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**MICROBIAL DEGRADATION OF CRUDE OILS**

**A thesis submitted for the degree of  
DOCTOR OF PHILOSOPHY  
IN THE  
UNIVERSITY OF KENT AT CANTERBURY**

**by**

**PAUL DOUGLAS GILBERT**

**Canterbury 1980**



**ACKNOWLEDGEMENTS**

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## ABSTRACT

The microbial degradation of two crude mineral oils and one synthetic crude oil was studied under in situ estuarine conditions and in the laboratory. The three oils differed widely in composition; the Athabasca synthetic crude was a light oil with a high saturate content, conversely its parent sand tar was a heavy oil low in saturates. The North Sea Forties crude represented an intermediate between these two extremes.

A new environmental experimental system was devised to yield quantitative data on oil degradation and this was deployed in the Medway estuary under both winter and summer conditions between December 1976 and July 1978. During both winter and summer experiments oil composition had a major influence on the rate and extent of oil loss which followed the order, synthetic crude > Forties crude > sand tar. This order conforms to predictions made on the basis of the fractional composition of the oils. The saturate fraction of the Forties crude and sand tar was most extensively degraded during the second summer experiment. Temperature rather than inorganic nutrient supply appeared to limit degradation during the winter months; degradation was more rapid during the summer months. Microbial colonization of the oils was predominantly bacterial and was more rapid during the summer. The overall colonization pattern however was similar for each oil throughout both winter and summer experiments.

The laboratory experiments using supplemented media broadly

confirmed the degradation patterns found during the in situ experiments. However, higher initial degradation rates for all the oils were found at 25°C in supplemented medium than those occurring in the in situ summer experiments. The laboratory experiments also revealed the interesting phenomena of novel hydrocarbon synthesis and the occurrence of intracellular hydrocarbon inclusions during growth on the Forties crude and synthetic crude respectively.



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**CHAPTER 1**

**INTRODUCTION**

## INTRODUCTION

1.1 GENERAL

Oil pollution of the sea is a subject of increasing environmental concern, stimulated by the expansion of offshore oil production and the supertanker accidents. In addition to this there is the more commonplace but less spectacular pollution encountered during docking operations and tank washings. Entry of oil into the marine environment does not stem solely from man's activities, natural inputs include submarine oil seeps and the release of biogenic hydrocarbons from marine biota. However during the last century there has been a rapidly increasing input, sometimes resulting in high local concentrations, due to man's exploitation of the world's oil reserves. One estimate of man's input (Petroleum in the Marine Environment, 1975) gave a value of  $6.113 \times 10^6$  metric tons/annum, compared to which the estimated contribution of natural seeps was only  $0.6 \times 10^6$  metric tons/annum. At the present time we have considerable knowledge of the fate of oil entering the sea, and of the individual physical, chemical, and biological processes affecting its breakdown and disappearance. However the relative contribution, interaction, and extent of these processes at work during in situ oil breakdown in the marine environment is an area where knowledge is lacking. This may be attributed to the difficulty of establishing meaningful experiments to monitor these processes. Several major reviews (Zobell, 1963, 1970, 1973a, Parker, Freegarde & Hatchard, 1971, Floodgate, 1972, Atlas & Bartha, 1973a, Hughes & McKenzie, 1975, 1976, Atlas, 1977, Colwell & Walker, 1977) and workshops



(American Petroleum Institute, 1970, 1973, Microbial Degradation of Oil Pollutants, 1973, Petroleum in the Marine Environment, 1975) have established the factors affecting the fate of crude oil in the marine environment and these will be discussed subsequently, however it is pertinent to consider first the nature of crude oils.

### 1.2 THE NATURE OF CRUDE OILS.

Crude oil is an extremely complex mixture of both hydrocarbon and non-hydrocarbon compounds. Different crudes vary widely in their composition depending upon their source, and even crudes from the same field may show considerable variation. The hydrocarbons form the major part of a crude oil and may be grouped according to their chemical structure, these include straight chain, branched, and cyclic alkanes, aromatics, and sometimes traces of alkenes. The cycloalkanes and aromatics occur as single and multiple ring structures including alkyl substituted forms. Multiple ring compounds containing aromatic and cyclo-paraffinic rings also occur; these may also be alkyl substituted.

The non-hydrocarbon components encompass oxygen, nitrogen, and sulphur containing compounds and metal chelated porphyrins. The oxygen containing compounds include naphthenic acids, phenols, carboxylic acids, esters and ketones. Nitrogen containing components occur as heterocyclic ring compounds, including pyridines, quinolenes, indoles, pyrroles and porphyrins. The sulphur containing compounds include alkylthiols, cycloalkylthiols



and sulphur aromatics.

Table 1 summarises the average amounts of the various classes of compound found in petroleum.

TABLE 1

Average Amounts of Major Hydrocarbons and Related Compounds Present in Different Petroleums. Zobell (1973a).

COMPONENT.	PERCENTAGE.
Aliphatic (alkanes)	15 - 35
Cycloparaffinic (cycloalkanes)	30 - 50
Aromatic (mono- & polynuclear)	5 - 20
Asphaltic (asphaltenes, heterocyclic compounds containing oxygen, sulphur or nitrogen)	2 - 15
Olefinic (alkenes)	nil

Crude oils are often described as heavy or light and this refers to their specific gravity; light oils have a specific gravity of around 0.75 and contain a higher percentage of lower boiling point fractions, these are less plentiful in heavy oils which have a specific gravity nearer to 1.0. The individual components of oil are often ascribed to fractions on the basis of their boiling points; however in most degradative studies oils are divided into fractions by column chromatography. These include saturate, aromatic, and asphaltene fractions. The saturate fraction contains normal-, branched-, cyclo- and alkylsubstituted cyclo-alkanes. The aromatic fraction may be further separated

into classes of monoaromatic, diaromatic, triaromatic, tetraaromatic and their alkylsubstituted forms, depending upon the sophistication of the procedure. The residue after the saturate and aromatic fractions have been removed is referred to as the asphaltene fraction or the polycyclic-aromatic, nitrogen-, sulphur-, and oxygen- containing compound (PNSO) fraction.

### 1.3 FACTORS AFFECTING THE DEGRADATION OF CRUDE OILS AT SEA.

#### 1.3.1 Weathering.

On entering the sea oil forms a surface slick which is then acted upon by physical and chemical forces. Microbial colonization will also occur, but at this early stage non-biological factors will make a greater contribution to any change in the oil. Meteorological conditions such as wind speed and wave action, together with the amount of oil spilt will determine the nature of the slick, which in turn will determine the rate and extent of chemical modification. For example, a heavy spill in rough conditions may result in the formation of a water in oil emulsion (chocolate mousse) which will reduce the extent of evaporation, photooxidation and dissolution due to the reduced area of oil exposed. In contrast, the formation of an oil in water emulsion may increase these processes due to a larger area of exposed oil.

Low boiling point compounds will be lost from the oil by evaporation at a rate that will depend upon meteorological conditions, especially temperature and wind speed. Reports on the extent of evaporative loss vary. For example, Pipel (1968) reported loss of those compounds with a boiling point below 150°C

but Brunnock et al (1968) analysing residual oil from the Torrey Canyon found compounds boiling below 200-300°C had been lost. In both investigations the losses were found to have occurred in a few days. In the case of a small slick, Kinney et al (1970) observed an even more rapid loss of those compounds with carbon numbers below C<sub>12</sub> within 8 hours. When oil is stranded above or within the intertidal area these losses are less rapid taking several months, whilst oil incorporated into marine sediments show little evaporative loss even at the C<sub>12</sub> level, (Blumer et al, 1973). Sivadier & Mikolaj (1973) investigated the extent of evaporative loss of small amounts of oil under in situ conditions and found that up to 20% by weight could be lost within 80 hours. Evaporative loss will be more substantial with the lighter oils as these possess a considerable low boiling point fraction.

Coincident with evaporation the more polar compounds present in the oil will dissolve and hence be leached out of the slick. Solubility of the alkanes decreases with increasing carbon number, hence it is the lighter fraction that will be lost, which is also most susceptible to evaporation. Wasik & Brown (1973) reported that the low boiling point aromatics were the most soluble which is similar to Frankenfeld's (1973) findings which also state that the light oils contain a greater soluble fraction than heavier oils.

In the presence of sunlight and oxygen chemical changes in oil composition occur: these autooxidation reactions are most extensive when a large surface area of oil is exposed and the

optimum wavelengths fall in the ultraviolet region of sunlight (300-350nm.) (Parker, Freegrade & Hatchard, 1971). Alkanes, alkenes and many aromatics undergo autooxidation initiated by the formation of hydroperoxides. The reactions are autocatalytic as the hydroperoxides themselves decompose to form free radicals. Decomposition of the hydroperoxides to hydroxyl compounds may be followed by further oxidation to aldehydes, ketones and carboxylic acids. The latter may leach out of the slick. Alternatively radical-radical combinations, condensation and esterification may lead to the formation of high molecular weight tars and gums which may prove more recalcitrant than their precursors. The rate of these photoactivated reactions will depend upon the surface area of oil exposed and the intensity and duration of sunlight. Furthermore the presence of certain metals found in oil and sea water may accelerate the process; conversely sulphur compounds and phenolics also present in oil may inhibit or terminate the reactions. Hansen (1975) investigated the photodecomposition of a Libyan crude under laboratory conditions and found that up to 10% by weight of the original crude oil was degraded within 19 days. The aromatic fraction appeared to be the most susceptible to photodegradation and branched chain alkanes were slightly more so than n-alkanes. Extrapolating from these findings to in situ conditions, it was calculated that a 0.4mm surface film of hydrocarbons would be photochemically degraded at a rate of 0.07%/day.

The product of weathering is termed a weathered oil which has altered properties compared to the fresh oil; the extent of

this alteration will depend upon the nature of the fresh oil. A lighter oil will show a greater overall loss and change in properties than a heavier oil (Atlas, 1975). The most apparent change in a weathered oil is an increase in specific gravity due to loss of the low boiling point compounds and an increase in the high boiling point compounds due to photooxidation. In some cases the increase in density may cause the oil to sink. The initial disappearance of slicks is attributed to weathering and emulsification rather than to microbial actions. Kinney et al (1970) noted the half life of small experimental and larger accidental slicks in Alaska's Cook Inlet to be of the order of a day with complete disappearance within three to four days. This rapid disappearance combined with the low water temperature (5°C) exclude the possibility of any significant microbial contribution.

#### 1.3.2. Microbial Degradation.

##### A. Distribution.

The metabolic capacity for hydrocarbon degradation is widely distributed amongst microbial populations in the environment, about 20% of free living species isolated having some capacity for hydrocarbon degradation (Quayle, 1968). Colonization and degradation of oil entering the marine environment is attributed to indigenous microbial populations on the basis of several observations. These include: i) enrichment of oil degrading populations after an oil spill or during the course of prolonged pollution, ii) isolation of oil degrading populations from oil recovered after exposure to the environment, iii) in situ



oxidation of labelled hydrocarbon substrates, iv) laboratory isolation of oil degrading populations from natural isolates, v) the occurrence in oil which has been exposed at sea for a considerable length of time of products resulting from microbial growth on oil.

A variety of bacteria, filamentous fungi and yeasts capable of growth on crude oil have been isolated from the marine environment. Table 2 gives examples of the main groups found, from which it can be seen that bacteria comprise the majority of the isolates. However there are also reports that algae (Walker et al, 1975d) and plankton (Corner, 1978) may play a role in oil degradation. Oil degrading microbial populations do not occur uniformly throughout the marine environment. Their numbers are dependent upon the degree of existing oil pollution and their location, which may be in sediment, at varying depths in the water column, in coastal waters or in the open ocean. Oil degrading bacteria comprise a small proportion of the total heterotrophic population in pristine environments, but in the presence of oil their relative numbers increase dramatically. Oil degrading bacteria are rare in the open ocean and are more commonly found in coastal waters, whereas oil degrading yeasts and filamentous fungi are only found in the immediate vicinity of land, (Zobell, 1970). Gunkel (1972) investigating the distribution of oil oxidizing bacteria in the North Sea found sediment to contain the highest numbers (a mean of  $1.61 \times 10^8/L$ ) which comprised 4.02% of the total heterotrophic population; the water column contained fewer numbers (a mean of

TABLE 2

Some Examples Of Natural Oil Degrading Isolates.

SOURCE	ISOLATES	REFERENCE
Coastal Waters (Marsejille)	191 Bacterial strains of genera: <u>Achromobacter</u> , <u>Alcaligenes</u> , <u>Psuedomonas</u> , <u>Acinetobacter</u> .	LePetit <u>et al</u> (1975)
Water & sediment (Chedabucto Bay Nova Scotia)	49 Isolates of genera: <u>Pseudomonas</u> , <u>Vibrio</u> , <u>Flavobacterium</u> , <u>Micro-</u> <u>coccus</u> , <u>Bacillus</u> , <u>Arthro-</u> <u>bacter</u> , <u>Nocardia</u> , <u>Coryne-</u> <u>bacterium</u> , <u>Beneckea</u> .	Cundell & Traxler (1973)
Estuarine water & sediment (N. Carolina)	Mainly fungi including <u>Cunninghamella</u> , <u>Aspergillus</u> <u>Cephalosporium</u> , <u>Penicillium</u> species.	Cerniglia & Perry (1973)
Water & sediment (Chesapeake Bay)	99 Bacterial strains class- ified by numerical taxonomy including <u>Pseudomonads</u> , <u>Actinomycetes</u> , <u>Micrococcus</u> sp. <u>Coryneforms</u> , <u>Nocardia</u> sp. <u>Enterobacteriaceae</u> .	Austin <u>et al</u> (1977)

$5.39 \times 10^4/L$ ) but the percentage of the total population varied more (0.52 - 16.8%). The numbers in surface films and at a depth of 1 metre fluctuated with means of  $2.47 \times 10^5$  and  $1.40 \times 10^5/L$  respectively. The numbers quoted by Atlas & Bartha (1973b) of 20/L and by Hughes & McKenzie (1976) of less than 40/L for non polluted waters are lower. As well as variation in numbers dependent upon location, Austin et al (1977) found certain strains to be specific to sediment only at given sampling sites; this was not found for strains present in the water column. Gunkel (1972) found that oil degraders were present throughout the year although fluctuations in numbers showed no yearly cycle. However temperature variations throughout the year may be important as at lower temperatures the diversity of bacterial strains comprising the mixed oil degrading population is decreased (Zobell, 1973b, Walker & Colwell, 1974a). The total number of oil degrading bacteria can be correlated with the degree of existing pollution, which suggests an in situ enrichment brought about by the oil. Atlas & Bartha (1973b) found a decrease in the numbers in surface water with increasing distance from polluted waters. Zobell & Prokop (1966) reported up to  $10^8$  oil degraders/g. of wet mud collected from a polluted site but numbers from non polluted sites were considerably lower. Furthermore existing oil pollution will increase the oil degrading component of the total heterotrophic population (Walker & Colwell, 1976a, Horowitz & Atlas, 1977b). Polluted sites also show a greater diversity of strains of oil utilizing bacteria than do pristine environments (Austin et al, 1977). Bacteria of varying

oil degrading capacity show distribution within a polluted site; Walker et al (1975a) found those in the water column were more effective and consistent in degrading oil than those isolated from sediment. Deep sea bacteria have been isolated from 4940 metres (Schwarz et al, 1975) which are capable of hydrocarbon degradation; however this is very slow due to the decreased temperature and increased pressure found at depth.

#### B. Metabolic Capacity.

It is not within the scope of this introduction to cover in detail the metabolic pathways for growth on hydrocarbons which have been elucidated in microorganisms, as this alone includes sufficient material for several introductions. Yet the pathways involved in the breakdown of pure hydrocarbons are important as they may provide an insight into the nature of microbial attack on a whole crude; therefore they will be discussed briefly.

All the major hydrocarbon classes found in crude oils are susceptible to microbial degradation and there are several major reviews covering each of these classes (Klug & Markovetz, 1971, Dagley, 1971, Pirnik, 1977, Perry, 1977) and their relative susceptibility to microbial degradation (Zobell, 1950, Higgins & Gilbert, 1978). The alkanes are the most readily degraded by a wide variety of microbial species, and within this group the normal alkanes from  $C_9 - C_{18}$  are the most susceptible. Growth on those above  $C_{18}$  is slower whilst those below  $C_9$  are sometimes toxic. Figure 1a summarizes the major routes for n-alkane degradation. Alkenes are less readily degraded than n-alkanes

but within this group terminal alkenes are more widely degraded than internal alkenes. Alkenes are rare in crude oils but may occur in refined oils. Most hydrocarbon degrading microorganisms cannot degrade branched chain alkanes, however some bacteria can degrade simple and multiple branched alkanes (King & Perry, 1975, Yamada et al, 1977).

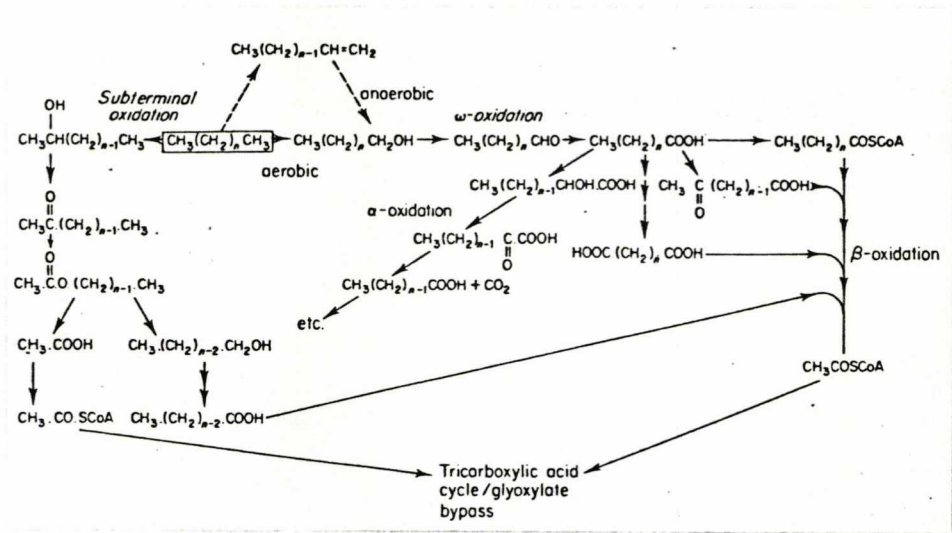
A narrower range of microorganisms are capable of growth on monoaromatic and simple polycyclic aromatic hydrocarbons, these will readily degrade alkanes but the converse is not always true. Figure 1b summarizes the initial conversion of benzene to catechol which represents a model for the initial attack on the more complex aromatics. Catechol is further degraded by ortho- or meta- ring cleavage which finally results in the formation of acetyl-CoA and succinate or acetaldehyde and pyruvate respectively. Simple benzene derivatives such as naphthalene, phenanthrene and anthracene are degraded by sequential ring cleavage, the initial attack being instigated by the formation of a dihydroxyl ring fission substrate, in an analogous manner to benzene degradation. Long chain n-alkylbenzene degradation is initiated by  $\omega$ -oxidation of the terminal methyl group followed by  $\beta$ -oxidation of the side chain. This may be followed by ring cleavage but in some cases the resulting short chain phenylalkonic acids are not further metabolized.

Unsubstituted cycloalkanes appear to be the most recalcitrant compounds; however alkyl substituted derivatives are more common in petroleum. These are more readily degraded, reflecting utilization of the alkyl substituent, and the resulting short



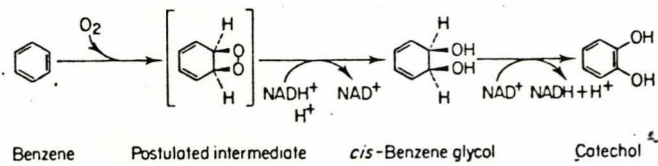
Figure 1.1

A



Major routes for n-alkane degradation

B



Conversion of benzene to catechol

chain acid derivatives themselves provide favourable substrates. Cooxidation may play a role in the initial attack on unsubstituted cycloalkanes (Beam & Perry, 1973, deKlerk & VanderLinden, 1974).

The complex nature of the asphaltene fraction precludes the detailed knowledge of biodegradation which exists for the hydrocarbon components. This fraction includes complex polycyclic aromatic and cycloalkane compounds as well as compounds containing both types of ring. Nitrogen and sulphur containing ring compounds are also present.

Initial microbial attack on hydrocarbons is most commonly by oxidation, involving complex multicomponent monooxygenase and dioxygenase enzymes. Therefore this is predominately an aerobic process. There are some reports of anaerobic degradation (Senez & Azoulay, 1961, Zobell & Prokop, 1966, Parekh *et al*, 1977). The ultimate products of hydrocarbon oxidation are  $\text{CO}_2$  and  $\text{H}_2\text{O}$  but in practice roughly half the carbon present in the substrate is converted into biomass. Furthermore partial oxidation products such as acids, ketones, aldehydes and esters may be released from cells. These are unlikely to accumulate in the environment as they themselves are susceptible to degradation. Several parameters are used to assess hydrocarbon degradation. These include oxygen consumption,  $\text{CO}_2$  evolution, disappearance of substrate, emulsification, microbial growth, the incorporation of labelled substrate and the evolution of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -labelled substrates.

### C. Degradation Of Whole Crudes.

Whilst knowledge of the degradation of pure hydrocarbons is helpful in predicting the overall breakdown of a crude oil,



this is of necessity a more complex process due to the wide variety of compounds present. Crude oil degradation is at best an uneven process occurring at variable rates; some compounds are selectively degraded whilst others remain virtually unaltered. There have been no reports of total degradation. In view of the complexity of crude oil it is not surprising that the majority of natural oil degrading isolates are mixed cultures. It would be hard to envisage a single microbial strain with the metabolic capacity to utilize all of the degradable compounds present. The microbiology of mixed culture growth on a complex substrate such as oil is again a complex process, involving such factors as competition, antagonism, succession and cooxidation. Cooxidation, which may be defined as the ability to oxidise a non growth supporting substrate, coincident with growth on another readily metabolised substrate, may be an important mechanism for initial microbial attack on the more recalcitrant compounds. Cooxidation of alkanes, aromatics and cycloalkanes has been demonstrated (Raymond et al, 1971, Higgins & Gilbert, 1978). Crude oil would provide an ideal mixture for cooxidation, containing simultaneously recalcitrant compounds and a wide variety of growth supporting substrates. The cooxidation products would probably be used rapidly by the diverse natural mixed microbial population.

There have been numerous laboratory studies on crude oil degradation using natural isolates, and these provide insight into the overall process. All of the fractional classes present in crude oils undergo degradation (Walker & Colwell, 1974b,

Atlas, 1975). However the n-alkanes are the most susceptible (Atlas & Bartha, 1972a, Soli & Bens, 1972, Zajic & Supplison, 1972, Jobson et al, 1972, Westlake et al, 1974, Horowitz et al, 1975). The most detailed analytical studies on crude oil degradation have been undertaken by Walker & Colwell (1974b) and Walker, Colwell & Petrakis (1975b), using computerised gas liquid chromatography/mass spectrometry. During a three week experiment in which a sediment inoculum was grown on a South Louisiana crude the saturates decreased by 83.4%, the aromatics by 70.5% whilst the asphaltenes increased by 28%. Some alkanes, ( $C_{28}-C_{36}$ ) were produced but this is more extensively reported elsewhere (Walker & Colwell, 1976b). Within the alkane fraction microbial degradation removed 96.4% of the normal and branched compounds, the remainder being mainly (95%) cycloalkanes of which most were 3, 4 & 5 ring structures. These were susceptible to degradation in order of the number of rings present:  $6 > 1 > 2 > 3 > 5 > 4$ . Within the aromatic fraction the percentage degradation decreased as the ring number increased from 1 to 5. Sulphur aromatics and mixed ring compounds containing one or more cycloparaffin rings were more recalcitrant. Studies on degradation rates (Walker, Colwell & Petrakis, 1976a) showed that all the component classes were degraded simultaneously but at different rates. Normal and branched alkanes were degraded most rapidly and consistently over the seven week period of the experiment. The cycloalkanes presented a more complex picture but their relative degradation rates followed the same order as their susceptibility to degradation. The aromatics showed a greater fluctuation in rates, the amounts

showing an initial decrease followed by an increase and finally a further decrease. The amount of asphaltenes decreased during early log phase, increased during stationary phase and finally decreased again.

The microbial degradation of  $^{14}\text{C}$ -labelled polycyclic aromatics by mixed populations from pristine and petroleum contaminated fresh water sediments has been investigated by Herbes & Schwall (1978). They reported that while two and three ring compounds were metabolized in both cases, the four and five ring compounds such as benz(a)anthracene and benz(a)pyrene were highly recalcitrant. This was so even in contaminated sediment which had received chronic polycyclic aromatic compound input.

During microbial growth on oils the asphaltene fraction is reported to increase in most cases, however knowledge of the degradation of this fraction is lacking. Microbial degradation of asphaltic and bituminous materials, mainly by bacterial soil isolates, has been observed (Traxler, 1962, 1966, Phillips & Traxler, 1963, Traxler et al, 1965). The extent of degradation varied, (3-25% in one week) depending upon the nature of the isolate; one pseudomonad reportedly degraded 90% in one month. During in situ incubation in soil microbial action was held responsible for the majority of the loss and in this case a Mycobacterium was isolated with a wide degradative capacity for different bituminous fractions.

A different approach to the study of the degradation of a whole crude was undertaken by Horowitz et al, (1975) using a sequential enrichment procedure. An efficient oil utilizing

marine bacterial isolate was grown on a Rostam crude, this degraded 66% of the oil (specifically the n-alkanes, of which 95% were degraded). Subsequent enrichment with the depleted residue provided two more bacterial isolates. When these were grown on the whole crude they did not effect significant degradation of the n-alkane fraction and their cell yields were not as high as for the primary isolate. However after their growth new paraffinic materials appeared. When the three strains were grown sequentially on the whole crude the secondary isolates degraded 3.8% and 13.2% respectively of the residue left by the primary isolate, the cell yields were very similar to those found in pure culture. This sequential growth resulted in 77.2% degradation of the oil. When all three isolates were grown simultaneously the primary isolate first dominated the culture, whereas in latter stages the secondary isolates were more numerous. Simultaneous growth resulted in 74% degradation of the oil. This study clearly shows the significance of succession during the growth of a mixed population. Furthermore when the primary isolate was grown in mixed culture with another efficient n-alkane utilizer competition was demonstrated. The primary isolate exhibited emulsification properties and the ability to attach to oil droplets which could have promoted its early dominance in the mixed culture.

Reports on the extent and rate of microbial oil degradation vary. This is not unexpected considering the complexity of the process and the diversity of environmental parameters that can affect the rate of degradation. Table 3 gives some examples of



the extent of oil degradation. Unless otherwise stated, the results shown in the table refer to the growth of a mixed isolate from environmental sources during batch culture in a supplemented medium. These laboratory conditions in some cases reflect an 'ideal' situation for oil degradation, with temperature or nutrient supply above values normally found in the environment. It is doubtful whether these were intended to be extrapolated to in situ conditions. The extent of oil degradation reported during simulated or genuine field experiments will be discussed in a later section. Figures quoted for oil degradation rates are sometimes extrapolated to the environmental situation. Zobell (1963) measuring oil oxidation by oxygen uptake and CO<sub>2</sub> evolution, extrapolated laboratory findings to give possible oxidation rates of 36 and 350g/m<sup>3</sup>/year at 5°C and 25°C respectively. Investigating rates at lower temperatures, a light paraffinic oil was found to be degraded at 0.13 - 0.9mg/L/day at -1.1°C on the basis of oxygen uptake. On a gravimetric basis (which takes into account oil converted into biomass) the average rate for a variety of crudes was 18mg/L/day at -1.1°C (Zobell, 1973b). Johnston (1970) used oxygen uptake as a measure of degradation in a system of oiled sand percolated with sea water at 10°C for several months. Degradation rates of 0.09g/m<sup>2</sup>/day and 0.04g/m<sup>2</sup>/day for heavily and lightly oiled sands respectively were reported. This degradation accounted for only 10% of the oil, the remainder decaying 'immeasurably slowly'. This was attributed to the anaerobic conditions rapidly established in the lower portions of the sand columns. Tausson & Shapiro (1934) quoted values of 250g/m<sup>2</sup> and

TABLE 3

Examples Of The Extent Of Microbial Oil Degradation Under Laboratory Conditions.

<u>OIL</u>	<u>EXTENT OF OIL LOSS</u>	<u>REFERENCE</u>
Bunker C	15% by weight in	Zajic & Supplisson
Fuel Oil.	11 days.	(1972)
S. Louisiana	50% by weight & G.L.C.	Soli & Bens (1972)
Crude.	data in 48 hours.	
Sweden Crude	60% by G.L.C. data	Atlas & Bartha
(Pure Culture).	in 14 days.	(1972a)
Rostam Crude.	74% by weight.	Horowitz <u>et al</u>
		(1975)
S. Louisiana	55% of saturate fraction	Kator <u>et al</u> (1971)
Crude.	by weight in 92 hours.	
S. Louisiana	50% by weight in 7 - 10	Walker <u>et al</u>
Crude.	days.	(1976b)
Several Crudes.	26 - 50% at 20°C	Atlas (1975)
	11 - 28% at 10°C	
	by weight in 42 days.	
Several Crudes.	11 - 82% by weight in	Walker <u>et al</u>
	28 days.	(1976c)
California Crude.	48% by weight & G.L.C.	Mechalas <u>et al</u>
	data in 48 hours.	(1973)
Kuwait Crude.	44% by weight in 27 weeks.	Gibbs (1975)
Several Crudes.	26.3 - 97.9% by weight	Zobell & Prokop
	in 30 days.	(1966)

100g/m<sup>2</sup> oxidized in 7 months for a crude and refined oil respectively, using a soil isolate in supplemented batch culture. Gibbs (1975) and Gibbs et al (1975) using a Kuwait crude and an oil/water system with semicontinuous addition of nutrients gave tentative rates of 30g/m<sup>3</sup>/year at 14°C and 11g/m<sup>3</sup>/year at 4°C. These figures took into account the rate limiting effect of nutrient supply and the natural capacity of sea water for nutrient turnover.

### 1.3.3. Crude Oil Composition.

Crude oils from different sources vary widely in their composition with respect to the main hydrocarbon and non-hydrocarbon classes. This variation is a major factor affecting their degradation by biological and non-biological processes. The loss due to weathering is more pronounced for the lighter crudes. Knowledge of the fractional composition of a crude and the susceptibility of its fractions to biodegradation can be helpful in predicting the rate and extent of degradation of the whole oil.

There have been several studies specifically investigating the relationship between crude oil composition and biodegradability. Jobson et al (1972) studied the degradation of 'high' and 'low' quality crudes by soil isolates. The 'low' quality crude contained more aromatics, asphaltenes and sulphur compounds but was lower in saturates than the 'high' quality crude. A mixed bacterial population which degraded primarily the n-alkane fraction under both mesophilic (30°C) and psychrophilic (4°C) conditions was isolated on the 'high' quality crude. This

population showed only limited degradation of the low quality crude. However a separate population isolated on the 'low' quality crude could degrade both this and the 'high' quality oil, demonstrating that enrichment of a degrading population is influenced by the composition of the crude used as a substrate. A similar study was undertaken by Westslake et al (1974) using four different crudes, three of which had similar n-alkane profiles but a differing total saturate content. The fourth, an Atkinson point oil, had an abnormally low n-alkane content. Mixed bacterial populations were isolated from soil for each oil at 4°C and 30°C and the nature of the population isolated was specific for each oil. Growth of the oil specific populations on any of the three oils of similar n-alkane profile caused little change in the generic composition of the population. Conversely, growth of the population isolated on Atkinson point oil on any of the other oils, caused a pronounced change in the population composition and vice versa. Growth on the three oils of similar n-alkane profile was at the expense of the n-alkanes whereas with the Atkinson point oil the aromatic fraction was concluded to support growth. The Atkinson point enriched population would readily utilize the other three oils but the converse was not true. Although the n-alkane content of the oil was important in determining its biodegradability this was not the sole factor; oils of similar n-alkane content did not demonstrate the same extent of degradation for this component. It was concluded that composition and amounts of the other fractions present could influence biodegradability. Walker et al (1976c) compared the

biodegradability of two crude oils and two fuel oils using estuarine isolates. Each oil supported a unique mixed population of bacteria, and in the case of the crude oils yeasts were also present in the population. The crude oil high in saturates but low in sulphur compounds, aromatics and asphaltenes was more extensively degraded (82%) than the comparative crude which was low in saturates but high in sulphur, aromatics and asphaltenes (51%). The two fuel oils showed similar degradation patterns dependant upon composition. The degradation of seven crude oils with specific gravities ranging from 0.771 to 0.896, during a 42 day incubation with microbial populations indigenous to sea water at 20°C and 10°C, was investigated by Atlas (1975). The extent of biodegradation varied between 50% and 26% at 20°C and in general decreased as the specific gravity increased; at 10°C the pattern differed and this will be discussed in a later section. Fractional analysis of the degraded oils showed that the saturate, aromatic and asphaltic fractions all underwent degradation. The lighter oils were more susceptible as they contained higher proportions of low molecular weight compounds than the heavier oils.

The impact on the microflora of oil entering the marine environment is not confined solely to the enrichment of an oil degrading microbial population; there may be deleterious effects such as growth inhibition and toxicity. Griffin & Calder (1977) investigated the toxic effect of the water soluble fraction of three crude and two refined oils on a marine *Serratia* strain. These fractions reduced growth rate and cell yield in a glucose/



yeast extract medium. The toxic effect appeared to be bacteriostatic, varied for each oil and could not be correlated to the total water soluble fraction of the oil, or to any oil fraction. A more toxic water soluble fraction was produced during weathering of the oils. This was attributed to the production of partial oxidation products during the weathering process.

The effect of a crude and a fuel oil on the heterotrophic microbial population isolated from a non-polluted site was investigated by Walker et al (1975c). They found that neither oil significantly affected the growth of yeasts or fungi, but the fuel oil limited the growth of heterotrophic bacteria. Despite the fact that the crude oil supported growth of proteolytic, lypolytic, chitinolytic and cellulolytic bacteria, comparison to non-oiled controls showed that the oil had a toxic effect. A more specific detrimental effect has been demonstrated on bacterial chemotaxis. Young & Mitchell (1973) found that a variety of chemicals, including benzene and toluene, inhibited the chemotatic response of four marine *Pseudomonads* to nutrient broth. Walsh & Mitchell (1973) showed inhibition of chemoreception in a motile marine bacterium by a variety of hydrocarbons including benzene, Kuwait crude and kerosene; however, the inhibition appeared to be reversible on removal of the hydrocarbon. Chemotatic inhibition may severely limit the efficiency of lytic bacteria in detrital recycling processes.

There are inhibitory, low molecular weight volatile compounds present in some crude oils that delay the onset of bacterial growth. Atlas & Bartha (1972b) using a Sweden crude and a marine

isolate, found that the lag before the onset of growth on a fresh oil increased with decreasing temperature; this was not so pronounced for a weathered oil. They concluded that the increase in lag was due to inhibitory low molecular weight compounds present in the oil, the volatility of which decreased with temperature and which were removed by weathering. This was confirmed by adding the volatile fraction to the weathered crude, which, if evaporation was prevented, completely inhibited growth. This effect is more pronounced with the lighter crudes as they contain higher proportions of these low molecular weight inhibitory compounds, and is dependant upon the rate of the weathering process.

Fatty acids may be produced during microbial growth on oils and these, in conjunction with crude oil, were demonstrated to have a toxic effect (Atlas & Bartha, 1973c). The short chain acids such as caprylic, lauric and myristic were more effective in inhibiting biodegradation. This effect was relieved by removing the acids by dialysis; hence in the environmental situation where these acids will rapidly diffuse away, this is not likely to be a significant factor.

#### 1.3.4. Environmental Parameters.

Following weathering and microbial colonisation, the subsequent degradation process at sea will be governed by environmental parameters such as temperature, nutrient supply, oxygen concentration and salinity, which influence the growth of the oil degrading population.

##### A. Temperature.

Temperature will affect the rate of growth of an oil utilizing

population and hence the rate of biological oil degradation. Zobell (1963) quoted a  $Q_{10}$  of about 3 for marine bacteria growing on a refined mineral oil between 0°C and 40°C. This is in good agreement with the  $Q_{10}$  of about 2.7 quoted by Gibbs et al (1975) for a mixed population growing on a Kuwait crude at 14°C and 4°C, which represent the range of temperature expected in British waters. Extrapolating this to the environmental situation and taking nutrient limitation into account, tentative estimates of oil degraded were 30 and 11g/m<sup>3</sup>/year at 14°C and 4°C respectively; the latter is somewhat lower than the value of 36.5g/m<sup>3</sup>/year at 5°C given by Zobell (1963). The effect of low temperatures on degradation is particularly important as about 90% of the oceans (by volume) are cooler than 5°C, and sea water temperatures in temperate climates are low during winter. Low temperature not only affects rates, but also the nature of the oil degrading population and the pattern of degradation.

There is considerable evidence for the existence of psychrophilic and psychrotolerant oil degrading populations in the environment. Zobell (1973b) isolated mixed psychrophilic bacterial populations from Alaskan soil, water and tundra muck that degraded nine different weathered crudes at -1.1°C, 4°C and 8°C. Using a purified crude it was shown that at lower temperatures there were fewer components of the mixed population. This phenomenon was also reported by Walker & Colwell (1974a).

Traxler (1973) isolated marine hydrocarbon utilizers from environments at 16 - 17°C and 2.5 - 5°C and investigated their

substrate specificity for a variety of hydrocarbons at 24°C, 16°C, 8°C and 0°C. The strains isolated from the 16 - 17°C environment exhibited growth at the higher temperatures but little growth at 8°C and 0°C. In contrast, those organisms from the 2.5 - 5°C environment grew well at the lower temperatures and also grew at 16°C and 25°C; these organisms were considered psychrotrophic. Robertson et al (1973) and Kinney et al (1970) reported the isolation of oil degrading bacteria from Alaska's Cook Inlet at the summer temperature of 5°C. In the latter report the biodegradation of Cook Inlet crude in unsupplemented sea water was reported to be essentially complete in a matter of months. Horowitz & Atlas (1977a) found up to 90% degradation of gasoline by biological and non-biological factors in five weeks, following an accidental spillage in an arctic fresh water system at a mean temperature of 5°C. Artificial fertilization and seeding with hydrocarbon degraders increased the rate, but the extent of degradation was improved by only 7%. The degradation of a Sweden crude oil by sea water isolates collected in summer (17.5°C) and winter (7.5°C) was investigated by Atlas & Bartha (1972b) at a range of temperatures from 0°C to 20°C. With both isolates, lower temperatures were found to increase the lag period and decrease the degradation rate. Some of the lag period could be removed by weathering the oil, and this has been discussed previously. The winter isolates gave higher degradation rates at the lower temperatures than the summer samples, but at the higher temperatures the rates with both isolates were comparable. These results are similar to

those found by Traxler (1973), namely that degradation by low temperature isolates occurs over the range of temperature found in the environment, whilst the high temperature isolates are ineffective at low temperatures.

Atlas (1975) showed that light oils displayed a greater reduction in biodegradative losses with drop in temperature than heavy oils. This can be attributed to the increased time taken for the volatile inhibitory components present in the light oils to evaporate at the lower temperatures. Analysis of the oils after degradation at 10°C and 20°C showed that saturate, aromatic and asphaltene fractions underwent loss. However, the aromatic fraction appeared to be more readily degraded at 20°C, whilst the converse was true for the saturate fraction. Branched chain alkanes were less readily degraded than straight chain alkanes at both temperatures. Westlake et al (1974) isolated mixed bacterial populations from soil on four different crude oils, under psychrophilic (4°C) and mesophilic (30°C) conditions. Although the composition of the population varied, depending upon temperature and type of crude oil, they did not report reduced generic diversity of the population at lower temperatures. In common with other studies it was found that psychrophilic isolates could degrade oil under both mesophilic and psychrophilic conditions. In contrast the mesophilic isolates only showed limited degradative ability at 4°C. Growth at both temperatures was primarily at the expense of the n-alkane fraction. However the branched alkanes were less readily utilized at the lower temperature. This phenomenon appears to be inherent in the



nature of the psychrophilic population, rather than an effect of temperature, since when the same population was grown at 30°C these compounds remained recalcitrant.

#### B. Nutrient Supply.

Crude oil as a substrate does not provide the inorganic nutrients necessary for microbial growth. These must be supplied from elsewhere if growth, and hence degradation, is to occur. Nutrient supply, and especially that of nitrogen and phosphorus, has been shown to limit growth rates in both fresh water and marine environments, but this effect does not appear to be so pronounced in soil (Raymond *et al*, 1976). The demand for nutrient supply is interrelated with temperature. At lower temperatures growth rates are slower and hence the demand for nutrient supply is reduced.

Inorganic nutrient limitation of hydrocarbon biodegradation was found in 25 oligotrophic lakes by Ward & Brock (1976) whilst in an eutrophic lake, nutrients only became limiting during the summer when optimum temperatures for degradation were established. At this stage higher degradation rates could be produced by addition of nitrogen and phosphorus. Gibbs (1975) examined the effect of nutrient limitation on the degradation of a Kuwait crude in sea water at 14°C. With unsupplemented sea water, nitrate and phosphate became undetectable within a week and following this the rate of degradation, as measured by oxygen uptake, was roughly proportioned to the rate of nitrate addition and utilization. The limiting effect of phosphorus was less pronounced above a P/N ratio of 0.02. At a lower temperature of

4°C, the rate of nitrogen supply was still a limiting factor (Gibbs et al, 1975). The rate limiting effect of the low concentrations of nitrogen and phosphorus found in sea water has been widely reported. Atlas & Bartha (1972c) found that unsupplemented sea water would support only 3% degradation of a South Louisiana crude. Neither nitrogen added as  $\text{NH}_4\text{NO}_3$  or  $\text{KNO}_3$ , nor phosphorus added as  $\text{Na}_2\text{HPO}_4$ , could alone significantly affect the extent of degradation. However, added together, 70% degradation was observed. Bridie & Bos (1971) reported that the degradation of a Kuwait crude in sea water over 5 - 10 days could be increased from 3 - 4% to 30% by addition of ammonia and phosphate. In contrast to Gibbs' findings, Mulkins-Phillips & Stewart (1974) reported that the nitrate concentrations they found in sea water were not growth limiting for a *Nocardia* species grown on a variety of oils. However this value exceeded the highest value for nitrogen concentration used by Gibbs. They concluded that phosphorus supply was more important. Reisfeld et al (1972) found that phosphate and ammonia were needed to supplement sea water in order to effect dispersion of an Iranian crude, by pure or mixed cultures. LePetit & N'Guyen (1976) demonstrated that the requirement for phosphorus was linked to salinity, in the case of six marine bacterial isolates. Higher concentrations of phosphate were needed to support optimum growth on hydrocarbons in brackish waters, than those required in water with salinity close to that of the open ocean. Knowledge of the growth limiting effect of the low concentrations of nitrogen and phosphorus present in sea water, has led to the development of oleophilic

fertilizers. Atlas & Bartha (1973d) screened a variety of oleophilic nitrogen and phosphorus sources for their ability to stimulate degradation of a Sweden crude. They found that paraffinised urea and octyl phosphate supported most extensive degradation, which was close to that found using water soluble nitrate and phosphate sources, in a closed laboratory system. In simulated field trials using open flow-through tanks, addition of water soluble nitrate and phosphate only gave a temporary increase in biodegradation, whereas oleophilic sources gave a steady increase in biodegradation of up to 60% in 42 days; in comparison, only 10% was degraded in unsupplemented water. Apart from nitrogen and phosphorus, the only other inorganic nutrient reported to limit biodegradation in sea water, is iron. Dibble & Bartha (1976) found that in non polluted, iron deficient waters, addition of water soluble chelated iron, or oleophilic ferric octate stimulated degradation of a South Louisiana crude.

#### C. Oxygen, Salinity And Depth.

Oil degradation is widely regarded as an aerobic process, hence oxygen supply is an important consideration. The amount of dissolved oxygen found in surface sea water is not likely to limit the degradation of thin slicks; however, this may not be true for chronic pollution and oxygen may be limiting in sediments.

Pierce et al (1975) found signs of anaerobiosis in estuarine beach sediments, following a spill of heavy fuel oil, during warm weather, which encourages higher growth rates and hence increased oxygen demand. Johnston (1970) investigating the

degradation of oil in sand columns percolated with sea water found that the layers beneath the oil rapidly became anaerobic and that recovery to aerobic status was slow and incomplete within four months. Similar findings were reported by Hughes & McKenzie (1976) when, after 40 weeks incubation in silt columns, only oil in the upper more aerobic portion of the column showed degradation; below this level the oil remained virtually unchanged. The extent of this anaerobic effect was governed by the amount of oil added to the column.

Salinity changes may affect the microbial population colonizing a crude oil and hence its degradation. The majority of fresh water microbial species do not survive in sea water, hence an increase in salinity will inhibit the growth of fresh water oil degrading populations. On the other hand a decrease in salinity to below 2 - 1.5‰ will inhibit the growth of marine populations. Marine psychrophilic bacteria are particularly sensitive to salinity changes, both salinity and low temperature are required for stability of cellular structure. The salt concentration can also influence the maximum growth temperature. Morita (1975) found that as the salt concentration was lowered so was the maximum growth temperature. The effect of pressure on psychrophiles is also important as low temperatures are found at depth. Pressures of over 500 atmospheres were found to inhibit DNA, RNA and protein synthesis and also amino acid transport. These effects may explain the low rates of hydrocarbon oxidation reported by Schwartz et al (1975) by bacteria at in situ low temperature (4°C) and high pressure (500 atmos-

pheres).

#### 1.4 ENVIRONMENTAL STUDIES

Compared to the extensive laboratory studies on the microbial degradation of crude oils, there has been little in situ environmental work. Although difficult to establish and maintain, environmental experiments are essential if the knowledge gained from laboratory work is to be verified under environmental conditions.

##### 1.4.1. Environmental Surveys And Accidental Spills.

Some knowledge of oil degradation in the environment has been gained from the investigation of polluted and non-polluted sites, and from the observation of accidental spills and their aftermath. The isolation of oil degrading microbial populations from polluted and non-polluted sites has shown that polluting oil may enrich for oil degraders, both in terms of numbers and effectiveness in degrading oil. However the exposure of organisms from a non-polluted site to crude oil has shown its possible toxic effect.

Aerial observation of small experimental spills and larger accidental spills in Alaska's Cook Inlet by Kinney et al (1970) gave the estimated apparent half life of a slick to be less than a day, with complete surface disappearance of even larger slicks within 3 - 4 days. It seems unlikely that microbial action was responsible for this disappearance, especially at the prevailing low surface water temperatures of about 5°C. The 5 knot current and 20 - 30 foot tides may have been major factors aiding disappearance.



The fate of spilled oil deposited on beaches is easier to monitor than that which remains at sea, and several studies have been carried out following accidental spills. Pierce et al (1975) examined the degradation over a one year period, of fuel oil spilled on an estuarine beach. They reported a rapid enrichment of hydrocarbon degrading bacteria between 4 and 16 days after the spill. This did not represent an increase in total numbers, but an increase in the proportion of oil degraders in the total heterotrophic population. Psychrophilic bacteria predominated in the winter months and were capable of oil degradation at 5°C, but they were absent during warmer periods. Conversely mesophiles were present during both winter and summer, but showed reduced degradative activity below 15°C. The degradation of the spilled oil presented a more complex picture than the microbial response. In the mid tide area the concentration declined rapidly within one week, indicating migration of the oil to the low tide sediment. The subsequent steady decline in concentration was attributed to microbial action. The situation in the low tide sediment was complicated by the influx of oil from the mid tide sediment. However, after one year oil was still present. The reduction in n-alkanes and aromatics coupled with a residue enriched in branched chain and cycloalkanes was indicative of biodegradation. Brunnock et al (1968) reported that oil buried in anaerobic layers of sand following the Torrey Canyon spill remained unchanged for at least one year. Blumer et al (1973) investigating the degradation of a distilled fuel oil in marine sediments following a

spill, reported little degradation after six months. N-alkanes began to decrease after ten months and were absent after two years, but branched chain alkanes, cycloalkanes and aromatics were still present. The environmental fate of oil stranded above or within the tidal zone, showed that evaporation was slow and complete only after several months. Microbial degradation of oil stranded above the tidal zone was very slow. In the intertidal zone degradation of n-alkanes was still slow by laboratory standards, those up to C<sub>27</sub> being degraded in three months, whilst branched- and cyclo-alkanes remained intact.

#### 1.4.2. Model Systems.

An alternative to in situ experiments is the use of model systems. Horowitz & Atlas (1977a) used a series of open flow-through tanks incubated in situ on the Alaskan coast and fed with sea water collected daily, to study the degradation of a Prudhoe crude. Addition of oil to the tanks caused an increase in bacterial numbers and an increase in the ratio of oil degraders to total heterotrophs. Addition of fertilizer in water soluble or oleophilic form supported higher cell numbers than the unfertilized control. In the latter case only 15% of the oil was lost in 51 days at a mean temperature of 6.7°C and this was considered to be mainly abiotic. In comparison, weekly addition of water soluble fertilizer or single addition of oleophilic fertilizer gave 25% and 32.5% oil loss. Analysis of the oils showed that little degradation had occurred, but that the pattern of fractional degradation was the same in all cases. The extremely limited degradation found was attributed to the

particularly cold conditions. Kator et al (1971) used large open tanks filled with sea water and incubated out of doors, to study the degradation of a South Louisiana crude. The initial rate of oil removal, in supplemented sea water with microbial seeding, was at least twice as large as evaporative loss. This was attributed to microbial degradation of n-alkanes of chain length below C<sub>15</sub>. Although organisms were present at the oil/water interface throughout the experiment, degradative losses returned to values comparable to evaporative loss after the initial period.

#### 1.4.3. In Situ Experiments.

There have been few in situ marine experiments to investigate the degradation of crude oils, presumably due to the difficulty of maintaining enclosed oil samples at sea. Those studies that have been undertaken generally use floating oil contained in rafts, tanks or boom enclosures or sunken oil in surface layers of the sediment. Hughes & McKenzie (1975), using a raft containing open-bottomed tanks with several crudes, reported rapid growth following artificial seeding with degradation of n-alkanes up to C<sub>22</sub> in two weeks. The problems of oil sinking and 'wash out' during gales prevented estimation of overall oil loss. Davies & Gibbs (1975) followed the fate of a Kuwait crude exposed at sea in a raft of open tanks over two years. Changes in the oil properties were the only evidence for degradation. These included a 50% decrease in n-alkane content and an increase in specific gravity, viscosity and asphaltene content. The contribution to these changes made by microbial action is not clear.

Olivieri et al (1976) used a system of floats to contain a Saria crude at sea for a period of 21 days. Total losses due to biotic and abiotic degradation were 40%, compared to 63% sustained by the addition of a paraffin supported fertilizer. Taking into account the paraffin content of the fertilizer which could be up to 20% of the recovered oil, the net microbial degradation above that of the unfertilized control was 38%. Fractional analysis of the recovered oils showed that 30% and 57% of the saturate fraction, and 14% and 44% of the aromatic fraction was degraded in the control and fertilized area respectively. In both cases the asphaltene fraction increased. Gatellier et al (1973) used in situ 'ecosystems' of floating polythene cylinders to study the degradation of a crude oil over several months, with the addition of dispersants and nutrients. The addition of the latter greatly enhanced degradation, the saturate fraction suffering the most substantial loss. Within this fraction the n-alkanes were more readily degraded than the branched chain alkanes. The aromatic fraction showed some degradation but the asphaltenes exhibited an increase.

