitated or disposed of afterwards. This process is often favoured as it may prove quicker than bioremediation in which time is needed for biodegradation to occur.

Incineration may prove a viable alternative in instances when low temperature burning of contaminated material will result in harmless or acceptable by-products. Special incinerators with advanced air quality control facilities need to be used, where high temperatures are needed or harmful by-products are produced. Mobile incinerators are available for oil spills where large quantities of soil need to be treated or for the incineration of solvent or PCB polluted soils. The applicability of incineration is dependant on whether appropriate equipment is available in the country or not.

The remediation of groundwater is possible by several of the methodologies mentioned already, as well as by air sparging and a “pump and treat” method. These last two types of remediation are less commonly used. In sparging, air is forced through a contaminated aquifer. Like soil vapour extraction, volatile compounds are evaporated and forced out of the water. A stream of air bubbles scours underground water and soil particles, removing contaminants to a vapour extraction system for treatment. An advantage of this system is that it can be used not only for the treatment of contaminated areas, but also as a barrier to prevent groundwater pollution from spreading.

“Pump and treat” is the removal and filtering of underground water. The reconditioned water is returned to the original site, however, this water often follows highly specific flow paths. This may result in certain underground portions being continually missed and therefore not remediated. This method is not commonly used although it is effective for any compound for which a filter is available. It is successful, for example, with dissolved oil. The process may however take years before the contaminant can be successfully removed. For any of these technologies,

quick and sometimes also effective elimination of a pollutant involves the removal of the effected material to an off-site treatment plant. Such efforts, although successful, are expensive and time consuming, warranting a closer look at bacterial bioremediation.

1.5 Background and Overview of the Study

Mining establishments have a long heritage in South Africa, perhaps none more so than those of the reef city of Johannesburg. Mining was the founding reason for the country's commercial centre and continues to be a major source of capital to this day (DOH 1998). It contributes 8% of the country's GDP, second only to manufacturing and 1.1% more than tourism (Spenceley *et.al.* 2002). Gold and platinum mining operations contribute 90% of jobs in the mining industry. However, mining, especially gold mining, is far from being environmentally friendly.

Of the many areas of environmental concern, one of the most worrying must be the pollution of groundwater. South Africa is historically a drought-prone country (Endfield *et al.* 2004). As such, the conservation of water, and in particular the prevention and treatment of water pollution, is of the highest priority. There are several bioremediation agents available for the treatment of contaminated water and soil (Aldrett *et al.* 1997) however, they have all, to our knowledge, been developed in the northern hemisphere and most have not been tested in South Africa. Furthermore, application of such products in an underground closed mine-environment has not been previously reported.

There was an obvious need for a suitable local bioremediation agent, specifically tailored for South African conditions. To this end, the basis for a bioaugmentation product suitable for use in underground mine-environments was

formulated from indigenous bacterial isolates. Not only are these the first bacteria, to our knowledge, isolated from subsurface mine soil/water for the remediation of hydrocarbons, but this will constitute, to our knowledge, the first South African developed and tested bioaugmentation agent.

An international bioaugmentation agent for the bioremediation of hydrocarbon-contaminated soil was compared to local sewage bacteria. The product was believed to contain a strain of *Pseudomonas fluorescens* which is known to biodegrade various types of organic pollutants, including surfactants (Taranova *et al.* 2002). A South African mining conglomerate, raised the possibility of utilizing this product in the bioremediation of their closed mine water system. Multiple hydrocarbon contaminants including various oils (gear oil; hydraulic oil and diesel fuel) and surfactants used in their cleaning, were the main pollutants of concern. Minimal research had been carried out on the microbiology or biodegradation potential of this product. The singular tests conducted up until this point were simple field experiments that confirmed only *visible* cleaning of oil-contaminated ground surfaces after application of the product.

An area of concern was whether or not the detergents being used in subsurface cleaning were biodegradable. A pilot study to ascertain whether the commercial bioremediation product (CBP) was able to reduce total surfactant concentration in a simple batch experiment was conducted. This was carried out for both pure surfactant and detergent-surfactant. A major focus of the project was the emphasis on public health and safety with respect to the bacteria used. As such the active bacterial degraders in the CBP were isolated and identified. The study was then further extended to isolate, identify and selectively utilise indigenous mine bacterial flora for the biodegradation of surfactants and the more recalcitrant mine hydrocarbon

contaminants. Subsequent quantification of the biodegradation ability of all relevant isolates was then carried out. The relevant extraction and GC-FID analysis methods for the various stages of the study had to be elucidated and optimised for the given samples.

With 11% of the water in South Africa being used by industry, including mining operations (WRI 2005), the need for effective, timely and cost-efficient water treatment relates not only to the environment, but also to mine profitability and ultimately job security. As such, this study, aimed at the rehabilitation of hydrocarbon contaminated mine soil and water is of great importance, highlighting not only the initiatives of “green industry” but also providing an applicable solution to a typically South African, yet global problem.

CHAPTER 2

MOTIVATION



2.1 Objective of the Study

The objective of the study was to create the basis for the first South African bioaugmentation product, to rehabilitate hydrocarbon contamination, by investigating the microbial and biodegradation properties of indigenous South African bacteria, isolated from a subsurface gold mine.

2.2 Aims of the Study

The study objective was achieved by fulfilling six project aims as follows:

1) Compare the ability of an international CBP to biodegrade anionic surfactants relative to that of sewage bacteria.
2) Identify the biodegrading bacteria active in the CBP.
3) Identify possible sources of other biodegrading bacteria.
4) Isolate and identify indigenous biodegrading bacteria from consistently polluted sites of the subsurface mine-environment.
5) Evaluate the bioremediation ability of the selected isolates.
6) Compare the efficiency of the new South African isolates relative to those from the internationally marketed CBP.

CHAPTER 3

EXPERIMENTAL DESIGN AND METHODOLOGY



3.1 Selection and Preparation of Bacterial Inoculum

Sewage samples were collected in 1 L screw top polypropylene containers, from Goudkoppies municipal sewage plant in Eldorado Park, south of Johannesburg, South Africa and refrigerated until inoculation (within 1 week). Samples were collected from the activated system outflow point and are referred to as activated sludge samples. Sewage was used as a standard to compare the biodegradation that would occur in the waste water treatment plant (WWTP) to that of the CBP. The dry powdered CBP, believed to contain *Pseudomonas* sp., was chosen as a source of specially selected biodegrading organisms. The product was imported from a bioremediation technology company in Denmark. The product is activated by the addition of water or a nutrient medium.

Initial flasks for the biodegradation experiments were prepared to contain a final bacterial concentration of approximately 5 to 6 log (CFU/ml) after inoculation. Flasks containing sewage, however, varied greatly due to the inherent heterogeneous nature of the samples. CBP was prepared according to manufacturer's instructions.

Soil and mine isolates were prepared for inoculation as follows: Bacteria were grown up overnight at 30ºC in 1/10 strength nutrient broth (NB) (Biolab, Midrand, South Africa), i.e. 0.2 g NB per 50 mL. Of this, 1 mL was centrifuged at 13 500 rpm for 3 minutes, the supernatant removed and the pellet resuspended in 0.85% saline. This was centrifuged at 13 500 rpm for 3 minutes, the supernatant discarded and the pellet resuspended in 1 mL minimal medium (see section 3.2). The resultant 1 mL (/400 mL minimal medium) inoculum was added to the biodegradation flasks.

3.2 Biodegradation Assays

All biodegradation batch experiments were carried out in duplicate, performed in previously acid-washed 500-mL Erlenmeyer flasks, rinsed with distilled water. Where surfactants were the analyte of interest, detergents were omitted from the cleaning procedure. These batch-flask type experiments are best suited for the fast assessment of biodegradation. They may also be used to great effect in judging the suitability of media, biodegradation rates and the biotoxicity of various compounds (Senguputa *et al.* 1995).

To each of four flasks, 400 mL of minimal medium was added (Lindsay *et al.* 2000). Minimal medium contained per litre: 8.8 g $Na_2HPO_4.12H_2O$; 3 g KH_2PO_4; 1 g NH_4Cl; 0.5 g NaCl; 1 mL 1 M $MgSO_4.7H_2O$; 2.5 mL of trace salts (containing 23 mg/L $MnCl_2.2H_2O$, 30 mg/L $MnCl_4.H_2O$, 31 mg/L H_3BO_3, 36 mg/L $CoCl_2.6H_2O$, 10 mg/L $CuCl_2.2H_2O$, 20 mg/L $NiSO_4$, 30 mg/L $Na_2MoO_4.2H_2O$ and 50 mg/L $ZnCl_2$).

All chemicals used, unless indicated otherwise, were purchased from Merck (Modderfontein, South Africa). All solutions, unless indicated otherwise, were prepared with distilled, double deionised water (Milli-Q RG, Milipore, Bedford, M.A., U.S.A.). Glassware used in microbiological assays was autoclaved at 121ºC and 20 Psi for 15 minutes, media were then added and re-autocalved for 15 minutes.

3.2.1 Surfactant biodegradation experiments

To two of these flasks (A and B), the equivalent amount of detergent (containing 200 mg of surfactant) was added. A typical industrial-grade detergent containing anionic surfactant was supplied by a local cleaning chemicals company. To the remaining two (C and D), 200 mg of pure sodium dodecylbenzene sulfonate

was added as a control. One of each of the detergent and surfactant flasks (A and C) was then inoculated with 4 mL of well-stirred sewage and the remaining flasks (B and D) with 4 mL of the CBP (prepared according to manufacturer's instructions) (Figure 3.7).

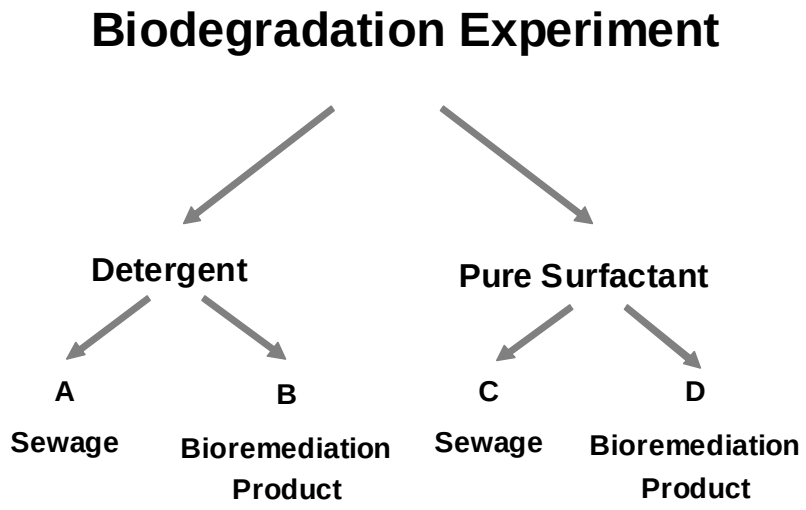


Figure 3.7 Batch-flask experiment set-up resulting in combinations of detergent and surfactant with each source of inoculum (sewage and the commercial bioremediation product).

Flasks were incubated at an average ambient temperature of 17ºC to simulate uncontrolled field temperature conditions, on an orbital shaker set at 180 rpm. Optical density (OD) readings were taken weekly for 28 days at 600 nm and samples frozen at -7ºC for later analysis by the methylene blue active substances test (MBAS) test (section 3.4).

3.2.2 Subsequent biodegradation experiments

Subsequent assays to assess the ability of isolates to utilise a specific sole carbon source were designed similarly to the initial surfactant biodegradation experiment (section 3.2.1). On each occasion the appropriate source of inoculum was added in combination with the desired carbon source as required:

a) CBP and detergent components – Each of the following detergent components was added into separate flasks: convanyl blue dye (0.0128 g); egg yellow dye (0.0128 g); ethylene diamine tetra-acetic acid (2.563 g); potassium hydroxide (25.64 g); sodium tripolyphosphate (76.92 g); sodium xylene sulfonate (12.82 g); triethanolamine (64.09 ml) and ethylene glycol monobutyl ether (89.74 ml). CBP inoculum was then added to each flask and incubated similarly to experiment 3.2.1 and OD readings taken thrice weekly for 28 days. The aforementioned components were of industrial grade, supplied by the detergent manufacturer. Flasks containing detergent components without inoculum were prepared as controls.

b) Sewage and detergent components – The above experiment a) was repeated but with sewage as the source of inoculum.

c) Soil isolates and gear oil – Isolates taken from continually polluted industrial sites were used to degrade an oil commonly found in the mine, Olef 460 gear oil (20 ± 5 mL /400 mL), setup as described previously (section 3.2.1). OD readings were recorded thrice weekly and flasks containing glucose (4 g /400 mL) and nutrient broth (1.2 g /400 mL) used as positive controls.

d) Soil isolates and SDBS surfactant – Experiment c) was repeated with SDBS surfactant (4 g /400 mL) as the sole carbon source.

e) CBP isolates and monograde engine oil – Isolates from the CBP were used to degrade Sasol topaz 30 monograde engine oil (25 ± 5 mL /400 mL) (Sasol, South Africa), setup as described previously (section 3.2.1). Hydrocarbon degrading bacterial populations were enumerated as described in section 3.3 and 1 mL samples frozen weekly, for later evaluation of biodegradation by GC-FID analysis (section 3.7).

f) Mine isolates and Sasol topaz 30 monograde engine oil – Experiment e) was repeated using the mine isolates as the source of inoculum.

3.3 Enumeration of Hydrocarbon Degrading Bacterial Populations

Weekly, 1 mL samples, taken from the biodegradation experiments, were serially diluted in 0.85% saline and 50 µL plated in duplicate onto Standard One Nutrient Agar (SONA) plates (Biolab) using the droplet plate technique (Lindsay *et al.* 1999). Plates were incubated at 25ºC for two days for sewage and CBP, and 30ºC for all other samples (after a pilot study to identify optimal incubation times) and colonies for the 100x and 1000x dilutions counted. All experiments were carried out in duplicate on two separate occasions.

3.4 Methylene Blue Active Substances Test

Spectroscopic analysis, specifically MBAS, was, for a long time, the most common technique used for the investigation of surfactant biodegradation. It remains a popular method for fast, general evaluation of biodegradation, although for the exact monitoring of breakdown products, more accurate methods, (e.g. LC-MS or HPLC with fluorescence detection) are favoured, time and analytical facilities permitting. Measurement of chemical oxygen demand (COD), dissolved organic

carbon (DOC), inorganic sulphate and $^{14}C/^{35}S$ tracer techniques are preferentially employed. Monitoring of $^{14}CO_2$ is a specific and sensitive technique allowing for quantitative evaluation unaffected by chemical interferences and microorganism metabolism. Combinations of the above are ultimately best, combined with a thorough knowledge of surfactant catabolism (as 100% biodegradation is seldom seen in the laboratory) (Schoberl 1989).

