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ENHANCED OIL DEGRADATION BY BACTERIA WHEN IMMOBILIZED TO AN OLEOPHILIC SUBSTRATUM

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by

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Dedicated to my parents, David and Joyce Wilson for their love, support and understanding over the last 26 years.

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Abstract

Three immobilization matrices, Biofix (kaolinite microspheres), Drizit (polypropylene fibres) and polyester polyurethane were used as substrata for use in bioremediation. Enhanced biodegradation of petrol (Slovene diesel) and Ekofisk crude oil occurred with immobilization of *Pseudomonas fluorescens* to Biofix and Drizit in freshwater and saltwater systems. When compared to free bacteria, immobilization resulted in; increased growth, accelerated ability of the cells to utilize oil, and enhanced biodegradation as determined by gas chromatography. In the freshwater systems Drizit immobilized cells reduced the lag phase to one day in comparison to six in a free system and increased biodegradation of the n-alkanes by 67 %. Immobilization resulted in enhanced production of a rhamnolipid biosurfactant over the first three days of incubation in comparison to free living bacteria which showed a lag phase of two days. All three supports were compared by; scanning electron microscopy, cell loading capacity, absorption of oil, their abilities to enhance oil biodegradation and the effect of drying and storage on the immobilized cells. Biodegradation of hydrocarbons by immobilized cells was dependent on the biocarrier, with polyurethane immobilized cells failing to enhance biodegradation of Ekofisk crude oil. Drizit was the most effective biocarrier tested and the most suitable immobilization substratum for use as a bioremediation agent. Investigation into the location of the genes for alkane degradation in immobilized *Pseudomonas fluorescens* was undertaken. Plasmid DNA was detected using gel electrophoresis, and caesium chloride-ethidium bromide gradient was carried out to confirm the presence of the plasmid, but no plasmid band was visualized. The successful immobilization system was scaled-up, optimized by supplementation with nitrates and phosphates and applied to microcosms that modelled environmental conditions. Enhanced biodegradation of Ekofisk crude oil was demonstrated on a larger scale, in an estuarine microcosm, with the immobilized systems showing an average degradation of 41.9 % in comparison to 7.1 % in the microcosm containing free indigenous bacteria.

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Abbreviations

ANOVA	Analysis of variance.
BP	British petroleum.
BSA	Bovine serum albumin.
CAM	Camphor.
CAP	Caprolactam.
CCC	Covalently closed circular.
CFU	Colony forming unit.
DCM	Dichloromethane.
ECC	English china clay.
EDTA	Ethylene diamine tetraacetic Acid.
FAD	Flavin adenine dinucleotide.
FID	Flame ionisation detector.
GC	Gas chromatography.
kbp	Kilo base pairs.
kDa	Kilodaltons.
LPS	Lipopolysaccharides.
MANOVA	Multilple analysis of variance.
N-alkanes	Normal-alkanes.
NAD	Nicotinamide adenine dinucleotide.
NADP	Nicotinamide adenine dinucleotide phosphate.
NAH	Naphthalene.
LPS	Lipopolysaccharides.
LSD	Least significant difference.
OCT	Octane.
PBS	Phosphate buffered saline.
Rb	Rubredoxin.

RbR   Rubredoxin oxido-reductase.

SAL   Salicylate.

SDS   Sodium dodecylsulphate.

STE buffer   Sodium tris(hydroxymethy) aminomethane ethylene diamine tetraacetic acid buffer.

TBE Buffer   Tris(hydroxymethy) aminomethane-borate ethylene diamine tetraacetic acid buffer.

TCA   Tricarboxylic acid.

TDP   Thymine diphosphate

TE Buffer   Tris(hydroxymethy) aminomethane ethylene diamine tetraacetic acid buffer.

TOL   Toluene.

TSB   Tryptone soy broth.

Tris. Cl   Tris(hydroxymethy) aminomethane chloride.

XYL   Xylene.

Chapter 1

General Introduction

1.1. Occurrence of petroleum pollution

Petroleum can be defined as a crude oil occurring in sedimentary rocks, consisting mainly of hydrocarbons. Fractional distillation separates the petroleum into petrol, paraffin, diesel oil and lubricating oil. Fuel oil, paraffin wax, asphalt, and carbon black are extracted from the residue (Collins 1989). The dispersion of petroleum in water, its movements, its chemical modifications and its persistence in the sea are influenced by the specific gravities (densities), chemical composition, quantity and state in which the petroleum is introduced into the sea (Zobell 1964). Petroleum pollution can be chronic (arising from a continuous or regular discharge) or acute (the result of one accidental spillage or intentional dumping), (Nelson-Smith 1972). Input of petroleum into the sea includes natural seepage (estimated at 0.6 million tons annually) and input by marine organisms, which can contain up to 1 g of hydrocarbons per kg fresh weight (Zobell 1964). As well as these natural sources there are several anthropogenic sources by which petroleum products may infiltrate into the sea. These include devastating accidents which occur periodically, e.g in 1989 the Exxon Valdez ran aground near Bligh Island, Alaska and released a large quantity of Prudhoe Bay crude oil (Button et al. 1992) and in 1993, the Braer went aground on Shetland releasing light crude, fuel oil and diesel (Dixon 1993; Bayfield 1994). Upwelling of oil can occur when new oil wells are being submerged (Floodgate 1972; Nelson-Smith 1972), and there is the problem of discharge of oil from ships, ports and oil terminals (chronic pollution). This type of pollution transpires mainly from cargo, passenger and navy ships, (although even small motor boats emit oil into the sea from bearings and exhausts). The problem arises because these ships use tanks alternatively for fuel oil and saltwater ballast. When the fuel oil has been used, the tanks are filled with seawater to maintain stability. When more fuel is taken into the tanker the oil-polluted seawater is discharged into the environment (Zobell 1964; Nelson-Smith 1972).

Hydrocarbons form up to 98 % of some crude oils and are compounds whose molecules contain only carbon and hydrogen atoms. However, crude oil contains carbon and hydrogen and hydrocarbon derivatives containing oxygen, sulphur, and nitrogen or a combination of these (Nelson-Smith 1972). Petroleum hydrocarbons can be split into four groups;

1) Paraffins or alkanes, are saturated compounds having the formula C_nH_{2n+2}, they do not have multiple bonds between carbon atoms e.g methane and ethane (Solomon 1994) and may have straight or branched chains. The term 'normal' alkanes (n-alkanes) indicates that the compound contains an unbranched chain of carbon atoms. The distinction is made due to the occurrence of alternative 'branched chain' structures of the same formula. At room temperature the n-alkanes range from gases (CH_4 to C_4H_{10}) to liquids (C_5H_{12} to $C_{16}H_{34}$) and then to solids ($C_{17}H_{36}$ upwards). Physical properties depend on the molecular weight and shape of the compound (Hanson 1984)
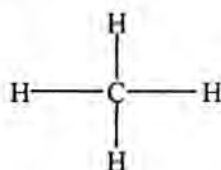
a) methane - CH_4 b) ethane - C_2H_6
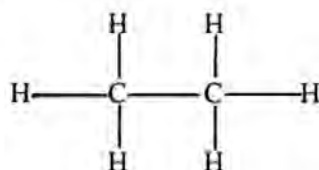
Figure 1.1. Structure of the n-alkanes methane and ethane (Hanson 1984).

2) Naphthenes (cycloparaffins), these are also saturated, but the ends of the chain are joined to form a ring structure, two hydrogen atoms are thus eliminated, giving the general formula C_nH_{2n} (Solomon 1994).

3) Aromatics, These are unsaturated cyclic compounds based on the benzene ring (figure 1.2) with resonating double bonds and which therefore have six less hydrogens per ring than the corresponding naphthene (Solomon 1994).
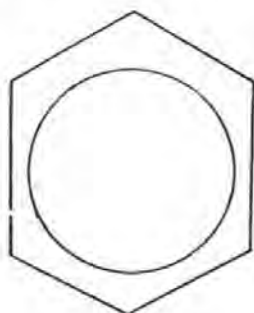
a) the benzene ring   b) benzene
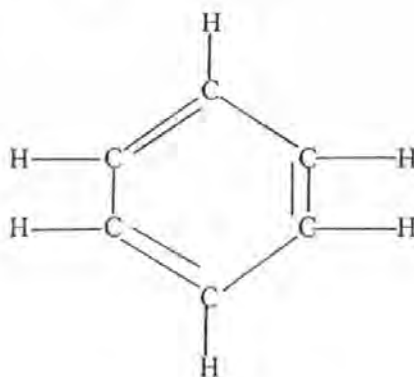
Figure 1.2. Structure of the benzene ring and benzene (Grundon and Henbest 1969).

4) Alkenes, these are unsaturated non-cyclic compounds with two or fewer hydrogen atoms for each carbon, with the general formula C_nH_{2n}. They may form straight or branched chains (Nelson-Smith 1972). The presence of a single double bond in a hydrocarbon is indicated by replacing the terminal -ane in the name of the parent hydrocarbon by -ene e.g $H_2C=CH_2$ - ethene (Hanson 1984).

1.2. Fate of oil in the marine environment.

The boiling point, specific gravity and viscosity of the oil are the physical properties of a petroleum mixture which determine its fate. Components of low boiling point evaporate rapidly, and some components mainly lower aromatics and alkanes, and non-hydrocarbon constituents are fairly water soluble and will be leached out when an oil is spilt thinly over an agitated sea (Nelson-Smith 1972). Petroleum spilled on water floats on the surface to form slicks especially if the oil is rich in polar components, in which case it spreads until a small quantity may cover an area of several square metres. The floating oil is moved by the wind and by ocean currents and drifts, which determine the destination and to some extent the persistence of the oil on the water. Some of the material will find its way onto beaches where the oily material will be subjected to the weathering action of sunlight and water, the final fate ashore depending upon the properties of the beach (Bayfield and Conroy 1994; Fusey and Oudot 1984). Some of the oil may be readily absorbed by clay, silt and other particulate matter in the sea which results in the oil eventually sinking to the bottom (Floodgate 1972). The persistence of oil on the seabed depends on whether it is buried by sedimentation before being broken down by auto-oxidation or microbial activity.

Little biodegradation takes place after hydrocarbons are buried in sediments due to the presence of anaerobic conditions (Zobell 1964).

The rate of evaporation of oil on aqueous surfaces depends on the weather, low temperatures tending to depress evaporation and high wind speeds increasing it. Depending on the original composition of the oil, up to 50 % can be lost in a few hours, but the amount of oil lost decreases exponentially with time (Floodgate 1984). Although the small aliphatic and aromatic hydrocarbons may dissolve, solubility decreases with increased molecular weight and most petroleum hydrocarbons are not very soluble in water (Floodgate 1972). Alkanes and aromatic hydrocarbons can be subject to atmospheric oxidation or photo-oxidation by sunlight and many of the oxidation products will be water-soluble (Nelson-Smith 1972). The resulting mixture is different in composition from the original, being richer in metals such as nickel and vanadium and having a higher content of waxes which are esters of long chain carboxylic acids with long-chain alcohols (Ryles et al. 1980) and asphaltenes (Floodgate 1984).

As a result of wind and wave action, a process collectively known as weathering, oil-in-water and water-in-oil 'mousse' emulsions form. 'Mousse' refers to large accumulations of emulsified oil in 'globs' up to 1 m in diameter (Nelson-Smith 1972; Davis and Gibbs 1975; Atlas 1981; Cooney 1984). The slicks in the form of oil-in-water emulsions increase the surface area of the oil available for microbial attack (Leahy and Colwell 1990). However, as water-in-oil 'mousse' emulsions form, there is a decrease in surface area to volume ratio resulting in limited oil/water interfacial area. Davis and Gibbs (1975) found that large accumulations of 'mousse' weathered extremely slowly with no net loss of hydrocarbons over 2 years. If weathering continues extensively then the hydrocarbons can be seen to occur as tar balls, which are very resistant to microbial attack (Atlas 1981).

From the evidence presented it is clear that the persistence of oil spilt in the environment depends on the quantity and quality of the oil and on the properties of the affected area. Under one set of

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environmental conditions the hydrocarbons may persist whereas under another set of conditions the same hydrocarbons can be completely degraded within a few days.

1.3. Microbial degradation of petroleum pollutants.

Photo-oxidation and evaporation play a small part in the degradation of oil, but it is accomplished mainly by microorganisms (Zobell 1964: Gunkel and Gassman 1980) which have been described as the principle agents of self purification (Fukushima *et al.* 1978). Many species of marine and soil bacteria are endowed with the ability to oxidize petroleum hydrocarbons and their derivatives (Zobell 1964). The ability of microorganisms to degrade hydrocarbons is not restricted to any particular group, but bacteria and yeasts appear to be the most prevalent hydrocarbon degraders in marine environments (Cooney and Summers 1976). The most widespread genera of hydrocarbon-utilizing microorganisms in aquatic environments are *Pseudomonas. Achromobacter, Arthrobacter, Micrococcus, Norcardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium, Candida, Rhodococcus, Rhodotorula, Aspergillus, Mucor. Fusarium* and *Penicillium* (Boulton and Ratledge 1984; Sorkhoh *et al.* 1990). The range of abilities to degrade hydrocarbons is considerable, and includes straight and branched chain alkanes through to aromatic hydrocarbons. However no single organism has been recorded as capable of utilizing all degradable hydrocarbons as growth substances (Cerniglia 1984; Singer and Finnerty 1984)

Most of the petroleum which enters the sea eventually undergoes degradation but the rate at which it occurs is slow and does not provide any great relief when accidents occur, or to the problem of chronic pollution (Atlas and Bartha 1972b). There have been many reasons put forward as to why degradation of oil in the natural environment occurs at such a slow rate, some of which were discussed in section 1.2. The rate of microbial petroleum biodegradation in the environment is also influenced by the kinds and abundance of microorganisms present and the physiological capabilities of those populations. A population's previous history of exposure to hydrocarbons will determine how fast hydrocarbon inputs will be degraded (Leahy and Colwell 1990; Atlas 1991a). In

unpolluted environments hydrocarbon degraders generally constitute less than 1% of the microbial community (Atlas 1981), but previous exposure of an ecosystem to oil pollution results in hydrocarbon degraders representing approximately 1-10% of microorganisms.

Biodegradation of hydrocarbons by bacteria involves the oxidation of the substrate by oxygenases for which molecular oxygen is required (section 1.4), (Floodgate 1972; Singer and Finnerty 1984), thus insufficiency of dissolved oxygen can result in slow rates of hydrocarbon degradation. The rate of bacterial oxidation is also influenced by temperature due to its effect on the physical nature and chemical composition of the oil (Floodgate 1972; Gibbs *et al.* 1975; Atlas 1991a).

Lastly, the lack of essential nutrients in the sea affects the rate and extent of degradation of petroleum in the marine environment (Atlas and Bartha 1972a; Bartha and Atlas 1973; Gibbs *et al.* 1975; Dibble and Bartha 1976; Atlas 1991a). Several investigators have reported that concentrations of available nitrogen and phosphorous are severely limiting to microbial hydrocarbon degradation (Atlas 1981; Floodgate 1984; Leahy and Colwell 1990). The carbon compounds present as a result of an oil spill act as a new source of food and once the indigenous species of bacteria become acclimated they start to degrade the oil and the population expands. This depletes existing supplies of nutrients, especially nitrates and phosphates, and results in unfavourable levels of nitrates and phosphates in relation to carbon (Atlas and Bartha 1972a).

1.4. Alkane assimilation and degradation.

Studies of the biochemistry of hydrocarbon degradation tend to focus on alkanes with a chain length of 10-18. Hydrocarbons of chain length C_6 to C_8 tend to be cytotoxic to most cells and alkanes of C_{18} chain length and longer are solid at room temperature. Hydrocarbons are insoluble in water, and this poses a problem in their utilization by microorganisms, due to contact having to be made between two immiscible surfaces, the hydrocarbon droplet and the microbial cell (Ratledge 1987). The substrate must be transported through the aqueous phase to achieve cell substrate contact. Three mechanisms of hydrocarbon transport/uptake into microbial cells are

considered:

1) Hydrocarbon dissolved in the aqueous phase may be taken up directly, but this is naturally a low amount and is of negligible importance (Boulton and Ratledge 1984).

2) The direct contact of the cell with large hydrocarbon droplets (figure 1.3). The cells passively adsorb to the surface of the hydrocarbon drops without involvement of an enzymic reaction (Rosenburg et al. 1980); the affinity between the cell surface and hydrocarbon substrate being based on a lipophilic layer located at the cell surface (Kaeppelli and Fiechter 1976). Substrate uptake may take place by diffusion or active transport at the point of contact (Miura et al. 1977; Boulton and Ratledge 1984). The problem arises of how contact is achieved between a polar, hydrophilic cell surface and a non-polar hydrophobic substrate, and contact may therefore involve modification of the cell surface. When contact is used as the mechanism for uptake, the surface area of the substrate available for cell attachment is the limiting factor, and this limitation can be overcome by the production of biosurfactants by the microbes (Reddy et al. 1983; Gutnick and Minas 1987; Hommel 1994; Volkering et al. 1995). Surfactants cause greater dispersion of the water-insoluble hydrocarbons in the aqueous phase due to their amphipathic properties (Singh and Desai 1986) which therefore increases the surface area available for cell attachment (Goswami and Singh 1991).

3) The uptake of solubilized or pseudosolubilized hydrocarbon droplets much smaller than the cells (Kappelli and Finnerty 1980; Boulton and Ratledge 1984). This involves the action of a specific hydrocarbon solubilizer produced by the microbes. The solubilizer is substrate specific and therefore has the capacity to react rapidly with the substrate particles. The significance of the solubilizing factor in hydrocarbon transport lies in its capacity to produce readily assimilable submicron droplets rapidly and extensively (Reddy et al. 1982). Goswami and Singh (1991) studied two species of *Pseudomonas* growing on hexadecane. One species was dependent on mechanical agitation of the culture to produce hydrocarbon droplets, therefore increasing the surface area available for cell attachment. This dependency occurs due to a lack of production of

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hydrocarbon transport mediators, such as extracellular biosurfactants and solubilizers in the culture media, thus supporting the theory of uptake of hydrocarbon by direct contact. The other species produced an extracellular glycolipoprotein as a solubilizing factor and showed strong hydrocarbon emulsification leading to the conclusion that substrate uptake occurred from the pseudosolubilzed hydrocarbon.

Figure 1.3. Direct contact of cells (a) with large hydrocarbon droplets (b), taken by Normarski differential contrast microscopy by Dr G. Bradley, University of Plymouth, Plymouth, Devon.

The mechanisms discussed so far take the hydrocarbon substrate as far as the outer surface of the microorganism, after which the hydrocarbon has to be transported to the site of initial enzyme attack. The exact route of uptake of hydrocarbons into a cell still appears to be unresolved. Kennedy *et al.* (1975) stated that the uptake of hydrocarbons was an active process because hydrocarbon was taken up against a concentration gradient. Work carried out by Lindley and Heydeman (1986) also supports this theory and suggested that *Cladosporium resinae* adsorbed alkanes onto the outer surface of the cell passively; after adsorption onto the surface of the cell the energy-requiring transfer of unmodified alkane into the cytosol occurred. Energy may have been required for either active transport or for pinocytosis.

Witholt *et al.* (1990) suggested that uptake of hydrocarbon in *P. oleovorans* involved the modification of the outer-membrane lipopolysaccharide (LPS). The interaction of a hydrocarbon droplet with the bacterial cell may result in the LPS membrane moving away from the cell envelope and surrounding the droplet, thus acting as an emulsifying agent with the result that the hydrocarbon becomes water soluble. Alternatively outer membrane vesicles may be excreted with an emulsifier, which again results in the transfer of amphipathic macromolecules e.g LPS, to the alkane droplet allowing it to become water soluble. This mechanism appears to be a form of pinocytosis whereby the droplet is engulfed by the LPS, the droplet may then be drawn into the cell through pores created due to the modifications which have taken place at the cell surface with the formation of the LPS vesicles, or alternatively drawn into the cell by passive diffusion (Ratledge 1992). Ratledge (1987), hypothesised that the hydrocarbon becomes associated with surfactant produced by the cell which then enter the lipophilic outer part of the cell envelope together. The hydrocarbon can then dissociate from the complex in an amphipathic receptor or channel and partition into the membrane.

The growth of microbes on hydrocarbons can result in distinct alterations in cell morphology. These alterations can include the visible inclusions of hydrocarbon (Turner *et al.* 1980) possibly

because the uptake of hydrocarbons is faster than their oxidation (Watkinson 1980) and the synthesis of specific organelles and structures required for the metabolism of hydrocarbon substrates. Rosenberg *et al.* (1982) described the presence of numerous thin fimbrae (approximately 3.5 μm in diameter) on the cell surface of strains of *A cinetobacter* able to degrade hydrocarbons. A strong correlation was established between the distribution of fimbrae among various strains of microbes and the cells ability to adhere to hydrocarbons.

Kennedy *et al.* (1975) described intracytoplasmic inclusions in hydrocarbon grown *A cinetobacter sp.* and gas chromatography and x-ray diffraction showed these inclusions were unchanged hydrocarbon. Scott *et al.* (1976) described ultrastructural modifications and the sequestering of hydrocarbons resulting from growth on hydrocarbon substrates in a variety of bacteria and the yeast *Candida.* It has further been shown by Scott and Finnerty (1976) that a unique membrane exists in hexadecane-grown cells of *A cinetobacter* which surrounds the intracytoplasmic inclusions. The membrane contained significantly greater amounts of phospholipid and hexadecane than did the cytoplasmic or outer membranes derived from non-hexadecane-grown cells.

Once uptake has occurred the primary attack by bacteria on n-alkanes occurs at the terminal methyl groups, transforming the hydrocarbon into a primary alcohol. The metabolic attack is mediated by mono-oxygenases, as only one atom of molecular oxygen is assimilated into the product, the other atom ending up as water. The reaction uses a number of co-factors to complete the sequence.

$$R\text{-}CH_3 + O_2 + XH_2 \text{ ------- } R\text{-}CH_2\text{-}OH + H_2O + X$$
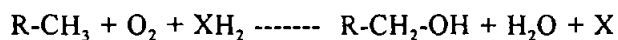
XH_2 and X are the reduced and oxidized forms of a redox co-factor. Both the rubredoxin and cytochrome P-450 systems mediate such oxidations resulting in the same primary oxidation. When mono-oxygenase is linked to rubredoxin, the rubredoxin is in turn linked to a rubredoxin reductase which is a flavo-enzyme. There are three separate proteins involved, the mono-oxygenase, rubredoxin and rubredoxin reductase. These catalyse the following two partial reactions.

i) Rubredoxin (Rb)-dependent mono-oxygenase.

$$R\text{-}CH_3 + Rb\text{-}Fe^{2+} + O_2 \longrightarrow R\text{-}CH_2OH + RbFe^{3+} + H_2O$$

ii) NADH-dependent rubredoxin oxido-reductase (RbR).

$$RbFe^{3+} + RbR\text{-}FAD + NADH \longrightarrow RbFe^{2+} + RbR\text{-}FADH_2 + NAD^+$$
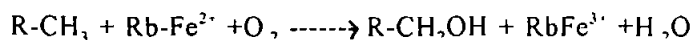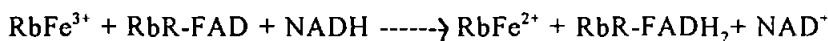
The cytochrome P-450 system is found in both eukaryotic and prokaryotic cells. In the above system cytochrome P-450 takes the place of rubredoxin and the reduction of the oxidized cytochrome P-450 is in turn linked to oxido-reductase which is linked to NADH or NADPH. Both systems are induced when cells are grown on alkanes and may be the rate limiting step explaining why droplets of unchanged alkane can be seen within cells (Ratledge 1978; Singer and Finnerty 1984; Ratledge 1992).

The primary alcohols are subsequently oxidized to aldehydes and then fatty acids. The oxidation of the primary alcohol to a fatty aldehyde occurs in bacteria by an NAD(P) - dependent alcohol dehydrogenase or a fatty alcohol oxidase.

$$R\text{-}CH_2OH + X \longrightarrow R\text{-}CHO + XH_2$$
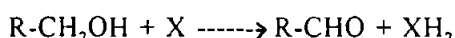
where X, in bacteria is NAD(P).

The fatty aldehyde is then oxidized to the corresponding fatty acid.

$$R\text{-}CHO + NAD(P)^+ \longrightarrow R\text{-}COOH + NAD(P)H$$
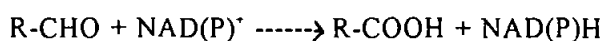
catalysed by a long chain aldehyde dehydrogenase (Ratledge 1992). The fatty acids are subsequently reduced by C_2 units by *beta*-oxidation until the C_4 level is reached with the final substrate being acetoacetyl - CoA ($CH_3CO.CH_2CO\text{-}S\text{-}CoA$) which is cleaved into two acetyl-CoA units (Ratledge 1992), which are fed into the tricarboxylic acid (TCA) cycle (figure 1.4).

Controversy surrounds the anaerobic oxidation of alkanes. It is often assumed that in the absence of molecular oxygen the hydrocarbon loses hydrogen to form a double bond between adjacent carbon atoms. Oxygen is introduced into the molecule by hydration across the double bond, while nitrate or sulphate ions act as terminal hydrogen acceptors (Floodgate 1972). Anaerobic degradation of hydrocarbons occurs but the rate at which it transpires in the natural environment is negligible (Grbic-Gallic and Vogel 1987; Lee and Levy 1989).

Alkane

⬇

Rubredoxin dependent mono-oxygenase
NADH-dependent Rubredoxin oxido-reductase

Alcohol

⬇

Fatty alcohol Oxidase or NAD(P) - dependent alcohol dehydrogenase

Fatty Aldehyde

⬇

Fatty Aldehyde Dehydrogenase

Fatty Acid

⬇

beta-oxidation

Two units of acetyl-CoA

⬇

TCA cycle
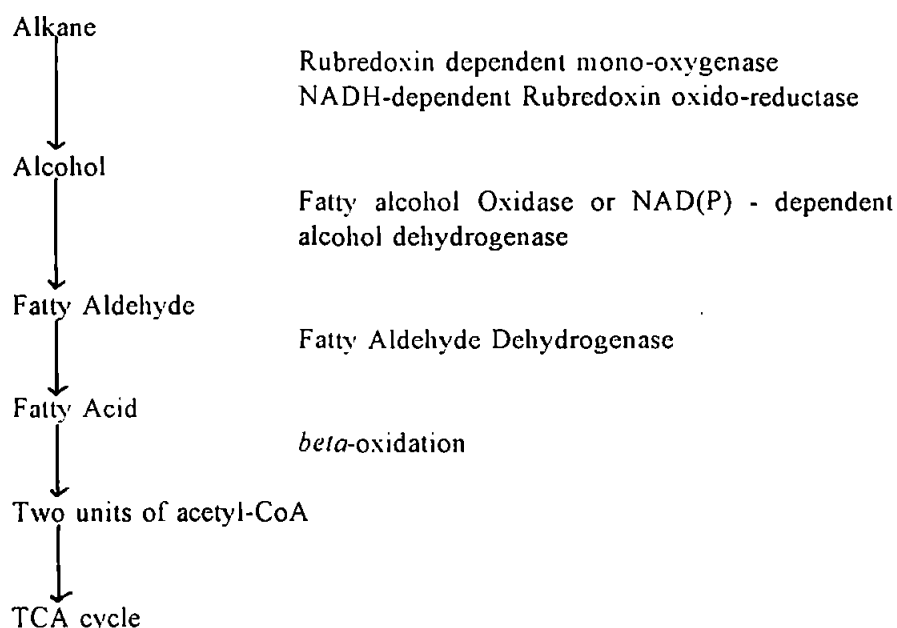
Figure 1.4. Summary diagram of alkane degradation.

1.5. Bioremediation of aqueous oil spills.

Oil spills are an emotive topic due to their visibility and impact. Oil is biodegradable but its degradation is limited by several factors in the natural environment as discussed in sections 1.2 and 1.3. Biotechnology can offer a way of reducing contamination by optimising biotic and environmental conditions so that biodegradation occurs as rapidly and completely as possible. Such biotechnology has been termed as "bioremediation" (Morgan 1991). Bioremediation can involve, for example the use of additives such as fertilizers or detergents to accelerate the natural

biodegradation process (Hoff 1992). Such bioremediation reduces ecological damage caused by an oil spill by accelerating the rates of petroleum biodegradation that form non-toxic end products, such as carbon dioxide and water (mineralization). If bioremediation is effective the persistence of biodegradable hydrocarbons is reduced so the compounds that may have persisted in the environment for decades can be biodegraded in months to years (Atlas 1991b). Three main types of bioremediation technologies are currently being used or developed for treatment of marine oil spills; nutrient addition, microbial seeding and the use of dispersion agents (Hoff 1992).

1.5.1. *Nutrient addition.*

Results from a study carried out by Atlas and Bartha (1972a) identified the low concentrations of phosphates and nitrates in natural seawater as the principal limiting factor of petroleum degradation as discussed in section 1.3. The simplest approach for enhancing oil biodegradation is the addition of nitrogen and phosphorous to improve the ratio of carbon:nitrogen and carbon:phosphorous (Olivieri *et al.* 1978; Atlas 1991a, 1991b). Nutrient addition can include a variety of application techniques as well as numerous commercial products (usually fertilizers). Products can be grouped into three basic categories: soluble inorganic nutrients, oleophilic formulations and slow release formulations (Hoff 1992).

Numerous laboratory and field studies have shown that attempts to overcome the nutrient limitations described generally lead to successful enhancement of oil biodegradation (Atlas and Bartha 1972a; Olivieri *et al* 1978; Atlas 1981; Floodgate 1984; Fox 1989; Atlas and Bartha 1992; Marty and Martin 1993). The largest study carried out on fertilizer application was on the contaminated shoreline of the Prince William Sound after the Exxon Valdez accident in 1989 where three typical fertilizer application strategies were tested (Pritchard and Costa 1991; Merski and Griffin 1993); an oleophilic formulation, a soluble inorganic nutrient addition and a slow-release formulation. Of these the most successful was the oleophilic fertilizer, Inipol EAP 22, an oil-in-water microemulsion. However, many questions still remain; for example, do these products appear to work better because the microbes are degrading the carbon in the product instead of the spilled oil?

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Lee and Levy (1989) concluded that the addition of an oleophilic nutrient source was ineffective because the microbes were preferentially 'eating' the organic components of the product. It is also possible that Inipol appears to be effective because it works initially as a chemical surfactant rather than a bioremediation agent. The visual effects seen after application of oleophilic products in the field may result from their surfactant properties in contrast to enhanced biodegradation (Hoff 1992).

The potential advantage of using nutrient addition as a form of bioremediation must be balanced against possible detrimental environmental effects. Some fertilizer products whose initial use is in a terrestrial setting may contain trace elements as micro-nutrients (e.g iron or mercury) which could have more significant toxicological effects than beneficial effects. Also, physical disturbance from the application process and from monitoring will have some impacts on the shoreline (Hoff 1992). The addition of nutrients as a means of bioremediation in marine environments is still in the experimental stage and any trials should include full monitoring programs to determine whether the effects are positive or detrimental (Pritchard 1991; Pritchard and Costa 1991).

1.5.2. *Microbial seeding.*

Adding microbes to a contaminated area, is known as 'seeding', and is carried out with the aim of enhancing biodegradation of a polluted area by strains of bacteria known to degrade hydrocarbons (Hoff 1992). If the culture is added in an active state rapidly following the spillage, the seeding process could reduce the initial lag period which is often seen with indigenous bacteria before acclimation (Atlas 1991a). Examples of microbial applications include; 1) the Apex Barges collision, Texas 1990, where approximately 2,650,000 l of partially refined oil was spilt into Galveston bay. A pre-mixed solution containing a microbial product and nutrient mix was applied, however no significant differences were found between treated and untreated sites. 2) a well blowout offshore of Seal Beach, California in 1990 released 1500 l of crude oil. A microbial product plus a fertilizer was added one week after the blowout, followed by an application of only fertilizer two weeks later. Samples taken from the site again showed no differences between treated and non-treated sites (Hoff 1992). Therefore, it appears there could be a number of factors that

may restrict the potential of an inoculum: Indigenous microbes are fully adapted to the environmental conditions, thus 'foreign' organisms must compete with indigenous organisms to survive in their new environment. The inoculum must remain in contact with the oil and avoid dilution which is very difficult in an open sea environment (Hoff 1992; Morgan 1991). Most microbial products either contain or the suppliers recommend the use of nutrient additions, which are needed to sustain the growth of the seed cultures, so concerns about potential toxicity of trace elements also apply here as described in section 1.5 (Atlas 1991a; Hoff 1992).

Spills can also be seeded with genetically engineered microorganisms. The genetic information for some enzymes involved in alkane and simple aromatic hydrocarbon transformation occurs on plasmids (Chakrabarty 1973; Chakrabarty *et al.* 1973; Chakrabarty 1985). Molecular contributions could include; the selection and recovery of novel microbes capable of more versatile degradation, followed by improvement of the biochemical performance and versatility of the microbial strains. The development of microbial strains and technology for environmental monitoring and control could be carried out with the selection and development of microbial strains with increased robustness and environmental fitness for application in suboptimal environments (Sayler 1991). Problems arise however with the controversy surrounding the release of such genetically engineered microbes into the environment (Sussman *et al.* 1988).

1.5.3. *Dispersion agents.*

High populations of oil degraders after a spill do not necessarily mean that rapid hydrocarbon degradation will occur, due to the problem of accessibility of hydrocarbons to the bacteria. Most hydrocarbon-degrading bacteria produce surfactants which naturally disperse oil (Section 1.4). Relatively low concentrations of surfactants added to the environment by man can enhance the aqueous dispersion of hydrocarbons by reducing the surface tension of a liquid medium (Zhang and Miller 1992). Surfactants can be synthetic (detergents) or natural, produced by plants, animals and many microorganisms (Zajic and Panchal 1976). Chemical dispersants have been widely used in marine oil spills but are believed to cause pollution problems themselves, as highlighted by the

Torrey Canyon spill, because of their toxicity and persistence in nature (Canevari 1969; Cook and Knap 1983; McIntosh 1989). The use of biosurfactants in the cleaning of oil-polluted areas such as the Prince William Bay after the Exxon Valdez spill in 1989, is presently not widely accepted and the short term effects of this type of treatment do not outweigh the additional toxic effects, meaning bioremediation by the use of dispersion agents is not widely carried out (Zajic and Panchal 1976; Bertrand *et al.* 1983; Foght *et al.* 1989; Atlas 1991a, 1991b; Fiechter 1992).

Harvey *et al.* (1990) demonstrated the production of a non-toxic and biodegradable, glycolipid emulsifier by a strain of *Pseudomonas aeruginosa* that could effectively reduce the surface tension of an oil-water interface and act as a dispersant of oil in water. Zhang and Miller (1992) demonstrated that mineralization rates could be increased significantly by rhamnolipid enhanced octadecane dispersion. This makes the use of biosurfactants look attractive because they are natural products and are therefore biodegradable, however work is still required on the fate and effect in the environment of the substantial quantities of rhamnolipid which would be required to disperse a spill.

1.6. Whole cell immobilization and its application to bioremediation.

"Immobilized cells can be defined as cells that are entrapped within or associated with an insoluble matrix"

(O'Reilly and Crawford 1989b).

Immobilized bacteria technology uses selected chemical-degrading bacteria enclosed on a support matrix (Heitkamp *et al.* 1990). In the immobilized state the cells are in an environment with optimal conditions giving protection against unfavourable circumstances (Fukui and Tanaka 1982; Rosevear *et al.* 1987; Koge *et al.* 1992). The reasoning behind immobilizing a biologically active system is to concentrate the system in a small area, allowing the enhancement of activity and also allowing the recovery and recycling of the system (Veliky 1984). Under many conditions immobilized cells have advantages over free cells or immobilized enzymes, allowing a high cell density to be maintained, and catalytic stability to be increased. For example Chibata *et al.* (1974)

investigated the immobilization of microbial cells for continuous production of L-aspartic acid due to immobilized aspartase being too unstable for industrial application. This study showed that immobilized cells were advantageous in comparison with immobilized aspartase for the industrial production of L-aspartic acid from ammonium fumarate. Lastly, in some systems increased tolerance of high concentrations of toxic compounds occurs with immobilization (Klein and Wagner 1978; O'Reilly and Crawford 1989b; Klein and Ziehr 1990).

The area of immobilization in the past has been dominated by the use of 'hydrogels' such as calcium alginate, which may be due to their ease of preparation and use (Coughlan and Kierstan 1988; Pras et al. 1989). However in the past decade a large number of polymers have been coming into use. Many forms of material can be applied to immobilisation, but in general the choice is between particles, sheets, fibres or membrane systems (Rosevear et al. 1987) and mainly organic materials of synthetic or biological origin (Klein and Wagner 1978). The choice of the support matrix is an important decision which is influenced by; 1) the binding capacity of the cells, 2) the accessibility of the substrate to the cells, 3) the rigidity of the support material. The support should have high mechanical strength and its regeneration should be possible. It should also be thermally and chemically stable and offer a high contact area with the surrounding medium (Adams et al. 1988). The polymers in use can be used to confer added strength or loading capacity in conjunction with another support material or used alone as a cell support. These polymers can include beads, cubes (confined to those polymers which require cutting to a suitable size and shape), membranes, fibres, porous glass and ceramics (Hackel et al. 1975; Adams et al. 1988; Omar and Rehm 1988; Salter et al. 1990; Salter and Kell 1991; Stormo and Crawford 1992) and celite particles (Caunt and Chase 1988).

There are several different methods of whole cell immobilization which can be classified as follows:

1.6.1. Adsorption.

Adsorption involves binding of the cell (e.g by Van der Waals or ionic binding) to a

support and can occur on any material of natural or synthetic origin which is biologically inert and non-toxic. Advantages include simplicity of biocatalyst preparation and the avoidance of using toxic chemicals or non-physiological conditions (Klein and Wagner 1978) Where adsorbtion does occur, immobilization simply requires that the cells are mixed with the support and uses the natural tendency of microbes to adhere to solid surfaces (Scott 1987). However this technique is one of the most sensitive to environmental changes and unless the interaction is fairly specific, desorbtion due to competitive binding can often equally be found, causing the release of surface-adsorbed cells (Rosevear 1984) Combining this technique with covalent bonding or crosslinking increases the stability of the system, crosslinking agents however can lower the activity or viability of the cells (Veliky 1984).

1.6.2. *Crosslinking.*

This includes both chemical and physical crosslinking. Chemical crosslinking is the covalent attachment of cells to each other via polyfunctional reagents like di-aldehydes or di-amines, but the toxicity of these chemicals puts limits onto the general applicability of these methods (Rosevear 1984; Veliky 1984). 'Physical' crosslinking includes aggregation or flocculation and pelletization which occurs where cells are in suspension at high concentrations for extended periods. These methods benefit from adherence forces of some microbial cultures with the result that particles can be very stable within a columnar bioreactor, allowing high biomass loadings with concomitant high reaction rates (Scott 1987; Coughlan and Kierstan 1988).

1.6.3. *Entrapment / Encapsulation.*

The entrapment method (Chibata *et al.* 1974; Scott 1987) is based on the confinement of cells within a lattice of a matrix or enclosing them in semi-permeable membranes. Methods of whole cell entrapment in polymeric porous networks are still of primary importance and these methods can be arranged in four categories:

1) Precipitation - non-specific secondary binding

2) Ion exchange gelation - Ionic bonding

3) Polycondensation - heteropolar covalent bonding

4) Polymerization - homeopolar covalent bonding

(Klein and Wagner 1978; Mattiasson 1983; Tramper 1985)

Covalent binding to supports can also be considered, but it is not very appropriate for the immobilization of cells as renewal of the cell wall near the site of attachment can permit release of live cells which had previously been bound (Rosevear 1984).

However, categorization of whole cell immobilization methods under one of these techniques is perhaps an oversimplification and in many cases more than one method is involved. None of these methods of immobilization has advantages for all whole cell immobilizations and therefore, for a specific process the choice of methods should be made on the basis of need, available materials and knowledge (Klein and Wagner 1978; Rosevear 1984; Veliky 1984; Tramper 1985; Klein and Ziehr 1990).

Immobilized biocatalysts catalyze biochemical reactions under more stabilized conditions than their free counterparts and processes often require the immobilization of not only one enzyme system for a simple reaction e.g hydrolysis, but also of multi-enzyme systems that mediate more complicated reactions for the synthesis and conversion of numerous compounds. Microbial cells and cellular organelles contain metabolic systems that catalyze such complicated reactions, therefore, immobilization of microbial cells and organelles in turn permits immobilization of multi-step and co-operative enzyme systems (Fukui and Tanaka 1982). Cells in theory are easy to immobilize, however their sensitivity to stress and the presence of many unwanted reactions and contaminants can make the immobilization of live cells disadvantageous unless a large part of a biosynthetic pathway is required (Rosevear 1984).

The objective of immobilizing whole cells or the immobilization of any catalytic system is to obtain an activity and stability which is high enough to give an economic or functional advantage over the free catalytic system, thus when talking about an increased catalytic activity the comparison between the activity of immobilized cells and the activity of the same number of free cells must be made. However, a serious constraint to increased productivity of immobilized aerobic cells which may have to be taken into consideration is the limitation on activity caused by reduced mass transfer of oxygen into the interior of the immobilization matrix, with the specific oxygen uptake rate of immobilized cells decreasing with increasing cell content of the immobilization substrata (Chang and Moo-Young 1988; Stormo and Crawford 1992). Also, inadequate transfer of nutrients through the outer layer of metabolising cells can seriously limit viability, as can the poor mass transfer of inhibitory products away from the cells which can result in changes in the microenvironment and therefore reduce viability. Other limitations may arise from a number of causes; Loss of catalytic activity during immobilization due to reduced accessibility of the substrate to the biocatalyst due to slow diffusion from the bulk phase to the active site, or total exclusion of large substrate molecules by a porous barrier. Deactivation due to changes in pH and temperature in the microenvironment and uncontrolled growth of immobilized cells can result in the sloughing off of clumps of cells into the fluid. Biofilms will often detach as the cells in the lower layers die and degrade. These problems may limit the amount of usable catalytic activity obtained from the immobilized cell under practical conditions (Rosevear 1984).

Immobilization is still an empirical technology, relying on systematic optimisation rather than theories. Cells can be unpredictable with their response to the immobilization process and the new microenvironment may change their morphology, primary metabolism and secondary metabolism, so each problem must be tackled individually with the desired effect in mind (Rosevear et al. 1987).

Whole cell immobilization is commonly used in fermentation processes in the food and pharmaceutical industries and for beverage production, biomedical products, biosensors, microbial production of chemicals and environmental waste facilities (Chibata et al. 1974; Takahashi et al.

1981; Fynn and Whitmore 1982; Veliky 1984; Rhodes *et al.* 1987; Scott 1987; Shi *et al.* 1987; Sueki *et al.* 1991). Interest has grown in immobilized bacteria technology for the biological treatment of chemical waste, with the immobilization of large numbers of chemical-degrading bacteria in an optimal environment giving rise to high chemical degradation rates. Thus, the immobilization of microbes could be a suitable method of localizing bacteria in contaminated areas with complementary increases in degradation (Omar and Rehm 1988; O'Reilly and Crawford 1989a, 1989b; Heitkamp *et al.* 1990; Stormo and Crawford 1992; Wiesel *et al.* 1993).

1.7. Study aims

The aims of this study were:

1) To establish whether enhanced biodegradation of oil occurs with immobilization and nutrient supplementation of *Pseudomonas fluorescens* in comparison to a free system of the same bacteria.

2) To compare a variety of commercially available support matrices; to determine their applicability as bioremediation agents and decide if the choice of biosupports affects the success of the application.

3) To determine the source of genetic information for alkane degradation in *Pseudomonas fluorescens*.

4) To scale-up and develop an optimized immobilization system based on the previous investigations.

Chapter 2

Enhanced Degradation of Petrol (Slovene Diesel) in an Aqueous System by Immobilized *Pseudomonas fluorescens*

2.1. Introduction

Diesel is a refined petroleum product which may be derived from high-boiling kerosene or low-boiling gas oils, which have been separated in a refinery by distillation of crude oil. Diesel-oils may have a high aromatic content and can thus be more toxic than some crude oils, causing lasting pollution problems (Nelson-Smith 1972).