The degradation of oil applied to surface sediment in a salt water marsh area was studied by Zobell & Prokop (1966). The oil was contained by cylindrical oil pens open at both ends to prevent loss by wind and water movement. The oil gradually disappeared over a nine week period, in some cases complete disappearance occurring. This was attributed to microbial action on the basis of significant enrichment of oil degraders, and the absence of bacterial growth and reduced oil loss in the

poisoned controls. The fate of sunken oil in surface sediments in oxygen rich and depleted zones was investigated by Hughes & McKenzie (1976) over a one year period. Rapid microbial colonization of the oil was reported at both stations during the first month, numbers of total heterotrophs and oil degraders reaching a maximum in 8 - 24 weeks, thereafter followed by a gradual decline. The inclusion of fertilizers had little effect on cell numbers. Analysis of the recovered oil showed that little change had occurred over one year; only the light alkanes up to undecane had been lost from the surface. Degradation may have been severely limited by the low surface area of oil available to microbial attack.

#### 1.5 CONCLUSION

The degradation of crude oils in the marine environment is a complex process involving both non-biological and biological factors. Initial physical alteration of the oil will affect the rate of degradation due to both these factors as a result of surface area of oil exposed. In the early stages the non-biological factors, including evaporation, dissolution and photooxidation probably are responsible for the majority of oil loss. These effects are more pronounced for lighter oils and may affect the susceptibility of the oil to subsequent microbial degradation. The product of these processes is termed a weathered oil.

Coincident with weathering the oil will be colonized by indigenous microbial populations, a proportion of which have



the metabolic capacity for growth at the expense of oil, hence effecting its degradation. Oil degrading microbes are not uniformly distributed throughout the marine environment. They are less numerous in the open ocean than in coastal waters, and are more numerous in locations subjected to long term oil pollution than in pristine environments. Although oil degrading yeasts and filamentous fungi are present in waters in the immediate vicinity of land, they are absent in more open waters. The majority of oil degrading isolates from the marine environment are bacterial. The initial microbial degradation rate, following colonization, will be dependent on the composition and numbers of the local indigenous population, which in turn is influenced by location.

The microbial degradation of a crude oil is an uneven process in terms of rates, degradation patterns and extent, and does not proceed to completion. The rate and extent of biodegradation is affected by the composition of the oil and environmental parameters. The lighter oils are degraded more rapidly and extensively than heavier oils, due to their higher content of low boiling fractions and n-alkanes which are more susceptible to microbial attack. The residue after degradation may contain long chain n-alkanes and a higher asphaltene content as a result of microbial growth. Temperature and the rate of nitrogen and phosphorus supply are two of the most important environmental parameters that can limit biodegradation rates. As the degradation process is predominantly aerobic, anaerobic conditions are almost totally inhibitory; salinity and depth

are other influential factors. The limited in situ studies to date have emphasised the lower rates of biodegradation encountered in the environment compared to those found in the laboratory.

The aim of this project was to study the degradation of three crude oils of widely different composition (Athabasca sand tar, Athabasca synthetic crude and North Sea Forties crude) under both laboratory and in situ conditions. Commercial exploitation of Athabasca sand tar and its derivative synthetic crude, is still in its infancy, but the extensive reserves of sand tar make this a highly probable oil source for the future. Knowledge of the degradation of these oils in the marine environment is lacking, and it is pertinent to obtain this before large scale transportation of the oils becomes commonplace. The Forties crude possesses properties intermediate between the first two oils, and its degradation in British coastal waters is especially relevant. The study of oil degradation under in situ conditions necessitated the development of equipment suitable for quantitative measurements of oil loss under marine conditions.

**CHAPTER 2**

**MATERIALS AND METHODS**

## MATERIALS &amp; METHODS

2.1 OILS

## 2.1.1 General

Three oils were chosen for study, an Athabasca synthetic crude, its parent sand tar and a North Sea Forties crude; all were gifts from British Petroleum Ltd. The sand tars of North Alberta, of which the Athabasca deposit is the largest, represent a vast and to date unexploited oil reserve. The estimated recoverable reserves from the Athabasca deposit alone,  $369.1 \times 10^9$  barrels (Pow et al, 1963), are in the same order as the total Middle East reserves,  $350 \times 10^9$  barrels. Sand tar occurs naturally as a bituminous coating surrounding sand particles with a consistency somewhat similar to putty. The deposits occur on the surface and at varying depths of up to 2000 feet and the yield of bitumen falls between 2 - 18%. Recovery methods include surface mining and steam/hot water extraction. The raw bitumen is a heavy viscous oil with a specific gravity of 1.025 but this can be thermally and catalytically upgraded to a synthetic crude with a specific gravity of 0.850 (Berkowitz & Speight, 1975), which is acceptable to most refineries. On the basis of present processes the reserves of  $369.1 \times 10^9$  barrels of bitumen could be converted to  $266.9 \times 10^9$  barrels of synthetic crude. Although at the present time commercial exploitation is limited to a few pilot scale plants, as 'conventional' oil reserves become depleted the sand tar deposits may become economically viable. In this event the synthetic crude is most likely to be transported as this is



acceptable to refineries, and hence will almost certainly find its way into the marine environment. Its synthetic nature would render it an environmentally 'alien' oil and therefore its susceptibility to biodegradation is a pertinent area of investigation. Although the extracted sand tar is not likely to be transported to conventional refineries, the biodegradability of this bitumen is interesting as it is such a poor quality oil in comparison to the synthetic crude. The North Sea Forties crude was chosen as an intermediate between the two extremes of the heavy and light oils and because of its particular relevance to British coastal waters.

#### 2.1.2 Extraction Of Sand Tar.

The sand tar was received in its natural state and prior to any investigation the bitumen was extracted. This was performed by refluxing with dichloromethane in a Soxhlet apparatus; the yield of bitumen was 13% by weight. The extreme viscosity of the raw bituminous extract prevented its use in laboratory batch culture experiments and rendered it unsuitable for the environmental system. The viscosity was reduced by removing most of the asphaltenes by agitation with large volumes of n-pentane. The pentane was subsequently removed by evaporation.

#### 2.1.3 Topping Of Oils.

To simulate evaporative losses which occur in the environment during the weathering process, all the oils were subjected to artificial evaporation before use in the laboratory or the environment. Small volumes (ca. 50 ml.) of synthetic and

Forties crude were sparged with air for 24 hours at room temperature. This was not suitable for the sand tar due to its high viscosity; this was left exposed to the atmosphere at room temperature for several weeks before use. The evaporative losses were 26%, 20% and 10% for the synthetic crude, Forties crude and sand tar respectively.

#### 2.1.4 Quantification Of Oils.

In both laboratory and environmental experiments, oil was quantified by infrared spectroscopy after the method of Simrad et al (1951). A Perkin-Elmer 237 infrared spectrometer (Perkin-Elmer Ltd., Beaconsfield, Bucks.) was used and samples were contained in either 10 mm path length quartz cuvettes or potassium bromide infrared cells employing a lead spacer to give a path length of 0.5 mm. The samples were contained in  $\text{CCL}_4$  and a reference of  $\text{CCL}_4$  was routinely included. The sum of peak heights above the base line at 3.50, 3.42 and 3.38  $\mu$ , representing  $-\text{CH}_3$ ,  $-\text{CH}_2$  and  $-\text{CH}$  stretching frequencies was taken as a quantitative measurement of the amount of oil. Standard curves of the sum of peak heights versus the mass of oil were constructed for each oil in both the environmental (Chapter 3, section 3.8) and laboratory systems. The amount of oil was determined by reference to the standard curve. As the spectrometer was used intermittently over a long period of time standards containing known quantities of oil were included in every analysis. Readings were corrected for any variation in the standard.



### 2.1.5 Analysis Of Oils.

Oils were analysed by gas liquid chromatography (G.L.C.) and liquid chromatography. G.L.C. was performed using a Pye series 104 gas chromatograph (Pye-Unicam Ltd., Cambridge) equipped with dual flame ionization detectors suitable for temperature programming. Glass columns of 2 m x 6.4 mm internal diameter containing a liquid phase of 3% OV-17 or 3% OV-1 on diatomite CLQ (J.J. Chromatography Ltd., Kings Lynn, Norfolk) were employed. These were used in pairs for temperature programme work, one column receiving the sample whilst the other compensated for changes in gas flow rate due to the changes in temperature. A temperature programme of 50 - 320°C rising at 8°C/minute and held isothermally at 320°C for 5 minutes was employed. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min; the flow rates for hydrogen and oxygen were 40 ml/min and 300ml/min respectively. The oil samples were in solution in CCL<sub>4</sub> and a sample volume of 3 microlitres was employed.

Liquid chromatography was performed using a dual phase column of silica and alumina gel after the method of Horowitz & Atlas (1977a), which is a scaled down application of the method of Coleman et al (1973). A glass column of 300 mm x 15 mm internal diameter was packed in the following manner. Silica A-R, CC4 (Mallinckrodt, St. Louis, U.S.A.) was fully activated (16 hr. at 260°C) and packed on top of aluminium oxide, W200 basic grade super 1, (ICN Pharmaceuticals GMBH & Co. W. Germany) fully activated (16 hr. at 420°C) in a 1:1

(W/W) ratio. Elution was performed with solvents pre-dried over magnesium sulphate and fractions were collected over magnesium sulphate for further drying. Fractions were reduced to small volumes by evaporation, transferred to pre-weighed beakers and evaporated to constant weight over several days at room temperature. The fractional composition of the oils was calculated on the basis of weight.

#### 2.1.6 Sterilization Of Oils.

Prior to use in experiments small volumes (ca. 15 ml) of the topped Forties crude and synthetic crude were filter sterilized using a Millipore type S.C. filter of pore size  $0.22\ \mu$  (Millipore S.A. Molsheim, France) and stored in sterile screw cap universals. Throughout use sterility checks were performed by plating the oil onto nutrient agar. The extreme viscosity of the sand tar precluded this method of sterilization, however sterility checks were performed as above.

## 2.2 MEDIA

### 2.2.1 Liquid Media.

Several types of liquid and solid media were employed, all were sterilized by autoclaving at  $121^{\circ}\text{C}$  for 15 minutes unless otherwise stated.

**Minimal Salts Medium:** The minimal salts medium of Mulkins-Phillips and Stewart (1974) was routinely used except in the case of the aerated batch culture with semi-continuous addition of nutrients (Chapter 6, section 6.3). This medium had the following composition in g/L:  $\text{K}_2\text{HPO}_4$ , 4.74;  $\text{KH}_2\text{PO}_4$ , 0.56;

MgSO<sub>4</sub>, 0.50; NaCl, 28.4; CaCl<sub>2</sub>, 0.1; NH<sub>4</sub>NO<sub>3</sub>, 2.5. The phosphate was prepared separately, adjusted to pH 7.8, autoclaved separately from the bulk medium and combined after cooling. Trace elements were prepared to the composition of Bauchop & Elsdon (1960), autoclaved separately and added to the bulk medium after cooling.

**Sea Water Medium:** Fresh sea water was collected from approximately seven miles off shore from the north Kent coast. This was stored in 30 gallon fibre glass tanks at 4°C in the dark, the sea water was allowed to age for at least two months before use in media. Prior to use the sea water was filtered (Whatman No.1, Whatman Ltd., Maidstone, Kent), buffered by addition of 2.65 g/L Trisma base (Tris-hydroxymethylaminomethane) and adjusted to pH 8.0 with HCL. The medium was supplemented by semi-continuous addition of nitrate and phosphate (Chapter 6, section 6.3). Phosphate (K<sub>2</sub>HPO<sub>4</sub>) and nitrate (KNO<sub>3</sub>) were prepared as 0.5 M solutions in distilled water and added aseptically via syringe.

**Suspending Medium:** A 0.05 M phosphate buffer adjusted to pH 7.8 and containing 28.4 g/L NaCl was used as a general suspending medium and diluent.

**Nutrient Broth:** Nutrient broth (Oxoid No. 2, Oxoid Ltd., Basingstoke, Hants.) was prepared as 25 g/L with the inclusion of 28.4 g/L NaCl.

#### 2.2.2 Solid Media.

The following solid media were used.

**Marine Agar:** Marine agar (Difco Laboratories, W. Molesey,



Surrey) was prepared as 55.1 g/L.

**Sea Water Nutrient Agar:** Sea Water nutrient agar was prepared by addition of 28 g/L nutrient agar (Oxoid) to a mixture of 75% filtered, aged sea water and 25% deionized water.

**Minimal Agar:** Minimal agar was prepared by the addition of 2% purified Bacto-agar (Difco) to the minimal salts medium.

**Oil Agars:** Oil agars were prepared in several different ways and these are described in a later section (Chapter 3, section 3.10).

**Potato Dextrose Agar:** Potato dextrose agar (Difco, 39 g/L added to 75% aged sea water) was used for the quantification of fungi and yeasts from the marine environment.

### 2.2.3 Media Used For Identification.

**Medium For The Determination Of Carbohydrate Metabolism:** This was a modification of the classical Hugh & Leifsons medium adapted for use with marine bacteria (Leifson, 1963). The medium had the following composition (% W/V: casitone, 0.1; yeast extract, 0.01;  $(\text{NH}_4)_2 \text{SO}_4$ , 0.05; tris buffer, 0.05; purified agar, 0.3; and phenol red, 0.001. Ingredients were dissolved at double strength in distilled water, the pH adjusted to 7.5 and then autoclaved. The medium was diluted with an equal volume of aged sterile sea water and glucose added to give a final concentration of 1.0% W/V.

**Sugar Medium:** Sugar medium was prepared with the following composition (g/L): peptone, 10; NaCl, 28.4. Bromothymol blue was prepared as an alkaline solution of 1 g. in 25 ml of 0.1MNaOH and added as 12 ml/L. The medium was adjusted to

pH 7.5 and autoclaved; sterile glucose solution was added to give a final concentration of 1% W/V.

### 2.3 ISOLATION OF OIL DEGRADERS FROM ESTUARINE SEDIMENT

#### 2.3.1 Location And Sampling.

Surface sediment was collected in sterile screw cap universals from the intertidal area on the Isle of Grain in the Medway estuary (Figure 2.1) during October 1975. Samples were transported back to the laboratory in a cooled, insulated container and enrichment was initiated within 2 hours of sampling.

#### 2.3.2 Enrichment.

Sediment suspensions (2 ml) were used as inocula for 250 ml aliquots of minimal salts medium contained in 500 ml capacity conical flasks. Either sterile synthetic crude, Forties crude or sand tar was added to the medium (0.04%, V/V) as the sole carbon source. The medium was magnetically stirred at room temperature. After 48 hours 2 ml of the enrichment culture was transferred to a second identical enrichment medium containing the respective oil (0.4%, V/V) and was incubated for a further 48 hours. Cultures were observed by phase contrast microscopy and by gram stain throughout the enrichment period. At the end of the second enrichment, 10 ml of culture was centrifuged at 3000 r.p.m. for 20 minutes. This produced separation into a surface 'oily' pellicle and a cell pellet, both of these as well as uncentrifuged culture were plated onto marine agar and incubated at room temperature. Colony types were grouped on the basis of morphology and separated by serial plating until pure

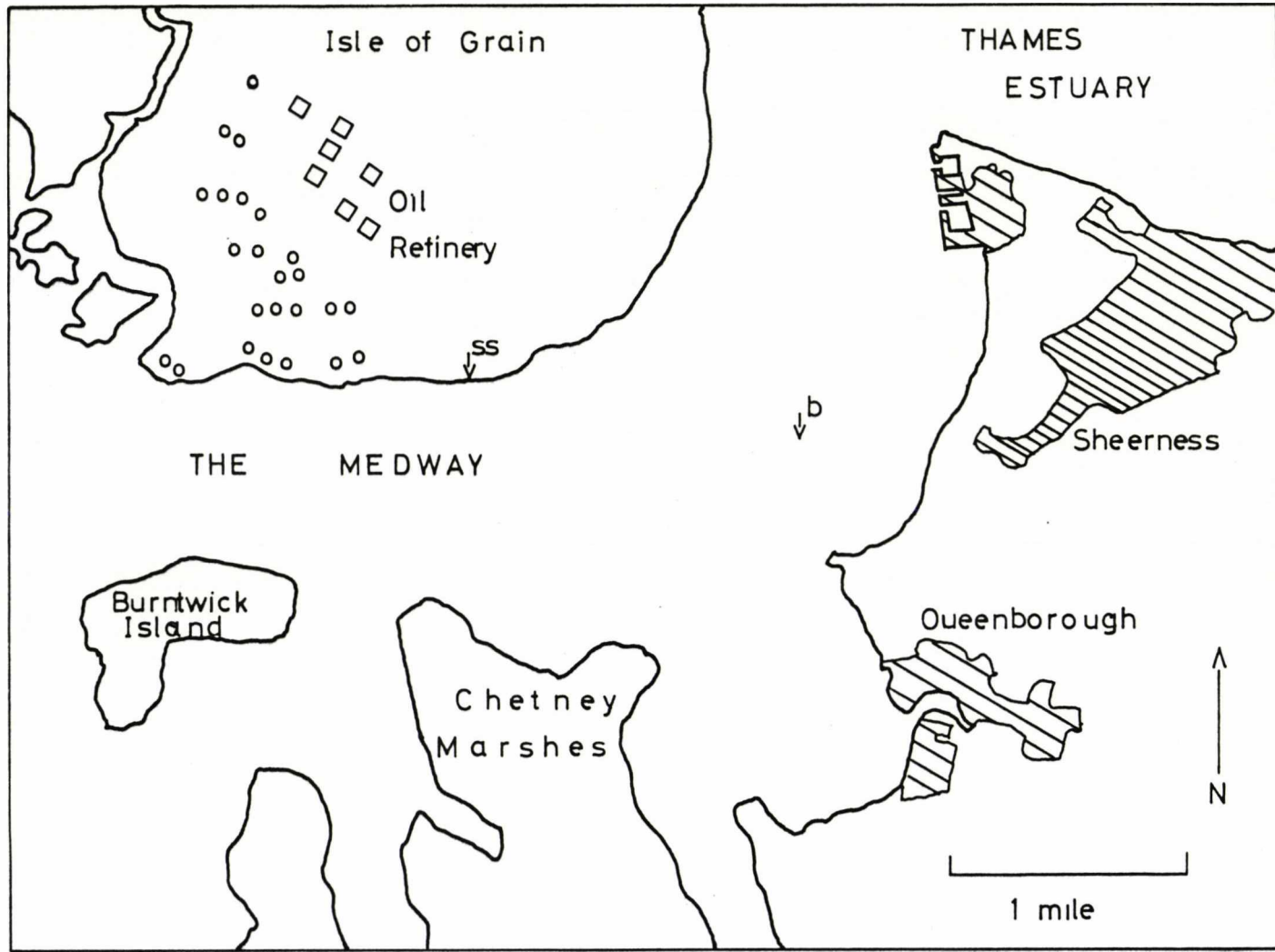
FIGURE 2.1

Position of the buoy used for attachment of experimental equipment and of the sampling site in the Medway estuary.

Position of the Queenborough Spit buoy (b) arrowed.

SS - sampling site.





cultures were obtained. Strains were maintained on marine agar slopes stored at 4°C.

#### 2.4 BATCH CULTURE STUDIES

Two methods of batch culture were employed:

- i) one litre volumes, magnetically stirred with forced aeration,
- ii) 150 ml volumes shaken at 180 r.p.m. on an orbital flat bed shaker.

##### 2.4.1 Batch Culture (1L) With Forced Aeration.

Three different systems were used, one for the assessment of microbial growth on oils (Chapter 6, section 6.2), a second for monitoring degradation of oils with semi-continuous addition of nutrients (Chapter 6, section 6.3). The third employed the technique of silica adsorbed oils (Chapter 6, section 6.7).

The apparatus used for the assessment of microbial growth (Chapter 6, section 6.2) consisted of 2 litre conical flasks, an example of which is shown in Plate 2.1b. The culture was sparged with sterile compressed air which had been previously saturated with water vapour to minimise evaporation. The flow rate was approximately 300 ml/min. The culture was magnetically stirred at room temperature and sampling was performed by restricting the air outlet, thus forcing a sample of culture through the sampling port. One litre of minimal medium was added to each flask and sterile, topped oil was added at 0.1% V/V by sterile glass syringe via the addition port. The inoculum consisted of a mixed culture of strains A<sub>1</sub> & A<sub>2</sub> for the synthetic crude and a mixed culture of strains N<sub>1</sub>, N<sub>2</sub> & N<sub>3</sub> for the Forties crude and

PLATE 2.1

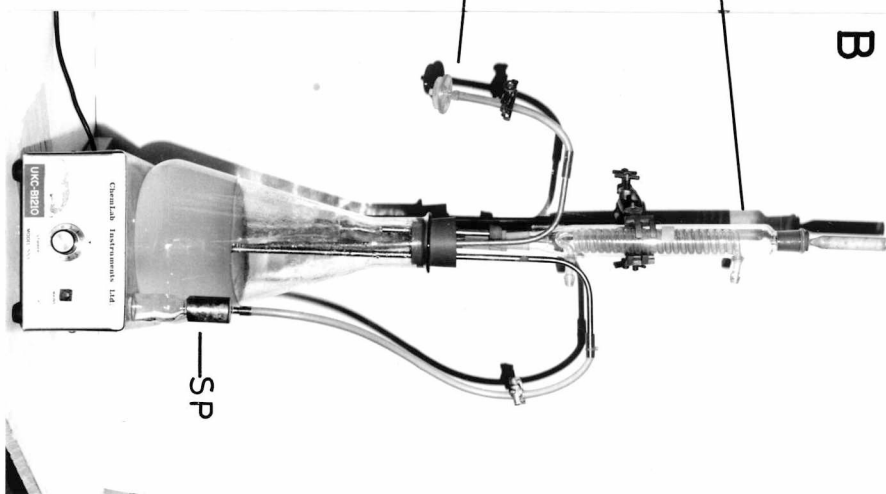
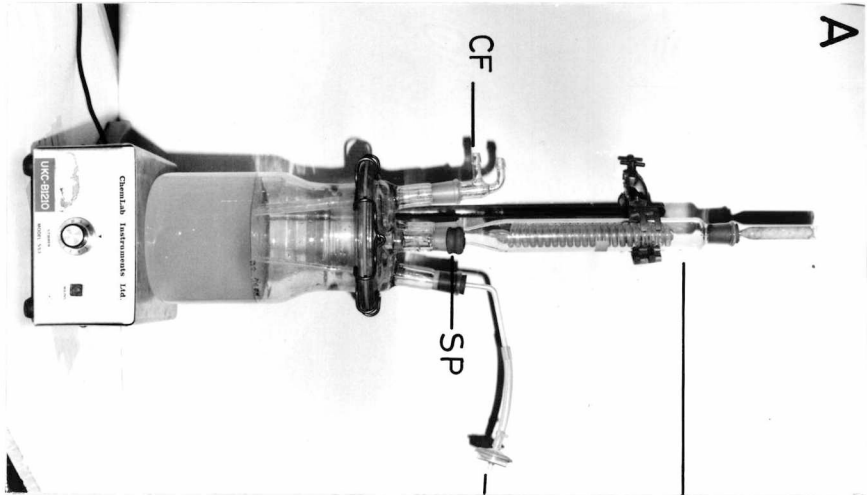
Equipment employed for batch culture (1L volume)  
with forced aeration.

Ai - air inlet.

Ao - air exit.

SP - sampling port.

CF - cold finger.





sand tar. The inocula were prepared by harvesting a 16 hour culture in nutrient broth of each of the strains (3000 r.p.m. for 15 mins.). The cell pellets were washed twice in suspending medium and finally diluted with the same to approximately equal turbidity (by eye). One ml aliquots were then mixed to give the requisite mixed culture and the inoculum was then introduced by sterile syringe via the addition port. The sampling system employed was not suitable for quantitative measurements of oil degradation by infrared spectroscopy. The assessment of microbial growth on samples withdrawn from the vessels was performed in three ways as follows. Direct counts were performed under phase contrast using a haemocytometer (Improved Neubauer, B.S. 748, Weber, England) and viable counts were performed in duplicate on marine agar. The third method employed an optical density measurement and the cells were prepared as follows. Oil was removed from the sample by extraction with  $\text{CCl}_4$ ; this required centrifugation to remove the emulsion formed during extraction. This resulted in the formation of a thin cell layer at the interface of the organic and aqueous phases, the cells were resuspended in the aqueous layer after the organic solvent had been removed by aspiration. Any remaining traces of organic solvent present in the aqueous layer were removed by evaporation under vacuum. The aqueous layer was then centrifuged and the cell pellet resuspended in a known volume of suspending medium. In some cases at this stage the cell suspension was not homogeneous but this could be rectified by several passages through a 25 gauge syringe needle. Preliminary scanning of cell

suspensions indicated an optimum absorbance at 460 n.m. and subsequently the optical density was recorded on a Pye-Unicam SP500 spectrophotometer at this wavelength. Although during the extraction with  $\text{CCl}_4$  cell viability decreased drastically observation of the cell suspension under phase contrast and by gram stain did not reveal any significant alteration in cell morphology.

The apparatus used for monitoring the degradation of oils with semi-continuous addition of nutrients (Chapter 6, section 6.3) consisted of a 2 litre glass chemostat vessel, an example of which is shown in Plate 2.1a. The culture was sparged with sterile compressed air saturated with water vapour, with a flow rate of approximately 300 ml/min. The culture was magnetically stirred and temperature controlled by means of a 'cold finger' connected to a circulating water bath maintained at  $25^\circ\text{C}$ . This provided a working temperature within the vessels of  $26 - 27^\circ\text{C}$ . One litre of Tris buffered (pH 8.0) sterile, aged sea water was added to each flask. This medium was supplemented by the addition of phosphate ( $\text{K}_2\text{HPO}_4$ ) and nitrate ( $\text{KNO}_3$ ) using a sterile syringe via the addition port. These were prepared as 0.5 M solutions and added in equimolar amounts to give an initial concentration of  $500\mu\text{M}$  in the medium. This represented roughly a 100 fold increase in the concentration of phosphate found in sea water during the environmental experiments (Chapters 4 & 5). Phosphate was assayed on a daily basis (samples were diluted 100 fold and assayed by the method used for environmental samples, section 2.8, and the requisite amount of stock phosphate solution



added to maintain the level at approximately  $500\mu\text{M}$ . Nitrate was added on an equimolar basis. The requisite inocula for each oil were prepared in an identical fashion to that used for the 2 litre conical flask experiments. Oil was added as a sonicated emulsion (M.S.E. type 150W sonicator, M.S.E. Ltd., Crawley, Sussex), in a small volume of sterile sea water to give a final concentration of 0.1% V/V. Samples were withdrawn from the vessel using a 12", 19 gauge syringe needle fitted to a 5 ml capacity glass syringe with Luer lock fitting. The sampling port was sealed with a suba-seal. Duplicate samples were withdrawn for oil analysis and a single sample for viable count. The oil analysis on each sample was performed in the following manner. The 5 ml of sample was transferred to a 10 ml glass stoppered centrifuge tube. This was followed by 2 ml of  $\text{CCl}_4$  which had been passed through the syringe and needle used for sampling to remove any oil adhering to the surface. The mixture was shaken thoroughly for 2 minutes, centrifuged (3000 r.p.m. for 5 minutes) to break any emulsion formed, and the organic layer transferred to a 2 ml capacity screw cap glass vial. These were stored at  $4^\circ\text{C}$  prior to analysis. The single extraction provided excellent separation (by eye) of both oils into the organic solvent, no visible traces of oil colour remaining in the aqueous layer. To confirm this, a second extraction was performed on each oil with a further 2 ml of  $\text{CCl}_4$ . This extract did not exhibit any oil colour and infrared analysis did not reveal any measurable amounts of oil present. Therefore routinely only a single extraction was used. The

extracted oil samples were quantified by infrared spectrometry (section 2.1.4) using 10 mm path length quartz cuvettes. The duplicate samples agreed very closely with one another throughout the experimental period with both oils. In most cases any variation fell within that found when identical samples were repeated. This extraction procedure was performed on samples containing both oil and cells, hence any contribution to the amount of oil quantified by hydrocarbon or lipids extracted from the cells was assessed. A pure culture of strain A<sub>1</sub> was grown on heptane vapour to a cell density of approximately  $10^8$ /ml. A sample (5 ml) of this culture was extracted in an identical fashion to that described and the extract quantified by infrared. This did not reveal any measurable amounts of hydrocarbon. The sterile control experiments required the addition of Tween 80 (polyoxethylenesorbitolmonoleate, final concentration, 0.02% V/V) to promote the stable emulsion required for the method of sampling employed. This was not necessary during the experiments in which microbial growth occurred as this promoted emulsion formation. Tween 80 possesses an infrared spectrum in the region used for oil quantification (3.38 - 3.50 $\mu$ ), however when a 0.02% V/V aqueous solution was extracted with CCl<sub>4</sub> no measurable spectrum was observed. This suggests that the Tween 80 partitions preferentially into the aqueous phase. This was further confirmed by extracting two emulsions of Forties crude (0.1% V/V) with CCl<sub>4</sub>, one of which contained Tween 80 (0.02% V/V). The resultant infrared spectra were found to be identical; this confirmed that Tween 80 did not contribute any



measurable component to the spectrum used for oil quantification. The entire system above was not suitable for use with the sand tar as its extreme viscosity prevented the formation of a homogeneous emulsion (either by sonication or as a consequence of microbial growth) which was an essential prerequisite for the system.

The silica adsorbed system (Chapter 6, section 6.7) was employed to give a direct comparison of the degradation rates between all three oils. The aim of the system was to produce a uniform suspension of oil on silica in the medium, analogous to a homogeneous emulsion. The oils were adsorbed onto silica as follows. Silica (Sigma silica gel type H, particle size 10 - 40  $\mu$ ) was preconditioned at 260°C for 16 hours. One gram was mixed with oil diluted in CCl<sub>4</sub> (1 ml oil in 4 ml CCl<sub>4</sub>) until a homogeneous slurry was formed. The CCl<sub>4</sub> was allowed to evaporate over several days at room temperature with intermittent stirring of the slurry. When this had assumed a 'powdery' form and no trace of CCl<sub>4</sub> remained the entire quantity was added to the medium giving a final concentration of 0.1% V/V of oil. The apparatus used was identical to that shown in Plate 2.1a except that the sampling port was sealed with a replaceable ground glass stopper. The conditions of culture and inocula were identical to those used for microbial growth on oils with semi-continuous addition of nutrients, except that minimal medium was used instead of aged sea water. Samples were withdrawn using a sterile 5 ml capacity pipette via the sampling port. Duplicate samples were used for oil analysis and a single

sample for viable count. Samples (5 ml) were used for oil analysis but prior to extraction with  $\text{CCl}_4$  the sample was treated with 0.5 ml of 5M NaOH at  $80^\circ\text{C}$  for 2 - 3 minutes to facilitate release of the oil from the silica. As with the emulsion studies a single extraction with  $\text{CCl}_4$  proved adequate, and the duplicate samples agreed very closely with one another throughout the experiment. Viable counts were carried out in duplicate on sea water nutrient agar and incubated at  $25^\circ\text{C}$ .

#### 2.4.2 Batch Cultures (150 ml) In Shake Flasks.

These batch culture studies were performed in 250 ml capacity conical flasks, containing 150 ml of minimal medium and shaken on a flat bed orbital shaker at approximately 180 r.p.m. The cultures were maintained at  $25^\circ\text{C}$  except for the study of degradation at low temperature ( $4^\circ\text{C}$ ).

In the case of degradation of the synthetic crude by strains  $A_1$  and  $A_2$  (Chapter 6, section 6.4) the oil was added as a sonicated emulsion (final concentration, 0.1% V/V) with the addition of Tween 80 (final concentration, 0.02% V/V) to promote a stable emulsion. Inocula were provided by a washed cell suspension harvested from a 16 hour nutrient broth grown culture of strains  $A_1$  or  $A_2$ . The effect of Tween 80 on the growth of strains  $A_1$  and  $A_2$  was investigated in the following manner. Strains  $A_1$  and  $A_2$  and a mixed culture of both were grown on nutrient broth, nutrient broth plus Tween 80 (final concentration, 0.02% V/V) and Tween 80 alone in minimal medium (final concentration, 0.02% V/V). Growth was monitored by optical density at 600 n.m. over a 48 hour period. Tween 80 alone did



not support any measurable growth and the nutrient broth and nutrient broth plus Tween 80 cultures showed closely similar growth patterns. The experimental flasks and controls were sampled in duplicate and duplicate samples for oil analysis were taken from each flask; a single sample was taken for viable count. Samples were withdrawn by sterile 5 ml capacity pipette. For oil analysis, 5 ml of sample was extracted with 2 ml of  $\text{CCl}_4$  and the extract quantified by infrared spectroscopy. Duplicate samples from the same flask agreed very closely with one another throughout the experiment but the samples from duplicate flasks showed a variation of 2 - 10%. Viable counts were performed on sea water nutrient agar in duplicate.

In the case of oil degradation at low temperatures and degradation of photooxidized oils (Chapter 6, sections 6.5 and 6.6 respectively) the oil was added and extracted in a different fashion. The oils were added as sonicated emulsions (no emulsifying agent was included) and an entire flask and its contents (with the exception of the cotton wool bung) were extracted at each sampling time. Flasks were sampled in duplicate and extracted twice with two consecutive 30 ml volumes of  $\text{CCl}_4$ . Oil present in the extract was quantified by infrared spectroscopy. In the case of the degradation of photooxidized oils the variation between duplicate flasks ranged from 2 - 20%; this was slightly improved with the oil degradation at low temperatures where the variation was 2 - 12%. Viable counts were performed in duplicate on sea water nutrient agar and incubated at  $25^\circ\text{C}$  and  $4^\circ\text{C}$  for the photooxidation and low temperature experiments respectively.

#### 2.4.3 Isolation Of Intracytoplasmic Inclusions From Strain A<sub>1</sub>.

Intracytoplasmic inclusions were isolated from strain A<sub>1</sub> (Chapter 6, section 6.4) after the method of Scott & Finnerty (1976). Strain A<sub>1</sub> was grown in 2 litres of minimal medium containing synthetic crude oil (0.1%, V/V). The cells were harvested at the time of lowest oil content in the medium (4 days) by centrifugation at 11,000 x g for 20 minutes at 4°C. The cell pellet was washed five times with suspending medium to remove extracellular hydrocarbons. Cells were broken by French Press (3 cycles) and unbroken cells removed by centrifugation at 6000 x g for 30 minutes at 4°C. The supernatant was centrifuged at 78,000 x g to separate the inclusions from cellular membranes. The inclusions formed a white band at the top of the tube and these were removed with a pasteur pipette. The contents of the inclusions were extracted by shaking and mild sonication in the presence of CCl<sub>4</sub>.

#### 2.4.4 Photooxidation Of Oils.

A sample (5 ml) of fresh 'untopped' oil was pipetted into a 10 cm diameter watch glass and placed approximately 10 cm from a model 12 bacteriocidal ultraviolet light (Hanovia Lamps Ltd., Slough, Bucks.). The power output of the lamp was approximately 3.0 watts at 254 n.m; samples were irradiated for 24 hours.

### 2.5 GROWTH ON PURE HYDROCARBON SUBSTRATES

The ability of several strains to grow on pure hydrocarbon substrates (Chapter 6, section 6.8) was tested in liquid media. All tests were performed in minimal medium and in duplicate,



the method of culture depended upon the type of hydrocarbon used as the sole carbon source. The hydrocarbons that might have proved toxic in all but low concentrations were introduced to the medium in the vapour phase. These included the aromatics, cycloalkanes and the n-alkanes heptane, octane and nonane. Centre well shake flasks of 250 ml capacity containing 100 ml of medium and approximately 5 ml of hydrocarbon substrate in the centre well were shaken at 180 r.p.m. on an orbital flat bed shaker at 25°C. The inoculum was provided by a single colony of a pure culture of the strain grown on sea water nutrient agar. Growth as indicated by turbidity was checked on a daily basis and also the level of the more volatile substrates in the centre wells. In those cases where growth occurred, purity checks were performed on sea water nutrient agar.

Tests for growth on the remaining alkanes were performed in tubes, each containing 10 ml of minimal medium with the substrate added as 2 drops from a pasteur pipette (in the case of n-octadecane this was immediately after autoclaving while the substrate was still liquid). All substrates were autoclaved before use and inocula were provided by single colonies from plates; tubes were incubated at 25°C. In those cases where growth occurred purity checks were performed on sea water nutrient agar.

## 2.6 IDENTIFICATION OF ENVIRONMENTAL ISOLATES.

A limited number of morphological and biochemical tests were performed for the presumptive identification of the environmental isolates (Chapter 6, section 6.1). The results were

compared to the determinative schemes of Scholes & Shewan (1964) and Cowen & Steel (1974). The tests were performed in duplicate (with the exception of colonial morphology and Gram stain) as follows.

- i) Colonial Morphology: this was observed on well separated colonies after 48 hour incubation on marine agar at 25°C.
- ii) Motility: this was observed by phase contrast microscopy on a wet film preparation after a 16 hour incubation in nutrient broth at 25°C. Strains were only scored as non-motile after three serial transfers.
- iii) Gram Reaction: heat fixed smears were stained by Gram stain (Kopeloff & Beermans modification, 1922) and observed under oil immersion.
- iv) Catalase: colonies were picked from marine agar plates with a glass rod and tested in H<sub>2</sub>O<sub>2</sub> (10 Vol).
- v) Oxidase: colonies were picked from marine agar plates with a glass rod and tested against filter papers soaked in a fresh 1.0% solution of tetramethyl-p-phenylene-diamine dihydrochloride.
- vi) Acid And Gas From Glucose: 10 ml of glucose peptone water was inoculated by single colony from plates. Tubes were incubated at 25°C and any colour change was observed by reference to a sterile control incubated in an identical fashion. Production of gas was detected by Durham tube.
- vii) Carbohydrate Metabolism: 10 ml of medium was inoculated by deep wire stab with single colonies from plates. An anaerobic jar was used to maintain anaerobic conditions



rather than the use of sterile paraffin as a sealant. Both aerobic and anaerobic tubes were incubated at 25°C and any colour change compared to sterile controls incubated in an identical fashion.

viii) Sensitivity to Compound O/129: lawns of growth were prepared on marine agar plates and incubated at 25°C in the presence of filter paper discs impregnated with compound O/129 (2:4 diamino-6:7 di-isopropyl pteridine, 150 g/disc). Sensitivity was indicated by a zone of inhibition surrounding the filter paper disc.

## 2.7 MICROSCOPY

Both light and electron microscopy were used for the examination of samples.

### 2.7.1 Light Microscopy.

Sections of filters recovered from in situ experiments (Chapter 3, section 3.9) were observed by phase contrast using a wet film preparation and by bright field using a general purpose stain (Anon, 1965). The stain had the following composition per litre. Aqueous phenol (5%), 60 ml; ethyl alcohol, 5 ml; glycerol, 4 ml; basic fuchsin, 125 mg; methylene blue, 120 mg. Prior to use the stain was filtered and samples were treated in the following manner. The sections were stained for 5 - 10 minutes, repeatedly washed with distilled water, air dried and then placed in immersion oil to render the section translucent. Sections were observed under bright field using oil immersion.

Suspensions of bacteria shaken from filters and samples from laboratory experiments were observed under phase contrast or by Gram stain under bright field. Examination of cells for the presence of intracellular lipid was performed using a Sudan Black stain (Burdon, 1946) and observed under bright field.

#### 2.7.2 Electron Microscopy.

Scanning electron microscopy was used to observe the surface of filters recovered from the in situ experiments. Sections of filters were prepared for microscopy by a modification of the method of Arnold et al (1971) used for the preservation of fine structure. Samples were prepared by treating them with the following series of fixation steps:

- i) 0.1% gluteraldehyde in sea water for 1 hour.
- ii) 2% " " " " " " "
- iii) Desalination through a graded series from 100 - 10% sea water all containing 2% gluteraldehyde.
- iv) S-collidine buffered osmium tetroxide for 30 mins.
- v) Dehydration through a graded alcohol series of 25 - 100%.

Alternatively sections were fixed in osmium vapour for 16 hours coincident with air drying. Sections were coated with a gold film using an E.5000 S.E.M. coating unit (Polaron Equipment Ltd., Watford, Herts.) and examined using a Cambridge Sterioscan 600 scanning electron microscope (Cambridge Instruments Ltd., Cambridge).

Transmission electron microscopy was employed for the observation of intracellular inclusions and for quantitative measurements on plasmid D.N.A. (Chapter 6, sections 6.4 and 6.9



respectively). Samples for the observation of inclusions were prepared by the following method. The cell suspension was fixed by addition of glutaraldehyde (25% V/V) followed by harvesting and resuspension in osmium tetroxide. The fixed sample was dehydrated through a graded alcohol series and embedded in Spurr's resin (TAAB Laboratories, Reading, Berks.). Sections were cut using an L.K.B. Ultratome III (L.K.B. Products AB, Bromma, Sweden) and mounted on copper grids. Sections were stained with uranyl acetate (60°C, 30 min.) followed by lead citrate (room temperature, 10 min.). Sections were viewed under an AEI 801A transmission electron microscope (GEC-AEI Electronics Ltd., Manchester).

Isolation and preparation of plasmid D.N.A. was performed after the method of Warner et al, (1977). Cells were grown in nutrient broth and harvested in late logarithmic phase, then lysed by treatment with lysozyme and sodium lauryl sulphate. The cleared lysate was subjected to isopycnic density gradient centrifugation in a caesium chloride gradient with ethidium bromide. Plasmid D.N.A. was identified by fluorescence under U.V. irradiation and harvested through a syringe needle inserted through the side of the polycarbonate centrifuge tube. The ethidium bromide was removed by extraction with caesium chloride saturated isopropanol and the caesium chloride removed by dialysis. The resultant D.N.A. was prepared for microscopy by the aqueous spreading technique followed by platinum/palladium coating. Electron micrographs of the plasmid D.N.A. were projected onto paper and the lengths of open circles measured using

a map measurer (curvimeter). Absolute values for length were obtained by comparison to electron micrographs of a diffraction grating replica of 2160 lines/mm. projected and traced in a similar fashion. Estimates of the molecular weight of the plasmid molecules were then made by assuming that 1  $\mu\text{m}$  of D.N.A. has a molecular weight of  $2.07 \times 10^6$ .

## 2.8 MEASUREMENT OF ENVIRONMENTAL PARAMETERS

**Temperature:** Surface water temperature was taken at the time of each sampling in close proximity to the buoy using a spirit filled thermometer ( $-10 - 50^{\circ}\text{C}$  graduated in  $1^{\circ}\text{C}$ ).

**Salinity:** Surface water samples were collected at the time of each sampling in 500 ml capacity screw cap polythene containers. The specific gravity of the samples was measured with a Gallenkamp soil hydrometer (Gallenkamp & Co. Ltd., London) previously calibrated for specific gravity readings by a series of sucrose solutions of known specific gravity. Specific gravity readings were converted to salinity (S‰) by use of a standard curve (Knudsen, 1901).

**Phosphate:** Phosphate concentrations were determined by the method of Murphy & Riley (1963). This employs a complex reagent of molybdic acid, ascorbic acid and trivalent antimony resulting in the formation of a phosphomolybdate complex and its subsequent reduction to a highly coloured blue compound, the extinction of which is measured at 885 n.m. Surface water samples from the immediate vicinity of the buoy were collected in previously rinsed 250 ml capacity polythene screw cap bottles and analysed



within 2 hours of collection. Determinations were made in quadruplicate and the phosphate concentration evaluated by comparison to a standard curve prepared for each batch of reagents. All determinations were performed using acid washed glassware to eliminate interference due to residual phosphates from detergents.

**Nitrate:** Several methods of nitrate determination were tested but none yielded consistent results. Automated nitrate analysis was kindly performed by Shell Research Ltd. (Sittingbourne, Kent) using a Technicon Autoanalyser II. This method relies upon reduction of nitrate to nitrite under alkaline conditions and the formation of an azo-dye which is measured at 520 n.m. Samples for nitrate analysis were collected in the same way as those for phosphate analysis.

**Dissolved Oxygen:** A modification of the classical Winkler procedure (Strickland & Parsons, 1965) was employed. A manganese solution followed by a strong alkali is added to the sample, any dissolved oxygen rapidly oxidizes an equivalent amount of divalent manganese to basic hydroxides of higher valency states. When the solution is acidified in the presence of iodine the oxidized manganese again reverts to the divalent state and iodine, equivalent to the original dissolved oxygen content of the water is liberated. The iodine is titrated with standardised thiosulphate solution. Surface water samples were collected in pre-rinsed 600 ml capacity glass bottles which were capped with suba seals under water to exclude any air bubbles. Within 15 minutes of sampling 2 ml of manganous sulphate reagent

followed by 2 ml of alkaline iodide solution were injected into the bottle via the suba seal. Acidification and titration were completed on return to the laboratory. Titration was performed with a standardised thiosulphate solution using a 10 ml burette graduated in 0.02 ml units. The sample was magnetically stirred and a starch indicator employed near the end point; a mean of five determinations was taken for each sample.

## 2.9 MATERIALS

Carbontetrachloride (analytical reagent grade) was obtained from Fisons, Loughborough, Lancs and pure hydrocarbons from B.D.H. Ltd., Poole, Dorset. Tween 80, silica gel type H, lysozyme and sodium lauryl sulphate were obtained from Sigma London Ltd., Kingston-upon-Thames, Surrey. All other chemicals were of the best commercial quality and were supplied by Fisons or B.D.H. Ltd. Oil samples were gifts from B.P. Ltd. (p.41).



CHAPTER 3

DEVELOPMENT OF THE ENVIRONMENTAL SYSTEM

## DEVELOPMENT OF THE ENVIRONMENTAL SYSTEM

3.1 SITES

Originally, two locations were chosen for in situ experiments, one in the Medway estuary (Figure 2.1) and the other approximately 7 miles off-shore from the north Kent coast. The site near Sheerness in the Medway was chosen as this is subjected to oil pollution; there is oil tanker traffic to and from the Isle of Grain oil refinery, and the estuary is heavily used by shipping docking at Sheerness and ports further upstream. This site also had the advantage of numerous channel marker buoys to which equipment could be attached. The offshore site in the vicinity of the red sands was chosen to represent an area less heavily polluted by oil. The site was inshore of the main shipping lanes yet its 7 mile distance from land rendered it virtually free from pleasure craft. The channel marker buoys on station at this offshore site were not suitable for attachment of equipment, nor was permission forthcoming from Trinity House. To overcome this problem a buoy was constructed from a 45 gallon oil drum modified to provide suitable attachment points and filled with urethane foam to prevent sinking in the event of puncture or slow leakage. The buoy was held on station by a 5 cwt concrete sinker attached by a  $\frac{1}{4}$ " diameter stainless steel cable; the buoy was positioned on station in mid December 1976. On return to the site in early January 1977 no trace of the buoy could be found, and the site was subsequently abandoned.



### 3.2 ESTABLISHMENT OF AN OIL FILM AT SEA

In an ideal experimental system, the oil should be present as a surface film open to the atmosphere to allow evaporation and photochemical changes and with free interchange of sea water beneath the film to permit dissolution, colonization and a supply of inorganic nutrients. In practice, containing an oil film in this state has many problems, in particular wind and wave action may cause the oil to be 'washed out' of equipment. If the size of the containing apparatus is increased in an attempt to prevent this, more substantial anchoring apparatus is required to withstand the harsh meteorological conditions invariably found at sea. As well as 'wash out' there is also the problem of oil adhering to the sides of the equipment and hence being removed from the surface film. These disadvantages prevent uniform sampling and hinder quantitative measurements of total oil loss, although qualitative measurements of changes in oil composition can be made. In view of these problems, maintenance of oil at sea in the form of a free floating film was not attempted. Instead, oil was adsorbed onto an inert surface and exposed in this fashion. Although this is a departure from the floating film it has the advantages of containing the oil uniformly, loss due to 'wash out' is minimised and can be estimated, and uniform sampling is facilitated. The presence of oil as a coating film over solid particles is not an unnatural occurrence in the environment.

### 3.3 ADSORPTION OF OILS

Despite the artificial nature of this process the aim was to produce a thin, uniform, microbially accessible film of oil approximating to that occurring naturally, but contained by its adhesion to the inert supporting matrix. The choice of supporting matrix was critical to the success of such a system; ideally the matrix should fulfil the following criteria:

- a) it should possess a uniform integral structure of large surface area, not containing detachable fibres nor exhibiting significant structural alteration on adsorption of oil.
- b) it should be able to adsorb oil uniformly and be chemically resistant to organic solvents used for oil extraction e.g.  $\text{CCl}_4$ .
- c) it should be a biologically inert support neither providing a substrate nor proving inhibitory to growth, and it should not act as a supply of inorganic nutrients.
- d) it should be sufficiently robust to withstand sea conditions and be corrosion resistant.

Several supports were investigated; glass fibre wool was an inert adsorptive support, yet it possessed detachable fibres and did not adsorb the oil uniformly. Microscopic examination revealed that the fibres tended to clump together with oil films between them, but there was not uniform coating of individual fibres. These disadvantages would prevent uniform sampling. Glass fibre filters and paper filters had similar disadvantages of uneven adsorption and a non integral structure, permitting



the loss of individual fibres. Membrane filters provided a more suitable support since they have a uniform integral structure of large surface area, with no detachable fibres. They are commercially available with different chemical composition, pore size and diameter; a Millipore type SC filter composed of mixed esters of cellulose acetate and cellulose nitrate, of  $8\mu$  pore size and 47 mm diameter was chosen for the following reasons. This type readily adsorbed oil uniformly and was resistant to  $\text{CCl}_4$ , while the filters composed of hydrophobic materials such as P.T.F.E. or P.T.F.E. + polythene did not have such good adsorptive properties. The large pore size was chosen to allow microbial access throughout the filter, and the large diameter permitted several sections to be cut from one filter for analysis. After adsorption of oil, examination of the filters by scanning electron microscopy, and quantification by infrared spectroscopy of the amount of oil present on identical sections cut from one filter showed that oil had been adsorbed uniformly over the filter. Examination of transverse sections of oiled filter showed that oil was present throughout the filter matrix and not simply confined to the surface. Unfortunately the pore size was reduced after adsorption which could restrict microbial access. Considering the worst possible hypothetical situation when only oil at the filter surface is available for attack plus maximum thickness of filter (range  $90\text{--}170\mu\text{m}$ ), the surface area/volume ratio is 11.76. A spherical oil droplet with the same ratio would have a radius of 0.26 mm. The maximum thickness of the filter is not unrepresentative of

some naturally occurring oil films.

#### 3.4 EQUIPMENT FOR THE MAINTENANCE OF OILED FILTERS AT SEA

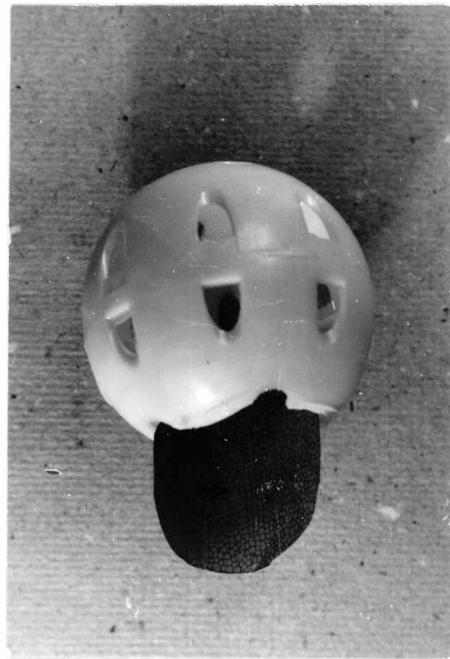
Equipment was designed to maintain oiled filters at sea while permitting free interchange with the environment, yet affording some protection against weather conditions. It should also permit simple, rapid sampling, which could be effected from a small craft in moderate seas. Each oiled filter was first enclosed in plastic practice golf balls (obtained locally); this afforded some protection and ensured the filters did not adhere to one another or to the sides of the equipment. Filters were cut to a 33 mm diameter disc and inserted into the balls through a flap cut in the surface (Plate 3.1 A & B) such that they formed a diaphragm equatorially. The flap was sealed with a hot blade and each ball numbered for identification. Up to twenty of these sample units were then placed in the apparatus, shown in Plate 3.1 C & D, designed to be attached to existing buoys and to contain samples under in situ sea conditions. Most of the materials needed for construction were obtained locally, except for the sampling port which was a modified BDH 'safepack' container. The material used to construct the lid was particularly important as this received considerable buffeting against the attachment buoy due to wave action. Initial trials using thin plastic resulted in rapid destruction of the lid and loss of samples; subsequently lids were cut from 5 mm thick plastic sheeting and these proved sufficiently strong.