Biodegradation of anionic surfactant was monitored using a variation of the MBAS test (Chikitela *et al.* 1995) in which anionic surfactant is complexed by cationic methylene blue dye (Figure 3.8). The dye-surfactant complex is extracted into an organic phase and the UV absorbance measured at 652 nm. A linear relationship between absorbance and surfactant concentration is seen below ~2.0 ppm. Concentrated detergent samples were hence diluted 5000 fold prior to testing.

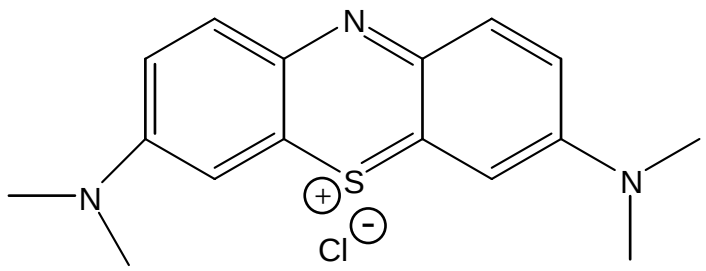


Figure 3.8 Line diagram of the cationic dye, methylene blue.

SDBS standards of 0, 0.4, 1.5, 2.0 and 2.5 ppm were prepared in test tubes from a 10.0 ppm stock solution. Standards and samples were then treated in an identical manner: The pH of the solution was adjusted to between 8 and 9 with 0.02 M NaOH, testing using universal indicator strips. Methylene blue (2 mL) and chloroform (2 mL) were then added to each tube. Samples were shaken vigorously

and the phases allowed to separate. The organic layer was removed and a second 2 mL chloroform extraction performed on the aqueous phase. The combined organic extracts were washed with 10 mL wash solution (500 mL water, 41 mL 6 N H_2SO_4 and 50 g $NaH_2PO_4.H_2O$ diluted to a total volume of 1000 mL) and extracted with a final 2 mL chloroform. UV absorbance of chloroform extracts was measured at 652 nm on a Varian Series 634 double beam UV-Visible spectrophotometer. Samples were analyzed for MBAS in duplicate on three separate occasions and the results averaged.

3.5 Isolation and Identification of Bacteria

3.5.1 Isolation

The CBP mixed bacterial culture used as a source of inoculum was inoculated into 50 mL 1/10 strength NB and incubated overnight at 25ºC. A loopful of this broth was streak plated onto vile red bile glucose (VRBG) agar (Oxoid, Midrand, South Africa) and grown for 24 hours to isolate presumptive Gram-negative *Pseudomonas* species. Isolates were then purified by successive streak plating onto NA at 25ºC and identified either using the BD BBL Crystal Identification Kit (Becton Dickinson and Company, U.S.A.) and/or by PCR and 16S rDNA gene sequence analysis.

For Gram-positive isolates, the same procedure as above was used by plating directly onto NA and streaking 3 successive times to achieve a pure culture.

For soil isolates and mine soil/water isolates a similar procedure was used: the mixed-bacterial-culture soil and water samples were inoculated into 400 mL minimal medium spiked with 25 ± 5 mL monograde engine oil to specifically isolate for hydrocarbon degrading bacteria. Cultures were incubated at 30ºC overnight and pure culture attained by streaking 3 successive times onto NA. Isolates were then identified using PCR and 16S rDNA analysis.

3.5.2 DNA isolation, PCR and 16S rDNA sequencing

DNA was extracted from each isolate using a modified boiling method described by Scarpellini *et al*. (2004). For each isolate, one colony from stock tryptone soy agar (TSA) plates (Biolab), was boiled for 20 minutes in 40 μL sterile, filtered water and 20 μL chloroform, then centrifuged at 14 000 rpm for 5 minutes. This supernatant contained the DNA template for the PCR reactions.

The primer set used for the amplification of 16S rDNA was U1392R (5'-ACG GGCGGT GTG TRC-3') (Lane *et al*. 1991, Ferris *et al*. 1996, McGarvey *et al*. 2004) and Bac27F (5'-AGA GTT TGA TCM TGG CTC AG-3') (Inagaki *et al*. 2003, McGarvey, *et al*. 2004) in combination with 2 times PCR Master Mix (Fermentas Life Sciences), according to the manufacturer's instructions, and yielding a product of approximately 1300 bp. PCR amplifications were performed using the following conditions: initial denaturation of template DNA at 94°C for 3 minutes, followed by 35 cycles consisting of denaturation (94°C, 30 s); annealing (60°C, 45 s); extension (1 min 30 s, 72°C) and a final 7 minute extension at 72°C. The purified PCR product was sequenced on an ABI Prism 310 Genetic Analyzer and the resulting sequences analysed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against 16S rDNA sequences from GenBank (GenBank database of the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/). A phylogenetic tree, highlighting the clustering of the isolates was constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft).

3.6 Microwave Extraction and GC-FID Analysis

Samples frozen from the biodegradation experiments were allowed to thaw gradually to room temperature, centrifuged for 30 seconds at 25 000 rpm using a MSE Mistral 1000 bench-top centrifuge to remove any bacterial cells or cell remnants. To 6 mL of dichloromethane, 4 mL of the supernatant was added. A

microwave assisted extraction (MAE) was performed using a Multiwave 3000 with Rotor 8SOLV Microwave System (Anton Paar, Austria). MAE has consistently proven to be a faster and more efficient method, for the extraction of hydrocarbons, than the traditional Soxhlet extraction technique (Barnabas *et al.* 1995, Kok *et al.* 1996, Rozario *et al.* 1997, Dean *et al.* 2000). The extraction was optimized from EPA method 3546 (2005), for the specific samples of interest. MAE was carried out for 35 minutes (5 min ramp, 10 min hold and 20 min cooling) in total, at 1400 W power (maximum temperature, 80ºC and pressure, 0.5 bar/s) (Anton Paar 2006).

The organic phase was removed and filtered through anhydrous magnesium sulphate and glass wool into a sample vial. Of this extract, 1 μL was quickly injected into the GC injection port set at 270°C. Helium carrier gas, hydrogen and air at a flow rate of approximately 4 – 7 mL/min was used with a Zebron ZB5 column, stationary phase 100% dimethylpolysiloxane and dimensions 30 m x 53 mm x 1.5 μm. The detector was set at a temperature of 270°C and the oven programmed to ramp directly from 70°C to 220°C, hold for 20 minutes, then ramp to 250°C and hold there for 30 minutes.

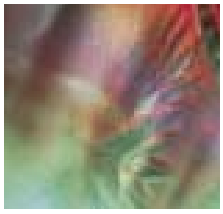
This GC analysis method was optimized from the Modified DRO Method for determining Diesel Range Organics (DNR 1995). The modified DRO method is based upon EPA test methods 8000, 8100, 3510, 3520, 3540 and 3550, for evaluating solid waste (EPA SW-836), and ASTM (American Society for Testing and Materials) method D 3328 "Standard Methods for Comparison of Waterborne Petroleum Oils by Gas Chromatography". GC-FID analysis was optimized for the specific samples being studied and additional studies consulted in the development of the methods used, for example Reddy *et al.* 1999, Wang *et al.* 2003 and Lai *et al.* 2004).

3.7 Scanning Electron Microscopy

Isolates were visualized using a JSM-840 scanning electron microscope (SEM) (bioworld.com). Sample cells were fixed in 3% (v/v) aqueous gluteraldehyde overnight at room temperature and dehydrated in an ethanol series ranging from 20% – 100% (v/v) in 10% increments. Each dehydration lasted 10 minutes and was performed at room temperature. Samples were then subjected to critical point drying, mounted and coated with carbon and gold-palladium (10 nm) for 10 minutes.

CHAPTER 4

THE STUDY AREA



4.1 Mine Location

The location of the study area was a typical South African gold mine on Gauteng's west rand near the border with Northwest province (Figure 4.9). Kloof mine is situated almost half-way between Westonaria and Carltonville, approximately 60 km south west of Johannesburg (Infomine 2006, Mining Technology 2006) (Figure 4.10). The Kloof gold mine is divided into three sections, namely Kloof, Libanon and Leeudoorn (operating 5 shafts) employing almost 15 000 people.



Figure 4.9 A map of Gauteng including Westonaria and Carltonville with Kloof indicated in red, approximately 60 km from Johannesburg.

Between the Witpoortjie Fault to the east and the Bank fault to the west lies the “West Wits” goldfield, in which Kloof is situated. This is part of the Archaean-age Witwatersrand Basin that is composed of 6 km thick argillaceous and arenaceous sedimentary rocks. Gold in this region usually occurs in an elemental state with pyrite and carbon, in the form of quartz pebble conglomerate reefs. Gold production comes mainly from the Ventersdorp Contact Reef at depths of 2.5 – 3.7 km and secondarily from the Kloof, Libanon and Main reefs. Kloof is the highest grade gold mine in South Africa.

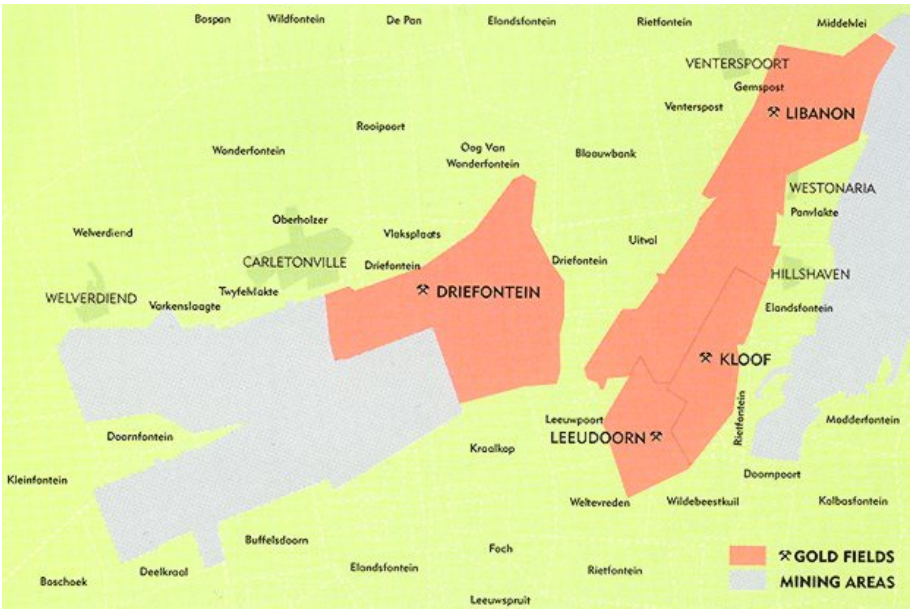


Figure 4.10 A map of the towns and mines surrounding Kloof mine. Red blocks indicate gold fields.

4.2 The Mine Water System

The specific environment considered for the purposes of this study is a recycled mine-water treatment system. The mine-water system is a closed water system as is illustrated in Figure 4.11. Water is used for cooling, washing, as an aid in

mining, for hydroelectric-power and for use by the staff (referred to as service). All of this water is circulated through the various channels, collected at main drains and treated to lower pH. A flocculant is added and thereafter the water is pumped to the filter plant. This is where all of the waste is filtered off and the clean water returned to the mine system for use. Anti-corrosive/anti-scaling agents are added, namely DREWSPERSE 867-Z (Sud-Chemie) and two microbiocidal agents, BIOSPERSE Br and BIOSPERSE 301 (Sud-Chemie).

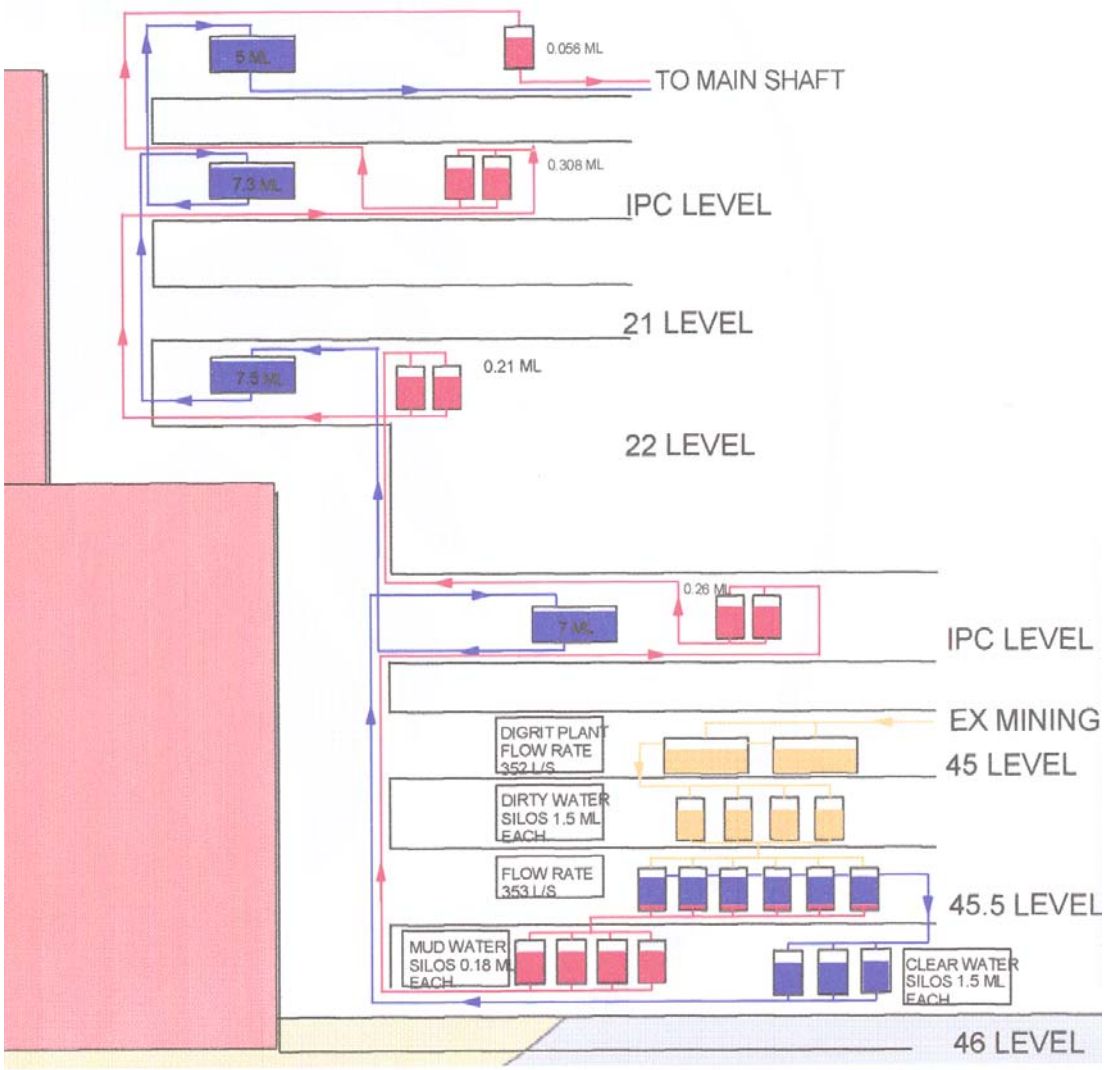


Figure 4.11 The water pumping system at Kloof #4 shaft of the mine used in the study (picture supplied by the mine).

