Biodegradability of Chemically-Dispersed Oil

A report produced for the Minerals Management Service (MMS), Alaska Department of Environmental Conservation (ADEC) and United States Coast Guard (USCG).

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Executive Summary

Operational experience shows that conventional oil spill response techniques, such as mechanical recovery, remove only a small fraction of oil during major oil spills. By contrast, the use of dispersants can enable significant fractions of oil to be removed from the sea surface by dispersing the oil into the water column. However, the use of dispersants raises concerns over the fate and impact of chemically-dispersed oil. This is particularly important as there is an increasing demand for responders to assess the net environmental benefit of an operational response to an oil spill.

Whilst much research has been undertaken on the effect of dispersants on the fate of dispersed oil, the results have often been conflicting. Some workers have proposed that dispersants have little effect on oil biodegradation, some suggest a positive effect and some note a negative effect (Robichaux & Myrick, 1972; Mulkins-Phillips & Stewart, 1974; Traxler and Bhattacharya, 1979; Foght & Westlake, 1982; Lee *et al.*, 1985; Litherathy *et al.*, 1989). Therefore further research remains to be undertaken in order to determine the fate and impact of dispersed oil in order to provide responders with crucial information when choosing an appropriate response option.

To investigate the effect of chemically-dispersed oil, a programme of research was commissioned by the Minerals Management Service (MMS), Alaska Department of Environmental Conservation (ADEC) and the U.S. Coast Guard (USCG). This project builds on a continued programme of research on the fate of dispersed oil. In the initial research project, which was undertaken on behalf the Department of the Environment, Transport and the Regions (DETR), experiments were conducted to study the interactions between bacteria, oil, various dispersants and suspended solids (Swannell & Daniel, 1999). The results of the research indicated that dispersants can enhance the rate of biodegradation of dispersed oil (with Forties crude oil), this is investigated further in this research project. The previous studies involved the construction of laboratory microcosms simulating marine conditions, which were shown to produce oil dispersion patterns similar to those seen in the field. In addition, a technique allowing the sampling of dispersed oil droplets in the microcosms, without perturbing their distribution, was developed by the project team. The techniques developed in the previous research projects were also used in this research project.

The experiment conditions were set to be directly relevant to the conditions encountered during a spill in the Prince William Sound (low nutrient levels and sea temperatures of 8°C). These temperature conditions and nutrient levels are lower than those used in the previous experiments for the DETR (which were undertaken in U.K. conditions, i.e. sea temperatures of 15°C). This allowed the effect of reducing the nutrient level and sea temperature to be investigated. In addition the tests were undertaken with a different oil and dispersant; Alaska North Slope crude oil (ANS) and Corexit 9500, allowing the investigation of the effect of oil type and the potential to directly apply the results to an oil from the U.S.

The results of the this research project indicate that:

- The mechanisms of the interaction between bacteria and dispersed oil correlates with the findings of the previous DETR work. Oil droplets were rapidly colonised by bacteria leading to the formation of clusters of oil droplets and bacteria trapped in a gel-like matrix. With time, the size of the clusters increased and they sank at the bottom of the microcosms, presumably because of a decrease in buoyancy due to oil biodegradation.

- Oil degraders proliferated in all biologically-active microcosms. Maximum microbial growth rates for the dispersant tests were approximately one fifth of those observed with Forties crude oil at 15^{o}C, presumably reflecting slower growth on oil at the lower temperature of 8^{o}C. In the absence of the dispersant, the onset of colonisation was delayed, although microbial growth rates and population sizes were greater than had been observed previously with Forties crude oil. This difference probably reflects the greater natural dispersion seen with ANS at 8^{o}C, than was seen with Forties at 15^{o}C.

- The utilisation of hexadecane as a carbon source leading to the formation of carbon dioxide and water is called hexadecane mineralization. The hexadecane mineralization results suggest that *n*-alkanes biodegradation was occurring in the natural dispersion tests. They also suggest that in presence of dispersant the rate of mineralisation was substantially increased. This is observation is supported in part by the oil chemistry analysis, although again, less oil degradation was observed with ANS at 8^{o}C than was found with Forties at 15^{o}C.