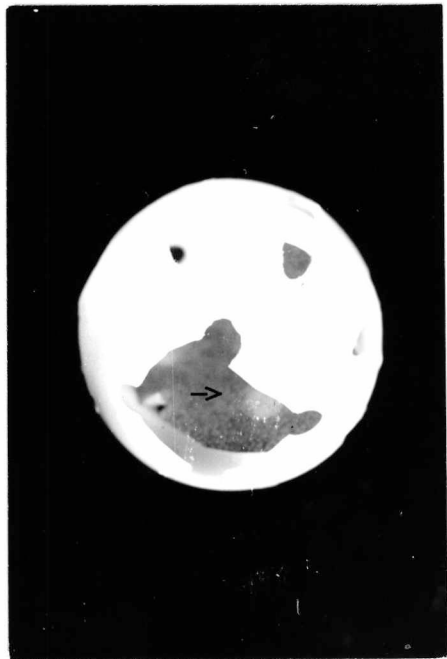
PLATE 3.1

Equipment for the maintenance of oil samples under in situ conditions.

- A - oiled filter (arrowed) partially inserted into practice golf balls.
- B - oiled filter (arrowed) positioned to form a diaphragm equatorially within the practice golf ball.
- C&D - equipment used to contain up to 20 of the sample units (oiled filter within practice golf ball) which was attached to the Queenborough Spit marker buoy.



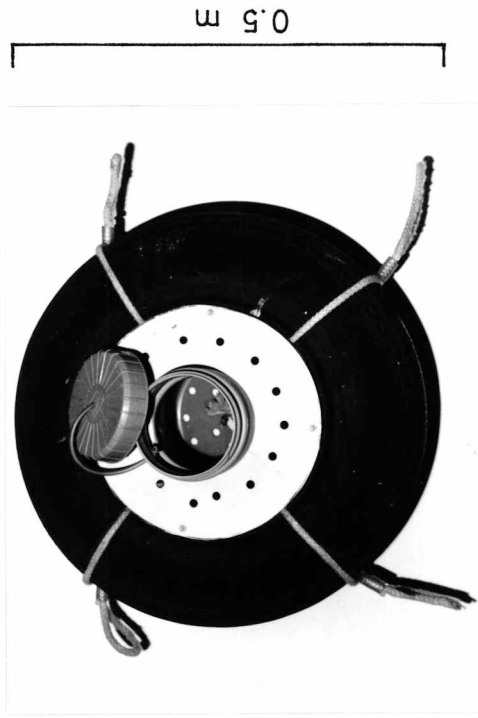
A



B



C



D



### 3.5 ESTABLISHMENT OF EQUIPMENT ON STATION

Permission was obtained from Capt. G. Baskerville of the Medway Ports Authority to use the Queenborough Spit channel marker buoy for attachment of the equipment. This buoy was chosen as it is situated in an open stretch of the estuary and could be reached without crossing the major shipping channel, (Plate 3.2). Four experimental units were attached to the buoy in each case by two synthetic fibre lines approximately 1 metre long (Plates 3.4 A & B). This allowed the equipment to float free of the buoy which minimised the damage due to buffeting that was experienced when the equipment was attached hard up against the buoy. Oiled filter sample units were then introduced through the sampling port; one piece of equipment was used for each oil and the fourth contained control filters.

### 3.6 SAMPLING

The buoy was reached using a variety of small craft powered by a 4 h.p. outboard engine, of which a 10ft. Avon Redseal inflatable dinghy proved to be the safest and most reliable (Plate 3.5 B). Sampling was attempted at regular intervals during experimental periods but this was dependent upon weather conditions; routine sampling was not attempted in sea conditions above force 5 (Beaufort scale) or in poor visibility. However samples could be obtained in seas of force 6 - 7. In some cases during prolonged periods of bad weather, sampling was carried out from a launch kindly provided by the Medway Ports Authority. Sample units (oiled filter within ball) were withdrawn via the

PLATE 3.2

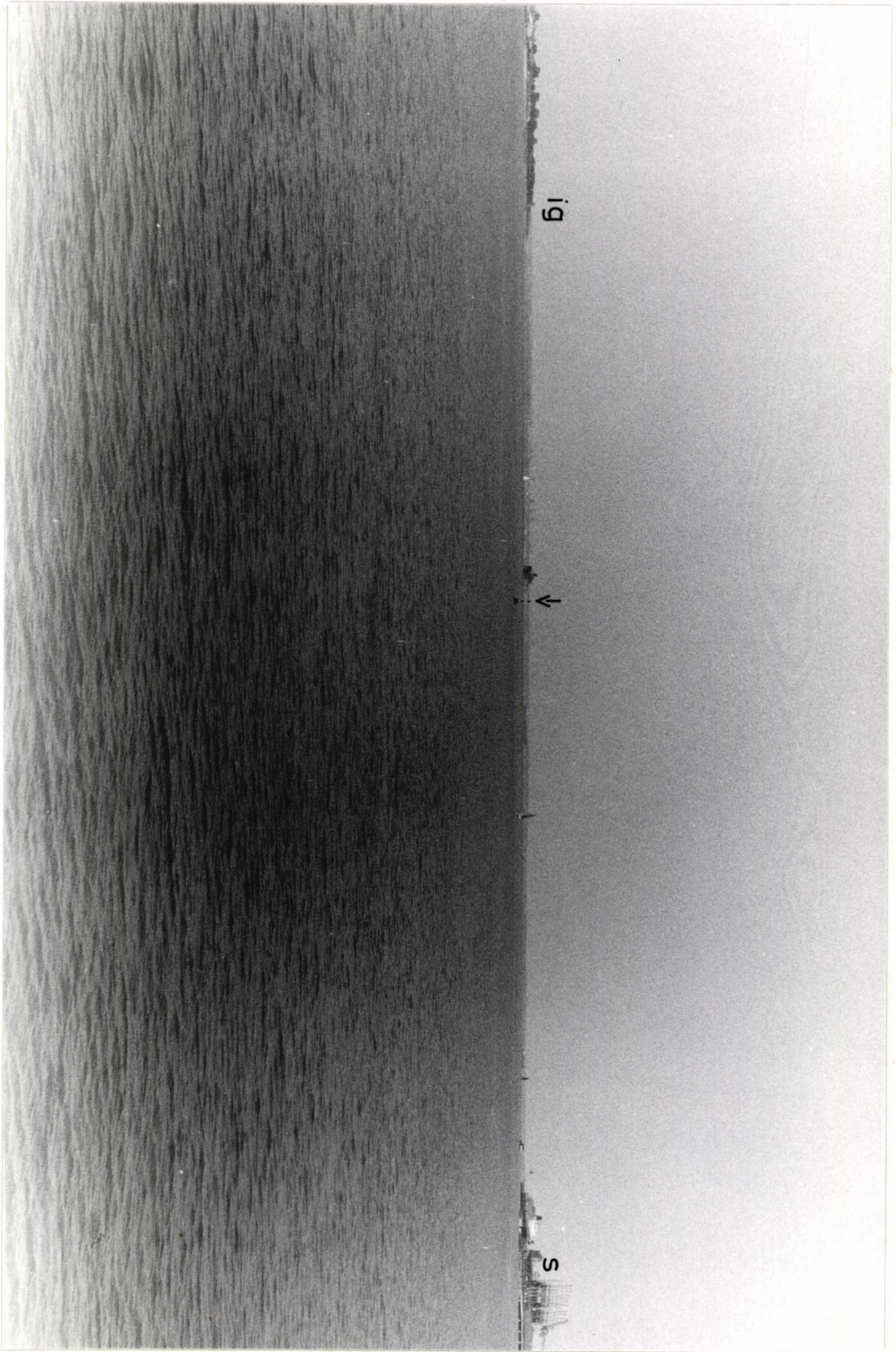
Position of the Queenborough Spit channel marker buoy used for the attachment of the experimental equipment in the Medway estuary.

ig - Isle of Grain.

s - Sheerness (Isle of Sheppey).

Position of buoy arrowed.





61

S

PLATE 3.3

Queenborough Spit buoy with attached experimental  
equipment.

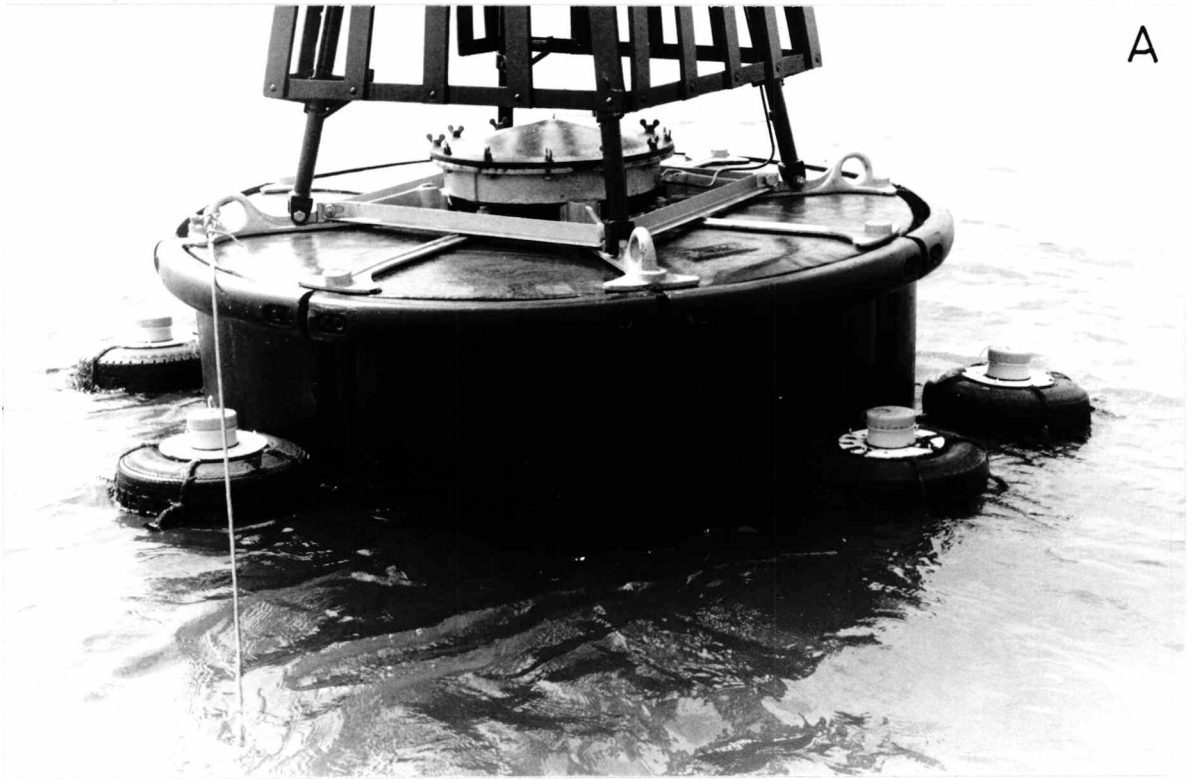




PLATE 3.4

Attachment of experimental equipment to the Queenborough  
Spit buoy.

- A - distribution of the four units of experimental  
equipment about the buoy.
- B - detail of the method of attachment of the  
equipment (securing lines arrowed) to the buoy.



A



B

sampling port and immediately placed in separate 250 ml capacity screw cap containers rinsed out and half filled with sea water collected at the time of sampling; examination of samples was initiated within two hours of collection.

### 3.7 PERFORMANCE OF THE EQUIPMENT

The equipment could be transported by small craft and positioned on station rapidly, (within about 40 minutes). The enclosure of oiled filters in practice golf balls proved satisfactory. This facilitated sampling; enabled individual filters to be identified, and their buoyancy maintained filters at the water surface. The overall system was extremely robust and has withstood gales of up to force 11 - 12 experienced during late December and early January 1977/78. Samples were recoverable after 80 days exposure at sea. The occasional loss of complete units of equipment was due to breakage of the securing lines. The oiled filters remained relatively intact over the exposure periods compared to the control filters which showed some disintegration. This difference may have been due to some structural binding effect provided by the coating oil film. Some biological fouling of the filters and equipment was experienced over prolonged exposure during the summer months, (Plate 3.5 A); however this was not sufficient to restrict seriously the flow of water through the equipment.

### 3.8 QUANTIFICATION OF OILS ON FILTERS

Prior to exposure, oiled filters were prepared by the

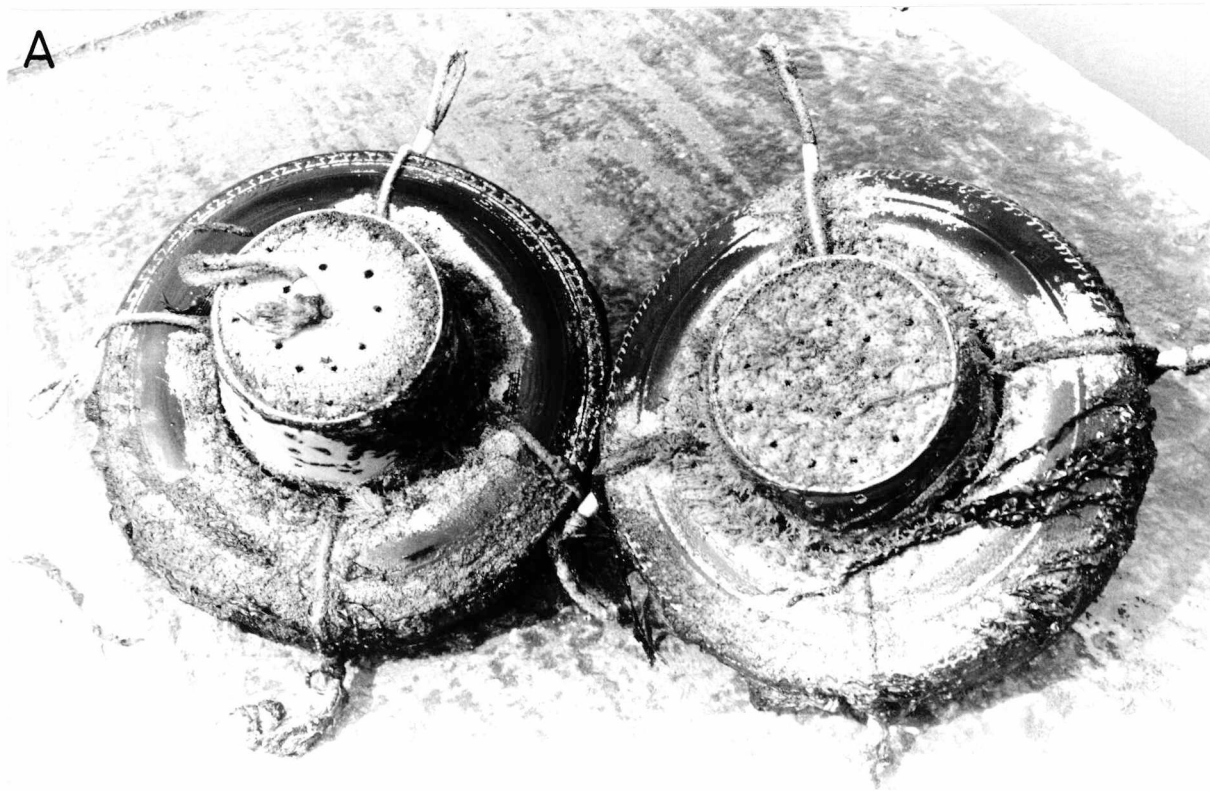


PLATE 3.5 A

Examples of biological fouling of the experimental equipment after 72 days in situ exposure during the summer months.

PLATE 3.5 B

Craft used for routine sampling of the in situ experiments.



following method. Filters were weighed, placed in clean numbered petri dishes, then immersed in 'topped' filter sterilized oil (except for the sand tar which was too viscous to be filtered), blot dried and returned to the petri dishes. After drying fully for at least three days at room temperature the filters were re-weighed and hence the amount of oil adsorbed determined. The synthetic crude and Forties crude could be adsorbed without dilution but the sand tar required dilution with  $\text{CCl}_4$  to achieve even adsorption. In practice rapid, even adsorption was facilitated if all the oils were first diluted in the ratio 3:1 (V/V) with  $\text{CCl}_4$ . The amount of oil adsorbed onto each filter was very similar; for example the standard deviation from the mean in a series of 16 filters prepared for each oil was as follows:

Forties crude	$0.13325\text{g} \pm 0.00824\text{g}$
Synthetic crude	$0.12933\text{g} \pm 0.00434\text{g}$
Sand tar	$0.109708\text{g} \pm 0.00607\text{g}$

On recovery from in situ experiments two identical 15 mm diameter discs were cut from each filter with a cork borer; these were allowed to dry at room temperature in a clean petri dish prior to extraction. Each disc was extracted with two consecutive 1 ml volumes of  $\text{CCl}_4$ , the extracts were pooled and then subjected to infrared spectroscopy (Chapter 2, section 2.1.4) in a 0.5 mm path length potassium bromide liquid cell. The amount of oil was quantified as described below. For each weathered oil a standard curve of mass of oil adsorbed onto filters versus sum of peak heights at 3.38, 3.42 and 3.50 $\mu$  was



constructed. This was achieved by preparing a graded series of filters with different masses of oil adsorbed, cutting 15 mm diameter discs from each, followed by extraction and quantification by infrared spectroscopy. The mass of oil on the 15 mm disc was determined by dividing the total amount of oil adsorbed on the entire filter by the ratio of the surface area of the filter to that of the disc. By reference to the standard curve the amount of oil on discs cut from recovered filters was determined. This was expressed as a percentage of that originally adsorbed prior to exposure.

### 3.9 ASSESSMENT OF MICROBIAL COLONIZATION OF OILED FILTERS

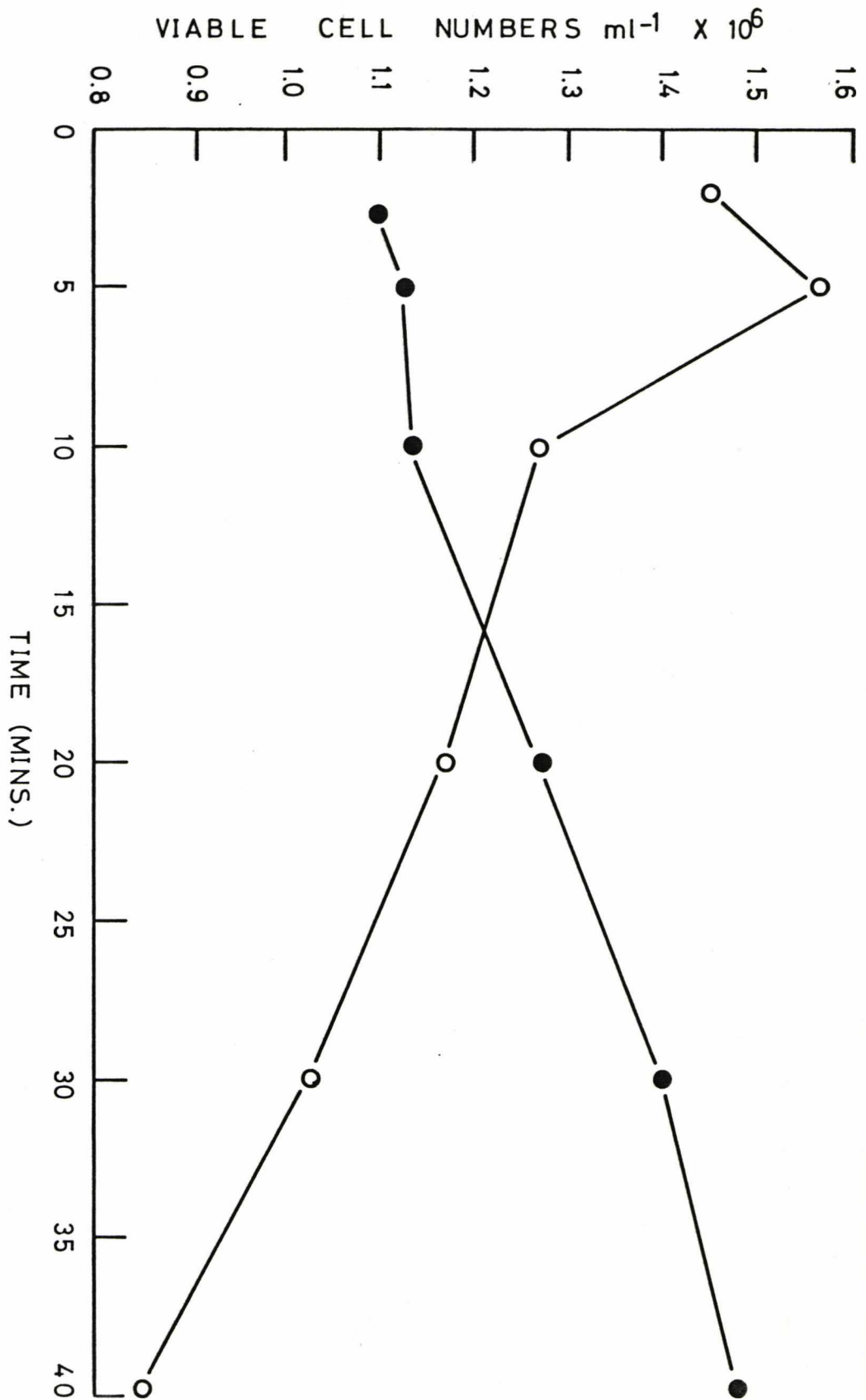
This was performed in three ways, namely, direct observation by light microscopy, scanning electron microscopy and viable counts on solid media. Observations by phase contrast, and by bright field illumination using the general purpose filter stain, were performed on sections of recovered filters shortly after sampling. These gave a qualitative assessment of growth. Scanning electron microscopy enabled the filter surface and any microbial colonizers to be examined in closer detail. Before performing viable counts, colonizers had to be freed from the filter surface, to produce a suspension suitable for serial dilution. Two methods were employed, mild sonication, (1 second pulses of 10 $\mu$  peak to peak), and vigorous shaking (800 cycles/min. with or without 1 mm glass beads), using a Gallenkamp wrist action shaker. Figure 3.1 summarises the efficiency of shaker method, from which it can be seen that shaking in the presence



FIGURE 3.1

Efficiency of removal of bacteria from an oiled filter surface.

- - viable cell numbers recovered by shaking at 800 cycles/min.
- - viable cell numbers recovered by shaking at 800 cycles/ min. in the presence of 1 mm dia -meter glass beads.



of beads for 5 minutes was most suitable. Sonication reduced recovery of viable cell numbers by an order of magnitude compared to shaker treatment. Therefore, routinely, 15 mm diameter discs were cut from recovered filters, placed in stoppered 25 ml capacity conical flasks containing 10 ml of sterile suspending buffer and 6, 1 mm diameter glass beads, and shaken for 5 minutes. Serial dilutions in sterile suspending buffer and plating onto sea water agar and oil agar was then performed. All glassware, suspending buffer and plates were cooled at 4°C before use. Plates were incubated at 15°C for at least one week before counting, and were then retained at room temperature for several days to allow for the appearance of further colonies.

### 3.10 OIL AGARS

Oil agars were prepared as a means of testing individual isolates for the ability to grow on crude oil as the sole carbon and energy source, and for direct enumeration of oil degraders by viable count. Several different methods of preparation were attempted and each was assessed by the degree of growth of a known oil degrading bacterial strain supported by each agar in comparison to that supported by minimal agar alone. Emulsions of oil in minimal agar were prepared by adding sterile 'topped' oil to agar just before pouring and shaking vigorously or by adding a sonicated emulsion of oil in sterile distilled water and mixing gently. Neither method supported significant growth. Growth on oil vapour, provided by an oil saturated filter placed



in the petri dish lid, was also sparse. Two methods of introducing the oil onto the surface of the agar as a film gave significantly better growth than the minimal agar controls. In the first, minimal agar plates were dried for several days at room temperature until they acquired a faint wrinkled appearance which indicated the surface was dry and porous. Approximately 0.1 ml of a 1:1 (V/V) mixture of sterile, weathered Forties crude and  $\text{CCl}_4$  was spread over the surface of the plate with a conventional glass spreader. The porous surface quickly adsorbed the mixture and the plates were dried for several minutes to allow the  $\text{CCl}_4$  to evaporate; plates were used only when no trace of  $\text{CCl}_4$  remained. In the second method, plates were treated in an identical way except the surface was spread with an oil emulsion in sterile distilled water in the ratio 1:3, (V/V), oil:water with Tween 80 added in the ratio 1:10, (V/V), Tween 80:oil, to promote a stable emulsion. Both these oil agars could be used for conventional plating out techniques. Plate 3.6 shows the growth obtained on the agar spread with the oil emulsion. The location of the oil film on the surface prevented the use of the spread plate technique for viable count. Instead the method of Miles & Misra (1938) was adopted. The plates spread with oil emulsion proved more suitable for this technique as they permitted the 0.02 ml drop to spread over the agar surface in the normal way which facilitated counting of the resultant colonies. Plates spread with the oil in  $\text{CCl}_4$  film prevented this natural spreading and consequently the resultant colonies were grouped in a smaller area and in some cases

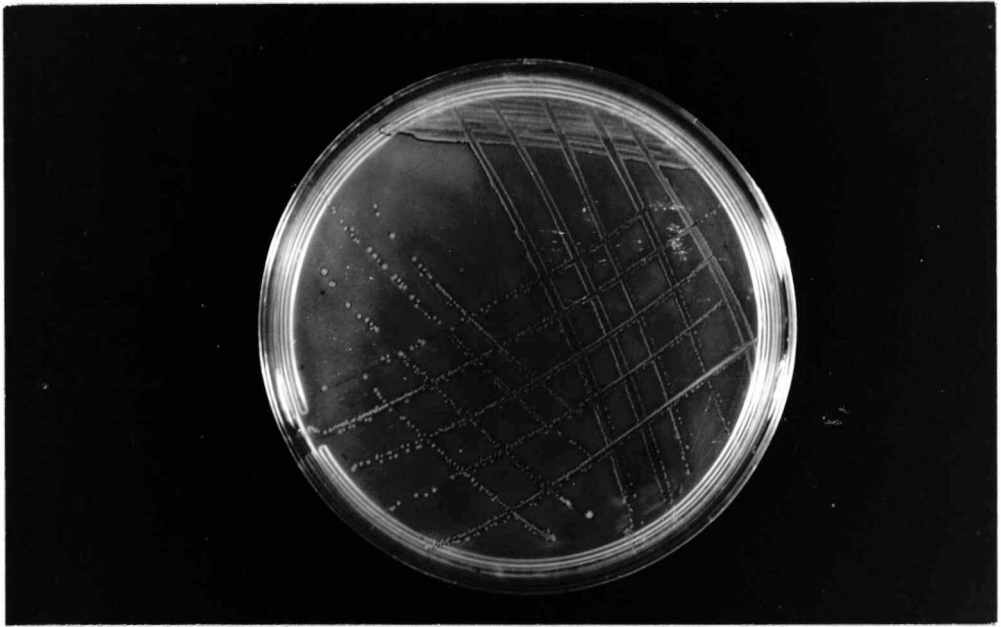


PLATE 3.6

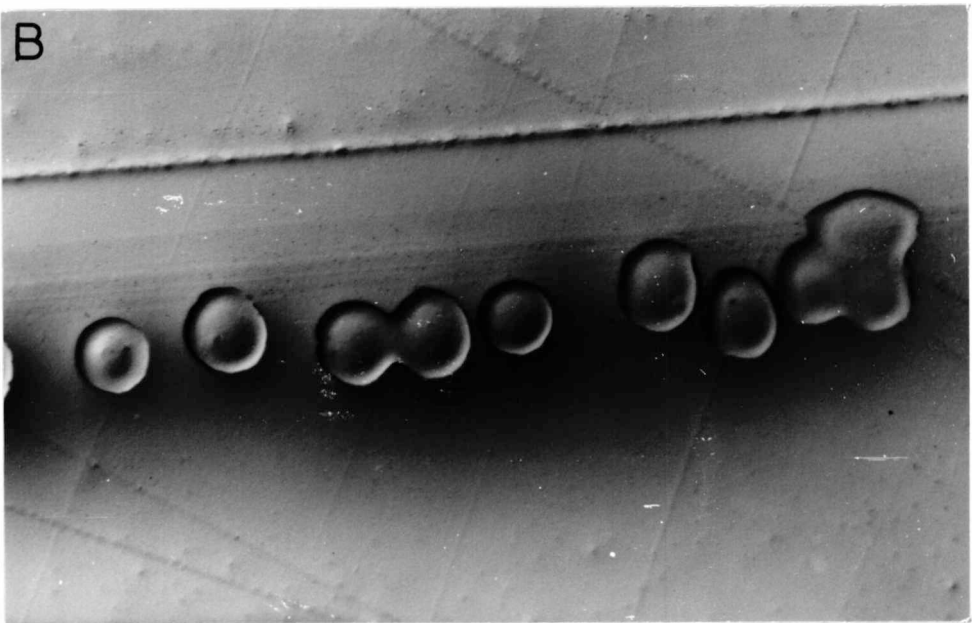
Examples of growth on oil agar.

- A - growth of strain N<sub>3</sub> after 3 days on minimal agar spread with an emulsion of Forties crude.
- B - detail of colonies on the surface of the above agar(x 30).

A



B



required a plate microscope for accurate counting.

### 3.11 CONTROLS

Two systems were used, one in situ and the other in closed flasks in the laboratory. At the same time that oiled filters were exposed to the environment blank filters were exposed in an identical way in a separate experimental unit. These were sampled at regular intervals to estimate the degree of microbial colonization of non-oiled filters and to ascertain if oil, sometimes present in the estuary, was adhering to the filters. The laboratory controls were an attempt to estimate the loss of oil from filters due to non-biological action such as dissolution and 'wash off' and to determine the change in fractional composition after this process. Oiled filter discs (15 mm diameter) were incubated in 2L flasks containing 600 ml of sterile aged sea water and ampicillin (0.11 mg/ml). Flasks were shaken on an orbital shaker, (100 r.p.m.), at 4 and 13°C (which represent the approximate in situ surface water temperatures during winter and summer respectively) and oil remaining on the discs, sampled at regular intervals, quantified by infrared spectroscopy. Sterility checks were performed at the time of each sampling. For fractional analysis, oiled filters enclosed in practice golf balls were incubated for 7 days in 6L of sterile, aged sea water and ampicillin, (0.08 mg/ml), magnetically stirred in a 10L vessel. Filters were extracted with  $\text{CCl}_4$  and the recovered oil subjected to column chromatography, (Chapter 2, section 2.1.5).

CHAPTER 4

IN SITU EXPERIMENTS UNDER WINTER CONDITIONS



## IN SITU EXPERIMENTS UNDER WINTER CONDITIONS

### 4.1 RESULTS

Two in situ experiments of 50 and 80 days duration respectively were performed under winter conditions, (23/12/1976 - 9/2/1977 and 30/11/1977 - 17/2/1978). Both experiments were carried out using the equipment previously described which was attached to the Queenborough Spit buoy.

Although samples were recovered at intervals during the experimental periods, bad weather prevented collection until the 13th day of the first experiment. Furthermore the complete apparatus containing the Forties crude samples was lost from station between days 23 and 36 of the second experiment. This was due to breakage of the securing lines. The apparatus was replaced, but this equipment subsequently broke up after a further 26 days and the samples were lost.

On recovery of the samples, 15 mm diameter discs were cut from the filters and the amount of oil remaining quantified, (Chapter 3, section 3.8) the results for the first and second experiments are shown in Figures 4.1 and 4.2 respectively. The extent of oil loss from the filters due to non-biological factors such as 'wash off' and dissolution was estimated by the laboratory controls previously described (Chapter 3, section 3.11). The results for these controls carried out at 4°C showed that oil loss from the filters was essentially complete within 7 days whereafter no appreciable further loss occurred. The extent of leaching was different for each oil: 50, 10 and 7% was lost for the synthetic crude, Forties crude and sand tar respectively.

FIGURE 4.1

Percentage of oil remaining on oiled filter discs of 15 mm diameter during the first winter experiment of 50 days duration.

- - Sand tar.
- - Forties crude.
- △ - Synthetic crude.

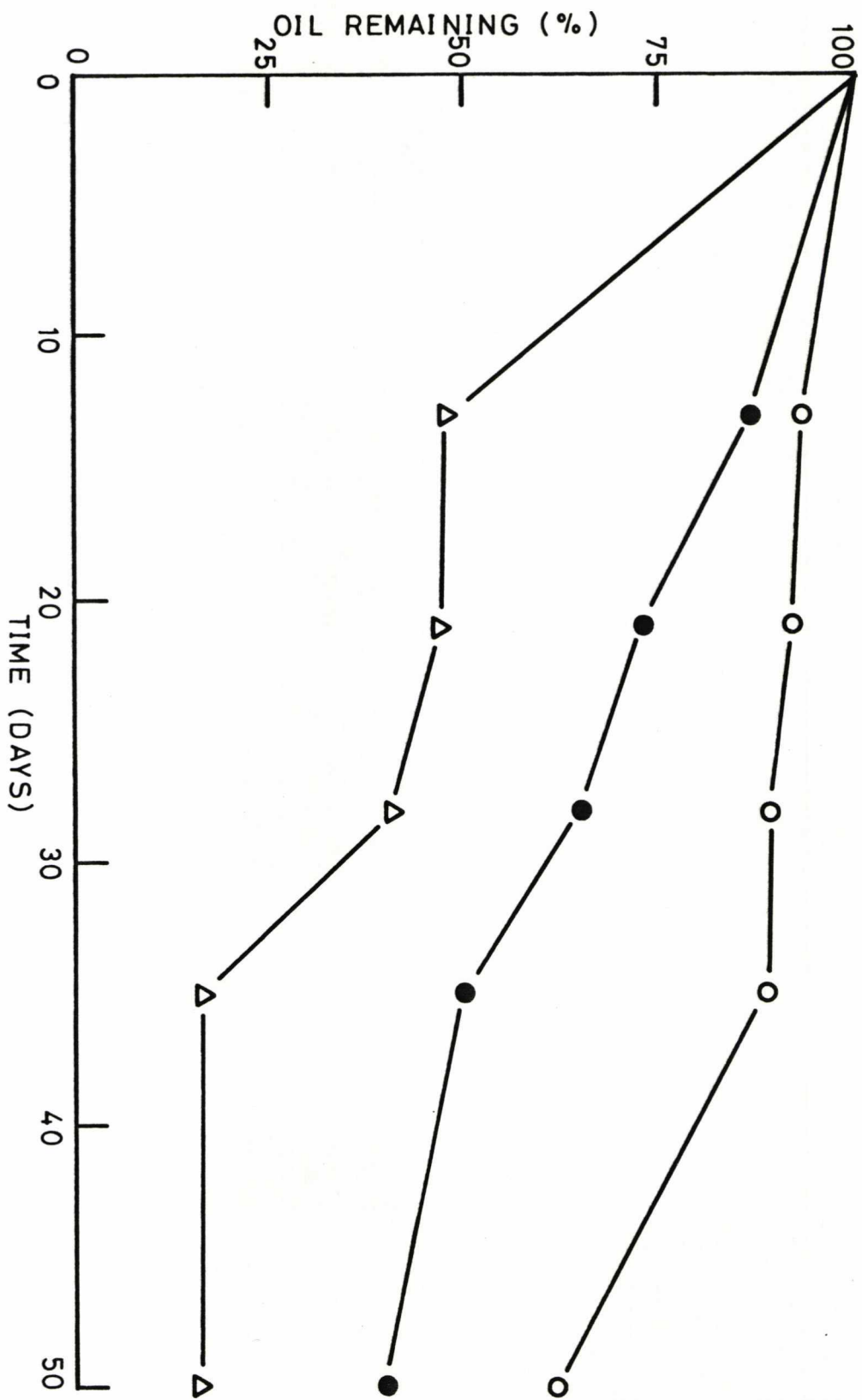
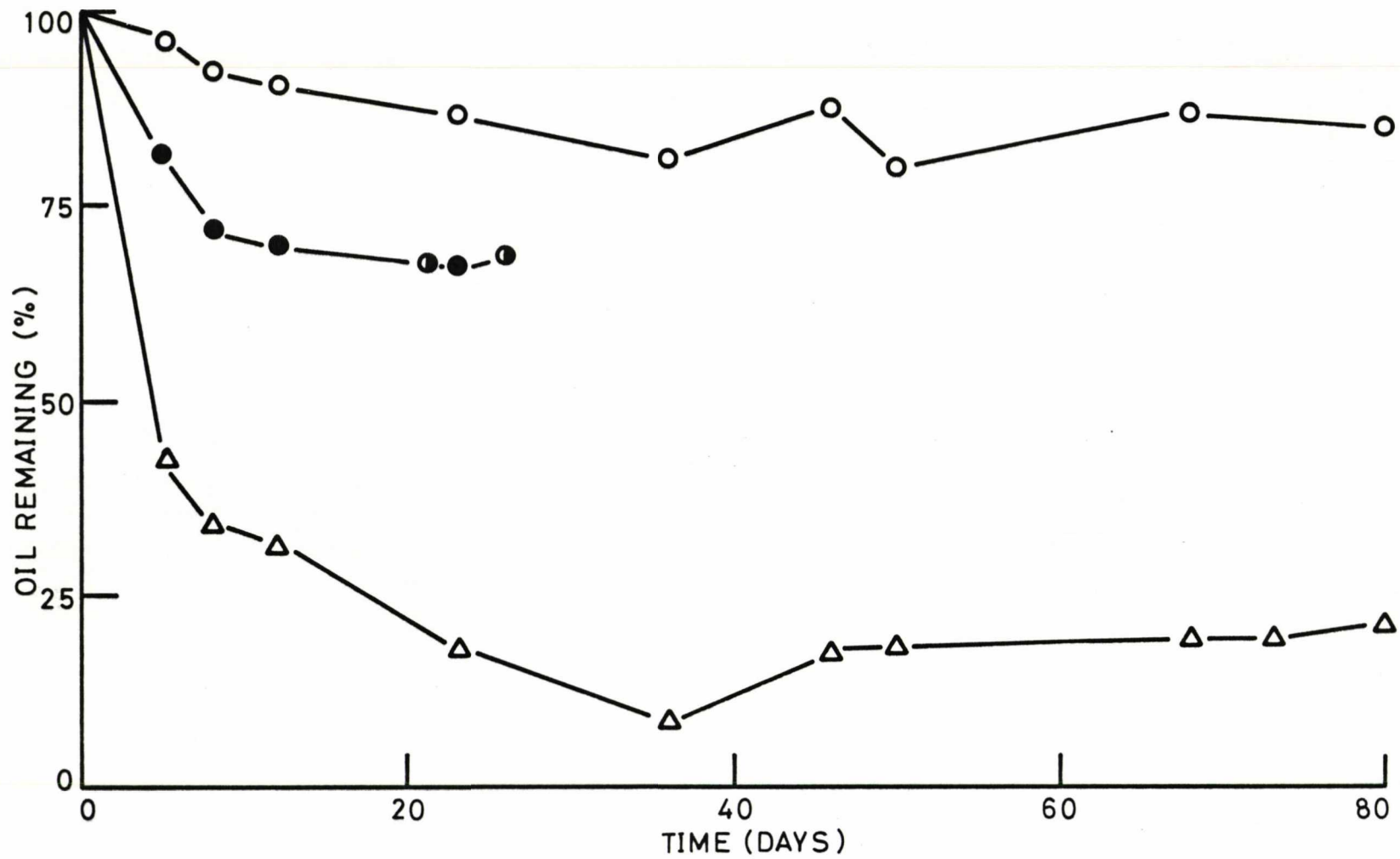


FIGURE 4.2

Percentage of oil remaining on oiled filter discs of 15mm diameter during the second winter experiment of 80 days duration.

- O - Sand tar.
- - Forties crude.
- ⊙ - Forties crude samples (replacement apparatus).
- Δ - Synthetic crude.





Gas chromatographic profiles of the synthetic and Forties crude after early exposure during the second winter experiment are shown in Figures 4.3 and 4.4 respectively, with fresh, 'topped' and oils leached in sterile sea water shown for comparison. The blank control filters exposed during both experiments did not reveal any significant adsorption of oil from the environment.

During the first experiment, the extent of microbial colonization of the oiled filters was estimated by conventional spread plate technique on sea water agar. Representatives of each colony type found were then transferred onto oil agar to test for oil utilization ability. The total oil utilizing microbial population isolated from each oil is shown in Figure 4.5. This population could be resolved into three separate bacterial strains distinguished on the basis of colony type and morphology; their relative numbers for each oil are shown in Figures 4.6, 4.7 and 4.8. Presumptive identification (Chapter 2, section 2.6) revealed strain 1 to be a marine Pseudomonad, strain 2 a Flavobacterium species, but strain 3 remains unidentified; all were designated psychrotrophic. During the second experiment microbial colonization of the oiled filters was determined by direct plating onto sea water agar and onto oil agar. The total number of oil utilizers are shown in Figure 4.9 and the total heterotrophic and oil utilizing population for each oil in Figures 4.10, 4.11 and 4.12. Throughout both experiments there was no evidence of filamentous fungi, but a small number of non oil utilizing yeasts (ca. 20 - 70 per 15 mm diameter disc) were isolated on potato dextrose agar. The blank control filters supported only a small

FIGURE 4.3

Gas chromatographic profiles of the synthetic crude recovered at intervals during the second winter experiment.

- A - fresh oil.
- B - topped oil.
- C - oil leached in sterile sea water for 7 days.
- D - oil leached in sterile sea water for 14 days.
- E - oil recovered after 5 days.
- F - oil recovered after 8 days.

Temperature increases from right to left across the profile. All profiles were performed under identical and are directly comparable.



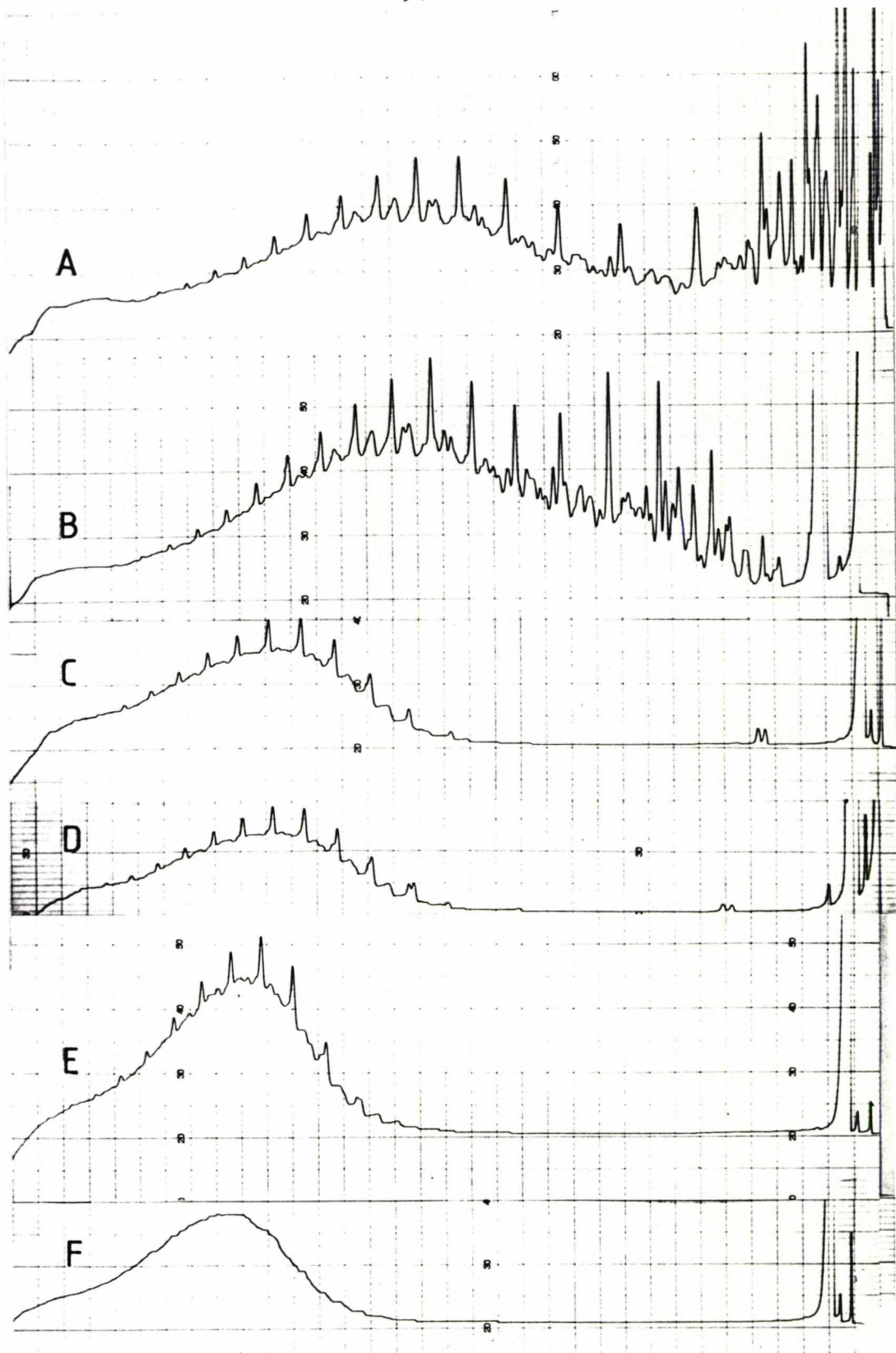




FIGURE 4.4

Gas chromatographic profiles of the Forties crude recovered at intervals during the second winter experiment.

- A - fresh oil.
- B - topped oil.
- C - oil leached in sterile sea water for 7 days.
- D - oil leached in sterile sea water for 14 days.
- E - oil recovered after 5 days.
- F - " " " 8 " .
- G - " " " 12 " .
- H - oil recovered after 26 days.

Temperature increases from right to left across the profile. All profiles were obtained under identical conditions and are directly comparable.

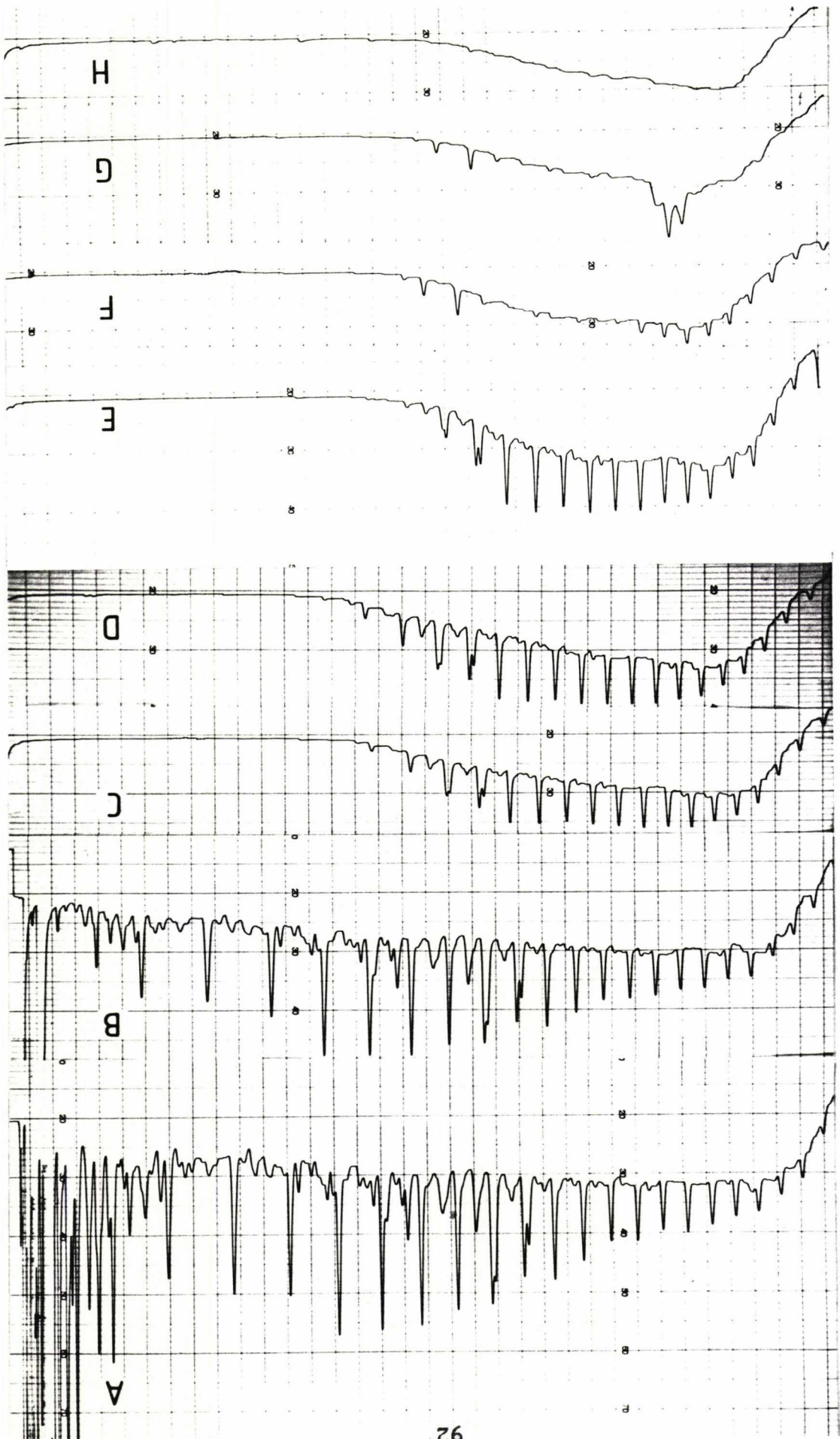


FIGURE 4.5

Log<sub>10</sub> of viable oil degrading bacterial cell numbers isolated from 15mm diameter discs of oiled filter during 50 days of the first winter experiment.

- O - isolates from sand tar.
- - isolates from Forties crude.
- Δ - isolates from synthetic crude.