The Kloof Mine #4 shaft has been used as a model for this study. The main filter plant pumps approximately 120 ML of clean treated water per day for Kloof mine as a whole. Water is pumped from the main shaft filter plant water tank (on the surface) through the fridge plants (90 L/s at 5ºC) to one of three 5 ML chilled water storage dams. From here the water is directed along two lines, A and B. Line A is designated for "service water" e.g. drinking water. Line B services "chilled water" and "hydro power". Chilled water is pumped to a 7.5 ML storage dam on level 22 and then down a sub-shaft to a 7 ML storage dam on IPC level. From IPC level it can be distributed to all the sub-levels (39 – 45). Hydro power water is fed from the chilled water dam to level 21 through a turbine at 120 L/s. It is then fed to a pressure-reducing station on level 22 where accumulated pressure from the surface is reduced from 18 MPa to 9 MPa. Water from level 22 pressure-reducing station is directed to levels 39 to 45. On each of the levels water is utilised, then feeds into the drain system to dirty water silos on level 45 where lime is added to decrease pH. Dirty water is directed to the settlers where mud is settled off and stored in mud water silos. The clear water from the settlers goes to the clear water silos on level 45.5. This constitutes the downward portion of the water's journey. From the clear water silos water is pumped to the IPC level storage tanks, from there to level 22, and from level 22 to the upper IPC level and back up to main shaft and the surface. About 18 – 24 ML per day is pumped within the #4 shaft water system. (Information on the mine-system was supplied by the South African gold mine in question.)

4.3 Cost-Benefit Analysis of Mine Bioremediation

There are numerous methods available for dealing with the various pollutants associated with mining. Some of these are compared in Table 4.1. Currently, the mining conglomerate treats contaminated water and soil as follows: Soil is washed with surfactants to remove pollutants. This waste water filters into the drain system

including all other polluted water and is recycled and purified in the water-filtration system. The main method of water decontamination in this system is a mechanical method, filtration (mentioned in the remediation technology section 1.4.2). Pollutants that cannot be removed from soil/rock by washing are covered over with a thin layer of powdered concrete and therefore not treated in any respect.

The main contaminants in the system are engine oil, diesel fuel oil, hydraulic oil, transmission oil, transformer/mineral oil and gear oil, as well as the detergents used to wash rock, machinery and surfaces within the mine. The total monthly cost of treating the water at Kloof mine was approximately R130 000 (2005) and, for the mining company as a whole, runs into over half a million rand per month.

A more cost-effective and environmentally friendly solution, eradicating the need for disposal of filtered pollutants, is sought for water purification in this mining water system. This study aims to develop a bacterial remediation suitable for use in the mine, particularly the subsurface areas. Current cost estimates for the bioremediation agent on a laboratory scale are approximately R195.53 / 1 L (R132 15 g salts + R2, 1 L water + 10 g organisms + R6.26 agar + R6.27 plate + R32.00 broth + R5.00 miscellaneous + labour R12 / 1 hr). This is an inflated value. Produced on a larger scale this estimate could be halved. Implementation in the mine will – at first – be more expensive than the current filtration remediation methods used, however in the long run, it should prove cheaper. This is because the cost of dumping the filtered waste will be elliminated: the pollution will have been degraded. Furthermore, with the contamination completely biodegraded there is no risk of future litigation or legal dispute over the responsibilities of disposal. The implications of this will be discussed further under “CHAPTER 7 FUTURE WORK”.

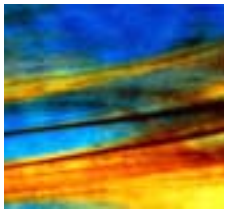
Table 4.1 Cost-benefit analysis of some remediation technologies currently used in South Africa.

Solution	Time-Span	Approximate Cost	Cost-Benefit Estimate
1) Natural attenuation (i.e. no treatment: simply allowing nature to take its course)	Months to years	None	zero cost; exceptionally simple and easy to implement but too slow and of low benefit;
2) Biostimulation (i.e. addition of fertilizers to increase biodegradation by naturally occurring microorganisms)	Months	~R60 /m^3	moderate cost; moderate speed, moderately easy to implement and high benefit;
3) Bioaugmentation (i.e. addition of exogenous micro-organisms and fertilizers to hasten biodegradation)	Weeks to months	~R150 /m^3	fairly moderate cost; moderate speed, moderately easy to implement and high benefit;
4) Oxidation (i.e. chemical oxidation of pollutant)	Days to weeks	~R200 /m^3	moderate to high cost (depending on contaminant); relatively fast speed, moderate benefit but more difficult to implement;
5) Disposal (i.e. dumping of polluted material)	Days to weeks	~R2000 /m^3	high cost; fast speed and easy to implement but low environmental benefit;
6) Incineration (i.e. combustion of polluted material)	Days to weeks	~R5000 /m^3	high to exceptionally high cost (depending on contaminant); fast speed but may be difficult to implement and of low environmental benefit;

The remediation treatment methods mentioned in Table 4.1 are currently in use at South African mining operations, in various forms and stages. However, the majority of mining establishments currently favour disposal or incineration: partly because these methods have been in place for many decades and partly because, although the costs are high, the legal implications after disposal are often transferred to a third party, therefore absolving the mines of accountability.

CHAPTER 5

RESULTS AND DISCUSSION



5.1 Pilot Study: Commercial Bioremediation Product

5.1.1 Commercial bioremediation product efficiency – Oil

A pilot study was conducted by the manufacturer of the CBP, to evaluate its effectiveness to biodegrade crude oil. Preliminary biodegradation tests were carried out on Nigerian crude oil-contaminated soil samples. Crude oil pollution is of critical environmental concern in Nigeria where oil spills are common due to the volatile political climate and the lack of proper safety measures (Aprioku 2003, Gramling *et al.* 2006). GC-FID analysis was carried out on untreated hydrocarbon-contaminated soil extracts, revealing light (200 – 800 s) and heavy (900 – 1200 s) hydrocarbons (Figure. 5.12). After 30 days of treatment with the CBP analysis detected no hydrocarbon peaks, even the characteristic UCM was no longer visible (Figure 5.13). Gravimetric analysis would have been necessary to quantify the resin and asphaltene fractions however as these were not of particular interest, this was not done.

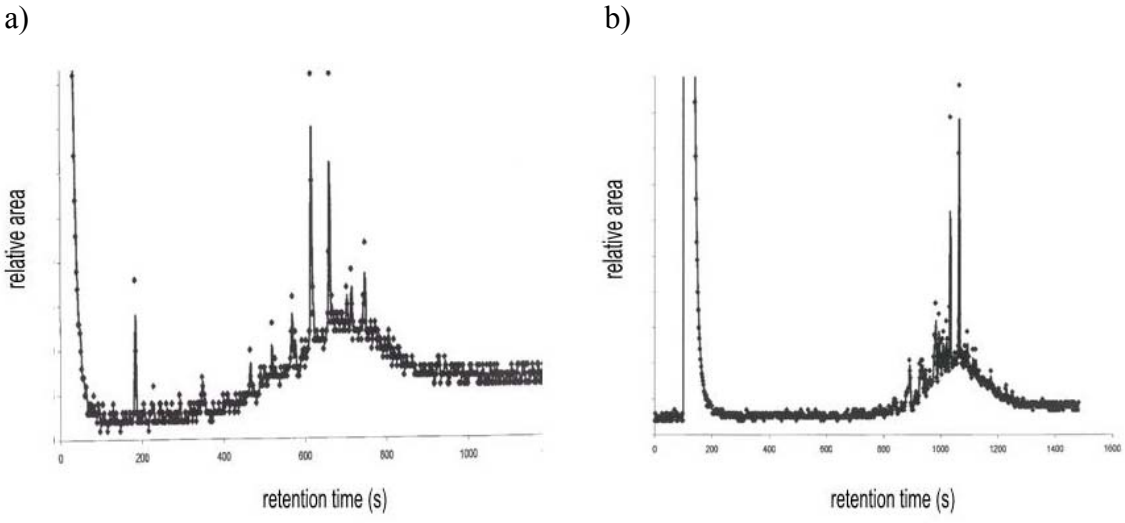


Figure 5.12 Chromatogram of a) light-hydrocarbon and b) heavy-hydrocarbon crude oil-contaminated Nigerian soil prior to application of any form of bioremediation treatment.

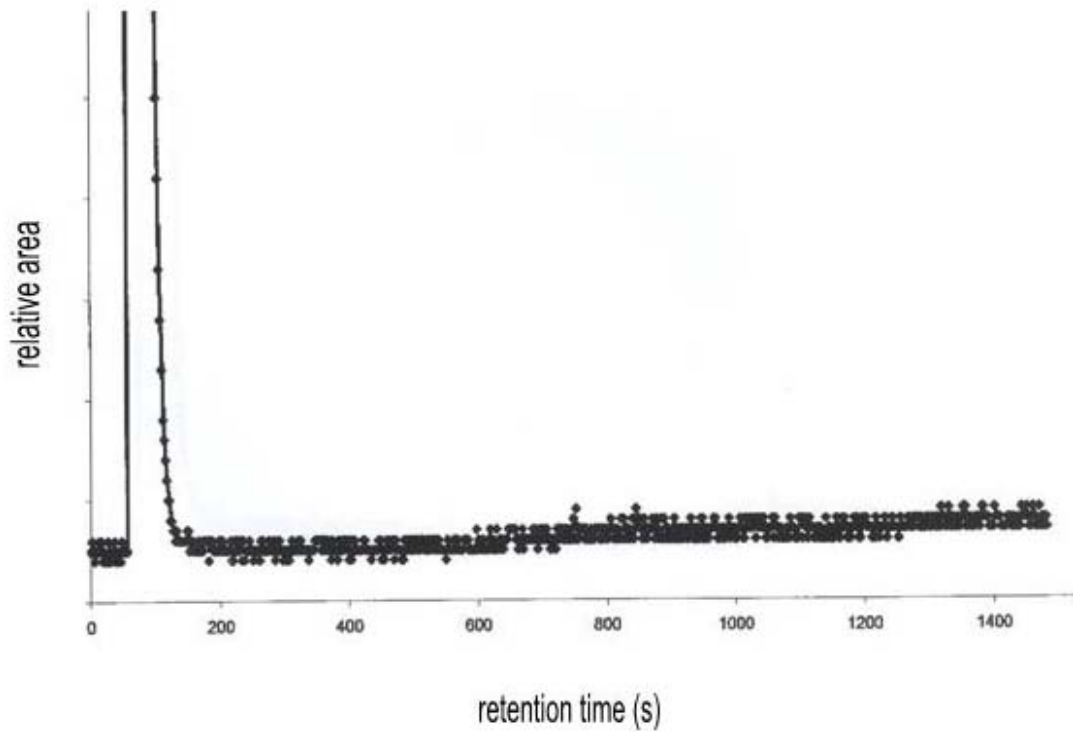


Figure 5.13 Chromatogram of hydrocarbon contaminated Nigerian soil, after one 30 day treatment with the commercial bioremediation product. All crude oil fractions have been completely degraded.

This established the ability of the CBP to successfully biodegrade petroleum hydrocarbons to carbon dioxide, water, salts and oxidized byproducts and biomass. The product therefore demonstrated its potential as a bioaugmentation agent for the remediation of hydrocarbon compounds.

Due to these promising results, an extension to the pilot study was designed to test a) the action of the CBP on surfactants (a target molecule of interest in the test site) and b) the biodegradation ability relative to that observed in a standard WWTP.

5.1.2 Commercial bioremediation product efficiency – Surfactant

Surfactant biodegradation by the CBP was compared to that observed under standard WWTP conditions. An anionic surfactant, specifically a LAS, was selected as it is the major class of surfactant in use, accounting for 27% of world market consumption and because of its characteristic structure (see section 1.3.1). SDBS was selected as a representative of a simple LAS molecule.

Degradation assays were performed on an industrial-grade detergent (flasks A and B) and the SDBS surfactant only, from that detergent (flasks C and D) as per method 3.2.1. The CBP and sewage were used as comparative sources of inoculum. Activated sludge was selected as the source of sewage inoculum as it contains the highest numbers of autotrophic and heterotrophic bacteria, 95%, and higher organisms (protozoa and fungi) 5%. Microbial degradation is well documented to occur primarily during the activated sludge treatment process (Berna *et al*. 1989, Painter *et al*. 1989). Percentage biodegradation over the course of the experiment was calculated by monitoring surfactant concentration using the MBAS test method 3.4 (Figure 5.14).

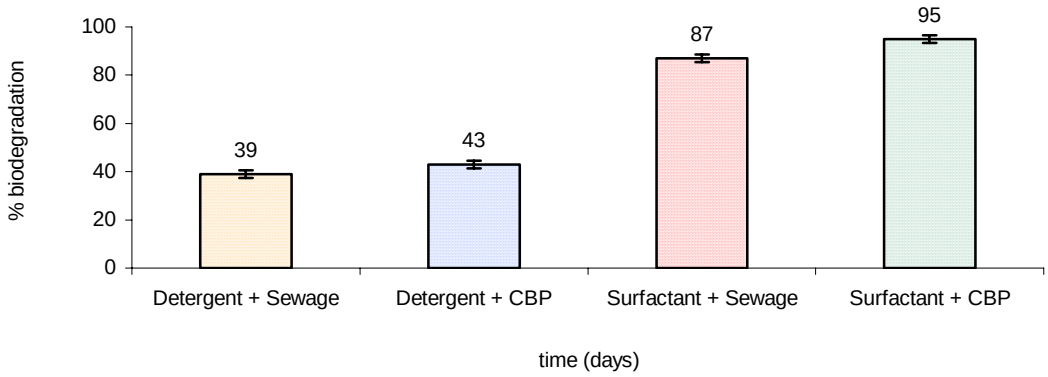


Figure 5.14 Percentage biodegradation of surfactant and detergent samples treated with two different sources of inoculum, activated sewage sludge and the commercial bioremediation product (CBP).