The previous research undertaken for the DETR has shown that micro-organisms can promote the physical dispersion of oil by colonising and biodegrading dispersed droplets (Daniel & Swannell, 1998). Addition of dispersants has been shown to stimulate the colonisation and biodegradation of dispersed oil even at the low nutrient levels (Swannell & Daniel, 1999). This finding suggested that the dispersion (physical or chemical) and biodegradation of oil reduces the impact of the oil on the marine environment, potentially having important implications for the management of oil spills in relation to the policy for dispersant use in the event of an oil spill incident. An additional finding from this research was that the indigenous hydrocarbon-degrading marine micro-organisms were shown to promote oil dispersion and biodegradation of both Forties or Gullfaks crudes provided that sufficient nutrients were available for microbial growth. However, this was a relatively slow process with substantial oil biodegradation being completed within 4 to 6 weeks depending on the nature of the oil tested. The application of dispersants to an oil slick at sea had a dual environmental benefit, stimulating both oil dispersion and oil biodegradation, an observation consistent with the findings of Varadaraj *et al.* (1995) and others (Mulyono *et al.*, 1994).

The previous work (Swannell & Daniel, 1999) with Forties crude oil at 15^{o}C suggested that in addition to increasing the amount of oil present in the water column for biodegradation, Corexit 9500 stimulated the growth of the hydrocarbon-degrading population, leading to increased oil biodegradation in comparison to natural dispersion. However, the work undertaken in this research project with ANS produced results which were different from those observed with Forties weathered crude oil at 15^{o}C. Fundamentally there was evidence that at 8^{o}C the addition of dispersants stimulated microbial colonisation of dispersed oil droplets and promoted microbial decomposition of components of the oil. However, the

effect was small in comparison to previous observations and this may be related to oil type and the lower temperature.

In conclusion, there is evidence that dispersant addition promotes the biodegradation of ANS crude oil at 8ºC. However, the effect is much smaller than that observed with a more dispersible and biodegradable oil (Forties) at 15ºC (Swannell & Daniel, 1999). From the results of the present work, it is not possible to conclude whether this is due to the different nature of the oil or to the effect of temperature or to a combination of both parameters. Therefore in future work, it will be important to determine the controlling parameter to gain a full understanding of the effect of dispersants on the fate of dispersed oil.

A recommended extension of this work would be to run a set of experiments with ANS crude oil at 15ºC, or with Forties crude oil at 8ºC to determine which of the parameters is reducing the effect of dispersant on the biodegradation of oil under simulated marine conditions. The recommended next phase of the research is to validate the findings from both the laboratory based projects with large scale experiments at sea. This is essential before the results can be used with confidence to contribute to the development of strategies for dispersant use.

Contents

1 Introduction

Research on the effect of dispersants on the fate of dispersed oil has often been conflicting, with some workers proposing that dispersants had little effect on oil biodegradation, some suggesting a positive effect and some noting a negative effect (Robichauz & Myrick, 1972; Mulkins-Phillips & Stewart, 1974C; Traxler and Bhattacharya, 1979; Foght & Westlake, 1982; Lee *et al.*, 1985; Litherathy *et al.*, 1989). It has been suggested that dispersants tend to increase oil biodegradation by increasing the surface area for microbial attack, and encouraging migration of the droplets through the water column making oxygen and nutrients more readily available (Mulyono *et al*., 1994: See Diagram 1). However, some dispersants were also found to have a toxic effect on microbial processes retarding the rate of oil decomposition (Mulyono *et al.*, 1994; Varadaraj *et al.*, 1995). Varadaraj *et al.* (1995) studied this dual capability of dispersants of increasing the surface area of dispersed oil and affecting the growth of hydrocarbon-degraders, and concluded that both of these factors influenced the effectiveness of dispersants at promoting oil decomposition. They found that increasing the sorbitan content of their dispersant stimulated oil biodegradation by acting as a nutrient source for the growth of hydrocarbon-degraders.