The problem of accessibility of the oil to the bacteria due to limited oil/water interfacial area, is often caused by the formation of water-in-oil emulsions (section 1.2); this makes contact between the hydrophobic oil and the polar hydrophilic cell surface more difficult. Interest is developing in immobilized bacteria technology as a method to treat oil pollution in water to overcome this problem. Section 1.4 describes the three mechanisms of hydrocarbon uptake to microbial cells and immobilized bacteria technology is used in this study to enhance the second mechanism of uptake described (the direct contact of the cell with large hydrocarbon droplets). The biosupport is used as a bioremediation agent to act as a possible intermediary between the oil and the cell surface, increasing contact between the two and also increasing the surface area of the substrate available for cell attachment with the aim of increasing the rate of degradation (Rosevear *et al.* 1987; Heitkamp *et al.* 1990).

The biosupports proposed for use in this study as bioremediation agents were two commercially available materials;

1) Biofix C2, a biosupport, specifically designed as a whole cell support (English China Clay (ECC) International, John Keay House, St Austell, Cornwall, England) made from specially treated slurry of a highly purified kaolinite ($Al_2O_3.2SiO_2.2H_2O$) formed into hollow microspheres by spray drying. After spray drying the particles are calcined, producing a rigid, singly-perforated, hollow

microsphere with non-porous walls of intermeshed mullite needles and amorphous silica. The particles are then etched (to remove the amorphous silica which is generally present in a glassy form) by sodium hydroxide at 80-100°C. After etching, each particle consists of mullite needles joined together into a 3D lattice, or 'bird's nest' which has a large volume of inter-connecting voidage. The resulting walls are porous and enable nutrients and products to diffuse freely. Biofix granules are extremely strong and resistant to chemical attack and can be heated up to 1000°C without loss of surface area or porosity. Due to their inert ceramic nature (table 2.1), the materials are non-toxic (Salter *et al.* 1990).

Table 2.1. Typical properties of Biofix C2.

Product form	hollow porous microspheres
particle size	50 - 70μm
cavity entrance	20μm
mean pore size	90nm
surface area/m^2g^{-1}	9.5
density (g cm^{-3})	0.55
pore volume (cm^3 g^{-1})	
wall	0.45
internal cavity	1.4
% void volume	
wall	22%
internal cavity	70%
pH stability	1 - 14
thermal stability	1000°C
crush strength	>55MPa

(Adams *et al.* 1988; Salter *et al.* 1990)

2) Drizit (Darcy products ltd, Invicta Works, East Malling, Kent, England), a biosorbant, made from 100% polypropylene loose fibres. It is a hydrophobic, non-toxic material which will absorb most liquids which do not mix with water. The product is designed primarily for the recovery of spilt oil but is used in this study not only as an absorbent but also as an immobilization matrix.

A salt tolerant, oil-degrading *Pseudomonas* is used throughout this study. The genus *Pseudomonas* contains gram-negative rods whose long axis is straight or curved and 0.5-1.0 μm by 1.5-5.0 μm in length. The *Pseudomonas* species have cell walls and membranes typical of gram-negative bacteria, differences however can be found in the chemical compositions among the *Pseudomonas* species and between strains of the same species. Generally they are motile bacteria, and most species have one or several unsheathed, polar flagella per cell. They are aerobic using oxygen as the terminal electron acceptor although in some cases nitrate can be used as an alternate electron acceptor (reducing nitrate to N_2O or N_2), allowing growth to occur anaerobically (Krieg and Holt 1984).

The *Pseudomonas* species are catalase positive and usually oxidase positive, although a few species give a weak positive oxidase reaction. Nutritional characterization of the *Pseudomonas* species has been based on their ability to grow in simple minimal media at the expense of a single carbon compound. Only a few species require the addition of organic growth factors. Growth occurs from 4-43°C, and for most species the optimum is around 30°C. All species can grow well at neutral or alkaline pH (7.0-8.5) and most are incapable of growth at pH 6.0 or below. Nutrition in the *Pseudomonas* species is of the chemoorganotrophic type, but some species can also live under autotrophic conditions using CO and/or H_2 as energy sources. All of the *Pseudomonas* species have a functional TCA cycle, oxidizing substrates to CO_2 and most hexoses are degraded by the Entner Doudoroff pathway rather than glycolytically. They are nutritionally a highly versatile genus, sugars and alcohols representing a small fraction of the potentially utilizable range of carbon sources, for example the fluorescent *Pseudomonas* species being able to use approximately 80 different substances as their carbon and energy sources (Krieg and Holt 1984).

The *Pseudomonas* species are common inhabitants of soil, freshwater and marine environments where they play an important role in the mineralization of organic matter. Some are major animal and plant pathogens for example *P. mallei* is a specialised mammalian parasite.

The *Pseudomonas* species have received a lot of increasing attention from bacterial geneticists (with *Pseudomonas aeruginosa* being the most extensively studied) due to their widespread occurrence, their nutritional and biochemical versatility and the simplicity of the conditions required for their cultivation in the laboratory. Plasmids are important components of the genetic make up of the *Pseudomonas* species. Some of them act as fertility factors, some known as R plasmids may confer resistance to various agents, or some may give the cell the capacity for the degradation of unusual carbon sources, thus contributing to the nutritional versatility of many members of the genus (Boronin 1992, Krieg and Holt 1984).

The fluorescent *Pseudomonas* species are the most extensively studied subgroup of this genus, and are primarily characterized by their ability to produce water-soluble, yellow-green fluorescent pigments. The fluorescent *Pseudomonas* species were first described by Flugge in 1886 as cited by Stanier *et al.* (1966), who recognized two biotypes, distinguishable on the character of gelatin liquefaction, now known as *Pseudomonas fluorescens* (liquefying) and *Pseudomonas putida* (non-liquefying). Since then many other species of fluorescent *Pseudomonas* have been named. Among the most well known of the *Pseudomonas* species soluble pigments are pyoverdin (fluorescent) and pyocyanin. The structure of pyocyanin is well known, but pyoverdin has only been partially characterized. The main component of pyoverdin is a quinoline chromophore linked to a cyclic peptide whose composition may be different according to the species and Meyer and Hornsperger (1978) as cited by Krieg and Holt (1984) have demonstrated that pyoverdins play a part in iron transport (Krieg and Holt 1984; Prescott *et al.* 1990; Singleton 1995). In media with low iron content fluorescent pigments are produced in large quantities and the fluorescence varies from white to blue-green upon excitation with ultraviolet or visible radiation. For maximum excitation a wavelength of around 400nm should be used, but the pigments are still able to fluoresce intensely

at lower wavelengths, a property that is useful for a differentiation of pyoverdins from other fluorescent compounds of different chemical nature (Krieg and Holt 1984).

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This study investigates enhanced *in vitro* biodegradation of petrol (Slovene diesel) supplied by the University of Ljubljana, 'Jozef Stefan' Institute, Ljubljana, Slovenia, in a freshwater system after eight days incubation and in a saltwater system after three days incubation with an oil degrading bacterium, *Pseudomonas fluorescens* when immobilized to Biofix and/or Drizit in comparison to a free system of the same bacteria.

2.2. Materials and methods

2.2.1. Bacterial isolates.

A salt-tolerant oil-degrading *Pseudomonas* species isolated from a contaminated metal working fluid by Beech and Gaylarde in 1989, and identified by the API 20 NE strip (La Balme-les Grottes, 38390 Mantalieu-Vercieu-France) as *Pseudomonas fluorescens* was used throughout this study. The strain was identified as *Pseudomonas fluorescens* using an API 20 NE strip and confirmed by the growth of the strain on sorbitol.

P. fluorescens was maintained in liquid nitrogen, subcultured onto nutrient agar (Oxoid) and incubated at 30°C for 24 hours. *P. fluorescens* was subcultured from the plates into nutrient broth (100 ml, Oxoid) and grown for 24 hours in Erlenmeyer flasks (250 ml) at 30°C. These broth cultures were used as the inoculum for each of the experiments.

2.2.2. Media

Tryptone Soy broth (TSB, Oxoid) was used as the nutrient source for the bacteria after immobilization and 0.01 M phosphate buffered saline (PBS), pH 7.3 (Oxoid, Dulbecco 'A' tablets) to wash the cells. The minimal medium, pH 7.2, used for the eight day freshwater experiment contained (g l^{-1}): NH_4Cl, 5.0; NH_4NO_3, 1.0; anhydrous $NaSO_4$, 2.0; K_2HPO_4, 3.0; KH_2PO_4, 1.0 and $MgSO_4.7H_2O$, 0.1 dissolved in order in distilled water. The minimal medium used for the eight day freshwater experiment and artificial seawater (Marine salts, TAP, Filton Bristol) used for the three day saltwater experiment were supplemented with 0.1% v/v petrol (Slovene diesel) as the carbon source for the biodegradation studies. In the three day incubation experiment some flasks containing saltwater were supplemented with 1 mM disodium hydrogen phosphate (BDH) and 10 mM potassium nitrate to allow for nutrient optimization (Section 1.5.1).

2.2.3. *Whole cell immobilisation*

2.2.3.1. Cell loading of Biofix.

A column consisting of a 5 ml syringe bunged with glass wool, connected to a reservoir at the top and a peristaltic pump from the bottom was packed with Biofix C2 suspended in PBS. The buffer was allowed to drain from the column and 100 ml of an overnight culture of *P. fluorescens* was added to the reservoir and allowed to recycle around the system for 2-3 h (1.5 ml min^{-1}). The column was flushed twice with 100 ml of PBS. TSB (100 ml) was added to the reservoir and the Biofix incubated at room temperature for 24 h. The Biofix was flushed twice with 100 ml PBS, 1 g was removed from the column and placed in 5 ml PBS and sonicated (Transsonic, T310, Camlab) for 2 min. The Biofix was removed and the bacteria now in free suspension were centrifuged at 13000 rev min^{-1} (12 x 1.5 ml head, MSE micro-centaur) for 3 minutes and resuspended in PBS. The amount of protein per gram of Biofix was determined by the Folin method (Lowry *et al.* 1951) modified by the addition of 2% (w/v) deoxycholic acid (Maddy and Spooner 1970). A standard curve was prepared using bovine serum albumin (BSA, appendix 3).

2.2.3.2. Cell loading of Drizit.

Drizit (2 g, Darcy Products Ltd) was added to an overnight culture of *P. fluorescens* and placed on an orbital shaker (120 rev min^{-1}) for 24 h at room temperature. After 24 h the Drizit was removed and washed twice in 10 ml PBS, 1 g of Drizit was placed in 5 ml of PBS and sonicated (Transsonic, T310, Camlab) for 2 mins. The Drizit was removed and the bacteria now in free suspension were centrifuged at 13000 rev min^{-1} (12 x 1.5 ml head, MSE Micro-centaur) and resuspended in PBS. The amount of protein per gram of Drizit was estimated by the Folin method as described above.

2.2.4. *Biodegradation of petrol (Slovene diesel) in a freshwater system.*

Bacteria in free suspension were centrifuged at 13000 rev min^{-1} (12 x 1.5 ml head, MSE Micro-centaur) and resuspended in PBS. Resuspended free bacteria and Drizit and Biofix immobilized bacteria were added to give 365 µg protein 100 ml^{-1} minimal media in 250 ml

Erlenmeyer flasks. An uninoculated control flask was prepared to account for abiotic, volatilization, and extraction losses of the diesel. A control with no carbon source was also prepared to ensure that no degradation of the biosupport had occurred. The flasks were incubated at 24°C on an orbital shaker (100 rev min^{-1}) for eight days. All treatments and analyses were performed in duplicate.

2.2.5. *Biodegradation of petrol (Slovene diesel) in a saltwater system.*

Bacteria in free suspension were centrifuged at 13000 rev min^{-1} (12 x 1.5 ml head, MSE Micro-centaur) and resuspended in PBS. Resuspended free bacteria and Drizit immobilized bacteria were added to give 365 μg protein 100 ml^{-1} seawater or 100 ml^{-1} seawater supplemented with nutrients (section 2.2.2) in 250 ml Erlenmeyer flasks. An uninoculated control flask was prepared to account for abiotic, volatilization, and extraction losses of the diesel. The flasks were incubated at 24°C on an orbital shaker (100 rev min^{-1}) for three days. All treatments and analyses were performed in duplicate.

2.2.6. *Petrol (Slovene diesel) extraction from a freshwater system and analysis.*

After eight days incubation, residual petrol (Slovene diesel) from the solid matrices of samples was recovered by extraction with 10 ml of dichloromethane (DCM, BDH) and the aqueous phases of incubated samples was recovered by extraction with 20 ml DCM after which the glassware was rinsed with 10 ml of DCM. The extracts from each sample were combined and concentrated by rotary evaporation (40°C) to approximately 10 ml after which the remaining DCM was removed under a gentle stream of pure nitrogen. Quantitative analysis was performed by gas chromatography (GC) using a Carlo Erba 4160 gas chromatograph with on column injection (0.5 μl samples) and a flame ionization detector (FID). Separation was achieved using a fused silica capillary column (0.32 mm by 30 m, DB-5, J and W Scientific, 91 Blue Ravine Road, Folsom, CA, 95630-4714). The oven temperature was increased from 50°C to 300°C at a rate of 6°C min^{-1} and held at 300°C for 10 min. Hydrogen was used as the carrier gas at a flow rate of 1.5 ml min^{-1} and the FID detector's temperature was kept at 330°C. Quantification of individual hydrocarbons was

made by measurement of the GC peak areas with a Shimadzu CR3-A integrator, and by a comparison of these peaks with the response of a known concentration of the internal standard Squalane. Percentage biodegradation of alkanes C_{12} - C_{18}, pristane and phytane was calculated by comparing the response of incubated samples, after correction for non-biological losses, to that of a petrol (Slovene diesel) sample which had not been exposed to any experimental conditions.

2.2.7. Petrol (Slovene diesel) extraction from a saltwater system and analysis.

Residual petrol (Slovene diesel) from the solid matrices and the aqueous phases of incubated samples were extracted at three days as detailed in section 2.2.6.

2.2.8. Cellular Growth of Pseudomonas fluorescens in free, and Drizit and Biofix immobilized freshwater Systems.

Duplicate samples of the free suspension of bacteria from each flask were centrifuged at 13000 rev min $^{-1}$ (12 x 1.5 ml head, MSE Micro-centaur) and resuspended in PBS. Cellular growth was then estimated using the Folin method, as previously (section 2.2.3.1), over an 8 d period.

2.2.9. Cellular growth of Pseudomonas fluorescens in free, and Drizit immobilized saltwater systems.

The absorbance (590 nm) of duplicate samples from the free suspension of bacteria in each flask was measured on a spectrophotometer (CE 1010, Cecil) over the three day incubation period.

2.2.10. Statistical analysis.

Multifactor analysis of variance (MANOVA) and a multiple range test (least significant difference- LSD) were carried out using Statgraphics, version 6.1, to determine any significant differences between the level of protein or absorbance at 590 nm in each system over time.

2.3. Results

2.3.1. *Growth of Pseudomonas fluorescens in a free and immobilized freshwater system incubated with petrol (Slovene diesel) as the carbon source.*

The study investigated enhanced *in vitro* biodegradation of petrol (Slovene diesel) in an aqueous system by *P. fluorescens* when immobilized to Biofix and Drizit in a freshwater system and Drizit in a saltwater system in comparison to a free system of the same bacteria. After eight days incubation of the bacteria in the three freshwater systems with petrol (Slovene diesel) as the sole carbon source there was a significant difference ($p = 0.01$) between the levels of protein in the free suspension of each system, with protein levels varying significantly ($p = 0.0$) over time within each system and over time between systems ($p = 0.004$). After three days incubation of the bacteria in the saltwater systems with petrol (Slovene diesel) as the sole carbon source there was a significant difference between the absorbance of the aqueous suspension in each system ($p = 0.0002$), a significant difference in the absorbance of the aqueous suspension on each day ($p = 0.000$), and a significant difference in the absorbance of the aqueous suspension in each system over time ($p = 0.0003$).

2.3.2. *Degradation of petrol (Slovene diesel) by free Pseudomonas fluorescens in a freshwater system.*

The average protein concentration of the free system increased from 3.64 µg ml^{-1} at the beginning of the experiment to a final level of 34 µg ml^{-1}. There was an initial lag phase until day six after which growth occurred (figure 2.1). Degradation of the n-alkanes, C_{12} to C_{18} and branched-alkanes, pristane and phytane were quantified using gas chromatography analysis. 52% of C_{12} and 11.6% of C_{13} had been degraded by the free suspension of *P. fluorescens*, no degradation of n-alkanes C_{14}-C_{18} and branched alkanes pristane and phytane occurred (table 2.2). Figure 2.2 shows the average degradation of C_{12}-C_{18} by each system per µg protein after eight days incubation. A significant difference ($p = 0.0$) was found between the level of degradation achieved by the three systems per µg of protein. A follow-up test (LSD) revealed that the free system had degraded significantly less carbon per µg protein than the Drizit and Biofix systems.

2.3.3. *Degradation of petrol (Slovene diesel) by Drizit and Biofix immobilized* Pseudomonas fluorescens. *in a freshwater system.*

Figure 2.1 shows typical growth curves of *P. fluorescens* immobilized to Biofix and Drizit over an 8 day incubation period. Protein (364 µg) was immobilized to the Biofix and Drizit and added to the experimental flasks; therefore at time zero no protein was present in free suspension. In the Biofix inoculated flasks there was an initial lag phase until day 4 after which growth occurred giving a final concentration of 39.5 µg ml^{-1} protein in the free suspension. The amount of protein present on the biocarrier remained constant over the incubation period.
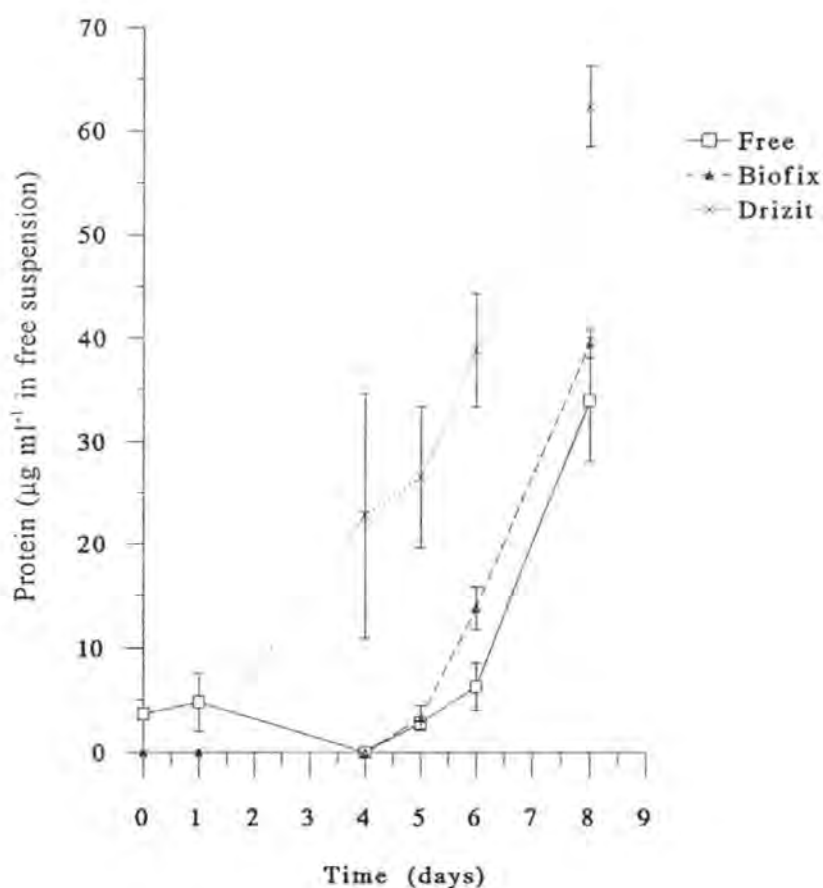
Figure 2.1. Typical growth curves of *Pseudomonas fluorescens* in the aqueous suspensions of free and immobilized freshwater systems, grown on petrol (Slovene diesel) over an eight day period. Bars indicate standard error.

Gas chromatographic analysis showed that immobilization of cells on Biofix enhanced degradation of the n-alkanes, C_{12} - C_{18} in comparison to unexposed petrol (Slovene diesel), and increased biodegradation of the n-alkanes by 38.8 % in comparison to the free system, enhancing degradation of n-alkanes C_{13} - C_{18} in comparison to the degradation rate of the free suspension of *P. fluorescens* (table 2.2). The lag phase was reduced to 1 day with Drizit immobilized cells, after which rapid cell growth occurred giving a final protein concentration of 62.3 μg ml^{-1} in the free suspension. The amount of protein in the biocarrier remained constant throughout the incubation period.

Table 2.2. Percentage carbon degradation of petrol (Slovene diesel) after eight days incubation with *Pseudomonas fluorescens* in free and immobilized freshwater systems.

System	*percentage carbon degraded								
	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	pristane	phytane
Free	52.3	11.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Biofix	14.8	41.6	63.3	40.8	64.5	64.0	46.6	0.0	0.0
Drizit	24.5	52.4	87.7	85.7	100	93.6	89.0	0.0	0.0

*figures are mean values of two samples and are adjusted to allow for photodegradation and volatilization using the uninoculated control.

Gas chromatographic analysis showed that immobilization on Drizit enhanced degradation of n-alkanes, C_{12} - C_{18} in comparison to unexposed petrol (Slovene diesel), increased biodegradation of the n-alkanes by 67 % in comparison to the free system and enhanced degradation of n-alkanes C_{13} - C_{18} in comparison to the free and Biofix degradation rates for these alkanes (table 2.2). However, although a significant difference (p = 0.0) was found between the level of degradation per μg of protein in the three systems, a follow-up test (LSD) and figure 2.2 clearly indicates there was no significant difference in the level of average percentage degradation per μg protein achieved between the Drizit and Biofix systems.
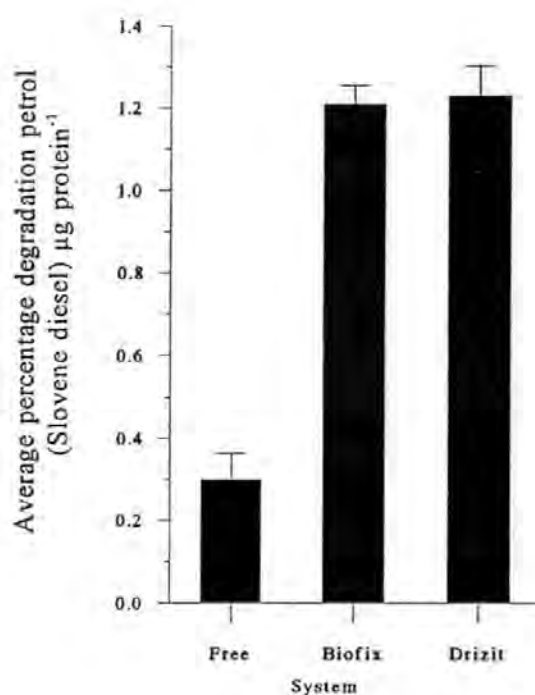
Figure 2.2. Average percentage degradation of C_{12} - C_{18} in petrol (Slovene diesel) μg protein^{-1} of *Pseudomonas fluorescens* after eight days incubation in a freshwater system. Bars indicate standard error.

Figure 2.3 shows the gas chromatographic pattern obtained for unexposed petrol (Slovene diesel) and patterns obtained after degradation by free and immobilized bacteria. The tracings show a decrease in hydrocarbons C_{12} - C_{18} when incubated with cells immobilized on Drizit, in comparison to Biofix immobilized cells, free cells and untreated petrol (Slovene diesel). Volatility accounted for 59% of C_{12} and 35% of C_{13} disappearance. The n-alkanes C_{14} to C_{18} were not affected by volatility but were subject to extensive biodegradation by immobilized bacteria. Branched chain alkanes, pristane and phytane were not affected by volatility or degradation by the bacteria in any state. Uninoculated controls for all three systems showed no increase in protein concentration and no decrease in n-alkanes and branched alkanes from the GC analysis after the eight day incubation period.
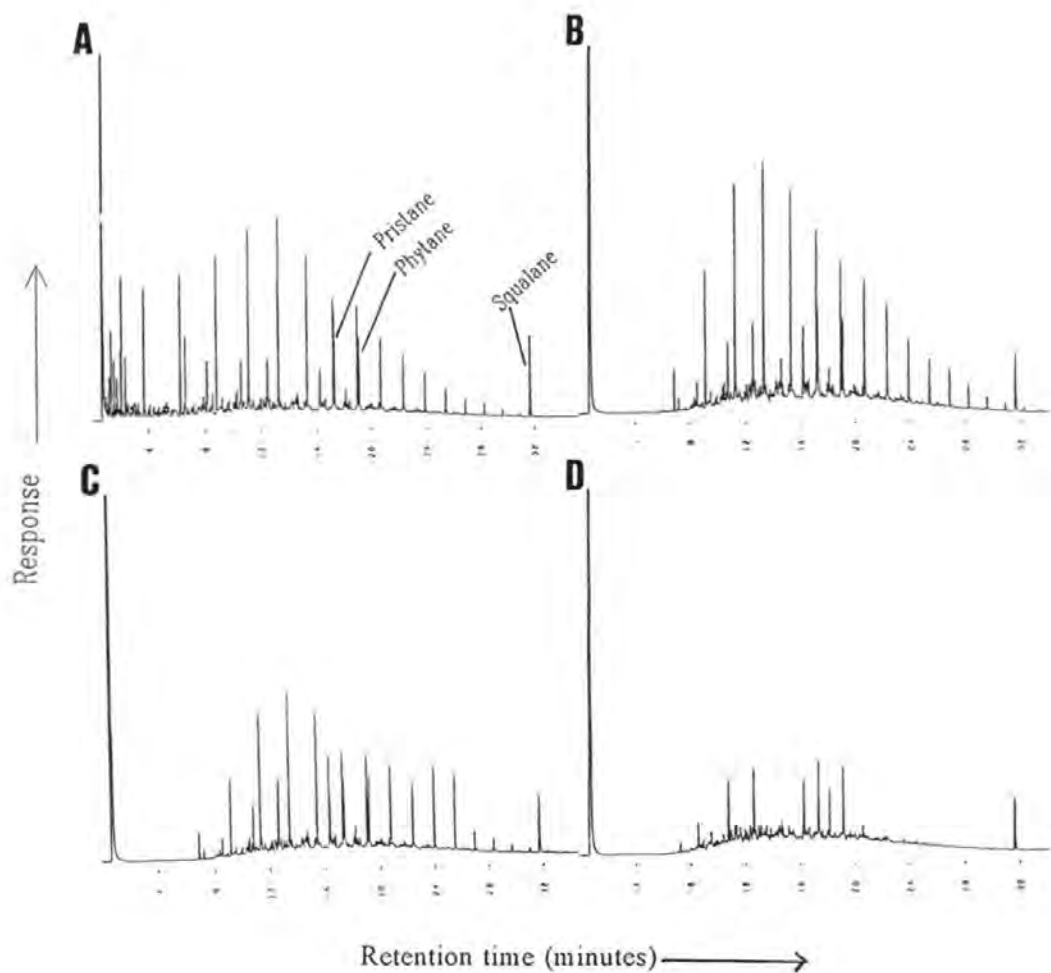
Figure 2.3. Typical gas chromatographic patterns for petrol (Slovene diesel). A) Unexposed. B)Incubated with free cells for eight days in a freshwater system. C)Incubated with Biofix immobilized cells for eight days in a freshwater system. D)Incubated with Drizit immobilized cells for eight days in a freshwater system.

2.3.4. Degradation of petrol (Slovene diesel) by free Pseudomonas fluorescens in a saltwater system.

After a two day lag phase in both the free systems (with and without nutrient supplementation) the average absorbance of the aqueous suspension of the free system without nutrient supplementation increased to a level little above the control, with a final absorbance of 0.061. In the free system with nutrient supplementation the average absorbance of the aqueous suspension had a final value of 0.087. The level and rate of growth in both the free systems were below that seen in the immobilized systems (figure 2.4). Degradation of the n-alkanes C_{13}-C_{18}, and branched alkanes pristane and phytane were quantified using gas chromotography analysis. In the free system without nutrient supplementation an average degradation of 39.8 % was achieved, a level below that seen in all the other systems.. In the free system with nutrient supplementation an average degradation of 72.4 % was achieved, a level close to that seen in the two immobilized systems (Table 2.3).

Table 2.3. Percentage carbon degradation of petrol (Slovene diesel) after three days incubation with *Pseudomonas fluorescens* in free and immobilized saltwater systems.

System									
	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	Pristane	Phytane	Average
Free	66	24	22.5	55.4	45.8	41.8	34.5	28	39.8
Free + nutrients	91	89.4	78.8	83.3	80.3	80.5	36.6	39.5	72.4
Immobilized	92.2	81.2	77.7	82.1	82.3	68.5	60.3	45.7	73.8
Immobilized + nutrients	93	93	80.8	89.6	84	68.4	60.5	23.9	74.15

*Percentage Carbon Degraded (header spanning the data columns)

*figures are mean values of two samples and are adjusted to allow for photodegradation and volatilization using the uninoculated control.
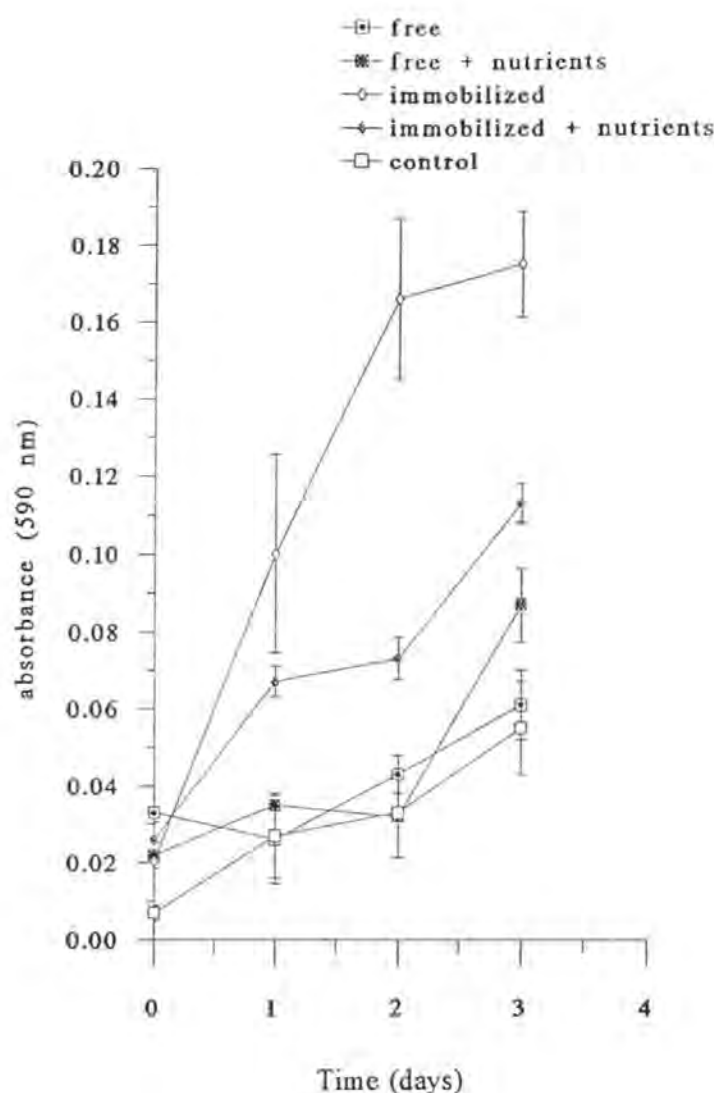
Figure 2.4. Growth curves of *Pseudomonas fluorescens* in the aqueous suspensions of free and immobilized saltwater systems, grown on petrol (Slovene diesel) over a three day incubation period. Bars indicate standard error

2.3.5. *Degradation of petrol (Slovene diesel) by Drizit immobilized* <u>Pseudomonas fluorescens</u> *in a saltwater system.*

The average absorbance of the aqueous suspension of the immobilized system without nutrient supplementation increased from 0.0205 to 0.175 and with nutrient supplementation from 0.026 to 0.113, the system supplemented with nutrients showing a lower level and rate of growth. No lag phase was observed in the growth cycles of the immobilized systems (figure 2.4). After three days incubation the two immobilized systems had degraded quantities of the n-alkanes C_{13}-C_{18}, and branched alkanes pristane and phytane. In the immobilized system without nutrient

supplementation an average of 73.8 % of petrol (Slovene diesel) had been degraded and in the immobilized system with nutrients an average of 74.15 % (table 2.3).

Statistical analysis (ANOVA) showed a significant difference between the degradation levels in the four saltwater systems for n-alkanes C_{14} ($p = 0.02$), C_{15} ($p = 0.0125$), C_{17} ($p = 0.006$) and C_{18} ($p = 0.0109$). A multiple range test (LSD) showed there was a significant difference between the free system and the free system with nutrient supplementation, the immobilized system and immobilized system with nutrient supplementation, the free system having degraded significantly less of each of the above n-alkanes in by weight ($\mu g\ mg^{-1}$ petrol (Slovene diesel)) than the other systems.

2.4. Discussion

The results presented show that enhanced biodegradation of petrol (Slovene diesel) occurred with immobilization of an oil degrading *P. fluorescens* in a freshwater system. Higher levels of growth and degradation of the n-alkanes C_{12} - C_{18} were achieved by *P. fluorescens* in the immobilized state compared to free living bacteria. This increase in activity can be seen when comparing the growth curves of *P. fluorescens* in the three different living states. In the free system there was a lag phase of six days during which no growth occurred, after which the protein concentration of the suspension increased for the remaining two days. Immobilization of cells on Biofix reduced the lag phase to 4 days and Drizit immobilized cells reduced the lag phase to one day. It appears therefore that the process of immobilization accelerates the ability of the cells to initially utilize the petrol (Slovene diesel) in a freshwater system.

In the saltwater system higher levels and rates of growth were achieved by *P. fluorescens* in the immobilized state, both with and without nutrient supplementation, compared to a free system of bacteria with and without nutrient supplementation. This increase can be seen when comparing the growth curves of *P. fluorescens* in the four different living states. In both the free systems there was a lag phase of two days after which growth occurred. Immobilization of *P. fluorescens* to Drizit eliminated the lag phase in both the immobilized systems Therefore, in a saltwater system as shown in the freshwater system the process of immobilization accelerates the ability of the cells to initially utilize the diesel.

The addition of nutrients to the free saltwater system increased the level of growth and n-alkane degradation achieved at day three in comparison to the levels achieved by a free system with no nutrient supplementation, however the nutrient supplementation had no effect on the length of the lag phase. This increase in growth and biodegradation with nutrient supplementation in saltwater is in accordance with numerous laboratory and field studies which have shown that nutrient

supplementation generally leads to successful increases in growth and enhancement of oil biodegradation (Atlas 1972b; Olivieri *et al* 1978; Atlas 1981; Floodgate 1984; Fox 1989; Atlas and Bartha 1992; Marty and Martin 1993).

There was no significant difference in level of growth and biodegradation of petrol (Slovene diesel) achieved after three days incubation of immobilized *P. fluorescens* with and without nutrient supplementation. The addition of nutrients was expected to enhance growth with a concomitant enhancement of biodegradation as shown in the two free systems. It is possible that the immobilization of *P. fluorescens* to Drizit renders the nutrients added to the saltwater inaccessible to the cells. Drizit is hydrophobic and oleophilic, and may therefore adsorb the petrol (Slovene diesel) but repel the saltwater and therefore any added nutrients. Ideally nutrients should be adsorbed to the matrix or be oleophilic so they adsorb to the oil and are therefore readily accessible to the bacteria. The addition of nutrients in saltwater systems is essential to establish optimum ratios of nitrates and phosphates to the carbon source and thus allow enhanced bioremediation (section 1.5.1). It is possible that if accessibility of the immobilized cells to these nutrients is not improved biodegradation may not be further enhanced in a saltwater system by immobilization.

In the saltwater systems, significantly higher levels of biodegradation of the n-alkanes C_{14}, C_{15}, C_{17} and C_{18} were achieved by the nutrient supplemented free system, immobilized system and nutrient supplemented immobilized system in comparison to the unsupplemented free system. Therefore the immobilization of *P. fluorescens* in a saltwater system significantly increases the level of degradation achieved in comparison to a free system but not in comparison to a free system with nutrient supplementation.

The process of petrol (Slovene diesel) degradation requires the immobilization of not only one enzyme system but of a multi-enzyme system that mediates complex reactions in the degradative pathway of n-alkanes (section 1.4). Whole cell immobilization permits the immobilization of these multi-enzyme systems. (Fukui and Tanaka 1982). The aim of immobilizing cells was to reduce the

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problem of the limited oil-water interface caused by the insolubility of alkanes in water. Limited solubility of alkanes means that the concentration of solubilized molecules, or the bioavailability of the alkanes, is too low to meet the needs of the microorganism (Zhang and Miller 1992). Therefore, adaptations are necessary to allow uptake to occur at a rate sufficient to satisfy the growth requirements. Pseudo-solubilization may occur, in which a surfactant is produced by the cells to decrease the surface tension of the hydrocarbon (section 1.4), (Ratledge 1992; Hommel 1994), and *Pseudomonas* species are known to produce biosurfactants for hydrocarbon utilization (Tagger *et al.* 1983). It is possible that immobilization on an oleophilic biosupport may either remove the need for the cell to produce surfactant by forming an intermediary between the cell and the oil or result in enhanced production of the surfactant due to earlier contact between the two. These theories would account for the extended lag phase seen in the growth curve of the free system in both the freshwater and saltwater systems, during which time the cell may be making contact with the oil and subsequently producing the surfactant at a later stage in the growth cycle. Succeeding investigations study the hypothesis that surfactant production is affected by cell immobilization.

The petrol (Slovene diesel) was seen to adsorb to the biosupport, bringing the cell into closer contact with the carbon source, thus increasing the bioavailability of the hydrocarbons to the cell. This has also been shown by Heinrich and Rehm (1981) using *Fusarium moniliforme* in the adsorption culture techniques of trickle flow columns and fixed-bed reactors, where contact between mycelia and alkane droplets were ensured and growth was therefore improved.

In the freshwater systems, the Drizit immobilized system contained significantly more protein in free suspension than the Biofix system, however, no significant difference was found in the levels of degradation of petrol (Slovene diesel) by Biofix and Drizit immobilized cells per μg protein although Drizit immobilized cells reduced the lag phase more than the Biofix immobilized cells. These results are in accordance with several studies carried out using immobilized bacteria. Takahashi *et al.* (1981) showed enhanced phenol removal from wastewater using *Aureobasidium*

pullans adhered to fibrous supports, O'Reilly and Crawford (1989b) illustrated enhanced degradation of pentachlorophenol by polyurethane immobilized *Flavobacterium* cells and Omar and Rehm (1988) showed enhanced degradation of an alkane mixture by *Candida parapsilosis* and *Penicillium frequentans* immobilized on granular clay.

Enhanced biodegradation of petrol (Slovene diesel) also occurred with the immobilization of *P. fluorescens* in a saltwater system in comparison to a free system of the same bacteria under identical conditions. It is interesting however that the same significant enhancement of biodegradation of petrol (Slovene diesel) was not achieved with immobilization of *P. fluorescens* either with or without nutrient supplementation in a saltwater system in comparison to a free system with nutrient supplementation, although a reduction in lag phase was observed with immobilization as seen in the freshwater systems. Due to the two day lag phase and the lower level of growth achieved in the free system with nutrient supplementation a lower level of biodegradation would have been expected. Further studies are required to establish why enhanced biodegradation does not occur with immobilization and nutrient supplementation in a saltwater system.

The results of this study have implications for the bioremediation of water polluted by compounds having limited water solubility or those present at low concentrations which results in poor availability of the compound for the microorganisms. The careful choice of biosupport will be essential for successful development of specific/further applications. The biosupports used in this study served as effective intermediaries between the petrol (Slovene diesel) and the microbes, increasing the biodegradation of the n-alkanes.

Chapter 3

A Comparison of Bacterial Immobilization Substrata

3.1. Introduction

The effectiveness of two commercially available materials, the biosupport, Biofix C2 and

the biosorbant, Drizit in enhancing the degradation of petrol (Slovene diesel), has been

demonstrated (chapter 2). The aims of this study were to extend the comparison of these materials

as biocarriers and look at the use of a third commercially available biosorbant, polyester

polyurethane (Putnams, Devon, England) as a bioremediation agent in a defined aqueous system.

Polyurethanes are a class of polymers synthesized as a result of the reactions between compounds

containing an isocyanate group (R-N=C=O) and compounds containing a hydroxyl group (O'Reilly

and Crawford 1989b). The three materials were compared by six criteria;

3.1.1. *Scanning electron microscopy.*

Scanning electron microscopy permits the observation and characterization of heterogeneous

organic and inorganic materials and surfaces. The area to be examined is irradiated with a finely

focused electron beam. The signals of importance produced when the electron beam impinges on

a specimen surface are the secondary and backscattered electrons, since these vary as a result of

differences in surface topography as the electron beam is swept across the specimen. The

secondary electron emission is confined to near the beam impact area, permitting images to be

obtained at relatively high resolution. The three dimensional appearance of the images is due to

the large depth of field of the scanning electron microscope, as well as to the shadow relief effect

of the secondary electron contrast (Goldstein *et al.* 1992).

3.1.2. *Normarski differential interference contrast microscopy.*

Normarski differential interference contrast microscopy is a form of differential interference

using special polarizing prisms arranged according to a design by Normarski. Differential interference is a double beam interference in which two wavetrains fall on the object plane or image plane and are separated laterally by a distance similar to the minimum resolvable distance. Normarski differential interference contrast microscopy is suitable for the observation of living cells and microorganisms without staining because it is non-invasive. Using this type of illumination, edges or abrupt changes in refractive index are picked out and contrast is increased due to the shadowing effect. The images that are produced appear three-dimensional because one side of the specimen appears lighter than the other as if light was falling on it and casting shadows (Rawlins 1992).

Scanning electron microscopy or Normarski differential interference contrast microscopy were used to illustrate and allow comparison of the structure of each support and to provide evidence for the immobilization of *P. fluorescens*.

3.1.3. *Cell loading capacity of Drizit, Biofix and polyester polyurethane.*

The efficiency of cell immobilization of each biocarrier was investigated to determine the loading capacity of each matrix.

3.1.4. *Absorbtion of Ekofisk crude oil to Drizit, Biofix and polyester polyurethane.*

It has been speculated (chapter 2) that immobilized bacteria technology results in enhanced levels of degradation due to increased contact between the immobilized bacteria and the absorbed oil. Thus for a substratum to act as an efficient bioremediation agent in this manner it is important that the substratum has a high absorbency of oil, therefore the absorbency of oil to each substratum was investigated.

3.1.5. *Oil degradation by Drizit, Biofix and polyester polyurethane immobilized* <u>*Pseudomonas*</u> <u>*fluorescens*</u>.

The level of oil degradation achieved by *P. fluorescens* when immobilized to each biocarrier

was compared to determine which matrix resulted in the highest level of enhanced degradation.

3.1.6. *The effect of drying, and storage over time on the recoverability of Drizit, Biofix and polyester polyurethane immobilized Pseudomonas fluorescens.*

There are several basic objectives of the preservation of cultures; to keep the cultures viable, uncontaminated, unchanged in their properties, and to have adequate and appropriate systems for replenishing stocks when necessary. The practical requirement is whether the culture will grow again after a period of preservation and frequently recovery of a culture is simply an all-or-none result. The methods have to be safe, reliable and useful for the storage of a wide range of microorganisms. The only way to be sure that a method of preservation is better than another is by viability counts made before preservation and compared with those obtained on recovery after storage. Drying of cultures is widely used as a method of preservation (Hill 1981). Dehydration reduces the metabolic state of the organisms, however not all organisms will survive drying methods without added protective agents. Three methods of drying were compared in this study to determine their effects on immobilized cells;

1) Freeze drying or lyophilization which is a process in which water vapour is removed from a frozen product by sublimation. Freeze drying causes little shrinkage of the cell and results in a completely soluble product that is easily rehydrated. Chemical changes are minimized by preventing concentration of solutes and also by virtue of the lowered temperature, which reduces the rate of chemical reaction (Rudge 1991).