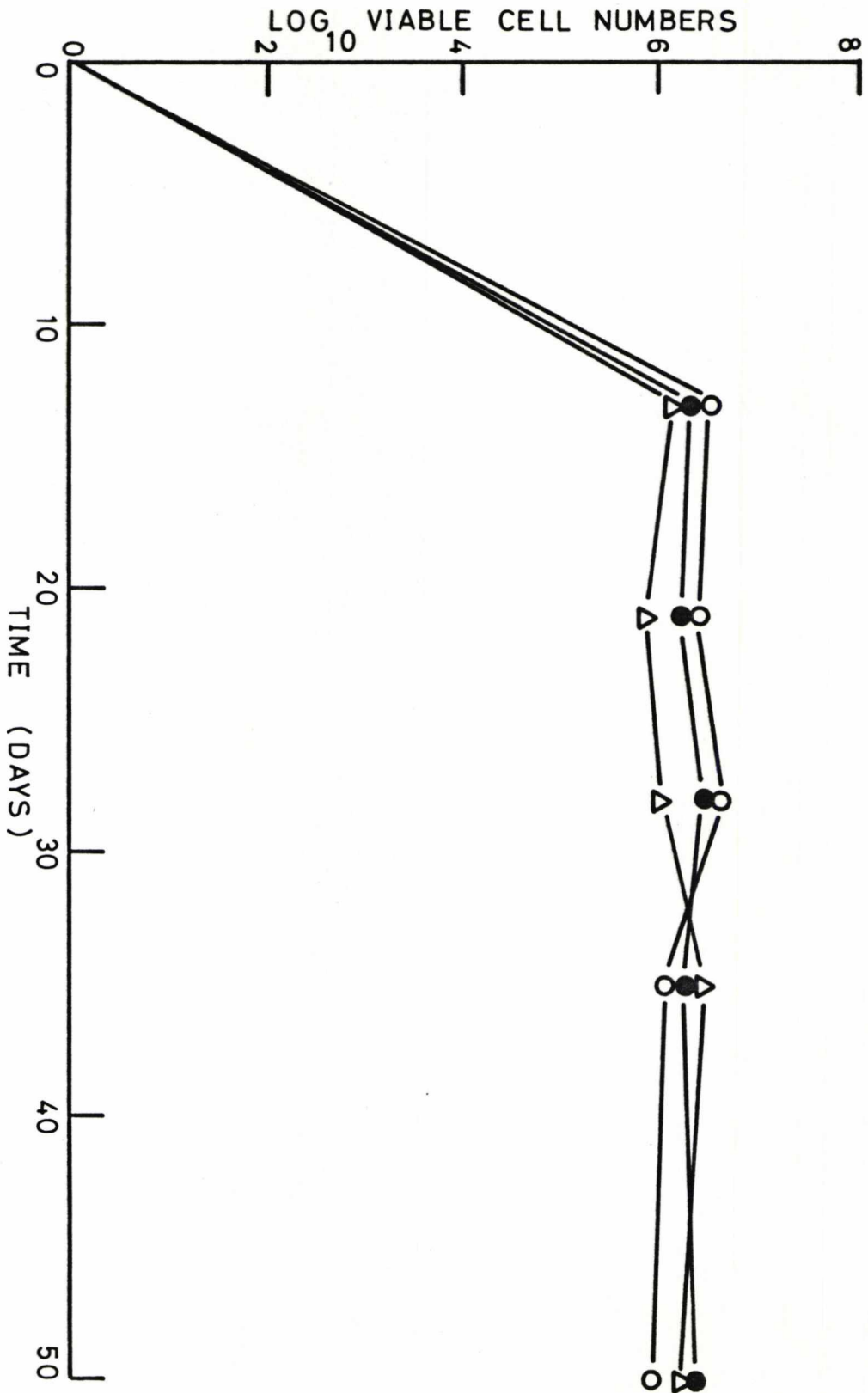




FIGURE 4.6

Log<sub>10</sub> of viable bacterial cell numbers of the three strains comprising the total oil degrading population isolated from 15mm diameter discs of sand tar coated filters during 50 days of the first winter experiment.

- ▽ - strain 1.
- - strain 2.
- - strain 3.

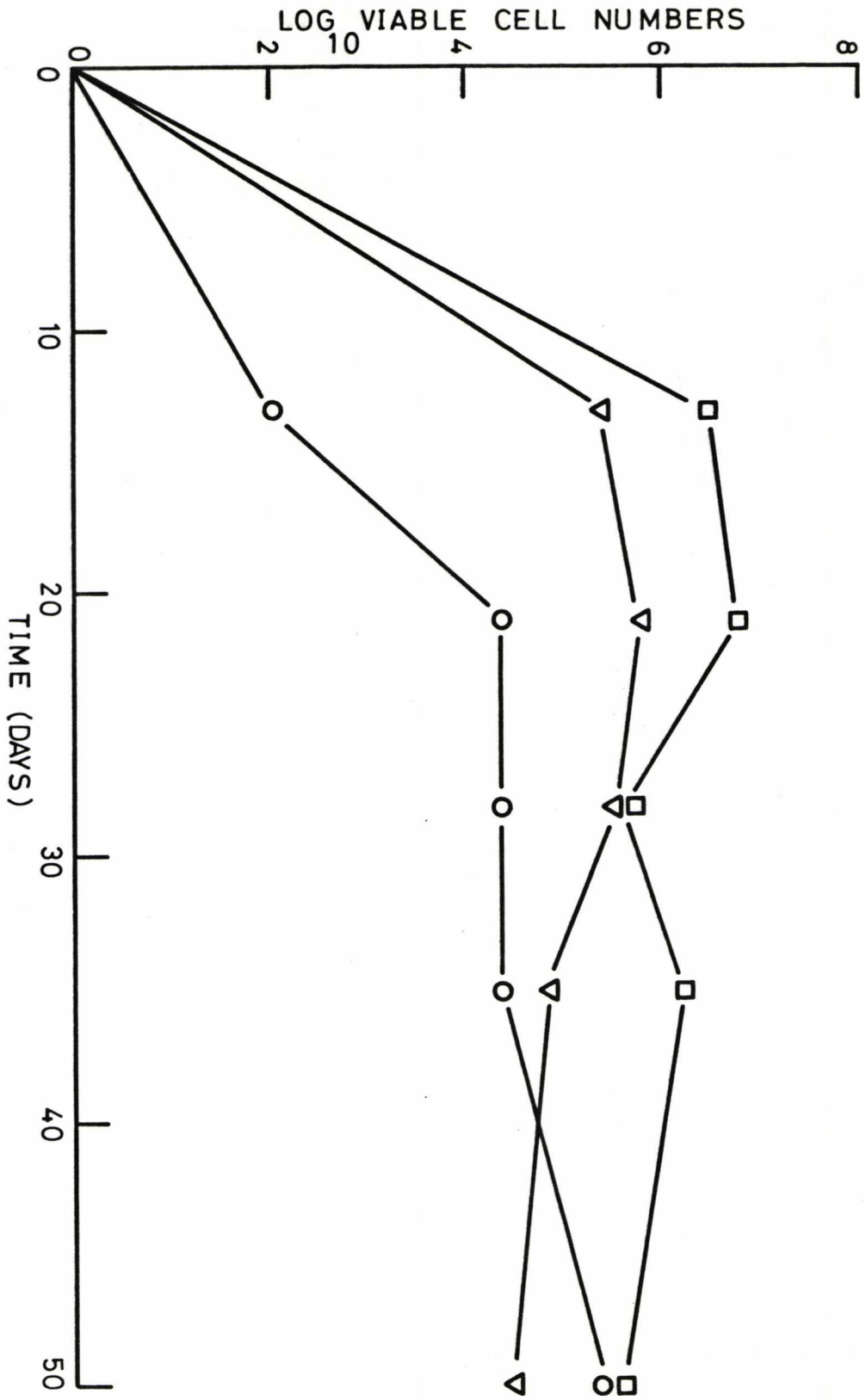


FIGURE 4.7

Log<sub>10</sub> of viable bacterial cell numbers of the three strains comprising the total oil degrading population isolated from 15mm diameter discs of filter coated with Forties crude during 50 days of the first winter experiment.

- ▽ - strain 1.
- - strain 2.
- 0 - strain 3.

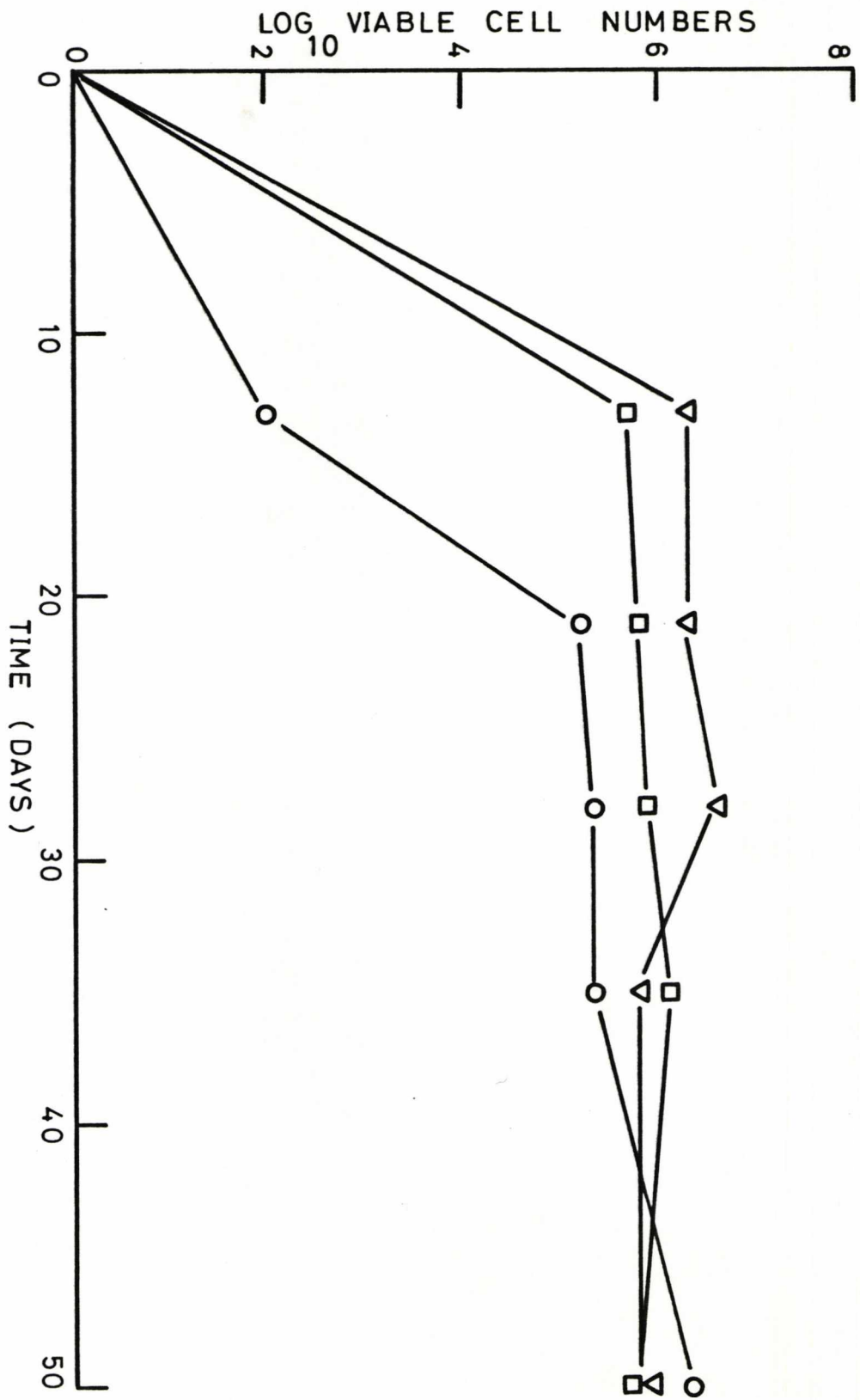




FIGURE 4.8

$\text{Log}_{10}$  of viable bacterial cell numbers of the three strains comprising the total oil degrading population isolated from 15mm diameter discs of filter coated with synthetic crude during 50 days of the first winter experiment.

▽ - strain 1.

□ - strain 2.

○ - strain 3.

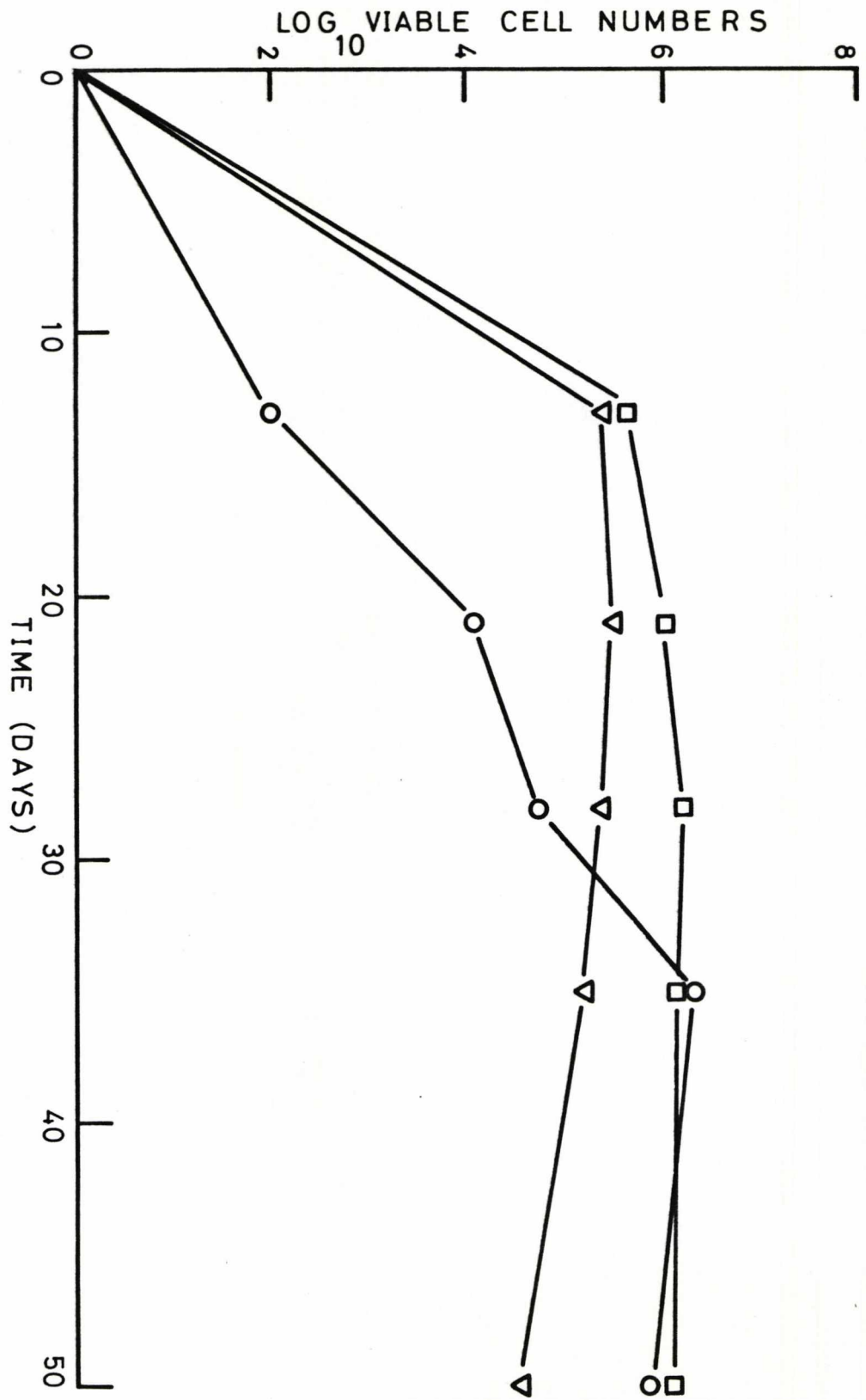


FIGURE 4.9

Log<sub>10</sub> of viable oil degrading bacterial cell numbers isolated from 15mm diameter discs of oiled filter during 80 days of the second winter experiment.

- - isolates from sand tar.
- - isolates from Forties crude.
- ◐ - isolates from Forties crude (replacement samples).
- △ - isolates from synthetic crude.

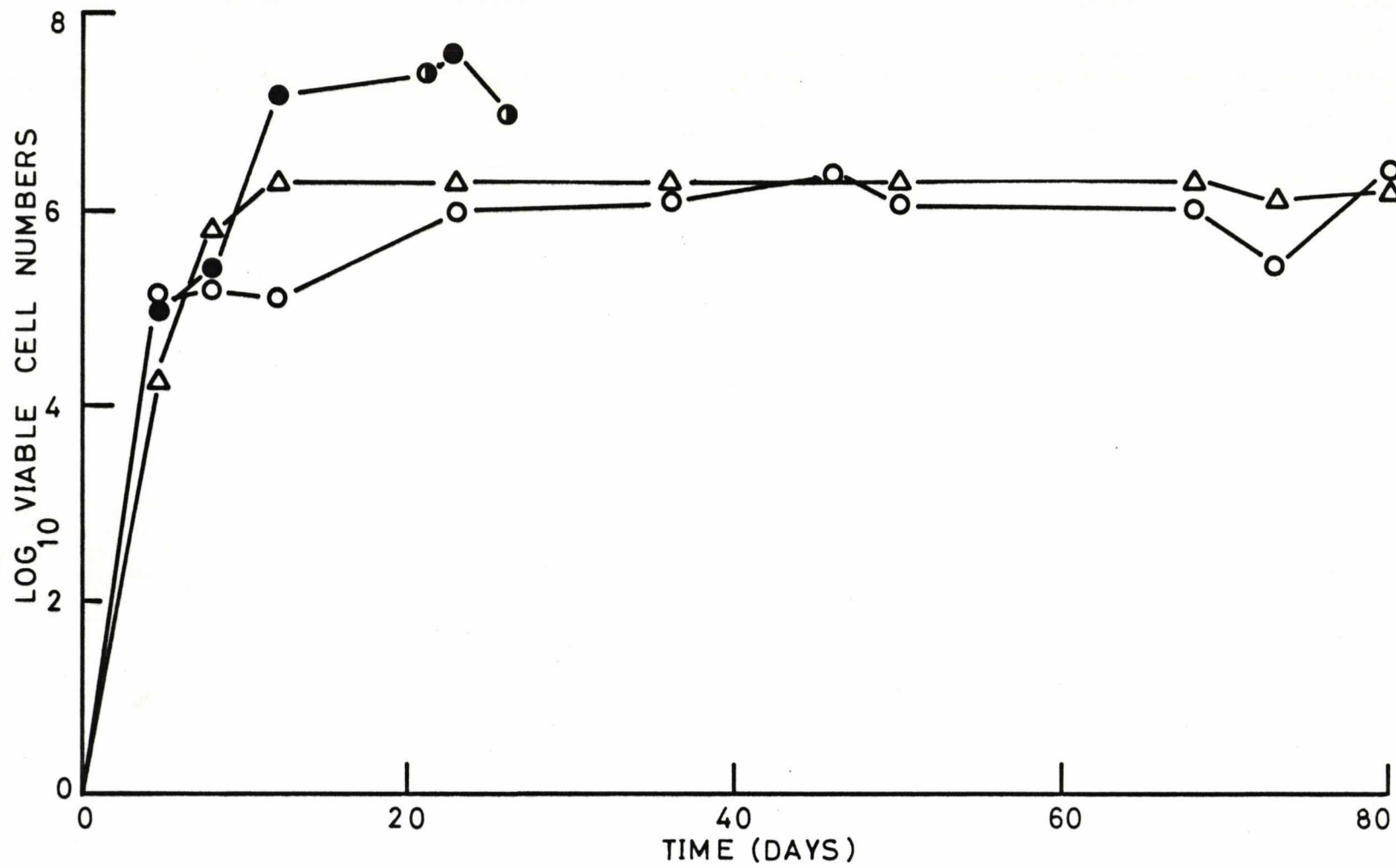
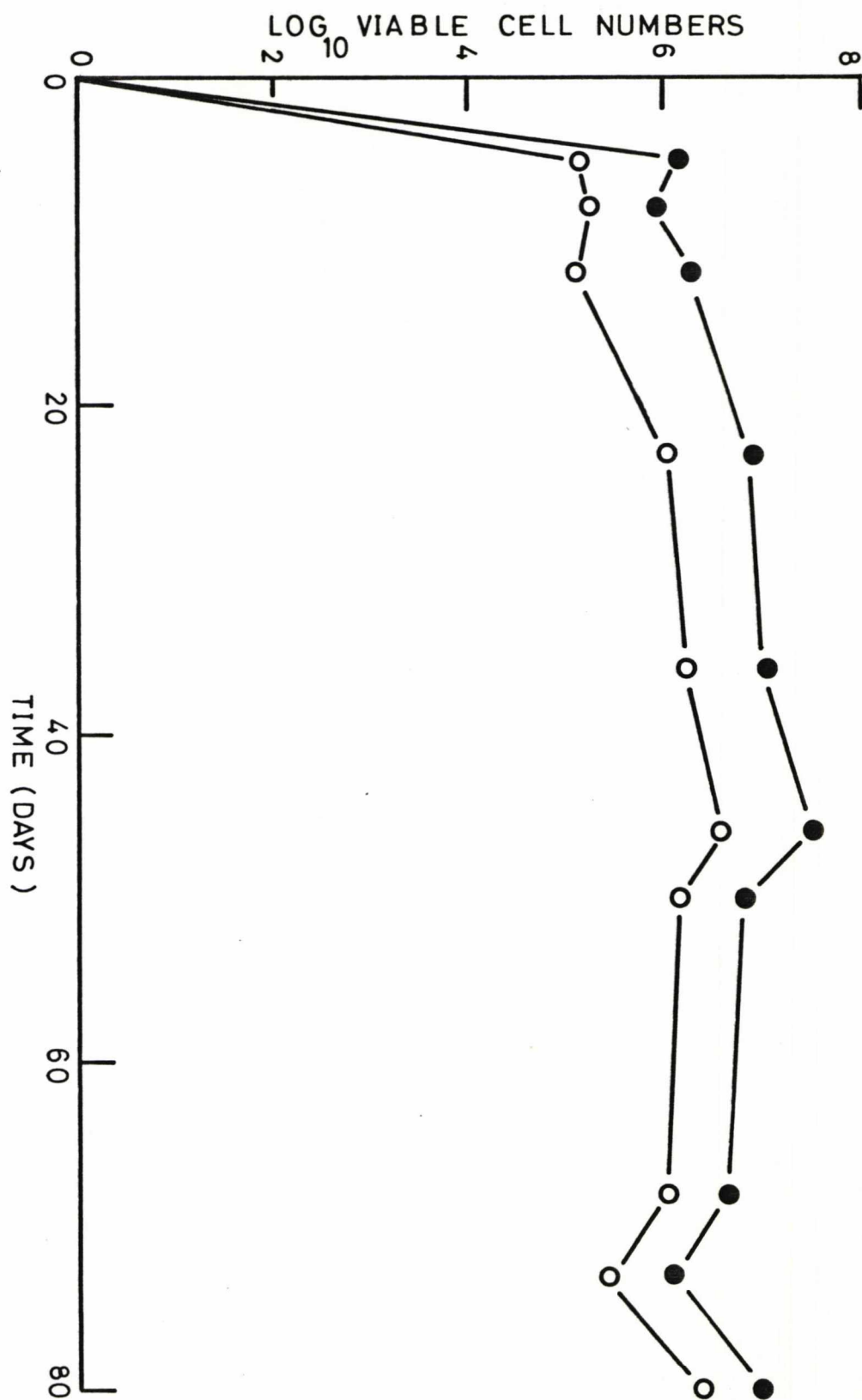




FIGURE 4.10

Log<sub>10</sub> of viable cell numbers for the total hetero-  
-trophic and oil degrading bacterial populations isolated from  
15mm diameter discs of filter coated with sand tar during 80  
days of the second winter experiment.

- - total heterotrophic population.
- - oil degrading population.



**FIGURE 4.11**

Log<sub>10</sub> of viable cell numbers for the total heterotrophic and oil degrading bacterial populations isolated from 15 mm diameter discs of filter coated with Forties crude during 80 days of the second winter experiment.

- - total heterotrophic population.
- - " " " (replacement samples).
- - oil degrading population.
- - oil degrading population (replacement samples).

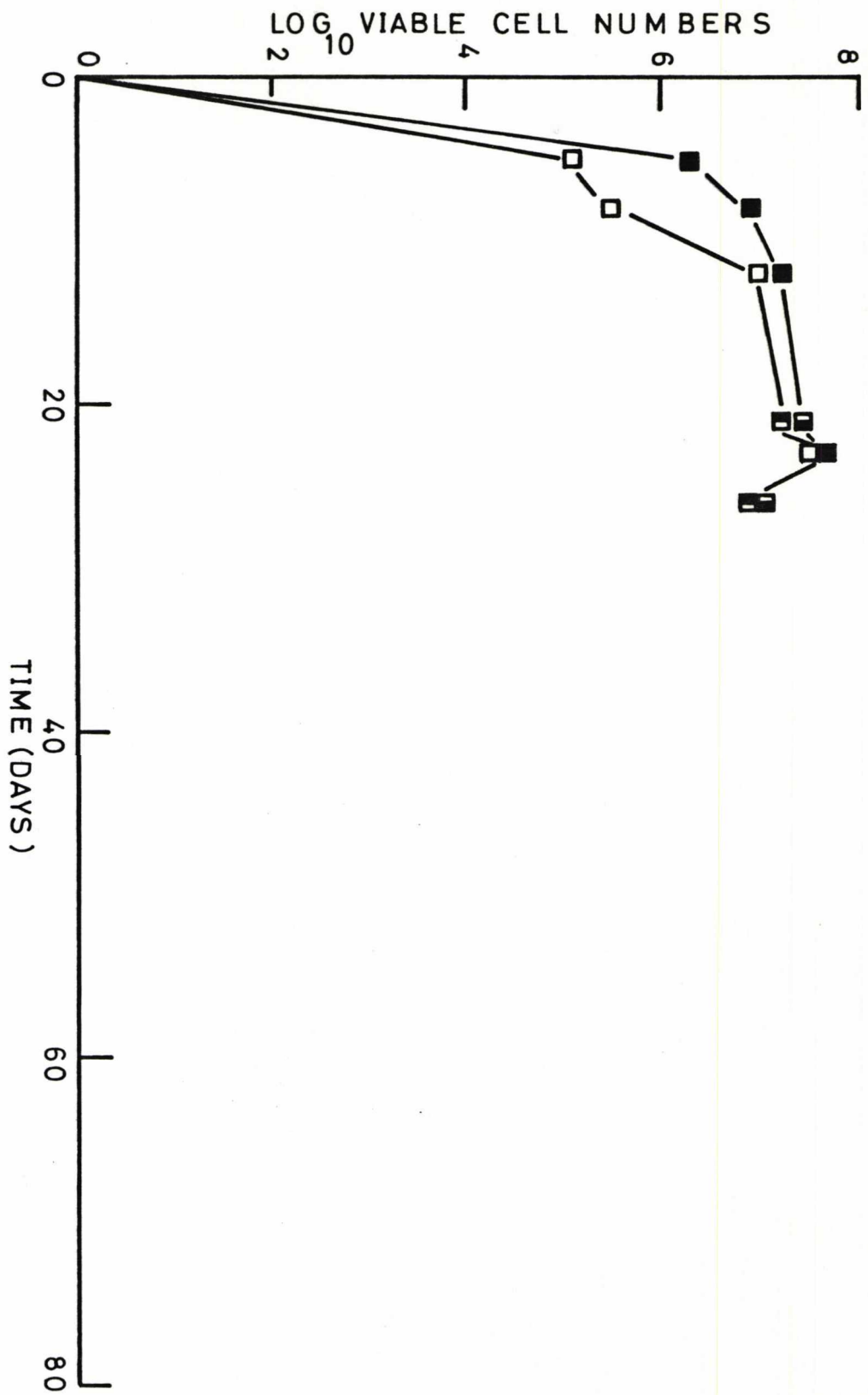


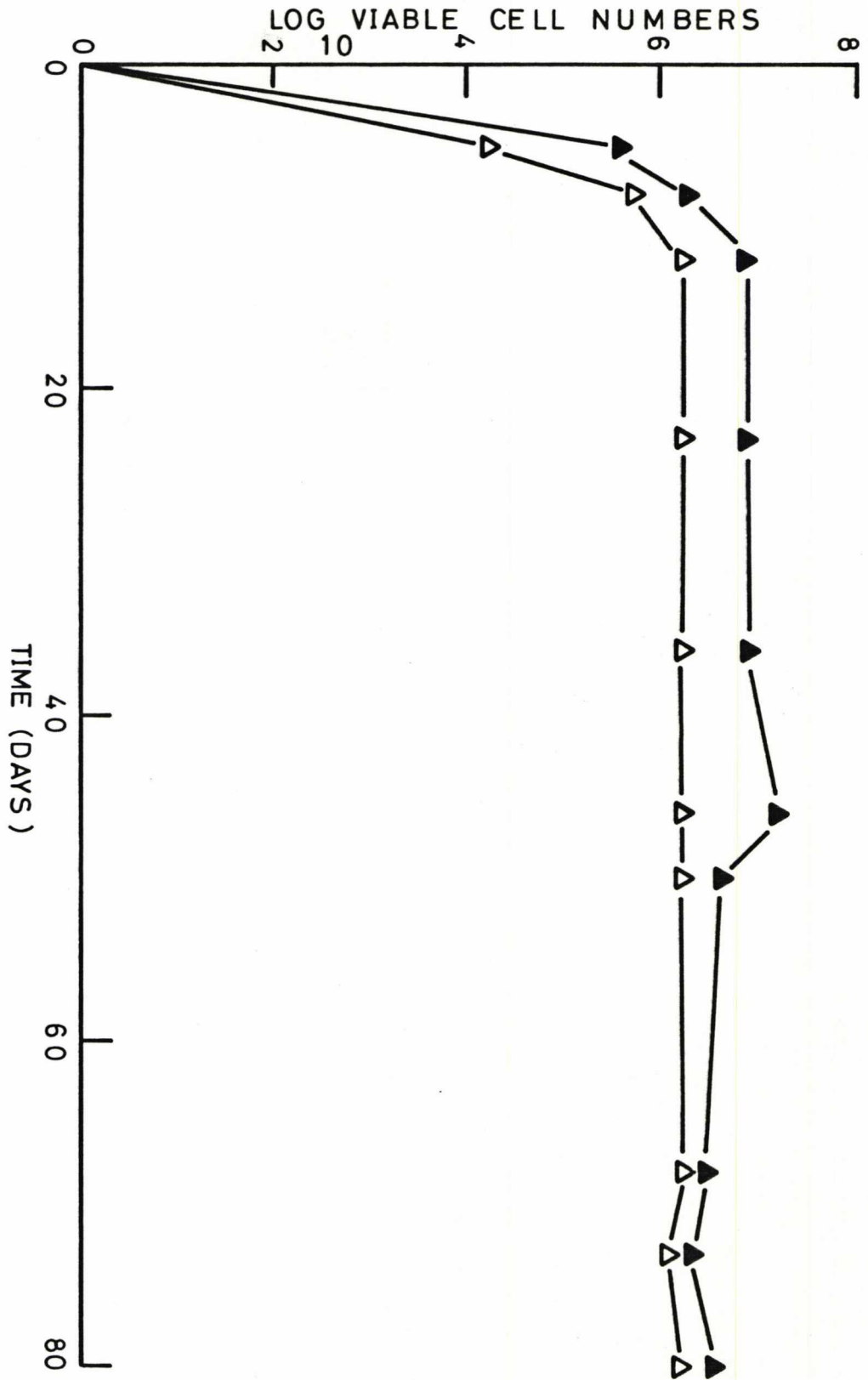


FIGURE    4.12

Log<sub>10</sub> of viable cell numbers for the total hetero-  
-trophic and oil degrading bacterial populations isolated from  
15mm diameter discs of filter coated with synthetic crude during  
80 days of the second winter experiment.

▲ - total heterotrophic population.

△ - oil degrading population.



bacterial population (ca. 100 per filter disc). In both experiments light microscopic observation of recovered filters by phase contrast and with stained samples confirmed the colonization patterns revealed by viable counts. Scanning electron micrographs proved inconclusive in identifying bacteria present on the surface of the oiled filters compared to those taken during early exposure in the summer experiments (Chapter 5, Plate 5.2). The surface of filters coated with Forties crude and sand tar maintained a similar appearance throughout both winter experiments, (Plate 4.1 C & D), however that of the synthetic crude coated filters rapidly returned to the appearance of a non-oiled filter, (Plate 4.1 E). Plates 4.1 A and 4.1 B show a blank filter and an oiled filter respectively, prior to exposure for comparison. The surface of the Forties crude coated filter exposed for 13 days during the first winter experiment (Plate 4.2 A) indicated that the colonizing bacteria may have been embedded in an extracellular matrix, hence obscuring definition. This was investigated by taking scanning electronmicrographs of the suspension formed after bacteria were shaken from the filter surface as previously described (Chapter 3, section 3.9). Glass coverslips were coated with the suspension, fixed and coated in an identical fashion to the sections of filter. The micrograph, (Plate 4.2 B) shows an amorphous material in which individual bacteria could not be defined, although viable counts indicated a cell concentration of  $2.5 \times 10^5$ /ml. Plate 4.2 C shows the surface of the filter from which the bacteria had been removed by shaking.

The temperature and phosphate concentration of the sea water



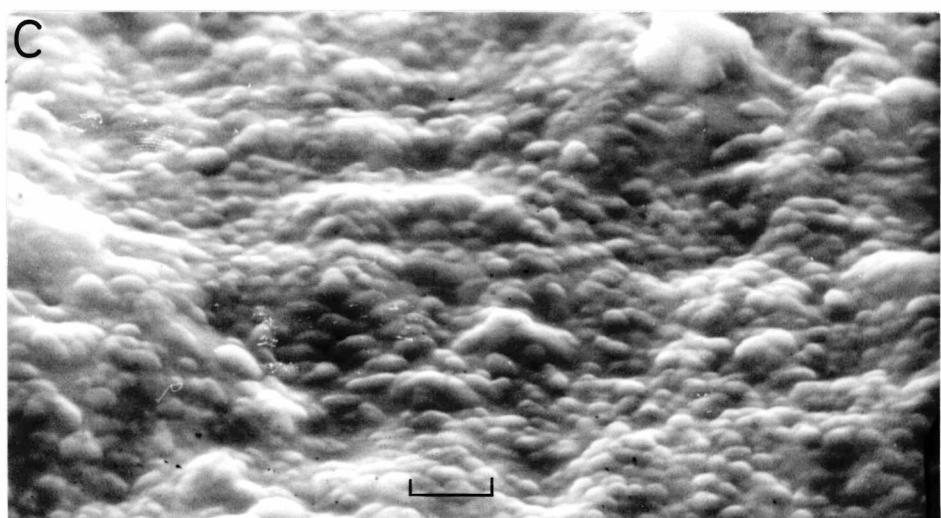
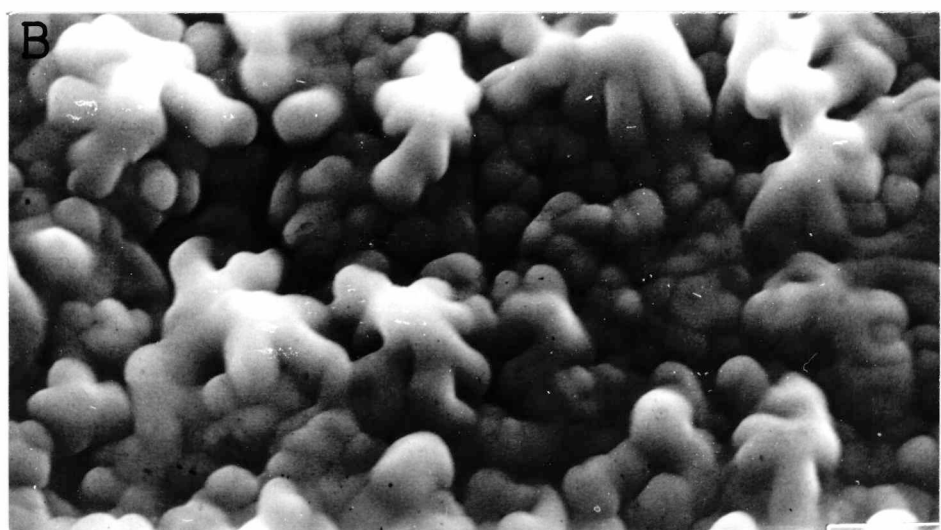
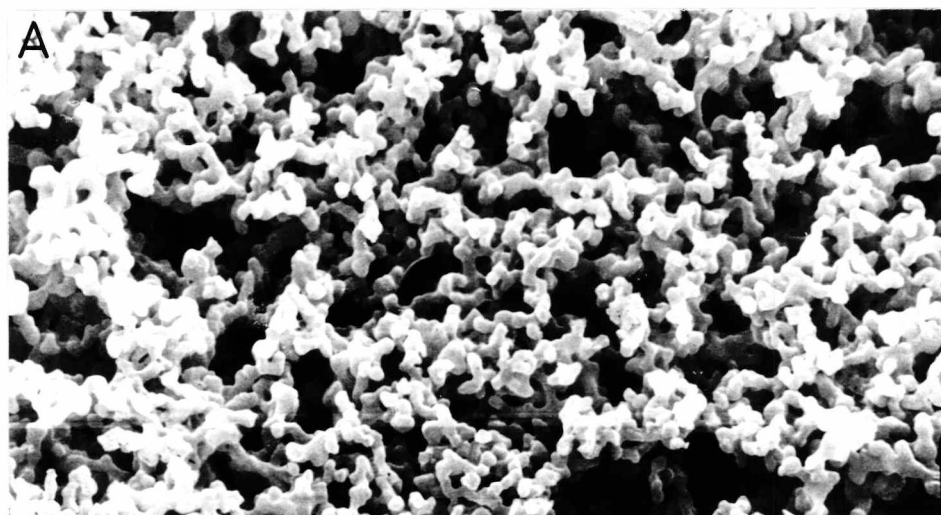
PLATE 4.1

Scanning electronmicrographs of the surface of the filters exposed during the first winter experiment.

- A - uncoated filter.
- B - filter coated with Forties crude prior to exposure.
- C - filter coated with sand tar (28 days exposure).
- D - filter coated with Forties crude (35 days exposure.).
- E - filter coated with synthetic crude (13 days exposure).

Bar marker represents  $4\mu$ .





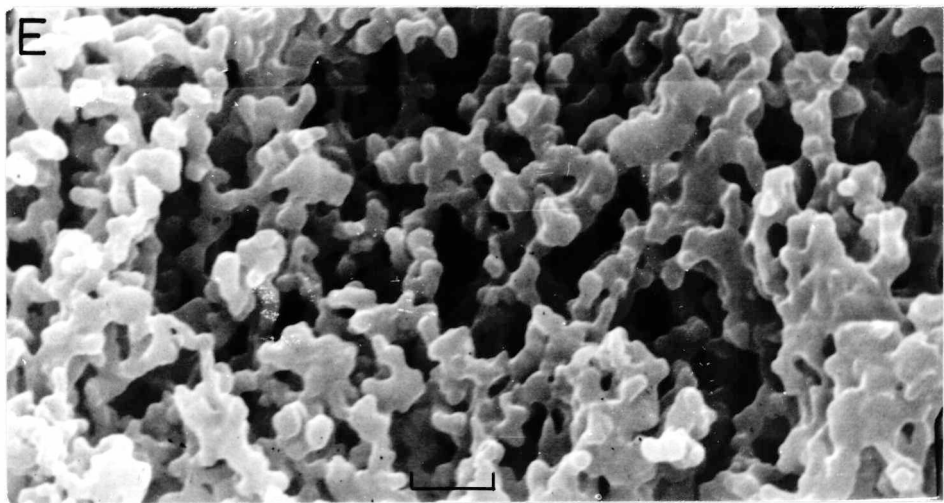
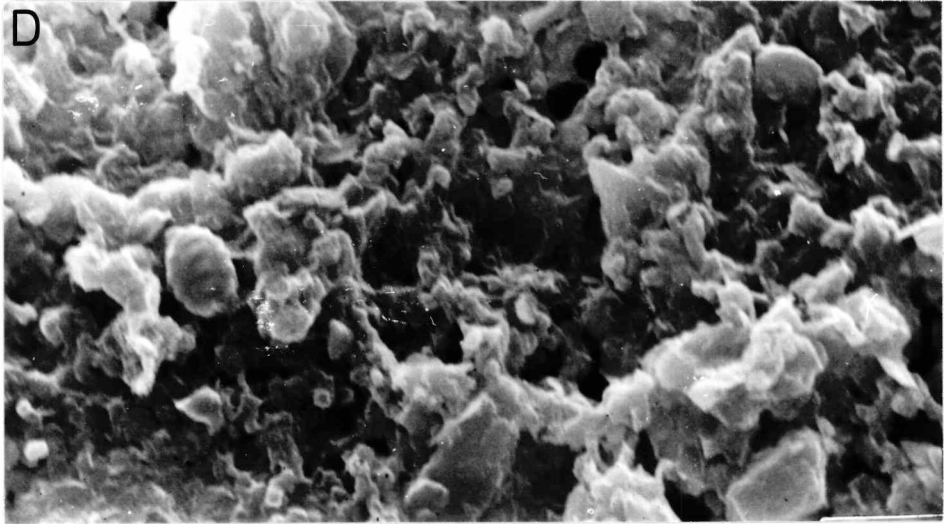
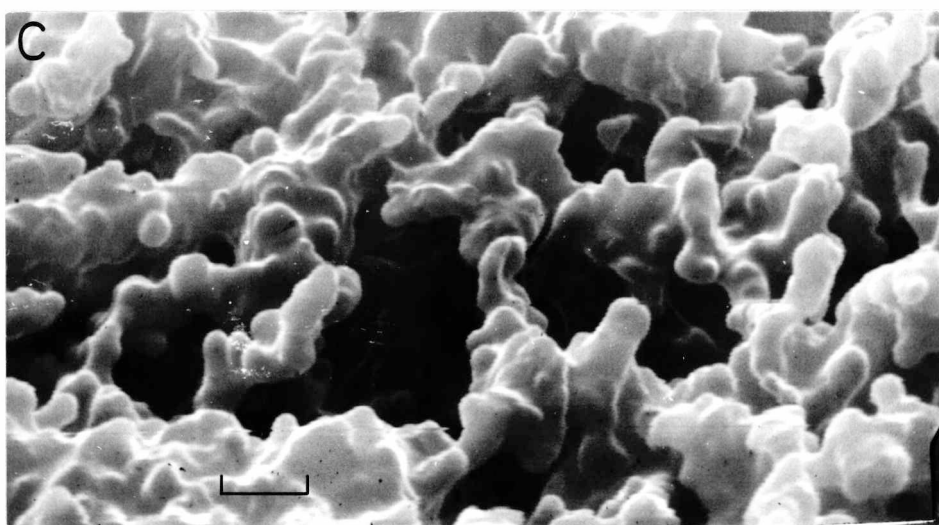
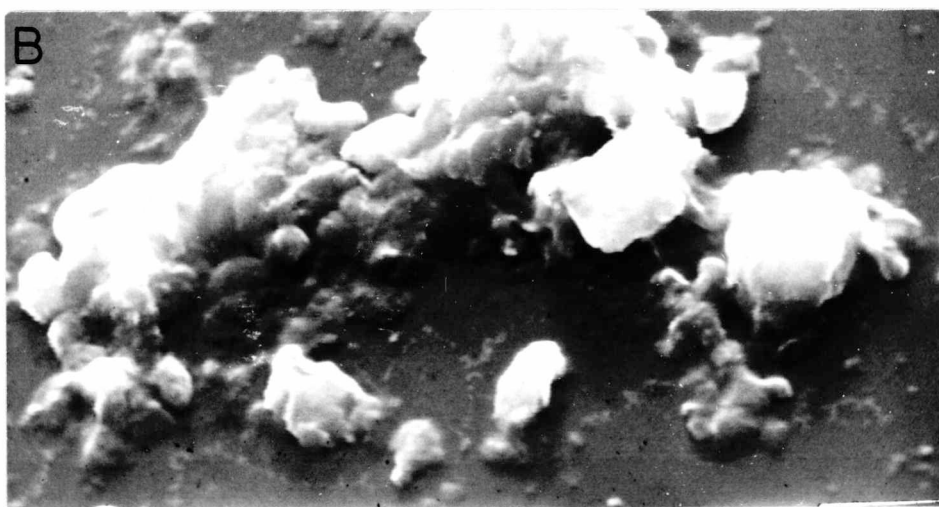
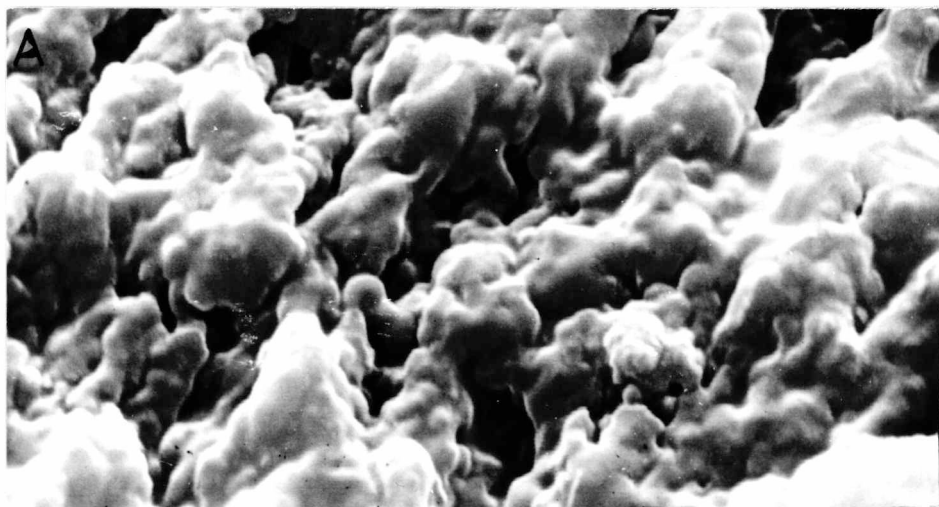


PLATE 4.2

Scanning electronmicrographs of the surface of filters coated with Forties crude after 13 days exposure during the first winter experiment.

- A - filter directly after recovery.
- B - amorphous material produced after the filter was shaken to remove adhering bacteria.
- C - surface of the filter after shaking.

Bar marker represents  $4\mu$ .





varied little during both experiments. During the first experiment the mean temperature was 5°C, (range, 4 - 6°C) and the mean phosphate concentration 4.8µM, (range, 4.5 - 5.0µM). During the second experiment these values were 6°C, (range, 5 - 7°C) and 5µM, (range, 4.5 - 5.2µM) for temperature and phosphate concentration respectively. The total heterotrophic bacterial population isolated from surface water at the times of sampling during the second winter experiment varied between  $5.3 \times 10^3$  -  $5.4 \times 10^4$  viable cells/ml with a mean of  $2.5 \times 10^4$  viable cells/ml. The oil degrading component of this population varied between 0.1 - 10% of the total heterotrophic population.

#### 4.2 DISCUSSION

The pattern of oil loss from the filters was similar during both experimental periods, although the rates of oil loss were uneven, the overall rate and extent of oil loss was in the order, synthetic crude > Forties crude > sand tar. In the case of the synthetic crude the large loss due to non-biological factors found in the laboratory controls could explain the initial, rapid rate of oil loss from the filters under in situ conditions. Loss of oil from the filters during in situ experiments above that found in the controls was attributed to microbial colonization and degradation of the oil, on the basis of the high numbers of oil degrading bacteria isolated from oiled filters in comparison to the non oiled control filters. Coincident with true biodegradation oil may also have been lost due to the formation and dissolution of more polar, intermediate breakdown products,

and by solubilization of undegraded oil mediated by microbially produced surfactants. Unfortunately the system cannot distinguish between these; however the nature of the colonized surface may tend to minimise the latter. The relative rate and extent of biodegradation of the three oils found during both experiments conformed to predictions that can be made on the basis of oil composition. The synthetic crude is the lightest oil with the largest saturate fraction, and this was degraded most rapidly and extensively. At the other extreme, the sand tar is the heaviest oil with the lowest saturate content, and this was degraded least rapidly and extensively. The Forties crude represents an intermediate between the two, which was reflected in the rate and extent of its degradation. The apparent increase in the amount of oil on the synthetic crude filters after the 36th day of the second experiment was coincident with a change in the infrared profile of the oil. For each oil the amount of oil present on identical discs cut from the same filter was very similar, indicating that degradation was occurring uniformly over the filter surface, within the area of discs cut and was not restricted to specific localities.

From the gas chromatographic profiles it can be seen that dissolution and 'wash off' occurring with the controls removes a substantial proportion of the lower molecular weight profile, and that this loss was essentially complete within 7 days, as the profile obtained after 14 days shows no further change. The profiles of the oils exposed at sea reveal a similar loss of the lower molecular weight components, and the reduction of the



higher molecular weight profile to an unresolved basal envelope within a short period of time; this is indicative of biodegradation. It is interesting to note the appearance of high molecular weight peaks in the Forties crude after 12 days of the second experiment; these are similar to peaks found at the same time during degradation of this oil in laboratory studies.

The pattern of microbial colonization of the oiled filters was similar in both experiments. Colonization was essentially complete by the time of first sampling, (13 days) in the first experiment; thereafter the number of oil utilizing bacteria isolated from each oil, per disc of filter, remained relatively constant. The total number of bacteria supported by each oil was similar, and the relative numbers of the three bacterial strains comprising the total, exhibited similar colonization patterns for each oil. Strain 3 (unidentified), consistently displayed a slower rise to the steady maximum numbers attained by strains 1 and 2. The recovery of samples at 5, 8 and 12 days during the second experiment enabled a more detailed examination of the initial colonization period. For the Forties and synthetic crude, cell numbers rose rapidly during the first 5 days and then more slowly between the 5th and 12th days to reach a broad maximum at this time. In the case of organisms isolated from the sand tar, this maximum was established between the 12th and 23rd days. During the same period the numbers isolated from the Forties crude were higher than for the other two oils. Unfortunately loss of the equipment precluded determination of whether these high numbers were maintained, or whether they returned to similar

values found for the other oils. The establishment of replacement samples and equipment enabled recovery of samples after a further 21 and 26 days. The cell numbers isolated at these times are of the same high order as those found prior to the loss of the first equipment. Similar numbers of bacteria were isolated from each disc for each oil during both experiments, although the degradation rates of the oils were different. This suggests that the numbers may be dependent, not upon the type of oil, but on the surface area available for colonization, and the extent to which bacteria may be removed from the filters by 'wash off' or protozal grazing.

In the case of the synthetic crude, the reversion of the filter surface to a non-oiled appearance during early exposure confirms the laboratory control findings that a substantial amount of oil was lost from the filters by non-biological processes.



CHAPTER 5

IN SITU EXPERIMENTS UNDER SUMMER CONDITIONS

IN SITU EXPERIMENTS UNDER SUMMER CONDITIONS5.1 RESULTS

Two in situ experiments of 17 and 72 days respectively were performed under summer conditions, (1/5/1977 - 17/5/1977 and 9/5/1978 - 10/7/1978). The procedure was identical to that of the in situ winter experiments and the Queenborough Spit buoy was again used for attachment.

Samples were recovered at intervals during the experimental periods and the amount of oil quantified (Chapter 3, section 3.8). The results for the first and second experiments are shown in Figures 5.1 and 5.2 respectively. Gas chromatographic profiles of the synthetic crude and Forties crude during early exposure in both experiments are shown in Figures 5.3 and 5.4 respectively. The extent of non-biological oil loss from the filters was estimated by laboratory controls at 13°C and these gave closely similar results to those carried out at 4°C i.e. 50, 12, and 8% loss for the synthetic crude, Forties crude and sand tar respectively within 7 days, but little loss thereafter.

At the conclusion of the first experiment, (17 days) oil was extracted from the remaining filters and subjected to column chromatography. The fractional composition of these oils is shown in Figure 5.5; insufficient synthetic crude was recovered to permit accurate analysis. The fractional composition of the three oils after 'topping' and after leaching in sterile sea water are also shown for comparison. During the second experiment, oil was recovered at 10, 36 and 72 days for fractional analysis, the results of which are shown in Figure 5.6;

FIGURE 5.1

Percentage of oil remaining on oiled filter discs  
of 15mm diameter during 17 days of the first summer experiment.

- - sand tar.
- - Forties crude.
- △ - synthetic crude.