Research conducted by National Environmental Technology Centre (NETCEN) for the Department of the Environment, Transport and the Regions (DETR) has shown that micro-organisms can promote the physical dispersion of oil by colonising and biodegrading dispersed droplets (Daniel & Swannell, 1998). Addition of dispersants has been shown to stimulate the colonisation and biodegradation of dispersed oil even at the low nutrient levels (Swannell & Daniel, 1999). This finding suggested that the dispersion (physical or chemical) and biodegradation of oil reduces the impact of the oil on the marine environment, potentially having important implications for the management of oil spills in relation to the policy for dispersant use in the event of an oil spill incident.

For the initial research undertaken for the DETR experiments were conducted to study the interactions between bacteria, oil, various dispersants and suspended solids (Swannell & Daniel, 1999). Microcosms simulating marine conditions were constructed which were shown to produce oil dispersion patterns similar to those seen in the field. A technique allowing the sampling of dispersed oil droplets in the microcosms, without perturbing their distribution, was also developed by the project team. Through this research a series of studies were carried out utilising a number of different oils and dispersants. These included:

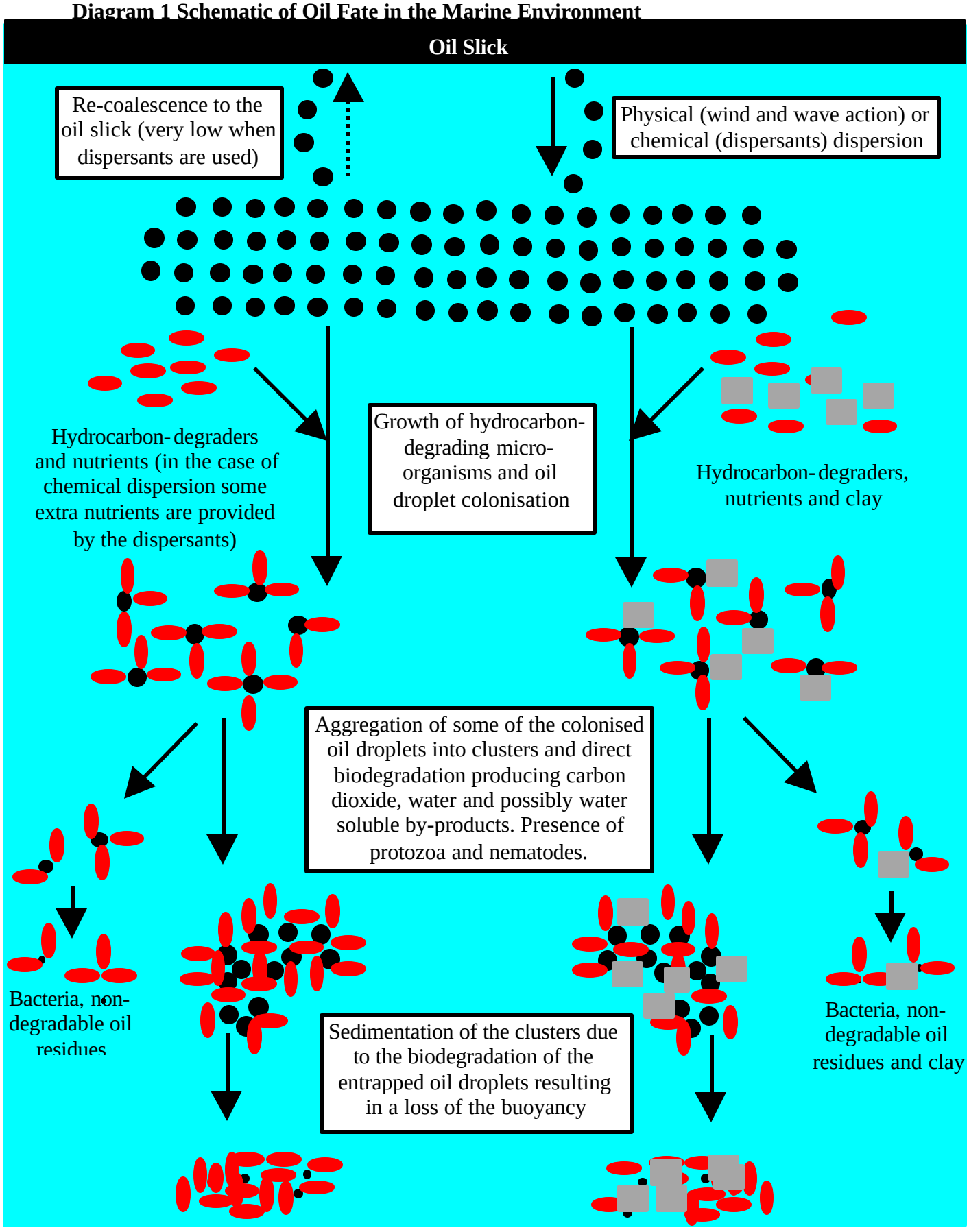
- Weathered Forties crude oil (the oil spilled during the *Sea Empress* incident in 1996), in the absence of dispersants, using artificial and natural seawater. The effect of nutrients on oil dispersion and degradation was also considered.
- Weathered Gullfaks crude oil (the oil spilled during the *Braer* incident in 1993) studied in the same manner as Forties crude oil in natural seawater.
- The dispersants Corexit 9500, Enersperse 1583, Finasol OSR-51 and Dasic Slickgone LTSW with weathered Forties crude oil in natural seawater.
- The effect of clay oil interaction on promoting oil dispersion. This refers to suspended clay sized mineral particles found in natural seawater, commonly found in coastal waters. Clay oil flocculation has been implicated in helping to remove oil from shorelines following the *Exxon Valdez* incident in 1989 (Bragg and Yang, 1995).