SDBS was biodegraded 95% by the CBP, the highest levels of biodegradation observed. By contrast, sewage bacteria biodegraded SDBS by 87%. Detergent-surfactant was degraded 43% by the CBP and 39% by sewage bacteria (the lowest levels of biodegradation observed). A sterile control showed no loss of surfactant. Calculated standard error for the experiment was at maximum 0.04% for 3 replicates (Table 5.2) hence error bars for the corresponding graph were multiplied by 100 to make them visible in Figure 5.14.

Table 5.2 Calculated standard error observed for percentage biodegradation of surfactant and detergent-surfactant by sewage and commercial bioremediation product (CBP) inocula.

Sample	Percentage Error (%)	Standard Error
Detergent and Sewage	0.01	39 ± 0.00530
Detergent and CBP	0.02	43 ± 0.00990
Surfactant and Sewage	0.04	87 ± 0.0223
Surfactant and CBP	0.02	95 ± 0.0229

Corresponding bacterial numbers were concomitantly monitored (Figure 5.15). Bacterial numbers were generally higher in the assays containing pure SDBS. Bacterial numbers were also usually elevated where the inoculum was CBP. Bacteria in the presence of detergent showed a decrease in bacterial numbers, days 1 – 7, (compared to that of the initial inoculum prepared) followed by a period of recovery demonstrated by slowed growth, days 7 – 14. Microbial counts began to decline after two weeks, at which stage the death phase was entered – this follows the typical trend of a general growth curve, except for the sewage inoculum with surfactant substrate, which continued to grow. This is potentially due to nutrients, even ubiquitous surfactants, already present in the sewage. With these added nutrients available and without the inhibitory effects of the detergent, growth continued to increase. It is

believed that had the experiment been extended, numbers would have declined shortly thereafter.

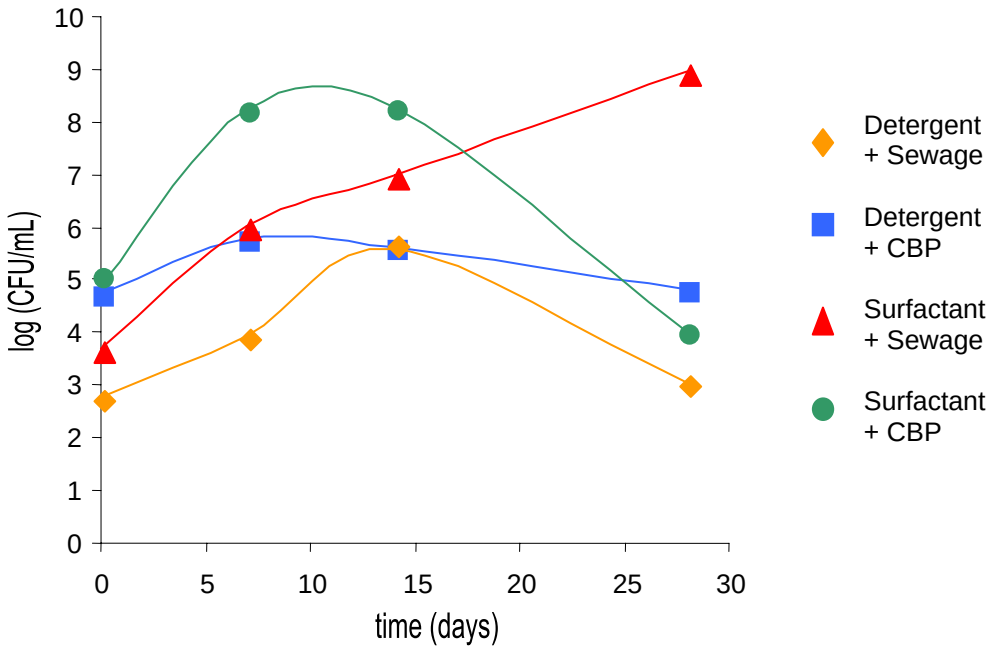


Figure 5.15 Bacterial growth in detergent and surfactant for two sources of inoculum, activated sludge and the commercial bioremediation product (CBP) monitored over 28 days.

A persistent result was obtained for sewage bacteria grown on detergent substrate: an increase in surfactant concentration prior to biodegradation. This was in contrast to the expected result: a gradual decrease in surfactant concentration over the course of the experiment (Figure 5.16). It was postulated that the increase was due to the production of biosurfactants stimulated by the detergent and an abundance of nutrients in the sewage (Figure 5.17). Further study is, however, necessary to confirm this observation.

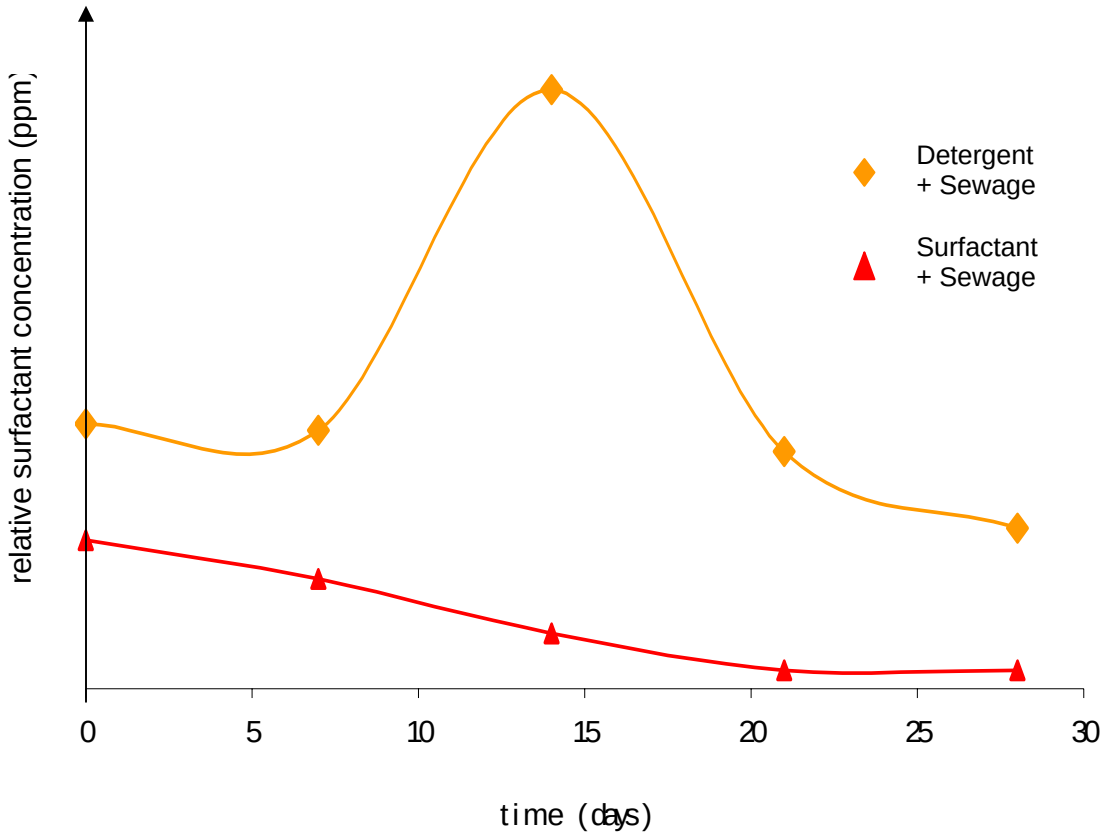


Figure 5.16 Relative changes in the concentration of anionic surfactant over the 28 day biodegradation experiment period for sewage inoculum.

Biosurfactants are thought to improve substrate bioavailability, by increasing water solubility when hydrocarbon-like molecules are solubilised in the hydrophobic cavities of biosurfactant micelles (Ron *et al.* 2001). Biosurfactants are naturally produced by many bacteria and emulsify hydrocarbons (Koch *et al.* 1991, Neu 1996) thereby increasing the interaction between the cells and their substrate (Baldi *et al.* 1999). They accumulate at the interface of immiscible liquids, decreasing surface tension. This increased hydrocarbon surface area means greater bioavailability and therefore biodegradation of insoluble compounds (Leahy *et al.* 1990). *Pseudomonas fluorescens*, the bacterium believed to be active in the CBP, is just one example commonly known to produce biosurfactants (Persson *et al.* 1998). Addition of

chemical surfactants has also been shown to increase crude oil biodegradation by approximately 60% (Van Hamme *et al.* 1999).

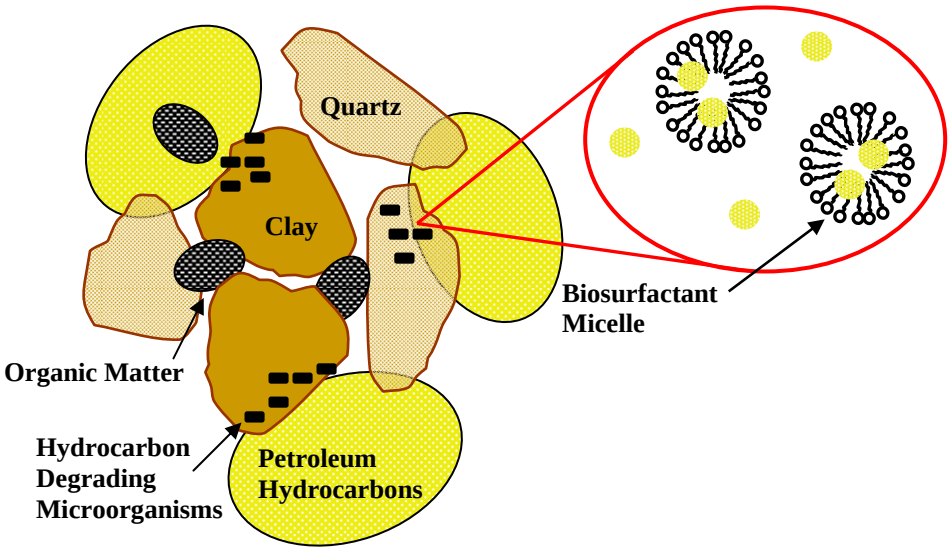


Figure 5.17 A model of a soil particle contaminated with hydrocarbons. Degrading bacteria and the biosurfactants they produce are visible at the interface with the hydrocarbon droplets (adapted from Schramm *et al.* 2003).

From Figure 5.14 is can be summarized that pure surfactant alone was shown to undergo 50% higher biodegradation on average, than detergent-surfactant and the CBP enhanced biodegradation by 6% on average, compared to sewage inoculum. Further, the CBP *did* increase overall levels of biodegradation, although not by the marked level expected. It was anticipated, based on previous results, that the CBP would increase biodegradation by 20 – 50%. This result suggested that some elements in the detergent may have inhibited biodegradation. As such, an experiment to assess the level of inhibition by the various components of the industrial detergent was designed. (Supporting data for this section can be found in Appendix A.)

5.2 Detergent Inhibition of Bacterial Growth

To test the inhibitory/stimulatory effects of the detergent components, each was added individually to a flask, as the sole energy/carbon source, and bacterial growth (rated on an absorbance scale [A_{600}] from 0.2 to 3) estimated once the stationary phase was entered, after 10 days (Table 5.3), as per method 3.2.2.

Table 5.3 Commercial bioremediation product (CBP) and sewage bacterial growth in the presence of each detergent component.

Component	CBP growth	Sewage growth
Covanyl blue dye	+ +	−
Egg yellow dye	+ +	−
EDTA	+	−
Potassium hydroxide	δ +	−
Sodium tripolyphosphate	δ +	−
Sodium xylene sulfonate	+ +	δ δ +
Triethanolamine	δ +	−
Ethylene glycol monobutyl ether	δ δ +	−

+ + $A_{600} > 3$; + A_{600} 2 – 3; δ + A_{600} 1 – 2; δ δ + A_{600} 0.2 – 1; − $A_{600} < 0.2$ after 10 days.

The results confirmed those seen previously: that biodegradation by the CBP was higher than that displayed by sewage bacteria; CBP bacteria were able to utilise more components of the detergent, whereas sewage bacteria metabolised only the surfactant sodium xylene sulfonate (SXS) significantly. CBP bacteria also utilised the SXS to a much greater extent. In general, SXS enhanced bacterial growth.

Measurements of components' concentrations would ideally be required to definitively conclude this.

The detergent formulation showed a moderately inhibitory effect in general when compared to SXS surfactant alone: low levels of growth were observed in the presence of potassium hydroxide, sodium tripolyphosphate (STPP), ethylene glycol monobutyl ether (EGMBE) and triethanolamine (TEA). Thus not only the choice and type of surfactant, but the overall detergent formulation impacts on its biodegradability.

STPP could easily be replaced with sodium-based carbonates, citrates and silicates. This complexing agent ("builder" or "softener") – as is its function in the detergent – poses the greatest environmental concern from the point of eutriphication. Once in solution, neither STPP nor potassium hydroxide should prove greatly bacteriocidal due to dilution effects. EGMBE is a common detergent solvent, particularly in spray cleaners. It is known to be toxic and easily absorbed through the skin. Other, non-petrochemical-based solvents, such as pine oil and *D*-limonene, are not only renewable vegetable oils but are readily biodegradable (aerobically and anaerobically), less toxic to aquatic life and additionally enhance the cleaning-action of the detergent. TEA, not surprisingly, decreases biodegradation activity as it is an antimicrobial (MacLeod *et al.* 1954). Here again, pine oil has antimicrobial properties which would be diluted on its disposal and is biodegradable and renewable compared to TEA which is toxic and non-biodegradable.

The detergent could be improved by the replacement – or complete elimination – of those components which were less favourable towards growth, with more stimulatory components. A separate project has been designed in which various

alternative detergent components are being synthesised and tested for their relative biodegradability. Changes in surfactant chemical structure are being correlated with changes in biodegradability.

5.3 Identification of Commercial Bioremediation Product Isolates

The four isolates comprising the CBP, designated CBP.1 – 4, were identified using 16S rDNA gene sequence analysis as per method 3.6.2. The CBP isolates were found to be homologous (percentages given below) to the following:

- CBP.1 (EF450111)* – *Bacillus subtilis* (DQ131589)* 100%
- CBP.2 (EF450112) – *Bacillus licheniformis* SK-1 (AF411341) 95%
- CBP.3 (EF450113) – *Pseudomonas putida* DSS2 (DQ304685) 95%
- CBP.4 (EF450114) – *Bacillus pumilus* CERIbio 02 (DQ207559) 99%

* GenBank accession numbers are given in brackets.