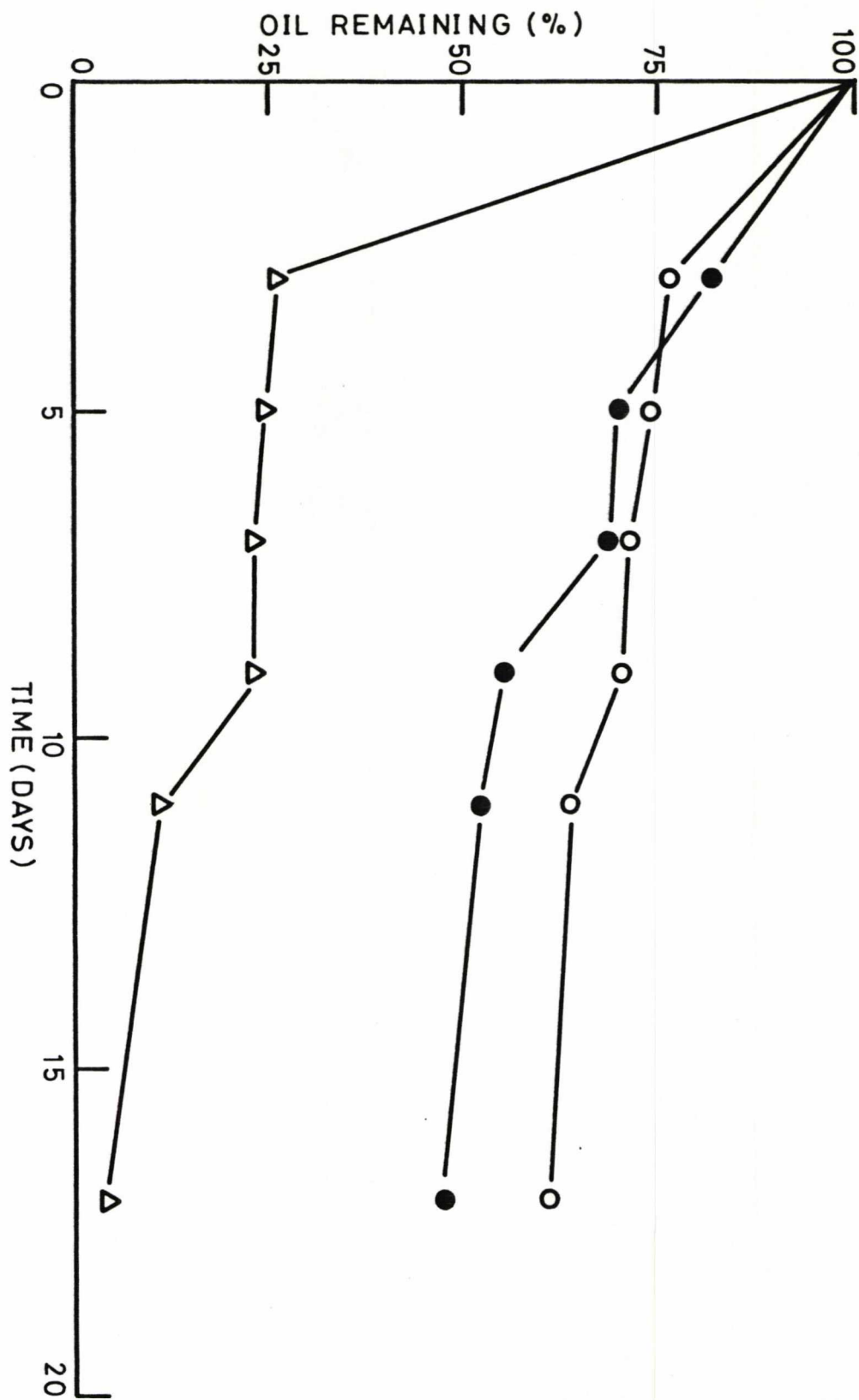




FIGURE 5.2

Percentage of oil remaining on oiled filter discs  
of 15mm diameter during 72 days of the second summer experiment.

- - sand tar.
- - Forties crude.
- △ - synthetic crude.

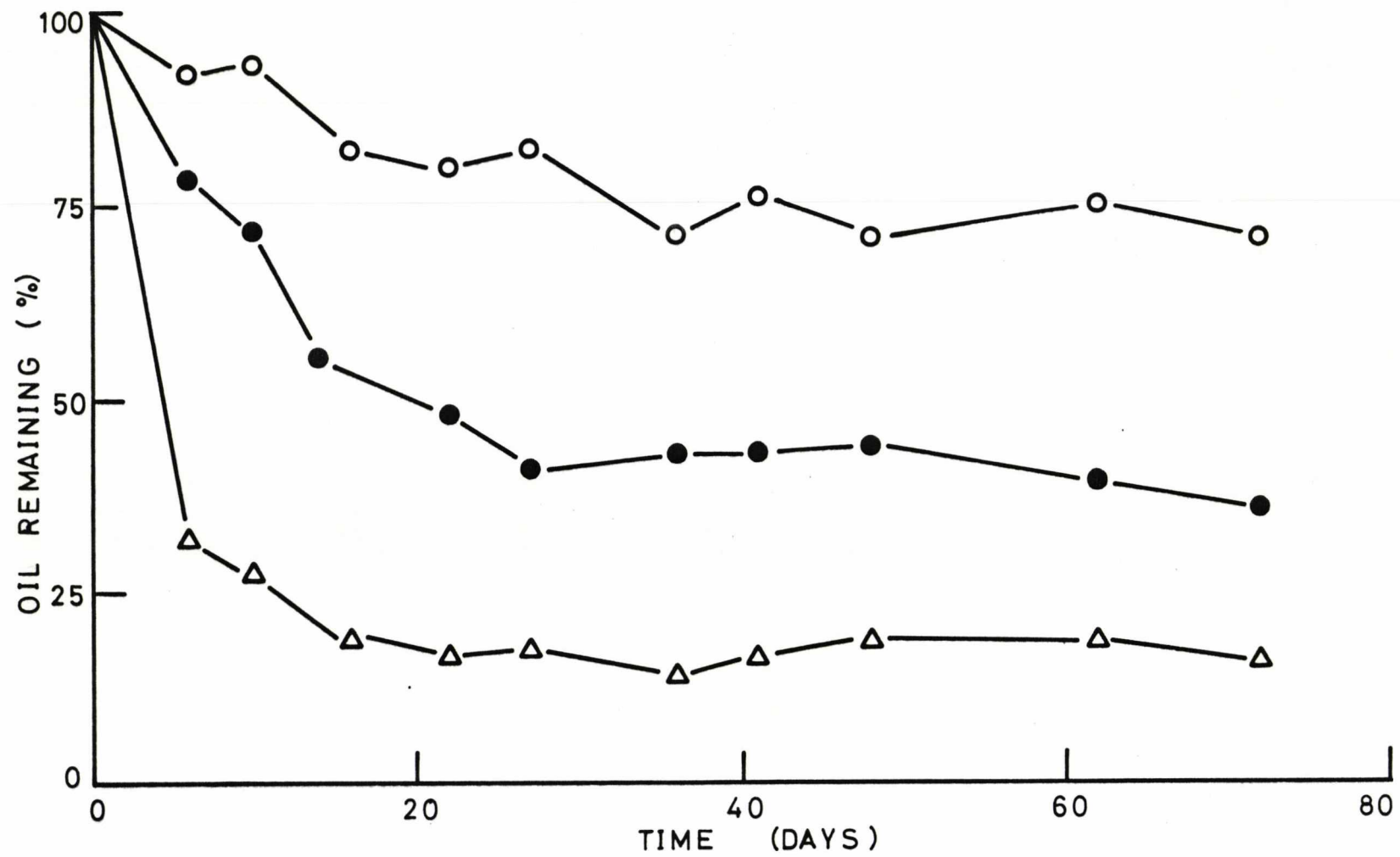


FIGURE 5.3

Gas chromatographic profiles of the synthetic crude recovered at intervals during the summer experiments.

- A - fresh oil.
- B - topped oil.
- C - oil leached in sterile sea water for 7 days.
- D - oil leached in sterile sea water for 14 days.
- E - oil recovered after 3 days (1<sup>st</sup> experiment).
- F - oil recovered after 6 days (2<sup>nd</sup> experiment).

Temperature increases from right to left across the profile. All profiles were obtained under identical conditions and are directly comparable.

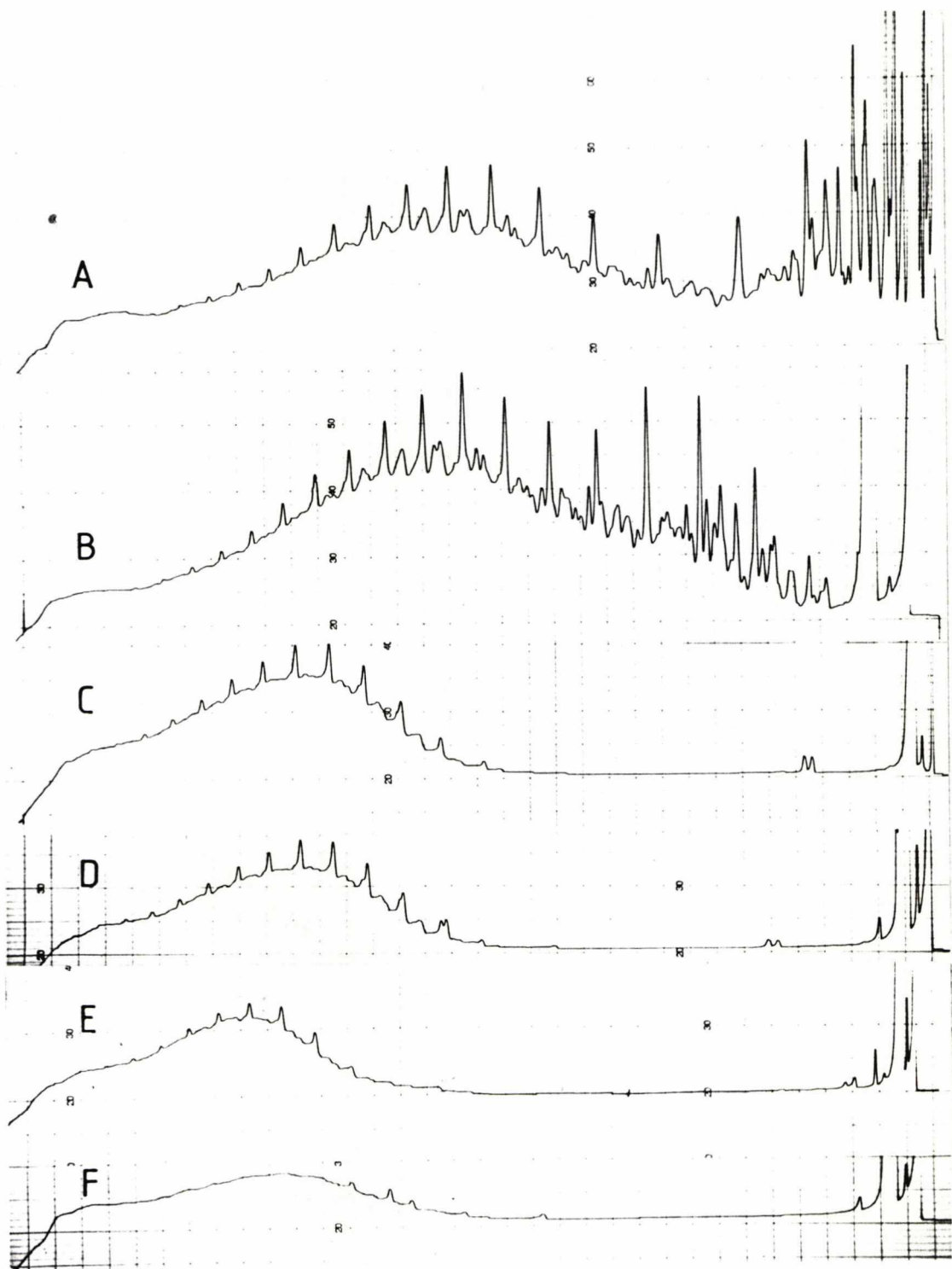




FIGURE 5.4

Gas chromatographic profiles of the Forties crude recovered at intervals during the summer experiments.

- A - fresh oil.
- B - topped oil.
- C - oil leached in sterile sea water for 7 days.
- D - oil leached in sterile sea water for 14 days.
- E - oil recovered after 3 days (1<sup>st</sup> experiment).
- F - " " " 6 days (2<sup>nd</sup> experiment).
- G - oil recovered after 10 days (2<sup>nd</sup> experiment).

Temperature increases from right to left across the profile. All profiles were obtained under identical conditions and are directly comparable.

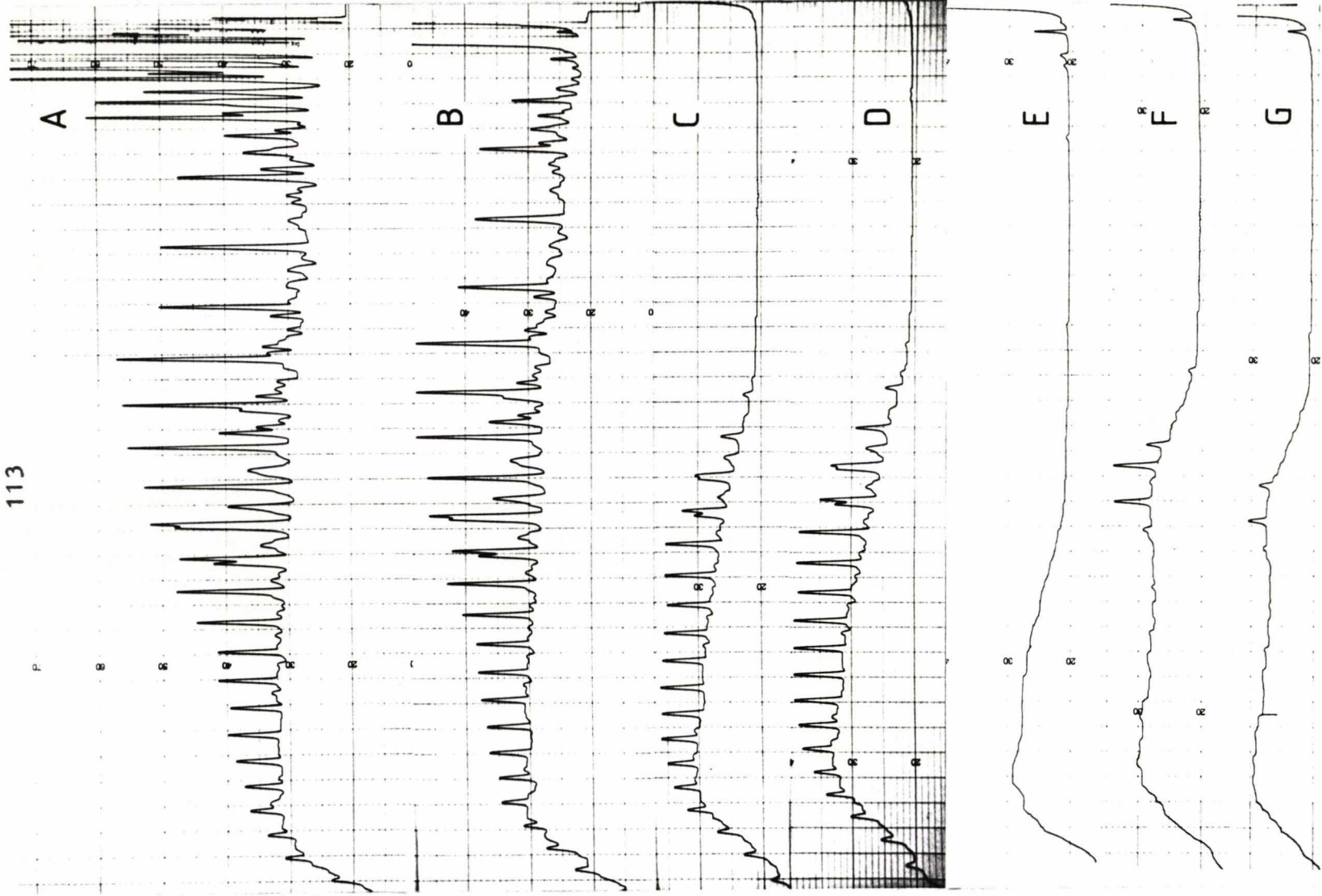


FIGURE 5.5

Fractional composition of oils prior to exposure in in situ experiments and of oil that had been exposed at sea for 17 days during the first summer experiment.

- A - topped synthetic crude.
- B - synthetic crude leached in sterile sea water for 7 days.
- C - topped Forties crude.
- D - Forties crude leached in sterile sea water for 7 days.
- E - Forties crude after 17 days exposure.
- F - topped sand tar.
- G - sand tar leached in sterile sea water for 7 days.
- G - sand tar after 17 days exposure.

s - saturate fraction ;      m - monoaromatic fraction ;  
d - diaromatic fraction ;    p - polycyclic- aromatic,  
nitrogen, sulphur, and oxygen containing compound  
fraction.

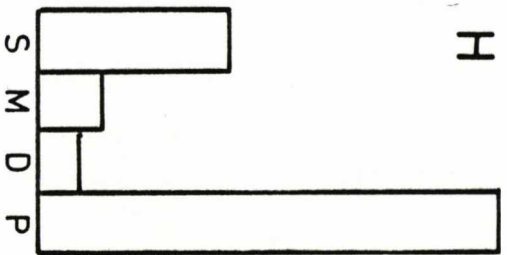
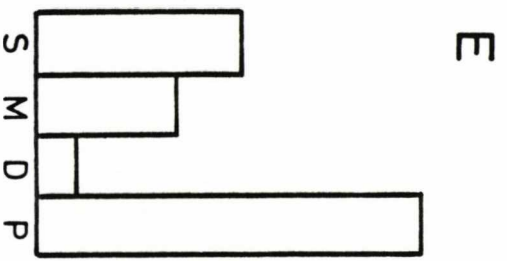
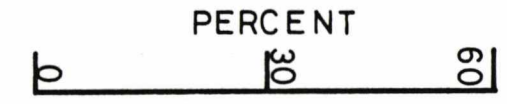
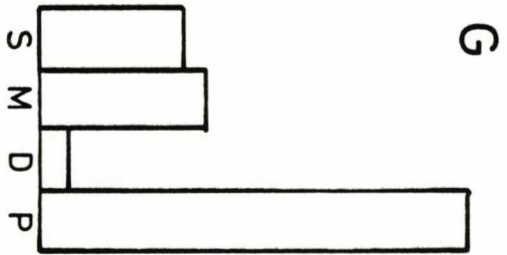
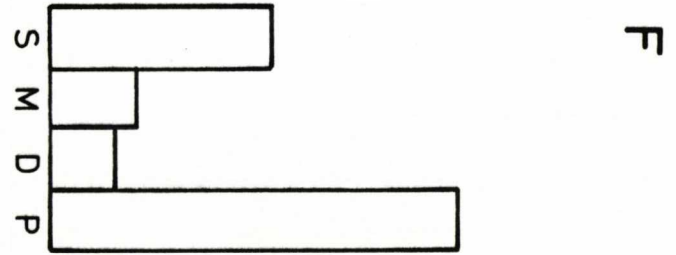
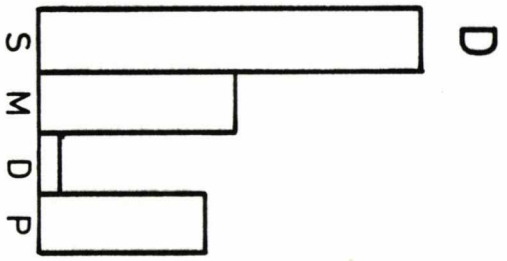
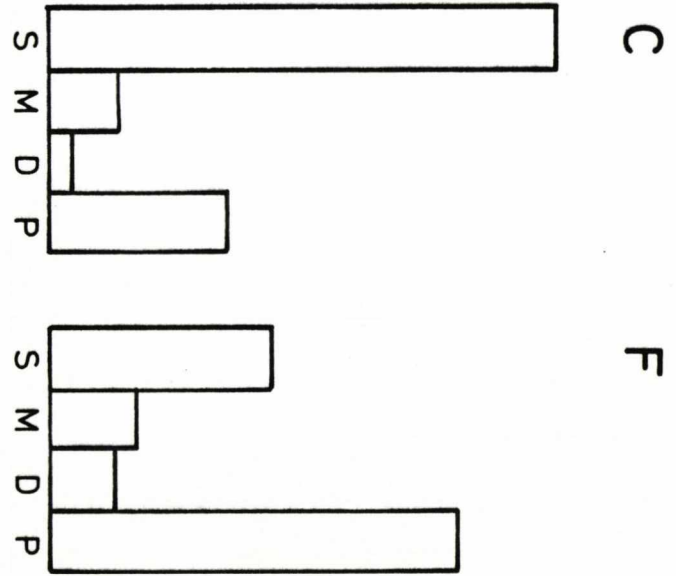
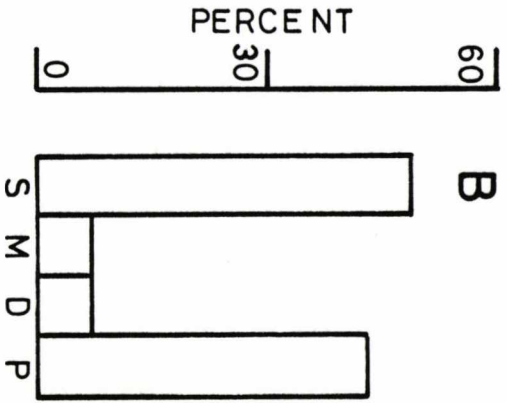
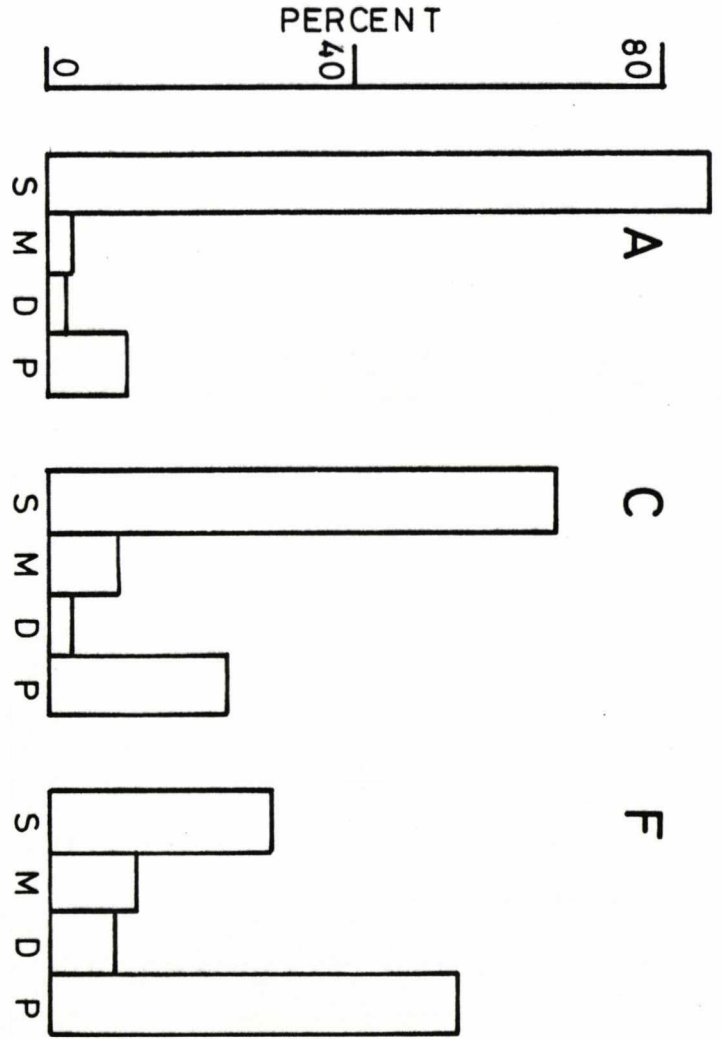




FIGURE 5.6

Fractional composition of oils recovered at intervals during 72 days of the second summer experiment.

Forties crude: A - 10 day exposure.

B - 36 day exposure.

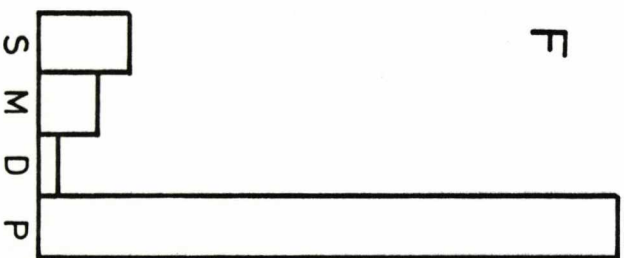
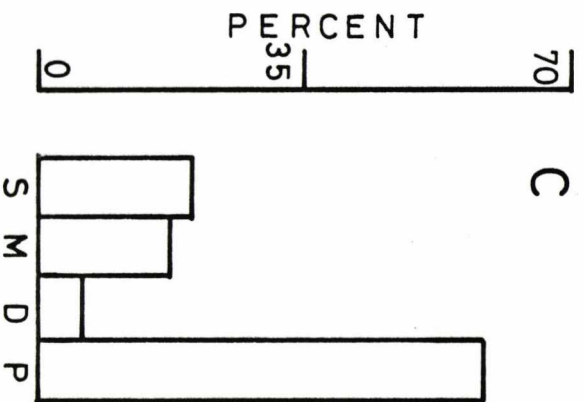
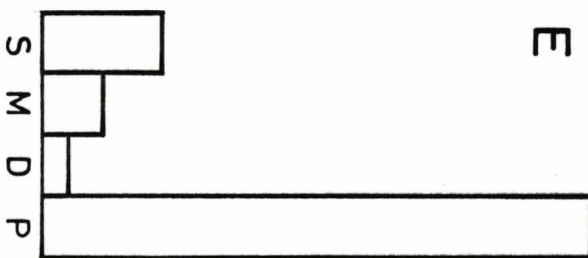
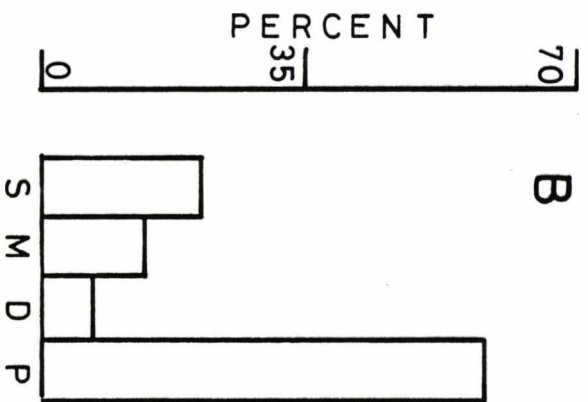
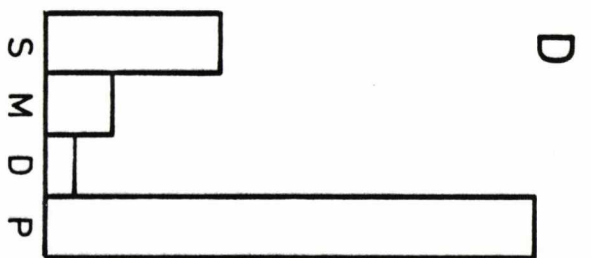
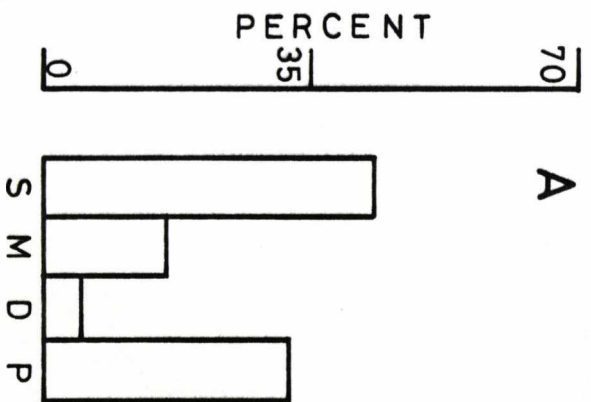
C - 72 day exposure.

Sand tar: D - 10 day exposure.

E - 36 day exposure.

F - 72 day exposure.

s - saturate fraction ; m - monoaromatic fraction ;  
d - diaromatic fraction ; p - polycyclic- aromatic ;  
nitrogen, sulphur, and oxygen compound containing fraction.



insufficient synthetic crude was recovered to permit accurate analysis.

As in the winter experiments the blank control filters did not show any detectable adsorption of oil from the environment.

Throughout the first experiment microbial colonization of the oiled filters was determined by the same method used during the first winter experiment; i.e. direct plating onto sea water agar and then transfer to oil agar to test for oil utilizing ability. Figure 5.7 shows the total oil utilizing microbial population isolated from each oil. Three separate bacterial strains distinguished on the basis of colony type and morphology were isolated from each oil as in the first winter experiment; their relative numbers for each oil are shown in Figures 5.8, 5.9 and 5.10. During the second experiment microbial colonization was estimated by direct plating onto sea water agar and oil agar; Figure 5.11 shows the total numbers of oil utilizers isolated from the three oils. Figures 5.12, 5.13 and 5.14 show the total numbers of oil utilizers and heterotrophs for each oil. As under winter conditions, throughout both experiments there was no evidence for filamentous fungi and only a small number of non oil-utilizing yeasts were isolated. The blank control filters supported the same small bacterial population (ca. 100 per filter disc). Light microscope observations confirmed the colonization patterns revealed by viable counts. Unfortunately, fouling by macrobiota during the latter stages of the second summer experiment hampered observation. Plate 5.1

FIGURE 5.7

Log<sub>10</sub> of viable oil degrading bacterial cell numbers isolated from 15mm diameter discs of oiled filter during 17 days of the first summer experiment.

- O - isolates from sand tar.
- - isolates from Forties crude.
- △ - isolates from synthetic crude.



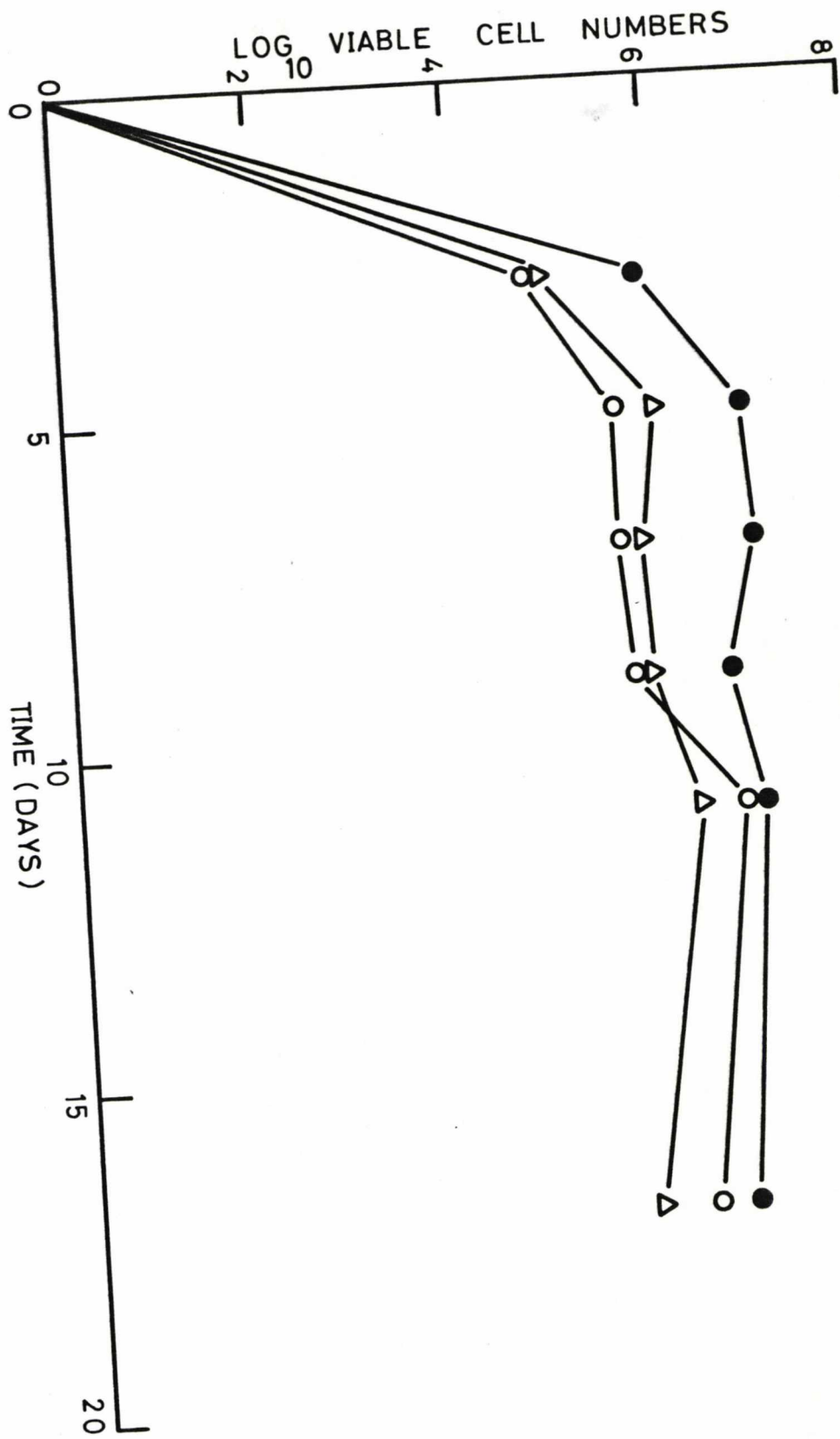


FIGURE 5.8

$\text{Log}_{10}$  of viable bacterial cell numbers of the three strains comprising the total oil degrading population isolated from 15mm diameter discs of filter coated with sand tar during 17 days of the first summer experiment.

- ∇ - strain 1.
- - strain 2.
- - strain 3.

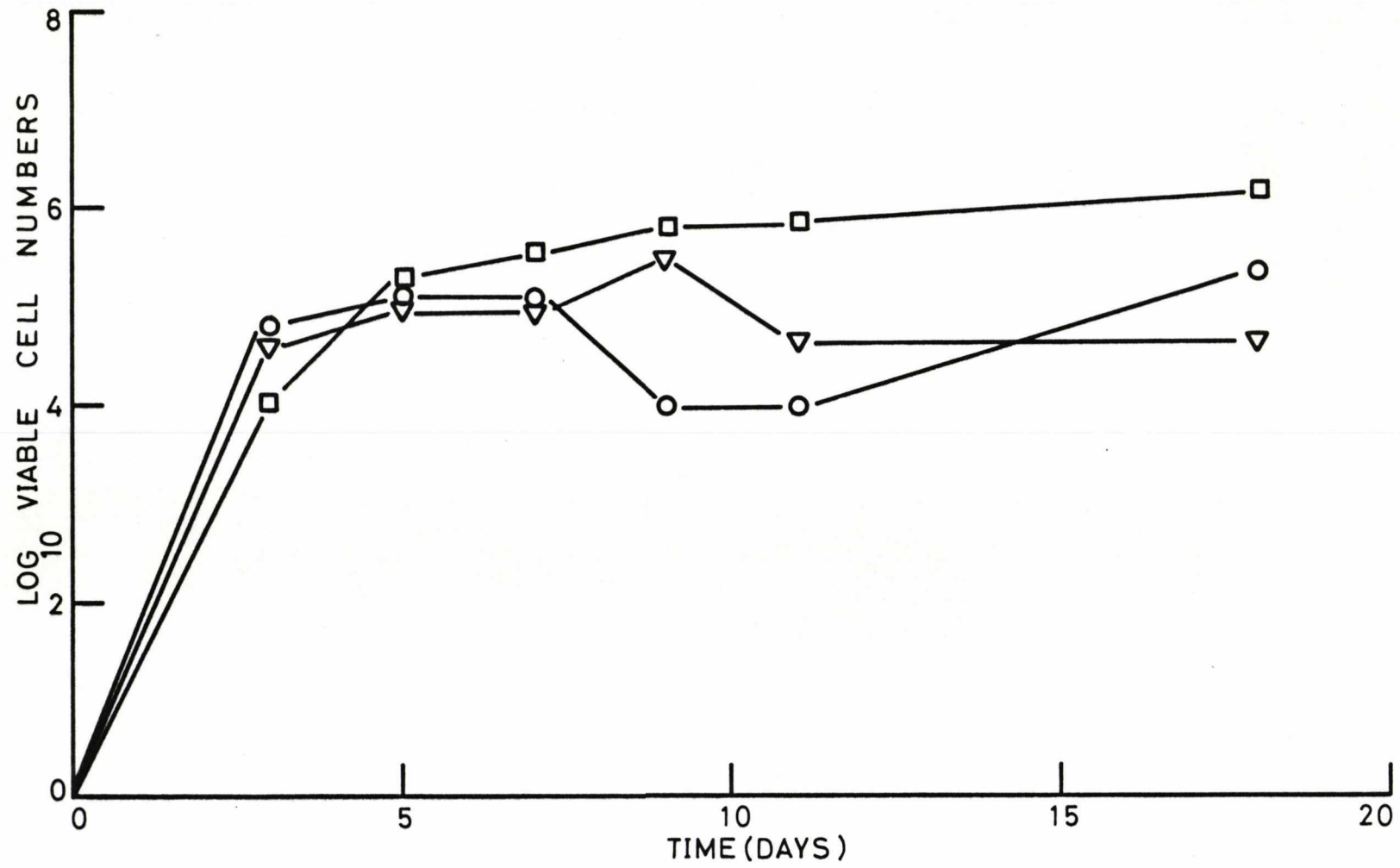


FIGURE 5.9

Log<sub>10</sub> of viable bacterial cell numbers of the three strains comprising the total oil degrading population isolated from 15mm diameter discs of filter coated with Forties crude during 17 days of the first summer experiment.

- ▽ - strain 1.
- - strain 2.
- 0 - strain 3.



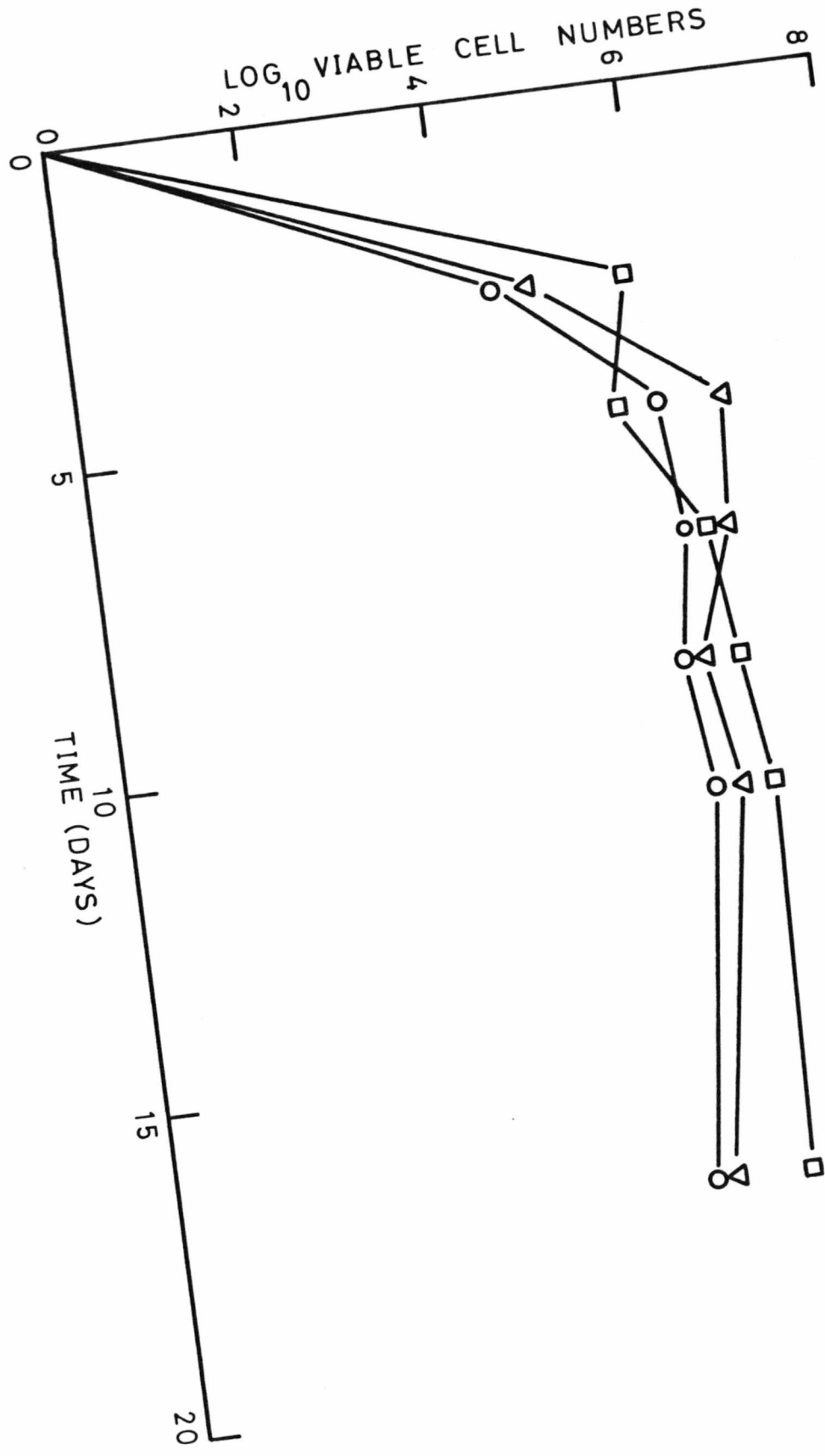


FIGURE 5.10

$\text{Log}_{10}$  of viable bacterial cell numbers of the three strains comprising the total oil degrading population isolated from 15mm diameter discs of filter coated with synthetic crude during 17 days of the first summer experiment.

- ▽ - strain 1.
- - strain 2.
- - strain 3.

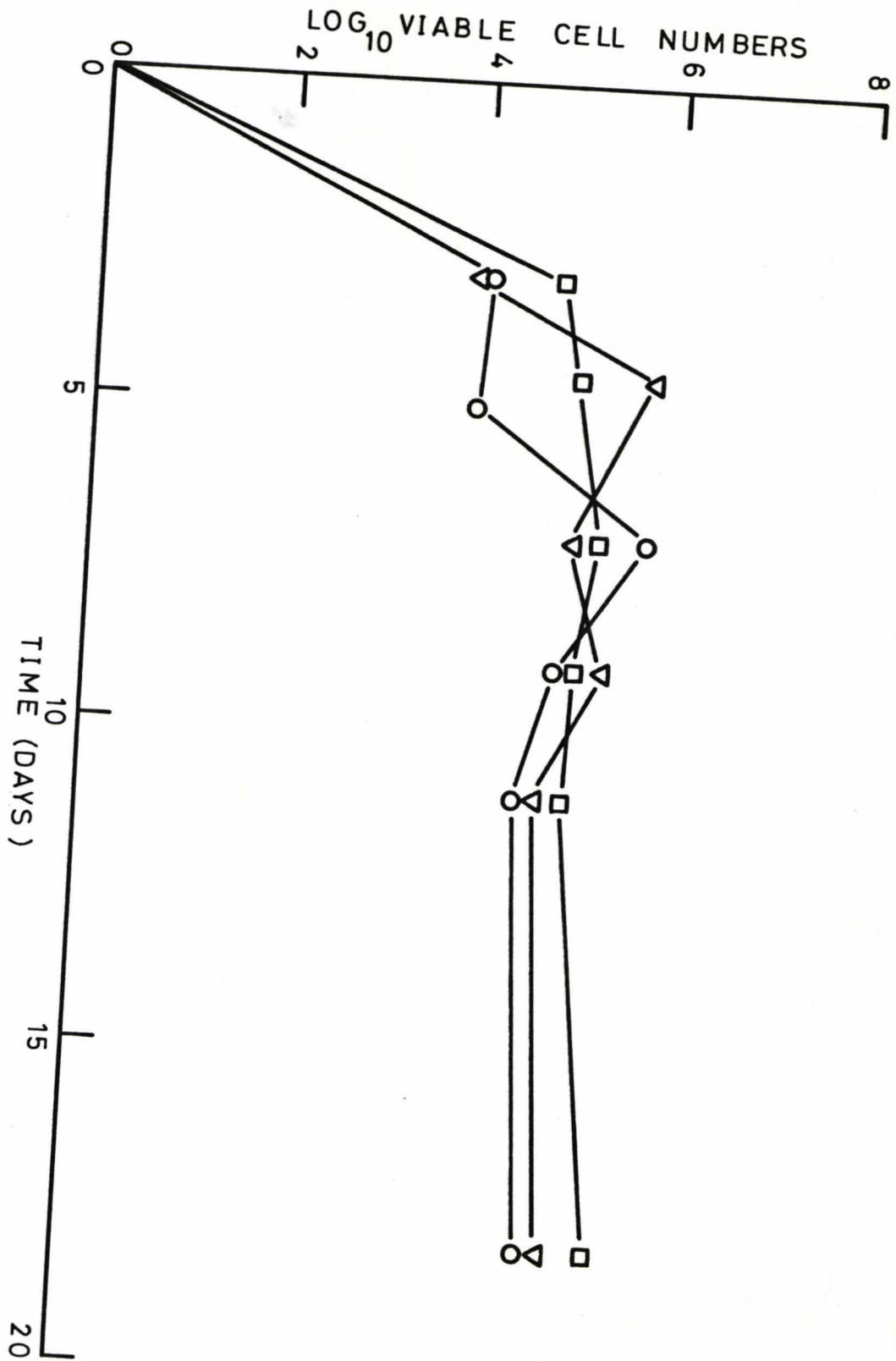


FIGURE 5.11

Log<sub>10</sub> of viable oil degrading bacterial cell numbers isolated from 15mm diameter discs of oiled filter during 72 days of the second summer experiment.

- O - isolates from sand tar.
- - isolates from Forties crude.
- △ - isolates from synthetic crude.



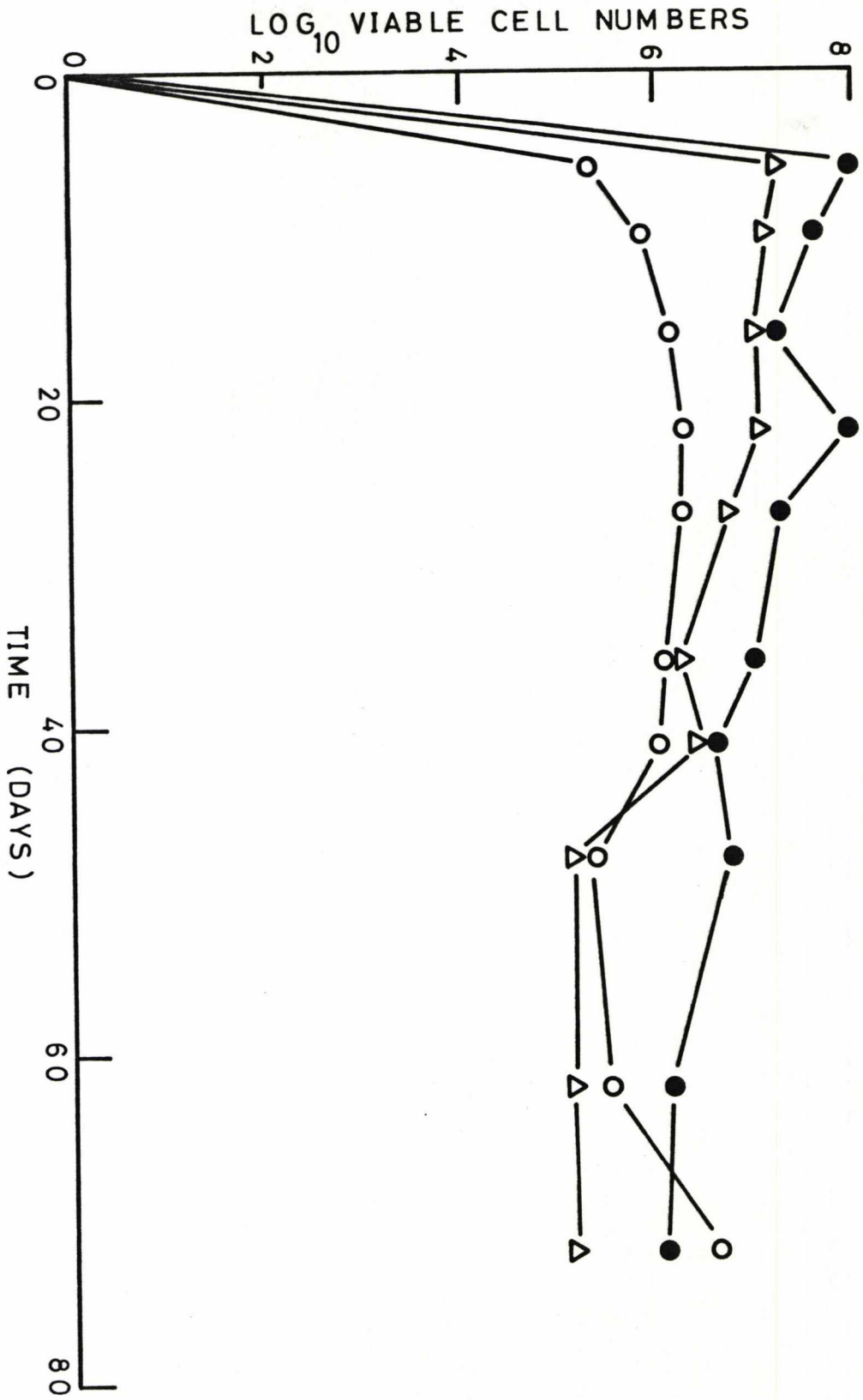


FIGURE 5.12

Log<sub>10</sub> of viable bacterial cell numbers for the total heterotrophic and oil degrading populations isolated from 15mm diameter discs of filter coated with sand tar during 72 days of the second summer experiment.

- - total heterotrophs.
- - oil degraders.

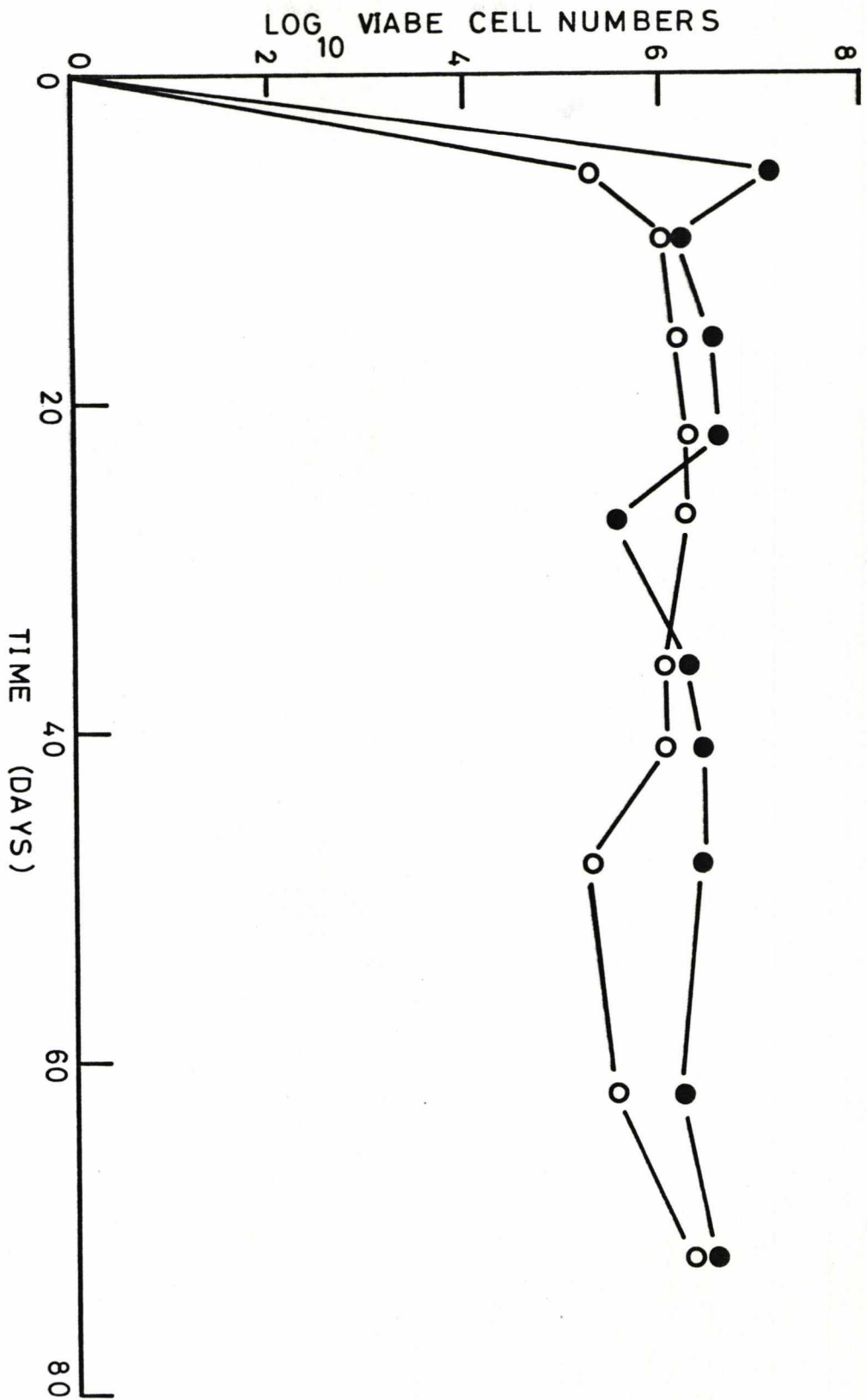


FIGURE 5.13

Log<sub>10</sub> of viable bacterial cell numbers for the total heterotrophic and oil degrading populations isolated from 15mm diameter discs of filter coated with Forties crude during 72 days of the second summer experiment.