2) Drying at room temperature, (Malik 1991).

3) Drying at 55°C. Protective agents are normally used to suspend cells to be dried in order to protect against drying injury, in this study no such agents were used, to determine whether the immobilization substrata themselves conferred any protection during dehydration.

3.2. Materials and methods

3.2.1. *Bacterial isolates.*

The details of the bacterial isolates used in this study are described in section 2.2.1.

3.2.2. *Media*

Artificial seawater (Marine salts, TAP) was supplemented with 1% (w/v) of Ekofisk crude oil (Lindsey oil refinery, South Humberside, England) for the biodegradation studies carried out with polyurethane immobilized cells. The minimal medium, pH 7.2, used for the Biofix and Drizit biodegradation studies contained (g l^{-1}): NH$_4$Cl, 5.0; NH$_4$NO$_3$, 1.0; anhydrous NaSO$_4$, 2.0; K$_2$HPO$_4$, 3.0; KH$_2$PO$_4$, 1.0 and MgSO$_4$.7H$_2$O, 0.1 dissolved in order in distilled water. The minimal medium was supplemented with 0.1% v/v petrol (Slovene diesel) as the carbon source. Tryptone Soy broth (TSB, Oxoid) was used as the nutrient source for the bacteria after immobilization and 0.01 M phosphate buffered saline (PBS), pH 7.3 (Oxoid, Dulbecco 'A' tablets) to wash the cells.

3.2.3. *Whole cell immobilisation*

3.2.3.1. Cell loading of polyester polyurethane.

Polyurethane blocks (1 cm cubes) were added to an overnight culture of *P. fluorescens* and placed on an orbital shaker (100 rev min^{-1}) for 24 hours at 24°C. Nutrient supplementation was carried out by squeezing and releasing blocks in nutrient broth (Oxoid) plus 1 mM disodium hydrogen phosphate (BDH) and 10 mM potassium nitrate (BDH). Non-supplemented blocks were squeezed and released in distilled water to allow for similar cell washout in both supplemented and non-supplemented cubes.

A polyurethane cube was sonicated (Transsonic T310, Camlab) for 2 minutes in 10 ml of PBS and the polyurethane cube removed. Viable counts were carried out on the remaining suspension and the optical density of the suspension found at 540 nm. A calibration curve was plotted of calculated viable count, (colony forming units - cfu) against the number of cubes (appendix 1) and using a free overnight culture of *P. fluorescens*, viable count, cfu ml^{-1} was plotted against calculated

optical density at 540 nm (appendix 2).

3.2.3.2. Cell loading of Biofix and Drizit.

The method detailing the cell loading of Biofix and Drizit is given in section 2.2.3.

3.2.4. *Scanning electron microscopy.*

Cells were immobilized to Biofix, Drizit (section 2.2.3) and polyurethane (section 3.2.5) and specimens of the three biocarriers mounted on brass specimen stubs, sputter coated with gold (6 nm thickness) and observed under a Jeol JSM 5300 scanning electron microscope.

3.2.5. *Normarski differential interference contrast microscopy.*

Specimens of the three biocarriers were mounted on slides and viewed under a Vanox Olympus research microscope (model AHBT) by Normarski differential interference contrast microscopy using a Normarski analyzer

3.2.6. *Cell loading capacity of Drizit, Biofix and polyester polyurethane.*

Drizit and polyurethane (non-supplemented) immobilized cells were incubated at 24°C in TSB on an orbital shaker (100 rev min⁻¹) for eight days. Biofix immobilized cells were incubated at 24°C in TSB in the column for eight days. Samples (1 g) were removed daily in triplicate and washed in 10 ml of PBS. The samples were then placed in 5 ml PBS and sonicated (Transsonic T310, Camlab) for 2 min. The immobilization substrata were removed and the bacteria now in free suspension were centrifuged at 13000 rev min⁻¹ (12 x 1.5 ml head, micro-centaur) for 3 minutes and resuspended in PBS. The amount of protein per gram of immobilization substratum was estimated by the Folin method (Lowry *et al.* 1951) modified by the addition of 2% deoxycholic acid (Maddy and Spooner 1970).

3.2.7. *Absorption of Ekofisk crude oil to Drizit, Biofix and polyester polyurethane.*

Duplicate non-inoculated substrata samples (1 g) were placed in excess Ekofisk crude oil and allowed to absorb to their maximum capacity. The substrata were removed from the oil and any excess allowed to drain off and subsequently reweighed.

3.2.8. *Degradation of petrol (Slovene diesel) by Pseudomonas fluorescens in free, and Biofix and Drizit immobilized freshwater systems and a Drizit immobilized saltwater system.*

Details of the method for the degradation of petrol (Slovene diesel) in free, and Biofix and Drizit immobilized freshwater systems is given in section 2.2.4 and in a Drizit immobilized saltwater system in section 2.2.5.

3.2.8.1. *Extraction and analysis of petrol (Slovene diesel).*

Sections 2.2.6 and 2.2.7 gives details of the method on extraction and analysis of petrol (Slovene diesel) from the freshwater and saltwater systems.

3.2.8.2. *Cellular growth of Pseudomonas fluorescens in free, and Drizit and Biofix immobilized freshwater systems and Drizit immobilized saltwater system.*

Sections 2.2.8 and 2.2.9 give details of the methods on cellular growth of *P. fluorescens* in free, and Drizit and Biofix immobilized freshwater systems and Drizit immobilized saltwater systems respectively.

3.2.9. *Degradation of Ekofisk crude oil by Pseudomonas fluorescens in free and polyester polyurethane immobilized systems (non-supplemented and nutritionally-supplemented).*

P. fluorescens immobilized on polyurethane, non-supplemented or supplemented with nutrients (section 3.2.3), or bacteria in free suspension, centrifuged at 13000 rev min^{-1} (12 x 1.5 ml head, MSE, micro-centaur) for 3 minutes and resuspended in PBS, were inoculated into 100 ml of artificial seawater (marine salts, TAP) in 250 ml Erlenmeyer flasks to give the same cfu 100 ml^{-1}, using the calibration curves (appendices 1 and 2) to determine the amount of inocula. The flasks

an orbital shaker (100 rev min⁻¹) for 20 days. All treatments and analyses were performed in duplicate

Uninoculated controls were prepared to account for abiotic, volatilization and extraction losses of the carbon source.

3.2.9.1. *Extraction and analysis of Ekofisk crude oil.*

Residual Ekofisk crude oil was recovered after 20 days from the solid matrices of incubated samples by extraction with 10 ml of dichloromethane (DCM, Oxoid) and the aqueous phases of incubated samples were recovered by extraction with 20 ml DCM after which the glassware were rinsed with a further 10 ml DCM. The extracts from each sample were combined and concentrated to approximately 10 ml by rotary evaporation (40°C) after which the remaining DCM was removed under a gentle stream of pure nitrogen. Quantitative analysis was performed by gas chromatography using a Carlo Erba 4160 gas chromatograph with on column injection (0.5 µl samples) and a flame ionization detector. Separation was achieved using a fused silica capillary column (0.32 mm by 30 m, DB-5, J and W Scientific, 91 Blue Ravine Rd, Folsom, CA, 95630-4714). The oven temperature was increased from 50°C to 300°C at a rate of 6°C min⁻¹ and held at 300°C for 10 minutes. Hydrogen was used as the carrier gas at a flow rate of 1.5 ml min⁻¹ and the FID detector's temperature was kept at 330°C. Quantification of individual hydrocarbons was made by measurement of the GC peak areas with a Shimadzu CR3-A integrator. Peaks produced from n-alkanes C_{12} - C_{17} from Ekofisk crude oil were compared to the response of the branched chain hydrocarbon Pristane and C_{18} to Phytane. Percentage biodegradation of alkanes C_{12}- C_{18} were calculated by comparing the response of biodegraded samples, after correction for non-biological losses, to that of an unexposed sample.

3.2.9.2. *Cellular growth of* Pseudomonas fluorescens *in free and polyester polyurethane immobilized systems.*

Viable counts were carried out on the aqueous suspensions of the free systems and

immobilized systems and on bacteria immobilized to polyurethane cubes. Polyurethane immobilized bacteria were released from the solid matrices by sonication (Transsonic T310, Camlab) for 2 min in 5 ml of PBS. Viable counts were carried out on nutrient agar (Oxoid) in duplicate, at time zero and after 20 days.

3.2.9.3. *Weight of Ekofisk crude oil after 20 Days incubation with* Pseudomonas fluorescens *in free and polyester polyurethane immobilized systems.*

Residual Ekofisk crude oil from incubated samples was recovered as previously (section 3.2.8.1) and rotary evaporated to approximately 10 ml. The samples were then dried overnight, by the addition of approximately 1 g of anhydrous sodium sulphate. After drying the samples were filtered through DCM pre-wetted filter paper to remove the sodium sulphate, and the filter paper washed with a further 5-10 ml of DCM. The samples were further rotary evaporated to a volume of approximately 3 ml and transferred to pre-weighed universal bottles. The remaining DCM was removed under a gentle stream of pure nitrogen. Ekofisk samples were reweighed at intervals and blow-down discontinued when a constant mass was achieved. The final weight of Ekofisk was recorded.

3.2.10. *The effect of drying and storage over time on the recoverability of Drizit, Biofix and polyester polyurethane immobilized* Pseudomonas fluorescens

Drizit, Biofix (100 mg samples) and 1 cm^3 polyurethane blocks (nutrient supplemented and non-supplemented) immobilized cells were dried; 1) at room temperature for 1 week, 2) at 55°C in an oven (model OV - 160, Galtenhampton) for 24 hours, 3) from frozen (-4°C for 24 hours) in a freeze dryer (Edwards, Super Modulyo) over 24 hours. After drying the substrata were stored in a dessicator containing anhydrous silica gel for 3 months.

3.3.10.1. *Recoverability of immobilized* Pseudomonas fluorescens *after drying and storage.*

Samples (100 mg) of Drizit, Biofix and 1 cm^3 polyurethane blocks containing immobilized *P. fluorescens*, dried under the stated conditions were removed from storage at, 1 week, 2 weeks,

2 months and 3 months. Immobilized bacteria were released from the solid matrices by sonication (Transsonic T310, Camlab) for 2 minutes in 5 ml of PBS. The substrata were removed from the PBS and viable counts carried out on the remaining suspension, on nutrient agar (oxoid) in duplicate.

3.3.11. *Statistical analysis.*

Multifactor analysis of variance (MANOVA) and a multiple range test (least significant difference- LSD) was carried out on the cell loading and storage data. The remaining data was analysed using one way analysis of variance and the LSD test. All statistics were carried out using Statgraphics, version 6.1, to determine any significant differences in the data.

3.3. Results.

This study compared Biofix, Drizit and polyester polyurethane as immobilization matrices and their ability to be used as bioremediation agents.

3.3.1. *Scanning electron microscopy.*

Figures 3.1 and 3.2 show the specific structure of the immobilization matrices, Biofix and polyurethane using scanning electron microscopy. Figure 3.1 illustrates the classic 'bird's nest' structure of Biofix, as described by Salter *et al.* (1990) and in some particles bacteria can be seen immobilized within the matrix. Figure 3.2 illustrates the structure of polyurethane which can be seen as a porous matrix with a network of orifices. In this sample fractures can be seen in the walls of the material, which may have occurred during the scanning electron microscopy or during preparation of the polyurethane cubes. Figure 3.3 illustrates the colonisation of the polyurethane, the bacteria having formed loose aggregations of organisms on the surface of the mesh. Figure 3.4 illustrates the fibrous structure of Drizit.

3.3.2. *Normarski differential interference contrast microscopy.*

Figure 3.5 illustrates the structure of Drizit and the colonization of the matrix using Normarski differential interference contrast microscopy, where the bacteria trapped between the entwined fibres can be seen as the pink granular effect.

Figure 3.1. Structure of the biosupport Biofix.

Figure 3.2. Structure of the biosupport polyester polyurethane.

3.1

0008   25KV   X200 100μm WD12

3.2

0010 3.0KV   X40 100μm WD10
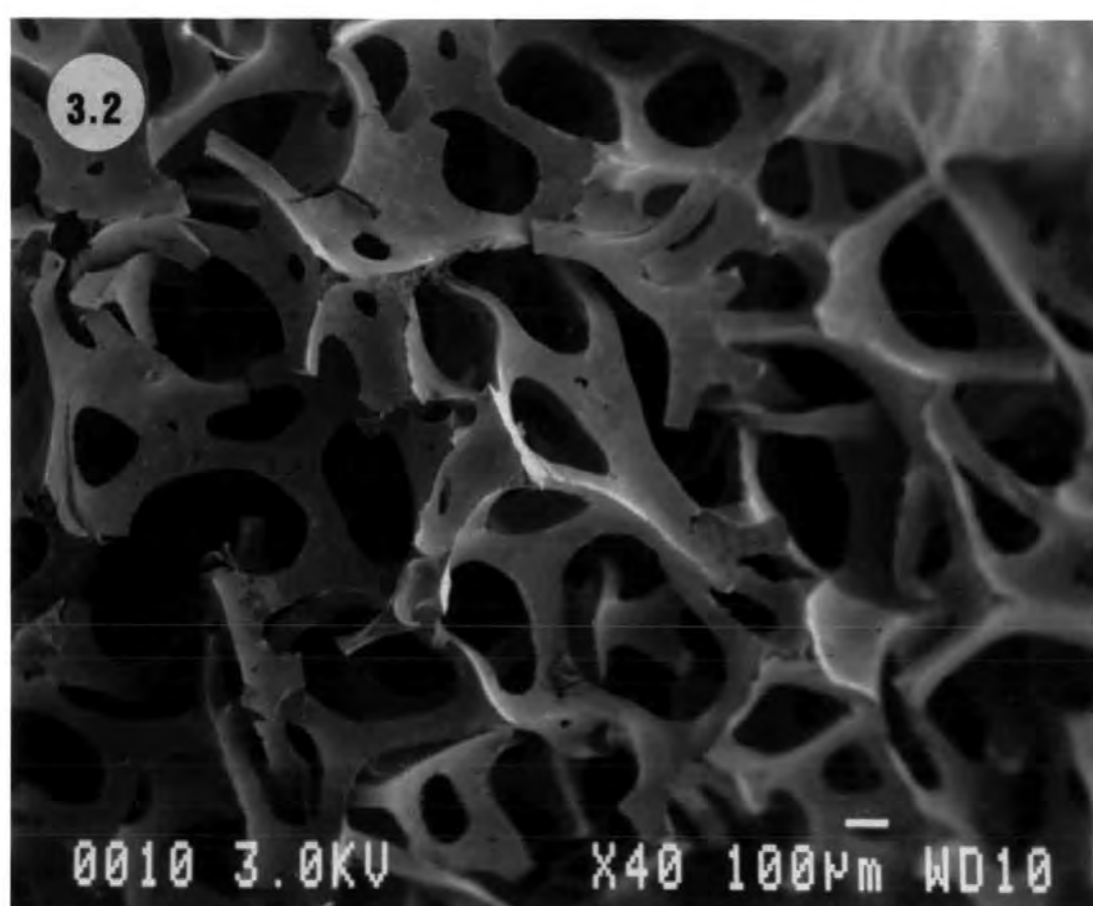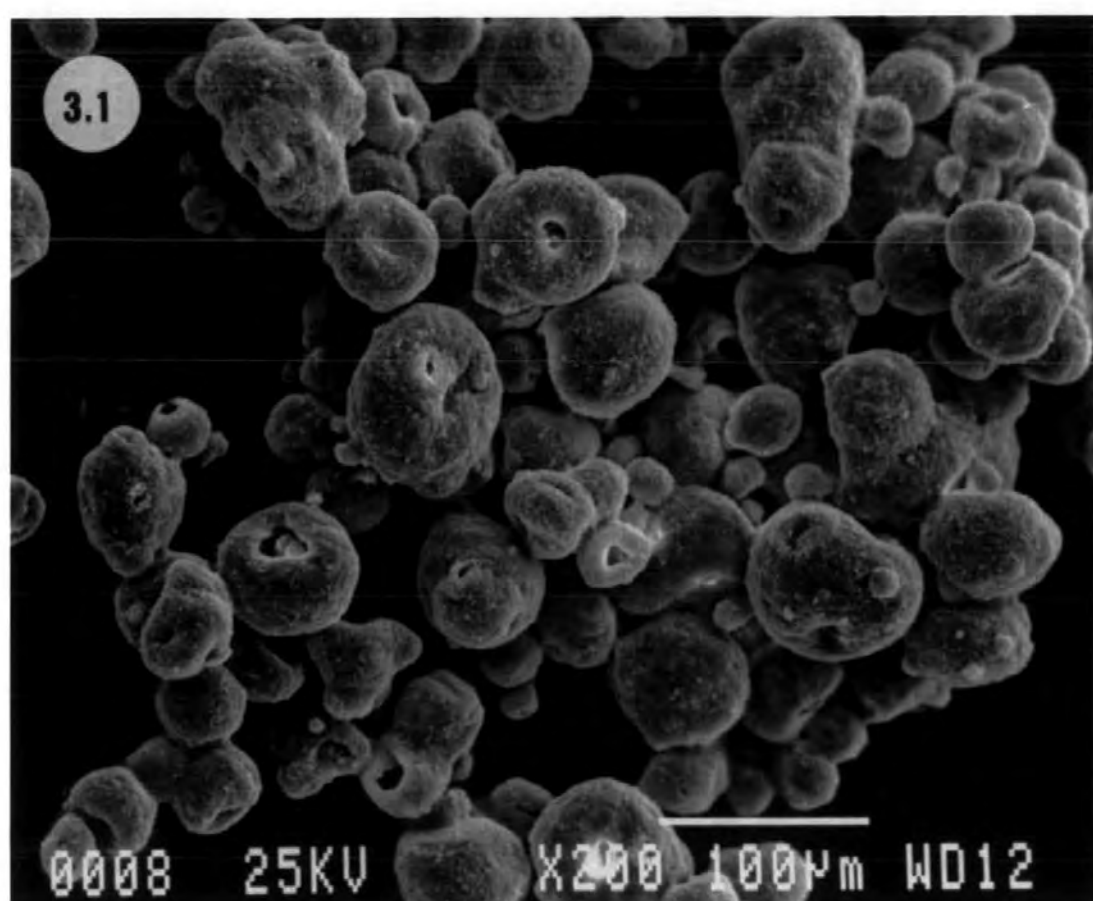
Figure 3.3. Polyester polyurethane with bacteria adsorbed to the surface.

Figure 3.4. Structure of the biosupport Drizit.

3.3

25KV   X7,000   1μm   WD10

3.4

0011 3.0KV   X75 100μm WD10

3.5

×100   0.1mm
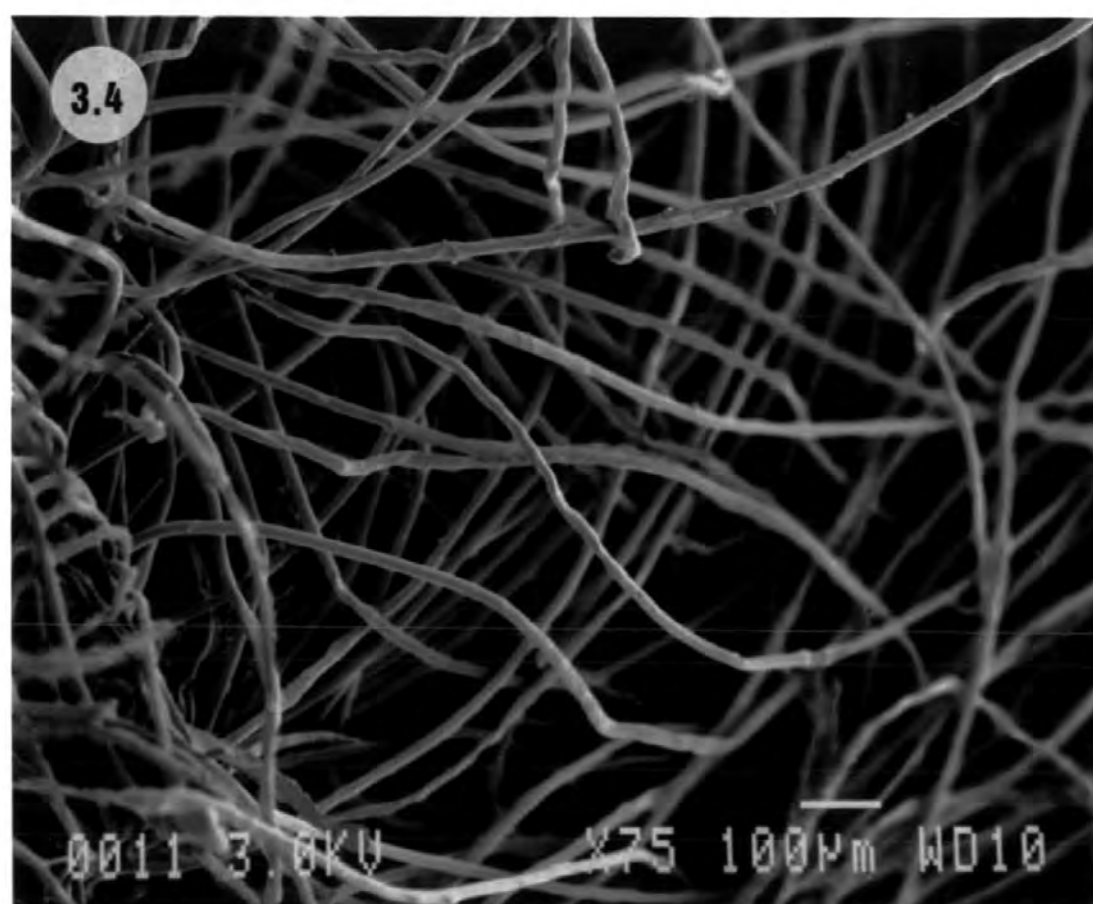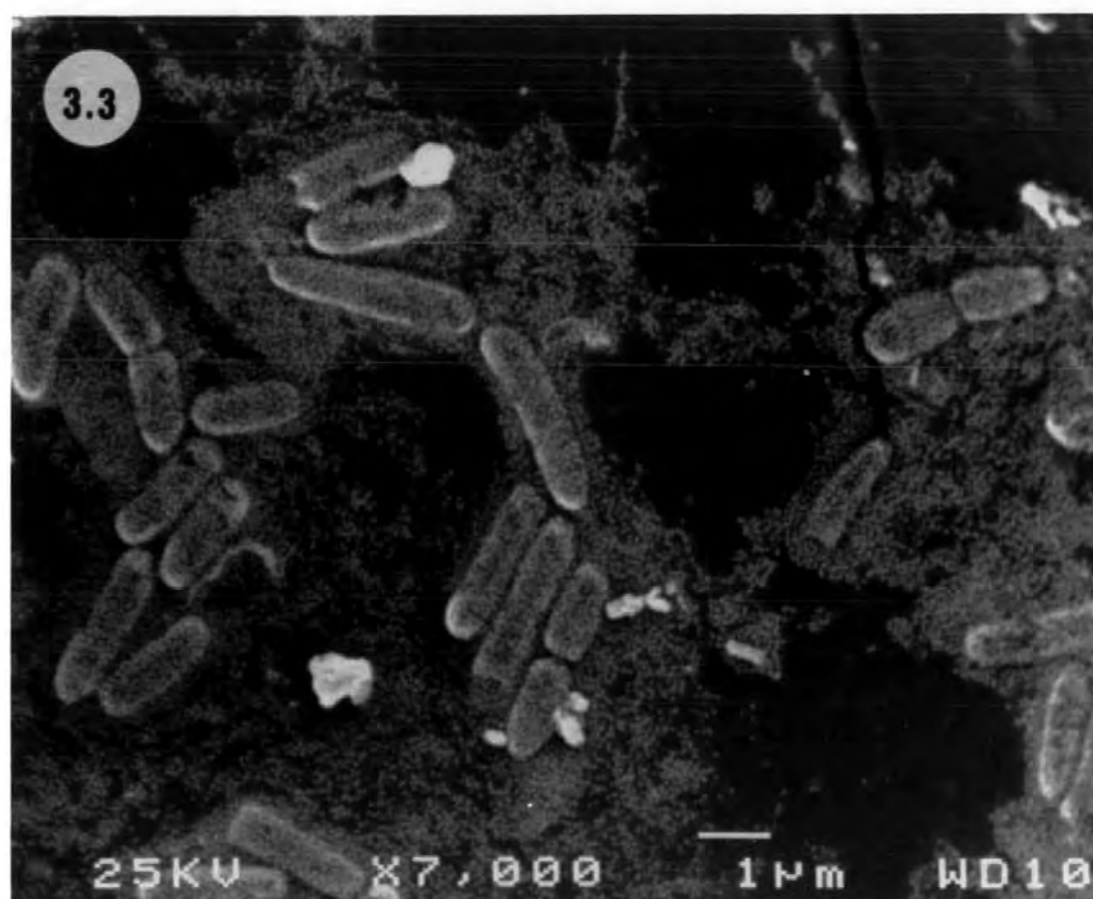
3.3.3. Cell loading capacity of Drizit, Biofix and polyester polyurethane

Figure 3.6. shows the effect of extending the loading phase over eight days on the immobilized protein content of Biofix, Drizit and polyurethane. There was a significant difference in the amount of protein immobilized in each system over time ($p = 0.0$) and a significant difference in the amount of protein immobilized between each system over time ($p = 0.0$). Polyurethane immobilized the highest amount of protein (23.1 mg protein g^{-1} substratum) compared to Biofix and Drizit. After day two the amount of protein per gram of polyurethane fell, the polyurethane was subsequently washed in PBS and the nutrients replenished. The amount of protein immobilized per gram of polyurethane then returned to a level comparable to that found at day two. After day six the immobilized protein content of polyurethane started to decline.
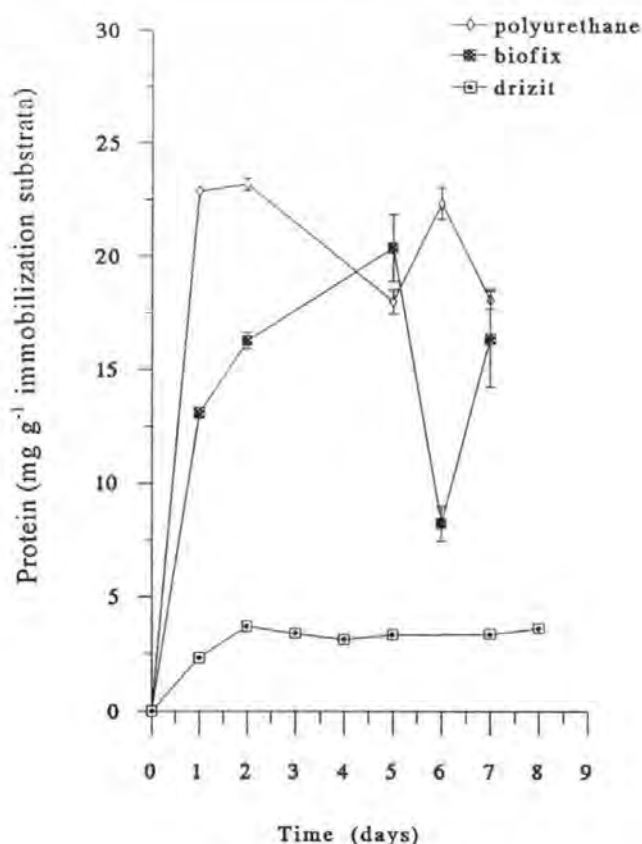
Figure 3.6. Effect of varying the loading phase on the amount of protein immobilized on Biofix, Drizit and polyester polyurethane. Bars indicate standard error.

Biofix immobilized cells reached a maximum of 20.37 mg protein g^{-1} substratum. At day five the cells were washed in PBS and the nutrient supply replenished, this caused a decline in the amount of protein immobilized, after which (day seven) the level rose. Drizit immobilized cells reached a maximum of 3.75 mg protein g^{-1} substratum. Although the level was lower than that of Biofix and polyurethane, the amount of protein immobilized was very stable over the eight days of incubation, showing less fluctuation than that found in Biofix and polyurethane immobilized protein.

3.3.4. *Absorption of Ekofisk crude oil to Biofix, Drizit and polyester polyurethane.*

The amount of Ekofisk crude oil absorbed varied significantly (p = 0.0007) with the different immobilization substrata. Polyurethane absorbed the highest amount of the crude oil, (10.72 g Ekofisk g^{-1} substratum), Drizit absorbed 7.49 g Ekofisk g^{-1} substratum and Biofix absorbed the least, 1.6 g Ekofisk g^{-1} substratum (figure 3.7).
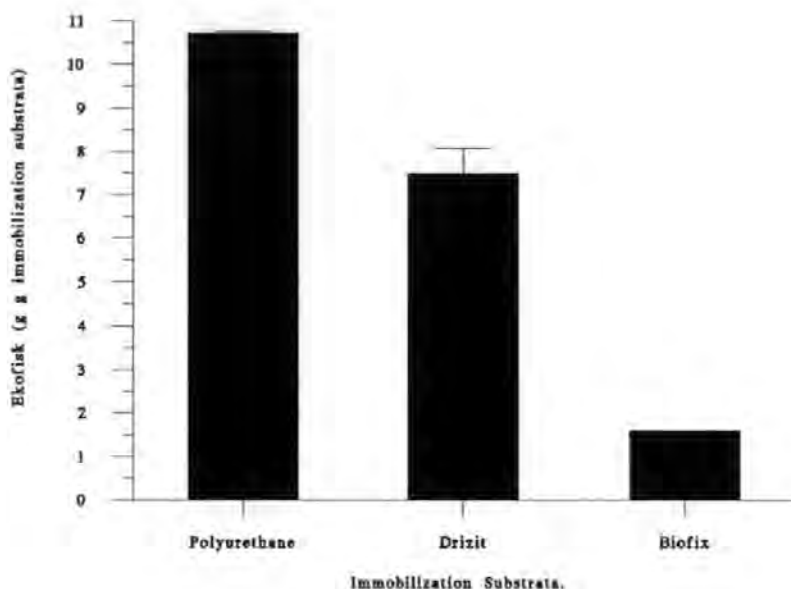
Figure 3.7. Absorption of Ekofisk crude oil to Biofix, Drizit and polyester polyurethane. Bars indicate standard error.

3.3.5. *Degradation of petrol (Slovene diesel) by* <u>*Pseudomonas fluorescens*</u> *in free, and Biofix and*

Drizit immobilized freshwater systems and Drizit immobilized saltwater system.

For details of the results of this investigation refer to section 2.3 .

3.3.6. *Degradation of Ekofisk crude oil by* <u>*Pseudomonas fluorescens*</u> *in free and polyester*

polyurethane immobilized systems (non-supplemented and nutritionally supplemented).

3.3.6.1. *Cellular growth of* <u>*Pseudomonas fluorescens*</u> *in free and polyester polyurethane immobilized*

systems.

After 20 days incubation of the bacteria with Ekofisk crude oil as the only carbon source,

the viable number of bacteria remaining in the free system and in the polyurethane immobilized

system without nutrient supplementation (table 3.1a), and with nutrient supplementation (table 3.1b)

were determined.

Table 3.1. Average viable counts of *Pseudomonas fluorescens* grown on Ekofisk crude oil for 20 Days,
a) in free suspensions of seawater and immobilized on polyester polyurethane, b) immobilized on polyester
polyurethane with and without nutrient supplementation.

	system	Average (n=4) Viable counts (cfu ml^{-1})	Standard Error
a)	free	2.75×10^{13}	2.75×10^{13}
	immobilized	3.14×10^{11}	1.65×10^{11}
b)	immobilized	2.37×10^{10}	5.1×10^{9}
	immobilized + nutrients	6.34×10^{11}	3.25×10^{11}

There was no significant difference between the viable counts in the free and the polyurethane immobilized system and no significant difference between the viable counts in the supplemented and the non-supplemented polyurethane immobilized systems. Due to the fact that the difference between the viable counts in the supplemented and the non-supplemented polyurethane immobilized systems were nearly significant (p = 0.085) at the 0.05 significance level, a least significant difference test (LSD) was carried out. This test revealed a significant difference between the viable counts in the two systems, the polyurethane immobilized system with nutrient supplementation having a significantly higher viable count.

3.3.6.2. *Weight of Ekofisk crude oil after 20 Days incubation with Pseudomonas fluorescens in free and polyester polyurethane immobilized systems.*

The weight of Ekofisk crude oil added to each system before incubation and at the end of the 20 day period was recorded. Figures 3.8 and 3.9 illustrate the weight of oil (g) degraded over 20 days. A significant difference (p = 0.027) was found in the weight of oil lost from the control, the free and polyurethane immobilized (non-supplemented) system over 20 days. A follow-up test, LSD, was carried out on the data which showed there was a significant difference between the weight lost in the control and free system and the control and the polyurethane immobilized (non-supplemented) system. The free and polyurethane immobilized system having lost significantly more Ekofisk crude oil by weight than the control. There was no significant difference in the weight of Ekofisk crude oil lost in the control, the supplemented and the non-supplemented polyurethane immobilized systems.
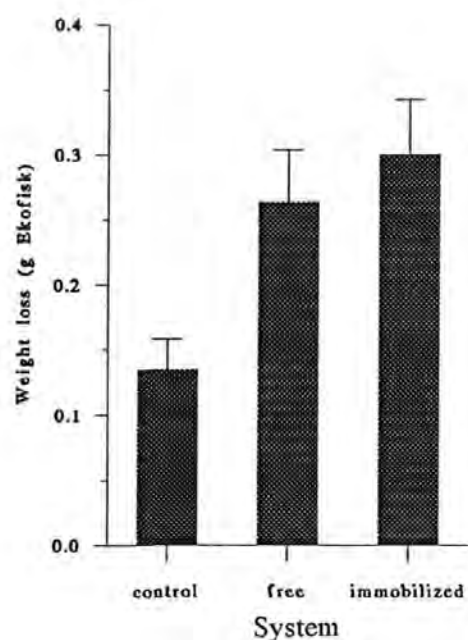
Figure 3.8. Average weight loss of Ekofisk crude oil after 20 days incubation with *Pseudomonas fluorescens* in seawater in free and polyester polyurethane immobilized systems. Bars indicate standard error.
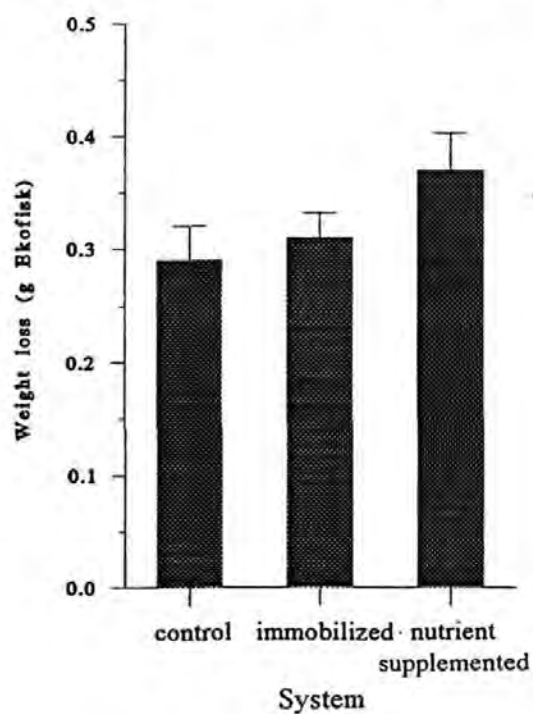
Figure 3.9. Average weight loss of Ekofisk crude oil after 20 days incubation with *Pseudomonas fluorescens* in seawater immobilized on polyester polyurethane with and without nutrient supplementation. Bars indicate standard error

3.3.6.3. *Ekofisk crude oil analysis.*

The extracted Ekofisk crude oil samples were run through the gas chromatograph. Table 3.2 shows the percentage of each carbon degraded after 20 days. There was no significant difference between the amount of C_{12} - C_{18} degraded in any of the systems.

Table 3.2. Percentage carbon degradation of Ekofisk crude oil after 20 days incubation with free *Pseudomonas fluorescens* and *Pseudomonas fluorescens* immobilized on polyester polyurethane with and without nutrient supplementation in seawater systems.

system	*Percentage carbon degraded						
	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}
control	69.8	70.2	65	63.3	76.1	69.5	68
free	53.4	49	47	50.3	82.2	61	64.3
immobilized	34.1	20.2	16.6	19.4	57.9	49.4	52.4
Control	78.1	67.9	65.7	69	81	76	75.5
Immobilized	90.6	80.4	74.4	71	79	72.1	72.2
Immobilized (supp)**	85.8	81	80	76.5	82	76.7	77.7

*Figures are mean values of two samples and are adjusted to allow for volatilization and extraction errors.
**Nutrient supplemented

3.3.7. *The effect of drying, and storage over time on the recoverability of Drizit, Biofix and polyester polyurethane immobilized Pseudomonas fluorescens.*

After drying polyurethane immobilized cells at room temperature and storing at this temperature for 1 week, high numbers of cells were recovered from the polyurethane, both with nutrient supplementation and without. The number of cells recovered fell from 2×10^9 cfu cube^{-1} to 2.95×10^8 cfu cube^{-1} and 1.17×10^9 cfu cube^{-1} to 1.74×10^8 cfu cube^{-1} respectively (figure 3.11). The numbers of cells recovered from Drizit and Biofix fell from 1.48×10^7 cfu 100 mg^{-1} to 1×10^2 cfu 100 mg^{-1} and 2.69×10^6 cfu 100 mg^{-1} to 1×10^3 cfu 100 mg^{-1} respectively (figure 3.10). In all

cases there was a significant difference between the number of cells recovered before drying and storage in comparison to the numbers recovered after drying and 1 weeks storage (p = 0.0). Over the period of storage, cells maintained on polyurethane with nutrient supplementation were recoverable for the full 3 months with a final count of 3.89×10^2 cfu cube^{-1}. In the polyurethane system (no supplementation) and Drizit system cells were not recoverable after 2 weeks and in the Biofix system after 2 months.

After drying polyurethane (non-supplemented and supplemented) immobilized cells and Drizit immobilized cells at 55°C and storing for 1 week at room temperature no cells were recovered (figure 3.10b and figure 3.11). Biofix maintained recoverable cells for 2 months, but after drying and 1 weeks storage the level of recoverable cells had fallen significantly (p = 0.0) from 2.69×10^6 cfu 100 mg^{-1} to 2.34×10^2 cfu 100 mg^{-1} (figure 3.10a).

Recoverable cells were maintained on all four support matrices after freeze drying and storage for 1 week at room temperature, in all cases there was a significant difference between the number of cells recovered at time zero and at 1 week (p = 0.0). Recoverable cells were maintained for 3 months in the two polyurethane matrices (figure 3.11), with the nutrient supplemented system producing more recoverable cells than the same system with no supplementation. In the Biofix system (figure 3.10a) the cells were not recoverable after 2 weeks (8.5×10^1) and in the Drizit system (figure 3.10b) after 2 months (2.4×10^1 cfu 100 mg^{-1}).

a)
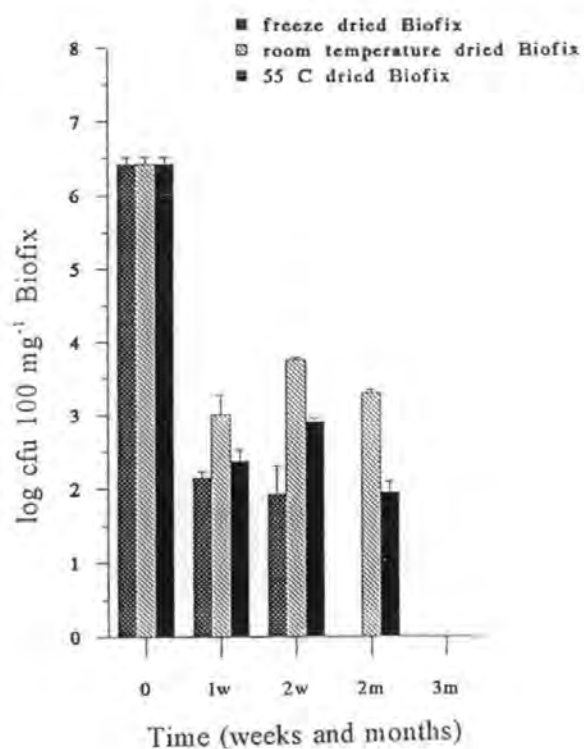
b)
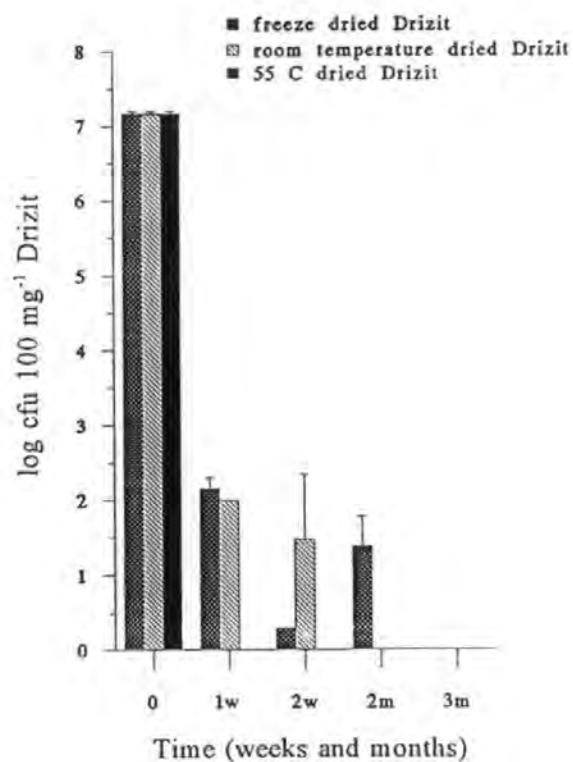
Figures 3.10. Average viable counts of *Pseudomonas fluorescens* immobilized on a) Biofix and b) Drizit, after drying and storage for 3 months. Bars indicate standard error.
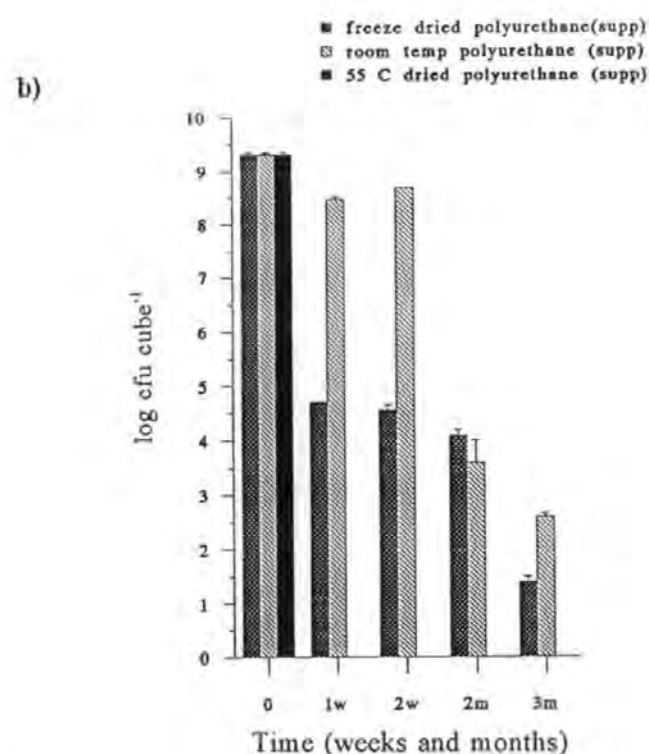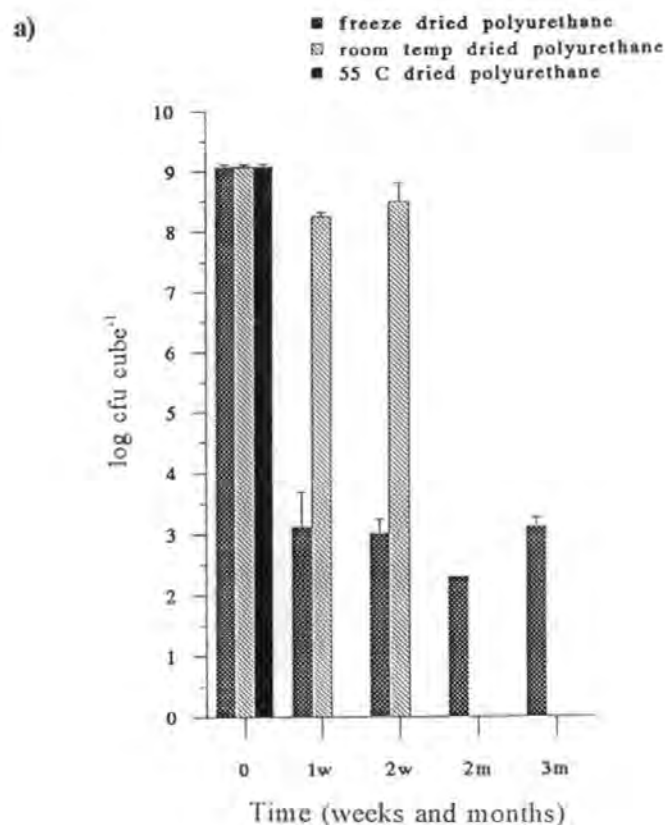
Figure 3.11. Average viable counts of *Pseudomonas fluorescens* immobilized on polyester polyurethane a) Non - supplemented b) Nutrient supplemented, after drying and storage for 3 months. Bars indicate standard error.