Three of the four isolates were homologous to the genus *Bacillus*. Due to the air recirculation system and the enclosed nature of the mine in which miners would be in close proximity to the CBP, it was felt that these isolates may pose a potential health risk. Typical *Bacillus* symptoms are similar to those of food poisoning: nausea, diarrhoea and vomiting (Dubouix *et al.* 2005). It is especially associated with soil, hence the concern with respect to the subsurface mine situation.

At this point a special note must be made: the bacterial composition of the CBP did not appear to be entirely stable. At two different points during the investigation, two other, possibly contaminating bacteria were identified. These were

presumptively identified as being homologous to *Bacillus cereus* and *Pantoea agglomerans* (formerly *Erwinia herbicola*), pictured in Figure 5.18.

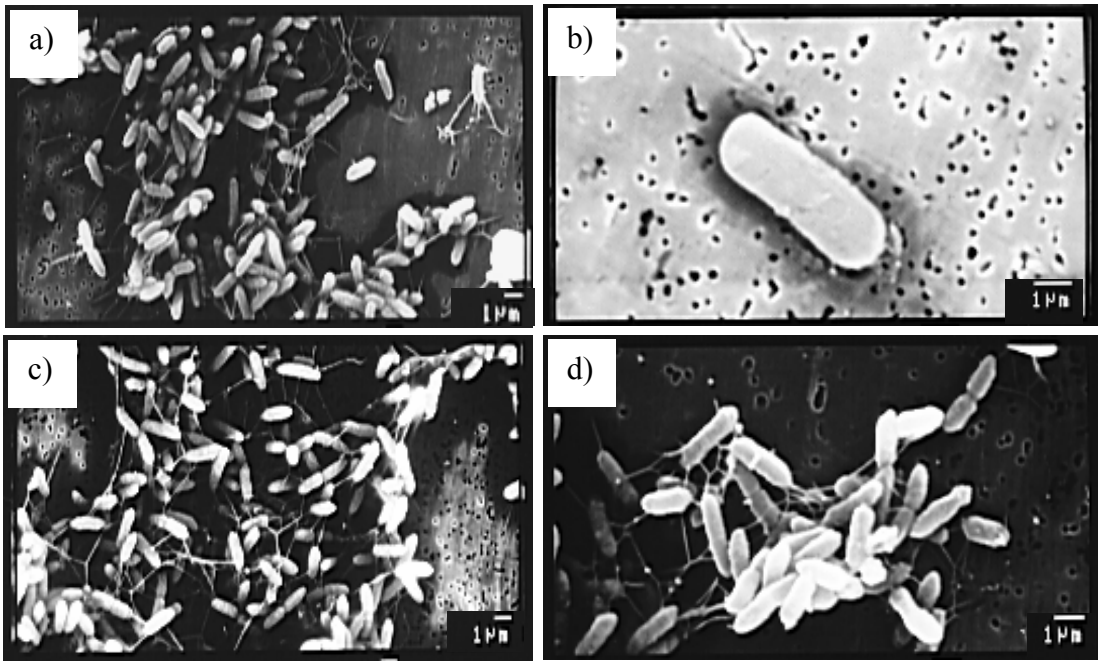


Figure 5.18 Scanning electron micrographs of *Bacillus cereus* (a and b) and *Pantoea agglomerans* (c and d), isolated from the commercial bioremediation product.

As these were only present on two separate occasions it was suspected that contamination may have occurred during packaging. As this was a recurring result despite aseptic techniques, the contamination was not ascribed to the analysis procedures. New samples of CBP were ordered and it is from these that the final identification given above was made. Whether these bacteria a) intentionally formed part of the CBP formula, b) were "contaminants" post-production or c) the bacterial composition of the CBP varied depending on the batch, all three instances are of concern. The bacterial composition of any augmentation product should be known

and monitored during the manufacturing process. Further, it should not promote the growth of potential pathogens such as *Bacillus cereus* which may prove a health risk and would prohibit its continued use. As such, new isolates were preferentially sought from areas of high hydrocarbon contamination.

5.4 Selection of Alternative Isolates

Alternative isolates were sourced from various soils known to contain perennially high levels of hydrocarbon contamination – specifically, contaminated diesel fuel industrial sites. These isolates, designated PS. 1 – 7, were tested for their ability to degrade not only surfactant but also Olef 460, a gear oil endemic in the mine-environment (method 3.2.1). The growth curves of two representative isolates are shown in Figure 5.19 a and b. Bacterial growth was rated on an absorbance scale (A_{600}) to allow the quick selection of suitable isolates.

With either surfactant or oil as the sole carbon source, exceptionally low levels of growth were observed: absorbance did not surpass 0.400 for growth in surfactant and 0.700 for growth in oil. Furthermore, considerable growth was not observed until 2 (oil) to 3 (surfactant) weeks into the degradation experiment. Controls were set up using glucose and nutrient broth to observe whether bacterial growth was indeed occurring at any significant level (Figures 5.20 a and b). Significant growth was observed inside 7 days for both nutrient sources with high to very high levels of growth observed. Bacteria quickly entered an exponential growth phase with the shape of the graph characteristic of a typical growth curve. The environmental isolates, accustomed to periods of starvation, may also have been initially overwhelmed by the high nutrient levels. It was deduced that the lack of growth in the oil/surfactant experiments may be due to a lack of bacterial adaptation to the various hydrocarbon substrates. Although these isolates were selected from areas of characteristically high hydrocarbon pollution, the nature of the specific

hydrocarbons was significantly chemically different, such that the isolates may not have been enzymatically equipped to optimally degrade the oil/surfactant. Although pre-adaptation of these isolates could have been performed, to economise on time, new isolates were sourced directly from the mine soil and water of interest. (Supporting data for this section can be found in Appendix B.)

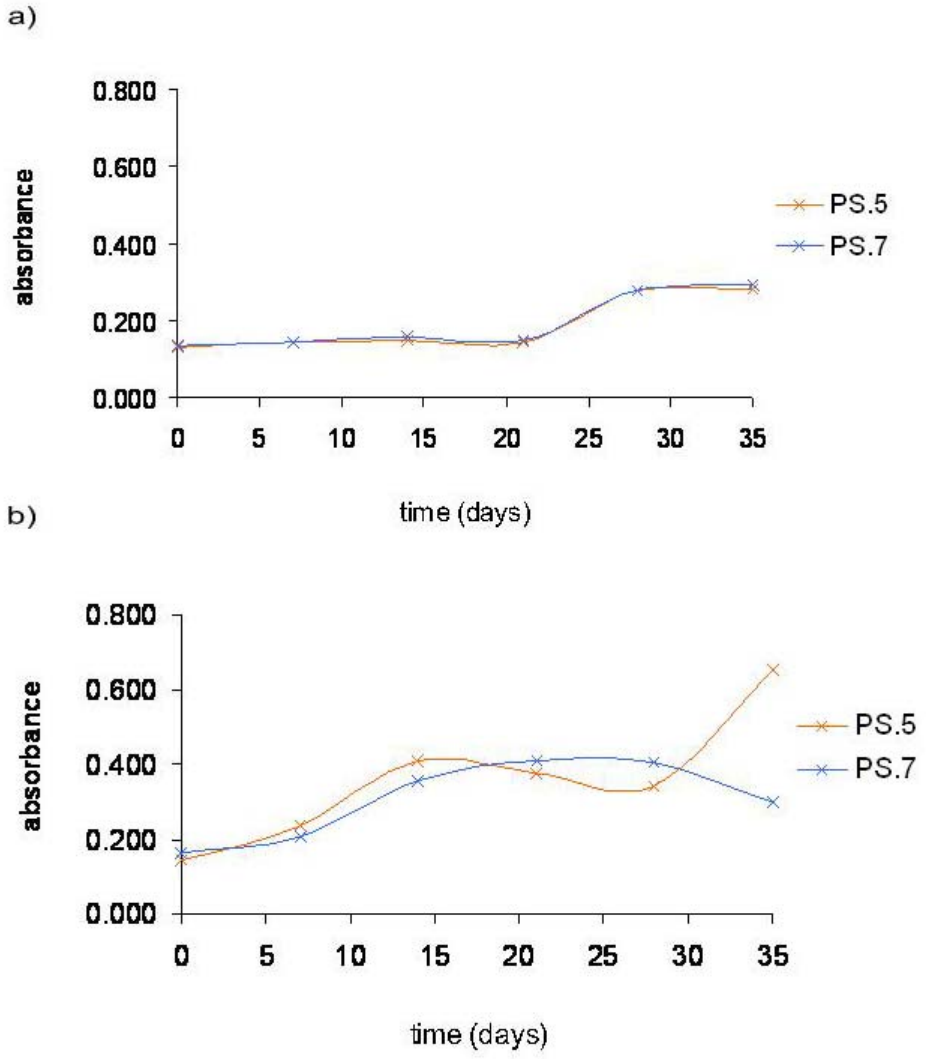


Figure 5.19 Bacterial growth of two of the alternative isolates (PS.5 & PS.7) grown on a) surfactant substrate and b) gear oil as the sole carbon source, monitored over 35 days.

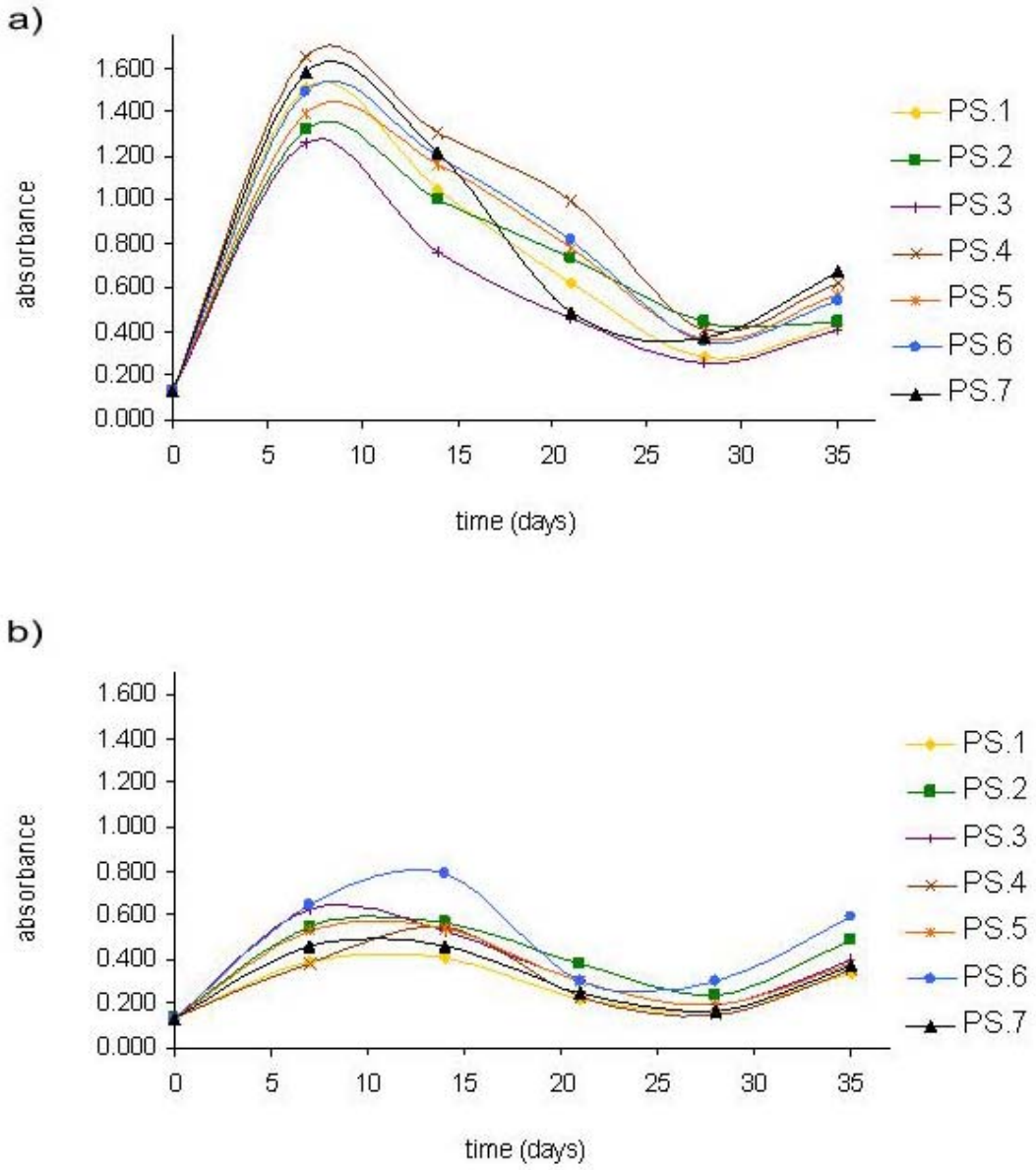


Figure 5.20 Bacterial growth for each of the alternative isolates with a) glucose and b) nutrient broth as the sole carbon source, monitored over 35 days.

5.5 Isolation of Mine Bacteria

Bacteria were isolated directly from hydrocarbon contaminated mine soil and water. Samples of mine soil and water were collected and three of the most heavily contaminated were selected for bacterial isolation (method 3.6.1). Two of the samples came from Level 23 Fast Haulage and the third from the Main Slope Working Area (Figure 21). In areas where soil and water were present, both were sampled and used.

a)



b)



c)



Figure 5.21 Sampling sites from which hydrocarbon degrading bacteria were isolated from soil and water: a) Level 23 Fast Haulage soil, b) Level 23 Fast Haulage water and c) Main Slope Working Area soil.

The six mine isolates, designated M.1 – 6, were identified using 16S rDNA gene sequence analysis as per method 3.6.2. They were found to be homologous (percentages given below) to the following:

- M.1 (EF450115)* – *Citrobacter freundii* 7 (DQ294285)* 99%
- M.2 (EF450116) – *Pseudomonas pseudoalcaligenes* (AB231158) 99%
- M.3 (EF450117) – *Pseudomonas aeruginosa* PD100 (AY825034) 99%
- M.4 (EF450118) – Trichloroacetic-acid-degrading bacterium TCAA1 (AF532186) 98%
  – *Flavobacterium* sp. (AY230767) 97%
- M.5 (EF450119) – *Enterobacter* sp. Tar-11 (AY744934) 97%
- M.6 (EF450120) – *Acinetobacter junii* T3943D1 (DQ298039) 99%

* GenBank accession numbers are given in brackets.

(Samples M.1 – M.5 were taken from Level 23 Fast Haulage; sample M.6 was taken from the Main Working Area. Samples M.1 – M.2 were water and samples M.3 – M.6 were soil.)