- - total heterotrophs.
- - oil degraders.



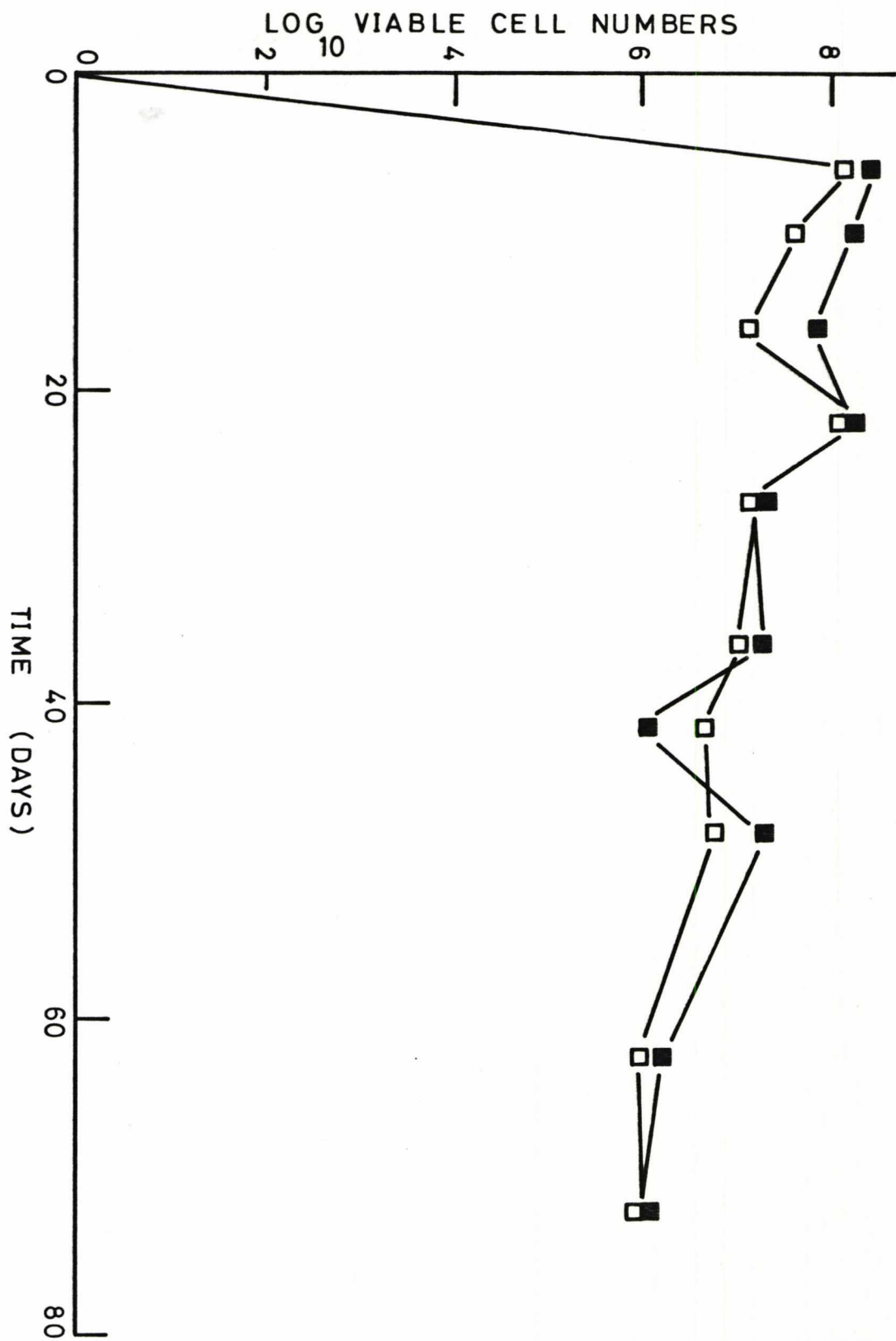


FIGURE 5.14

Log<sub>10</sub> of viable bacterial cell numbers for the total heterotrophic and oil degrading populations isolated from 15mm diameter discs of filter coated with synthetic crude during 72 days of the second summer experiment.

- ▲ - total heterotrophs.
- △ - oil degraders.

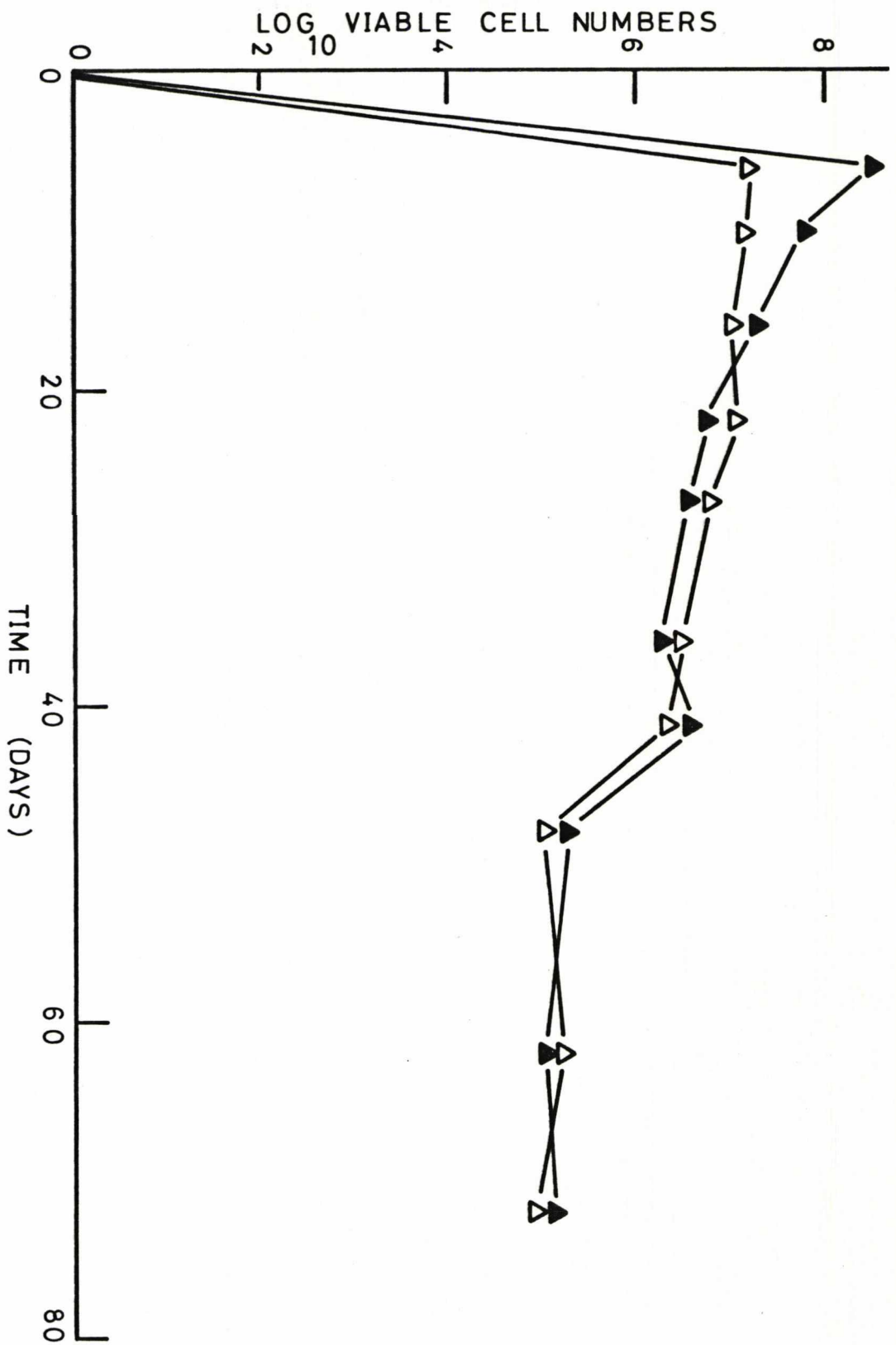


PLATE 5.1

Some examples of scanning electronmicrographs of macro- and micro-colonizers found on the surface of the Forties crude coated filter after 72 days exposure during the second summer experiment.

- A. - barnacle, Balanus spp.
- B. - marine phytoplankton, Coscinodiscus spp.

Bar marker represents 400 $\mu$  and 4 $\mu$  in A and B respectively.





shows some examples of macrocolonizers. Scanning electronmicrographs of the oiled filter surface after exposure during both summer experiments generally proved inconclusive in revealing the presence of bacteria. However the surface of the Forties crude coated filter after a 3 day exposure during the first summer experiment was exceptional in clearly demonstrating bacteria on the filter surface, (Plate 5.2). The surface of the synthetic crude coated filters exhibited a rapid return to a non-oiled appearance during early exposure in both summer experiments as was the case in the winter experiments. The appearance of the 15 mm diameter discs cut from filters coated with Forties crude or sand tar recovered at intervals during the second summer experiment is shown in Plate 5.3. The gas chromatographic profiles of sand tar are shown in Figure 5.15b, this shows the absence of peaks above an unresolved basal envelope. Figure 5.15a shows the gas chromatographic profile of a mixture of n-alkanes produced under identical conditions as those for oil samples.

The temperature and phosphate concentration of the sea water varied little during both experiments. During the first experiment the mean temperature was  $12^{\circ}\text{C}$ , (range,  $11 - 13^{\circ}\text{C}$ ), and the mean phosphate concentration  $5.0\mu\text{M}$ , (range,  $4.8 - 5.2\mu\text{M}$ ). During the second experiment these values were  $14^{\circ}\text{C}$ , (range,  $13 - 15^{\circ}\text{C}$ ) and  $4.3\mu\text{M}$ , (range,  $3.6 - 5.3\mu\text{M}$ ) for temperature and phosphate concentration respectively. The salinity of the sea water taken at the time of sampling throughout both experiments and at intervals over a period including low and

PLATE 5.2

Scanning electronmicrographs of the surface of filters coated with Forties crude recovered during the first summer experiment.

A&B - sample recovered after 3 days.

C - sample of control filter (no coating oil) recovered after 3 days.

Bar marker represents 4 for B & C and 10 for A.



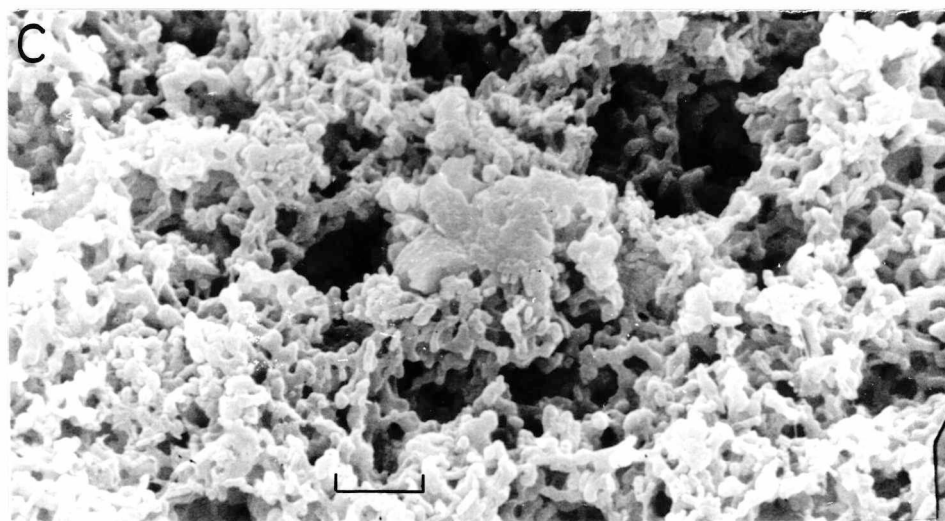
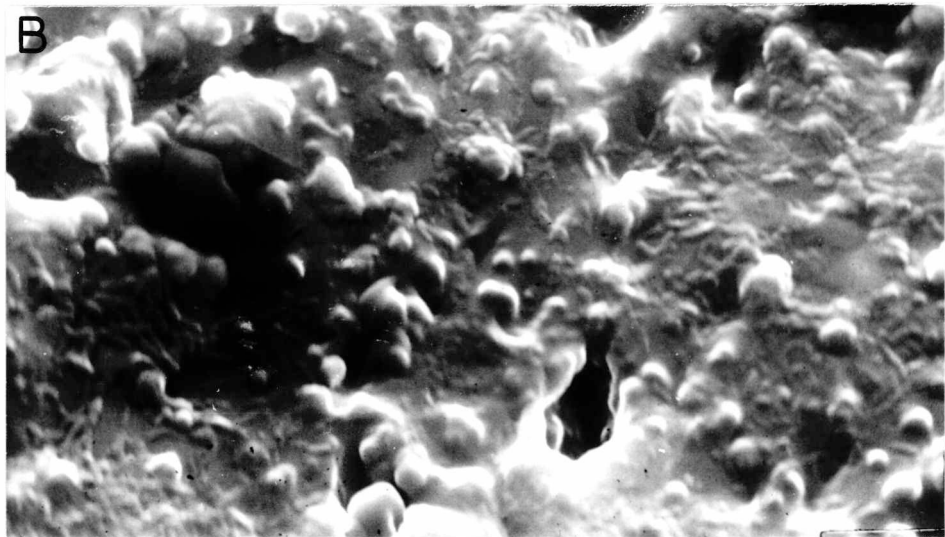
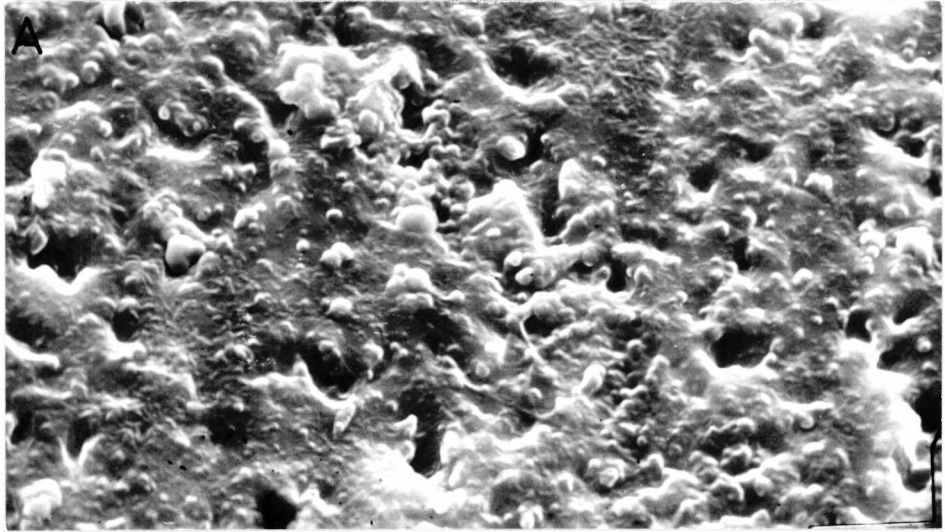




PLATE 5.3

The appearance of duplicate discs (15mm diameter) cut from filters coated with either Forties crude or sand tar recovered at intervals during the second summer experiment.

A - filters coated with sand tar.

B - filters coated with Forties crude.

Legend: figures represent the time (days) after which samples were recovered.

6	10	16
22	27	36
41	48	62
	72	

Bar marker represents 15mm.

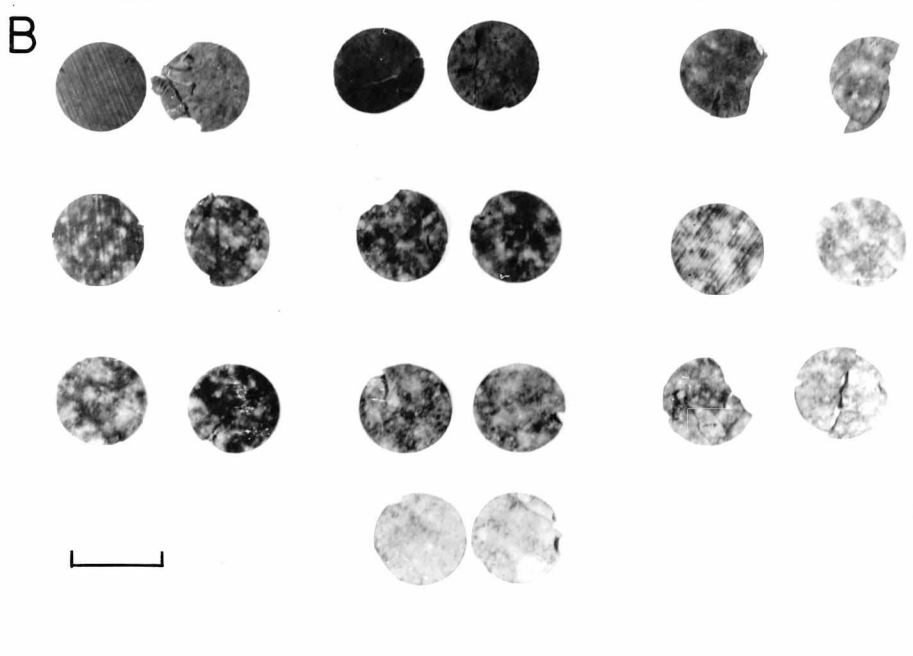
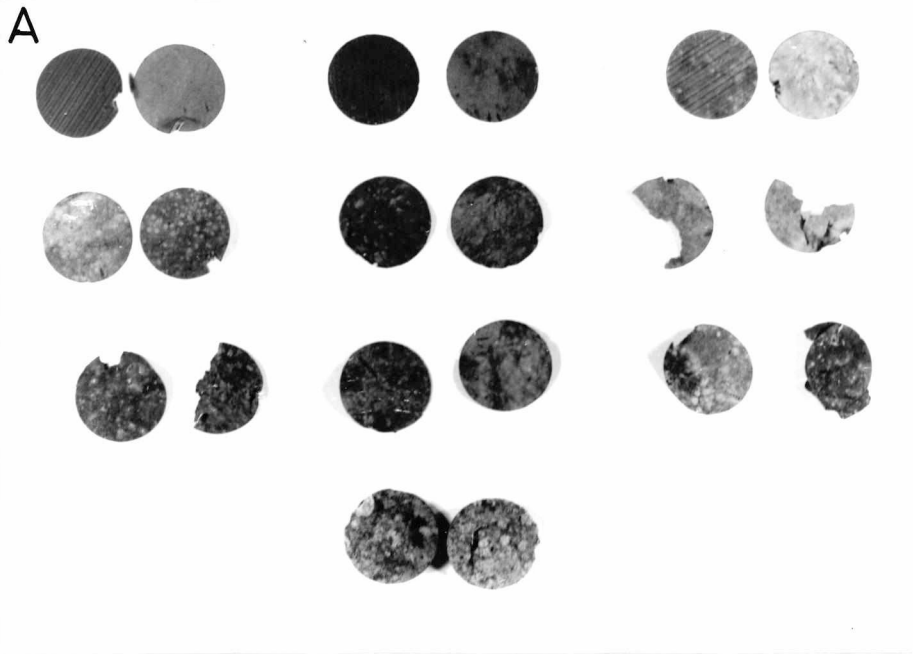
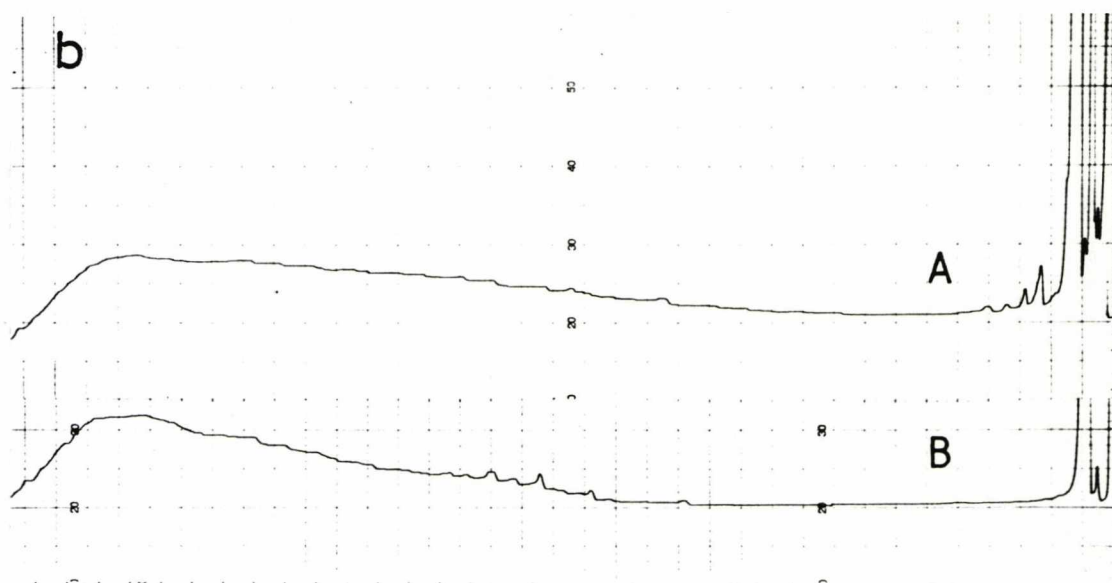
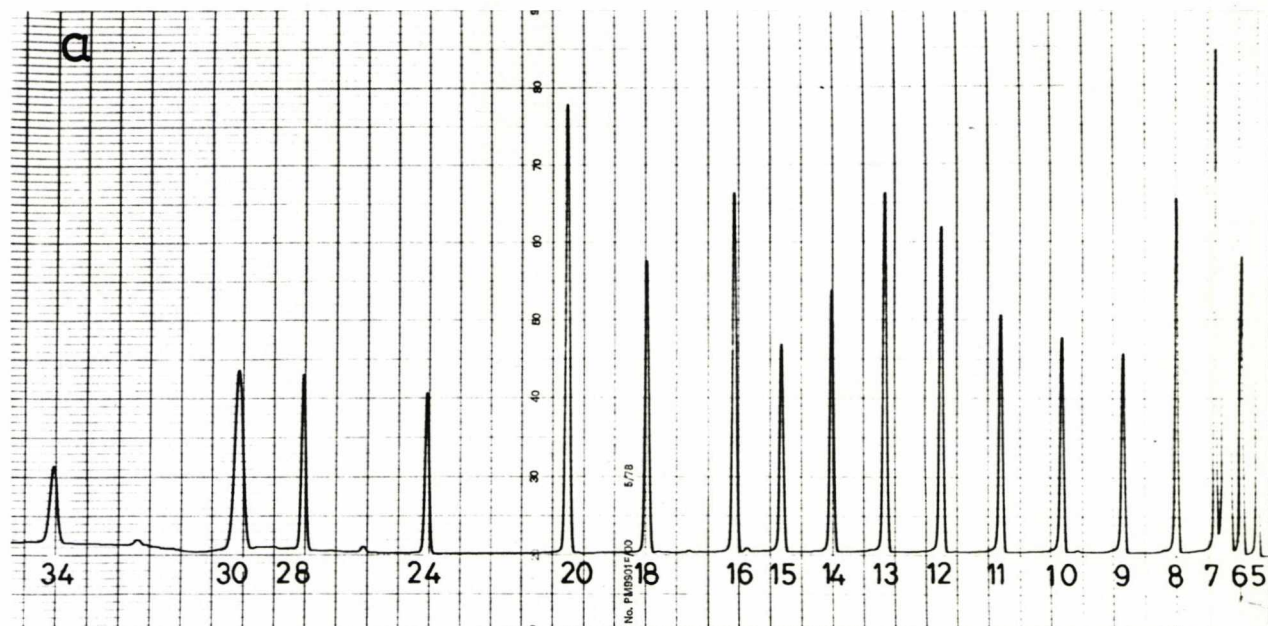


FIGURE 5.15

Gas chromatographic profiles of the sand tar and a mixture of n-alkane standards.

- a - profile of a mixture of n-alkane standards  
the number under each peak refers to the  
carbon number of the compound.
- b - A, fresh sand tar.  
B, topped sand tar which had been leached in  
sterile sea water for 7 days.

Temperature increases from right to left across the profile. Both profiles were performed under identical conditions and are directly comparable.





high tides, varied little (33 - 34 S%). Determination of the oxygen content of the surface water at the times of sampling throughout the second summer experiment never gave values below 85% air saturation. The nitrate concentration of sea water samples taken at intervals during the second summer experiment fell below the limit of detection of the auto-analyser which was  $6\mu\text{M}$ . The total heterotrophic bacterial population isolated from surface water at the times of sampling during the second summer experiment varied between  $9.8 \times 10^3$  -  $8.3 \times 10^4$  viable cells/ml with a mean of  $4.2 \times 10^4$  viable cells/ml. The oil degrading component of this population showed the same wide degree of variation found in the winter experiments, i.e. 0.1 - 10% of the total heterotrophic population.

## 5.2 DISCUSSION

The pattern of oil loss from the filters was similar to that found during winter experiments i.e. synthetic crude > Forties crude > sand tar in both summer experiments. The results of the non-biological controls (oils leached in sterile sea water) at  $13^\circ\text{C}$  again show a substantial loss of the synthetic crude; this was confirmed by the initial, rapid loss of this oil from the filters during early exposure in the summer experiments and by the electronmicrographs of the filter surface taken at this time. The synthetic crude exhibited an apparent increase in amount with concomitant change in infrared profile after the 16th day of the second summer experiment, whereas this occurred after the 36th day of the second winter experiment. With hind-

sight it would have been interesting to see if there was a similar occurrence after the 17th day of the first summer experiment. The gas chromatographic profiles of the synthetic crude and Forties crude during early exposure in the summer experiments are similar to those found in the winter periods. They show the same loss of the low molecular weight profile found in the non-biological controls, and also the reduction of the high molecular weight profile to an unresolved basal envelope. The latter was reached more rapidly in the summer experiments indicating an increased rate of biodegradation. The fractional composition of the oils recovered at the end of the first summer experiment show that the saturate fraction of the Forties crude sustained the most degradation. In the case of the sand tar this was true for the monoaromatic fraction; in both oils there was a relative increase in the polycyclic aromatic nitrogen- sulphur- and oxygen-containing compound, (PNSO) fraction. Fractional analysis of the Forties crude at intervals during the second summer experiment showed a progressive decrease in the saturate fraction and relative rise in the PNSO fraction. The change in the fractional composition of the sand tar was similar, unlike that of the first summer experiment in which the monoaromatic fraction was most extensively degraded. The pattern of decreasing saturate fraction and relative rise in PNSO fraction is similar to compositional changes found during microbial degradation of oil under laboratory conditions (Chapter 1, section 1.3.2C).

Microbial colonization of the oiled filters followed a



similar course during both summer experiments; showing a progressive rise in cell numbers to a maximum population which was maintained for the rest of the experiment. This was most clearly demonstrated in the first summer experiment during which frequent samples were taken over the initial period of colonization. The three bacterial strains comprising the total oil degrading population during this experiment showed similar colonization patterns for each oil. However, unlike the first winter experiment, strain 3 did not exhibit a delayed rise to maximum cell numbers. During the second summer experiment the maximum cell numbers isolated from the synthetic crude and Forties crude were higher than in the first experiment, but these declined slightly during the later stages of the experiment.

#### 5.2.1 Comparison Of Winter and Summer Experiments.

The uneven nature of the rate of oil loss from the filters hampers comparison of rates under winter and summer conditions; however Figures 5.16, 5.17 and 5.18 show the different rates of loss for each oil under both conditions. In the case of the sand tar, oil was lost from the filters more rapidly during the summer periods, but loss during the first summer experiment was markedly more rapid than during the other experiments. During the second winter and summer experiments the pattern of oil loss showed a similar fluctuation; this was not so pronounced in the first experiments.

The Forties crude showed a more rapid loss during the summer periods and both these experiments gave similar results. Comparison of the two winter experiments was precluded by

FIGURE 5.16

Comparison of oil loss from filters coated with sand tar during winter and summer experiments.

- - first winter experiment.
- - second winter experiment.
- ▲ - first summer experiment.
- △ - second summer experiment.



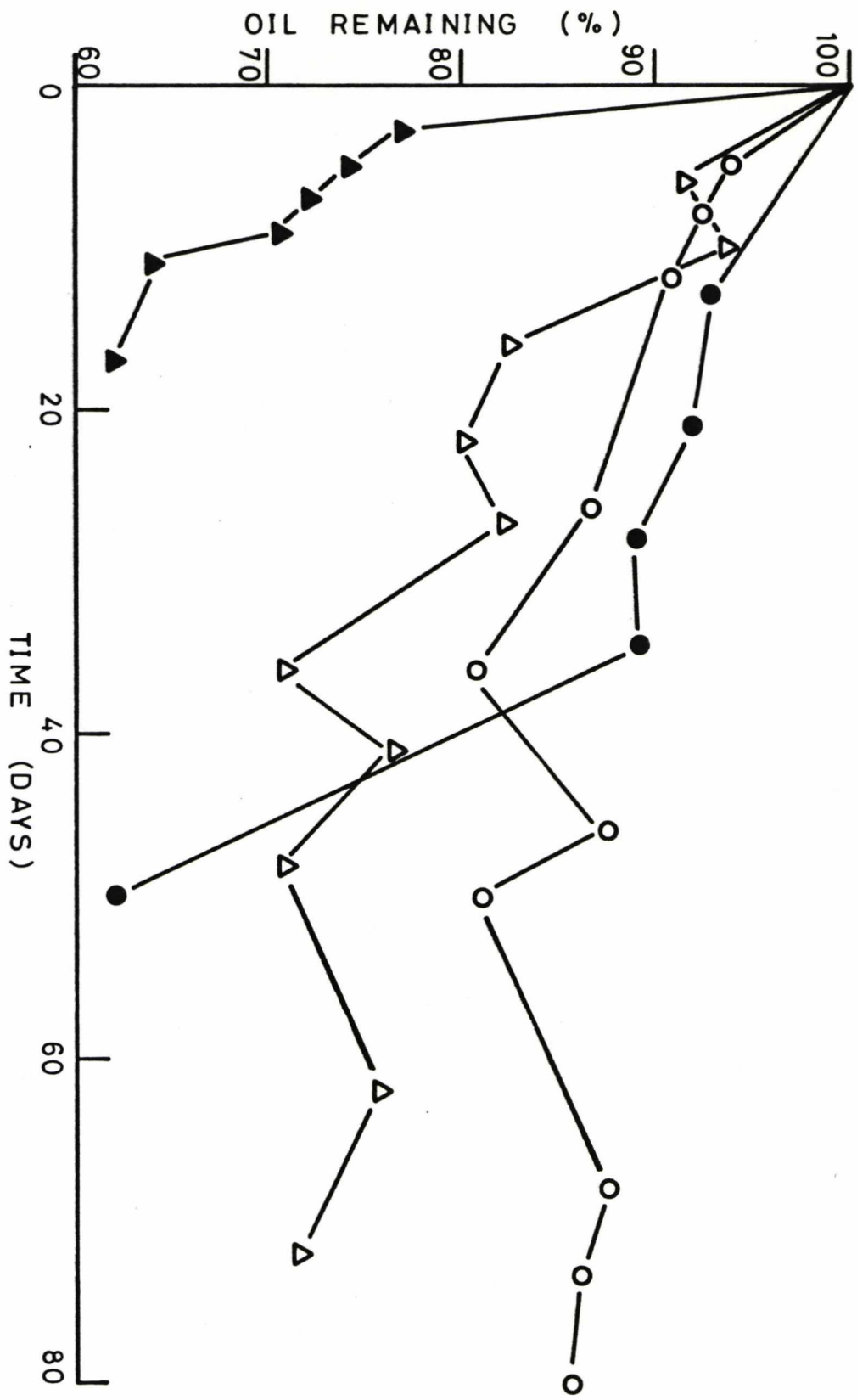


FIGURE 5.17

Comparison of oil loss from filters coated with Forties crude during winter and summer experiments.

- - first winter experiment.
- - second winter experiment.
- ◐ - second winter experiment (replacement samples)
- ▲ - first summer experiment.
- △ - second summer experiment.

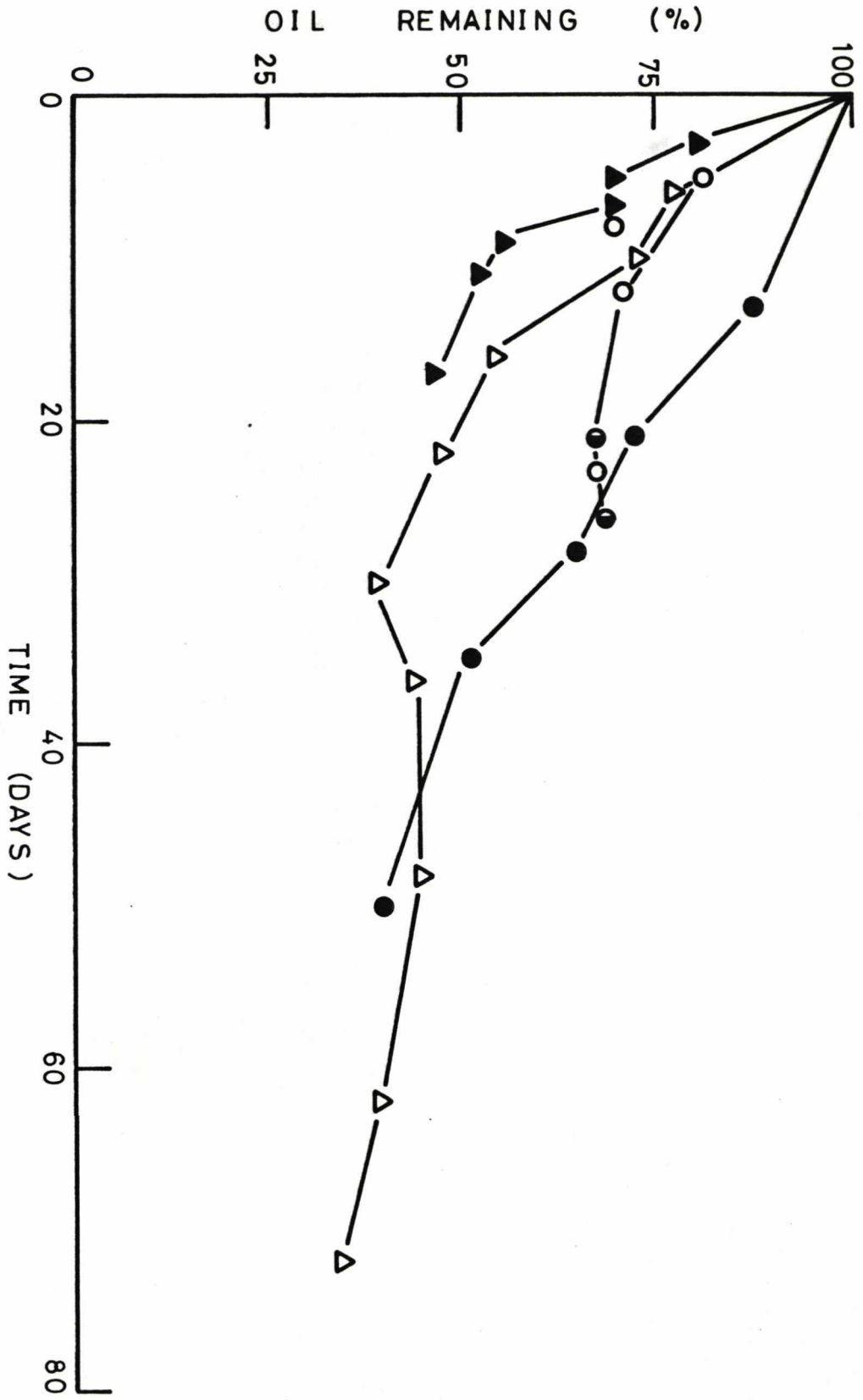
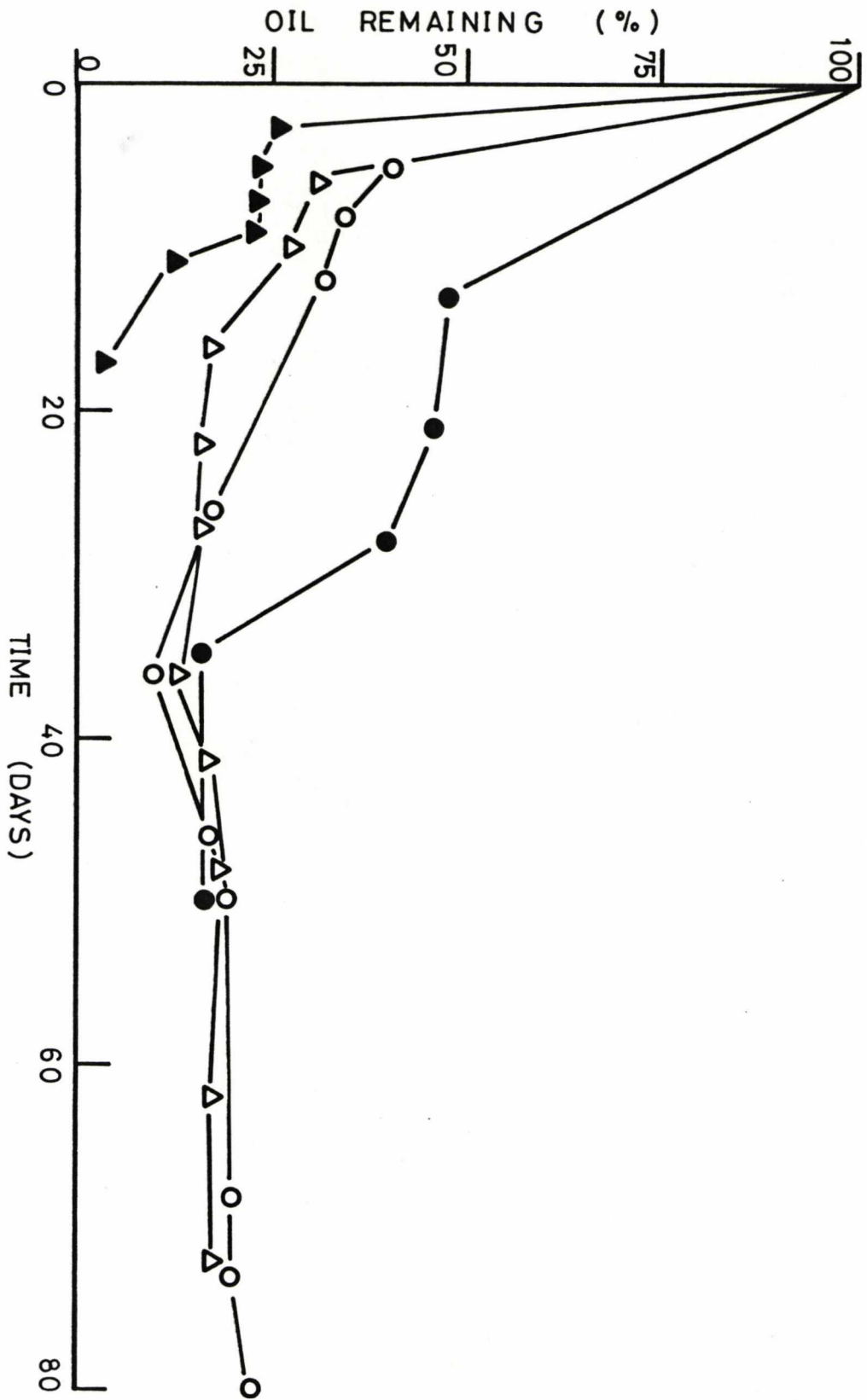


FIGURE 5.18

Comparison of oil loss from filters coated with synthetic crude during winter and summer experiments.

- - first winter experiment.
- - second winter experiment.
- ▲ - first summer experiment.
- △ - second summer experiment.





successive loss of equipment and samples during the second winter experiment.

In the case of the synthetic crude, all experiments showed a high initial loss of oil, which could be mainly attributed to non-biological 'wash off' on the basis of the laboratory controls (oil leached in sterile sea water). The slower rate observed during the first winter experiment may reflect the delay of 13 days before the first sample was taken. After the initial period the rate of loss was reduced but this was greater during the summer periods. During the later stages of the experiments the amount of oil remaining on the filters was approximately constant. The first summer experiment was an exception to this; however, it was concluded after only 17 days.

Although loss of oil from the filters was more rapid under summer conditions, the relative rates between the oils were the same during winter and summer experiments, i.e. synthetic crude > Forties crude > sand tar. This conformed to predictions that can be made on the basis of oil composition and hence shows the importance of composition in influencing breakdown rates despite different temperatures. The gas chromatographic profiles obtained under winter and summer conditions confirm that degradation was occurring more rapidly at the higher temperatures of the summer experiments.

Similar colonization patterns were found for each oil during both winter and summer experiments. However, in the latter maximum cell numbers were obtained more rapidly. These maximum numbers attained on each oil were similar in all experi-

ments, with the exception of those isolated from the Forties crude which were consistently higher in the second winter and second summer experiments. Despite the different rates of breakdown dependent upon oil composition and temperature, the maximum cell numbers isolated were remarkably similar. This suggests that these numbers were influenced by other parameters such as the surface area available for colonization and the extent and rate to which bacteria were removed from the filter surface by natural processes. Assuming the dimensions of colonizing bacteria to be  $1\mu \times 0.5\mu$  and the two surfaces of a 15 mm disc colonized by a monolayer of cells, a rough calculation shows that the disc could support  $7.1 \times 10^6$  cells. This value is an underestimate as the surface of the filter is intricate offering a larger surface area (perhaps as much as an order of magnitude larger) than a flat disc, and the cells are unlikely to exist as a monolayer. However, comparison of the maximum populations actually found during in situ experiments to this value indicate that the filters were heavily colonized.

The materials comprising the filters, (mixed esters of cellulose acetate and cellulose nitrate) could conceivably provide a substrate for microbial growth. However, during all the in situ experiments the blank control filters supported only a small population throughout the period. If the filters were supporting microbial growth a steady rise in cell numbers, similar to that found with the oiled filters, would be expected. Even if the total blank filter population were filter degraders, their numbers were small in comparison to that supported by



oiled filters. Furthermore, with the possible exception of the synthetic crude coated filters, the surface presented for microbial colonization was that of the coating oil and not filter material.



CHAPTER 6

DEGRADATIVE EXPERIMENTS UNDER LABORATORY CONDITIONS

## DEGRADATIVE EXPERIMENTS UNDER LABORATORY CONDITIONS

6.1 ISOLATION OF OIL DEGRADING MICROORGANISMS

Oil degrading microorganisms were isolated by conventional enrichment procedures (Chapter 2, section 2.3.2) from surface sediment samples collected from the intertidal area approximately half a mile downstream from the Isle of Grain oil refinery in the Medway estuary. At the time of sampling thin surface oil films were present in patches over the exposed sediment.

Microscopic observations revealed sparse growth on the three oils during 48 hours incubation at room temperature in the first enrichment flasks. On transfer to the second enrichment flasks, abundant growth was evident after a further 36 hours; growth in the control flask (no oil added) was sparse throughout the enrichment period. Centrifugation of the enrichment culture produced a floccular surface pellicle which was composed of bacteria surrounding oil droplets. For each oil, plating onto marine agar either direct from the enrichment culture, or using the surface pellicle or cell pellet produced after centrifugation gave identical mixed cultures. Once resolved into pure culture, strains were transferred to oil agar to test for oil utilization ability. This procedure gave two oil utilizing bacterial strains isolated on synthetic crude, designated  $A_1$  and  $A_2$  and three oil utilizing bacterial strains isolated on both Forties crude and sand tar designated  $N_1$ ,  $N_2$  and  $N_3$ . No filamentous fungi or yeasts were isolated on any of the three oils, and there was no microscopical evidence for their presence during the enrichment period. The colonial appearance of the



five strains on marine agar is summarized below.

- A<sub>1</sub>: low convex, entire, 1mm diam., white.  
 A<sub>2</sub>: domed, undulate, 3mm diam., cream/translucent, mucoid.  
 N<sub>1</sub>: spreading, cream/translucent.  
 N<sub>2</sub>: domed, entire, 3mm diam., white, mucoid.  
 N<sub>3</sub>: domed, entire, 1mm diam., white.

Table 6.1 gives the results of preliminary tests performed for presumptive identification. Strains N<sub>1</sub> and N<sub>2</sub> are motile, oxidase positive, fermentative, only produce acid in glucose and are sensitive to O/129; these characteristics are consistent with Vibrio species. Strains A<sub>1</sub> and N<sub>3</sub> are non-motile, oxidase negative, produce no conclusive reaction in Hugh & Leifsons medium, produce an alkaline reaction in glucose peptone water and are resistant to O/129. These characteristics are hard to place in the determinative scheme of Scholes & Shewan (1964), but they are more consistent with the properties of the Moraxella -Acinetobacter group outlined in Cowen & Steel (1974). Since good growth was obtained without addition of blood serum, these two strains are more probably Acinetobacter species. Strain A<sub>2</sub> can be tentatively assigned to an Alcaligines species but if this were so a more alkaline reaction would have been expected in glucose/peptone water. More detailed identification of the strains was kindly performed by Dr. J. Leigh and Mr. P. West of the Public Health Laboratories in Maidstone and the results are shown below.

**TABLE 6.1** Results Of Tests Performed On Oil Degrading Isolates  
 A<sub>1</sub>, A<sub>2</sub>, N<sub>1</sub>, N<sub>2</sub>, & N<sub>3</sub> For Presumptive Identification.

	A <sub>1</sub>	A <sub>2</sub>	N <sub>1</sub>	N <sub>2</sub>	N <sub>3</sub>
Gram	GNC/B	GNR	GNR	GNR	GNR
Motility	-	+	+	+(Weak)	-
Catalase	+	+	+	+	+
Oxidase	-	+	+	+	-
Hugh & Leifson	ND	ND	F	F	ND
Glucose/	ALK	NVC	A+	A+	ALK
Peptone	G-	G-	G-	G-	G-
O/129	R	R	S	S	R

- GNR - Gram negative rod.  
 GNC/B - Gram negative cocco/bacilli  
 ND - Not detected.  
 F - Fermentative.  
 A - Acid production.  
 ALK - Alkaline reaction.  
 NVC - Growth but no visible change.  
 R - Resistant.  
 S - Sensitive.  
 G - Gas production.



- A<sub>1</sub> Acinetobacter spp.  
A<sub>2</sub> Marine pseudomonad/Alcaligines spp.  
N<sub>1</sub> Vibrio alginolyticus.  
N<sub>2</sub> Atypical Beneckea natriegens.  
N<sub>3</sub> Acinetobacter spp.

## 6.2 ASSESSMENT OF MICROBIAL GROWTH ON OILS.

Three methods were used to assess the microbial growth of the mixed cultures isolated from estuarine sediment during aerated batch culture on each of the three oils. The methods were direct count, viable count on solid media and an optical density method (Chapter 2, section 2.4.1). Figures 6.1, 6.2 and 6.3 show the results for the synthetic crude, Forties crude and sand tar respectively.

## 6.3 MICROBIAL GROWTH ON OILS DURING AERATED BATCH CULTURE WITH SEMI-CONTINUOUS ADDITION OF NUTRIENTS.

The inocula used for these experiments were composed of a mixed culture of strains A<sub>1</sub> and A<sub>2</sub> for growth on the synthetic crude and a mixed culture of strains N<sub>1</sub>, N<sub>2</sub> and N<sub>3</sub> for growth on the Forties crude. Both experiments were carried out for 60 days using aerated batch culture at 26° - 27°C with semi-continuous addition of phosphate and nitrate (Chapter 2, section 2.4.1). Figures 6.4 and 6.5 show the results for the oil remaining (determined by infrared spectroscopy) and the number of viable cells/ml at intervals during the experiment for the synthetic crude and Forties crude respectively. Both experiments

FIGURE 6.1

Assessment of microbial growth during a 24 day aerated batch culture at 25°C of a mixed culture of strains A<sub>1</sub> and A<sub>2</sub> on synthetic crude oil.

○ - total count.

● - viable count.

△ - optical density method.

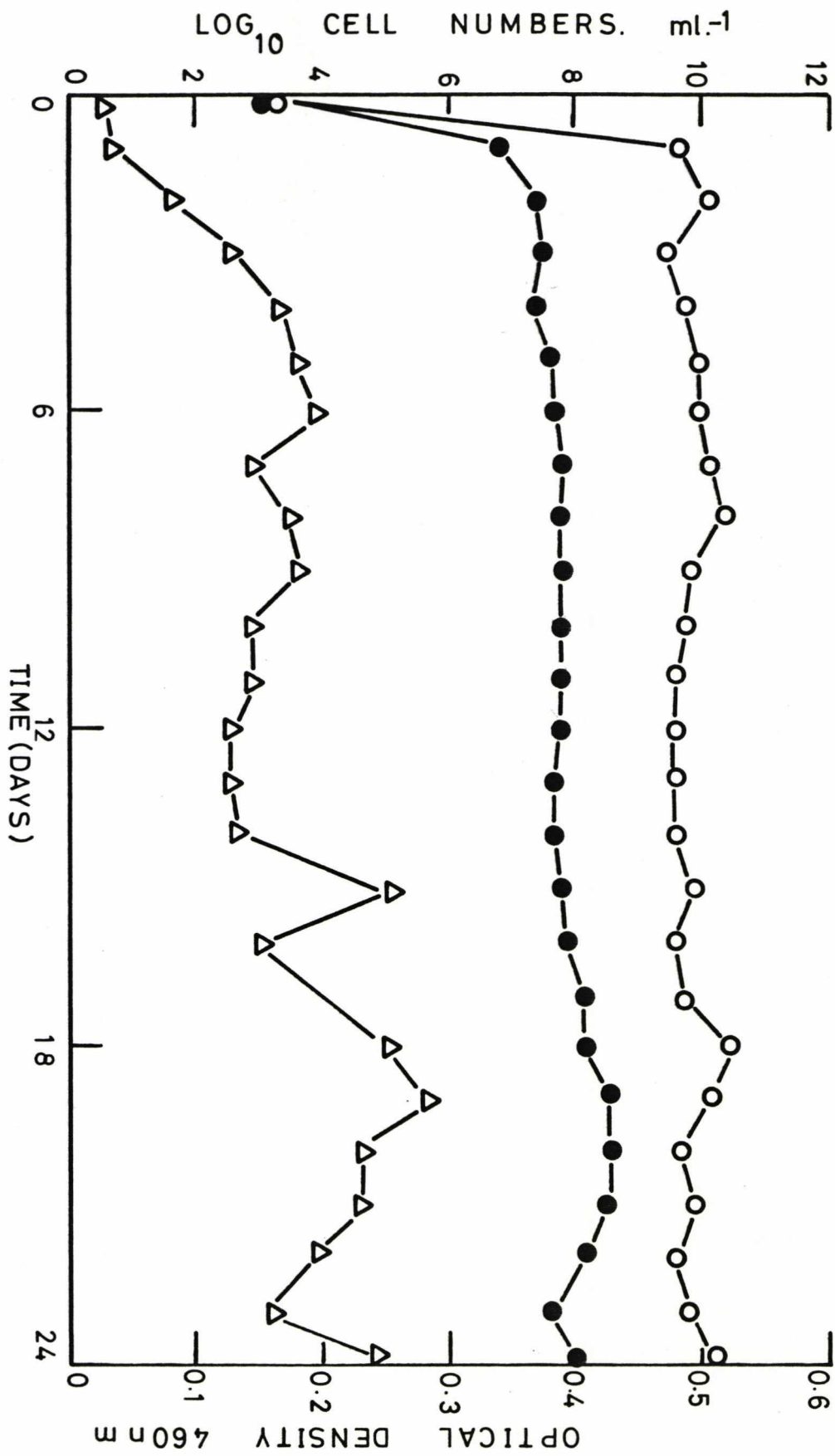


FIGURE 6.2

Assessment of microbial growth during a 24 day aerated batch culture at 25°C of a mixed culture of strains N<sub>1</sub>, N<sub>2</sub> and N<sub>3</sub> on Forties crude oil.