A number of findings were drawn from these studies. The indigenous hydrocarbon-degrading marine micro-organisms were shown to promote oil dispersion and biodegradation of both Forties or Gullfaks crudes provided that sufficient nutrients were available for microbial growth. However, this was a relatively slow process with substantial oil biodegradation being completed within 4 to 6 weeks depending on the nature of the oil tested. The application of dispersants to an oil slick at sea had a dual environmental benefit, stimulating both oil dispersion and oil biodegradation, an observation consistent with the findings of Varadaraj *et al.* (1995) and others (Mulyono *et al.*, 1994). The success of the dispersant at stimulating oil biodegradation was apparently dependent on its ability to promote the growth of indigenous hydrocarbon-degrading micro-organisms, again supporting the contention of Varadaraj *et al.* (1995). The presence of suspended clay particles substantially increased the rate of oil dispersion and subsequent biodegradation, presumably by providing surfaces on which the micro-organisms could grow and maintain higher populations than that observed in seawater in the absence of clay. This finding concurred with the observations made during the *Sea Empress* and *Exxon Valdez* incidents, *i.e.* that microniches of clay, microbes and oil form naturally under marine conditions (Lunel *et al.*, 1995; Lee *et al*., 1997; Bragg and Yang, 1995). These mixtures are neutrally-buoyant and may promote the decomposition of oil residues.

In all the studies described, the micro-organisms quickly colonised the small dispersed oil droplets (<10 µm) causing some to aggregate into clusters (micro-niches) of oil droplets, bacteria, protozoa and nematodes. The buoyancy of the clusters decreased over time as the entrapped oil was biodegraded. Consequently, the clusters sank to the bottom of the microcosms but only after substantial biodegradation. The time it takes for this process to be completed is probably dependent on the concentration of available nutrients, the amount of physical dispersion of the oil, the initial concentration of the hydrocarbon-degrading microbial population and the temperature. In our experimental microcosms, removal of the readily biodegradable (alkanes and 3 rings PAHs) oil residues was complete within 2-6 weeks, depending on the experimental conditions. The presence of dispersants or suspended clay minerals significantly stimulated this process, both by promoting microbial growth by between 10 and 100 times and by increasing the number of dispersed droplets in the water column by similar amounts. Thus, it was suggested that the presence of these materials promoted the rate of oil biodegradation. The findings of the research to date have been used to prepare a schematic diagram of the fate of oil in the water column (Diagram 1).