Arbitrarily rooted phylogenetic trees (Figures 5.22 & 5.23) were constructed showing the clustering of the mine isolates and those from the CBP. As the mine bacteria were isolated directly from the polluted sites in question, they may prove useful for bioaugmentation, especially those clustering with bacteria related to bioremediation studies, e.g. *Flavobacterium* sp. and Trichloroacetic-acid-degrading bacteria. Hence an experiment was designed to test compare the ability of the newly isolated mine bacteria to biodegrade hydrocarbons relative to the CBP isolates.

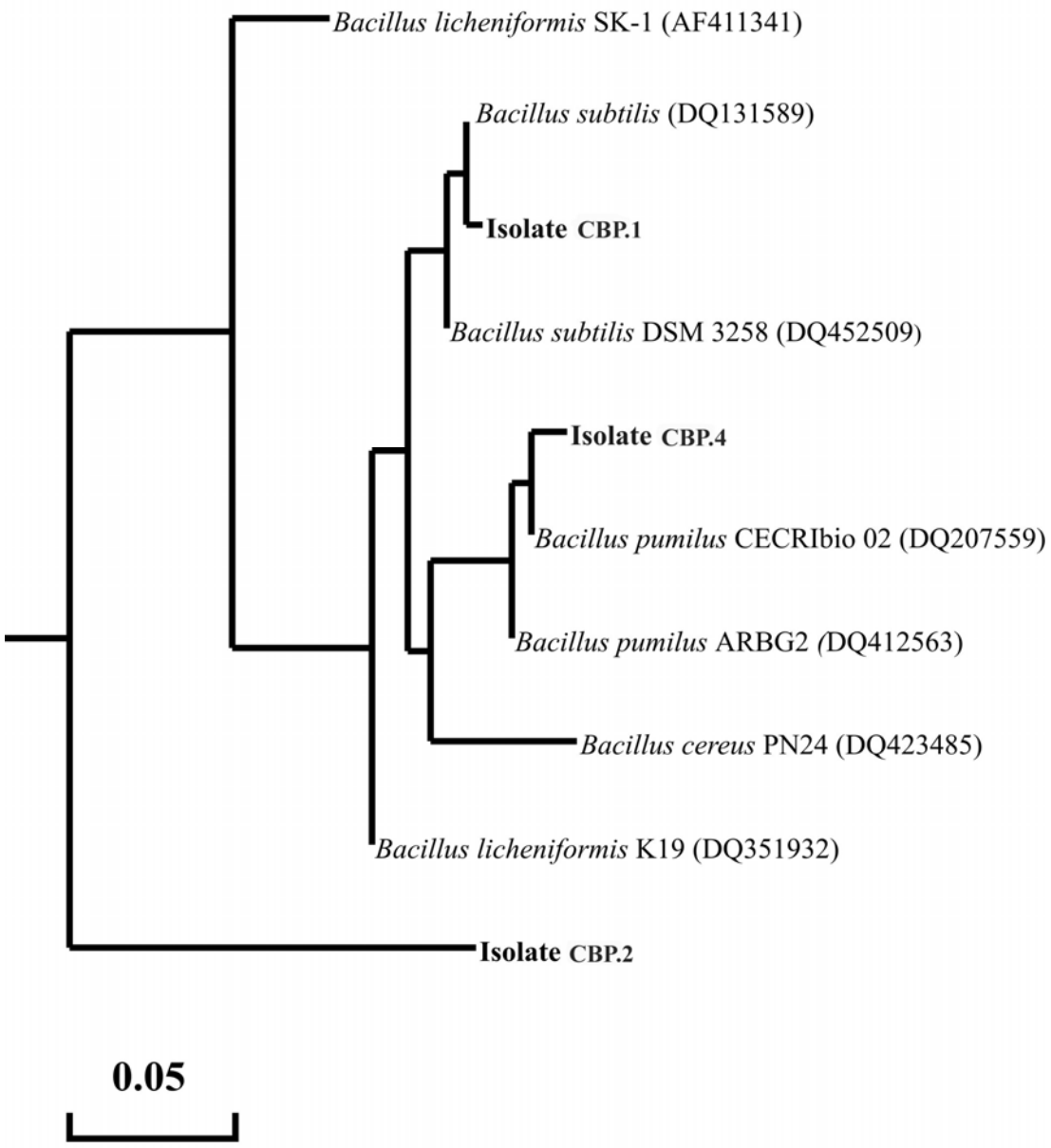


Figure 5.22 Phylogenetic relationships as revealed by 16S rDNA gene sequence analysis highlighting the clustering of the 3 Gram-positive isolates used in this study. The tree is arbitrarily rooted, with branch lengths proportional to the estimated genetic distance between strains (0.05%) (vertical distances are insignificant).

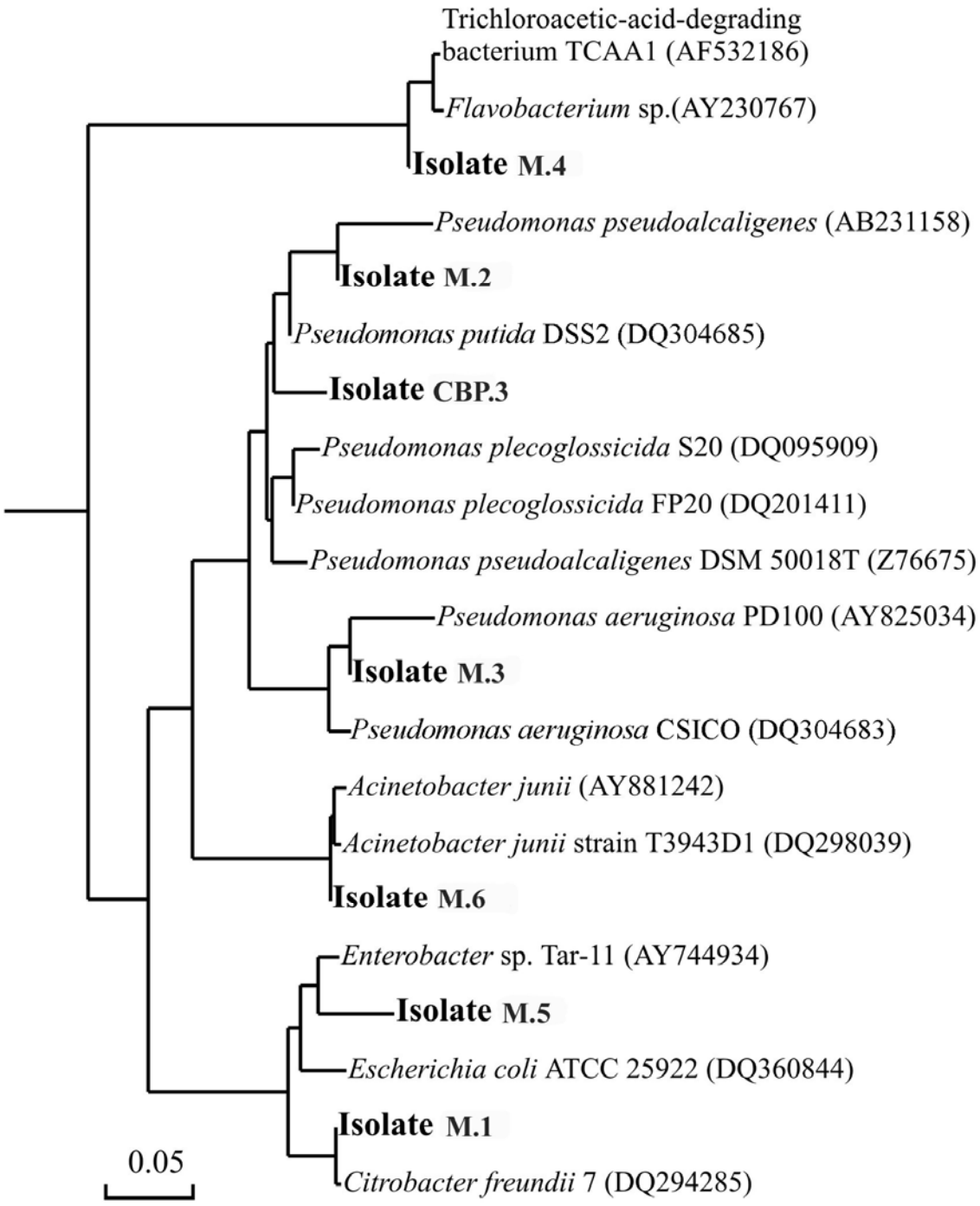


Figure 5.23 Phylogenetic tree as revealed by 16S rDNA gene sequence analysis highlighting the clustering of the 7 Gram-negative isolates used in this study. The tree is arbitrarily rooted, with branch lengths proportional to the estimated genetic distance between strains (0.05%) (vertical distances are insignificant).

5.6 Biodegradation of Monograde Engine Oil by Mine Isolates and Commercial Bioremediation Product Isolates

Each of the mine isolates and CBP isolates was tested with monograde engine oil as a sole carbon substrate. This oil was selected as an example of an old-type, low-grade, "non-engineered" engine oil because it should prove the most difficult to biodegrade. A batch-flask experiment was set-up in duplicate to evaluate the biodegradation efficiency of each of the isolates individually (method 3.2.1). Results are reported as a percentage growth increase over the 21 day biodegradation period (Figure 5.24). The results take into account the speed with which maximum growth was reached; the higher percentages indicate maximum growth attained, in the shortest possible time.

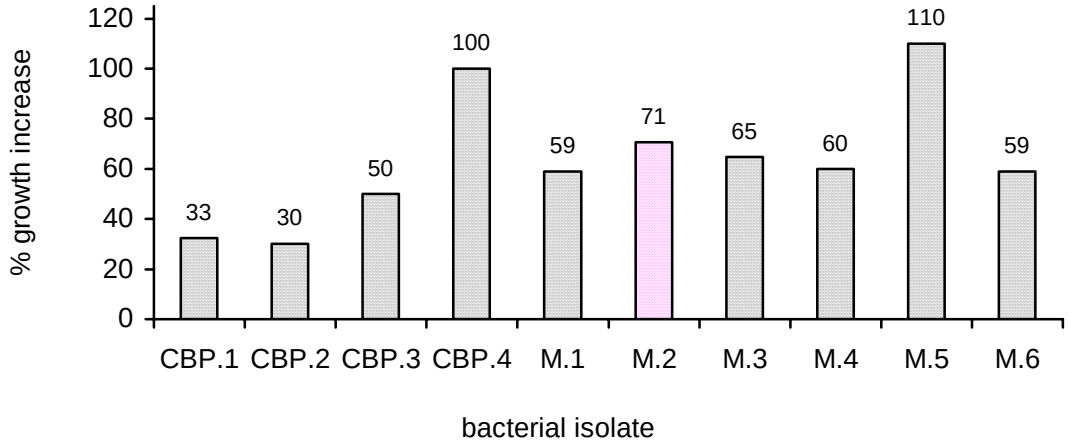


Figure 5.24 Percentage increase in growth for each of the oil biodegradation study isolates, taking into account the maximum growth reached and the time taken to reach that level. (The mine isolate showing average growth, M.2, is highlighted.)

The growth curve for the mine isolate showing average growth (highlighted in Figure 5.24) is given in Figure 5.25. A typical growth curve with a growth phase (week 1), stationary phase (week 2) and death phase (week 3) can clearly be observed. The remaining isolates displayed similarly characteristic growth curves but with varying maxima reached in various times. Error bars are relatively large due to the nature of the sample: bacteria adhere to the oil such that when a dilution is done to calculate bacterial numbers, the percentage of bacterial adhesion varies, resulting in large relative errors each time.

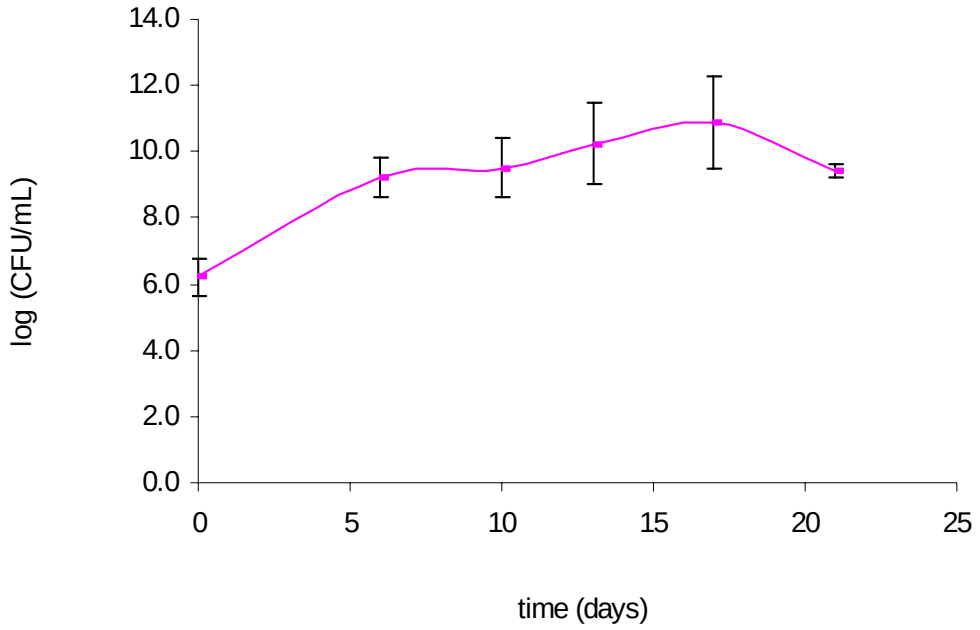


Figure 5.25 Bacterial growth for isolate M.2 with monograde engine oil as the sole carbon source.

Biodegraded oil of isolate M.2 was selected for further analysis by GC-FID (Figure 5.26). Three peaks of interest are highlighted by arrows: two of these peaks disappeared completely after 21 days of biodegradation by isolate M.2 (i.e. 100% biodegradation); the other peak decreased by 99.60% (pink arrow). The area changed

from 542.0 prior to application of the bacterial isolate to 2.147 post biodegradation. In Figure 5.26 b) the vertical scale has been increased 10 fold to allow the peak at 25.80 s to be visible. The broad base of the dichloromethane solvent peak is also visible in the chromatograms. Due to the viscosity of the monograde engine oil, which made analysis difficult, the solvent peak was unable to be completely resolved from the analyte peaks. Despite numerous attempts, use of alternative columns and changes to the GC programme, the solvent peak was never successfully separated from the analyte peaks. No other peaks were observed after the first 30 s, with chromatograms run for 3 hours in total. When compared to sterile control data it was clear that these reductions were not as a result of physical weathering.