3.4. Discussion.

This study compared three supports as immobilization substrata and their ability to be used as bioremediation agents. Scanning electron microscopy or Normarski differential interference contrast microscopy were used to illustrate and allow the comparison of the structure of each support. Figures 3.1 - 3.5 provide information on the composition of the supports and evidence for the immobilization of bacteria to each substratum, although no specific mechanisms involved in the attachment of the bacteria to the biocarrier can be indicated.

With knowledge of the structure of each biocarrier, interest arises on the extent to which each support can immobilize bacteria. A previous study by Salter *et al.* (1990) has characterized the cell loading of Biofix C2 by varying the volume of suspension used to load the column and an investigation has been carried out to estimate the biomass within the packed column. However, no work has been carried out to compare the loading capabilities of different biocarriers over time.

The results presented in this study demonstrate that by extending the loading phase of the biocarrier the amount of protein immobilized can be significantly increased. After two days incubation the amount of protein immobilized on polyurethane fell, this may have been due to all available nutrients being utilized or the build-up of toxic waste, resulting in cell sloughing. Therefore at day two, the polyurethane was washed in PBS and the nutrients replenished. This caused the amount of protein immobilized to return to a level close to that obtained after 2 days incubation.

Biofix appeared to reach maximum capacity of immobilized protein after 5 days. At day 5 the substratum was washed in PBS, due to the formation of a film of bacteria across the surface of the Biofix in the syringe, preventing the efficient pumping of inocula around the system. This resulted in a dramatic fall in the amount of protein immobilized, indicating washout of bacteria from the substratum may have occurred. After the initial drop the level of immobilized protein rose, approaching the maximum reached at day 5.

Drizit immobilized cells reached their maximum capacity at day 2, the maximum reached was lower than that achieved by polyurethane and Biofix. No washing or nutrient replenishment was carried out on the biocarrier due to the low level of immobilization achieved. Little fluctuation in the level of immobilized protein was seen compared with Polyurethane and Biofix from day 2 to 8.

The absorbency of oil to each substrata was investigated and the three biocarriers showed a significant difference in their absorbtion of Ekofisk crude oil. Due to the porous nature of polyester polyurethane, its level of absorbtion of Ekofisk crude oil was higher than that of Biofix and Drizit. Biofix had the lowest level of absorbtion of Ekofisk crude oil. Using an absorbent matrix may not only increase the availability of the oil to the bacteria but reduce the toxicity of some oils to the bacteria, for example O'Reilly and Crawford (1989b) have shown that the absorbtion of pentachlorophenol to a polyurethane matrix containing immobilized cells protects the cells from pentachlorophenol toxicity.

Immobilization of *P. fluorescens* on polyurethane and incubation with Ekofisk crude oil in seawater for 20 days, did not result in any enhancement of biodegradation, with or without nutrient supplementation in comparison to the free system and the uninoculated control. There was no significant difference in the viable counts after 20 days incubation between the free system and the polyurethane immobilized system and between the supplemented and non-supplemented polyurethane immobilized systems. The least significant difference, follow up test carried out on the supplemented and non-supplemented systems showed a significant difference between the viable counts in the two systems, the polyurethane immobilized system with nutrient supplementation having a significantly higher viable count. However, no concomitant enhancement of biodegradation of Ekofisk crude oil was observed with the increase in viable count. The supplemented nitrates and phosphates were added to nutrient broth before being added to the seawater culture, the addition of nutrient broth gave the bacteria an alternative, more accessible carbon source than that found in the non-supplemented system. This carbon source was however present in small quantities (1 ml nutrient broth 100 ml^{-1} seawater), so it would appear that the

bacteria may also have been obtaining their carbon from another source. The only other degradable source of carbon in the immobilized systems was that of the polyurethane itself, but this was present in both the supplemented and non-supplemented systems. When comparing the viable counts and percentage degradation in the free and immobilized systems, there was no significant difference in the viable counts in the two systems but the free system showed an increase in degradation of the n-alkanes in Ekofisk crude oil. This data indicates that the immobilized bacteria may have had access to an alternative carbon source, with the result that the viable counts in the free and immobilized systems were very similar. Studies have shown that polyurethane is degradable by fungi (Evans and Levisohn 1968; Kaplan *et al.* 1968; Jones and Le-Campion Alsumard 1970; Filip 1979; Pathirana and Seal 1985) and bacteria (Hedrick and Crum 1968; Cameron *et al.* 1988; Jansen *et al.* 1991). Further studies are required to investigate the degradation of polyurethane by *P. fluorescens*.

A significant difference in the weight of Ekofisk crude oil lost after 20 days incubation was found between the polyurethane immobilized (non-supplemented) system, the free system and the control. A follow up test (least significant difference) showed a significant difference between the weight of oil lost in the control and free system and the control and the polyurethane immobilized system. The free and polyurethane immobilized system lost significantly more Ekofisk crude oil by weight than the control. These results indicate that more oil was degraded when incubation with the bacteria took place, but that the process of immobilization on polyurethane did not affect the degradation rates. When analysis of the individual carbons was examined no significant difference was found between the amount of C_{12} - C_{18} degraded in any of the systems. This would indicate that any differences found in weight loss may have been due to extraction errors.

Previous work (chapter 2) however, has reported enhanced biodegradation of petrol (Slovene diesel) with immobilization of *P. fluorescens* on Biofix and Drizit in a freshwater system and Drizit in a saltwater system. Higher levels of growth and degradation of the n-alkanes C_{12} - C_{18} in petrol (Slovene diesel) were achieved by immobilized *P. fluorescens* when compared with free living

bacteria. For example, in the freshwater systems the free system had a lag phase of six days during which no growth occurred. Immobilization of cells on Biofix reduced the lag phase to four days and Drizit immobilized cells reduced the lag phase to one day. It appears therefore that the process of immobilizing cells to Biofix and Drizit, in contrast to polyester polyurethane (nutrient supplemented and non-supplemented) accelerates the ability of the cells to initially utilize the petrol (Slovene diesel), and also results in a higher level of utilization of this carbon source.

The three biocarriers were compared in their ability to maintain viable cells after drying by different mechanisms and storage over three months. Results indicate differences depending on the matrix and the drying mechanism used. Drying polyurethane immobilized cells (nutrient supplemented and non-supplemented) at room temperature and storage at this temperature for 1 week maintained high levels of recoverable cells in comparison to the two other biocarriers. However, this mechanism did not result in complete removal of moisture from the polyurethane until approximately 1 month, with resulting contamination of some of the cubes. High levels of residual moisture in the dried product may have detrimental effects (Malik 1991) and levels of available nutrients, substantial enough to increase the survival period may have been maintained in an accessible form in the polyurethane but with concomitant contamination. This problem was not encountered with the Drizit and Biofix, which were dry after the 1 week storage period, but a lower number of cells were recovered in comparison to the polyurethane. The addition of nutrients to the polyurethane sustained the recovery of cells for the three month storage period which did not occur in any other matrix. This survival may also be attributed to the remaining moisture on the cubes causing a level of available nutrients elevated above that seen in the other biocarriers. In comparison, in the unsupplemented polyurethane matrix recovery was only maintained for 2 weeks and Drizit and Biofix cells were recovered for 2 weeks and 2 months respectively.

No cells were recovered from polyurethane (supplemented and non-supplemented) and Drizit after drying at 55°C and storage for 1 week, possibly due to the inactivation of micro-organisms by heat (Strange 1976). Biofix was the only biocarrier to maintain recoverable cells after drying at 55°C

and storage for 1 week, which may indicate Biofix can convey protection to cells from excessive heat. The support is known to be thermally stable to 1,000°C and can be autoclaved without loss of surface area, porosity or activity (Salter *et al.* 1990). However, although recovery of the cells were maintained for 2 months, cell injury may still have occurred and further work is required to determine the effect of heat on Biofix immobilized cells.

After freeze drying and storage for 1 week, recoverable cells were maintained on all four support matrices. Recoverable cells were maintained for three months in the two polyurethane matrices, with the supplemented system generally maintaining a higher level of recovery, indicating that the supplementation of nutrients to the cells prolongs their survival. A significant decrease was seen in the number of recoverable cells after drying and storage for 1 week in all cases, but the polyurethane immobilized cells (supplemented and non-supplemented) showed no further large decreases in the number of cells recovered over the 3 month storage period.

The large differences seen in viability counts before and after drying could mean that the survivors may not be fully representative of the original culture (Snell 1991). Therefore, after any drying and storage the characteristics of the survivors should be compared to the original culture. This would include determining whether the cells have maintained their ability to not only degrade hydrocarbons but also to enhance degradation of hydrocarbons in comparison to a free system of the bacteria.

These data indicate that with the addition of nitrates and phosphates into the matrix, enhanced recovery of the cells after drying at room temperature and freeze drying is achieved. Further work is required to investigate whether the addition of nitrates and phosphates into Biofix and Drizit immobilized cells would, firstly increase cell recovery after drying and storage for 1 week and secondly extend the period of recovery during storage.

From the data presented, freeze drying appears to be the most suitable drying mechanism tested, with recoverable cells maintained on all the biocarriers for 2 weeks, on Drizit for 2 months and the two polyurethane systems for the full 3 months. Freeze drying is a standard technique for preserving free suspensions of bacteria and one major advantage of freeze-drying over many other preservation methods is that material can be kept stable over a period of many years without the need for special storage conditions (Hill 1981; Malik 1991; Rudge 1991). Bacteria preserved in this way usually have a suitable suspending fluid to prevent overdrying and to protect the bacteria from mechanical and chemical damage during both drying and storage. Protective agents were not included in this study during drying to determine whether the substrata conferred any protection during dehydration, the only substrata to convey any such protection was Biofix during drying at 55°C. A suitable protective agent frequently used is 10% (w/v) solution of skimmed milk containing 3% (w/v) *meso*-inositol or 5% (w/v) honey (Malik 1991). The incorporation of a protective agent into the substrata may further improve immobilized cell recovery rate, and further work is required to investigate the use of protective agents with immobilized cells. Alternatively, storing the dried product at a lower temperature may result in improved cell recovery, the higher the storage temperature the faster a product will degrade. Thus, the storage of dried cultures at lower temperatures will extend the shelf life, similarly, sealing the dried product under vacuum prolongs the shelf life (Malik 1991). Further work is required to test these methods on immobilized bacteria.

The data presented has shown that both the drying technique and the biocarrier are important factors when considering the drying and storage of immobilized cells. If immobilized cells are to be stored before use, both of these factors should be considered when determining which biocarrier would be the most suitable for use as a bioremediation agent.

The data demonstrate that the enhancement of biodegradation rates of hydrocarbons by immobilized cells appears to be greatly dependent on the biocarrier and culture conditions used. No enhanced degradation of Ekofisk crude oil occurred with polyester polyurethane immobilized cells and it can

be concluded that this material is unsuitable as a biocarrier for use in bioremediation. However, due to polyurethane having a high absorbency of oil and a high cell loading capacity it may be beneficial to carry out work on a more resilient polyurethane e.g a polyether, which is less susceptible to microbial attack (Jones and Le-Campion Alsumard 1970; Wales and Sagar 1991). Polyurethane may also be a suitable biocarrier for different strains of bacteria that do not have the ability to utilize such a diverse range of carbon sources.

In a freshwater system Biofix and Drizit achieved a significantly higher level and a faster rate of biodegradation than seen with free living bacteria, there was no significant difference between the levels of biodegradation per µg protein achieved by the two immobilization systems, although the Drizit immobilized cells reduced the lag phase more than the Biofix immobilized cells (section 2.4). A lower capacity of protein was immobilized to Drizit over the eight day period in comparison to Biofix, but a more stable level was achieved. Drizit immobilized cells also absorbed a greater amount of Ekofisk crude oil per gram of substratum than Biofix, was an easier material to handle in comparison to Biofix and the process of cell immobilization more straightforward and cost effective. In conclusion, all the data discussed above indicates that Drizit was the most effective biocarrier tested and the most suitable immobilization substratum used as a bioremediation agent under the conditions stated. The careful choice of biosupport, picked to suit the task required will be essential for the successful development of specific/further applications.

Chapter 4

The Effect of Immobilization on the Production of Biosurfactant (Rhamnolipid) by *Pseudomonas fluorescens*

4.1. Introduction.

The rate of biodegradation of hydrocarbons is dependent on many different factors (section 1.2), with the ability of the bacteria to synthesize enzymes capable of metabolizing specific compounds being essential. If there are no microbes that have the enzymes capable of degrading the molecule, then the compound may be recalcitrant and remain in the environment. However, the presence of the necessary catabolic enzyme systems alone does not ensure rapid and efficient biodegradation. Hydrocarbon bioavailability is also an important consideration in overall rate of compound degradation as discussed previously in sections 1.4 and 2.4. The ability of hydrophobic compounds to be solubilized and transported to the intracellular location of the degradative enzyme systems, which involves movement across the cell wall and cell membrane barriers, may be the slowest and therefore the rate-limiting step in the process of biotransformation (Churchill *et al.* 1995). There is a three-way interaction involving biosurfactant production, the substrate and the cell. The effect of surfactant on the bioavailability of insoluble organic compounds can be explained by several different mechanisms.

1)A reduction in interfacial tension between the aqueous phase and non-aqueous phase causing the dispersion of non-aqueous phase liquid hydrocarbons and a resulting increase in contact area (only when liquid-phase hydrocarbons are present).

2)The formation of micelles (aggregates of 10-200 molecules)which can contain high concentrations of hydrophobic organic compounds, resulting in increased solubility of the pollutant.

3)Transfer of the insoluble organic compound from the solid phase to the aqueous phase, e.g interaction of the biosurfactant with solid interfaces and interaction of the compound with single biosurfactant molecules.

(Volkering *et al.* 1995)

For example, Zhang and Miller (1995) showed that the uptake of octadecane was faster than hexadecane in the absence of biosurfactant, but the reverse is true when biosurfactant is added to the system due to greater increase in the dispersion of the liquid alkane than that of the solid alkane.

Reddy *et al.* (1982) working with *Endomycopsis lipolytica (Saccharomycopsis lipolytica), Candida tropicalis* and a *Pseudomonas* strain, described how submicron droplets of hydrocarbon may be produced by three different mechanisms; 1) agitation. 2) the action of an emulsifier produced by the cells whose presence would stabilize and increase the production of submicron droplets. 3) the action of a specific solubilizer which is distinguished from the emulsifier by its specificity towards the growth substance. The solubilizer can produce readily available submicron droplets more extensively than the action of the emulsifier or agitation. However the emulsifier may help solubilization by increasing the surface area of the hydrocarbon available for interaction with the solubilizing factor (Reddy *et al.* 1983).

Most microorganisms produce biosurfactant mixtures that are structurally similar but have different physicochemical properties. Biosurfactant structure is a characteristic of the producing species and the available carbon source during growth. Biosurfactant structures may play different roles in hydrocarbon metabolism although it is not clear how structure affects degradation rates. It has been established that biosurfactant solubilization and dispersion of organic compounds which are related to interfacial tension are dependent on biosurfactant structure (Lang *et al.* 1984 as cited by Zhang and Miller 1995; Zajic and Mahomedy 1984).

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Biosurfactants typically have a hydrophilic and lipophilic moiety and depending on the concentration of the two and the growth conditions of the bacteria, biosurfactants may aggregate to form micelles or reversed micelles and accumulate at liquid/liquid, liquid/gas and liquid/solid interfaces. The term biosurfactant has also been used to describe compounds which do not reduce interfacial tension but may prevent oil drops from coalescing, these compounds usually have similar compositions to the cell wall or capsules and should be termed bioemulsifiers (Pines and Gutnick 1986; Gutnick and Minas 1987). The lipophilic portion of the biosurfactant is usually composed of the hydrocarbon (alkyl) tail of one or more fatty acids which may be saturated, unsaturated, hydroxylated or branched and which is linked to the hydrophilic group by a glycosidic, ester or amide bond. The hydrophilic moieties may be mono-, di or polysaccharides or simple carboxylate groups and it is this portion which is responsible for the degree of solubility of the biosurfactant in water Most biosurfactants are either neutral or negatively charged due to the presence of carboxylate groups (Haferburg *et al.* 1986).

In contrast to the number of alkane degrading microbes, there is only a limited number of bacteria and yeasts known to produce interfacially active compounds and most of them have been described in connection with growth of microorganisms on hydrocarbons (Hommel 1990). Among the most frequently occurring alkane utilizing microorganisms, the following genera are known as producers of biosurfactants or emulsifying polymers, *Pseudomonas, Acinetobacter, Arthrobacter, Candida* and *Rhodococcus.* (Rapp *et al.* 1979; Kretschmer *et al.* 1982; Reddy *et al.* 1982; Syldatk *et al.* 1985a, 1985b; Haferburg *et al.* 1986; Pines and Gutnick 1986; Gutnick and Minas 1987; Hommel 1990; Schulz *et al.* 1991; Bury and Miller 1993).

Biosurfactant producing microbes can be divided into three groups with respect to alkane utilization and the synthesis of extracellular lipids :

1. Microbes which produce biosurfactants exclusively during growth on alkanes.

2. Microbes which produce biosurfactants on both alkanes and water-soluble compounds.

3. Microbes which exclusively produce biosurfactants during growth on water soluble compounds (Haferburg *et al.* 1986).

The biosurfactant may be cell-bound in some cases and cell-free in others. It is also possible if the culture conditions are changed that the relative distribution of cell-bound and cell-free forms changes (Gutnick and Minas 1987). When the biosurfactants are extracellular they cause emulsification of the hydrocarbon. When they are cell wall-associated they facilitate the penetration of hydrocarbon to the periplasmic space (Lang and Wagner 1987)

Biosurfactants can then be grouped into classes depending on the character of the hydrophilic moiety; 1) glycolipids, 2) lipopeptides, 3) fatty acids, 4) phospholipids, 5) neutral lipids (Hommel 1990). Glycolipids are the group of biosurfactants produced by hydrocarbon-utilizing bacteria, included in the glycolipids are the sophorose and mannosyl-erthritol lipids produced by *Candida* (Haferburg *et al.* 1986; Hommel 1990), trehalose lipids produced by *Rhodococcus ertytropolis*, (Rapp *et al.* 1979; Kretschmer *et al.* 1982) and by *Acintomycetes* (Haferburg *et al.* 1986; Hommel 1990), and the rhamnolipids produced by *Pseudomonas* species (Itoh *et al.* 1971; Syldatk *et al.* 1985a, 1985b; Mulligan and Gibbs 1989; Zhang and Miller 1995) Some of the biosurfactants such as the sophorosides, rhamnolipids or peptidolipids should be attributed to secondary metabolism, others such as the trehalose esters are similar to existing cell wall constituents, and may be involved in cellular adaptation to hydrophobic growth substrates (Haferburg *et al.* 1986; Gutnick and Minas 1987; Hommel 1990). Subsequent paragraphs will concentrate on the production of glycolipids by the *Pseudomonas* species.

The sugar moiety of rhamnolipids produced by the *Pseudomonas* consists of one or two rhamnose (6-deoxy-L-mannose) units glycosidically linked with one or two β-hydroxydecanoic acid units. Reddy *et al.* in 1982 and 1983 indicated the extracellular lipid produced by *Pseudomonas aeruginosa* was composed of two compounds and with work carried out in 1965 by Edwards and Hayashi. as cited by Syldatk *et al.* (1985a) up to four rhamnolipids have been identified, R1-R4,

depending on the culture conditions used. R2 and R4 possess only one β-hydroxydecanoic acid in comparison with rhamnolipids R1 and R3 which contain two (Syldatk *et al.* 1985a). The critical micelle concentration of the purified rhamnolipids R1-R4 are in the range of 2 to 200 mg l^{-1} depending on the particular rhamnolipid, so these compounds are only required in small amounts to allow the growth of the *Pseudomonas* species on hydrocarbons (Syldatk *et al.* 1985a). Rhamnolipids produced by the *Pseudomonas* species are formed from cultures growing on hydrocarbons or water soluble carbon sources, and both nitrogen-limited growing cultures or resting cells on n-alkanes have been shown to produce high levels of lipids (Syldatk *et al.* 1985a, 1985b). Rhamnolipids are ionic biosurfactants which pseudosolubilize the alkane which in turn increases the surface area available, non-ionic biosurfactants like the trehalose lipids render the charged cell surface hydrophobic which should then facilitate the attachment and subsequent passive transport of alkanes into the cell (Rapp *et al.* 1979; Syldatk and Wagner 1987). Thus the role of biosurfactants is a complex process and data to present supports the idea that biosurfactants play an important role in alkane utilization by increasing the bioaccessibility of the hydrocarbon to the cell (Espeche *et al.* 1994; Iqbal *et al.* 1995; Zhang and Miller 1995).

This study attempts to follow the production of the biosurfactant rhamnolipid by *P. fluorescens* during growth on petrol (Slovene diesel) when in free suspension and during immobilized bacteria technology enhanced growth, to determine whether immobilization affects the production of biosurfactant by *P. fluorescens* and to investigate whether this could explain the enhanced degradation and acceleration of utilization of petrol (Slovene diesel) observed after immobilization (chapter 2). Studies using *Endomycopsis lipolytica (Saccharomycopsis lipolytica), Candida tropicalis* and a *Pseudomonas* strain, have shown ethylene diamine tetraacetic acid (EDTA) can strongly inhibit hydrocarbon pseudosolubilization (Rapp *et al.* 1979; Reddy *et al.* 1982; Reddy *et al.* 1983). This study also investigates the use of EDTA as an inhibitor of rhamnolipid production.

4.2. Materials and methods.

4.2.1. *Bacterial isolates.*

Details of the bacterial isolates used throughout this study can be found in section 2.2.1.

4.2.2. *Media.*

Details on the media used throughout this study can be found in section 2.2.2.

4.2.3. *Whole cell immobilisation.*

4.2.3.1 Cell loading of Biofix and Drizit.

The method on cell loading of Biofix and Drizit is detailed in section 2.2.3.

4.2.4. *Cellular growth and biosurfactant (rhamnolipid) production by free, and Drizit and Biofix immobilized Pseudomonas fluorescens.*

Bacteria in free suspension were centrifuged at 13000 rev min⁻¹ (12 x 1.5 ml head, MSE Micro-centaur) for 3 minutes and resuspended in PBS. Resuspended free bacteria or immobilized bacteria were added to give 2.5 mg protein 100 ml⁻¹ minimal media in 250 ml Erlenmeyer flasks. The flasks were incubated at 30°C on an orbital shaker (100 rev min⁻¹) for five days. Controls were set-up with no carbon source and all treatments and analyses were performed in duplicate.

4.2.4.1. *Cellular growth.*

Cellular growth in the free suspension of the free and immobilized systems was estimated daily in duplicate by measuring absorbance at 590 nm using a spectrophotometer (CE 1010, Cecil).

4.2.4.2. *Biosurfactant (rhamnolipid) extraction and measurement.*

Samples (5 ml) were removed daily in duplicate from flasks and rhamnolipid extracted using the method of Zhang and Miller (1992). Cells were removed by centrifugation at 9000 rev min⁻¹ (8 x 50 ml head, MSE, Europa 24m) for 20 minutes. The rhamnolipid was precipitated from

the supernatant by acidification (1 M H_2SO_4) to pH 2.0 and pelleted by centrifugation at 12,100 rev min^{-1} for 20 minutes. The pellet was dissolved in 0.05 M bicarbonate (pH 8.6, BDH) and the resulting suspension centrifuged at 12,100 rev min^{-1} for 20 minutes. The remaining precipitate was extracted three times with chloroform-ethanol (2:1, BDH) and then rotary evaporated (80°C) to remove the solvents. The remaining biosurfactant was dissolved in 2 ml of 0.05 M bicarbonate and rhamnolipid was quantified by measurement of L-rhamnose using the 6-deoxyhexose method (Chandrasekaran and BeMiller 1980). Sulphuric acid (BDH) solution (4.5 ml), (90 ml sulphuric acid added to 15 ml distilled water) was added to 1 ml biosurfactant solution and heated in boiling water for 10 minutes. After cooling, 0.1 ml of thioglycollic acid (Sigma) solution (0.1 g thioglycollic acid in 3 ml of water) was added, mixed and left in the dark for 3 hours. The absorbance was measured at 420 nm using a spectrophotometer (CE 1010, Cecil). Standard curves were prepared with L-rhamnose (BDH, appendix 4).

4.2.5. *Effect of EDTA on the growth and biosurfactant (rhamnolipid) production of free and Drizit immobilized* Pseudomonas fluorescens.

Bacteria in free suspension were centrifuged at 13000 rev min^{-1} (12 x 1.5 ml head, MSE Micro-centaur) and resuspended in PBS. Resuspended free bacteria or bacteria immobilized to Drizit were added to give 2.5 mg protein 100 ml^{-1} minimal media in 250 ml Erlenmeyer flasks. Minimal media was supplemented with 4 mM EDTA (Sigma) and compared to unsupplemented control flasks. The cultures were incubated at 30°C on an orbital shaker (100 rev min^{-1}) for five days and sampled for growth and rhamnolipid production daily as previously. All treatments and analyses were performed in duplicate.

4.2.6. *Statistical analysis.*

Multifactor analysis of variance was carried out using Statgraphics, version 6.1 to determine any significant differences in growth in each system over time.

4.3. Results.

4.3.1. *Cellular growth and biosurfactant (rhamnolipid) production by* <u>*Pseudomonas fluorescens*</u> *in free, and Drizit and Biofix immobilized systems.*

4.3.1.1. *Cellular growth.*

Figure 4.1 shows typical growth curves of *P.fluorescens* in the aqueous suspensions of the free, and Biofix and Drizit immobilized systems incubated with petrol (Slovene diesel) over five days, the graph illustrates the significant difference (p = 0.029), between the levels of growth in the free suspension of the four systems, with absorbance levels varying significantly over time within each system (p = 0.0) and between systems (p = 0.0). All four systems showed a small increase in absorbance and therefore growth until day one, after which the immobilized systems continued to increase significantly in absorbance, with the bacteria in free suspension from the Biofix system increasing at a faster rate than that in the Drizit immobilized system.
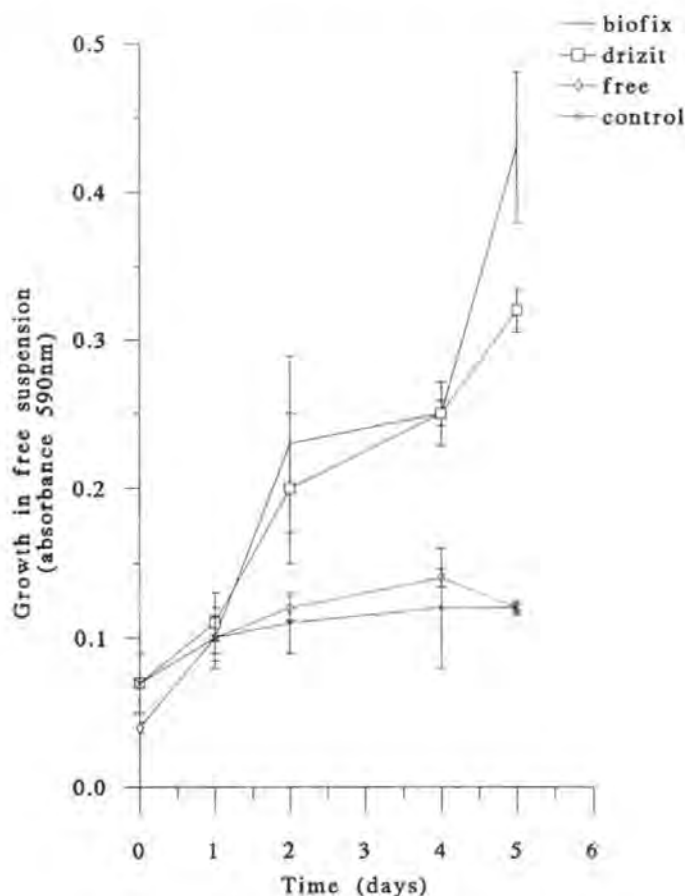
Figure 4.1. Typical growth curves of *Pseudomonas fluorescens* in the aqueous suspensions of free and Drizit and Biofix immobilized systems incubated with petrol (Slovene diesel). Bars indicate standard error.

The free system after the initial increase showed marginal growth, a little above the level of the control.

4.3.1.2. *Biosurfactant (rhamnolipid) extraction and measurement.*

Figure 4.2 shows the cumulative µg rhamnolipid ml^{-1} produced by *P. fluorescens* over four days and illustrates the difference in the amount of rhamnolipid produced in the four systems over the incubation period. There was sustained increase in total cumulative rhamnolipid production in the immobilized systems over the first three days of incubation in comparison to the free system and the control. The Biofix system showed an increased cumulative production in comparison to the Drizit system. The free system showed a lag phase of two days where the amount of rhamnolipid produced remained constant, after which the rate of production increased and by day four reaches a level comparable to that of the immobilized systems. The control showed a lag phase of three days after which the rate of production increased but to a lower cumulative level than in the free system.
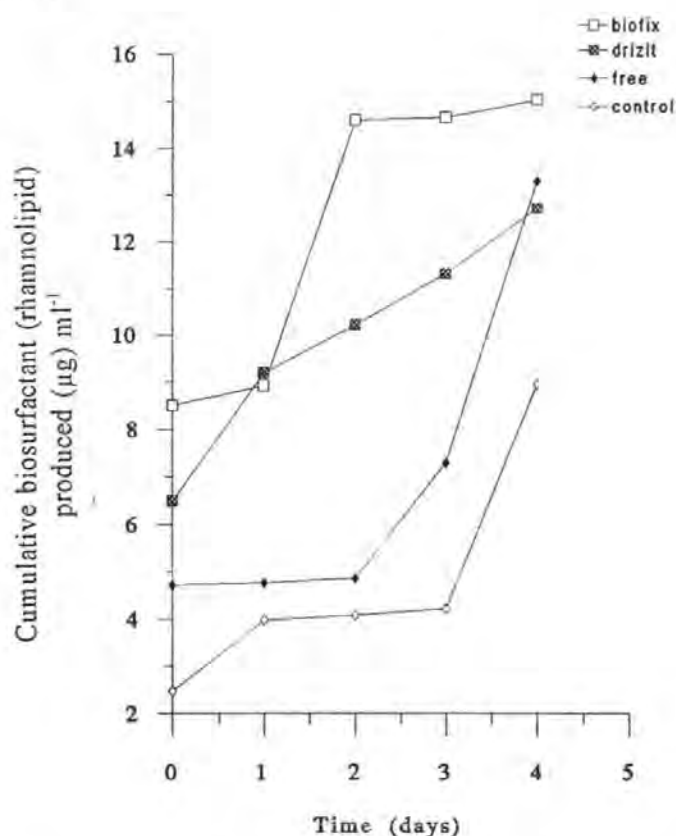
Figure 4.2. Cumulative µg biosurfactant (rhamnolipid) produced in aqueous suspension by *Pseudomonas fluorescens* in free and Drizit and Biofix immobilized aqueous systems incubated with petrol (Slovene diesel).

Figure 4.3 shows the cumulative μg rhamnolipid produced per optical density unit of broth culture. This shows that cellular rhamnolipid production in the immobilized systems is maintained at a higher level over the first four days of incubation and occurs at a faster rate for the initial two days than in the free and control systems. The Biofix system showed an increased rate of production until day three after which rhamnolipid production by the Drizit immobilized cells rose above that seen in the Biofix system. The free and control system showed a lag phase lasting two and three days respectively before any increase in production was seen. The level of rhamnolipid production per optical density unit of broth culture in the free system and the control remained at a lower level than that seen in the immobilized systems over the five days incubation period.
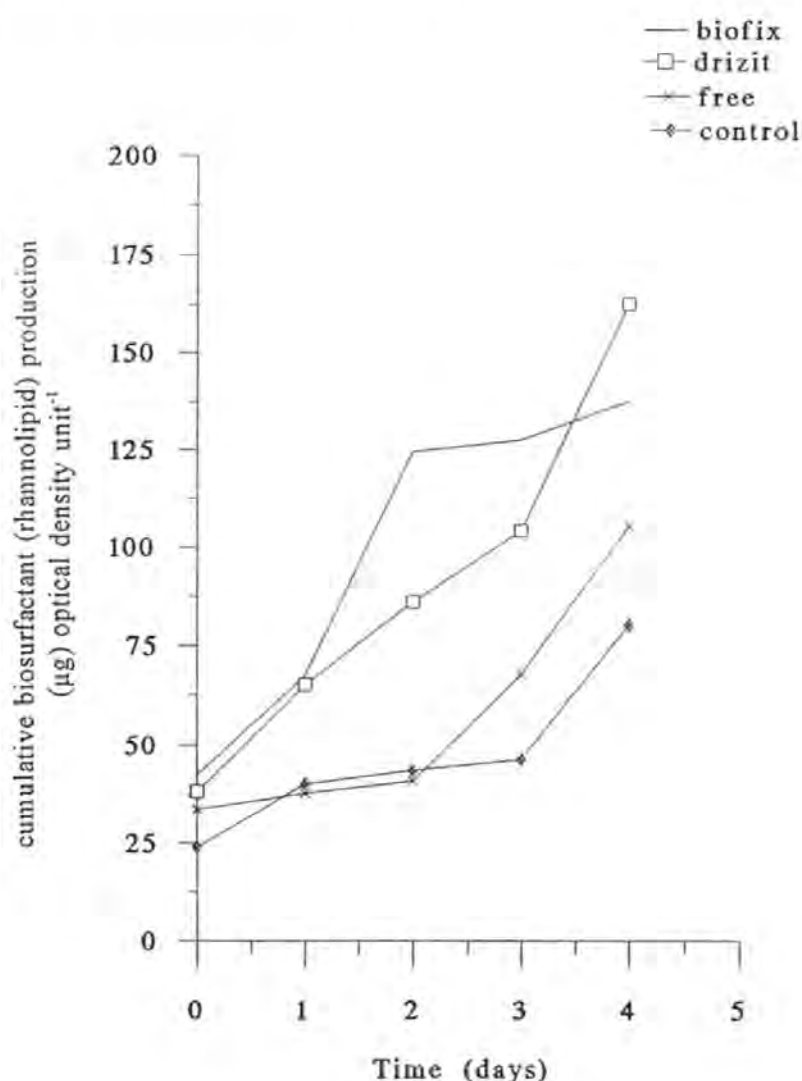
Figure 4.3. Cumulative μg biosurfactant (rhamnolipid) per optical density unit produced in the aqueous suspension of *Pseudomonas fluorescens* in free and Drizit and Biofix immobilized systems incubated with petrol (Slovene diesel)

4.3.2. *Effect of EDTA on the growth and biosurfactant (rhamnolipid) production of Pseudomonas fluorescens in free and Drizit immobilized systems.*

Growth in the aqueous suspensions of the free system and Drizit immobilized system with and without EDTA additions were measured over four days (figure 4.4). In the free system growth on each day was unaffected by the addition of EDTA, however in the immobilized systems growth was significantly decreased (p = 0.0). In the free system growth with and without EDTA supplementation followed a similar pattern until day three, after which the absorbance in the EDTA supplemented system fell. In the Drizit immobilized system with EDTA supplementation, growth until day two was higher than that seen in the unsupplemented system, after day two growth in both immobilized systems followed a similar pattern (figure 4.4). The rhamnolipid production in the free and Drizit immobilized system decreased with EDTA addition (figure 4.5).
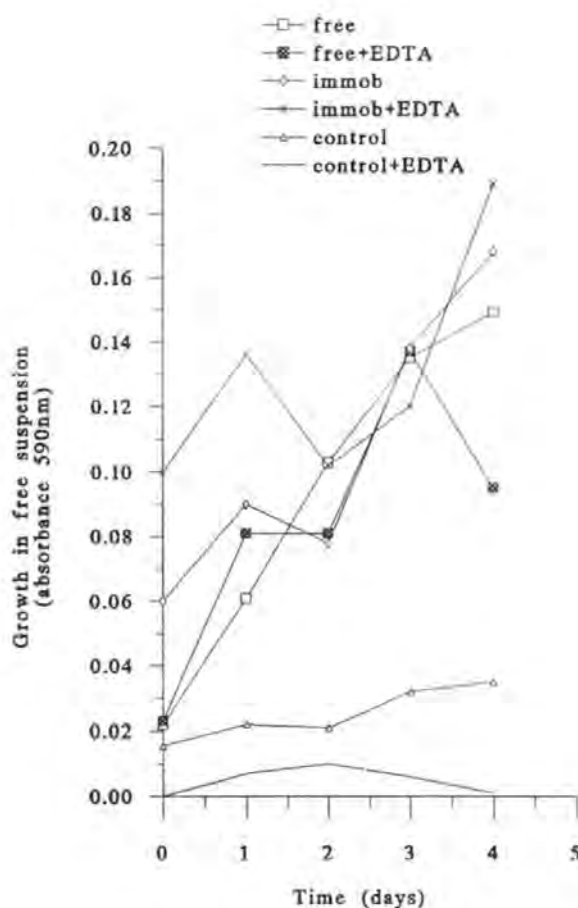
Figure 4.4. The effect of EDTA on the growth of *Pseudomonas fluorescens* on petrol (Slovene diesel) in the aqueous suspensions of free and Drizit immobilized systems.
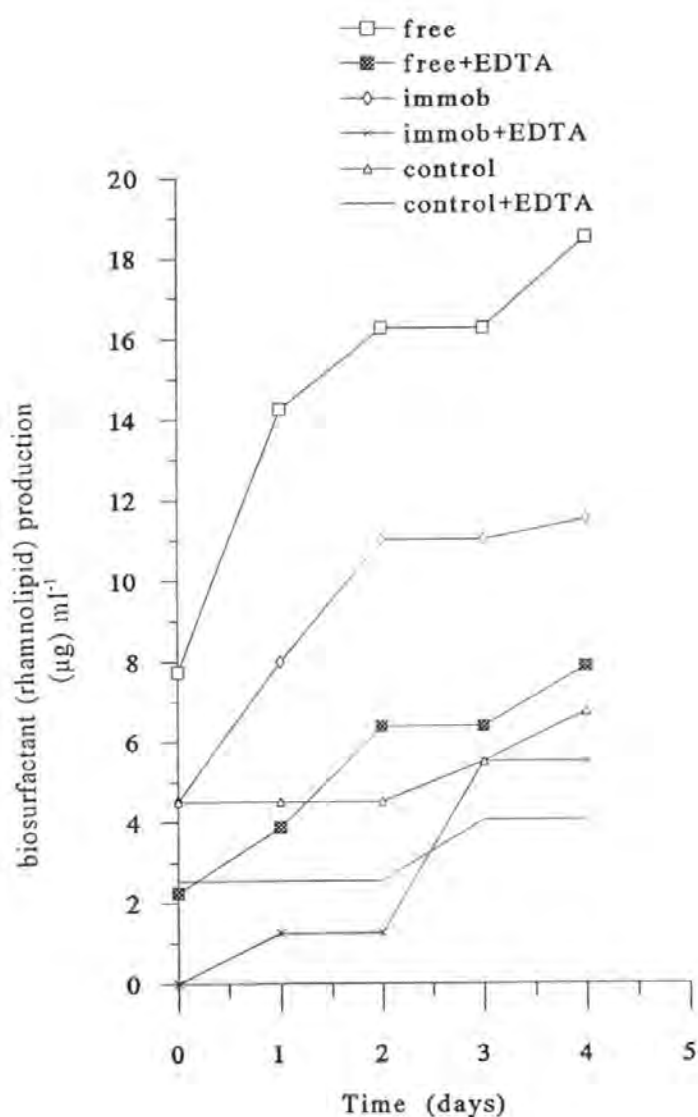
Figure 4.5. The effect of EDTA on the cumulative μg biosurfactant (rhamnolipid) produced in the aqueous suspensions of *Pseudomonas fluorescens* in a free and Drizit immobilized system incubated with petrol (Slovene diesel).

4.4. Discussion.

Previous data (chapter 2) has shown enhanced biodegradation of petrol (Slovene diesel) with immobilization of *P. fluorescens* on Biofix and Drizit. The aim of this study was firstly to investigate a possible reason why such enhancement of degradation and acceleration of utilisation occurred and secondly to determine whether immobilization affects the production of biosurfactant by *P. fluorescens*. Immobilization of bacteria to an oleophilic substratum could cause the cells to come into close contact with the oil earlier than in a free system, where cells are left to collide with hydrocarbon droplets at random. The enhanced degradation and utilization of petrol (Slovene diesel) previously shown may be due to the early contact made between the hydrocarbon and the cells through this process. If the bacteria use the production of a surface active agent to increase the bioavailability of the hydrocarbons, any early contact between cell and hydrocarbon due to immobilization may affect the production of these agents.

This study again demonstrates significantly higher levels of growth on petrol (Slovene diesel) by *P. fluorescens* when immobilized on Biofix and Drizit in comparison to free living bacteria. In the free system growth increased until day 1 after which marginal growth occurred. The Biofix and Drizit immobilized systems showed growth in their aqueous suspensions throughout the five day incubation period, with bacteria in the Biofix immobilized system growing at a faster rate for the first two days than in the Drizit system. These results indicate the bioavailability of the carbon source may have increased with immobilization, due to the increase in growth seen over the five day incubation period.

Rhamnolipid data collected were used to form graphs of cumulative rhamnolipid production over the incubation period, on the assumption made by Kaepelli and Fiechter in 1976 and Ratledge in 1987 (section 1.4). Briefly, they hypothesised that in an aqueous medium the biosurfactant and hydrocarbon were in association with each other and entered the cell envelope together. At this point it was assumed the hydrocarbon dissociated from the complex and partitioned into the membrane and the biosurfactant returned to the exterior of the cell. However the rate of

dissociation is not known and to alleviate discrepancies between newly synthesized biosurfactant and that released from the membrane, the biosurfactant isolated on each day was assumed to be newly synthesized.

P. fluorescens immobilized to Biofix and Drizit showed an increase in rhamnolipid production until day 3 in comparison to the free system. The free system showed a lag phase of two days where rhamnolipid production remained low and constant. During this period it can be assumed the cells were making random contact with the hydrocarbon droplets, utilizing them in preparation for the production of the surface active agent which comes from the utilization of the substrate itself (Watkinson 1980). Syldatk and Wagner (1987) and Hommel (1990) have described several general biosynthetic paths of the production of biosurfactants which include the formation of the hydrophilic and lipophilic moiety of the molecule. These include;

1)The hydrophilic and the lipid moiety are synthesized independently of the growth substrate.