Such findings are of crucial importance to the understanding of the fate of oil in the marine environment, and to devising responses to oil spill incidents that result in a net environmental benefit. The research undertaken herein aims to build on the research described above to further validate the laboratory studies and undertake studies on the rate of oil biodegradation at a temperature characteristic of Prince Williams Sound (PWS). Through these laboratory based microcosm studies the effects of temperature, nutrient content, oil type and dispersant formulation are investigated. The research is undertaken on behalf of the Minerals Management Service (MMS), Alaska Department of Environmental Conservation (ADEC) and the U.S. Coast Guard (USCG). The project team consists of NETCEN, the University of Newcastle upon Tyne, and the University of Alaska at Fairbanks.

Diagram 1 Schematic of Oil Fate in the Marine Environment



1.1 PURPOSE AND OBJECTIVES

The objective of this research project was to determine whether dispersant application not only reduces the risk of the oil polluting the shoreline, but also enhances the rate of removal of the oil from the environment. Alaska North Slope crude oil was selected for this phase of research. The temperature characteristics (regimes) consistent with Prince William Sound in Alaska were used in the microcosm experiments. In utilising the microcosm approach, this work was carried out in the context of an ecosystem approach to the management of the marine environment.

The specific objectives of the project were to investigate:

1. The fate of the chemical components of the dispersed oil over time.
2. The response of the microbial community in terms of rate of growth of hydrocarbon degraders and the relationship to rates of biodegradation.
3. The rate of biodegradation under laboratory conditions for naturally and chemically dispersed oil.

2 Materials and Methods

2.1 EXPERIMENTAL APPARATUS

Laboratory microcosms simulating marine conditions were constructed, based on the apparatus designed by Mackay and Szeto (1980). The microcosms consisted of three glass vessels, each large enough to contain 15 litres of seawater while still providing headspace (approximately 10 to 15 litres). As shown in Figure 1, these were sealed with thick metal lids containing sampling ports and air inlet and outlet points. Each lid had three sampling ports with taps, two of which were situated on a removable hatch. Two large section apertures were used as the air inlet and outlet. The air inlet consisted of a pipe at a 90° angle directing the air flow onto the water surface. This served to simulate wind and wave action. The air speed in the three microcosms was identical at 5.0 ± 0.5 m/s. To control the temperature throughout the experiments, the three microcosms were placed into an aquarium filled with chilled water. Water was recirculated through a refrigeration unit with a temperature of 8°C maintained throughout the experiments, representative of marine conditions around the Alaskan coasts. A photograph of the completed model is attached as Appendix 1.