- - total count.
- - viable count.
- △ - optical density method.



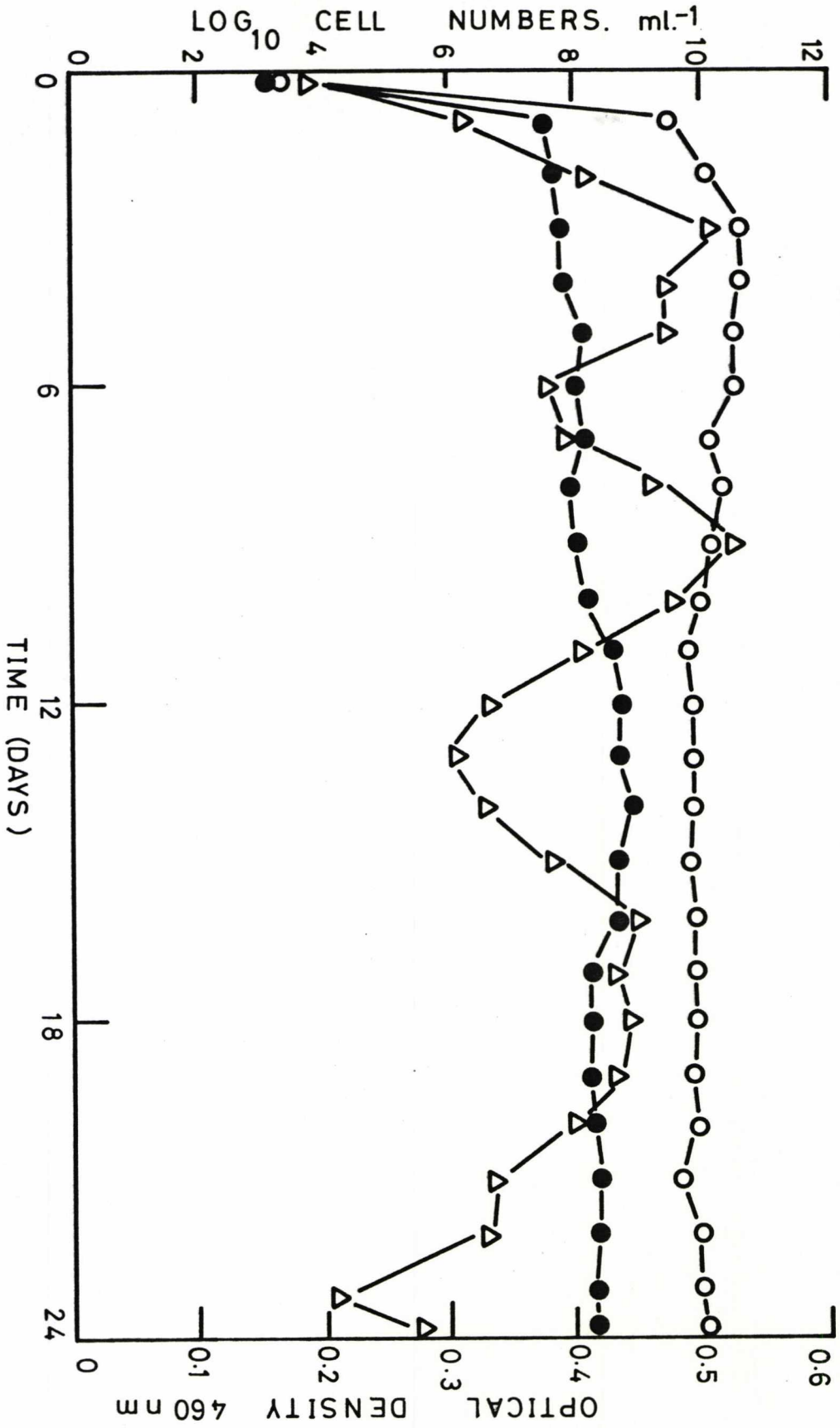


FIGURE 6.3

Assessment of microbial growth during a 24 day aerated batch culture at 25°C of a mixed culture of strains N<sub>1</sub>, N<sub>2</sub> and N<sub>3</sub> on sand tar.

- - total count.
- - viable count.
- △ - optical density method.

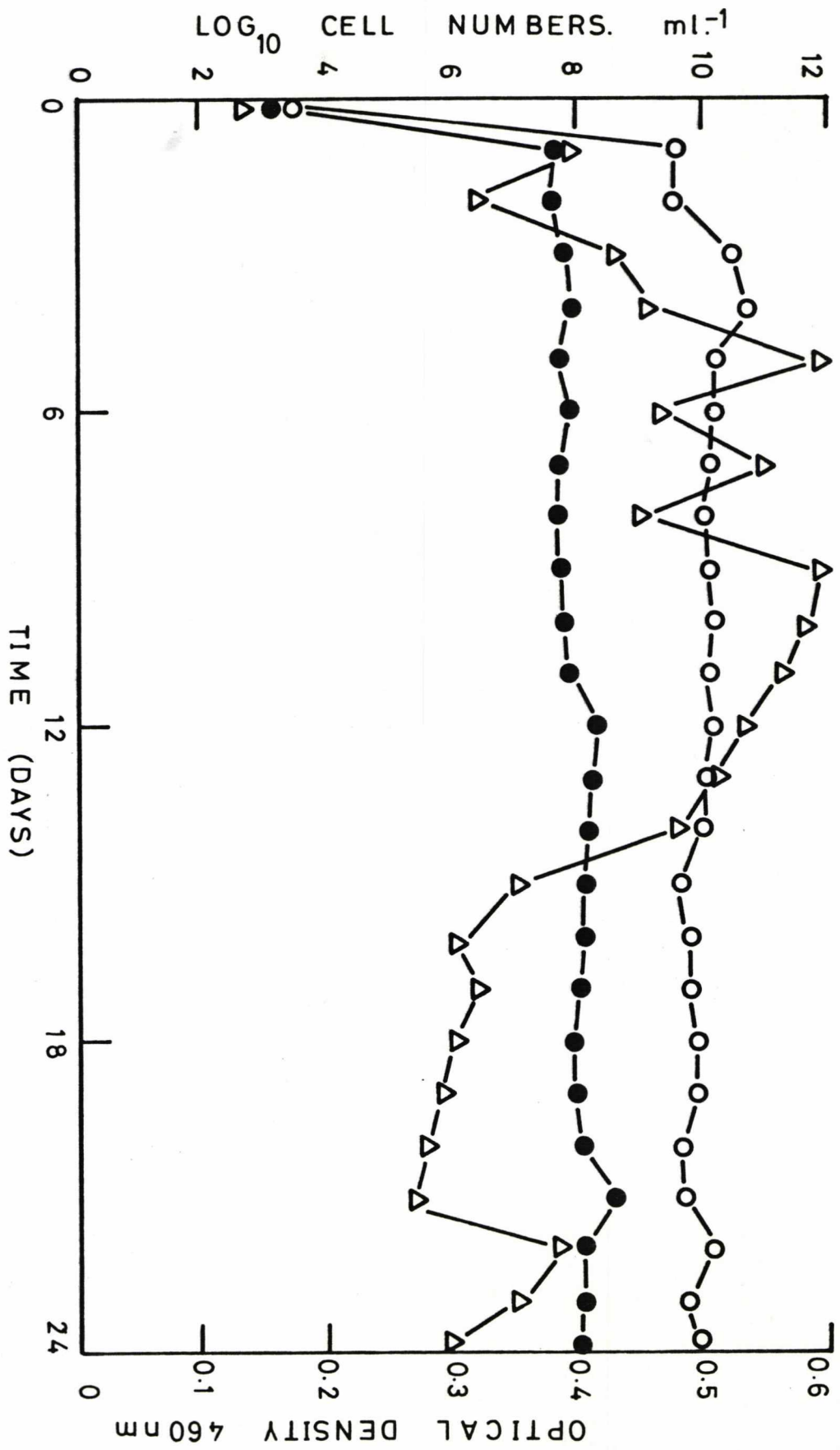


FIGURE 6.4

Oil remaining (%) and viable bacterial cell numbers ( $\log_{10}/\text{ml}$ ) during a 60 day aerated batch culture of a mixed culture of strains  $A_1$  and  $A_2$  on synthetic crude oil. Nitrate and phosphate were added semi-continuously throughout the experiment.

$\Delta$  - oil remaining.

O - viable bacterial cell numbers.



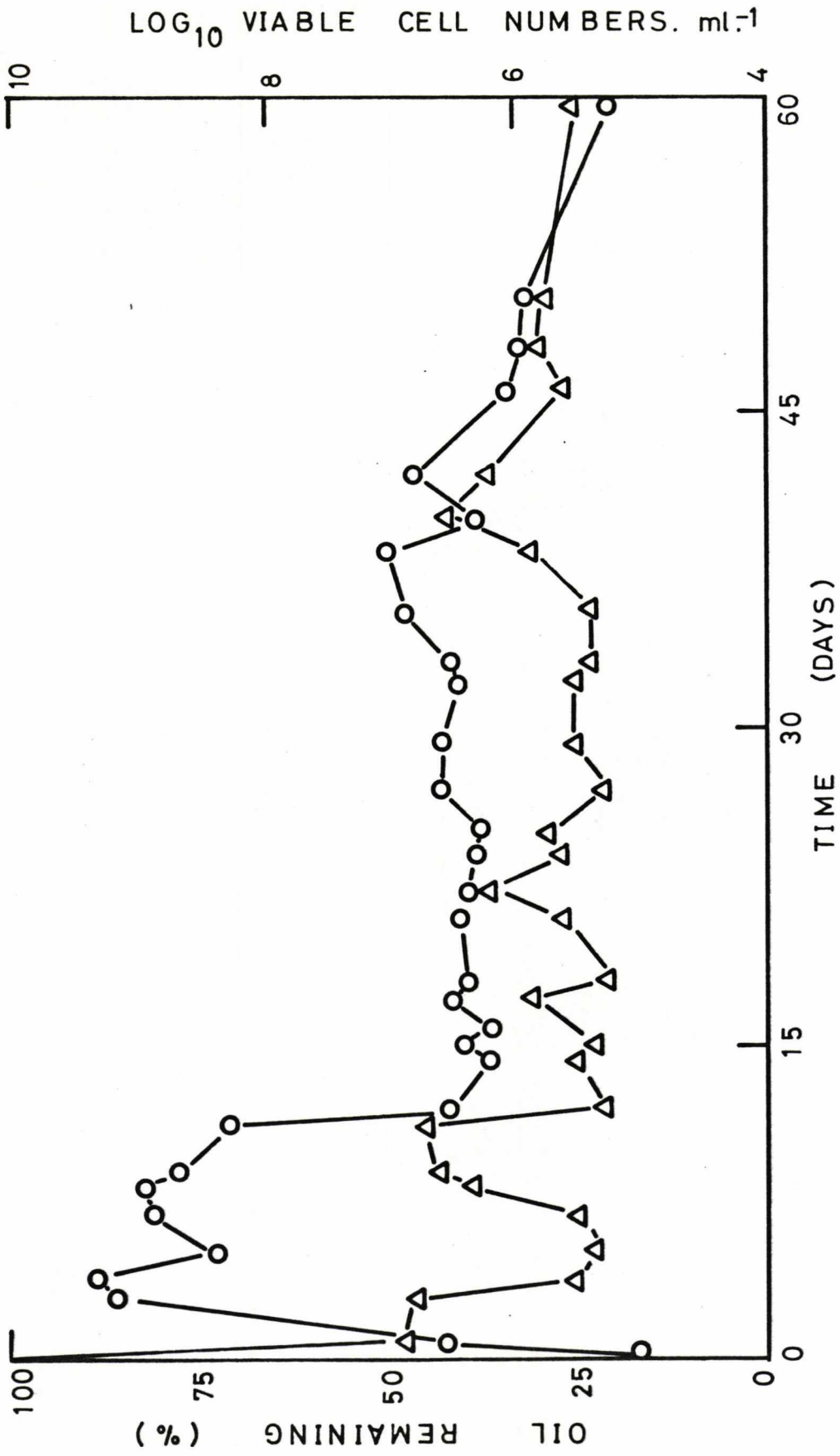
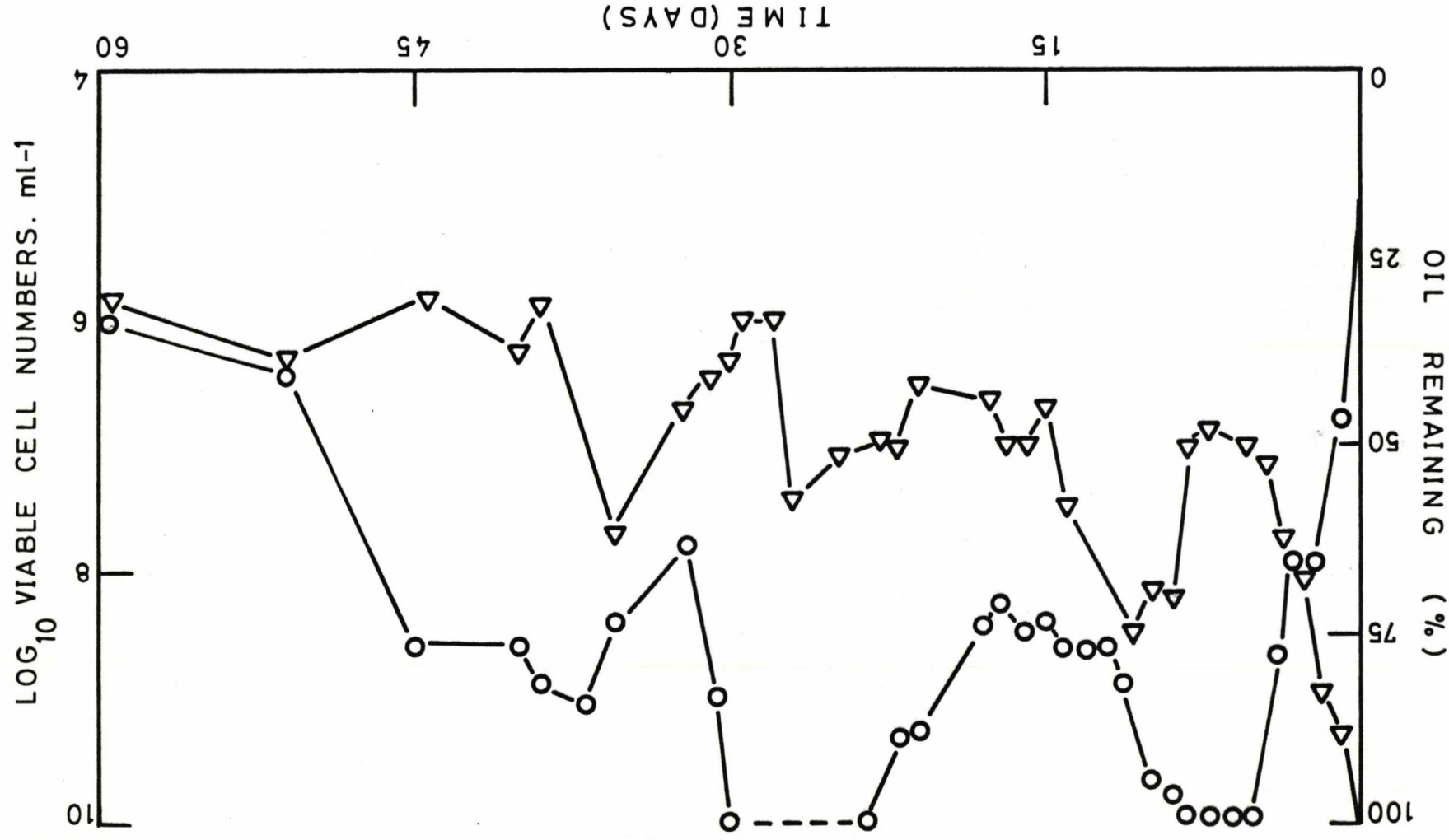


FIGURE 6.5

Oil remaining (%) and viable bacterial cell numbers ( $\log_{10}/\text{ml}$ ) during a 60 day aerated batch culture of a mixed culture of strains  $N_1$ ,  $N_2$  and  $N_3$  on Forties crude oil. Nitrate and phosphate were added semi-continuously throughout the experiment.

$\Delta$  - oil remaining.

O - viable bacterial cell numbers.



show a marked fluctuation in the amount of oil remaining and in the viable cell numbers/ml over the 60 day period.

Figure 6.6 (A-F) shows the gas chromatographic profile of oil recovered from the Forties crude experiment at 0, 8, 10, 12, 14 & 15 days; this covers the first period of apparent increase in oil remaining. It is interesting to note the appearance of novel high molecular weight hydrocarbon peaks not present in the starting oil between the 10th and 14th days. These are absent on the 8th and 15th days. Figure 6.6 (G & H) shows the gas chromatographic profile of oil recovered at 27 and 35 days respectively. Although there is an apparent increase in the amount of oil remaining the profiles do not reveal any novel hydrocarbon peaks. Novel hydrocarbon peaks in the same region of the profile but reduced in amplitude appeared after 12 days during the experiment to assess microbial growth on the Forties crude (section 6.2). In contrast to the Forties crude, gas chromatographic profiles of the synthetic crude recovered at times of apparent increase in the amount of oil remaining during this experiment, did not show any novel hydrocarbon peaks.

The control experiments to assess the extent of oil loss in the absence of microbial growth were carried out in an identical fashion except that Tween 80 was added (final concentration, 0.02%, V/V) to promote a stable emulsion. The aged sea water medium was not supplemented with nitrate or phosphate and streptomycin sulphate (final concentration, 0.67 mg/ml) was added to inhibit bacterial growth. The effect of Tween 80 on infrared quantification of oil is discussed in Chapter 2,



FIGURE 6.6

Gas chromatographic profiles of Forties crude at intervals during a 60 day aerated batch culture of strains N<sub>1</sub>, N<sub>2</sub> and N<sub>3</sub> at 26-27°C with semi-continuous addition of nitrate and phosphate.

- A - 0 day profile.
- B - 8 " " .
- C - 10 " " .
- D - 12 " " .
- E - 14 " " .
- F - 15 " " .
- G - 27 " " .
- H - 35 day profile.

Temperature increases from right to left across the profile. All profiles were obtained under identical conditions and are directly comparable.



section 2.4.1. The controls were sampled at 5 day intervals and checked for microbial growth by direct plating onto sea water nutrient agar. After 5 days the controls showed oil losses of 5% and 8% for the Forties crude and synthetic crude respectively and thereafter no appreciable further loss was incurred. Both controls were concluded on the 25th day as both became contaminated after day 20.

#### 6.4 DEGRADATION OF SYNTHETIC CRUDE BY STRAINS A<sub>1</sub> AND A<sub>2</sub>.

The degradation of the synthetic crude by pure cultures of strains A<sub>1</sub> and A<sub>2</sub> and by a mixed culture of the two, over a 20 day period in batch shake flask culture at 25°C is shown in Figure 6.7. Strain A<sub>2</sub> shows an even, progressive degradation pattern, whilst strain A<sub>1</sub> and the mixed culture show initial, rapid, apparent degradation followed by a relative increase in the amount of oil present. This was similar to the fluctuating degradation pattern found during growth in aerated batch culture (Figure 6.4). Figure 6.8 shows the pattern of growth of strains A<sub>1</sub> and A<sub>2</sub> in pure and mixed culture during the experiment. Strain A<sub>1</sub> shows a similar growth pattern in both pure and mixed culture, reaching maximum cell numbers when the amount of oil in the medium is at a minimum and thereafter showing a steady fall in numbers as the amount of oil apparently increases again. Strain A<sub>2</sub> showed lower cell numbers in mixed culture than in pure culture but the overall growth pattern was the same in both cases. The rise to maximum cell numbers was less rapid than with A<sub>1</sub> and was reached after 10 days; thereafter there

FIGURE 6.7

Oil remaining (%) during a 20 day batch culture at 25°C of strains A<sub>1</sub> and A<sub>2</sub> in pure and mixed culture on synthetic crude oil.

- O - strains A<sub>1</sub>+A<sub>2</sub>.
- Δ - strain A<sub>1</sub>.
- ▽ - strain A<sub>2</sub>.



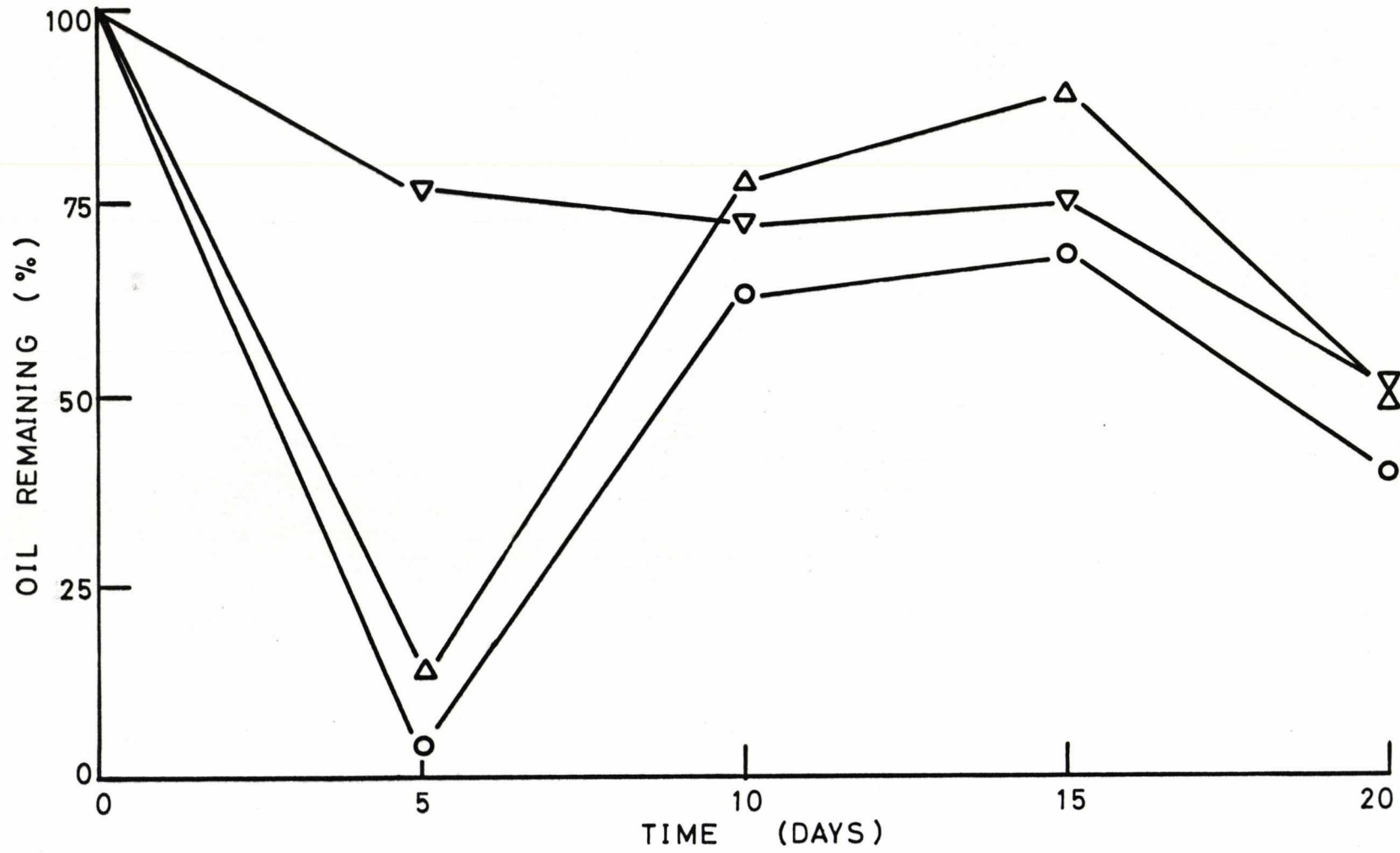
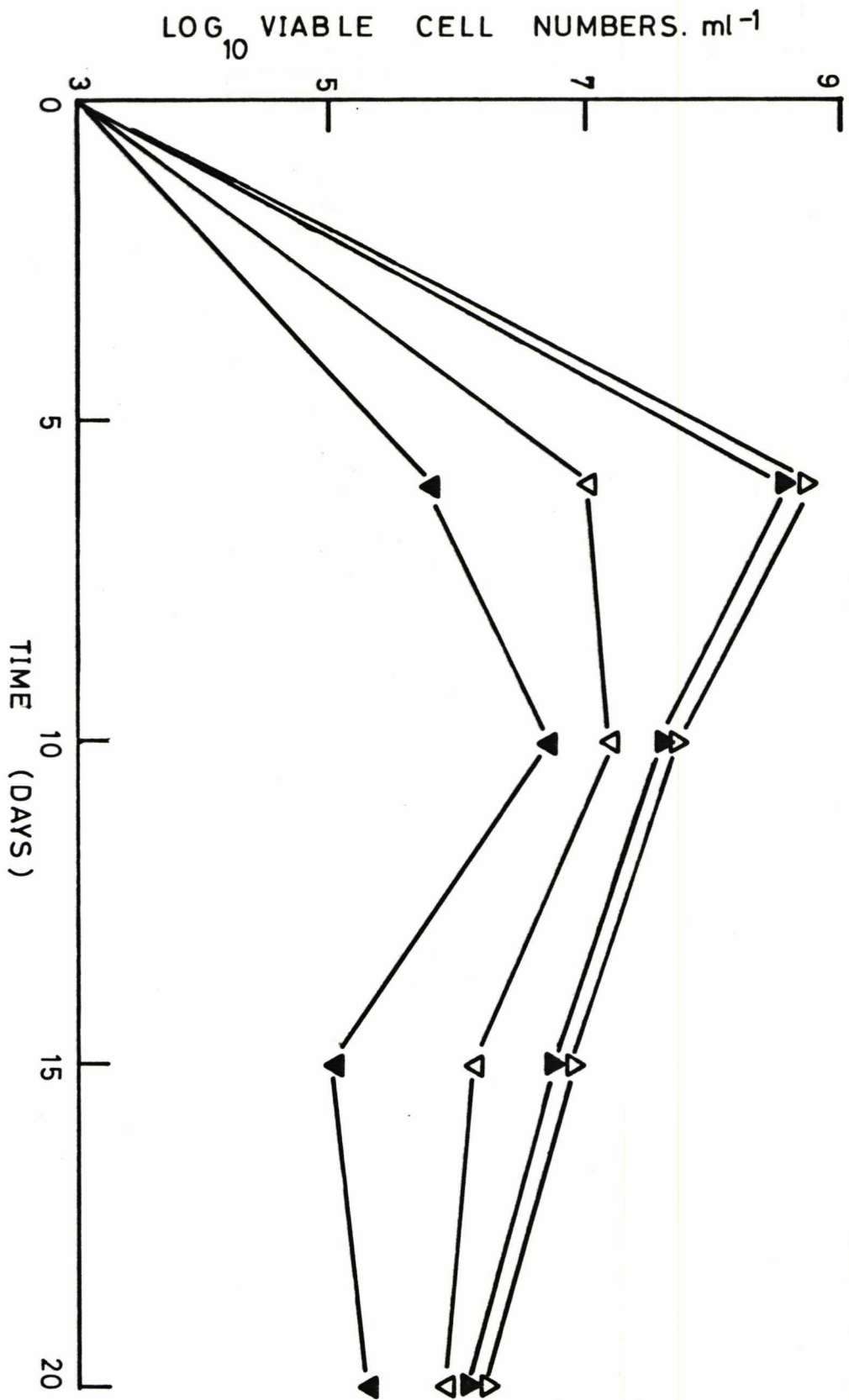


FIGURE 6.8

Viable cell numbers ( $\log_{10}/\text{ml}$ ) of strains  $A_1$  and  $A_2$  in pure and mixed culture during a 20 day batch culture at  $25^\circ\text{C}$  on synthetic crude oil.

- $\Delta$  - strain  $A_1$  in pure culture.
- $\blacktriangle$  - strain  $A_1$  in mixed culture.
- $\nabla$  - strain  $A_2$  in pure culture.
- $\blacktriangledown$  - strain  $A_2$  in mixed culture.



was a slow decline in cell numbers. Tween 80 (0.02%, V/V) was used in these batch culture experiments to promote a stable emulsion; the effect of Tween 80 on the growth of strains A<sub>1</sub> and A<sub>2</sub> is discussed in Chapter 2, section 2.4.2. The sterile controls showed a loss of about 10% at the time of first sampling and thereafter no further appreciable loss.

At the time of lowest oil concentration in the medium (5 days) pure cultures of strain A<sub>1</sub> were examined for the presence of intracellular hydrocarbon inclusions. Smears stained with sudan black and examined by light microscope failed to show any inclusions. An electronmicrograph of an example of a thin section of a cell is shown in Plate 6.1 A; this demonstrates the presence of inclusion bodies at the periphery of the cell. Plate 6.1 B shows an electronmicrograph of glucose grown cells in which these inclusions are absent. The intracellular inclusions were isolated at the time of lowest oil concentration in the medium (Chapter 2, section 2.4.3) and their contents examined by G.L.C. Figure 6.9 shows gas chromatograms of the contents of the inclusion bodies and of residual oil extracted from the medium at the time of harvesting. That of the inclusions has the characteristic profile of the synthetic crude except for an increase in a poorly resolved high molecular weight portion. The profile of the residual oil in the medium is similar to that of the inclusions except the high molecular weight portion is more resolved and certain mid molecular weight peaks are more evident.



PLATE 6.1

Transmission electronmicrographs of an example of a thin section of a cell of strain A<sub>1</sub> exhibiting intracellular inclusion bodies after growth on synthetic crude oil and that of a glucose grown cell for comparison.

A - growth on synthetic crude, (x100,000)

B - growth on glucose, (x63,000).

cw - cell wall.

n - nuclear material.

ib - inclusion body.

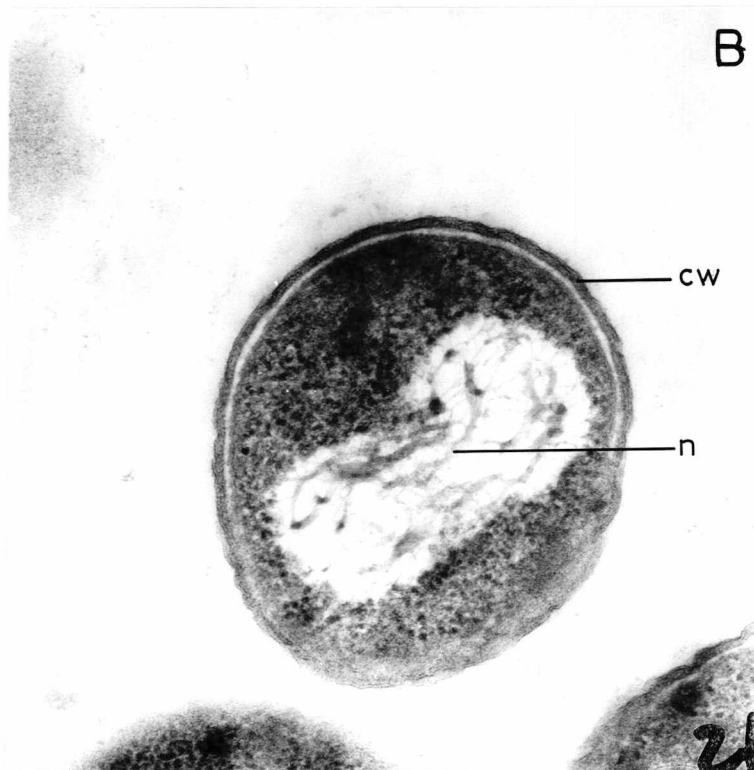
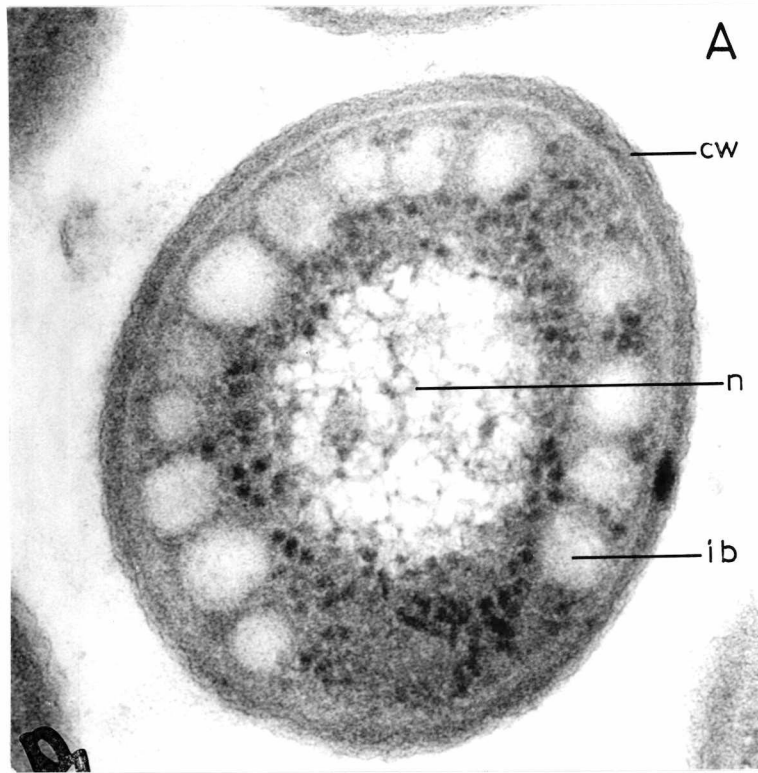
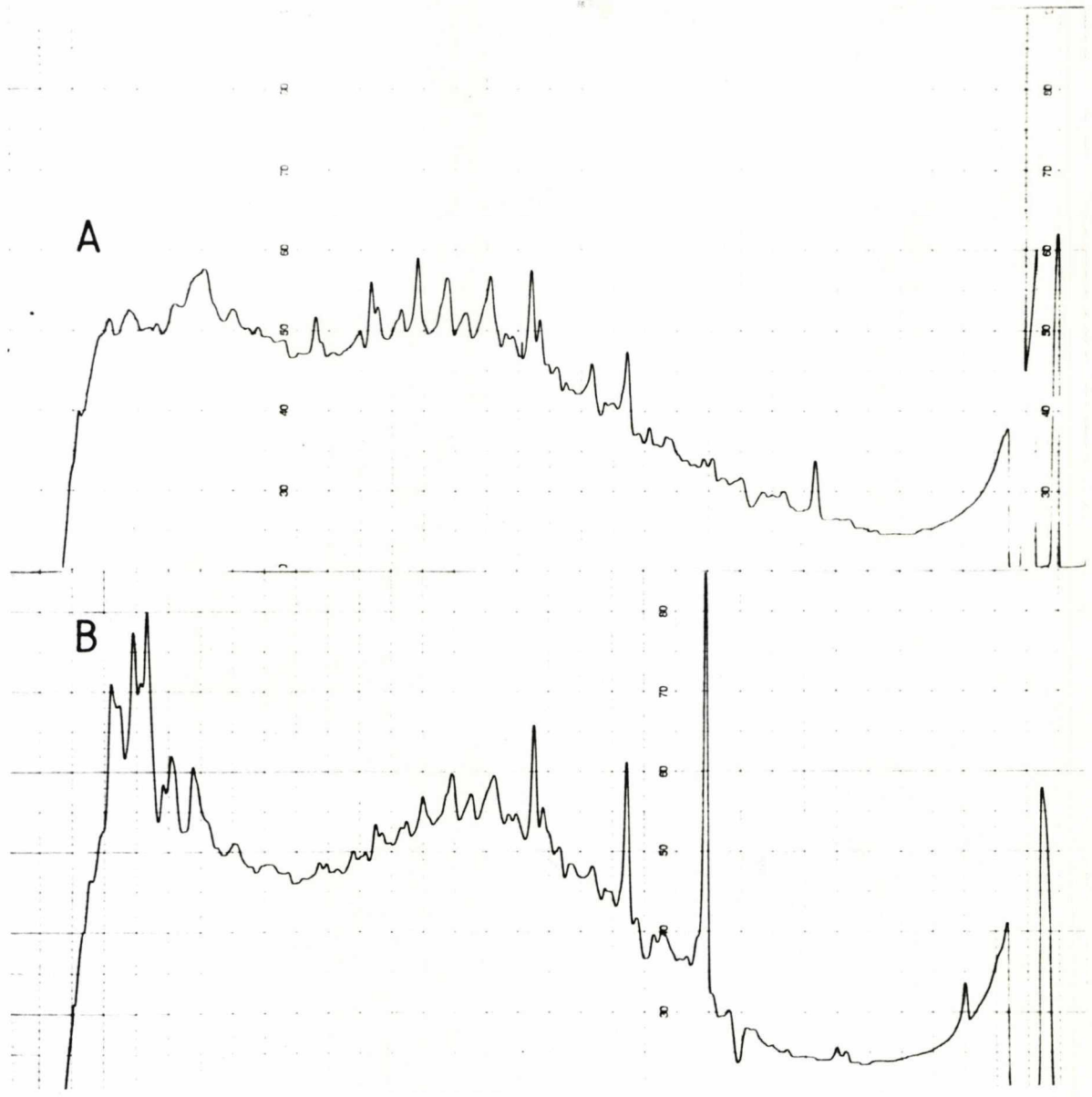


FIGURE 6.9

Gas chromatographic profile of the contents of intracellular inclusion bodies found in strain A<sub>1</sub> after growth on synthetic crude oil. A gas chromatographic profile of residual oil from the medium at the time of harvesting is shown for comparison.

- A - profile of the contents of the inclusion bodies harvested at the time of lowest oil content of the medium (5 days).
- B - profile of residual oil present in the medium at the time of harvesting.

Temperature increases from right to left across the profile. Both profiles were obtained under identical conditions and are directly comparable.





### 6.5 DEGRADATION OF OILS AT LOW TEMPERATURE.

The degradation of the three oils was monitored by infrared spectroscopy during a 74 day batch culture at 4°C. The inoculum for each oil was provided by a mixed bacterial population isolated from a filter coated with the respective oil which had been exposed at sea for 26 days during the second winter experiment (Chapter 4). The cultures were isolated and maintained at 15°C. Figures 6.10 and 6.11 show the oil remaining and viable cell numbers at intervals during the 74 day culture. The rate and extent of oil degradation follows the pattern found previously i.e. synthetic crude > Forties crude > sand tar. The sterile controls showed no appreciable loss of any of the three oils throughout the experiment at this low temperature. The cell numbers growing on each oil were closely similar throughout the experiment, rising to a maximum by 28 days and thereafter followed by a slight decline.

### 6.6 DEGRADATION OF PHOTOOXIDIZED OILS.

The degradation of photooxidized oils was compared to that of untreated oils during a 35 day batch shake flask culture at 25°C. The oils were photooxidized using a high intensity U.V. source of approximately 254 nm. (Chapter 2, section 2.4.4) and monitored by infrared spectroscopy. Gas chromatographic profiles of photooxidized and untreated synthetic crude and Forties crude showed no detectable difference. A mixed culture of strains A<sub>1</sub> and A<sub>2</sub> was used as an inoculum for the synthetic crude oils and a mixed culture of strains N<sub>1</sub>, N<sub>2</sub> and N<sub>3</sub> for the Forties crude

FIGURE 6.10

Oil remaining (%) during a 74 day batch culture  
at 4°C .

- O - sand tar.
- - Forties crude.
- △ - synthetic crude.

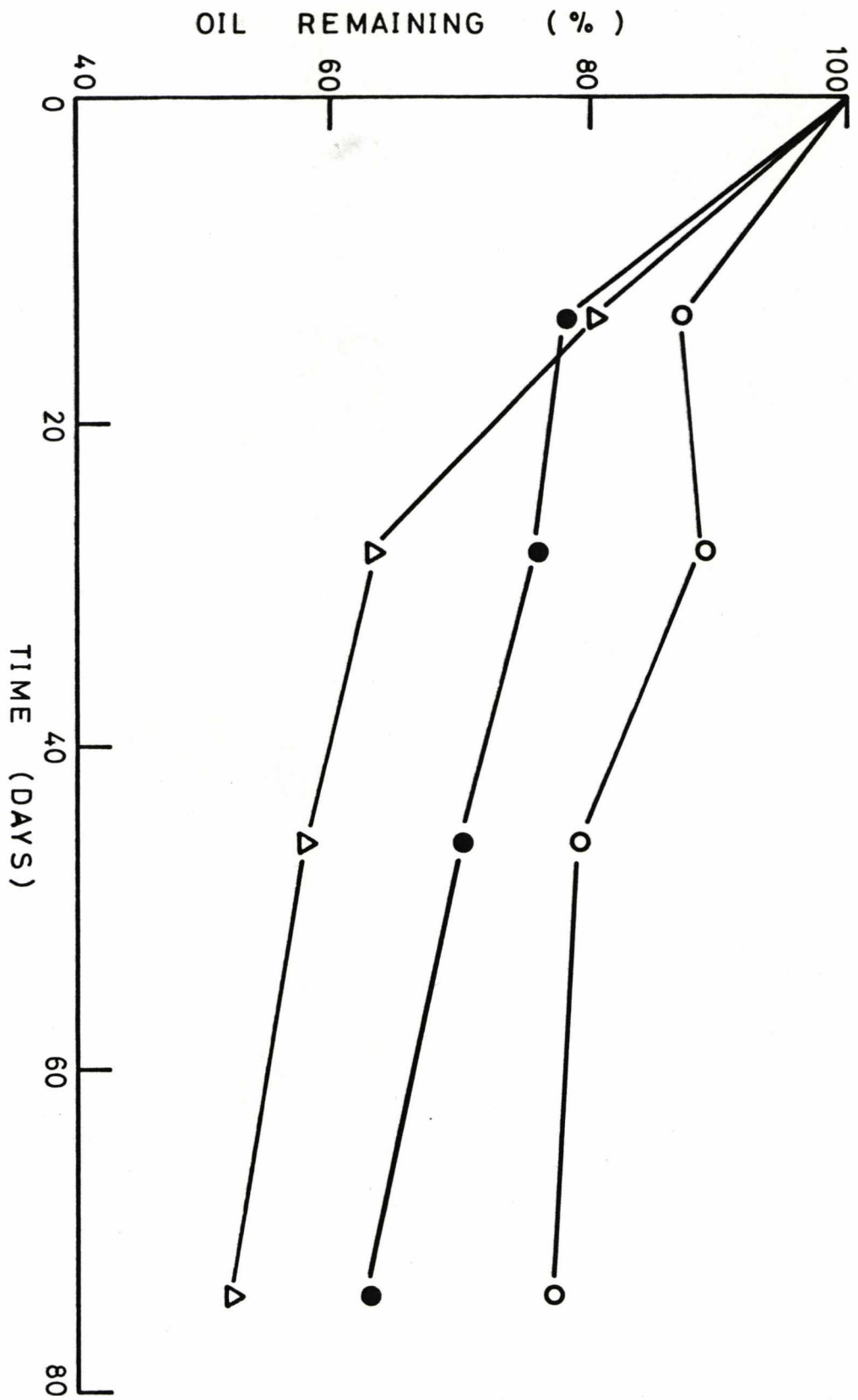
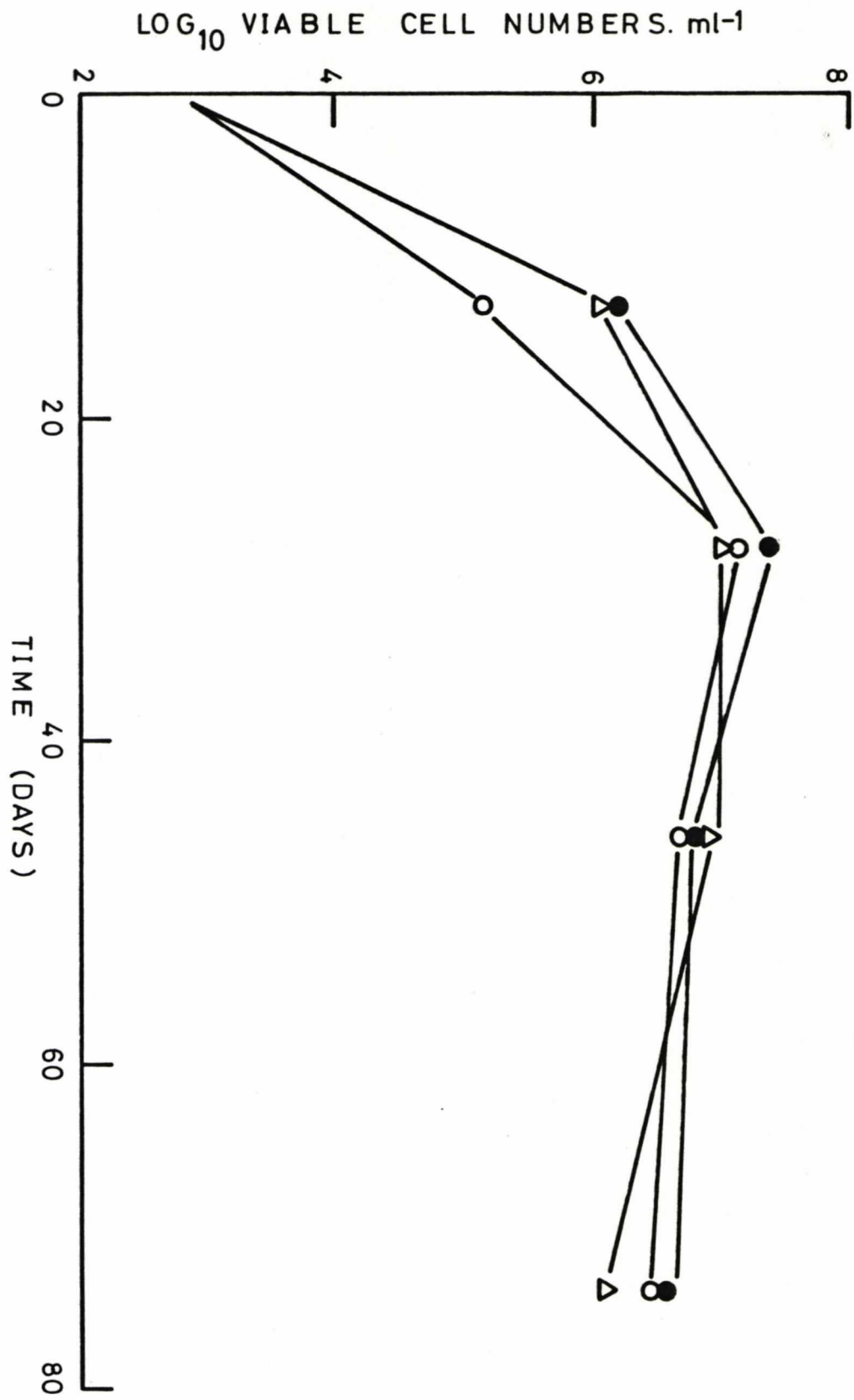


FIGURE 6.11

Viable bacterial cell numbers ( $\log_{10}$ ) during a 74 day batch culture on each of the three oils at 4°C .

- - sand tar.
- - Forties crude.
- △ - synthetic crude.





and sand tar. Figure 6.12 shows the rate and extent of degradation for the three oils which follow the familiar order of synthetic crude > Forties crude > sand tar. The photooxidized and untreated sand tar followed a closely similar course with the former showing slightly less degradation. This trend was reversed in the latter part of the Forties crude experiment. The photooxidized synthetic crude showed markedly less degradation than the untreated oil throughout the experiment, except at the conclusion where the values were similar. The sterile controls for the untreated oils showed no appreciable loss of oil throughout the experiment. Those for the photooxidized oils showed a similar pattern up to the 15th day after which they became contaminated. Microbial growth on the photooxidized and untreated oils is shown in Figure 6.13. For each oil the viable cell numbers isolated from photooxidized and untreated oils were closely similar. The overall pattern of growth consisted of a rise to maximum cell numbers within 6 days, followed by a steady decline.

#### 6.7 DEGRADATION OF SILICA ADSORBED OILS.

The three oils were adsorbed onto silica (Chapter 2, section 2.4.1) and 1L magnetically stirred batch cultures containing these oils were incubated for 60 days at 25°C. The strains used to inoculate each oil were identical to those mentioned above (section 6.6). Oil was quantified by the extraction of uniform samples followed by infrared spectroscopy; Figure 6.14 shows the degradation of the oils over the 60 day

FIGURE 6.12

Oil remaining (%) during a 35 day batch culture at 25°C on both photooxidized and untreated oils.

Photooxidized oils ; ■ - sand tar.  
● - Forties crude.  
▲ - synthetic crude.

Untreated oils ; □ - sand tar.  
○ - Forties crude.  
△ - synthetic crude.

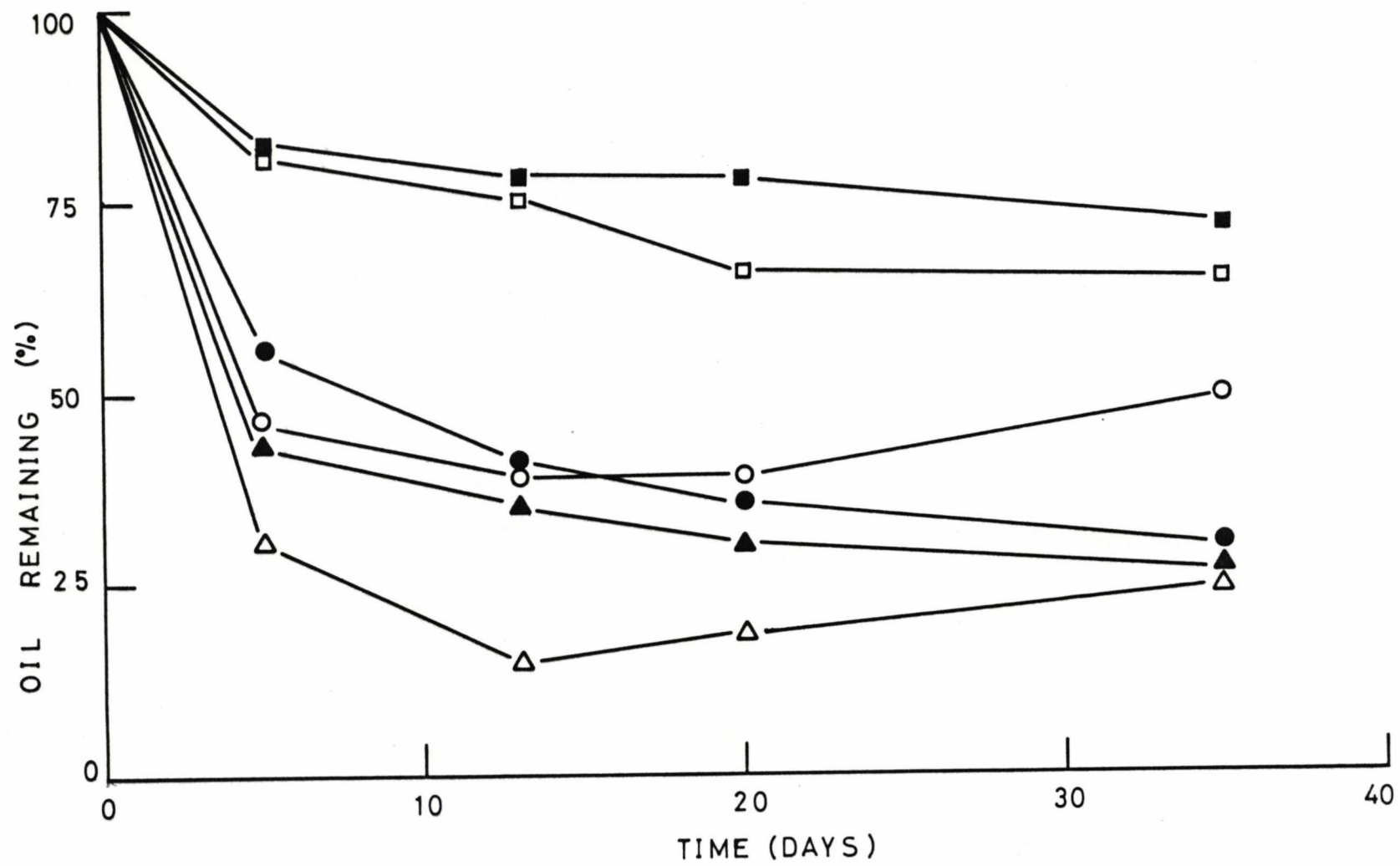




FIGURE 6.13

Viable bacterial cell numbers ( $\log_{10}/\text{ml}$ ) isolated from photooxidized and untreated oils during a 35 day batch culture at  $25^{\circ}\text{C}$  .