2)The synthesis of the lipid moiety depends on the hydrophobic carbon source.

3)The hydrophilic moiety depends on the carbon source.

4)Synthesis of both the hydrophilic and lipid moiety depends on the carbon source.

If *P. fluorescens* relies on any of the latter three mechanisms for the biosynthesis of biosurfactant, a lag phase would occur in its production as shown in section 4.3 and the biosurfactant would be produced later in the growth cycle as a secondary metabolite.

By day four of incubation the level of production of biosurfactant in the free system had reached a level comparable to that found in the immobilized systems. The Biofix and Drizit immobilized systems produced more rhamnolipid per optical density unit at an earlier stage in the growth cycle than in the free system, where there is a lag phase of two days. It is reasonable to assume that the substrate is being utilized sooner in the immobilized system than in the free, with the result that the

lag phase is shortened and the surface active agent is produced earlier. With the production of the surface active agent at an earlier stage in the growth cycle the hydrocarbon becomes available sooner resulting in the enhanced levels of degradation previously shown in chapter 2.

In the EDTA unsupplemented systems, the Drizit immobilized system showed enhanced growth in comparison to the free system until day 1. After day 1 there was no difference between the level of growth in the two systems. Also the free systems showed no lag phase in the production of rhamnolipid. These results are atypical, figures 4.1 and 4.2 and previous data (chapter 2) have clearly shown an increased rate and level of growth in a Drizit immobilized system and a lag phase in the production of rhamnolipid,

The growth of *P. fluorescens* was not significantly affected by the addition of EDTA in the free system, although there was a small decrease in growth with a concomitant decrease in rhamnolipid production. This leads to the conclusion that this bacteria may be able to rely on contact between the cell and oil droplets generated by mechanical agitation (Goswami and Singh 1991) resulting in similar levels of growth both with and without rhamnolipid production. These data indicate that the addition of EDTA, which chelates divalent and polyvalent metal ions may have removed a metal ion from the aqueous suspension of the free system which is important in the cells production of rhamnolipid, therefore inhibiting the cells production of this surface active agent. Reddy *et al.* (1982) have shown that the binding of Ca^{2+} by EDTA resulted in the bacteria's failure to produce biosurfactant.

It could be argued that the successful decrease in rhamnolipid production in the free system is due to an inhibitory effect of the EDTA on the metabolic machinery of the cells or through interference in the transport of cations across the cell membrane (Reddy *et al.* 1982). For example *Pseudomonas aeruginosa* cells are lysed rapidly by EDTA addition without osmotic protection in the medium. Treatment of this species with EDTA results in the release of proteins from the outer membrane and the release of lipopolysaccharide and loosely bound lipid (Krieg and Holt 1984)

However, growth in the free system did not appear significantly affected by EDTA addition in these studies. Reddy *et al.* (1982) reported that EDTA caused lysis of gram-negative bacteria but had no effect on EDTA-resistant *Pseudomonas*, gram positive bacteria, yeasts and moulds. It is also known that the cell envelope of hydrocarbon-degrading bacteria is highly lipophilic, with hydrocarbons accumulating in and around the cell envelope (Kennedy *et al.* 1975; Reddy *et al.* 1982). It is unlikely that EDTA can penetrate this highly lipophilic cell envelope due to its highly charged ionic state in aqueous solution. Wilkinson (1970) as cited by Krieg and Holt (1984) found a relationship between EDTA sensitivity and a high phosphorous content in the outer membrane, the phosphorous having a metal binding capacity which may bind the lipopolysaccharide to the other wall components. *P. fluorescens* was described as less sensitive to EDTA due to a lower content of phosphorous in the outer membrane.

If growth however was predicted to be affected by the addition of EDTA, it would be due to EDTA's property of chelating divalent and polyvalent metal ions. EDTA could be capable of binding all divalent and polyvalent ions at the correct pH (alkaline). However the media used was pH 7.2 and it is likely that only Ca^{2+}, with an optimum of pH 7.5 for stable complex formation would be bound extensively. The complexing efficiency of EDTA for Ca^{2+} however is lowered as the pH drops below 7.5 (Welcher 1958), at pH 7.2 a small amount of Ca^{2+} may have remained in the free state in the media and consequently satisfied the requirements of the cells for regulating some cytoplasmic membranes and ATPase activity (Kretsinger 1976 as cited by Reddy *et al.* 1982). EDTA has also been shown to have no direct effect on the adsorbtion of cells to oil drops, thus EDTA inhibition of growth cannot be attributed to interference of the contact of the cells with oil drops (Reddy *et al.* 1982).

In comparison however, a significant reduction in growth can be seen in the immobilized system with the addition of EDTA, with a corresponding decrease in rhamnolipid production. The decrease in biosurfactant production may be due to the addition of EDTA, resulting in chelation of Ca^{2+} needed for rhamnolipid production or the reduction in the population size due to the effect of

EDTA as explained above. It was expected that the immobilization substrata would confer protection to the bacteria from the EDTA. For example, several studies on the degradation of phenol have shown that immobilized bacteria can withstand higher concentrations than the free bacteria, the substratum acting as a 'buffer' (O'Reilly and Crawford 1989a; Levinson *et al.* 1994). In this case the presence of the substratum may have enhanced the effects of the EDTA on the cells by concentrating the EDTA around the immobilized bacteria resulting in a significant decrease in growth levels. Alternatively EDTA inhibition of growth could be interpreted as being due to reduced alkane solubilization due to reduced biosurfactant production by the cells. This is supported by data from the free system where a small decrease in growth was observed with the reduction in rhamnolipid production. It has been shown by Reddy *et al.* (1982) that if a culture is provided with 'artificially prepared solubilized alkane' the inhibitory effect of the EDTA on growth is removed. Further studies are required to determine the effects of EDTA on the metabolic machinery of *P. fluorescens* possibly by using soluble non-hydrocarbon substrates (Reddy *et al.* 1982).

In summary the results from this study indicate that the oil degrading *P. fluorescens* when immobilized to an oleophilic substratum may result in enhanced contact between the hydrocarbon and the cell. As mentioned previously, where bacteria use the contact of hydrocarbons for their utilization, in a free system availability of the substrate surface for cell attachment is the limiting factor. In the immobilized system this limiting factor is removed due to the oil absorbing to the substratum and increasing the surface area for cell attachment. Although the bacteria may be able to use contact as a means of growth on hydrocarbons, which is why a reduction in lag phase is seen in the immobilized system, the production of biosurfactant may still be an important factor to aid utilization and may explain the reduced growth levels seen in both systems when biosurfactant production is suppressed. It can be concluded that immobilization of *P. fluorescens* resulted in a combination of increased contact between the cell and hydrocarbon, resulting in enhanced levels of rhamnolipid production early in the growth cycle which in turn may have been responsible for the enhanced levels of degradation observed previously (chapter 2).

Chapter 5

Genetic Regulation of Hydrocarbon Degradation in *Pseudomonas fluorescens*

5.1. Introduction.

The nutritional diversity of the *Pseudomonas* species gives them the ability to utilize a wide range of organic compounds (section 2.1), indicating the cells are endowed with the genetic information to specify the enzymes required for the catabolic processes to breakdown these substances. Chakrabarty *et al.* (1973) carried out a study of the genes coding for the enzymes of catabolic pathways in several *Pseudomonas* species. They found that many of the genes were associated with extrachromosomal elements rather than with the chromosome. These extrachromosomal elements or plasmids, are usually covalently closed circular (ccc) deoxyribonucleic acid (DNA) molecules, although linear DNA plasmids, as well as single stranded plasmids (which are intermediates in replication), have been described (Hirochika and Sakaguchi 1982 as cited by Crosa *et al.* 1994; Barbour and Garon 1987; Keen *et al.* 1988; Gruss and Ehrlich 1989; Perng and Lefebre 1990). Plasmids exist independently of the hosts chromosomes and generally only have a few genes (in the region of 25-30) and their sizes range from 1 to more than 200 kilobase pairs (kbp), (Couturier *et al.* 1988). These molecules can be stably inherited without being linked to a chromosome and their presence is not essential to the bacteria, those without them being able to function normally (Prescott *et al.* 1990). Plasmids can be regarded as being of two types, the first group are unable to promote their own transfer by conjugation, and are present in multiple copies within the cell. The replicon of these plasmids is not linked to chromosome replication and cell division and therefore there is a high copy number. The second group (tending to contain the larger plasmids), can promote their own transfer by conjugation. They are present in only one or two copies per cell because their replication is controlled by the same mechanisms as that of the chromosome (Dale 1989).

Those *Pseudomonas* species known to degrade n-alkanes and aromatic hydrocarbons can carry the genes for the degradation of such hydrocarbons on either chromosomes or plasmids or a combination of the two. For example, toluene (TOL), octane (OCT), naphthalene (NAH), m- and p-xylenes (XYL), and camphor (CAM) have been shown to be plasmid encoded (table 5.1), (Williams and Franklin 1980). These plasmids are unique in that they define a set of genes involved in the biodegradation of an organic compound. They occur naturally and can be either transmissible or non-transmissible. In the case of the *Pseudomonas* species, transmissible plasmids although transmissible among most *Pseudomonas* species, have not been shown to be transferred to members of other genera (Chakrabarty 1976).

The amount of catabolic genetic information carried on these plasmids includes both the regulatory apparatus and the structural genes for the enzymes which convert the growth substrate to a simpler metabolite, which can then be metabolized by the chromosomally-coded enzymes. The plasmid-specified pathways can constitute the entire enzyme complement and if the cell has a plasmid of this type it enables the cell to derive carbon and energy from the oxidative metabolism of these and related substrates.

Table 5.1. Properties of some typical degradative plasmids.

Plasmid	Degradative Pathway	Transmissibility	Molecular Weight $(\times 10^6)$
NAH	Naphthalene	Conjugative	not determined
SAL	Salicylate	Conjugative	40, 55
CAM	Camphor	Conjugative	150
OCT	n-Octane	Non-conjugative	not determined
XYL	p- or m-Xylene	Non-conjugative	10
TOL	Toluene, p- or m-xylene	Conjugative	75

(Chakrabarty 1976)

Plasmid borne hydrocarbon degradative genes are often found in clusters (Chakrabarty 1985), for example, the genes for naphthalene degradation comprises eleven naphthalene structural genes in the plasmid pIG7 occurring in two operon clusters, nahA-F and nahG-K. A regulatory locus, nahR, has been localized between these two gene clusters, and the expression of the structural genes has been shown to be under positive control by this locus (Grund and Gunsalus 1983). This type of organization can also be seen in the toluene degradative genes on the TOL plasmid. These genes are arranged in two regulatory units. The xylABC operon codes for the genes of toluene oxygenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase and the xylDEGF operon codes for the genes of toluate oxygenase, catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase. On these two operons the expression of the structural genes is controlled positively by two regulatory genes, xylR and xylS. For the activation of the xylDEGF operons the gene products of both xylR and xylS are needed although if the inducer is m-toluic acid this operon can be activated by xylS alone. For the activation of the xylABC operon by xylR, toluene needs to be present. Similar positive control mechanisms and clustering of the structural genes have been identified in degradative pathways for other organic compounds such as the n-alkanes (Perry and Scheld 1968; Grund *et al.* 1975; Chakrabarty 1985).

Plasmids can offer considerable selective advantages to the cells if the plasmid hosts degradative pathways that convert complex organic compounds to a common metabolite. This selective advantage is seen when the environment is depleted of normally available simple organic compounds. Due to this selective ability Chakrabarty (1973) designated this class of naturally occurring plasmids the 'degradative plasmids' to differentiate them from the other types of plasmids e.g sex factors and drug resistance factors coded for by plasmids in other microbes. The degradative plasmids which have been identified are mainly in strains of *Pseudomonas*. The genetic origin of these degradative plasmids is unknown and information on such matters would help explain the evolution of the extreme nutritional versatility found in the *Pseudomonas* species

(Chakrabarty 1973).

Most degradative plasmids appear to be compatible with each other. Incompatibility is the inability of two plasmids, that have been introduced into a bacteria to be stably maintained in the same cell in the absence of selective pressure for both plasmids. Incompatible plasmids share some or all traits involved in replication or maintenance, and plasmids have been classified by being assigned to incompatibility groups, therefore, plasmids in the same group are incompatible. For example, attempts to accommodate the CAM and OCT plasmids have been unsuccessful as introduction of one led to the eventual segregation of the other (Chakrabarty 1973; Chakrabarty *et al.* 1973). CAM and OCT plasmids and SAL and TOL plasmids are not compatible and must therefore belong to the same incompatibility groups. However, when plasmids belong to different incompatibility groups, due to different compatibility characteristics several hydrocarbon degradative plasmids can be sustained in a single bacterial strain to form a multi-plasmid strain which has an amplified facility to utilize hydrocarbons (Chakrabarty 1976). Most of the *Pseudomonas* degradative plasmids belong to a limited number of incompatibility groups; P2, P7 and P9, e.g the naphthalene (NAH) plasmids belong to incompatibility groups P7 and P9. Many of the large degradative plasmids belong to the incompatibility group P2, and data suggests that these plasmids have evolved mechanisms which ensure their secure maintenance in the bacterial population, despite the metabolic load inflicted upon the host cell (Boronin 1992). However, it is not possible to compare plasmids from different species unless they can be transferred to a common host therefore, there is one set of groups for *Escherichia coli* plasmids and another for plasmids from *Pseudomonas* species (Dale 1989).

In the case of the CAM and OCT plasmids, genetic fusion of these incompatible plasmids is possible and may be due to the existence of plasmid-coded alcohol-dehydrogenating activity, which is also coded for chromosomally. This genetic 'redundancy' on the degradative plasmids may be the factor which allows recombination between the CAM and OCT to form the fused CAM-OCT plasmid (Chakrabarty 1973; Chakrabarty 1976). A SAL-TOL *in vivo* recombinant plasmid has also

been characterized in a strain from a mixed culture of bacteria harbouring various degradative plasmids (Furukawa *et al.* 1985).

Several strains of *Pseudomonas* are capable of growth on n-alkanes, oxidising them by the pathway detailed in section 1.4 and evidence of one such n-alkane degrading *Pseudomonas* species is detailed in chapter 2. Chakrabarty *et al.* (1973), Grund *et al.* (1975) and Witholt *et al.* (1990) described how *Pseudomonas* strains grow on n-alkanes due to the presence of the transmissible Oct plasmid. The OCT plasmid consists of two regions, the alkBAC operon which encodes structural genes and the alkR regulatory region. The alkBAC operon has been renamed the alkBFGHJKL operon since it encodes seven peptides. The alkR region encodes two peptides (alkST), one of which actually has an enzymatic rather than a regulatory function. The expression of alkB-L is positively regulated by the 99 kilodalton (kDa) product of the alkS gene (Figure 5.1).
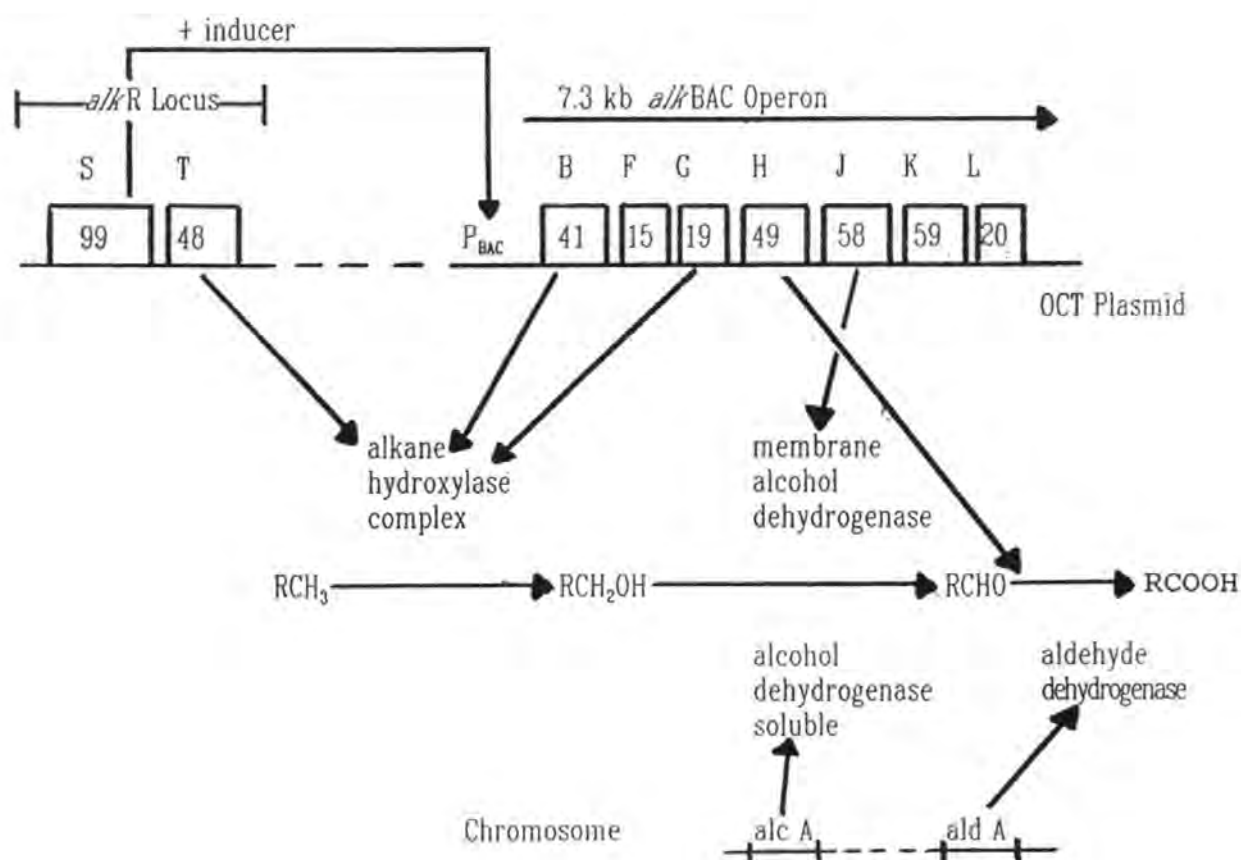
Figure 5.1. Genetics of n-alkane oxidation by *Pseudomonas oleovorans* (Witholt *et al.* 1990)

Witholt *et al.* (1990) described the alkane hydroxylase system of *Pseudomonas oleovorans* (Figure 5.2)

'The alkane hydroxylase system (A) consists of 3 proteins. The cytoplasmic membrane alkane hydroxylase component is a 45.7 kDa monooxygenase encoded by alkB. Rubredoxin is a 19 kDa cytoplasmic protein encoded by alkG. Rubredoxin reductase is a 48 kDa cytoplasmic flavoprotein encoded by alkT. After oxidation of alkanes to alkanols, these are further oxidized by alkanol dehydrogenases (B), which are encoded by the chromosome as well as the alk system (alkJ, alkK) and aldehydes are oxidized by aldehyde dehydrogenases (C), which are also encoded on the chromosome as well as the alk system (alkH). These enzymes together produce carboxylic acids, which are metabolized as carbon and energy source by the chromosomally encoded fatty acid degradation pathway (D)'
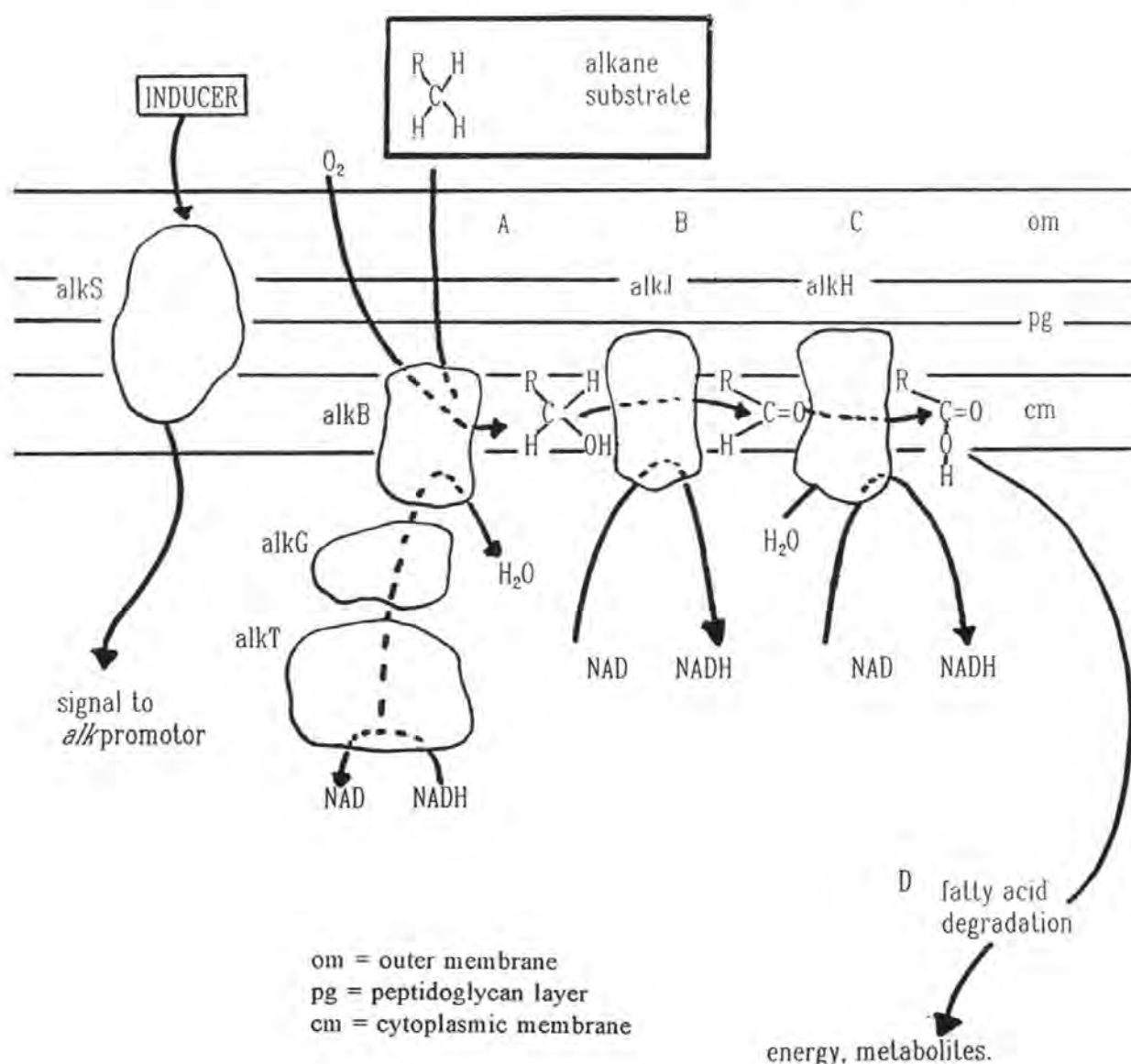
Figure 5.2. The alkane hydroxylase system of *Pseudomonas oleovorans*. (Witholt *et al.* 1990)

Grund et al. (1975) found that growth of *P. putida* on n-alkanes resulted in alkane oxidizing activity which is not found in glucose grown cells. They found that growth on a single alkane substrate (octane) induced oxidizing activity for all five alkanes which support the growth of this strain. It appeared therefore, there was a single induction mechanism where any alkane was equivalent to any other, both as an inducer and as an oxidation substrate. However the range of substrates is larger than the range of inducers and the substrate ranges of alkane-oxidizing bacteria may be limited by inducer specificity.

The OCT plasmid only codes for the first two steps in alkane oxidation and not the entire alkane to fatty acid pathway, which explains why the process is both plasmid and chromosomally encoded. However, alkane hydroxylation by itself would be sufficient to bring these substrates into the range of chromosomally determined cellular metabolism, so questions arise as to why the plasmid evolved with its own inducible primary aliphatic alcohol dehydrogenase ? (Grund et al. 1975)

Plasmids play a role in the adaptation and the procurement of new genetic traits of bacteria in response to pollution. Plasmid encoded pathways are ecologically beneficial because they provide genetically flexible systems which can be transferred between species. Gene transfer by plasmids was perceived when resistances to antibiotics were discovered to be mediated via R-plasmids. Gene transfer depends on bacteria survival, plasmid stability, available nutrients and cell numbers. Since aquatic environments are natural reservoirs for microbes, genetic interactions are possible if the appropriate conditions are present. It appears that some plasmids, for example plasmids specifying the dissimilation of chlorinated compounds may have evolved by the assembly of genes from various plasmids. If this is a general mode of evolution of plasmids, then it may be possible to allow the evolution of plasmids for the degradation of various toxic chemicals (Vertraete and Top 1992).

The isolation of new plasmids in the *Pseudomonas* species will continue to be paramount for the

understanding of conjugation and chromosome transfer. Also these plasmids constitute a vast gene pool in nature for the genetic diversity, expressed as biochemical versatility, which is characteristic of this genus.

This investigation determines whether immobilized *P. fluorescens* grown with diesel (British Petroleum - BP) as the sole carbon source, carries the genes for hydrocarbon degradation either on chromosomes, a plasmid or a combination of both. Knowledge of the genetic basis of immobilized *P. fluorescens* hydrocarbon degradation will allow its comparison to that found in a free living *P. fluorescens* and such studies may reveal further details on why enhanced biodegradation occurs with immobilization.

All techniques used require some means of gently lysing bacterial cells so that the plasmid DNA is preserved intact and can be physically separated from the chromosomal DNA. In this study this was attempted by using standard agarose gel electrophoresis to separate and identify the DNA. The electrophoretic migration rate of DNA through agarose gels is dependent upon several parameters:

1)The molecular size of the DNA.

2)The agarose concentration. Depending on the concentration of the agarose, a DNA fragment of a certain size migrates at a different rate (table 5.2).

3)The conformation of the DNA. DNA of the same molecular weight, depending on whether it is, closed circular, nicked circular, or linear will travel through agarose at different rates.

4)The applied current.

(Maniatis *et al.* 1982)

The location of DNA in the gel can be determined easily by staining the bands in the gel with low concentrations of ethidium bromide. Plasmids range in size from about 0.2 to 4 % of the bacterial chromosome and the separation of plasmid DNA is more easily achieved with smaller plasmids. The degree of difficulty in separation increases as the size of the plasmid increases. Large plasmids

also pose a problem in electrophoresis as they are prone to breakage during extraction (Crosa *et al.* 1994).

Table 5.2. Relationship between agarose concentration and efficient range of separation of linear DNA molecules.

Amount of Agarose (%)	Efficient range of separation of linear DNA molecules (kbp)
0.3	60-5
0.6	20-1
0.7	10-0.8
0.9	7-0.5
1.2	6-0.4
1.5	4-0.2
2.0	3-0.1

(Maniatis *et al.* 1982)

Due to problems encountered with extracting plasmids, especially in circumstances where large plasmids are encountered, a second method for large plasmid extraction devised by Board *et al.* (1992) was adapted and used to attempt to separate the plasmid DNA from the immobilized oil degrading *P. fluorescens*.

" There are many methods published for extracting and analysing plasmids from various bacteria but *Rhizobium* plasmids pose a problem because many are larger than 100 kbp and are prone to breakage during extraction. When this occurs the resultant large linear fragments are much more difficult to resolve by gel electrophoresis than are the relatively compact supercoiled molecules of closed circular coiled plasmids. Therefore methods involving *in situ* lysis of bacterial cells on the gel are most suitable" (Board *et al.* 1992).

Plasmids of known molecular weight were used as standards to determine the molecular weight of the unknown plasmid in all the methods of gel electrophoresis.

Purification of the plasmid DNA was attempted by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient. The rationale behind this method is as follows; the ethidium bromide binds to dsDNA by intercalating between the stacked base pairs, resulting in the molecules unwinding. Less ethidium bromide can bind to a circular molecule than to a linear molecule due to topological restraints, differentiating the molecules by density. Therefore the buoyant densities of closed circular plasmid DNA will be higher than other forms of double stranded DNA in saturating ethidium bromide thus, plasmid DNA forms a band separate from the other material after centrifugation (Figure 5.3). Therefore the closed circular plasmid DNA forms a band at one position in the centrifuge tube and nicked plasmid DNA, linear fragments of chromosomal DNA and other double stranded DNA form a band higher in the tube. Ribonucleic acid (RNA) forms a pellet at the bottom of the tube and any protein left floats to the surface (Walker and Gaastra 1983; Berger and Kimmel 1987).
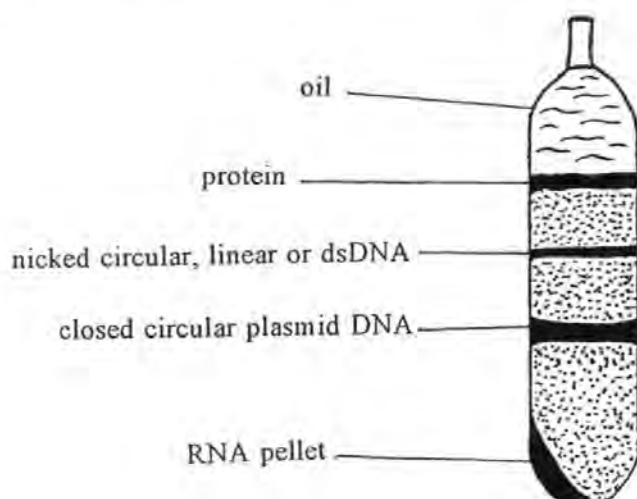
Figure 5.3. Purification of closed circular DNA by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients. Illustration of the bands formed by plasmid and chromosomal DNA (Maniatis *et al.* 1982).

5.2. Materials and methods.

5.2.1. *Bacterial isolates.*

An oil degrading *P. fluorescens* (section 2.2.1) was immobilized to Drizit (section 2.2.3.2) and after growth (section 5.2.2.), released from the matrix by sonication (Transsonic, T310, Camlab) for 2 minutes. The resulting free cells were used in this study to determine the presence of a plasmid responsible for hydrocarbon degradation.

5.2.2. *Media.*

Tryptone Soy broth (TSB, Oxoid) was used as the nutrient source for the bacteria after immobilization and 0.01 M phosphate buffer saline (PBS), pH 7.3 (Oxoid, Dulbecco 'A' tablets) to wash the cells. Minimal media, pH 7.2 containing (g l^{-1}); NH$_4$Cl, 5.0; NH$_4$NO$_3$, 1.0; anhydrous NaSO$_4$, 2.0; K$_2$HPO$_4$, 3.0; KH$_2$PO$_4$, 1.0 and MgSO$_4$.7H$_2$O, 0.1 dissolved in order in distilled water, supplemented with 0.1 % v/v diesel (British Petroleum, BP) as the sole carbon source, was used to grow the immobilized cells for 24 hours at 30°C prior to sonication (section 5.2.1).

5.2.3. *Harvesting and lysis of bacteria. Method 1.*

The cells were harvested (60 ml) by centrifugation (Denley, BR401) at 5000 g for 20 minutes and the supernatant discarded. The pellet was washed in 1 ml of ice-cold STE (0.1 M NaCl, 10 mM Tris.Cl (pH 7.8) and 1 mM EDTA) and resuspended in 100 μl of Solution I (50 mM glucose, 25 mM Tris.Cl, pH 8.0, 10 mM EDTA), containing 5 mg ml^{-1} lysozyme (Sigma). The suspension was incubated at room temperature for 5 minutes after which 200 μl of solution II (0.2 N NaOH, 1 % sodium dodecyl sulphate (SDS)), was added and mixed by gentle vortexing. The suspension was left to stand on ice for 5 minutes, after which 150 μl of solution III (3 M NaAcetate, pH 4.8) was added and the suspension was mixed by gentle inversion of the tube. After standing on ice for a further 5 minutes, the suspension was centrifuged (Heraeus bench centrifuge, sepatech) at 1300 rpm for 3 minutes to collect the precipitated plasmid DNA. The supernatant was discarded and the remaining pellet resuspended in 100 μl of solution IV (50 mM Tris-HCl, pH 8.0,

100 mM NaAcetate). The DNA was reprecipitated by the addition of 250 μl of cold ethanol and left on ice for 10 minutes. The suspension was recentrifuged (Heraeus bench centrifuge, septech) at 1300 rpm for 3 minutes to collect the precipitated plasmid DNA, the supernatant was discarded and the pellet resuspended in 10 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), (Birnboim and Doyle 1979)

5.2.3.1. *Isolation and sizing of plasmid DNA. Method I.*

Agarose gel (0.7 %), 100 ml final volume, was heated until the agarose dissolved, 10 ml of TBE buffer (0.5 x 0.045 M Tris-borate, 0.001 M EDTA), (Sambrook *et al.* 1989) was added and the solution cooled to 50°C, after which 10 ml of ethidium bromide stock solution (10 mg ml^{-1}) was added. The edges of a clean, dry, glass plate were sealed with tape and the warm agarose solution added and the well former clamped into position near one end of the gel. After the gel was set the comb and tape were removed and the gel mounted in an electrophoresis tank. Enough TBE buffer (containing 0.5 μg ml^{-1} of ethidium bromide) was added to the tank to cover the gel to a depth of about 1 mm (Maniatis *et al.* 1982) Samples (10 μl) were mixed with loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 15 % Ficoll (type 400; Pharmacia) in water), (Sambrook *et al.* 1989) 4μl and loaded into the wells of the submerged gel. The following samples were loaded; 1 kbp ladders (BRL), *P. fluorescens, P. putida* (NCIMB 12593, pkf 439), Lambda wild type DNA, 0.05 μg μl^{-1} (48 kbp), Control plasmid DNA pAX4 (6-7 kbp), (New England Biolabs). The gel was run at 150 volts for half an hour and then at 100 volts for 8 hours. After further staining with ethidium bromide the gel was examined under uv light at 240 nm.

5.2.4. *Harvesting and lysis of bacteria. Method II.*

Cells were harvested (400 ml) by centrifugation at 5000 g for 20 minutes (Denley, BR 401). The pellet was resuspended in 16 μl of 25 % w/v sucrose in 0.025 M l^{-1} Tris-Cl, pH 8.0, containing lysozyme (1 mg ml^{-1}) and RNase at 1 unit ml^{-1}.

5.2.4.1. *Isolation and sizing of plasmid DNA. Method II.*

A 0.6 % w/v agarose gel was prepared as described in section 5.2.3.1. The gel was poured with a solid insert in the place of the well former. Once the gel had set the solid insert was removed and replaced with a well former. The remaining trough was filled with 0.4 % (w/v) agarose in TBE buffer containing 1 % SDS and left to set (Board *et al.* 1992). Samples (10 μl) were mixed with 4 μl of loading buffer and loaded into the wells. The following samples were loaded; 1 kbp ladders (BRL), *P. fluorescens, P. putida* (NCIMB 12593, pkf 439), Lambda wild type DNA, 0.05 μg μl⁻¹ (48 kbp), Control plasmid DNA pAX4 (6-7 kbp), (New England Biolabs). The gel was run for four hours at 150 v. After further staining with ethidium bromide the gel was examined under uv light at 240 nm.

5.2.5. *Harvesting and lysis of bacteria. Method III.*

Details of the method for harvesting and lysis of the bacteria are given in section 5.2.4.

5.2.5.1. *Isolation and sizing of plasmid DNA. Method III.*

Details of the method for isolating the plasmid DNA is given in section 5.2.4.1. The following samples were loaded; 1 kbp ladders (BRL), *P. fluorescens, P. putida* (NCIMB 12593, pkf 439), Lambda wild type DNA, 0.05 μg μl⁻¹ (48 kbp), Control plasmid DNA pAX4 (6-7 kbp), (New England Biolabs). The gel was run at 75 v for 3.5 hours, at 40 v for 12 hours, at 1.5 hours at 70 v and a further four hours at 40 v. After further staining with ethidium bromide the gel was observed under UV light at 240 nm.

5.2.6. *Cesium chloride-ethidium bromide gradient.*

P. fluorescens was harvested and lysed as in section 5.2.4. The remaining volume of DNA solution was measured and for every ml of solution 1 g of cesium chloride was added. Ethidium bromide was added at a volume of 0.8 ml of solution (10 mg ethidium bromide ml⁻¹ H₂O), for every 10 ml of cesium chloride solution and mixed. The cesium chloride solution was transferred to a centrifuge tube and the remainder of the tube filled with light paraffin oil and the tube sealed. The

tube was centrifuged (Beckman ultracentrifuge, TL-100) at 110,000 g for 36 hours at 20°C. Plasmid DNA could then be retrieved by removing the cap from the tube, puncturing the tube with a syringe just below the plasmid DNA band and drawing of the DNA into the syringe (Maniatis *et al*. 1982).

5.3. Results.

This study attempted to determine whether immobilized *P. fluorescens* grown on diesel (BP) carried the genes for hydrocarbon degradation on a plasmid.

5.3.1. *Isolation and sizing of plasmid DNA. Method I.*

The results from this method were inconclusive. No plasmid DNA bands were detected from *P. putida* known to have a plasmid of 134 kbp or from *P. fluorescens.*

5.3.2. *Isolation and sizing of plasmid DNA. Method II.*

The results from this gel were inconclusive, the voltage was set too high and the gel was damaged by excessive heat production.

5.3.3. *Isolation and sizing of plasmid DNA. Method III.*

The gel from this run showed a band of plasmid DNA from *P. fluorescens* corresponding to a plasmid DNA band belonging to *P. putida* which is known to contain a plasmid 134 kbp in size (figure 5.4).

Figure 5.4. Agarose gel illustrating A) plasmid DNA band from *Pseudomonas fluorescens* B) plasmid DNA band from *Pseudomonas putida* (134 kbp).

5.3.4. *Cesium chloride-ethidium bromide gradient.*

Only one chromosomal DNA band was detected. No plasmid DNA band was observed.

5.4. Discussion.

Plasmids are ubiquitous in *Pseudomonas*, although the frequency of occurrence of plasmids varies in a particular species or groups of species. The frequency of this occurrence also depends on the ecological niche from which the bacteria were isolated. For example Boronin (1992) stated that the appearance of degradative plasmids in strains of *P. putida* and *P. fluorescens* were more frequent in bacteria isolated from areas containing xenobiotics. In nature the degradative plasmid-host combinations give rise to a greater diversity of bacterial strains capable of degrading particular xenobiotics.

The aim of this present study was to determine if the immobilized oil degrading *P. fluorescens* used throughout this research carried a plasmid which coded for hydrocarbon degradation. It was hoped to compare the genetic make-up of both free and immobilized *P. fluorescens* to establish any differences in their genetics for hydrocarbon degradation. With knowledge of a plasmid coding for hydrocarbon degradation, further insight may have been gained to help explain the enhanced biodegradation seen with immobilized *P. fluorescens*.

Problems were encountered in detecting a plasmid using the traditional agarose gel electrophoresis technique. Larger plasmids (> 100 kbp) may have had problems in moving through the 0.7 % gel, as indicated by the *P. putida* plasmid, which is 134 kbp in size, but on the gel no plasmid could be detected. The absence of this plasmid on the gel would indicate that either the plasmid DNA could not leave the wells in the gel due to their size, or the DNA extraction methods resulted in breakage of the plasmids. Breakage can result in large linear fragments which are more difficult to resolve by gel electrophoresis than the relatively compact supercoiled molecules of covalently closed circular plasmids (Board *et al.* 1992). Therefore a method of *in situ* lysis of the bacteria was used to prevent breakage of the plasmids during extraction. Most of the chromosomal DNA remains intact after lysis, therefore it remains in the well and less than 0.5 % of the total chromosomal DNA appears in the gel as a band of linear DNA (Crosa *et al.* 1994). Also a 0.4 %

agarose gel insert was used to make the wells, to allow easier movement of the plasmid DNA from this area.

Results obtained from method II, confirmed that this adapted *Rhizobium* plasmid extraction method could be used to separate large plasmids, due to the presence of a plasmid DNA band from the *P. putida* extraction. A band was also observed adjacent to this, from the well inoculated with *P. fluorescens* giving preliminary evidence of the presence of a plasmid. The band observed from the *P. fluorescens* may however, have been due to contamination from the *P. putida* well. The cesium chloride-ethidium bromide gradient was carried out to confirm the presence of plasmid DNA in *P. fluorescens*, but no band was visualized. This may indicate the absence of plasmid DNA in *P. fluorescens* or may have been due to problems encountered with extracting the DNA from the cells. Treatment of the DNA may have resulted in breakage of the plasmid DNA into linear fragments which may not have been resolvable from the chromosomal DNA, with the result of only one band of DNA being visualized. Also this procedure was originally devised for the purification of relatively small, supercoiled circular plasmids and therefore may not be appropriate for the purification of larger plasmids (Dale 1989).

It is possible that the genes for n-alkane degradation may be chromosomally borne in this strain of *P. fluorescens*. For example 70 strains of the genus *Pseudomonas* were identified as ε-caprolactam degrading strains and 10 % of them did not have the caprolactam (CAP) plasmid which determines degradation of ε-caprolactam. Also 14 out of 30 strains of *Pseudomonas* species growing on naphthalene, did not have the conjugative plasmids (NAH) controlling the oxidation of this compound (Boronin 1992), indicating that the absence of these plasmids does not rule out the ability of the bacteria to degrade these compounds.

Future work could involve, repeating the adapted *Rhizobium* plasmid extraction method and the cesium chloride-ethidium bromide gradient. If the presence of a plasmid is confirmed, the plasmid could be cured by treating the bacterial population with chemical or physical curing agents such

as acridine dyes, ethidium bromide, SDS, antibiotics or high temperature. Elimination or curing

of a plasmid is the optimal way to determine whether a genetic trait is dependant on the bearing

of that specific plasmid by the bacteria. A phenotype due to the presence of a plasmid will not be

expressed in cured cultures. Reintroduction of the plasmid carrying the genetic trait will result in

the re-establishment of the phenotype (Crosa *et al.* 1994). Thus the ability of immobilized *P.*

fluorescens to degrade n-alkanes after curing could be investigated. If the trait of n-alkane

degradation is lost after curing it can be assumed that the genes coding for the enzymes in n-alkane

degradation are associated with a plasmid rather than with the chromosome. If no plasmid is found,

it could be assumed that the genes for n-alkane degradation in this *Pseudomonas* are chromosomally

based and further work could involve locating these genes on the genome. Future work could also

include investigation into the degree of the occurrence of plasmids in the fluorescent *Pseudomonas*

species inhabiting distinct natural environments, and the phenotypic features determined by these

plasmids. Also the diversity of plasmids determining similar attributes and the diversity of the

plasmids that occur in the bacterial populations of particular species could be investigated. Further

work could also determine if immobilization results in earlier expression of oxidizing activity than

in free cells, due to the early contact made between immobilized cells and oil.

Chapter 6

Enhanced Biodegradation of Ekofisk Crude Oil by Immobilized Bacteria in a Microcosm Simulating an Estuarine Environment.

6.1. Introduction.

Bioremediation of an oil spill in an aqueous environment may require the introduction of large numbers of bacterial strains into the ecosystem. New technologies using microbial strains sometimes fail to produce the desired effect in the natural habitats because their survival and activity in the environment are not as predicted in the laboratory (Goldstein *et al.* 1985). To speculate upon the consequences of bioremediation treatments, microcosms can be used to model characteristics of the natural environment (Wagner-Dobler *et al.* 1992).

An estuarine site was selected for this study, to allow for optimization of an immobilized bacteria system in mild saline conditions. Water samples were collected in August, from the Erme estuary, situated in the South West of England on the South Devon Coast (figure 6.1). The estuary is found between Meadowsfoot Beach and Wonwell Beach near Mothecombe, Devon (figure 6.2). The last oil spill in the area occurred in 1990 when the tanker Rose Bay was involved in a collision with a fishing vessel. An estimated 1000 tonnes of crude oil was lost from the Rose Bay. Attempts were made at isolating crude oil degraders from the estuary and water samples were collected to set-up microcosms. A microcosm is an experimental system that attempts to bring an intact, minimally disturbed part of an ecosystem into the laboratory (Pritchard and Bourquin 1984). Simple microcosms are used to identify factors influencing the survival of introduced bacteria in natural waters. The advantage of microcosms are in the introduction of some of the complex interactions that are present in the environment while allowing a level of control that is impossible with field studies (Barkay *et al.* 1995). These microcosms allowed the effects of parameters, such as temperature, pH, predation and competition on the survival and activity of immobilized

indigenous or immobilized non-indigenous microbes to be established (Leser 1995).