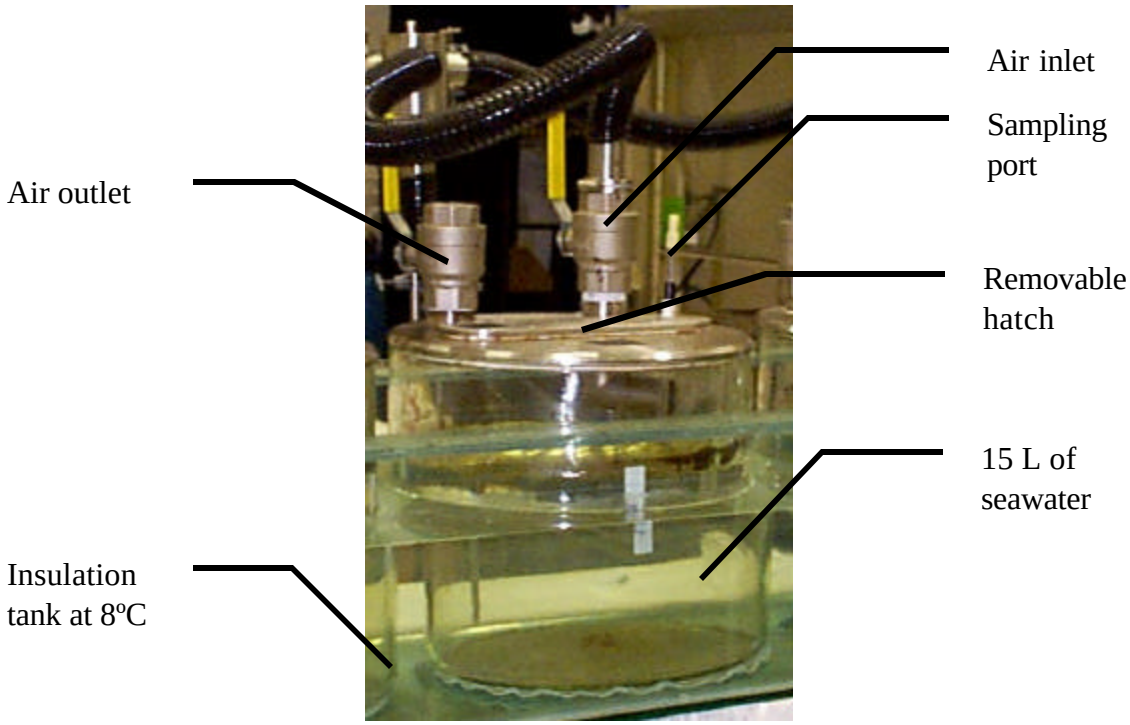


Figure 1. Schematic diagram of a microcosm

During previous studies investigating the biological fate of chemically-dispersed oil under simulated marine conditions, the model produced oil dispersion patterns similar to those seen in field work (Lunel, 1994). Herein, oil was added to the surface of 15 litres of seawater to achieve a final concentration of 100 ppm and allowed to disperse over the course of 15 minutes. At the end of this time, the droplet distribution was measured using the Phase Doppler Particle Analyser (PDPA). The oil droplet size distribution observed was similar to that found in the field, thus showing the model's ability to simulate oil dispersal at sea (Mitchell and Swannell, 1995).

2.2 MICROCOSMS DESIGN

To investigate the biodegradability of chemically-dispersed oil at sea, three microcosm experiments were carried out. For each experiment the design of the three microcosms was as follows:

1) Control containing 15 litres of seawater, approximately 3.7 g of weathered Alaskan North Slope (ANS) crude oil, approximately 0.370 g of Corexit 9500 (1 in 10 dispersant to oil ratio) and 4.5 g of mercuric chloride to ensure sterility throughout the experiment.
2) Dispersant test, identical to the control but without mercuric chloride.
3) Natural dispersion test, identical to the dispersant test but without dispersant.

The composition of the microcosms for each experiment is given in Table 1 below

	Microcosm	Seawater	ANS	Corexit 9500	Mercuric chloride
Experiment 1	Control	15 litres	3.751 g	0.381 g	4.5 g
	Dispersant test	15 litres	3.760 g	0.385 g	
	Natural dispersion test	15 litres	3.761 g		
Experiment 2	Control	15 litres	3.770 g	0.383 g	4.5 g
	Dispersant test	15 litres	3.824 g	0.384 g	
	Natural dispersion test	15 litres	3.678 g		
Experiment 3	Control	15 litres	3.760 g	0.377 g	4.5 g
	Dispersant test	15 litres	3.756 g	0.376 g	
	Natural dispersion test	15 litres	3.700 g		

Table 1. Composition of the Microcosms for Each Experiment

Natural seawater was used in the experiments. This was obtained from Eddystone Lighthouse, Plymouth in the UK.