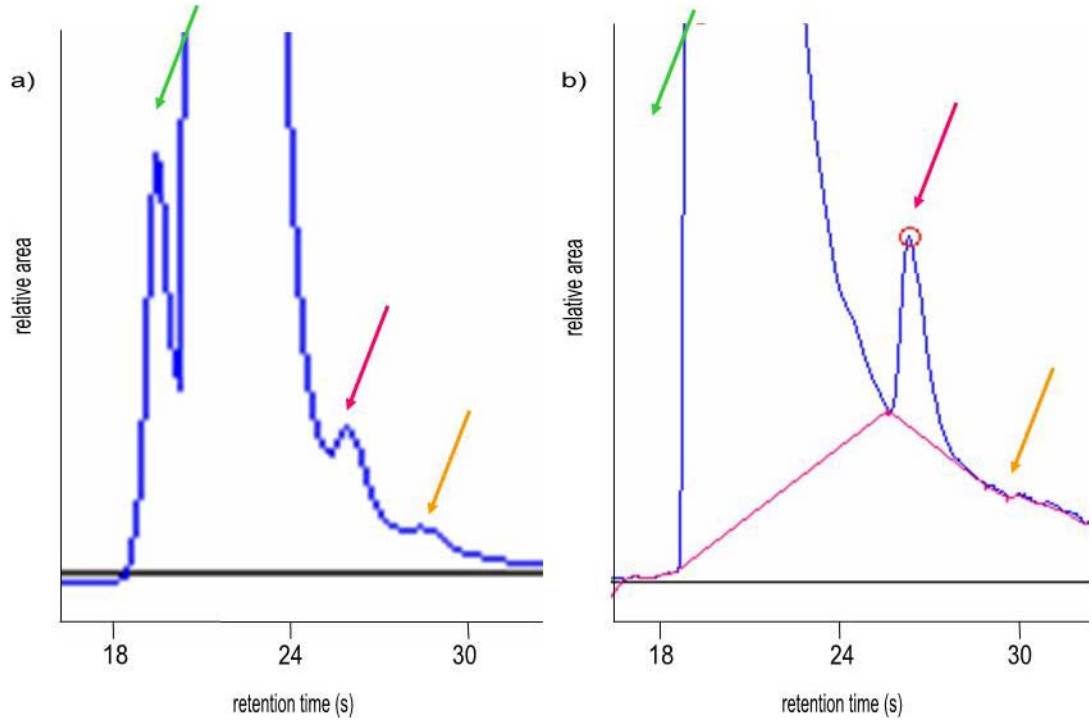


Figure 5.26 Chromatogram of engine oil from flask M.2, a) prior to biodegradation at time zero and b) after 21 days of biodegradation. The arrows indicate the retention times of the three hydrocarbon peaks of interest (18.96 s, 25.80 s and 28.38 s respectively).

Oil is emulsified by the bacteria into droplets and adheres to the glass walls of the flask (general observation for all bacteria although more prevalent for CBP.3 and M.1, 5 and 6 (See Appendix D)). The visible change in the oil can be clearly seen in Figure 5.27. Firstly, the oil is shown without any biodegradation, just after addition to the flask (Figure 5.27 a). Figure 5.27 b) shows the control flask after 21 days of aeration. The difference is clearly visible, slight emulsification has taken place at the interface between the medium and the oil. Figure 5.27 c) illustrates the difference after addition of a hydrocarbon-degrading bacterial isolate. The colour and consistency of the oil has changed completely and large-scale emulsification of the oil into droplets has taken place. The volume of oil has decreased by almost half (ultimate biodegradation) and is starting to lose its hydrophobic properties (primary biodegradation). This differs considerably from the control.

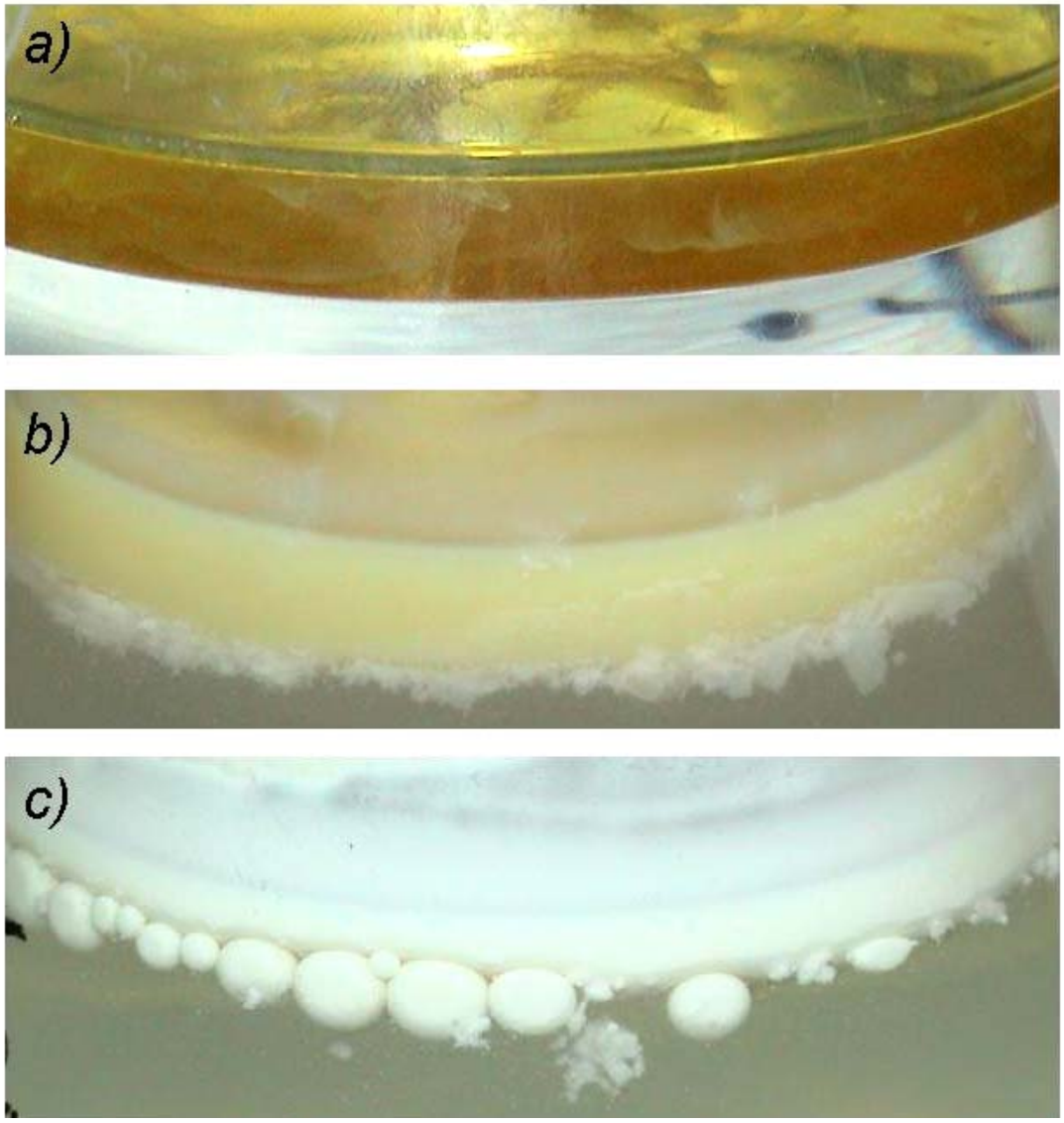


Figure 5.27 a) Monograde engine oil in minimal medium prior to biodegradation. b) Control flask with monograde engine oil in minimal medium after 21 days of aeration. c) Monograde engine oil after 21 days of biodegradation by isolate M.2. (Note the emulsification of the oil.)

On average, the mine isolates were 16% more effective at degrading monograde motor oil than the CBP isolates. This is based on the maximum percentage biodegradation realised in the shortest possible time. The CBP bacteria were identified as being homologous with *Bacillus subtilis, Bacillus licheniformis, Pseudomonas putida* and *Bacillus pumilus* (batch 1) and *Pantoea agglomerans* and *Bacillus cereus* (batch 2). Those isolated from the mine were identified as being homologous with *Citrobacter freundii, Pseudomonas pseudoalcaligenes, Pseudomonas aeruginosa, Flavobacterium* sp. / Trichloroacetic-acid-degrading bacterium, *Enterobacter* sp. and *Acinetobacter junii*. Almost all of these bacteria have previously demonstrated their biodegradation ability, under numerous sets of conditions:

Enterobacteriaceae are recognized hydrocarbon degraders (Neelam *et al.* 1987, Katsievela *et al.* 2005) and *Citrobacter freundii* is known to degrade biphenyl (Grishchenkov *et al.* 2002).

Gram-positive, catalase positive, oxidase negative, *B. subtilis* is an endospore-producing soil, obligate aerobe, anabolising a cyclic lipopeptide called surfactin, that degrades carbon chains of length C10 to C19 (Madigan *et al.* 2005). The most active biosurfactant discovered to date, it has been linked to the *sfp* nucleotide gene sequence, which can be cloned into some non-surfactant producing species (Ron *et al.* 2001). The surfactant decreases surface tension and increases emulsification. Up to 98% biodegradation within 24 hours has been demonstrated. Similar results have been observed for the addition of surfactant along with non-surfactant producers due to improved bioavailability (Kim *et al.* 2000). *B. subtilis* degrades pyrene and thermophillic varieties are able to degrade recalcitrant PCB e.g. chlorobenzoate, pentacholorphenol, trochloroethylene, and dioxin (Margesin *et al.* 2001). Although *B. subtilis* can contaminate food, it rarely causes food poisoning and is generally not considered to be a human pathogen (Ryan *et al.* 2004). This bacterium has many commercial applications as a fungicide, laundry detergent additive, in agriculture and

in the food industry (Gielen *et al.* 2004, EPA 2006). *B. licheniformis*, able to reduce nitrate and metabolise citrate, is closely clustered with *B. subtilis, B. cereus* and *B. pumilus* (Zhuang *et al.* 2002) which are common biodegraders, mostly by virtue of lipopeptide biosurfactant production (Barkay *et al.* 1999, Ron *et al.* 2001).

In general, *Bacillus* sp. have been identified as naphthalene and pyrene degraders (Naphthalene is often used as a model for PAH biodegradation (Ron *et al.* 2001, Zhuang *et al.* 2002)) and are known petroleum hydrocarbon degraders (Gazhali *et al.* 2004, Das *et al.* 2007).

P. putida is a common organic solvent degrader, completely safe for human exposure and has shown much success in bioremediation (Margesin *et al.* 2001). The biodegradation activity is plasmid encoded. *P. putida* and *Pseudomonads* in general degrade a range of compounds: PAH (for example naphthalene), salicylate, BTEX and phenol (Grimm *et al.* 1997, Hamann *et al.* 1999, Shim *et al.* 1999, Margesin *et al.* 2001). A chemotactic relationship with biphenyls is plasmid encoded (Grimm *et al.* 1997). Phenanthrene is toxic to *P. putida* (Cerniglia 1992). *P. pseudoalcaligenes* degrades PCB (Kumamaru *et al.* 1998).

In general, *Pseudomonas* sp. have been identified as petroleum hydrocarbon degraders (Neelam *et al.* 1987, Williams *et al.* 1994, Deschenes *et al.* 1996, Gazhali *et al.* 2004, Das *et al.* 2007). *P. aeruginosa* has proven more efficient than *B. subtilis* corresponding with the results seen for the biodegradation study (section 5.6) (Das *et al.* 2007).

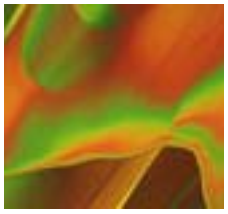
Pantoea agglomerans produces biosurfactant which aids in hydrocarbon biodegradation (Vasileva-Tonkova *et al.* 2007). *Acinetobacter* spp. are acknowledged hydrocarbon degraders (e.g. phenanthrene, fluoranthrene and pyrene), not least because of the production of the biosurfactant alasan (Barkay *et al.* 1999) which doubles the rate of mineralization of some compounds. Like other biosurfactants, its

mode of operation is increased substrate-bioavailability. Indeed, for this reason, all the genera mentioned in this discussion are often isolated concurrently at the same site and have been identified in many bioremediation studies (Cerniglia 1992, Cerniglia 1993, Atlas *et al.* 1995, Pieper *et al.* 2000, Bento *et al.* 2003, Bento *et al.* 2005). (Supporting data for this section can be found in Appendices C and D.)

Thus, the bacteria isolated from the subsurface mine soil and water are known hydrocarbon degraders that improved biodegradation when compared to the CBP isolates. This may suggest that simple biostimulation may be the best practice however, it has been shown that reintroduction of local isolates at a specific site is a highly effective and cost-efficient means of bioremediation (Korda *et al.* 1997). It is therefore proposed that these degrading bacteria successfully constitute the first South African bioaugmentation agent for the remediation of subsurface petroleum contamination. They may prove able to degrade hydrocarbons at other such sites. Extended field-testing will potentially confirm this and provide the added data needed to successfully round off this project.

CHAPTER 6

CONCLUSIONS



- The CBP was effectively quantified by GC-FID to biodegrade light and heavy hydrocarbon fractions of crude oil by 100%. The product may therefore have some application as a bioaugmentation agent in the bioremediation of crude oil.

- CBP biodegradation of sodium dodecylbenzene sulfonate by 95% was demonstrated, whilst detergent-surfactant was degraded by 43%. This was 6%, on average, better biodegradation than that observed by sewage bacteria. The product may therefore prove useful as a bioaugmentation agent for the bioremediation of LAS.

- Most of the chemical components of the detergent formulation were utilised by CBP bacteria, whereas sewage bacteria utilised only SXS significantly.

- Potassium hydroxide, STPP, EGMBE and TEA, in the detergent, were all found to inhibit bacterial growth to some extent. Replacement of some of these components may make the detergent more biodegradable and hence environmentally friendly.

- The bacteria contained in the CBP were isolated and found to be homologous to *Bacillus* sp., potentially posing some health risk.

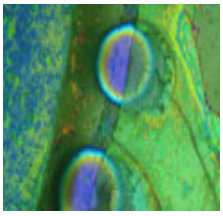
- Bacteria known to degrade diesel fuel oil, isolated from industrial contamination sites, were tested for their ability to biodegrade surfactant and gear oil. The chemical structures of the substrates were found to be sufficiently dissimilar, such that pre-adaptation of the isolates would have proven necessary.

- Hydrocarbon-degrading bacteria were therefore isolated directly from the mine wastes. None of these were identified as being homologous to *Bacillus* sp., therefore posing minimal health risk. They were tested for their ability to biodegrade a low-grade engine oil and were found to be 16% more efficient, on average, than the CBP isolates.