Enhanced biodegradation of petrol (Slovene diesel) has been illustrated using an immobilized bacteria system with an oil-degrading *P. fluorescens* immobilized to, Biofix and Drizit in a freshwater system and to Drizit in a saltwater system (chapter 2). This study attempted to optimize the immobilized bacteria system for a specific set of environmental conditions and scale-up the system used in the previous studies. An oil-degrading *P. fluorescens* or an oil-degrading bacterium isolated from the Erme estuary were immobilized to Drizit, and their abilities as bioremediation agents compared under a set of environmental conditions similar to those found at the estuary.
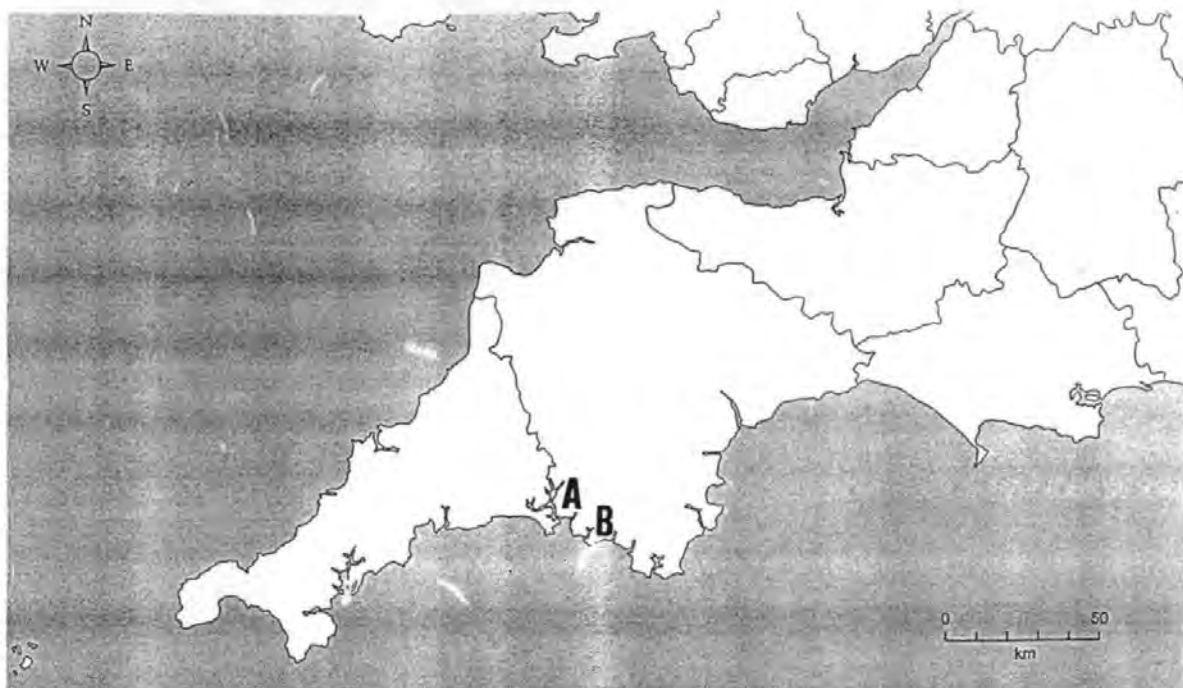
Figure 6.1. The location of Plymouth (A) and Mothecombe (B) in relation to the South West of England.
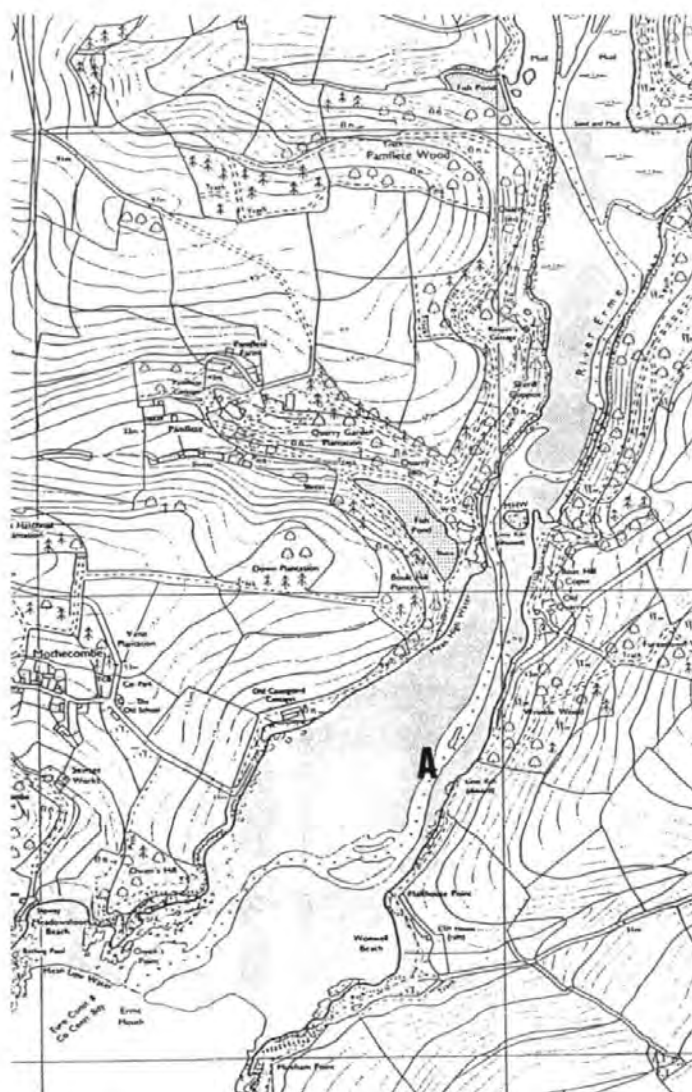
Figure 6.2. The location of the Erme estuary based on an ordance survey 1 : 10,000 map demonstrating the point of sampling (A)

6.2. Materials and methods.

6.2.1. *Bacterial isolates.*

A water sample (6 l) was collected from the Erme at low tide, and measurements of salinity (Atago hand refractometer, Chemlab Scientific products ltd), pH and temperature (Hach one pH meter, Camlab) taken at the estuary. Samples (100 ml and 500 ml) were filtered through 0.45 μm membrane filters (Whatman), and the membranes placed on oil medium No. 2 plates (Walker and Colwell 1975). Oil medium No. 2 was prepared by steaming 5 ml l^{-1} of Ekofisk crude oil, 1 g l^{-1} NH$_4$NO$_3$, 15 g l^{-1} of purified agar in artificial seawater (Marine Salts, TAP, Filton, Bristol), the steamed mixture was then sonicated (ultrasonic processor w-385, Heat systems, Ultrasonics inc.) for 5 minutes and autoclaved for 15 minutes at 121°C. After cooling to 45°C phosphates were added aseptically, using sterile solutions to yield a final pH of 7 (3 ml l^{-1} 10 % solution KH$_2$PO$_4$, 7 ml l^{-1}, 10 % solution K$_2$HPO$_4$ l^{-1}). The plates were incubated for 96 hours at 30°C, after which individual colonies were subcultured onto nutrient agar (Oxoid). Pure cultures were gram stained, and tested for the presence of cytochrome C oxidase, catalase and their ability to grow in minimal media (section 2.2.2) supplemented with 0.1 % v/v Ekofisk crude oil.

6.2.2. *Whole cell immobilization.*

6.2.2.1. Cell Loading of Drizit.

P. fluorescens and an Ekofisk, oil-degrading, gram-positive coccus isolated from the Erme estuary were immobilized on Drizit as detailed in section 2.2.3.2.

6.2.3. *Microcosm set-up.*

Erme estuary water samples were analyzed for levels of nitrates and phosphates using an autoanalyzer (Technicon, Appendix 7) and for levels of total carbon (TOC-5000, Shimadzu, Appendix 6). Four 12 l tanks were filled with 6 litres of untreated estuarine water from the Erme, and 0.1 % v/v Ekofisk crude oil was added. Nitrates (KNO$_3$) and phosphates (Na$_2$HPO$_4$) were optimized in two microcosms to give ratios of C:N and C:P of 10:1 and 30:1 respectively (Atlas 1991b). Immobilized bacteria; immobilized *P.fluorescens*, immobilized *P.fluorescens* supplemented

with nitrates and phosphates or the immobilized indigenous oil degrader supplemented with nitrates and phosphates, were added to 3 separate tanks to give 2.83 mg protein l^{-1} estuarine water. An uninoculated tank was prepared to allow for the degradation of Ekofisk crude oil by the indigenous population of bacteria. The microcosms were incubated in a constant temperature room (19°C) on magnetic stirrers for eight days.

6.2.4. *Extraction and analysis of Ekofisk crude oil.*

Samples (1 ml) were removed daily from the free suspensions of the microcosms and Ekofisk crude oil extracted and analysed using the method detailed in section 3.2.9.1.

6.2.5. *Cellular growth of Pseudomonas fluorescens , the indigenous oil degrader and total heterotrophs.*

Viable counts were carried out daily, in duplicate, over the eight day incubation period, on the aqueous suspensions of all the microcosms to give numbers of; 1) total heterotrophs using nutrient agar (Oxoid), 2) the indigenous oil degrader using nutrient agar (Oxoid), 3) *Pseudomonas* species using *Pseudomonas* agar base cm559 (Oxoid), supplemented with CFC supplement SR103 (Oxoid).

6.2.6. *Statistical analysis.*

Multifactor analysis of variance (MANOVA) and a multiple range test - least significant difference (LSD) was carried out using Statgraphics version 6.1, on the growth data to determine any significant difference between the numbers of total heterotrophs and *Pseudomonas* in and between each of the microcosms over time.

6.3. Results.

6.3.1. *Bacterial isolates.*

The salinity of the water at the Erme estuary was 32 %, pH 7.3 and the temperature 19°C.

Six bacteria were isolated from the estuary using membrane filtration and incubation on oil medium

No. 2 (Walker and Colwell 1975). The results of the identification tests can be seen in table 6.1.

Table 6.1. Identification results of six bacteria isolated from the Erme estuary.

Gram Stain	Oxidase	Catalase	Minimal media + Ekofisk
negative rod	-	+	Growth
negative rod	-	+	Growth
negative rod	+	+	Growth
negative rod	+	+	Growth
negative rod	+	+	Growth
positive coccus	-	-	Growth

The morphology of the positive coccus (mucoid and yellow) was distinct from the other bacteria

isolated from the Erme estuary and from *P. fluorescens* which allowed easy identification of the

strain in the growth studies.

6.3.2. *Analysis of Ekofisk crude oil.*

An oil-degrading *P. fluorescens* or an oil-degrading bacterium isolated from the Erme

estuary were immobilized to Drizit and their abilities as bioremediation agents compared over eight

days under a set of environmental conditions similar to those found at the Erme estuary. After

eight days incubation, enhanced biodegradation of C_{12} - C_{18} was seen in the microcosm

supplemented with nutrients and inoculated with *P. fluorescens* in comparison to all the other

microcosms (table 6.2), with an average biodegradation of 46.4 %. Enhanced biodegradation of

pristane and phytane was seen in the microcosm inoculated with immobilized *P. fluorescens* in

comparison to all the other microcosms (table 6.2), with an average biodegradation of 45.5 %. The microcosm inoculated with the immobilized indigenous species showed an average degradation of 33.8 % (table 6.2). All three immobilized systems showed an average degradation of 41.9 %, in comparison the uninoculated microcosm which showed marginal degradation of carbons C_{12}, C_{13}, C_{15}, C_{16}, C_{17} and no degradation of C_{14}, C_{18}, pristane and phytane, with an average degradation of 7.1 %.

Table 6.2. Percentage degradation of C_{12} - C_{18}, pristane and phytane in Ekofisk crude oil, after eight days exposure in estuarine microcosms. Microcosm 1 - uninoculated, microcosm 2 - immobilized *P. fluorescens*, microcosm 3 -immobilized *P. fluorescens* supplemented with nitrates and phosphates, microcosm 4 - indigenous oil degrader supplemented with nitrates and phosphates.

	percentage carbon degradation								
Microcosm	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	Pristane	Phytane
1	30.1	4.1	0	4.5	24.6	0.3	0	0	0
2	39.6	36.3	36.4	50.2	60.9	53.7	41.3	33.5	57.6
3	52.4	42.2	41.7	51.5	62.2	56.5	49.7	21.6	39.8
4	46	39.9	34.6	38.2	57.4	36.1	32.1	0	19.5

6.3.3. Cellular growth of *Pseudomonas fluorescens*, the indigenous oil degrader and total heterotrophs.

Growth data (day 0 to day 5) from the four microcosms were compared statistically. MANOVA showed a significant difference between the number of total heterotrophs in each of the four microcosm (p = 0.004), and a significant difference between the number of total heterotrophs in each of the four microcosms over time (p = 0.00). The LSD test showed a significant difference between the number of total heterotrophs in the microcosm inoculated with immobilized *P. fluorescens* and all the other microcosms, the microcosm inoculated with immobilized *P. fluorescens* having significantly more total heterotrophs than the other microorganisms.

In the two microcosms inoculated with immobilized *P. fluorescens* the growth of the total heterotrophs followed similar patterns for the first four days with the level of growth in the supplemented system below that seen in the unsupplemented system. After four days, growth in the supplemented microcosm declined (figure 6.3).
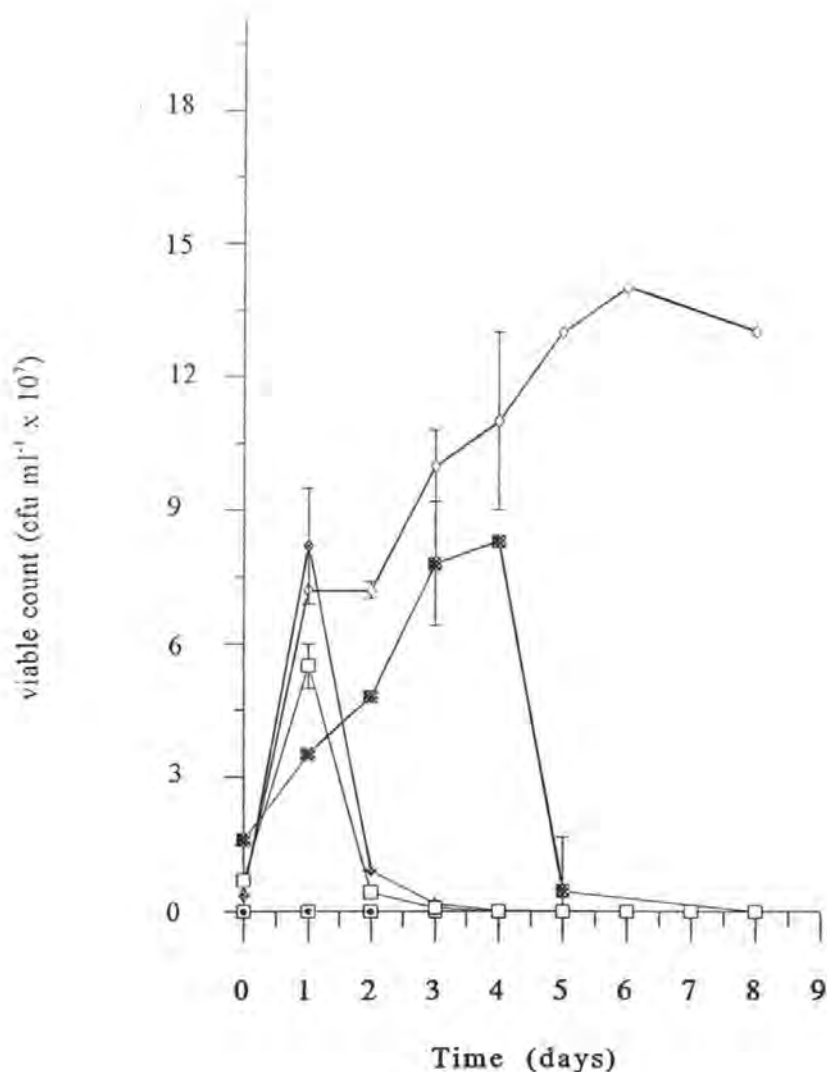
Figure 6.3. Growth of total heterotrophs over eight days in the aqueous suspensions of free and immobilized estuarine microcosms supplemented with Ekofisk crude oil. Bars indicate standard error. ▣ - uninoculated, ◇ - inoculated with immobilized *P. fluorescens*, ▩ - inoculated with *P. fluorescens* and supplemented with nutrients, ✦ - inoculated with the indigenous oil degrader and supplemented with nutrients, ▢ - growth of the indigenous oil degrader in the microcosms inoculated with the indigenous oil degrader and supplemented with nutrients.

The non-supplemented microcosm showed an increase in growth until day 7, after which a small decline was observed. Although growth in the uninoculated microcosm followed a typical growth pattern, marginal growth occurred. In the microcosm inoculated with the immobilized indigenous species, the total heterotrophs and the indigenous oil degrader showed an increase in growth from day 0 to day 1 after which growth fell for the remainder of the sampling period. The growth of the indigenous oil degrader was below that of the total heterotrophs for the eight day incubation period (figure 6.3).

MANOVA showed a significant difference between the number of *Pseudomonas* in each of the four microcosms (p = 0.00) and between the number of *Pseudomonas* in each of the four microcosms over time (p = 0.00). The LSD follow-up test showed a significant difference between the number of *Pseudomonas* in the microcosm supplemented with nutrients and inoculated with immobilized *P. fluorescens* and all the other microcosms. The microcosm supplemented with nutrients and inoculated with immobilized *P. fluorescens* having significantly more *Pseudomonas* than the other microcosms.

Figure 6.4 illustrates the growth of *Pseudomonas* in the four microcosms. In the two microcosms inoculated with immobilized *P. fluorescens* growth increased over the first three days of incubation. In the unsupplemented system growth continued to increase until day seven, while growth in the microcosm with nutrient supplementation fell from day 3 to day 7. In the uninoculated microcosm and the microcosm inoculated with immobilized indigenous oil degrader, low levels of *Pseudomonas* were seen throughout the eight day incubation period.

MANOVA showed a significant difference (p = 0.012) between the numbers of the indigenous oil degrader and the number of *Pseudomonas* in the microcosm supplemented with nutrients and inoculated with the immobilized indigenous oil degrader, and a significant difference between the numbers of the indigenous oil degrader compared to the number of the *Pseudomonas* in this microcosm over time (p = 0.00). The number of the indigenous oil degrader being significantly

higher than the number of *Pseudomonas* species. The number of the indigenous oil degrader were then statistically compared to the number of total heterotrophs (MANOVA) in this microcosm, no significant difference was found between the two ($p = 0.153$). The viable counts for the indigenous oil degrader and the total heterotrophs in the microcosm supplemented with nutrients and inoculated with immobilized indigenous oil degrader varied significantly with time ($p = 0.00$) although no significant difference was found between the viable counts of each organism over time ($p = 0.086$).
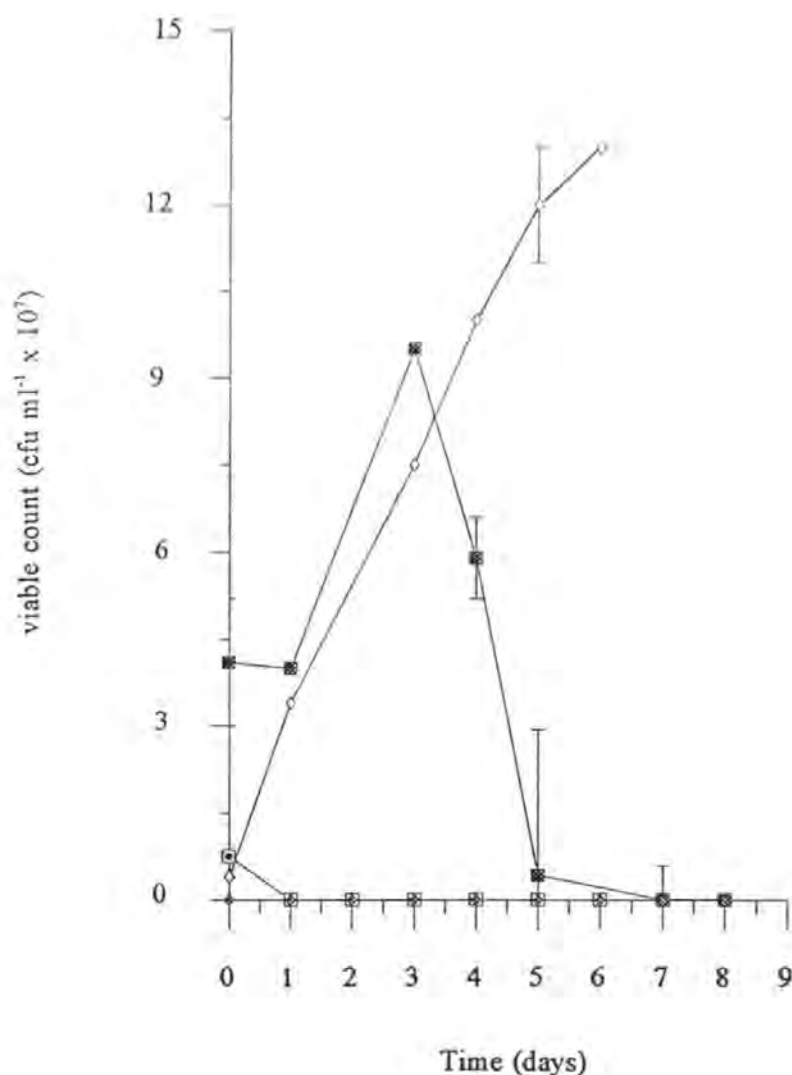
Figure 6.4. Growth of *Pseudomonas* species over eight days in the aqueous suspensions of free and immobilized microcosms supplemented with Ekofisk crude oil. Bars indicate standard error. ♦ - uninoculated, ◊ - inoculated with immobilized *P. fluorescens*, ■ - inoculated with *P. fluorescens* and supplemented with nutrients, ⊙ - inoculated with the indigenous oil degrader and supplemented with nutrients.

The number of *Pseudomonas* and total heterotrophs in the microcosm supplemented with nutrients and inoculated with immobilized *P. fluorescens* were statistically compared and a significant difference was found between the two (p = 0.037). A significant difference was also found between the numbers of the total heterotrophs and *Pseudomonas* in this microcosm over time (p = 0.0005). The number of total heterotrophs being significantly higher than the number of *Pseudomonas*. The viable counts of the *Pseudomonas* and total heterotrophs in the microcosm inoculated with immobilized *P. fluorescens* with no nutrient supplementation were also compared and no significant difference was found (p = 0.379). Lastly no significant difference was found between the viable counts of the *Pseudomonas* and total heterotrophs in this microcosm over time (p = 0.3483).

6.4. Discussion.

Enhanced growth and biodegradation of the n-alkanes C_{12}- C_{18}, pristane and phytane in Ekofisk crude oil occurred with immobilization of *P. fluorescens*, in an estuarine microcosm and a nutrient supplemented estuarine microcosm, compared to an uninoculated microcosm and a nutrient supplemented microcosm inoculated with the immobilized indigenous oil degrader. High numbers of *Pseudomonas* were found in the free suspension of the microcosms inoculated with immobilized *P. fluorescens*. Growth of immobilized cells within the matrix can result in the sloughing of cells into the medium as biofilms detach as the cells in the lower layers die and degrade (Rosevear 1984). This data provides evidence that surfaces provide good growth conditions for aquatic microbes, provide protection against predation (Ahl *et al*. 1995) and increase the availability of oil to bacteria. Increased growth due to immobilization has also been shown by Brettar and Hofle (1992), who found that the attachment of bacteria to particulate matter increased the survival of microbes introduced into mesocosms.

The degradation levels in the two microcosms inoculated with immobilized *P. fluorescens* followed similar trends to the results found in the saltwater study described in chapter 2, where similar levels of biodegradation were achieved in a saltwater system containing immobilized *P. fluorescens* both with and without nutrient supplementation. In this present study, the two microcosms inoculated with immobilized *P. fluorescens* (one supplemented with nitrates and phosphates) showed similar levels of biodegradation of Ekofisk crude oil. However, the system with nutrient supplementation showed significantly higher levels of growth of *Pseudomonas* species. This increase in growth with nutrient supplementation is not in accordance with findings in chapter 2 where no significant difference between the levels of growth in the two systems was found.

It was postulated in chapter 2 that further enhancement of the levels of biodegradation achieved with immobilization, may not have occurred in a saltwater system with nutrient supplementation because the nutrients added to the system may not have been available to the immobilized bacteria.

In this study the supplemented nutrients increased the growth of the *Pseudomonas* species but did not result in any further enhancement of biodegradation. This may indicate that a more accessible carbon source other than the Ekofisk crude oil was available to the immobilized bacteria when supplemented with nitrates and phosphates. The supplemented nutrients may however, be more accessible to non-immobilized bacteria than immobilized bacteria. This is illustrated by the data from this study where the number of total heterotrophs in the nutrient supplemented system was significantly higher than the number of *Pseudomonas* species, but in the system with no nutrient supplementation no significant difference was seen between the number of *Pseudomonas* species and the total heterotrophs.

The natural population of bacteria found at the Erme (the uninoculated microcosm), although adapted to the environmental conditions of the microcosms i.e temp, pH and salinity, achieved low levels of growth with concomitant low levels of biodegradation, possibly due to the absence of surfaces to provide protection against predation or to increase the availability of the crude oil. In the microcosm supplemented with nutrients and inoculated with the immobilized indigenous oil degrader, the level of biodegradation was lower than that achieved by the two microcosms inoculated with immobilized *P. fluorescens*, but higher than that achieved by the uninoculated microcosm. The number of the indigenous oil degrader was significantly higher than the number of *Pseudomonas* over the eight day incubation period (this provides evidence that the high numbers of *Pseudomonas* seen in the other microcosms must have been sloughed off from the immobilization substratum). However, there was no significant difference between the number of the indigenous oil degrader and the total heterotrophs. This demonstrates as mentioned previously, that sloughing off of the cells from the matrix may have occurred resulting in an increase in the indigenous oil degrader in the surrounding medium in relation to the numbers of total heterotrophs.

The growth and degradation levels achieved by the immobilized indigenous oil degrader were expected to be higher than the levels achieved in the other immobilized systems. The bacterium was originally isolated from the Erme estuary and the microcosms were set up using water samples

from the Erme and incubated in conditions similar to those found at the site of isolation. Indigenous microbes should be fully adapted to their environmental conditions and so enhanced levels of survival in similar conditions would be expected in comparison to a non-indigenous species. Several theories may explain the results obtained;

1) Each individual in a population must compete with others for resources and not all the individuals are likely to survive in any one habitat. Any individuals which are not as fit or are unable to adapt to the environmental conditions, may die. After being introduced to a new habitat not all cells will respond well to the existing environmental conditions, and despite their versatility no single bacterium has the ability to adapt to all accessible habitats of an ecosystem. When environmental conditions are extremely severe, individuals will be completely destroyed as a result of irreversible damage to their cells. When the conditions are less severe but unfavourable for growth and proliferation, cells may enter a stage of dormancy (Edmonds 1978).

2) The process of isolation of the indigenous oil degrader from the Erme and repeated transferral to obtain pure cultures may have affected the survival of the bacteria in the microcosm. For example genetic changes may have occurred during isolation, with the result that the bacteria were no longer fully adapted to the original conditions from which they were isolated. In comparison the indigenous *Pseudomonas* and other total heterotrophs were probably adapted to the environmental conditions presented to them in the microcosms, so the indigenous oil degrader or 'foreign' organism would have been expected to compete with the indigenous organisms to survive in the new environment.

3) Organisms grown axenically (i.e cultures of a single species or micro-organisms) on a simple sterilized medium are unlikely to behave in the same way in a complex environment where other organisms are present which compete for available nutrients.

Time restraints during this investigation meant only one sample of Ekofisk crude oil was removed

per microcosm on each day of the incubation period, which resulted in large variations in biodegradation levels between samples removed on different days from the same microcosm (appendix 5a-i). The microcosms were heterogenous environments similar to that found in the field. The crude oil was also heterogenous with the attributes of the oil differing across the surface of the microcosm, with a more viscous appearance around the edges of the tanks. One aim of the study was to compare the biodegradation achieved in each system daily over the incubation period. However, due to the fluctuations observed over the eight day incubation period (appendix 5a-i), the biodegradation levels achieved by each system on day eight of the incubation period were used for comparison. Modification of the sampling methods would reduce variation in the data and could include removing several samples on any one day, amalgamating these daily samples and then removing samples from the amalgamated oil to test by gas chromatography, reducing the error previously encountered due to the heterogeneity of the oil and the microcosms. Further work could include; 1) repetition of the above study incorporating the aforementioned modification, 2) the establishment of the availability of supplemented nutrients to immobilized bacteria and the effect of these supplements on biodegradation, 3) the use of oleophilic nutrients (Marty and Martin 1993; Churchill *et al.* 1995) with immobilized bacteria and their effect on biodegradation, 4) the possible use of nutrients adsorbed to the immobilization matrix (to maintain and prevent dilution of nutrients in the area of the spill) and their effect on biodegradation, 5) investigation into the production of biosurfactant by the free and immobilized indigenous oil degrader in comparison to free and immobilized *P. fluorescens*. This may give further insight into the role biosurfactant production plays in hydrocarbon biodegradation by immobilized bacteria.

The microcosms simulated estuarine environments, however it must be considered that the structure of the microbial community may have changed as a result of the confinement of the water. A flow-type system would have been more suitable than a batch system but was not available due to technological restrictions. This would have permitted for example, tidal action and the flow of nutrients. The implications of changes taking place in the microbial community is that, if selection on natural microbial populations in the microcosms differ from the natural system, the same may

be true for bacteria introduced into the model system. Care must be taken when microcosm results are extrapolated to the natural environment.

In summary, the results of this study illustrate enhanced biodegradation of oil can be achieved on a larger scale than previously demonstrated. An immobilized bacteria system can be applied to a specific set of environmental conditions with a resulting increase in biodegradation in comparison to free living and immobilized indigenous species. Thus, if further work shows that enhanced biodegradation can be achieved in mesocosms, by adapting the immobilization strategy to a specific ecosystem to give optimum conditions for enhanced biodegradation, then immobilized bacteria technology may have the potential to be applied in the field as a bioremediation agent.

Chapter 7.

General Discussion

The microbial degradation of oil pollutants is a complex process. With the increase in understanding of microbial hydrocarbon degradation processes in the environment and the identification of a number of rate limiting factors, it is now possible to develop strategies utilizing microbial hydrocarbon degrading activities for the removal of hydrocarbons from contaminated ecosystems. Current approaches in bioremediation include: 1) Nutrient addition (Atlas 1972a), involving the addition of nitrogen and phosphorous to improve the ratio of carbon:nitrogen and carbon:phosphorous (Olivieri et al. 1978; Atlas 1991a, 1991b). Nutrient addition includes a variety of application techniques and commercially available products which include soluble inorganic nutrients, oleophilic formulations and slow release formulations. 2) The use of microbial seeding which involves the addition of strains of bacteria known to degrade hydrocarbons to a contaminated area (Hoff 1992). These strains could also include genetically engineered microorganisms, problems arise however with the debate surrounding the release of such genetically engineered microbes into the environment. 3) The use of dispersion agents, low concentrations of surfactants added to the environment can enhance the aqueous dispersion of hydrocarbons by reducing the surface tension of a liquid medium (Zhang and Miller 1992), thus increasing the accessibility of hydrocarbons to the bacteria. In the strategy for bioremediation developed in this study, the availability of hydrocarbons to bacteria has been approached (Rosevear et al. 1987; Heitkamp et al. 1990; Atlas and Bartha 1992) which is one of the key issues preventing rapid biodegradation of petroleum products by microorganisms.

This study focused on the use of three immobilization substrata, Biofix, Drizit and polyester polyurethane and their use as bioremediation agents. It was proposed that through immobilization of bacteria to an oleophilic substratum, the problem of the availability of hydrocarbons to the bacteria would be reduced. The oil and bacteria would be brought into close proximity with each other, increasing the passive adsorption of cells to the surface of the hydrocarbon, therefore

increasing the accessibility of the hydrocarbon to the cell, with a final result of increased biodegradation.

It was successfully shown that enhanced biodegradation of petrol (Slovene diesel) and Ekofisk crude oil occurred with the immobilization of an oil degrading *P. fluorescens* to Biofix and Drizit in a freshwater system and to Drizit in a saltwater system. Immobilization increased bacterial growth and accelerated the ability of the cells to initially utilize the carbon sources in comparison to free living bacteria under the same conditions. This acceleration in hydrocarbon utilization was seen as a reduction in the lag phase, for example from six days in a free living system of bacteria to one day with Drizit immobilized cells in a freshwater system. It was speculated that these results were due to a combination of two factors; 1) increased contact between the cells and the hydrocarbon, due to the oil adsorbing to the biosupport, bringing the cell into closer contact with the carbon source, thus increasing the bioavailability of the hydrocarbons to the cell 2) increased production of a rhamnolipid biosurfactant over the first three days of the growth cycle in comparison to the free system which showed a lag phase of two days.

It was concluded that the enhanced levels of rhamnolipid production observed after immobilization were due to the increased contact between the cells and the hydrocarbon. It is not clear whether the early production of this surface active agent is of any benefit to the immobilized cells, or if it is only utilizable by those bacteria sloughed from the substratum into the surrounding medium and which still require the availability of the hydrocarbons to be increased by solubilization or emulsification (Reddy *et al.* 1982, 1983). If the immobilized bacteria can make no use of the rhamnolipid due to the adsorption of the oil to the substratum then the increase in biodegradation must be due to the increased contact between the bacteria and the hydrocarbons. The production of rhamnolipids by *Pseudomonas* is strictly regulated and a model for the regulation is given in figure 7.1. Research on *Pseudomonas aeruginosa* has revealed that the RhlR regulatory protein is activated by binding its autoinducers, which are produced by the RhlI autoinducer synthetase. All of the autoinducers known to date belong to the class of N-acylated homoserine lactones with acyl

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substituents of various lengths. At present it appears uncertain how the autoinducer-dependent regulatory systems of *Pseudomonas aeruginosa* influence the expression of their target genes, how they interact with each other or if the same system is present in other *Pseudomonas* species. The *rhlI* gene itself is under the control of the RhlR regulator. The binding of activated RhlR protein to target sites upstream of the *rhlA* promoter enhances transcription of the *rhlAB* operon, which encodes rhamnosyltransferase (Ochsner and Reiser 1995). The 28-kDa RhlR protein belongs to the LuxR family of transcriptional activators (Ochsner *et al.* 1994). The LuxR protein being the positive activator of the Lux regulon in the marine bacterium *Vibrio fischeri*. It has been assumed (Ochsner *et al.* 1994) that the RhlR protein belongs to a signal transduction pathway responsible for activating target genes in response to environmental stimuli such as nitrogen limitation.
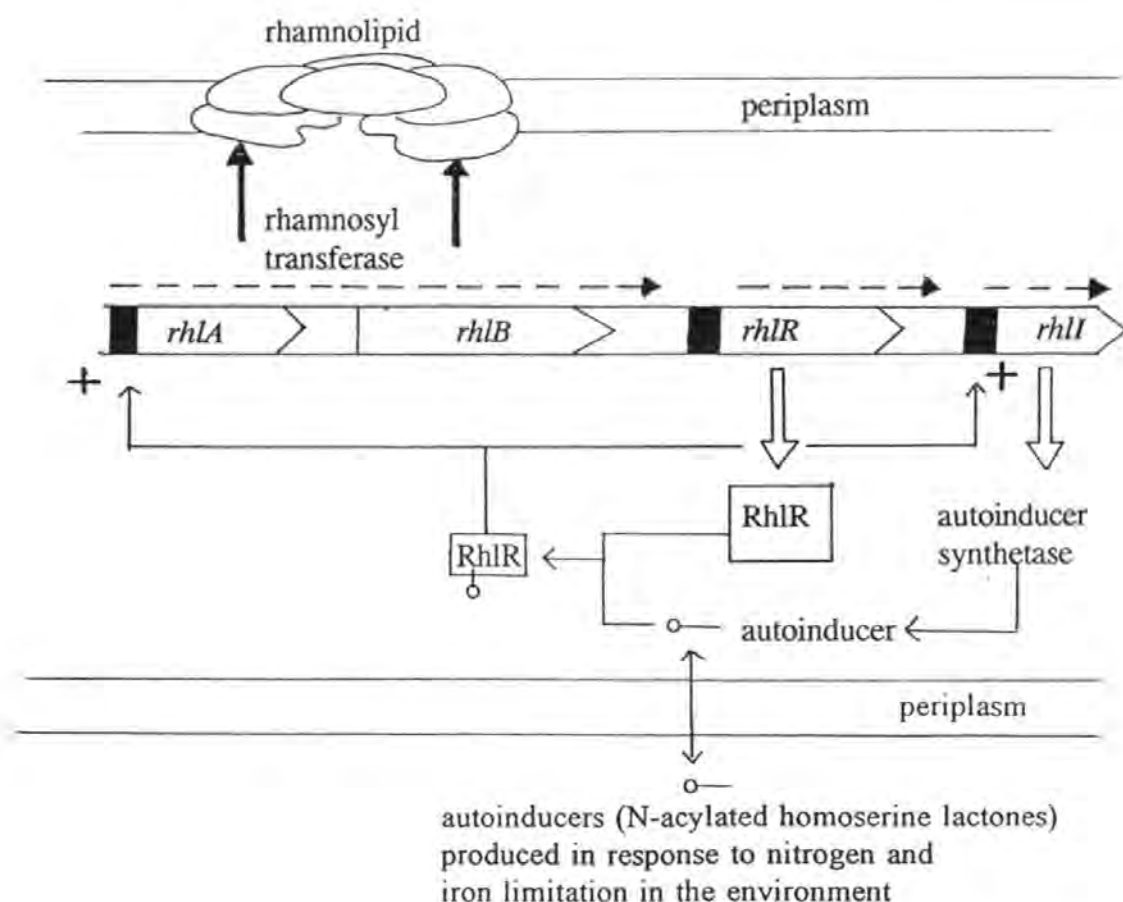
Figure 7.1. Regulation of rhamnolpid production in *Pseudomonas aeruginoas* (adapted from Ochsner and Reiser 1995).

Rhamnosyltransferase catalyzes the transfer of rhamnose from thymine diphosphate (TDP) alpha-L-rhamnose to the fatty acid B-hydroxydecanoyl-B-hydroxydecanoate (figure 7.2)

B-hydroxydecanoyl-B-hydroxydecanoate

TDP-L-rhamnose ─────────┼─────────→ TDP

L-rhamnosyl-B-hydroxydecanoyl-B-hydroxydecanoate

(rhamnolipid 2)

TDP-L-rhamnose ─────────┼─────────→ TDP

L-rhamnosyl-L-rhamnosyl-B-hydroxydecanoyl-B-hydroxydecanoate
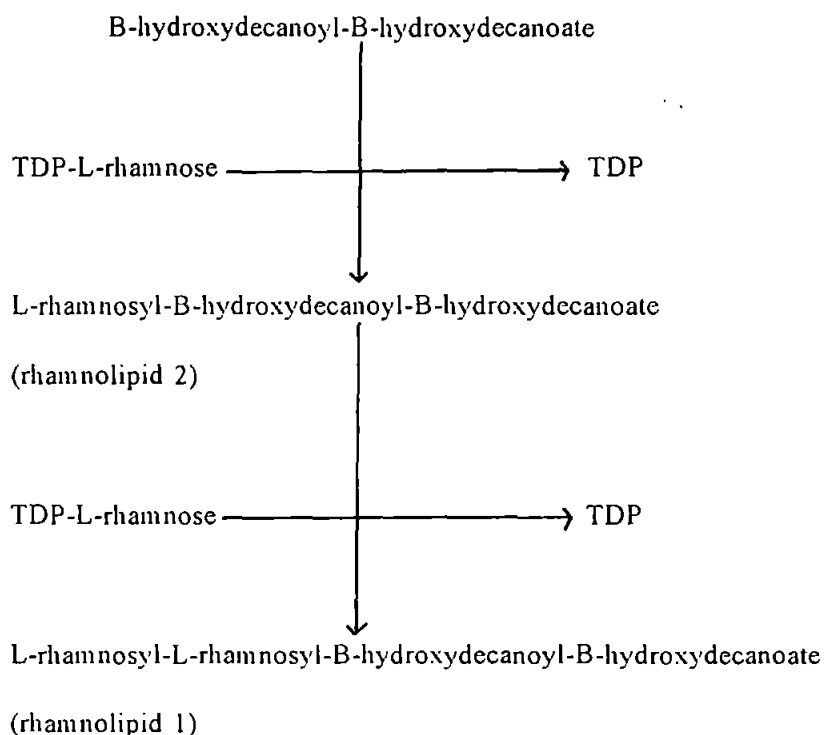
(rhamnolipid 1)

Figure 7.2. Synthesis of rhamnolipid by *Pseudomonas aeruginosa* (Haferburg *et al.* 1986).

Further work could involve investigation into the effect of immobilization on the genetic regulation of rhamnolipid production, with the aim to establish why immobilization results in early production of the rhamnolipid biosurfactant.

It was hoped to determine the location of the genes responsible for alkane degradation in immobilized *P. fluorescens* and to compare this with the genetics of free living *P. fluorescens*, with the aim to further explain the enhanced biodegradation which occurred with the immobilization of cells to an oleophilic substratum. Plasmid DNA was detected using gel electrophoresis, and cesium chloride-ethidium bromide gradient was carried out to confirm the presence of the plasmid. This process was unsuccessful and no band was visualized, therefore the results from this study were inconclusive and leaves scope for further research into determining whether immobilized *P.*

fluorescens carries the genes for hydrocarbon degradation either on chromosomes, a plasmid or a combination of both. If the degradative capabilities are coded for chromosomally then location of the genes on the chromosome could be determined. Further work could also investigate if induction of alkane oxidizing activity occurs earlier in immobilized cells than in free cells, due to the early contact made with hydrocarbons by immobilized bacteria. Previous investigations have observed a lag in the induction of alkane oxidizing activity by alkane substrates, which has been explained due to time needed for hydroxylase activity to form sufficient primary alcohol, for induction to occur (evidence suggests that primary alcohols are effective inducers because one end of the carbon chain mimics the unoxidized alkane molecule), (Grund *et al.* 1975), with immobilization this lag may be reduced due to the increased availability of the hydrocarbons. Grund *et al.* (1975) also discovered induction of alkane oxidizing activity by compounds that do not serve as substrates for either growth or respiration, for example dicyclopropyl methanol. Immobilized cells with an inducer of this type adsorbed to the matrix or the use of previously induced cells, ie. cells grown on oil before or during the immobilization process could result in earlier utilization of alkanes than already demonstrated in this study.

With the demonstration of enhanced biodegradation by immobilization of *P.fluorescens* to Biofix and Drizit, further investigation was carried out to determine whether the type of substratum used for immobilization had any effect on biodegradation. The three supports, Biofix, Drizit and polyester polyurethane were compared by; scanning electron microscopy or Normarski differential contrast microscopy, cell loading capacity, absorbtion of oil, their abilities to enhance oil biodegradation and the effect of drying and storage. The data in this study illustrated that enhanced biodegradation of hydrocarbons by immobilized cells appears to be greatly dependent on the biocarrier. The study showed that not all biocarriers are suitable for use as bioremediation agents of oil pollution, with polyurethane immobilized cells failing to enhance biodegradation of Ekofisk crude oil and it was concluded that this material was unsuitable as a biocarrier for application in bioremediation. It was speculated that the unsuitability of polyurethane may have been due to its degradability by bacteria (Hedrick and Crum 1968; Cameron *et al.* 1988; Jansen *et al.* 1991).

a high cell loading capacity and it was surmised that in future it may be beneficial to carry out work using a more resilient polyurethane, for example a polyether which is less susceptible to microbial attack (Jones and Le-Campion Alsumard 1970; Wales and Sagar 1991) or with an alternative strain of bacteria that does not have the ability to utilize such a diverse range of carbon sources. From comparison of the three substrata, Drizit was found to be the most effective biocarrier tested and the most suitable immobilization substratum for use as a bioremediation agent.