2.2.1 Oil Weathering

The ANS crude oil was weathered by distillation at 250ºC simulating the natural process of evaporation of the lighter fractions of the oil that are toxic to bacteria. During experimental spills of crude oil it has been noted that oils typically lose 20-30 % by weight after about one hour and 40-50 % by weight after several days, depending on the composition and environmental conditions (Walker et al, 1992; Buchanan and Hurford, 1988).

The same batch of weathered oil was used for each of the three experiments.

2.2.2 Nutrients Addition

On day 8 of each experiment, microcosms were amended with 1 mg/l N as sodium nitrate and 0.1 mg/l P as potassium dihydrogen orthophosphate. Day 8 was chosen for nutrient addition as the team estimated that the original seawater nutrient content would become limiting by that time. This reduced the likelihood that the biodegradation of oil by the indigenous microbial population would be limited by nitrogen or phosphorus over the course of the 35 days experiment.

2.3 SAMPLING STRATEGY AND ANALYSES

For each experiment, the following analyses were carried out:

- Oil droplet distribution for the small droplets (<2.5 to 15 µm) and large droplets (16 to 160 µm) at 12 time points (days 0, 1, 4, 7, 10, 14, 17, 21, 24, 28, 31 and 35 (Section 2.3.1)).
- Cluster distribution at 12 time points (days 0, 1, 4, 7, 10, 14, 17, 21, 24, 28, 31 and 35 (Section 2.3.2)).
- Most Probable Number (MPN) counts to estimate total hydrocarbon-degrading and dispersant-degrading populations at 8 time points (days 0, 4, 7, 10, 14, 21, 28 and 35 (Section 2.3.3)).
- Radiorespirometry to estimate the oil biodegradation rate at 6 time points (days 0, 2, 7, 14, 21 and 35 (Section 2.3.4)).
- Total Petroleum Hydrocarbons content (TPH) at 6 time points (days 0, 2, 7, 14, 21 and 35 (Section 2.3.5)).
- Oil chemistry analysis on days 0 and 35 (Section 2.3.5).
- Toxicity assessment by Microtox® analysis at 6 time points (days 0, 2, 7, 14, 21 and 35 (Section 2.3.6)).

The timetable for each experiment is presented in Appendix 2.

2.3.1 Oil Droplet Distribution by the Chamber Slide Method

A chamber slide was built using microscopic slides, cover slips and double-sided tape. It consisted of 2 cover slips sealed along two edges as shown on Figure 2 and presented a depth of approximately 0.35 mm.