Cells isolated from photooxidized oils:

- - sand tar.
- - Forties crude.
- ▲ - synthetic crude.

Cells isolated from untreated oils:

- - sand tar.
- - Forties crude.
- △ - synthetic crude.

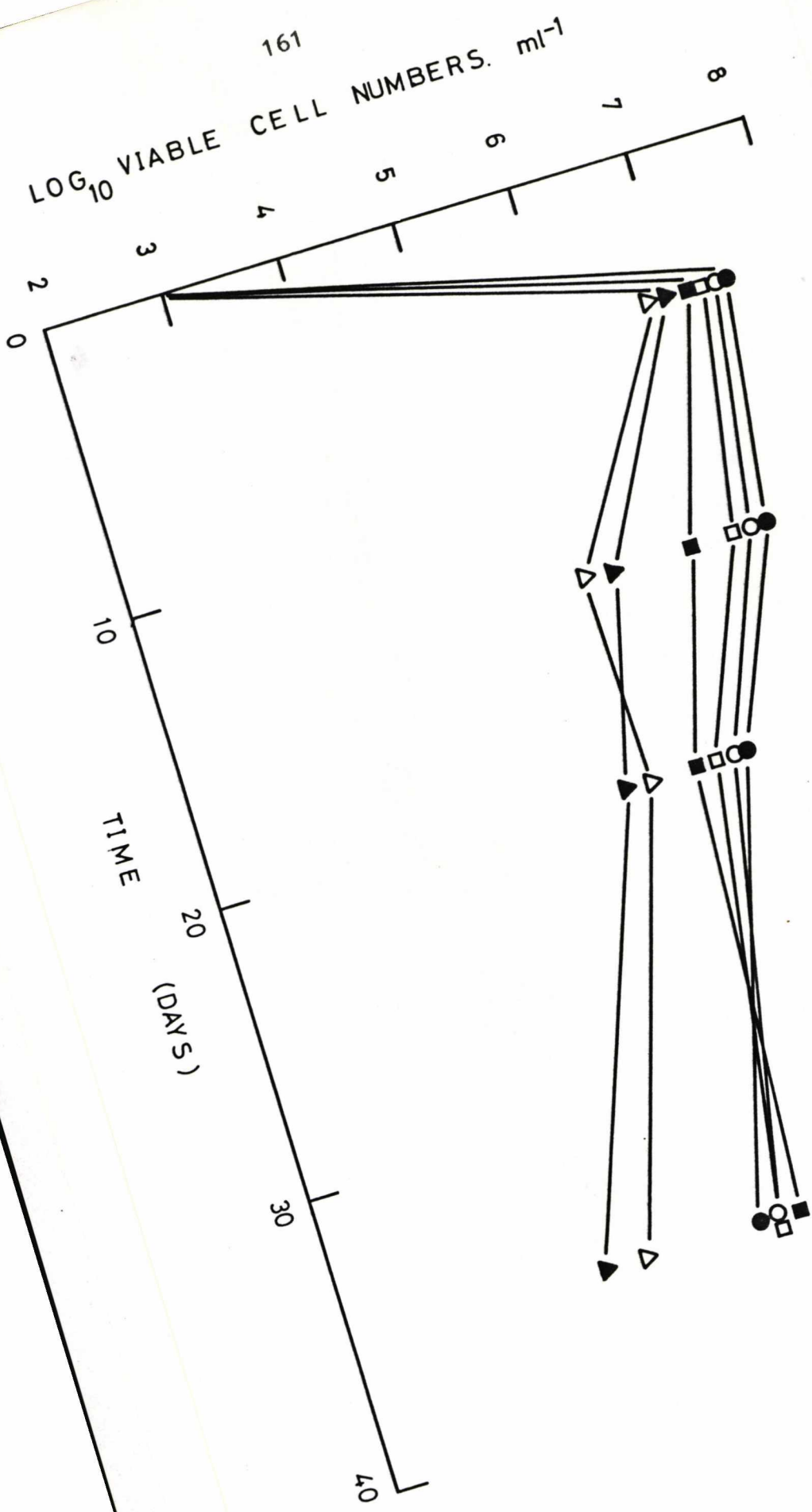
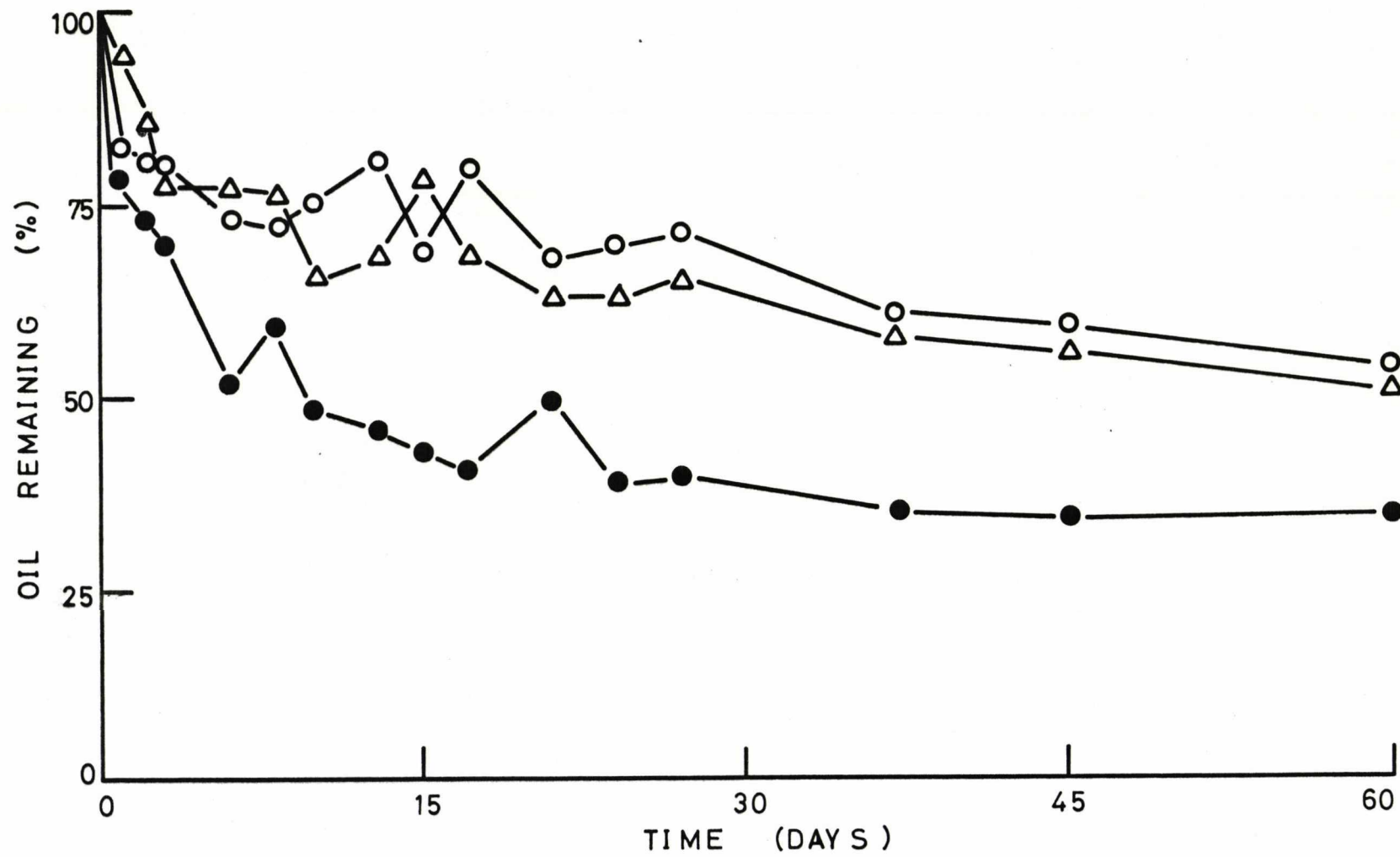


FIGURE 6.14

Oil remaining (%) of silica adsorbed oils during a  
60 day aerated batch culture at 25°C.

- - sand tar.
- - Forties crude.
- △ - synthetic crude.





period. This differs from previous findings in that the synthetic crude and sand tar showed similar degradation rates but the Forties crude markedly exceeded both of these. Figure 6.15 shows gas chromatographic profiles of the Forties crude at intervals up to 13 days during the experiment. There is a steady reduction in peak amplitude throughout the entire profile which suggests that there was simultaneous degradation of the range of different molecular weight compounds encompassed by the profile. The exception to this was certain mid-range molecular weight compounds whose peaks persisted until the 8th day but thereafter showed a decline. By the 13th day the profile was reduced to a predominately unresolved basal envelope and thereafter no significant change occurred. The gas chromatographic profiles of the synthetic crude are shown in Figure 6.16. These follow the same trend as the Forties crude showing steady, simultaneous degradation over the whole range of the profile. The absence of a resolved profile for the sand tar precluded the use of this method as a qualitative measure of degradation.

The sterile controls were carried out in an identical fashion except the flasks were shaken on an orbital shaker. The sand tar and Forties crude showed no appreciable loss, but the synthetic crude lost approximately 5% within 5 days but showed no loss thereafter. Viable counts on solid media did not reveal any microbial contamination and the loss may have been caused by oil washing off the silica support.

Figure 6.17 shows the microbial growth on the oils; this followed a similar pattern for each of the oils, except the

FIGURE 6.15

Gas chromatographic profiles of silica adsorbed Forties crude at intervals during the initial stages of a 60 day batch culture at 25°C.

- A - 0 day sample.
- B - 3 " " .
- C - 6 " " .
- D - 8 " " .
- E - 10 " " .
- F - 13 day sample.

Temperature increases from right to left across the profile. All profiles were obtained under identical conditions and are directly comparable.

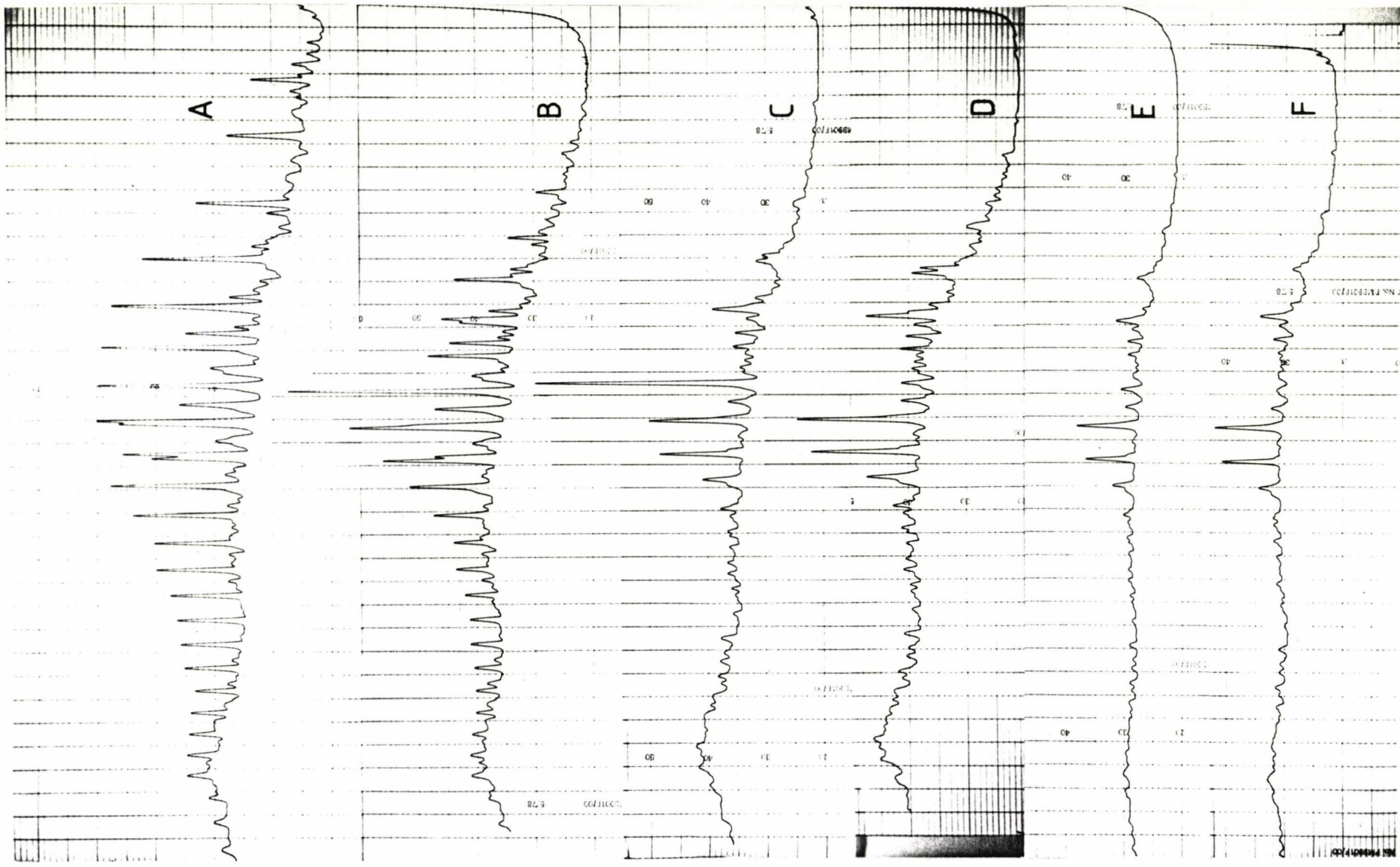


FIGURE 6.16

Gas chromatographic profiles of silica adsorbed synthetic crude at intervals during the initial stages of a 60 day batch culture at 25°C.

- A - 0 day sample.
- B - 3 " " .
- C - 6 " " .
- D - 8 " " .
- E - 10 " " .
- F - 13 day sample.

Temperature increases from right to left across the profile. All profiles were obtained under identical conditions and are directly comparable.



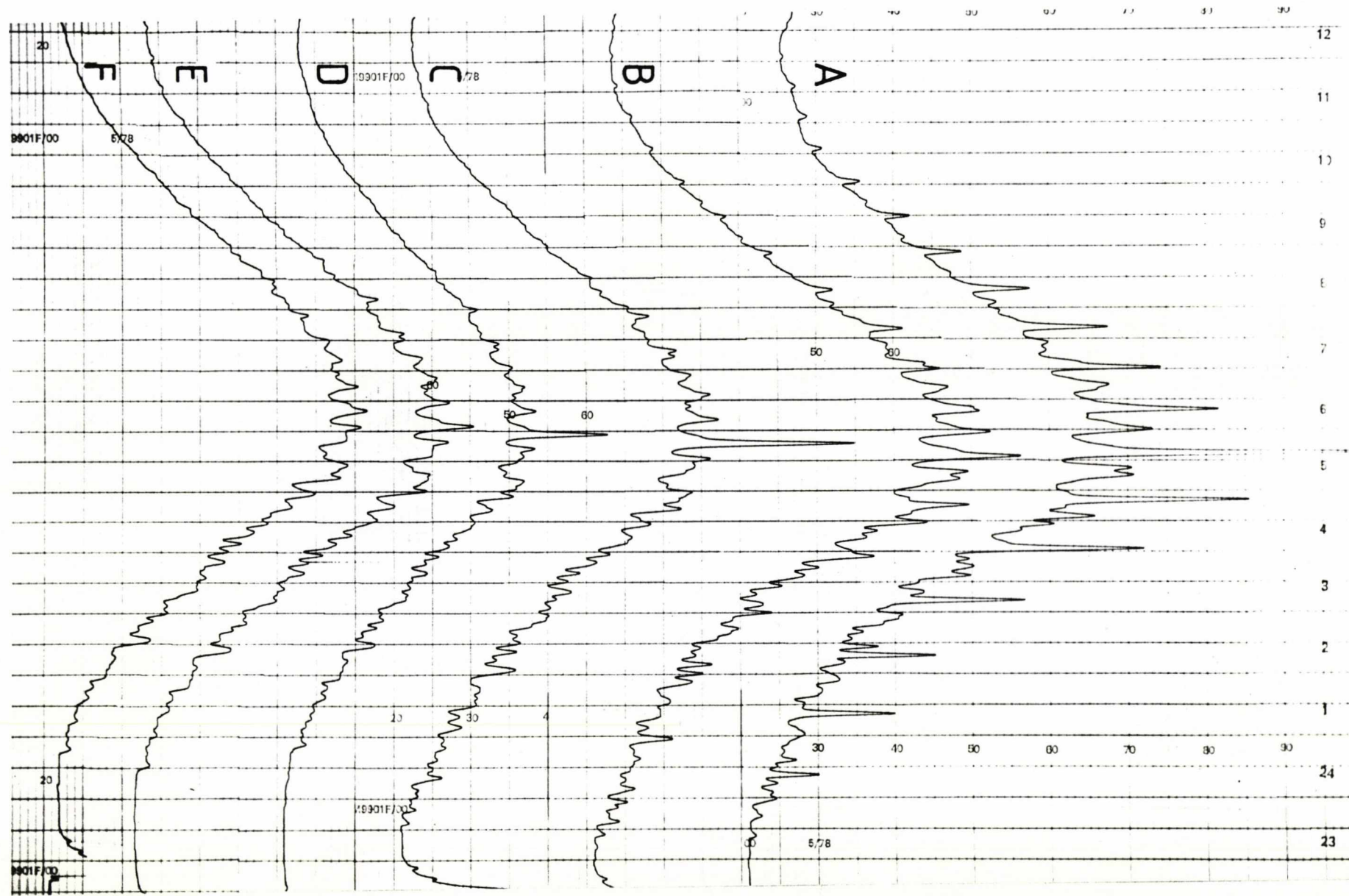
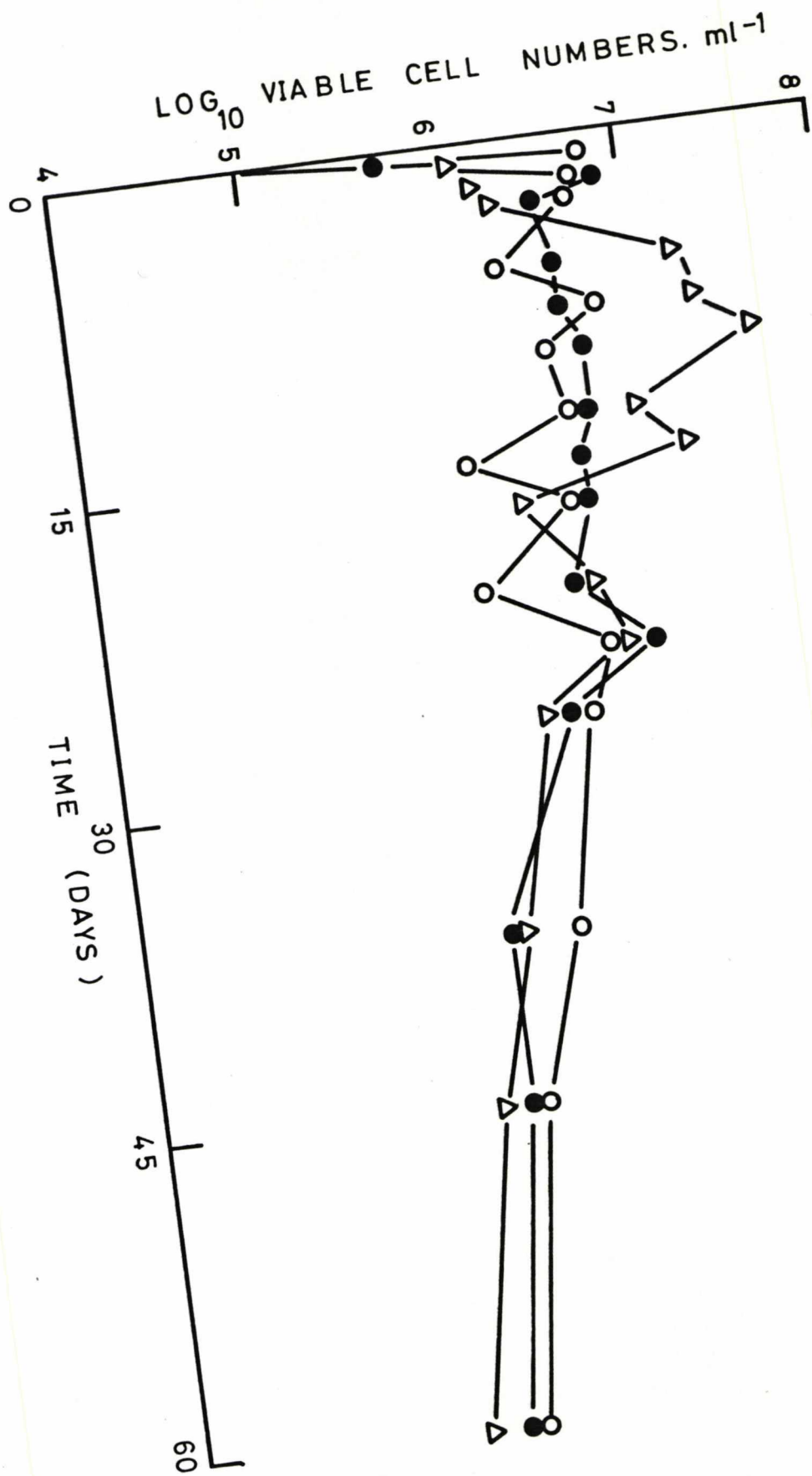


FIGURE 6.17

Viabile bacterial cell numbers ( $\log_{10}/\text{ml}$ ) isolated from silica adsorbed oils during a 60 day batch culture at 25°C.

- O - isolates from sand tar.
- - isolates from Forties crude.
- △ - isolates from synthetic crude.





cell numbers isolated from the synthetic crude were higher between the 6th and 16th days.

#### 6.8 GROWTH OF ENVIRONMENTAL ISOLATES ON PURE HYDROCARBON SUBSTRATES.

Strains  $N_1$ ,  $N_2$ ,  $N_3$ ,  $A_1$  and  $A_2$ , isolated as described (section 6.1), and strains PP and NST, isolated from oiled filters during in situ experiments, were tested for growth on a variety of pure hydrocarbon substrates. Strains PP and NST were selected as they showed excellent growth on oil agar. The incubations were performed at 25°C with shaking on an orbital shaker; the method of introduction of the substrate varied depending upon the type of hydrocarbon, as described previously (Chapter 2, section 2.5). Table 6.2 summarizes the results obtained. Strains  $N_3$ ,  $A_1$ , PP and NST grew on a wide range of n-alkanes whilst growth of strains  $N_1$ ,  $N_2$  and  $A_2$  was restricted to the higher n-alkanes from dodecane upwards. It is interesting to note that all of the strains grew on the multiple branched compound pristane. There was no visible growth on the simple aromatic or cycloalkane compounds that were tested.

#### 6.9 INVESTIGATION OF STRAINS $A_1$ AND PP FOR THE PRESENCE OF PLASMIDS.

Strains  $A_1$  and PP were chosen as both exhibited excellent growth on oil; the strains were prepared and examined for the presence of plasmids as previously described (Chapter 2,



**TABLE 6.2** Growth Of Environmental Isolates On Pure Hydrocarbon Substrates.

<u>Compound</u>	N <sub>1</sub>	N <sub>2</sub>	N <sub>3</sub>	A <sub>1</sub>	A <sub>2</sub>	PP	NST
Heptane	-	-	+	+	-	+	+
Octane	-	-	+	+	-	+	+
Nonane	-	-	+	+	-	+	+
Decane	-	-	+	+	-	+	+
Undecane	+	+	+	+	+	+	+
Dodecane	+	+	+	+	+	+	+
Tetradecane	+	+	+	+	+	+	+
Hexadecane	+	+	+	+	+	+	+
Octadecane	+	+	+	+	+	+	+
Pristane	+	+	+	+	+	+	+
Benzene	-	-	-	-	-	-	-
Toluene	-	-	-	-	-	-	-
M-Xylene	-	-	-	-	-	-	-
Cyclohexane	-	-	-	-	-	-	-
Methyl- Cyclohexane	-	-	-	-	-	-	-

+ Growth (turbidity)

- No growth

section 2.7.2). Strain A<sub>1</sub> exhibited a plasmid DNA band on the caesium chloride gradient and examination by electronmicroscopy revealed three size classes of plasmid with molecular weights as follows:

i.  $6.7 \pm 0.22 \times 10^6$  (30)

ii.  $8.5 \pm 0.18 \times 10^6$  (15)

iii.  $92 \pm 4.3 \times 10^6$  (2)

Examples of these are shown in Plate 6.2. Strain PP did not show a plasmid DNA band on the caesium chloride gradient.

#### 6.10 DISCUSSION

The site in the Medway estuary selected for the isolation of oil degrading microorganisms was chosen as it receives an input of oil from the Isle of Grain oil refinery. This should provide a natural enrichment for the indigenous oil degrading population. Samples were taken from the intertidal sediment as this receives surface oil films from the receding tide; these were evident in small patches at the time of sampling. Surface sediment was collected as oil degrading microorganisms should be most active in the aerobic layer and this is also in close proximity to deposited oil films.

The conventional enrichment procedure readily yielded a mixed microbial oil degrading population on each of the oils. It is interesting that the Forties crude and sand tar shared the same mixed population as generally, oils of different composition are utilized by separate mixed populations (Chapter 1, section 1.3.3). However enrichment on the synthetic crude

PLATE 6.2

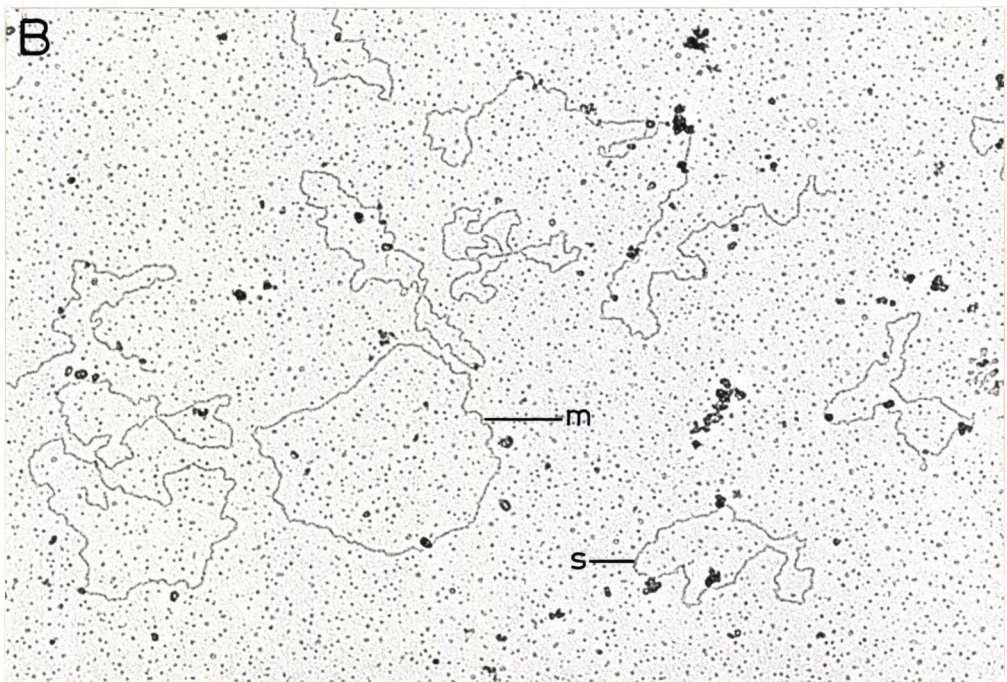
Transmission electronmicrographs of examples of the three size classes of plasmid DNA isolated from strain A<sub>1</sub>.

A - large plasmid  $92 \times 10^6$  molecular weight (x6,300).

B - m: medium plasmid  $8.5 \times 10^6$  molecular weight (x6,300).

s: small plasmid  $6.7 \times 10^6$  molecular weight (x6,300).







produced a separate mixed population; this may have been due to the large difference in composition between the synthetic crude and the other two oils. The absence of filamentous fungi and yeasts in the enrichment culture may have been caused by the pH of the medium (ca. 7.8) which could have inhibited their growth. With hindsight it would have been better to have performed separate enrichments at a lower pH (ca. 4 - 5). However, no oil degrading filamentous fungi or yeasts were isolated from the oiled filters exposed during in situ experiments, hence they may not play a significant role in oil degradation under these particular estuarine conditions. Presumptive identification of the bacterial isolates placed them in genera previously reported to contain oil degrading members.

Few of the conventional methods for quantitative assessment of microbial growth can be used successfully to monitor growth on oil. Dry weight determinations may prove inaccurate as it is not always possible to separate the cells from the oil substrate. An organic extraction of the oil/cell mixture can be used to quantify the amount of oil present but this in turn may alter the dry weight of the cells by removing their lipid components; although this was not found to be the case in cell and oil suspensions in liquid media (Chapter 2, section 2.4.1). Optical density measurements suffer from interference by oil droplets suspended in the medium, and the extent of this will depend upon the degree of emulsification. An added complication is the growth of cells in clusters around oil droplets. The method of Manfredini & Wang (1972), in which the hydrocarbon

phase is made water miscible by the addition of propionic acid, did not prove successful with any of the oils, as they could not be rendered water miscible. The three methods used to assess growth on each of the oils gave different results (section 6.2). In every case the total and viable counts followed a similar pattern of a rapid rise to maximum cell numbers which were broadly maintained for the remainder of the experiment. The total counts were consistently higher than the viable counts by 1 to 2 orders of magnitude; this may reflect inaccuracies in the methods of counting. During the viable count, inadvertent counting of oil droplets of a similar size to cells may have given rise to an overestimate of cell numbers. Conversely, the tendency of cells to surround oil droplets may have given rise to an underestimate of cell numbers during the viable count. Despite these factors the difference between total and viable cell numbers was remarkably consistent for each of the oils and throughout the experiment; and may reflect a real difference. In each case the optical density method gave fluctuating results throughout the experiment which bore little relation to the viable and total counts. This may have been caused by small amounts of residual oil in the cell suspension which could contribute a reading at 460 nm. The sand tar and Forties crude have a greater absorbance in this region than the synthetic crude and this may explain the lower optical density readings of the synthetic crude compared to the other two oils. Viable count was selected from the three methods for routine assessment of microbial growth and its use is



commonplace in many other studies on oil degradation.

The work of Gibbs (1975) has emphasized the important limiting effect of the low concentrations of inorganic nitrate and phosphate found in sea water. The semi-continuous addition of nitrate and phosphate during aerated batch culture (section 6.3) was an attempt to maintain relatively consistent high levels of these nutrients related to the changing pattern of growth on crude oil. The initial concentration of phosphate was  $500\ \mu\text{M}$  which was approximately 100 times the concentration found in sea water during the in situ experiments. Nitrate was initially added in the same concentration as the phosphate and then added in equimolar proportion to the subsequent phosphate additions. Uneven degradation rates on whole crudes are commonplace, but both the Forties crude and the synthetic crude showed apparent increases in the amount of undegraded oil at intervals over the 60 day experiment. The infrared method used to quantify the oil during these experiments only measures the total amount of oil and not changes in oil composition. Hence infrared alone could not be used to determine whether the apparent increases in undegraded oil were in fact due to oil alone or to other contaminating hydrocarbons. The gas chromatographic profiles of the Forties crude show the appearance of large quantities of novel hydrocarbons (between the 10th and 15th days) whose corresponding peaks occur at the high molecular weight end of the profile. Whether these hydrocarbons were present in the medium due to excretion or cell lysis or whether they were contained within the cells and released on  $\text{CCl}_4$

extraction, was not determined. However, extraction of cells grown on heptane vapour failed to give a significant infrared spectrum in the oil region; this would support the former explanation. The occurrence of novel high molecular weight hydrocarbons during microbial growth on oils has been previously reported (Chapter 1, section 1.3.2 C). No novel hydrocarbons could be detected during the other two periods of apparent oil increase which reached maxima at the 27th and 35th days. If novel hydrocarbons were produced at these times they may have been of sufficiently high molecular weight to escape detection by the gas chromatographic conditions employed. Those detected at the 14th day were close to the limit of detection at the high molecular weight end of the profile. Alternatively, the apparent increase at the 27th and 35th days may have an entirely different origin such as liberation of oil stored within the cell due to excretion or cell lysis.

The viable cell numbers isolated during the experiment exhibited the same marked fluctuation as the amount of oil present. The initial rapid rise to a maximum of  $10^{10}$  cells/ml was followed by a decline of 2 orders of magnitude. The first apparent oil increase was coincident with the fall in cell numbers and it may have been that the novel hydrocarbons were released during cell lysis or were extracted from dead cells. The second rise in viable cell numbers to  $10^{10}$ /ml and above was initiated after the novel hydrocarbons had disappeared from the medium so it was unlikely that these supported the second phase of growth. The third increase in cell numbers reached a



maximum at the 35th day but this was an order of magnitude less than the previous two increases and was thereafter followed by a decline.

The fluctuations in the amount of oil present and viable cell numbers during the 60 day experiment using the synthetic crude were not as great as those found during the Forties crude experiment. After an initial drop in the amount of oil there was an apparent rise between the 6th and 12th days, thereafter the fluctuations were small except for that between the 35th and 45th days. The absence of novel hydrocarbons in gas chromatograms suggested another origin for these fluctuations. The initial rise in viable cell numbers reached a maximum that was an order of magnitude less than that of the Forties crude and this was followed by a reduction by 2 - 3 orders of magnitude which was coincident with the first apparent rise in the amount of oil present. Thereafter, the viable cell numbers remained relatively consistent. The degradation of synthetic crude by pure and mixed cultures of strains  $A_1$  and  $A_2$  in batch culture (section 6.4) gave similar results to the initial stage of the 60 day aerated batch culture experiment. Strain  $A_1$  and the mixed culture showed a rapid decrease in the amount of oil present followed by an apparent increase, within the first ten days of the experiment, a similar pattern to that found during the initial stage of the 60 day culture. The maximum viable cell numbers reached by strain  $A_1$  in pure and mixed culture were of the same order of magnitude as the maximum cell numbers reached during the 60 day culture.

The presence of inclusion bodies in strain A<sub>1</sub> may explain the fluctuations observed in the amount of oil present in the medium. At the time of minimum oil content in the medium the cells exhibited inclusion bodies which contained a material with a very similar gas chromatographic profile to synthetic crude. It may have been that the initial decrease in oil present was the combination of degradation and storage of oil in intracellular inclusions. The release of oil from these inclusions during cell lysis may have given rise to the apparent increase in oil present in the medium. The viable cell numbers of strain A<sub>1</sub> decreased by roughly 1.5 orders of magnitude between the 5th and 10th day during which time the amount of oil in the medium apparently increased. Such a mechanism may have been active during the initial stages of the 60 day aerated batch culture experiment. Intracellular inclusion bodies containing lipid, exhibited during the growth of bacteria on hydrocarbons have been previously reported (Scott & Finnerty, 1976).

The pattern of oil degradation at low temperature (section 6.5) follows the familiar course found during the in situ experiments i.e. synthetic crude > Forties crude > sand tar. The pattern is more even than that found in the aerated batch culture experiments, but this may merely reflect the longer time intervals between samples employed in the low temperature experiment. The results from the low temperature experiment may be compared to those of the non-photooxidized oils whose incubation was carried out at 25°C, using the same replica sample procedure (Chapter 2, section 2.4.2), but the inocula in this case were



provided by the original isolates (section 6.1). With each of the oils the extent of degradation reached at the conclusion of the low temperature experiment (74 days) was exceeded at the time of first sampling of the 25°C experiment (5 days). This indicates a substantially slower rate of degradation at the lower temperature. The maximum viable cell numbers attained on each oil at 4 and 25°C were closely similar but these were reached within 5 days at 25°C compared to 23 days at 4°C.

Photooxidation has been reported to alter the composition of oils which may in turn influence their microbial degradation (Chapter 1, section 1.3.1). The wavelength of ultraviolet light used for photooxidation (254 nm.) and its intensity was not representative of the ultraviolet portion of sunlight which is closer to the region of 300 - 320 nm. However the shorter U.V. wavelengths tend to increase the alterations in composition due to photooxidation (Hansen, 1975) and this in turn should emphasize any changes in microbial degradation resulting from photooxidation. The gas chromatographic profiles of the photooxidized and untreated synthetic crude and Forties crude showed no detectable difference, and with hindsight it may have been wiser to have photooxidized the oils for a longer time. For the Forties crude and sand tar the treated and untreated oils showed very similar degradation patterns, but with the synthetic crude the treated oil showed markedly slower degradation. This would suggest that the photooxidation of the synthetic crude alters the composition of the oil in a manner unfavourable to microbial growth. Despite the difference in degradation the

viable cell numbers isolated from treated and untreated synthetic crude were similar throughout the experiment.

The aim of the degradative experiments using silica adsorbed oils (section 6.7) was to exploit the natural tendency of oils to adhere to surfaces; using this property a uniform suspension of silica particles with adsorbed oil was obtained in the medium. This permitted samples containing closely identical amounts of oil to be withdrawn from the culture vessel. This procedure is not normally applicable to non water miscible substrates as they are generally not dispersed uniformly within the medium. In the case of the synthetic crude and Forties crude emulsification provided a suitably uniform suspension, but this was not true for the sand tar whose high viscosity impeded any significant emulsification. Silica adsorption overcame this problem and the synthetic crude and Forties crude were treated likewise for comparison. Using this procedure the pattern of oil degradation was radically altered from that found previously. The order in rate and extent of degradation was found to be Forties crude > synthetic crude > sand tar and the difference between the synthetic crude and the sand tar was very slight. The degradation of the Forties crude was similar to that found during the experiment using photooxidized and untreated oils (section 6.6). This was also true for the sand tar. The synthetic crude however showed a much reduced rate and extent of degradation, hence silica adsorption appeared to be an acceptable method for the Forties crude and sand tar, but in some way inhibited degradation of the synthetic crude. Despite this the gas chromato-



graphic profiles show a steady degradation of the synthetic crude as well as the Forties crude. The viable cell numbers isolated from the silica adsorbed oils were slightly lower than those found in previous experiments. This may have been due to the attachment of cells to the oiled silica surface which was observed under phase contrast microscopy. This phenomenon would tend to give an underestimate of viable cell numbers.

The hydrocarbon substrate specificity of the primary isolates and of two strains isolated from oiled filters exposed in situ was restricted to the alkanes (section 6.9). This suggests that all of the isolates were growing at the expense of the saturate fraction of the oils. It is interesting that they all degraded pristane, a multiply branched hydrocarbon generally regarded as relatively recalcitrant. The lack of growth on simple aromatic and cycloalkane compounds may reflect the method of enrichment. For this whole crudes were used which contain a readily metabolizable alkane fraction. With hindsight it may have been advisable to have used a sequential enrichment procedure similar to that of Horowitz et al (1975).

The existence of plasmids coding for degradative pathways has been confirmed, Williams (1978) but the extent of their occurrence in natural populations is an area where knowledge is lacking. Of the three size classes of plasmid found in strain A<sub>1</sub> only the largest may code for enough information to be a possible degradative plasmid.

**CHAPTER 7**

**DISCUSSION**



## DISCUSSION

7.1 GENERAL

The aim of the environmental part of the project was to obtain quantitative measurements on oil degradation under in situ conditions and to assess the microbial contribution to this process. The majority of in situ investigations (Chapter 1, section 1.4.3) have used G.L.C. as a measure of microbial degradation. This has given mainly a qualitative assessment. Quantitative measurements on overall oil loss are difficult to obtain as it is hard to contain oil under in situ conditions, especially if the oil is present as a floating film allowing free interchange with the environment. Some oil may be lost from equipment by wind and wave action and unless this can be quantified, quantitative measurements on oil degradation are impossible. In order to overcome this problem, the oils used for in situ experiments throughout the project were contained by adsorption to an inert matrix (Chapter 3, sections 3.2 and 3.3). This had the disadvantage of departing from the natural free floating film but the small amount of oil present (ca. 100 - 140 mg. per 47 mm diameter filter disc) was adsorbed over a large surface area. This presented the majority of the oil in a form favourable to microbial colonization, a situation similar to that of a free floating film. The question of the filter material supporting microbial growth has been more thoroughly discussed elsewhere (Chapter 5, section 5.2.1). The equipment used to contain the oil samples at sea did not permit much light to reach the samples, hence any changes in

oil composition due to photooxidation would have been minimized. The oils were 'topped' before adsorption, however, to simulate natural evaporation occurring during weathering. The direct measurements on total oil loss can be most conveniently performed gravimetrically or by infrared spectroscopy. The former requires thorough drying and may incur errors due to the weight of salts left after evaporation and by the dry weight of macro- and microbiota colonizing the filter surface. Infrared spectroscopy may incur errors during extraction and by contaminating hydrocarbon from the environment or from the biological colonization. The blank control filters exposed during in situ experiments gave some assessment of the degree of contaminating hydrocarbon from the environment. That from microbial colonizers was not estimated directly but bacteria grown on heptane vapour provided little contamination (Chapter 2, section 2.4.1).

The data from the in situ experiments is presented as total oil loss from the filters. This encompasses loss due to physical factors such as 'wash off' and dissolution as well as loss mediated by microbial action (the latter is more fully discussed in Chapter 4, section 4.2). The non-biological controls (oils leached in sterile sea water) attempted to estimate oil loss from the filters by dissolution and 'wash off'. These were performed in the laboratory however, and were not subjected to identical conditions found during in situ experiments. Hence data from in situ experiments was not corrected for this loss, as it was not directly comparable. Nevertheless, these controls did give an estimate of the extent and nature of the non-biological



oil loss. They showed that the loss was different for each oil, occurred within a finite period of time and in the case of the Forties crude and synthetic crude were restricted to the low molecular weight G.L.C. profile of the oil. All of these attributes are consistent with loss due to physical factors. Loss of oil from the filters mediated by microbial action includes true biodegradation as well as secondary losses of metabolic intermediates and undegraded oil mediated by microbial surfactant production. Unfortunately these were indistinguishable.

Despite the limitations of the experimental system, the in situ experiments yielded reproducible data on three oils of widely different composition under both winter and summer conditions. Throughout all the experiments the rate and extent of degradation always followed the same order i.e. synthetic crude > Forties crude > sand tar. This shows the importance of oil composition in influencing degradation; a phenomenon that has been widely reported (Chapter 1, sections 1.3.2 C and 1.3.3). The order of degradation found follows the order of decreasing saturate fraction content of the oils i.e. the synthetic crude possessed the highest saturate fraction (86%) and the sand tar the lowest (28%). Again this conforms to predictions that can be made on the basis of previous findings. It is widely reported that the saturate fraction is the most susceptible to degradation and that oils with a high saturate fraction are more rapidly and extensively degraded than those with a low saturate content. Fractional analysis of the Forties crude and sand tar during the

second summer experiment confirmed that the saturate fraction sustained the greatest loss.

Two of the most important environmental parameters reported to influence in situ degradation rates are temperature and the supply of inorganic nitrogen and phosphorus (Chapter 1, sections 1.3.4 A and B). In the case of the in situ experiments the degradation rate in terms of oil loss from the filters was always higher at the higher temperatures found in the summer months. This was confirmed by the increased rate of microbial colonization and the increased disappearance of a resolvable G.L.C. profile at this time. This showed that at the inorganic nutrient status found in the estuary (which showed little variation between winter and summer experiments), temperature and not inorganic nutrient supply was limiting degradation during the winter months, as during the summer higher degradation rates were found. Whether this was true during the summer months was not determined and can only be confirmed by observing if higher rates can be obtained by supplementation with nitrogen and phosphorus (oleophilic fertilizers could be employed).

The limited diversity of the oil degrading microbial population colonizing the oiled filters is surprising. During both winter and summer experiments only three different bacterial strains were isolated and these appeared to be similar for each oil. This finding is contrary to those reported for conventional enrichment procedures in which crudes of different composition (Chapter 1, section 1.3.3) support different microbial populations. The absence of filamentous fungi and the paucity of yeasts



is consistent with previous reports (Chapter 1, section 1.3.2 A). The remarkably similar colonization patterns found throughout the in situ experiments may reflect a physical constraint on numbers, such as the area available for colonization. The scanning electronmicrographs of the filter surfaces suggest that within a short period of time (5 - 13 days) the colonizing bacteria became embedded in an extracellular matrix. This finding is commonplace when observing the attachment of bacteria from environmental isolates to surfaces.

The high numbers of oil degrading bacteria isolated from oiled filters was only indirect evidence for their participation in oil degradation. Direct evidence was provided by the G.L.C. profiles of oils recovered at intervals during the in situ experiments. The high molecular weight profile remaining after the low molecular weight profile had been removed by leaching steadily declined to an unresolved basal envelope; a pattern indicative of biodegradation. The changes in fractional composition of the Forties crude and sand tar i.e. a progressive decrease in saturate fraction and increase in PNSO fraction are again common consequences of microbial growth on oils. Furthermore the occurrence of novel high molecular weight peaks in the G.L.C. profile of the Forties crude (Figure 4.4 G) after 12 days exposure during the second winter experiment are similar to those produced after the same time during microbial growth under laboratory conditions (Figure 6.6). Taking all of these factors into consideration it is reasonable to assume that the oil degrading microbial populations isolated from the oiled filters

were directly responsible for degradation of the oil samples.

The total heterotrophic and oil degrading bacterial populations isolated from surface water samples in the vicinity of the buoy are high compared to previous reports (Chapter 1, section 1.3.2 A). This may reflect the position of the site in which tidal, wind and wave action in the shallow waters of an estuary may cause agitation and suspension of sediment material which in turn can increase numbers of bacteria in the water column.

The data gained from the in situ experiments was derived from small amounts of oil exposed in an estuarine location. This is not particularly relevant to large spills of oil in the open ocean where the number of indigenous oil degrading bacteria and the supply of inorganic nutrients will be less abundant. The data could be relevant however, to the small amounts of oil produced when a slick is dispersed, or to small amounts of oil released during long term pollution. In these cases the amount of oil is not likely to 'overload' the natural capacity of sea water to support its degradation. This is a situation similar to that of the in situ experiments.

The study of the degradation of the three oils under laboratory conditions was performed to complement the in situ experiments. The aim was to determine if degradation patterns and rates were affected when supplemented media and elevated temperatures (compared to the environment) were employed. Comparison of the in situ and laboratory experiments is only of limited value as the two represent very different systems.



The in situ experiments represented an 'open' situation in which there was a continuous supply of low levels of inorganic nutrients and of potential oil degrading microbial colonizers. Furthermore, the metabolic products of oil degradation were removed from the system. The closest analogy to this which could be constructed in the laboratory would be growth on oil using continuous culture. The batch culture system employed in the laboratory experiments was, in contrast, a closed system in which any metabolic products resulting from oil degradation remained in the system. Furthermore, the diversity of the microbial oil degrading population and the supply of inorganic nutrients was determined at the start of the experiment. With the exception of the silica adsorbed oils those in the laboratory experiments were present as an emulsion or as an oil film, whilst those in the in situ experiments were adsorbed to filters.

The microbial growth on oils during aerated batch culture with semi-continuous addition of nutrients (Chapter 6, section 6.3) did not offer much scope for comparison. With both the Forties crude and synthetic crude the pattern of oil degradation followed a markedly fluctuating course far above any fluctuation found during in situ experiments. The experiments did, however, reveal the interesting phenomena of the presence of intracellular inclusions and the production of novel hydrocarbons. In both experiments to study the degradation of photooxidized oils and the degradation of oils at low temperature (Chapter 6, sections 6.6 and 6.5 respectively) the rate and extent of degradation followed the order found during the in situ experiments i.e.

synthetic crude > Forties crude > sand tar. Comparing the results of oil degradation at 4°C to those of the in situ winter experiments it can be seen that the synthetic crude was apparently less extensively degraded in the former. However, during the in situ experiments up to 50% of the synthetic crude was removed from the filters by leaching and 'wash off' and this may explain the difference. If this is taken into account then the biological degradation at the conclusion of the in situ experiments was 33% (50 days) and 30% (80 days). That of the laboratory experiment was 48% (74 days) but this was in supplemented medium. Applying similar reasoning to the Forties crude, 27% was biologically degraded after 50 days of the first winter experiment, compared to 30% after 46 days of the laboratory experiment. During the second winter experiment 22% was biologically degraded after 26 days, compared to 24% after 28 days of the laboratory experiment. These figures show better agreement than those for the synthetic crude. The corresponding values for the sand tar at the conclusion of the second winter experiment (80 days) and the laboratory experiment (74 days) were 7% and 13% respectively. These figures are only broad comparisons but in the case of the Forties crude and sand tar do show some agreement.

Comparing the summer experiments to that of the degradation of the untreated oils at 25°C (Chapter 6, section 6.6) it can be seen that all of the oils were more rapidly degraded during the initial stages of the laboratory experiment than in either of the in situ experiments. This may simply reflect the use of a



supplemented medium and the higher temperature. After the initial period, however, the degradation rate was reduced, a phenomenon also found during the in situ experiments. This suggests a rapid utilization of the readily metabolizable components of the oil followed by slower growth on the more recalcitrant components.

The degradation of the silica adsorbed oils showed a different order to that found during in situ experiments and the laboratory experiments discussed above. This is more fully discussed elsewhere (Chapter 6, section 6.10).

In conclusion, it can be said that where comparable, the findings of the laboratory experiments broadly reflect the degradation patterns found during the in situ experiments. However, the supplemented medium and elevated temperatures in the former increased degradation rates. Considered independently the laboratory experiments show the important effect of the form in which oil is added to a microbial culture and the difficulty of assessing microbial growth supported by the oil. They also indicated interesting phenomena occurring during growth on oil such as novel hydrocarbon production and the presence of intracellular inclusions.

## 7.2 FUTURE WORK

Only a limited number of in situ experiments were performed during the project and only at specific times of the year. Experiments covering those periods omitted would provide a more complete picture, and would give a closer indication of the

influence of changing environmental conditions on degradation rates. The inclusion of oils containing oleophilic fertilizers could provide information on the limiting effect of inorganic nutrient supply especially during the summer months. The experimental system only monitored the fate of floating oil but did not provide any information on the fate of oil in estuarine sediments. It would be interesting to see if the system could be adapted to meet this situation. Unfortunately the equipment was only deployed at one site; an estuarine location subject to oil pollution. Deployment in a relatively pristine 'open' ocean site would provide a useful comparison but this would entail many logistical problems. The equipment used for the in situ experiments does have its limitations. Modifications, for example to permit more light to enter the system or to bring the samples closer to the natural state of a free floating film would be beneficial. The laboratory controls to assess non-biological oil loss from the filters were satisfactory, but no such controls were devised to differentiate between the different forms of microbially mediated oil loss. This is an important omission and merits further investigation although solutions to this problem are not readily forthcoming.

The phenomena of intracellular oil inclusions and novel hydrocarbon synthesis found during laboratory experiments have both been reported elsewhere in the literature. However, both these could benefit from further investigation, especially concerning the extent of their occurrence and their influence on the growth of mixed populations.



The presence of a possible degradative plasmid in strain A<sub>1</sub> was an interesting finding, however its function was not confirmed and this requires further curing experiments. This also raises the question of how commonly degradative plasmids occur in natural populations and what role they play in oil degradation. The concentration of oil degrading bacteria at the oil water interface could provide a favourable environment for plasmid transfer.

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