With evidence that immobilization resulted in enhanced biodegradation and having determined that Drizit was the most effective bioremediation agent tested, the successful immobilization system was scaled-up, optimized by supplementation with nitrates and phosphates to establish favourable carbon:nitrogen and carbon:phosphorous ratio's (Olivieri *et al.* 1978; Atlas 1991a, 1991b) and applied to microcosms that modelled an environmental condition. This approach involved using samples collected in the field that contained indigenous microbial populations and was carried out to produce results relevant to a 'real' situation. Enhanced biodegradation of Ekofisk crude oil was demonstrated on a larger scale, in estuarine microcosms. The results from this study indicate that this bioremediation strategy can be optimized to suit a specific set of environmental conditions with resulting enhanced biodegradation.

These microcosm studies also showed the effect of selected parameters; pH, temperature and salinity on the survival of the immobilized bacteria. The natural population of bacteria found at the Erme estuary (the uninoculated microcosm), although adapted to the environmental parameters, achieved low levels of growth with concomitant low levels of biodegradation. In comparison the immobilized, indigenous oil degrader and the immobilized *P. fluorescens* (supplemented and non-supplemented) achieved higher levels of growth and biodegradation, possibly due to the provision of surfaces to provide protection against predation and increased availability of the crude oil due to the oleophilic nature of the substratum. With the knowledge that the immobilized indigenous organism resulted in enhanced biodegradation in comparison to a free system, further work could be carried out to identify the indigenous oil-degrader and to determine if this species produces

biosurfactant to aid its utilization of hydrocarbons. A comparison of biosurfactant production by free and immobilized *P. fluorescens* and the free and immobilized indigenous oil degrader may reveal more information about the part biosurfactant production plays in biodegradation by immobilized bacteria.

To extend the knowledge on the behaviour of immobilized bacteria in the environment it would be necessary to assess the survival of immobilized bacteria when influenced by changing biotic and abiotic environmental factor's using mesocosm studies. Bacteria placed under stressful conditions such as in mesocosms, may not be culturable but may be physiologically active. Evaluation of the survival of these bacteria may have to be monitored by methods other than culture based ones. The combination of microcosm and mesocosm studies would enable a close examination of factors influencing the fate, activity and ecological effects of immobilized bacteria when introduced into a specific ecosystem. The investigations would also provide data relevant to risk assessment analysis on immobilized bacteria released into the environment.

Microbes require nitrogen and phosphorous and other mineral nutrients for incorporation into biomass and the availability of these in the area of hydrocarbon biodegradation is critical (Atlas and Bartha 1972a). Thus, supplementation of immobilized *P. fluorescens* with nitrates and phosphates in a saltwater system would have been expected to further enhance biodegradation, which did not occur. Further work could investigate the effect of different ratio's of C:N and C:P to establish if changing these ratio's enhances biodegradation. Alternatively, the nutrients may not have been fully available to the immobilized bacteria, a solution to this problem may involve the adsorbtion of the nutrients to the matrix or the use of oleophilic nutrients. Recent literature indicates that the use of oleophilic fertilizers cause substantial acceleration of biodegradation and appear to have no detrimental side effects (Bartha 1986; Pritchard and Costa 1991; Pritchard *et al.* 1992; Marty and Martin 1993). The concept of oleophilic fertilizers is based on the use of organic sources of nitrogen and phosphorous in a liquid carrier that is miscible with oil. In theory, when the liquid carrier is applied, the nutrients adhere to the oil (Pritchard *et al.* 1992). If this concept is used in

conjunction with immobilized bacteria technology, when the oil adheres to the substratum, the nutrients would be in close association with it, thus maintaining the oil, the nutrients and the immobilized bacteria in contact for sustained periods. Care must be taken however, to ensure that the liquid carrier is not biodegraded in preference to the oil (Hoff 1992).

The next step forward in using immobilized bacteria technology in bioremediation could involve the use of mixed immobilized cultures. The overall degradation of oil depends upon the generic composition of the microbial community and in particular the enzymes produced by the hydrocarbon degrading species (Atlas and Bartha 1992). Many investigators have suggested that mixtures of hydrocarbon degraders would be necessary in order to effectively degrade all of the hydrocarbons in a complex petroleum mixture (Atlas 1977). Alternatively a multi-plasmid strain with a variety of hydrocarbon degradative plasmids could be used. Such a strain was developed by Friello et al. (1975) and it could oxidize a variety of hydrocarbons including aliphatic, aromatic, terpenic and polynuclear aromatic hydrocarbons. In the presence of suitable inducers all the plasmid-specified pathways were induced and the hydrocarbon substrates oxidized simultaneously. This multi-plasmid strain was shown to grow much faster on crude oil than any bacteria containing just one of the plasmids. The combination of such a strain or a mixed culture with immobilized bacteria technology could result in significantly further enhanced levels of biodegradation.

There is sometimes however, a potential weakness with immobilized bacteria technology which may explain any difficulties encountered when trying to further enhance biodegradation of the n-alkanes with immobilized bacteria. This may arise from mass transfer problems. The high density of active material, places demands on the supply of molecules to the cells at the centre of the matrix. Inadequate transfer of nutrients and/or oxygen through the outer layer of metabolising cells can reduce activity resulting in cell death (Rosevear 1984; Rosevear et al. 1987). It has been suggested that changes in product formation or reduced activity can result from reduced oxygen supply (Mattiason and Hahn - Haegerdal 1982). When examining the three substrata used in this study predictions can be made in relation to problems that could occur with mass transfer into the

matrices. Biofix granules are hollow microspheres with porous walls which allow nutrients, oxygen and products to diffuse freely, therefore, it would appear unlikely that problems of mass transfer would occur with this substratum. Similarly, it would seem unlikely that Drizit immobilized cells would encounter mass transfer problems due to its fibrous nature, with many polypropylene strands and openings exposed to the environment. In contrast polyurethane consists of a porous matrix with a network of orifices through which nutrients, oxygen and products may encounter problems diffusing freely to the centre, possibly resulting in reduced activity, which could account for the lack of enhanced biodegradation with immobilization on polyurethane. Further work is required to investigate reduced activity in these substrata due to mass transfer problems.

Another factor which may account for the lack of enhancement with immobilization of cells on polyurethane could be the method of quantative analysis used to determine the amount of each of the n-alkanes present in Ekofisk crude oil before and after incubation. This involved comparing the peak areas of the n-alkanes to those of the branched alkanes pristane and phytane. Problems can occur if the branched alkanes are subjected to biodegradation with the result that 'false' ratio's may be obtained. These ratios may indicate that less of the n-alkanes have been degraded than in the uninoculated control where the quantity of pristane and phytane may not have been reduced to the same extent by photodegradation or volatilization. Due to these problems further work involving GC analysis incorporated the use of an internal standard. The internal standard, Squalane, had a retention time different from that of any sample component and its concentration was known. The advantage of using an internal standard is that absolute concentrations of components may be calculated individually giving more accurate results.

Another potential problem with immobilized bacteria technology raises the question that once the oil has adsorbed to the surface of the substratum, do the bacteria have the ability to utilize all fractions of the oil, or does the process of adsorption itself render some of the oil inaccessible? This would require investigation into the degradation of the aromatics, alkenes and naphthenes (cycloparaffins) by a species known to degrade these fractions in a free living system and to

establish if these fractions are still available when adsorbed to an immobilization substratum.

In summary, investigation into the use of immobilized bacteria technology in bioremediation has revealed the interaction of many factors which affect the biodegradation of oil using this strategy (figure 7.3). Immobilization ultimately results in the existance of two phases; an immobilized phase and a free phase, due to the sloughing off of cells from the substratum into the surrounding environment. These two phases may interact with the environment in different ways. Mineral nutrients, nitrates, phosphates, carbon and oxygen in the ecosystem are available for utilization by cells in both phases in varing degrees, for example in the immobilized phase it may depend on the substratum used due to problems of mass transfer caused by some matrices. The accessibility of oil to the cells depends on whether the cells are in the immobilized or free phase. In the immobilized state the accessibility of the oil is increased due to the adsorption of the oil onto the substratum increasing contact between the oil and the microbe and therefore enhancing utilization. In saltwater ecosystems supplementation with nitrates and phosphates may be essential to allow enhanced biodegradation of oil by both the free living and immobilized bacteria due to limitation of these nutrients in this environment. The free and immobilized bacteria are in competition for these nutrients and if the nutrients have been added directly into the free phase of the system, their accessibility to the immobilized cells may be reduced. Adherance of these nutrients to the substratum or the use of oleophilic nutrients may overcome these problems. In the immobilized state cells have been shown to produce enhanced levels of a rhamnolipid biosurfactant, at present it is still unclear whether the early production of this surface active agent is of any benefit to the immobilized cells or if it is only utilizable by those bacteria sloughed from the substratum. Those bacteria in free suspension will produce rhamnolipid but usually during the late exponential or the stationary phase of the growth cycle (Ochsner and Reiser 1995) and this production will increase the accessibility of the oil to the free cells due to increased solubilization. Immobilization of cells to a substratum will offer the cells protection from predation and toxicity (O'Reilly and Crawford 1989b).

EXTERNAL
ENVIRONMENT

INDUCER   ─O₂   OIL POLLUTION   NITRATES
                                          +
                                      PHOSPHATES

AQUEOUS
ENVIRONMENT

OIL DROPLETS   NUTRIENTS

biodegradation by
free cells

RHAMNOLIPID

PREDATION

adsorbtion of the
oil to the substratum

OLEOPHILIC
SUSTRATUM

SLOUGHING OF   enhanced biodegradation by immobilized cells -
CELLS   single or mixed cultures or genetically engineered
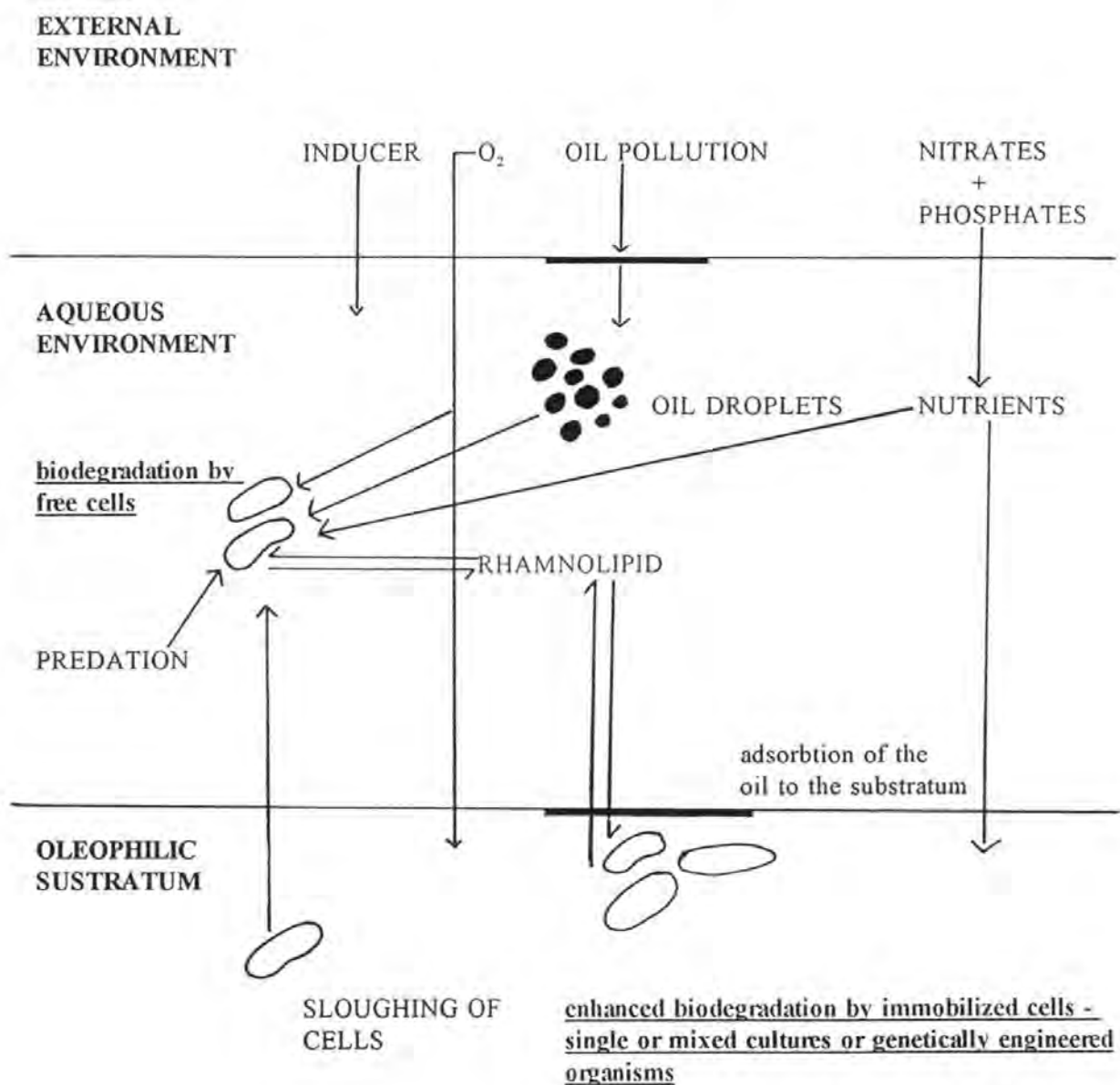   organisms

Figure 7.3. Summary diagram of an immobilized bacteria technology bioremediation strategy illustrating two phases of biodegradation.

The immobilization of cells to an oleophilic substratum could involve the use of single, mixed or genetically engineered organisms and lastly the use of induced cells or an inducer immobilized to the substratum may further enhance biodegradation due to earlier expression of the degradative genes. This would also be of benefit to those cells entering the free phase from the immobilized phase, giving them an advantage over the indigenous population which may not have expression of the genes coding for alkane degradation, especially if no prior exposure to hydrocarbons has occurred. The final result is an optimized system consisting of both free and immobilized cells adapted to degrading hydrocarbons and which consequently produces significant enhancement of biodegradation.

The use of bioremediation has become a major method employed in restoration of oil-polluted environments and immobilized bacteria technology is one strategy of bioremediation making use of natural microbial biodegradation activities. The use of immobilized bacteria technology has been applied in many areas of environmental decontamination e.g wastewater treatment (Linko and Linko 1984) and it may evolve as a technology for use in environments with low levels of contamination, with the substrata concentrating the contaminant into the vicinity of the cells. Advances in the aerobic and anaerobic treatment of wastewater, effluent and condensates using immobilized whole cells has been reviewed (Coughlan and Kierstan 1988), and work could be carried out to investigate the use of immobilized bacteria technology in the bioremediation of metal pollution. This study has shown that whole cell immobilization enhances biodegradation of hydrocarbons in an aqueous environment. These findings put this strategy of bioremediation in good stead for enhanced biodegradation of the less readily utilizable fractions and demonstrates the potential this treatment may have to stimulate the removal of, not only petroleum pollutants but also other pollutants from contaminated areas.

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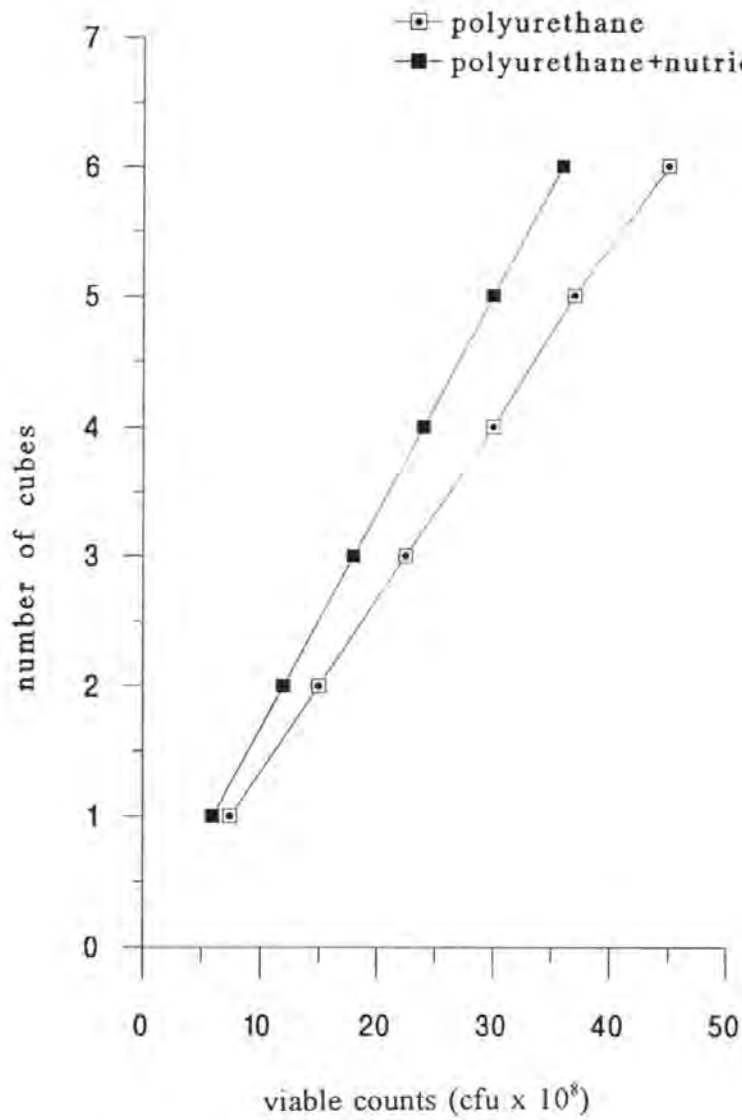
Appendix 1. Calibration curve of calculated viable counts (cfu x 10^8) of *Pseudomonas fluorescens* immobilized on polyester polyurethane cubes against number of nutrient supplemented or non-supplemented polyester polyurethane cubes.
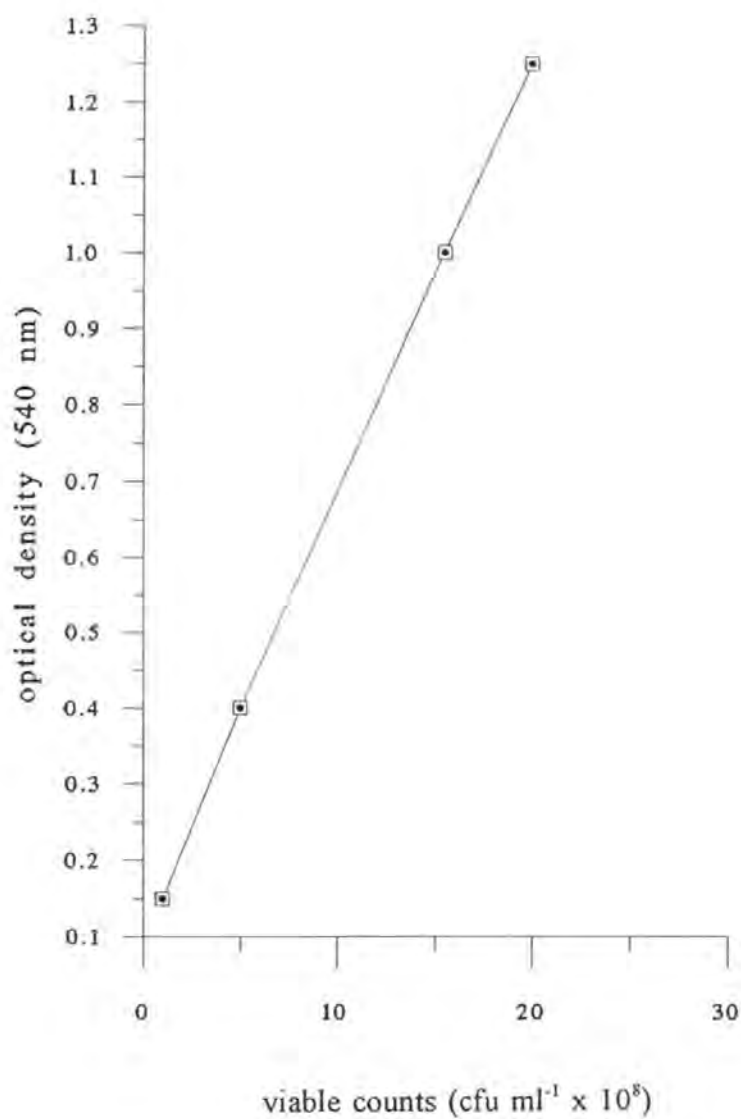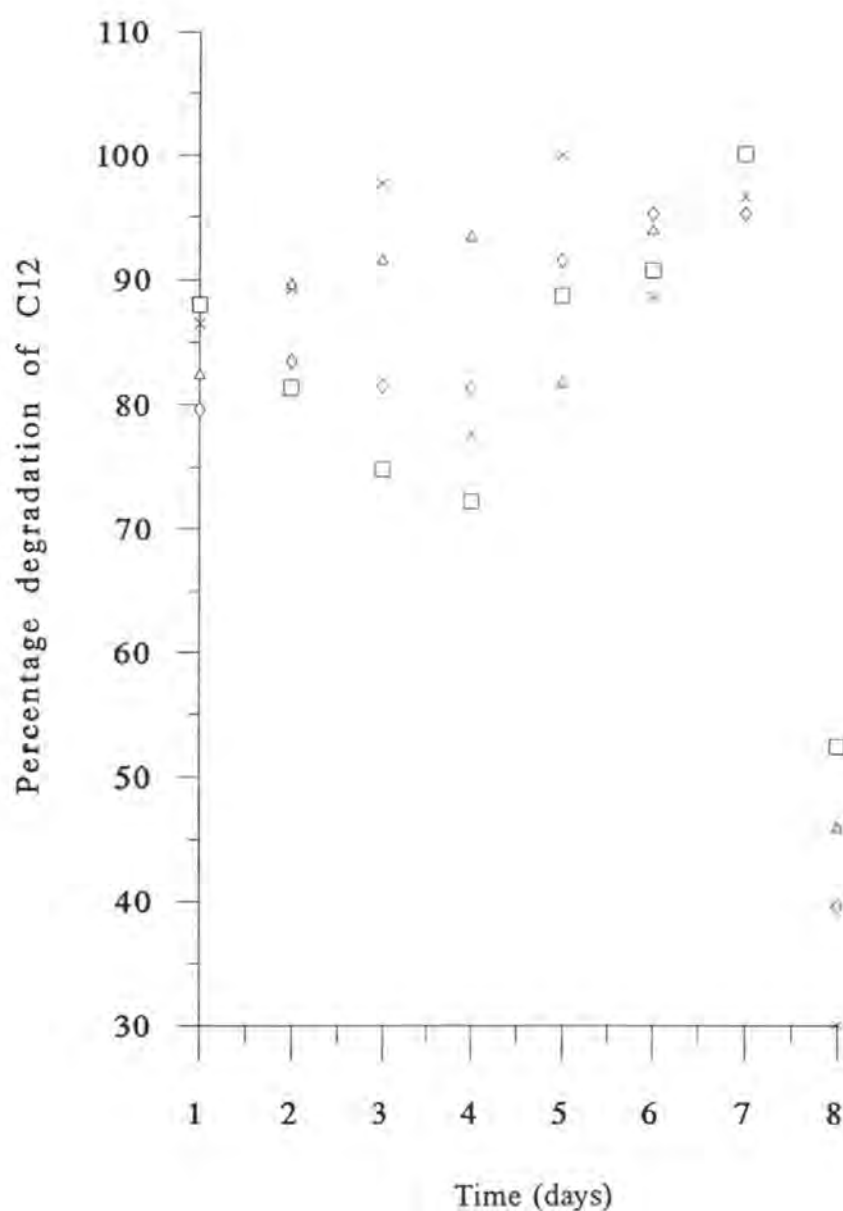
Appendix 2. Calibration curve of calculated viable counts (cfu ml^{-1} x 10^8) of a free system of *Pseudomonas fluorescens* against optical density (540 nm)
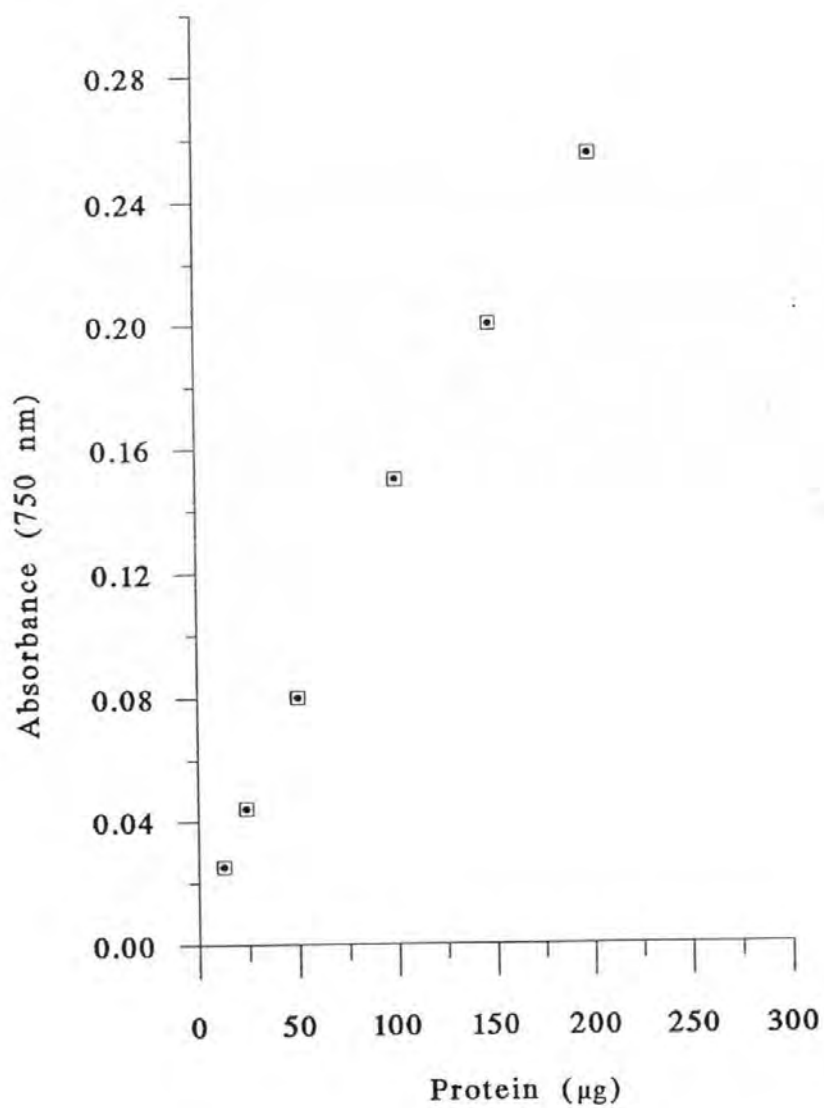
**Appendix 3.** Standard curve of protein (μg bovine serum albumin) against absorbance at 750 nm
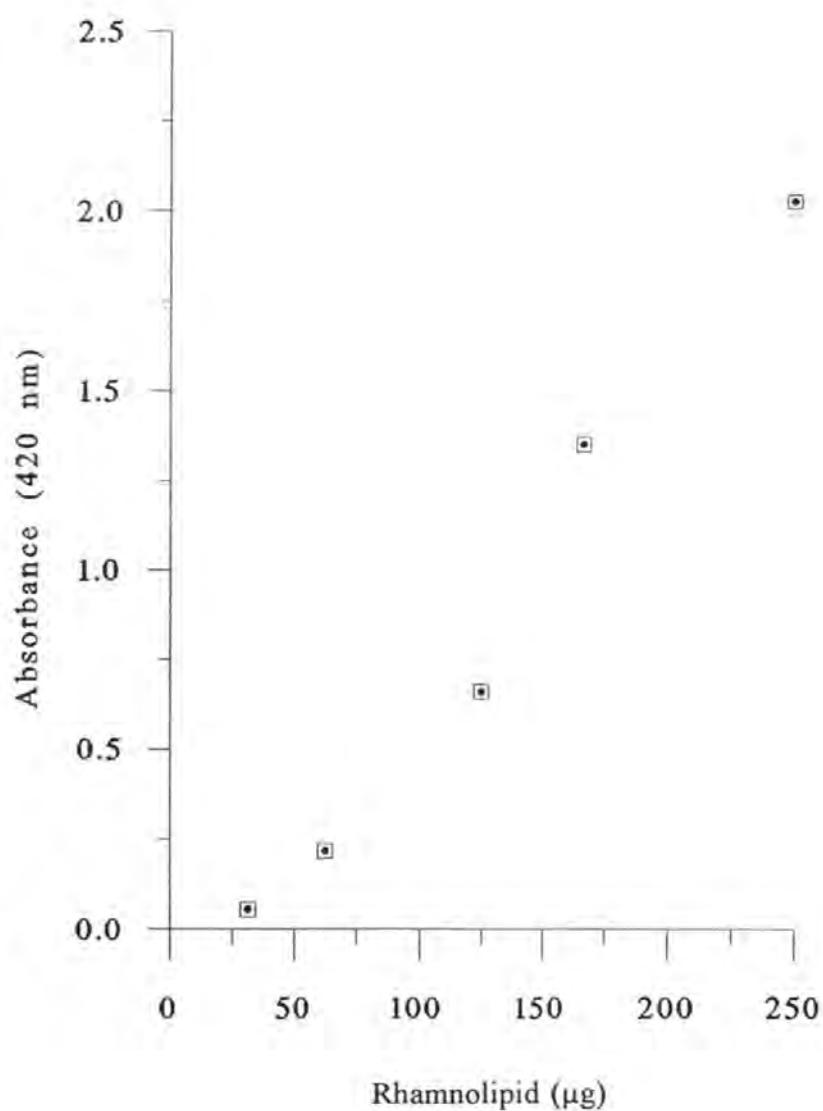
Appendix 4. Standard curve of biosurfactant (μg rhamnolipid) against absorbance at 420 nm.

Appendix 5a. Percentage degradation of C_{12} in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◇ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.
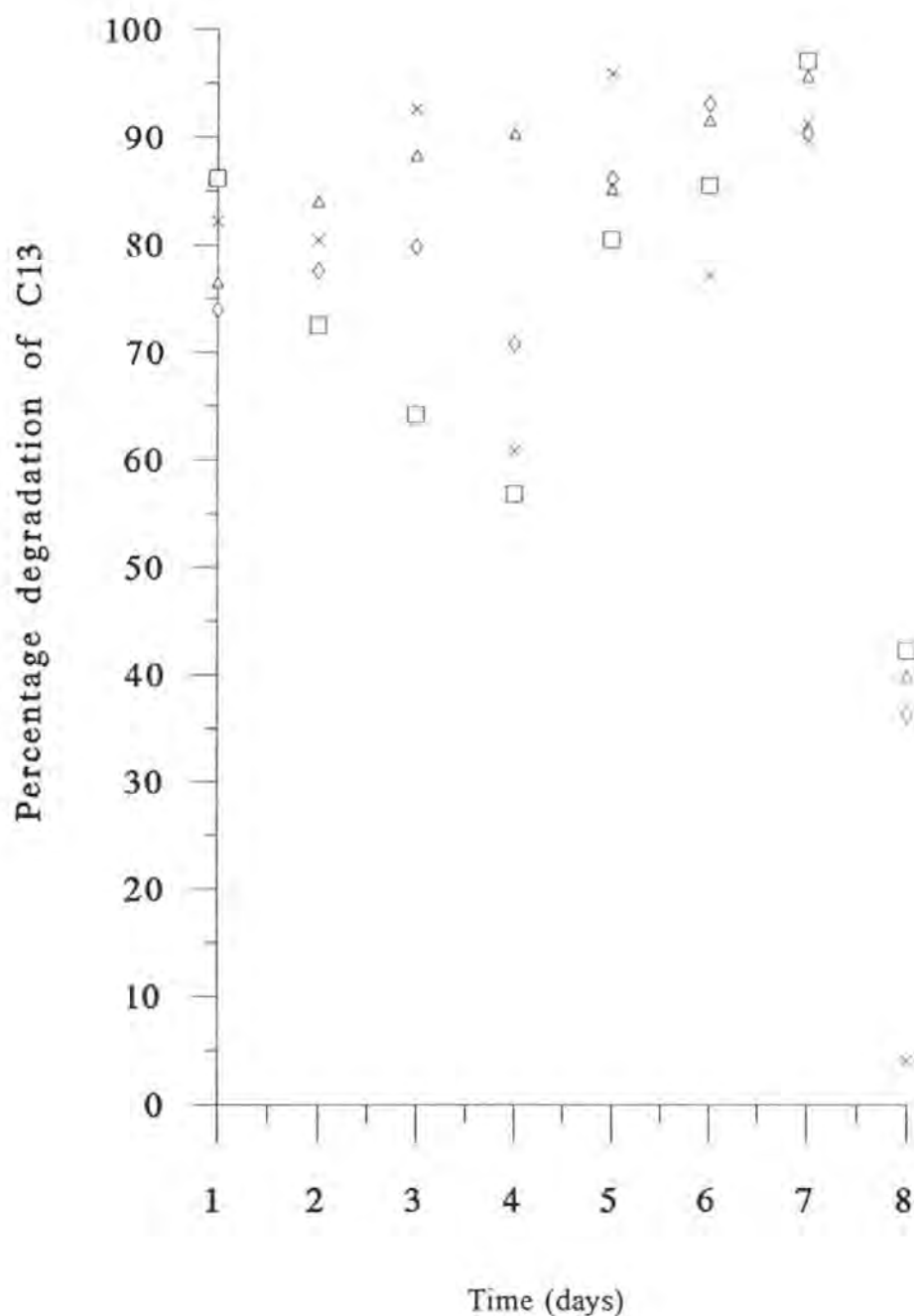
Appendix 5b. Percentage degradation of C_{13} in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◊ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.
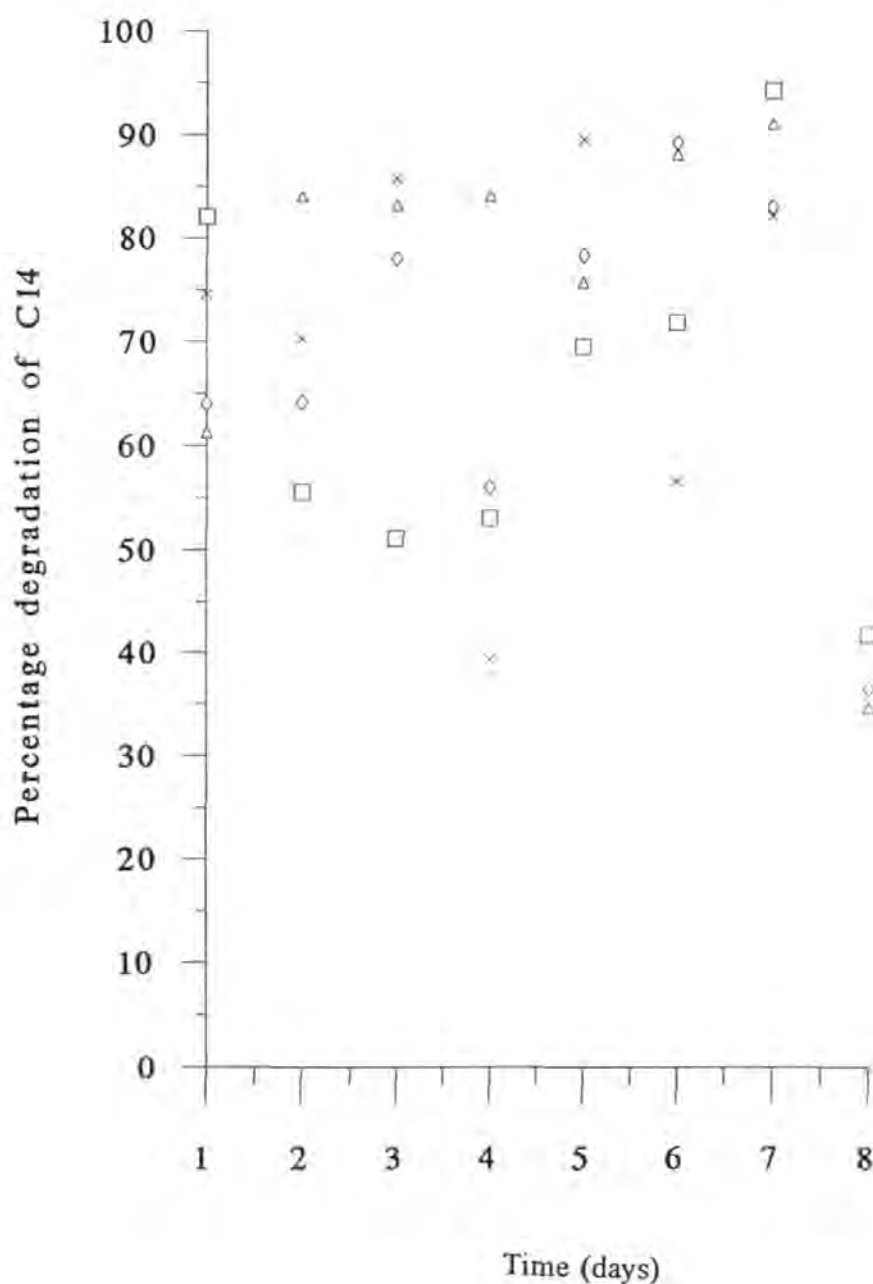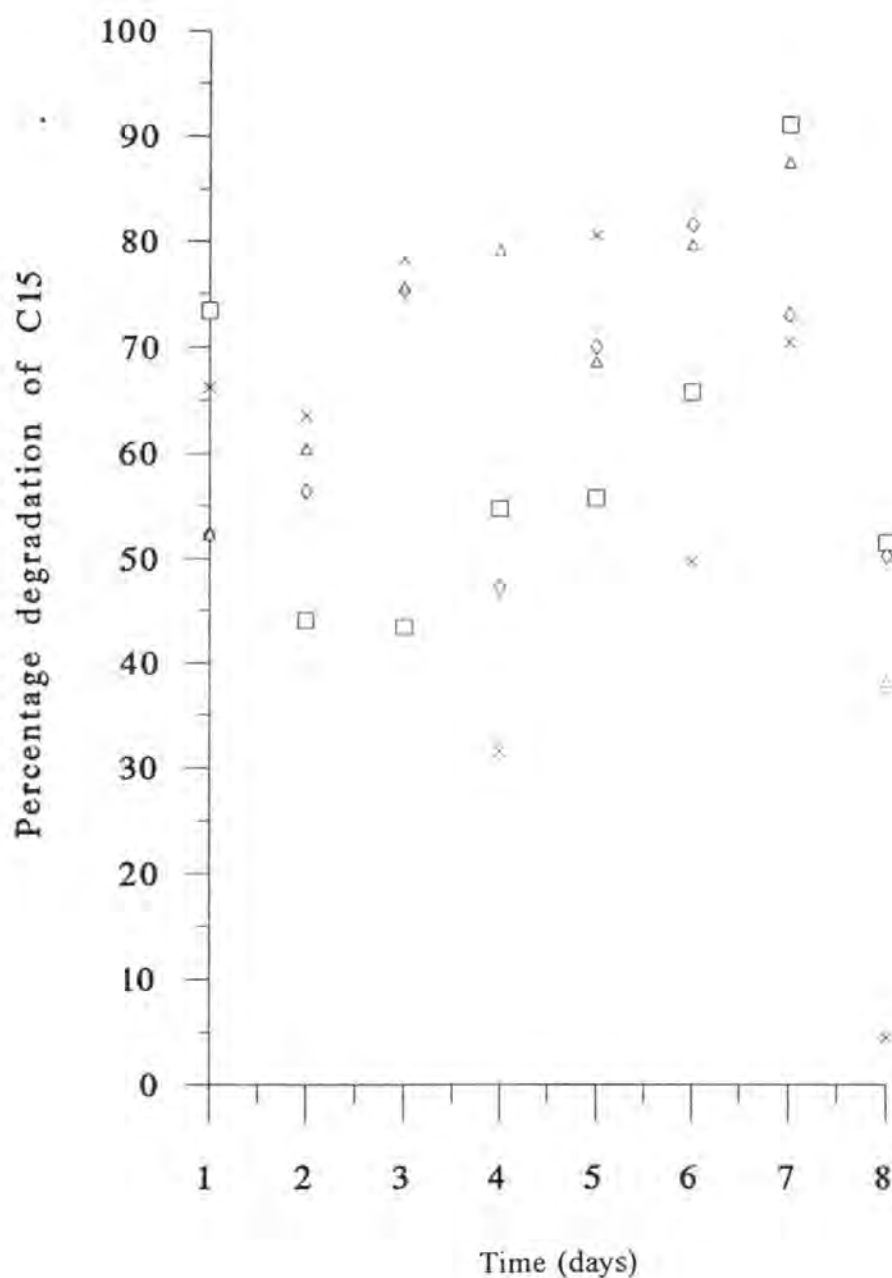
Appendix 5c. Percentage degradation of C_{14} in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◊ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.
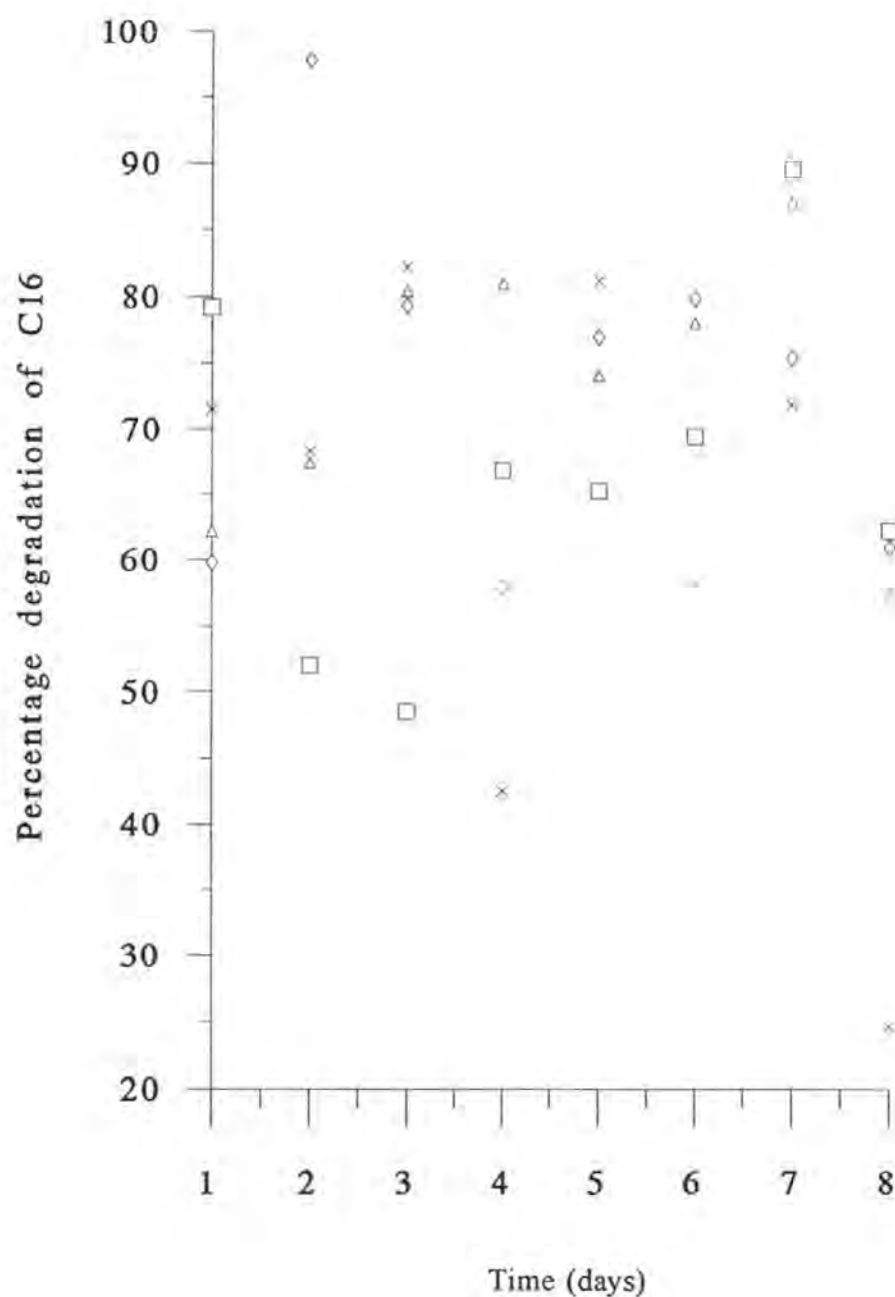
Appendix 5d. Percentage degradation of C_{15} in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◊ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.