- These new isolates may prove safe and efficient as the first South African isolated and tested bacteria for the bioremediation of hydrocarbon contamination via bioaugmentation.

CHAPTER 7

FUTURE WORK



The six mine isolates should be combined with absorbent material (e.g. straw or plant material), biosurfactants (e.g. surfactin) and nutrients (e.g. $MgNH_4PO_4$) (Swannell *et al.* 1996) to maximize biodegradation ability. This will result in the first South African-researched, tested and manufactured commercial bioremediation product for the clean-up of hydrocarbon contamination. It may also be worthwhile including some well-documented, *Gram-positive* biodegrading microorganisms (e.g. *Rhodococcus* sp.), as all of the mine isolates were Gram-negative (Van Hamme *et al.* 2001). Gram-positive and -negative isolates are known to "exhibit differential sensitivity" in the degradation of organic compounds although Gram-positive bacteria have been noted to be more susceptible to inhibition (Fuller *et al.* 1997).

A cost of R200 per litre was estimated in section 4.3 "Cost-Benefit Analysis of Mine Bioremediation". This is merely an estimate of the laboratory-scale cost to produce 1 L of inoculum for application to contaminated water or soil. That litre of inoculum could be used to seed a silo-like batch reactor for large-scale use in the mine water-treatment-system. If a small scale silo (5 m diameter × 15 m height) was used, this would yield a volume of approximately 1200 kL of inoculum. Additional air could easily be pumped into the silo via a simple plumbing setup. Extrapolating the amount of inoculum used in the biodegradation experiments, 1200 kL of inoculum should prove sufficient for the treatment of 120 ML of mine water (the amount pumped at Kloof per day). This decreases the estimated laboratory costs drastically, to only those required to maintain the culture and the bioremediation treatment plant. The cost of bioremediation would then be lower than those currently expended by the mine on traditional mechanical remediation methods.

Alternatively, the solid hydrocarbon waste that is collected by the Filter Plant could be treated on disposal. This would however then constitute an additional treatment cost over and above the R150 000 currently used to purify the mine's water. When this study was originally conceived, an accompanying doctoral study was designed to implement the augmentation agent in the mine in question. This will effectively provide the necessary large-scale experiments to accurately calculate the real costs involved and will hopefully be implemented in the near future.

CHAPTER 8

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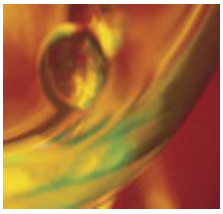

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CHAPTER 9

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CHAPTER 10

APPENDICES



10.1 Appendix A: MBAS Data

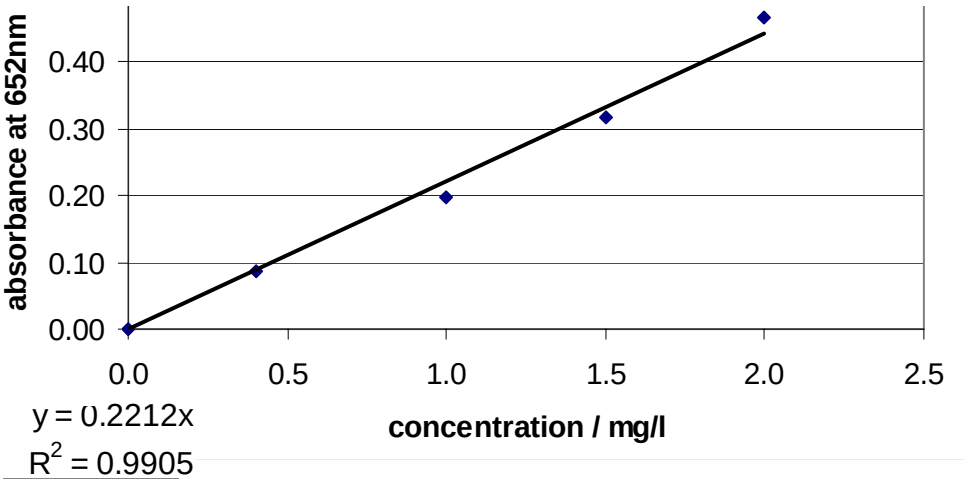


Figure 10.28 Standard curve of methylene blue dye, used for the MBAS test to quantify anionic surfactant in the batch-flask biodegradation experiments.

Table 10.4 Data for the biodegradation of surfactant and detergent-surfactant by two sources of inoculum, CBP and sewage.

	Microbial Counts (log CFU/mL)			
Day	Detergent + Sewage	Detergent + CBP	Surfactant + Sewage	Surfactant + CBP
0	2.78	4.73	3.71	4.88
7	3.92	5.77	6.04	8.24
14	5.60	5.61	6.99	8.28
28	3.05	4.81	8.96	4.02
	MBAS Absorbance 652 nm			
Day	Detergent + Sewage	Detergent + CBP	Surfactant + Sewage	Surfactant + CBP
0	0.130	0.127	0.0730	0.0720
7	0.127	0.122	0.0540	0.0190
14	0.294	0.131	0.0270	0.0110
21	0.117	0.113	0.0090	0.0200
28	0.0790	0.0720	0.0510	0.00360
	Calculated Surfactant Concentrations /ppm			
Day	Detergent + Sewage	Detergent + CBP	Surfactant + Sewage	Surfactant + CBP
0	3589	3504	2014	1987
7	3504	3367	1490	524.3
14	8113	3615	745.0	303.5
21	3208	3118	248.3	551.9
28	2180	1987	256	99.34
Overall % Degradation	39	43	87	95

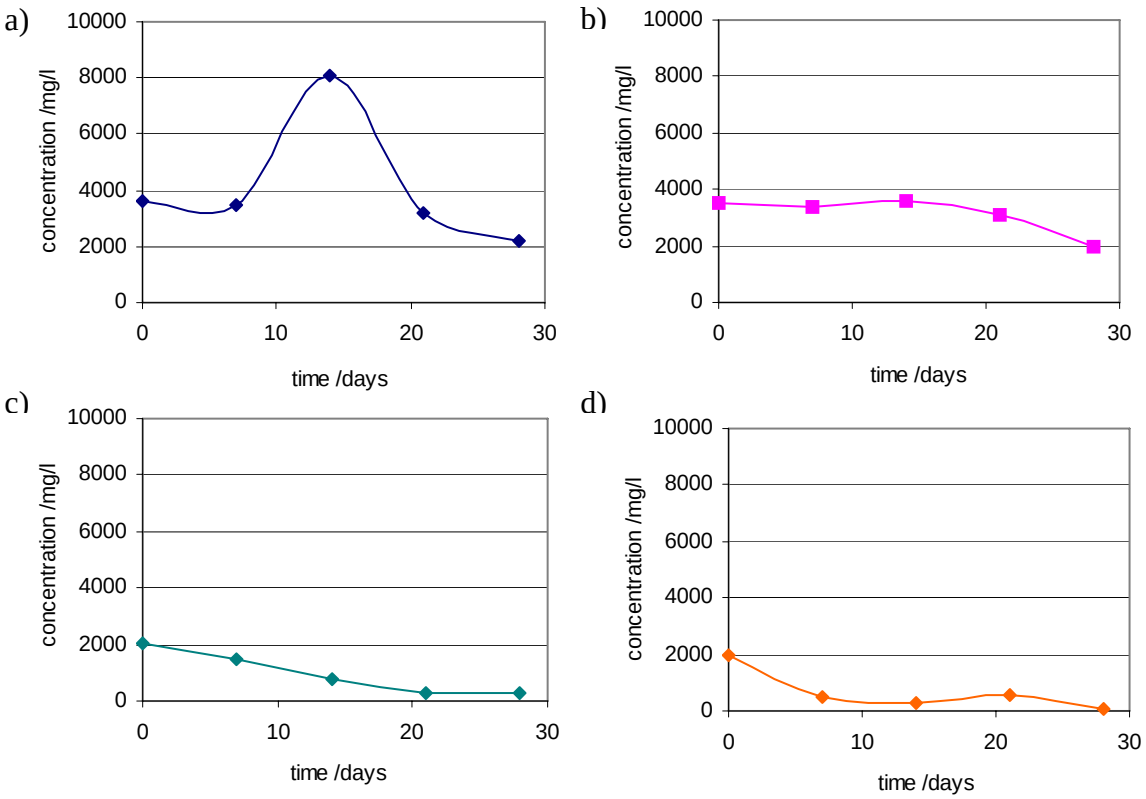


Figure 10.29 Composite of graphs for the change in surfactant concentration over the course of the biodegradation batch-flask experiment for a) detergent and sewage, b) detergent and CBP, c) surfactant and sewage and d) surfactant and CBP.

10.2 Appendix B: Identification of Alternative Isolates

The alternative isolates sourced from industrial areas contaminated with hydrocarbons were identified as being homologous to:

- PS.1 – *Pseudomonas aeruginosa*
- PS.2 – *Delftia* sp.
- PS.3 – *Stenotrophomonas maltophilia*
- PS.4 – *Pseudomonas aeurginosa*
- PS.5 – *Stenotrophomonas maltophilia*
- PS.6 – *Bacillus cereus*
- PS.7 – *Pantoea agglomerans*

10.3 Appendix C: GC Supporting Data

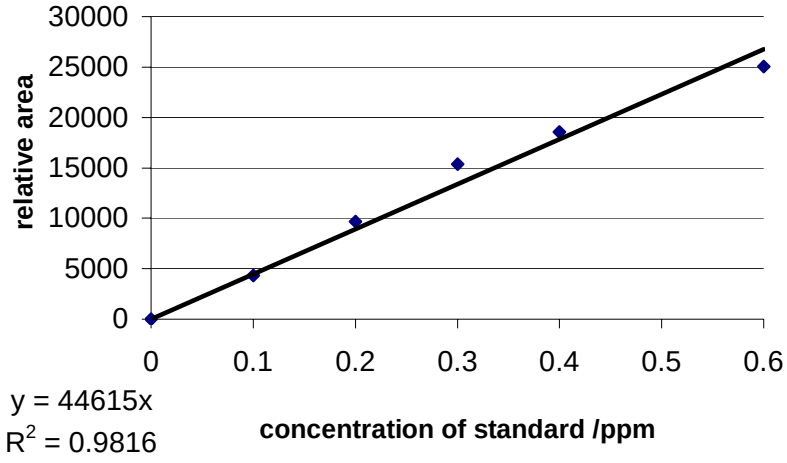


Figure 10.30 Standard curve of monograde engine oil correlating extraction recoveries from MAE and the GC-FID temperature program.

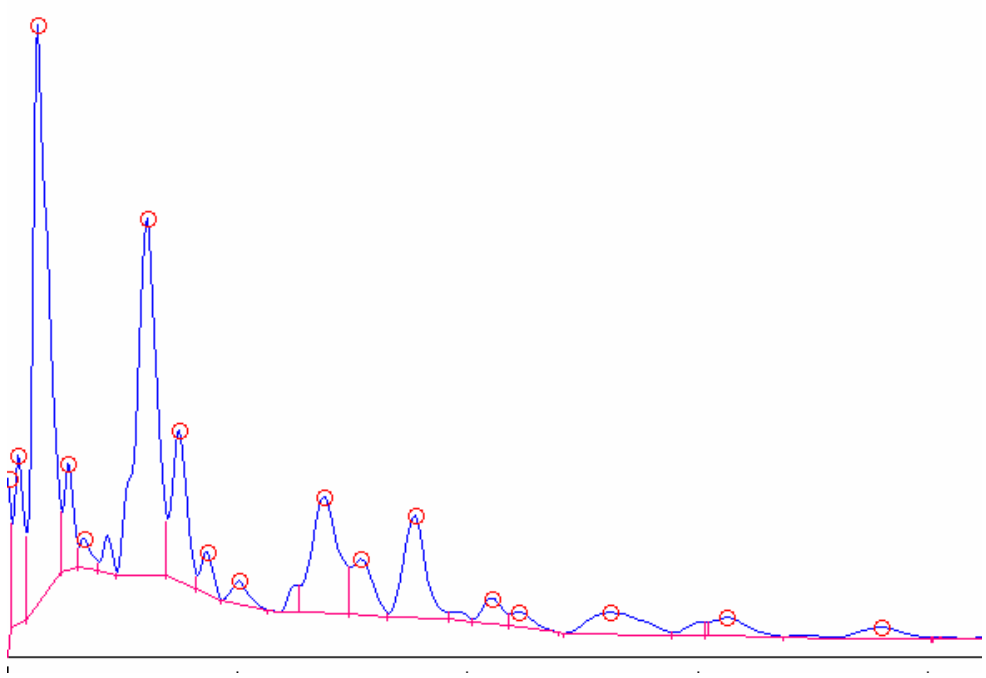
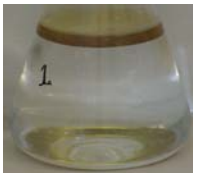
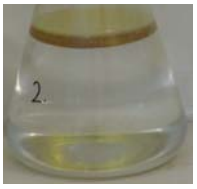
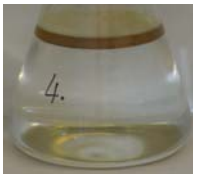
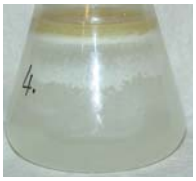
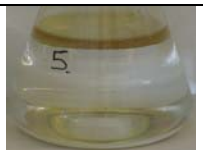
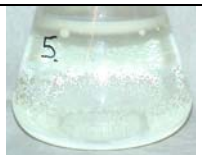
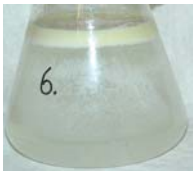
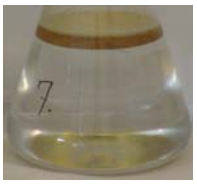
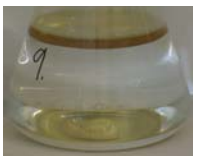


Figure 10.31 Chromatogram showing the resolution of the UCM of monograde engine oil into individual hydrocarbon peaks, correlating with optimization of the GC temperature program.

10.4 Appendix D: Biodegradation Photographs

Table 10.5 Photographs of the biodegradation batch-flask experiment, after addition of oil (Time Zero), after two weeks (Time Intermediate) and after four weeks (Time Final).

Isolate	Time Zero	Time Intermediate	Time Final
Control			
CBP.1			
CBP.2			
CBP.3			
CBP.4			

Isolate	Time Zero	Time Intermediate	Time Final
M.1			
M.2			
M.3			
M.4			
M.5			
M.6	