Appendix 5e. Percentage degradation of C_{16} in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◊ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.
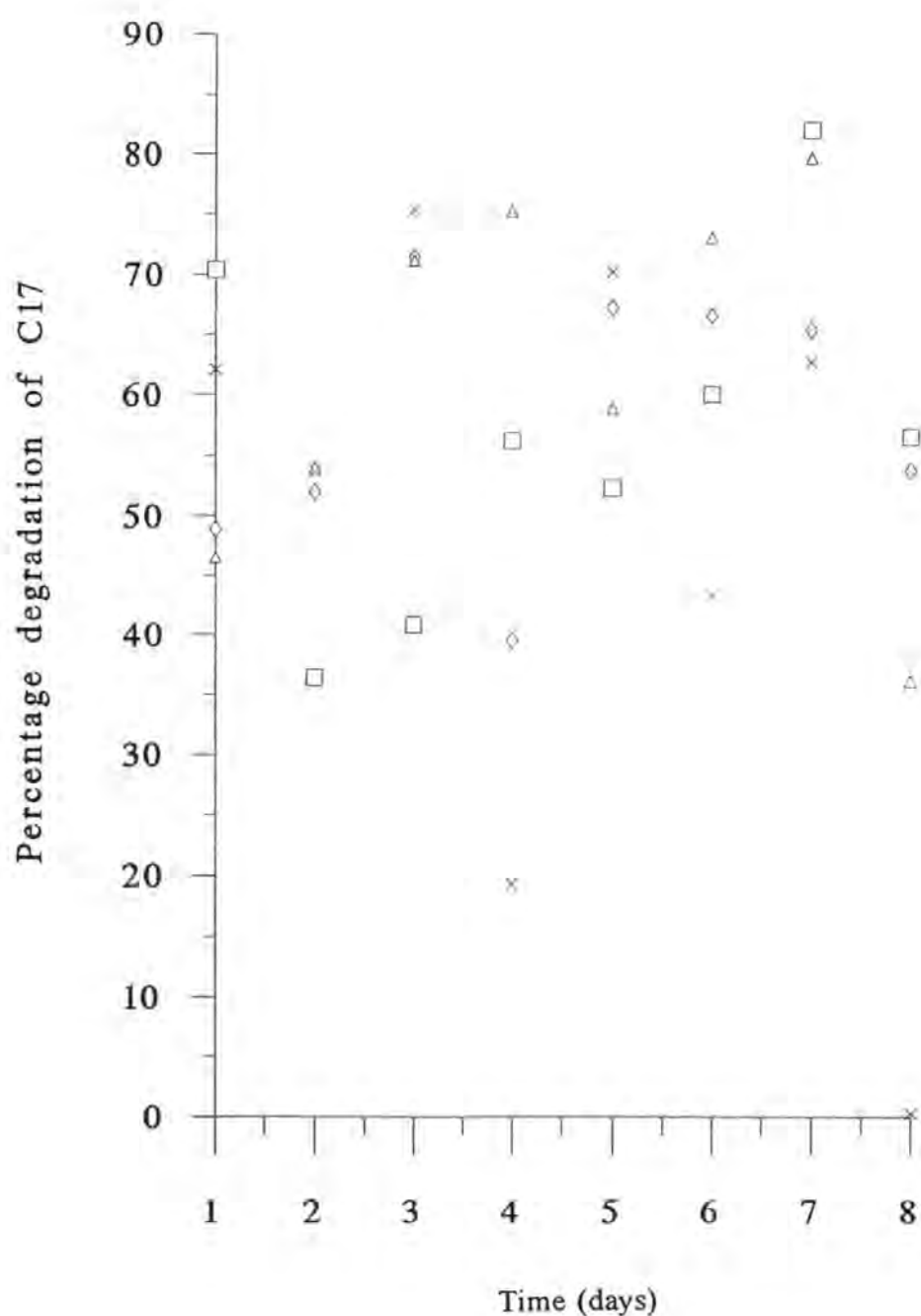
Appendix 5f. Percentage degradation of C_{17} in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◇ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.
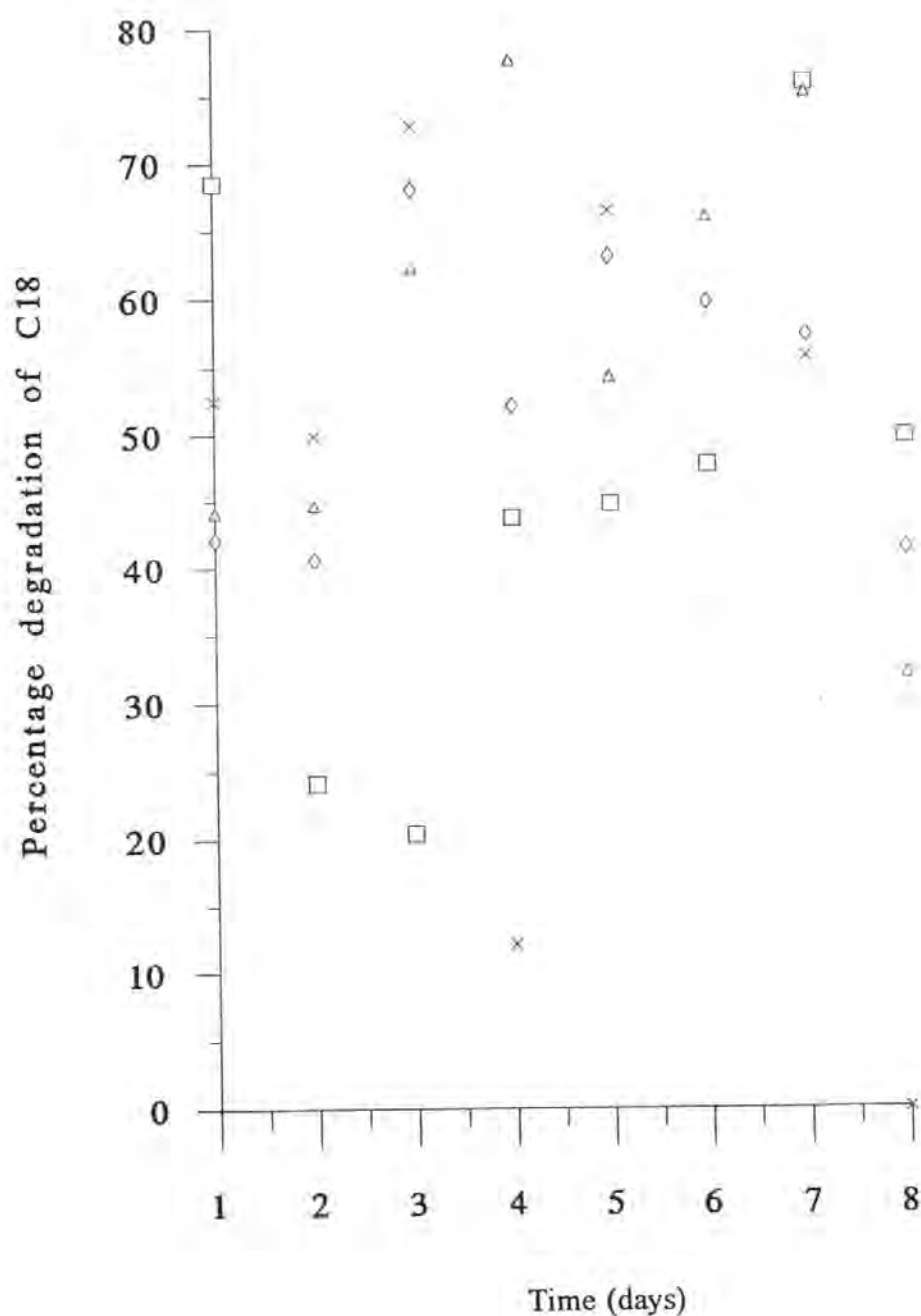
Appendix 5g. Percentage degradation of C_{18} in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◊ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, ▵ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.
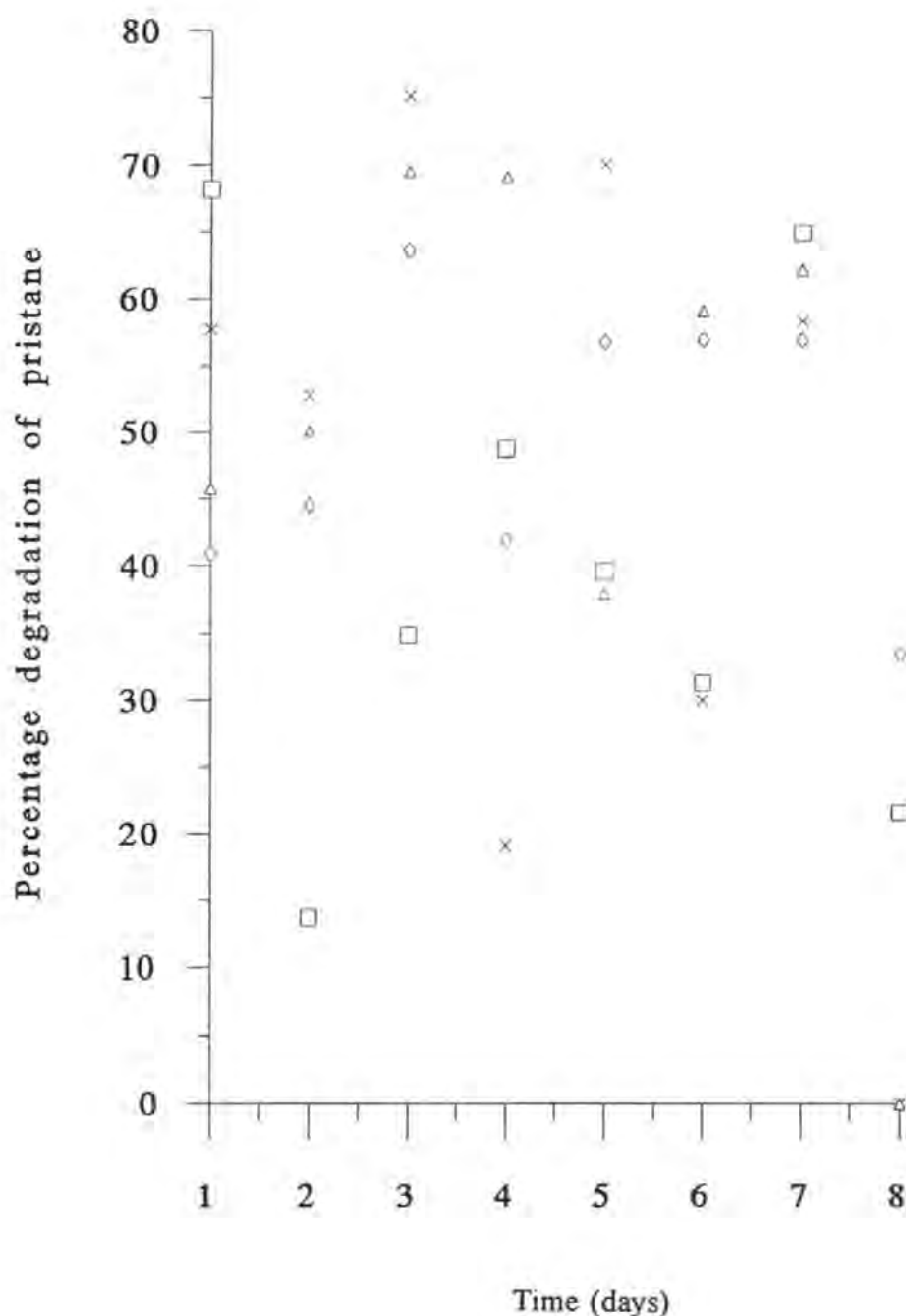
Appendix 5h. Percentage degradation of pristane in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ◊ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.

Appendix 5i. Percentage degradation of phytane in Ekofisk crude oil over eight days incubation in estuarine microcosms. × - uninoculated microcosm, ○ - microcosm inoculated with immobilized *Pseudomonas fluorescens*, □ -microcosm inoculated with immobilized *Pseudomonas fluorescens* and supplemented with nutrients, △ - microcosm inoculated with the immobilized indigenous oil degrader and supplemented with nutrients.
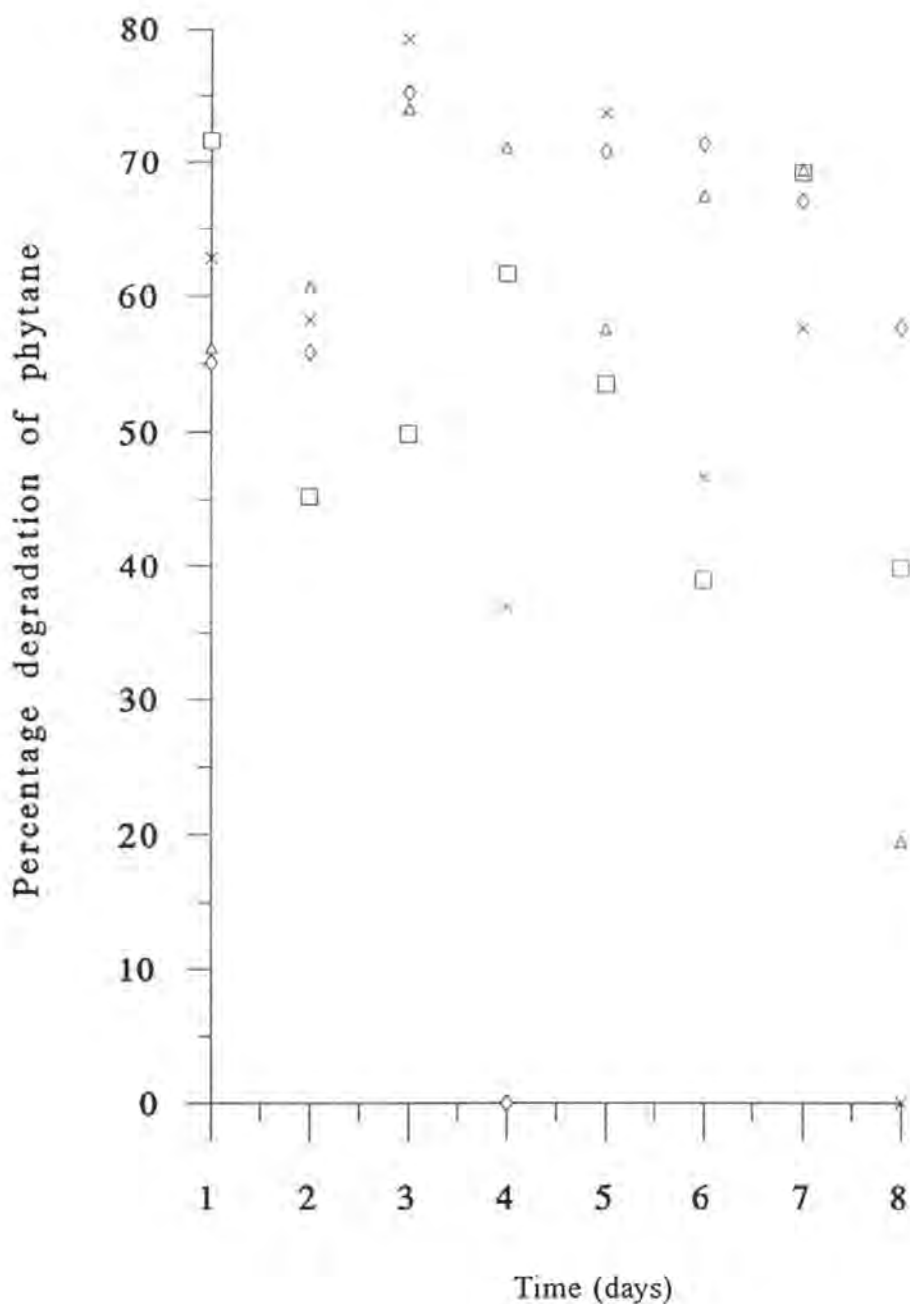
```
SAMPLE#   7   TC  VIAL:   7
[x 1,    30µl, C#  7, #WASH 4, SP   Omin]
   #  AREA ppm       C#  µl RG
   1-77449   36.68 H
   2 19688   46.62                05
   3 19540   46.27
   4 19466   46.10
   - - - - - - - - - - - - - -
   MN 19564   46.33
   SD   113  0.267
   CV  0.57 %
   COR CONC DIL   46.33 [x 1.0]
   COR CONC INJ   46.33 [x 1.0]

        DATE  06(JUN)-16-1995  11:14



SAMPLE#   8   IC  VIAL:   8
[x 1,    25µl, C#  8, #WASH 4, SP   Omin]
   #  AREA ppm       C#  µl RG
   1 38553   21.82
   2 37789   21.39
   3 37864   21.43
   - - - - - - - - - - - - - -
   MN 38068   21.54
   SD   421  0.238
   CV  1.10 %
   COR CONC DIL   21.54 [x 1.0]
   COR CONC INJ   21.54 [x 1.0]

        DATE  06(JUN)-16-1995  11:24
```

Total carbon = 46.33 mg l^{-1}.
Inorganic carbon = 21.54 mg l^{-1}.
Dissolved organic carbon = 24.79 mg l^{-1}.

Appendix 6. Level of total carbon in a water sample from the Erme estuary, detected using a TOC-5000, Shimadzu.

PO$_4$ Standard peak

N Standard peak

PO$_4$ Sample peak

N Sample peak
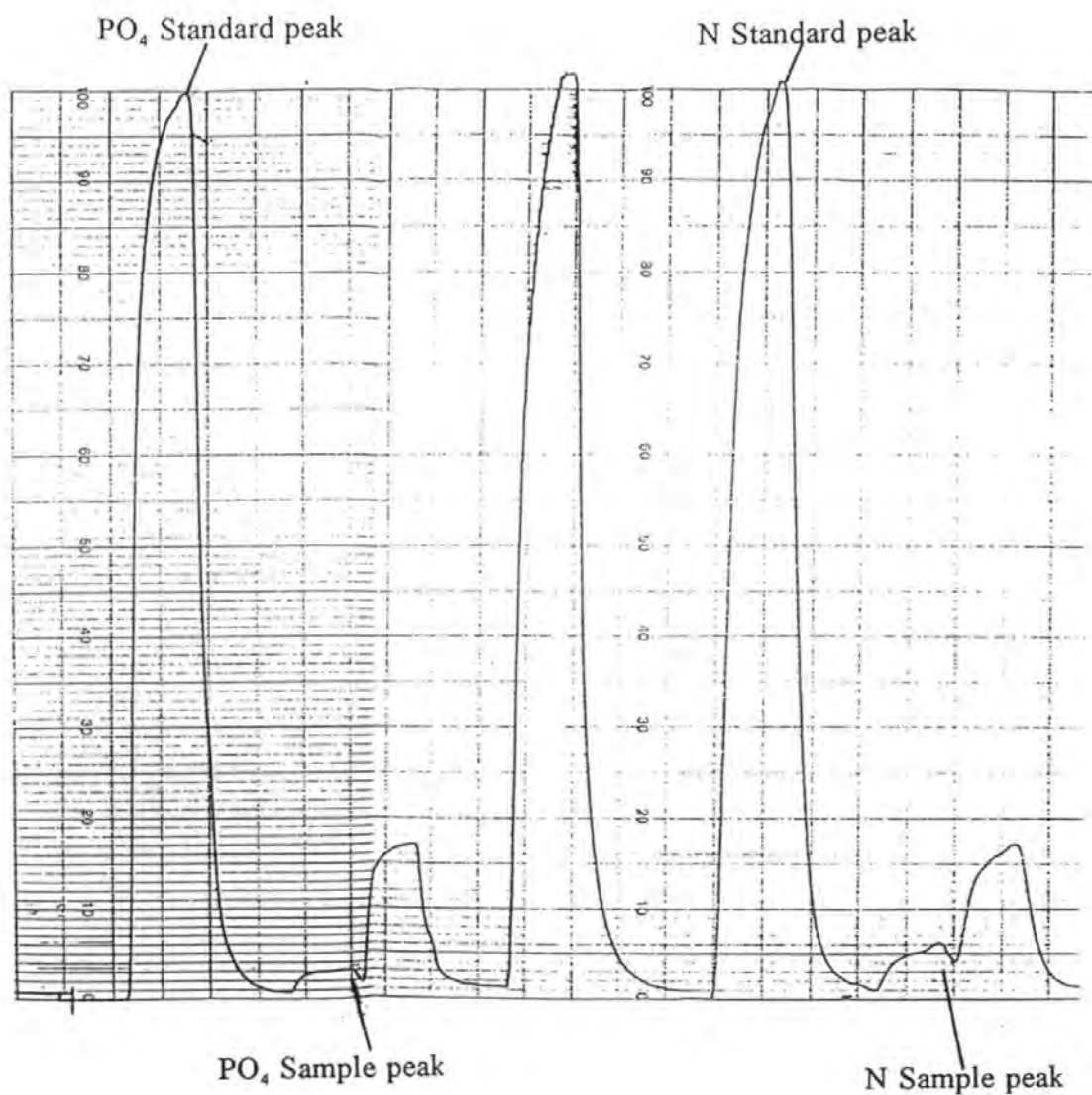
Standards:
PO$_4$ = 5.0 µg ml^{-1}.
N=20.0µgml^{-1}.

Samples:
PO$_4$ = 0.18 µg ml^{-1}.
N=0.13µg ml^{-1}.

Appendix 7. Level of nitrates and phosphates in a water sample from the Erme estuary, detected using an autoanalyzer (Technicon).

Enhanced degradation of petrol (Slovene diesel) in an aqueous system by immobilized *Pseudomonas fluorescens*

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N. G. WILSON AND G. BRADLEY. 1996. The rate of degradation of *n*-alkanes C_{12}–C_{18}, in petrol (Slovene diesel) in an aqueous system, by free and immobilized *Pseudomonas fluorescens* in shaking flasks was investigated. Cells were immobilized to a biosupport, Biofix, and a biosorbant, Drizit. Analysis of cellular growth of the free and immobilized bacteria over 8 d of incubation with diesel as the sole carbon source, showed a reduction in the lag phase in the immobilized cultures in comparison to the free system. The free system degraded 52·3% of C_{12} and 11·6% of C_{13}, but C_{14}–C_{18} were not degraded. In comparison to the free system and diesel which had not been exposed to experimental conditions (unexposed), the immobilized systems degraded significantly more of C_{13}–C_{18}. Biofix-immobilized cells degraded 14·8% of C_{12} and an average of 53·5% of C_{13}–C_{18}. Drizit-immobilized cells degraded 24·5% of C_{12}, 52·4% of C_{13} and an average of 91·2% of C_{14}–C_{18}. This study shows the successful use of immobilized bacteria technology to enhance the degradation of diesel in an aqueous system.

INTRODUCTION

Diesel is a refined petroleum product, which may be derived from high-boiling kerosene or low-boiling gas oils, which have been separated in a refinery by distillation of crude oil. Diesel-oils may have a high aromatic content and are thus more toxic than some crude oils, causing lasting pollution problems (Nelson–Smith 1972).

Most petroleum pollutants which enter the world's oceans eventually undergo degradation, but at a rate which is too slow to cope with harmful pollution incidents. Explanations for the slow degradation of oil in the natural environment include: the usually low counts of hydrocarbon-degrading micro-organisms in sea water, toxicity of some components, the limited oil–water interface, insufficiency of dissolved oxygen, suboptimal temperatures and lack of essential mineral nutrients (Atlas and Bartha 1972). Oil spills have traditionally been tackled using dispersant chemicals or by removing the oil physically from beaches and the sea surface (Aldous 1991). Artificial stimulation of biodegradation includes the use of adapted petroleum–degrading micro-organisms as an inocu-

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lum to the polluted area (Tagger *et al.* 1983) and the addition of fertilizers to accelerate the natural process of oil degradation (Anon. 1989).

The problem of accessibility of the oil to the bacteria due to limited oil/water interfacial area is often caused by the formation of water-in-oil emulsions. Biosurfactants produced by microbes are an important aspect in the uptake of hydrocarbons in this respect and artificial dispersants are an aid to overcoming this problem with the purpose of increasing surface area, therefore accelerating biodegradation (Leahy and Colwell 1990). However, in the past the use of dispersants with a degree of toxicity has resulted in a larger ecological impact than the oil spill itself (Atlas 1981).

Interest is developing in immobilized bacteria technology (IBT) as a method for the treatment of chemical waste (Heitkamp *et al.* 1990) and this method is applicable to the treatment of oil pollution in water to overcome the problem of limited oil/water interfacial area. IBT involves the immobilization of cells, that is the entrapment within or association of cells with an insoluble matrix (O'Reilly and Crawford 1989a). It is a term used to describe the restraint of a biologically active enzyme which is enclosed in a living cell, on a support material (Rosevear *et al.* 1987), and the method can utilize highly selected, chemical-degrading bacteria. In the

immobilized state, the cells are offered an optimal environment, giving the physical characteristics of the support material while still maintaining the level of biochemical activity of the free catalyst. The biosupport can act as an intermediary between the oil and the microbes, which can result in higher rates of reaction (Rosevear *et al.* 1987; Heitkamp *et al.* 1990).

Although whole cell immobilization is commonly used in processes for the microbial production of chemicals (Chibata *et al.* 1974; Fynn and Whitmoore 1982), only a few studies have addressed the use of immobilized cells to degrade toxic compounds (Omar *et al.* 1988; O'Reilly and Crawford 1989b). Data on degradation of hydrocarbons by immobilization has been published on immobilized fungi in oily sludge (Omar *et al.* 1990), but little work has been carried out on the degradation of *n*-alkanes in defined aqueous systems using bacteria immobilized on commercially available materials.

This study demonstrates enhanced *in vitro* biodegradation of petrol (Slovene diesel) in an aqueous system by an immobilized oil-degrading bacterium, *Pseudomonas fluorescens*, using two commercially available materials, a biosupport and a biosorbant as immobilization matrices.

MATERIALS AND METHODS

Bacterial isolates

The oil-degrading bacterial isolate used in this study was *Pseudomonas fluorescens*, originally isolated from a contaminated metal working fluid (Beech and Gaylarde 1989). The inoculum for each experiment was pregrown for 24 h in nutrient broth (100 ml, Oxoid) in 250 ml Erlenmeyer flasks at 30°C (overnight culture).

Media

The minimal medium used for the experiments contained (g l⁻¹): NH₄Cl, 5·0; NH₄NO₃, 1·0; anhydrous Na₂SO₄, 2·0; K₂HPO₄, 3·0; KH₂PO₄, 1·0 and MgSO₄.7H₂O, 0·1 dissolved in order in distilled water. The minimal medium was supplemented with 100 μl of Slovene diesel per 100 ml as the carbon source for the biodegradation studies. Tryptone Soya Broth (TSB, Oxoid) was used to grow the bacteria immobilized to the biosupport and 0·01 mol l⁻¹ Phosphate Buffered Saline (PBS), pH 7·3 (Oxoid, Dulbecco 'A' tablets), to wash the cells.

Whole cell immobilization on two different biocarriers

Cell loading of Biofix. The column consisted of a 5 ml syringe bunged with glass wool, connected to a reservoir from the top and a peristaltic pump from the bottom. The column was

packed with Biofix C1 (ECC International, UK) suspended in PBS. Biofix C1 is a biosupport made from kaolinite ($AlO_3.2SiO_2.2H_2O$). The buffer was allowed to drain from the column and 100 ml of an overnight culture of *Ps. fluorescens* was added to the reservoir and allowed to recycle around the system for 2–3 h (1·5 ml min⁻¹). The column was flushed twice with 100 ml of PBS. TSB (100 ml) was added to the reservoir and the Biofix incubated at room temperature for 24 h. The Biofix was flushed twice with 100 ml of PBS, 1 g was removed from the column and placed in 5 ml of PBS and sonicated (Transsonic T310, Camlab) for 2 min. The Biofix was removed and the bacteria now in free suspension were centrifuged at 13 000 rev min⁻¹ (12 × 1·5 ml head, MSE micro-centaur) and resuspended in PBS. The amount of protein per gram of Biofix was determined by the Folin method (Lowry *et al.* 1951) modified by the addition of 2% (w/v) deoxycholic acid (Maddy and Spooner 1970).

Cell loading of Drizit. Drizit (2 g, Darcy Products Ltd, UK), a synthetic (100% polypropylene), hydrophobic biosorbant, was added to an overnight culture of *Ps. fluorescens* and placed on an orbital shaker (120 rev min⁻¹) for 24 h at room temperature. After 24 h the Drizit was removed and washed twice in 10 ml of PBS, 1 g of Drizit was placed in 5 ml of PBS and sonicated (Transsonic T310, Camlab) for 2 min. The Drizit was removed and the bacteria now in free suspension were centrifuged at 13 000 rev min⁻¹ (12 × 1·5 ml head, MSE Micro-centaur) and resuspended in PBS. The amount of protein per gram of Drizit was estimated by the Folin method.

Biodegradation experiments

Bacteria in free suspension were centrifuged at 13 000 rev min⁻¹ (12 × 1·5 ml head, MSE Micro-centaur) and resuspended in PBS. Resuspended free bacteria and immobilized bacteria were added to give 365 μg protein per 100 ml minimal medium in 250 ml Erlenmeyer flasks. An uninoculated control flask was prepared to account for abiotic volatilization and extraction losses of the diesel. A control with no carbon source was also prepared to ensure that no degradation of the biosupport had occurred. The flasks were incubated at 24°C on an orbital shaker (100 rev min⁻¹) for 8 d. All treatments and analyses were performed in duplicate.

Diesel analysis

Residual diesel from the solid matrices of incubated samples was recovered by extraction with 10 ml of dichloromethane (DCM, BDH) and the aqueous phases of incubated samples recovered by extraction with 20 ml of DCM, after which the glassware was rinsed with 10 ml of DCM. The extracts

from each sample were combined and concentrated by rotary evaporation (40°C) to approximately 10 ml, after which the remaining DCM was removed under a gentle stream of pure nitrogen. Quantitative analysis was performed by gas chromatography using a Carlo Erba 4160 gas chromatograph with on-column injection (0·5 µl samples) and a flame ionization detector. Separation was achieved using a fused silica capillary column (0·32 mm by 30 m, DB-5, J and W Scientific, CA, USA). The oven temperature was increased from 50°C to 300°C at a rate of 6°C min^{-1} and held at 300°C for 10 min. Hydrogen was used as the carrier gas at the flow rate of 1·5 ml min^{-1} and the FID detector's temperature was kept at 330°C. Quantification of individual hydrocarbons was made by measurement of the GC peak areas with a Shimadzu CR3-A integrator, and by a comparison of these peaks with the response of a known concentration of the internal standard Squalane. Percentage biodegradation of alkanes C_{12}–C_{18} was calculated by comparing the response of incubated samples, after correction for non-biological losses, to that of a diesel sample which had not been exposed to any experimental conditions (unexposed).

Cellular growth

Duplicate samples of the free suspension of bacteria from each flask were centrifuged at 13 000 rev min^{-1} (12 × 1·5 ml head, MSE Micro-centaur) and resuspended in PBS. Cellular growth was then estimated using the Folin method, as previously, over an 8 d period.

RESULTS

Degradation of diesel by *Ps. fluorescens* in a free system

After 8 d of incubation of the bacteria in the free system, with diesel as the sole carbon source, the average protein concentration of the free suspension increased from 3·64 µg ml^{-1} at the beginning of the experiment to a final level of 34 µg ml^{-1}. There was an initial lag phase until day 6 after which growth occurred (Fig. 1). Degradation of the *n*-alkanes, C_{12}–C_{18} and branched-alkanes, pristane and phytane, were quantified using gas chromatography analysis. Fifty-two per cent of C_{12} and 11·6% of C_{13} had been degraded by the free suspension of *Ps. fluorescens*, no degradation of *n*-alkanes C_{14}–C_{18} and branched alkanes pristane and phytane occurred (Table 1).

Degradation of diesel by immobilized bacteria

Figure 1 shows typical growth curves of *Ps. fluorescens* immobilized to Biofix and Drizit over an 8 d incubation period. Protein (364 µg) was immobilized to the Biofix and Drizit
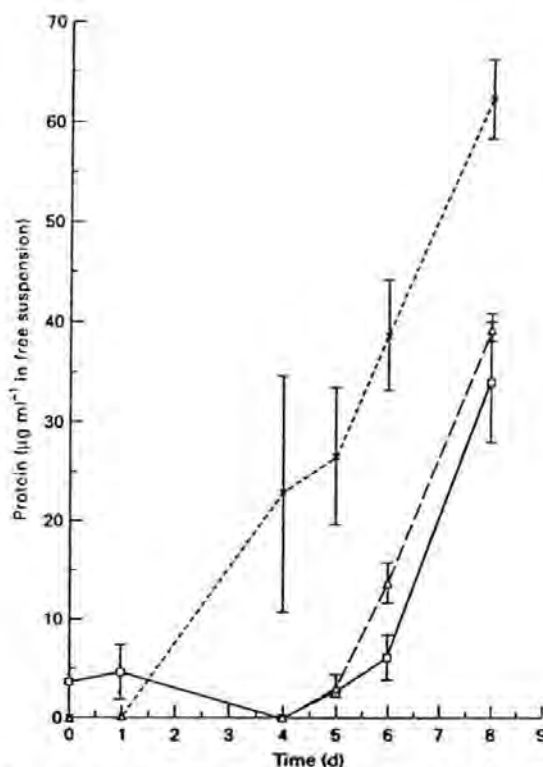

Fig. 1 Typical growth curves of *Pseudomonas fluorescens* in the aqueous suspensions of free and immobilized systems, grown on petrol (Slovene diesel) over an 8 d period. Bars indicate standard error. □, Free; △, Biofix; ×, Drizit

and added to the experimental flasks. Therefore, at time zero, no protein was present in free suspension. In the Biofix-inoculated flasks there was an initial lag phase until day 4 after which growth occurred giving a final concentration of 39·5 µg ml^{-1} protein in the free suspension. The amount of protein present on the biocarrier remained constant over the incubation period. Gas chromatographic analysis showed that immobilization of cells of Biofix enhanced degradation of the *n*-alkanes C_{12}–C_{18} in comparison to unexposed Slovene diesel, and enhanced degradation of *n*-alkanes C_{13}–C_{18} in comparison to the degradation rate of the free suspension of *Ps. fluorescens* (Table 1).

The lag phase was reduced to 1 d with Drizit-immobilized cells, after which rapid cell growth occurred giving a final protein concentration of 62·3 µg ml^{-1} in the free suspension. The amount of protein in the biocarrier remained constant throughout the incubation period.

Gas chromatographic analysis showed that immobilization on Drizit enhanced degradation of *n*-alkanes C_{12}–C_{18} in comparison to unexposed Slovene diesel and degradation of *n*-

System	Percentage carbon degraded*								
	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	Pristane	Phytane
Free	52·3	11·6	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Biofix	14·8	41·6	63·3	40·8	64·5	64·0	46·6	0·0	0·0
Drizit	24·5	52·4	87·7	85·7	100·0	93·6	89·0	0·0	0·0

Table 1 Percentage carbon degradation of petrol (Slovene diesel) after 8 d of incubation with *Pseudomonas fluorescens* in free and immobilized aqueous systems

*Figures are mean values of two samples and are adjusted to allow for photodegradation and volatilization.

alkanes C_{13}–C_{18} in comparison to the free and Biofix degradation rates for these alkanes (Table 1).

Figure 2 shows the gas chromatographic pattern obtained for unexposed Slovene diesel and patterns obtained after degradation by free and immobilized bacteria. The tracings show a decrease in hydrocarbons C_{12}–C_{18} when incubated with cells immobilized on Drizit, in comparison to Biofix immobilized cells, free cells and untreated Slovene diesel. Volatility accounted for 59% of C_{12} and 35% of C_{13} disappearance; the n-alkanes C_{14}–C_{18} were not affected by volatility but were subject to extensive biodegradation by immobilized bacteria. Branched-chain alkanes, pristane and phytane, were not affected by volatility or degradation by the bacteria in any state.

Uninoculated controls for all three systems showed no increase in protein concentration and no decrease in n-alkanes and branched alkanes from the GC analysis after the 8 d incubation period.

DISCUSSION

The results presented in this study show that enhanced biodegradation of diesel occurs with immobilization of an oil-degrading *Ps. fluorescens*. Higher levels of growth and degradation of the n-alkanes C_{12}–C_{18} were achieved by *Ps. fluorescens* in the immobilized state compared to free-living bacteria. This increase in activity can be seen when comparing the growth curves of *Ps. fluorescens* in the three different living states. In the free system there was a lag phase of 6 d during which no growth occurred, after which the protein concentration of the suspension reached a final level of 34 μg ml^{-1} on day 8. Immobilization of cells on Biofix reduced the lag phase to 4 d and resulted in the free suspension of the system having a final protein concentration of 39·5 μg ml^{-1}. Drizit-immobilized cells reduced the lag phase to 1 day and resulted in the free suspension of the system having a final protein concentration of 62·3 μg ml^{-1}. It appears therefore that the process of immobilization accelerates the ability of the cells to initially utilize the diesel.

The results of the gas chromatography show that immobilized cells degrade higher levels of alkanes, the tracings in Fig. 2 show a decrease in hydrocarbons C_{13}–C_{18} when incubated with immobilized cells in comparison to free cells and unexposed Slovene diesel after 8 d incubation. These traces do not take into account photodegradaton or volatilization. However figures in Table 1 are adjusted accordingly and still show a significant decrease in hydrocarbons C_{13}–C_{18} when incubated with immobilized cells in comparison to free cells and unexposed diesel.

The process of diesel degradation requires the immobilization of not only one enzyme system but of a multi-enzyme system that mediates complex reactions in the degradative pathway of aliphatic hydrocarbons. Whole cell immobilization permits the immobilization of these multi-enzyme systems (Fukui and Tanaka 1982).

The aim of immobilizing cells was to reduce the problem of the limited oil–water interface, caused by the insolubility of alkanes in water. Limited solubility of alkanes means that the concentration of solubilized molecules, or the bioavailability of the alkanes (Zhang and Miller 1992), is too low to meet the needs of the micro-organism. Therefore, adaptations are necessary to allow uptake to occur at a rate sufficient to satisfy the growth requirements. Pseudosolubilization may occur, in which a surfactant is produced by the cells to decrease the surface tension of the hydrocarbon (Ratledge 1992; Hommel 1994); other *Pseudomonas* species are known to produce biosurfactants for hydrocarbon utilization (Tagger *et al.* 1983). It is possible that immobilization on an oleophilic biosupport removes the need for the cell to produce surfactant by forming an intermediary between the cell and the oil. The diesel was seen to adsorb to the biosupport, bringing the cell into closer contact to the carbon source, thus increasing the bioavailability of the hydrocarbons. This theory would account for the extended lag phase on the growth curve from the free system, during which time the cell may be producing the surfactant in preparation for hydrocarbon utilization. Further studies are required to compare surfactant production in free and immobilized systems.

The enhancement of biodegradation of hydrocarbons by immobilized cells appears to be dependent on the biocarrier
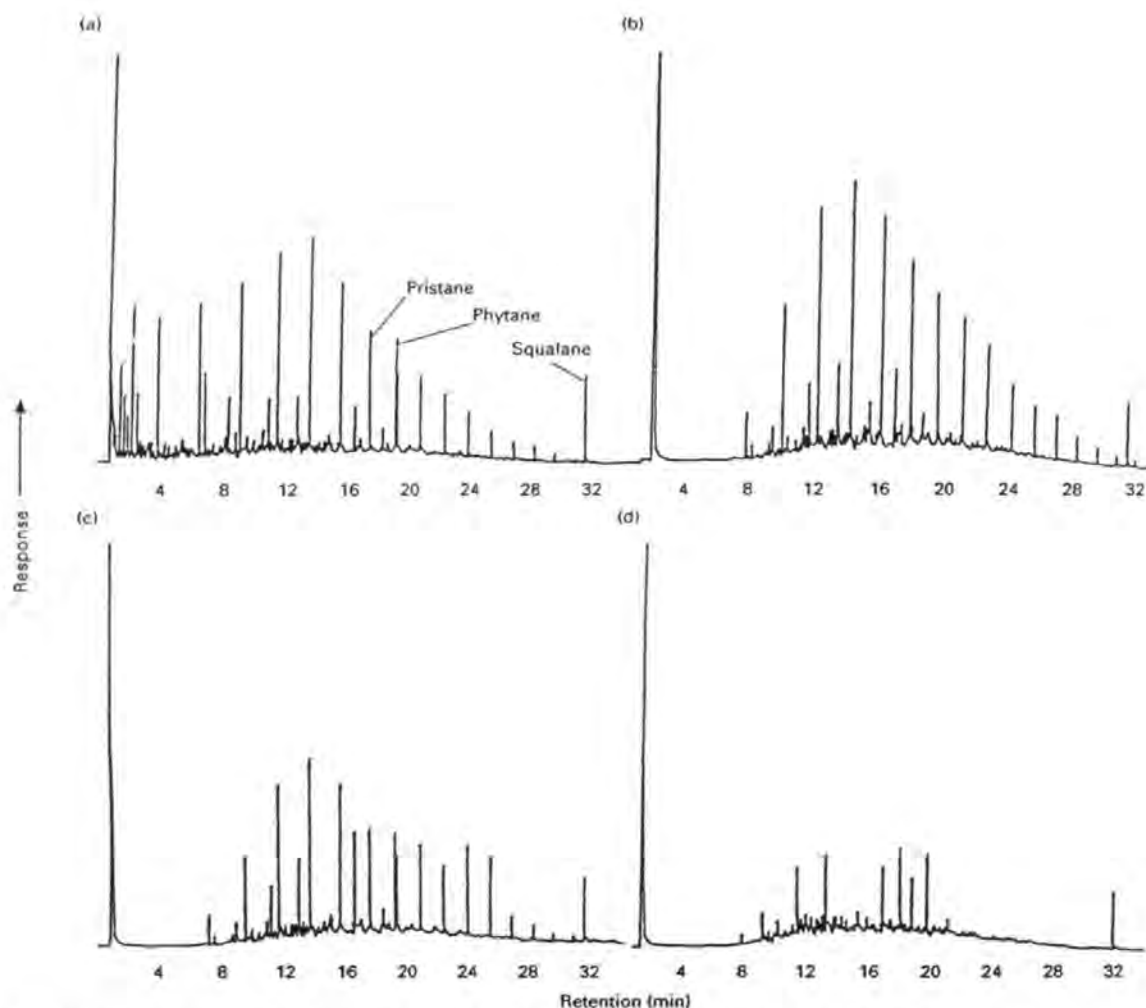
Fig. 2 Typical gas chromatographic patterns for petrol (Slovene diesel). (a) Unexposed; (b) incubated with free cells for 8 d; (c) incubated with Biofix-immobilized cells for 8 d; (d) incubated with Drizit-immobilized cells for 8 d

used. The Drizit-immobilized cells reduced the lag phase more than the Biofix-immobilized cells and also degraded a higher percentage of the n-alkanes C_{12}–C_{18} (Table 1). However, further work needs to be carried out to compare the two different immobilization methods, by characterization of the immobilization procedures (Klein and Wagner 1978) and their optimization to suit the prevailing conditions.

The results of this study have implications for the bio-remediation of water polluted by compounds having limited water solubility or those present at low concentrations which results in poor availability of the compound for the micro-organisms. The careful choice of biosupport will be essential for successful development of specific/further applications.

The biosupports used in this study served as effective inter-mediaries between the diesel and the microbes, increasing the biodegradation of the n-alkanes C_{12}–C_{18}.

ACKNOWLEDGEMENTS

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