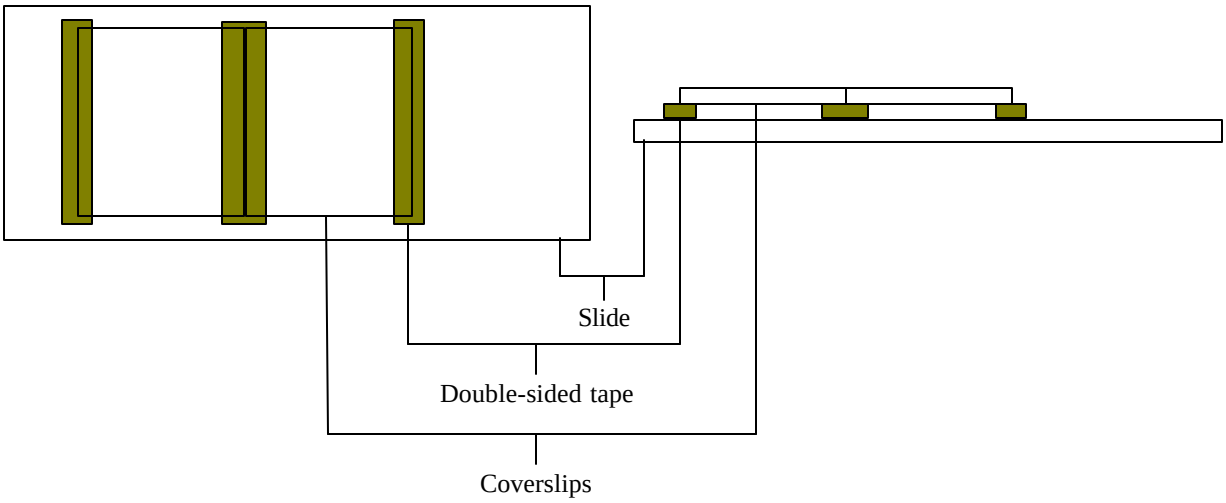


Figure 2. Schematic Diagram of the Chamber Slide

The sample spaces were filled with freshly reconstituted artificial seawater using a micropipette and the volume of each chamber recorded. Following, the slide was suspended vertically, diametrically opposite to the air inlet, and at 50% of the depth of the water in the microcosm, such that the openings faced the flow of water. The slide was inserted through the oil slick using a plastic sleeve, ensuring minimal oil loss from the microcosm and minimal deposit of oil on the coverslips. This ensured good optical conditions for the enumeration of oil droplets. After 10 minutes, the slides were removed from the microcosms via the plastic sleeve and wiped clean of oil before microscopic analysis. This method was based on that of Delvigne and Sweeney (1988), but with elimination of osmotic pressure and long settling times, which might affect the oil droplet distribution.

A Zeiss microscope with epifluorescence optics was used for the experimental observations. UV light was projected down through the lens of the microscope, with the resulting fluorescence of the oil observed through the ocular. Epifluorescence microscopy was crucial for enumeration of droplets less than 5 µm in diameter as it allowed the elimination of background, since background particles that are not oil, such as air bubbles, do not fluoresce.

2.3.1.1 Small Oil droplet distribution

At 400× magnification and using a simple oil droplet diameter measurement with a calibrated ocular graticule, droplets of a diameter varying from <2.5 to 15 µm were counted. In each of the 2 cells, 200 oil droplets were enumerated with the number of microscopic fields necessary to reach that number recorded. The volume and area of each cell of the slide and the number and area of microscopic fields was recorded with the results expressed as a number of oil droplets per ml.

2.3.1.2 Large Oil droplet distribution

The distribution of large oil droplets with diameters ranging from 16 to 160 µm was quantified using the same method as that used for the measurement of the small oil droplets distribution. However, instead of 400×, a 63× magnification was used. The same chamber slide was used for both small and large oil droplets distribution tests.

2.3.1.3 Statistical interpretation of data

The hypothesis underlying the statistical evaluation of droplet distribution is that the sampling method results in a random distribution of droplets observed. The accepted test for randomness is in agreement with the Poisson series. The use of this series as a mathematical model requires four definite conditions (Elliot, 1983), which are:

- The probability of any given point in the sampling area being occupied by a particular individual droplet is constant and small. Consequently, there is a high probability of any given point not being occupied by an individual droplet.
- The number of individual droplets per sample must be well below the maximum possible number that could occur.
- Presence of an individual droplet at any given point must not affect the likelihood of another individual droplet occurring nearby.
- Samples must be small relative to the population.

The two first points were satisfied, as the amount of dispersed oil in the microcosm resulted in a sparse distribution of droplets in the sample area with approximately 1 to 100 droplets per field. The third point, requiring that the sampling method did not cause droplet break-up or droplet coalescence, was satisfied by the chamber method employed. Since the samples represented very small volumes (approximately 200 µl) compared to the total volume (15 l), the fourth condition was satisfied.

The droplets in the chamber slides should therefore follow a Poisson distribution. This means that the statistical error in the number of droplets in any size range can be expressed by the following equation:

$$Percentage\ of\ \ count\ \ indicating\ 95\%\ confidence = \frac{2 \times \sqrt{Numberof\ dropletscounted}}{Numberof\ dropletscounted}$$

2.3.2 Cluster Distribution

A new method was developed in order to assess the concentration of clusters of oil droplets and bacteria trapped in a gel-like matrix. The method consisted of taking a water sample from the microcosms (approximately 20 ml) in a glass Petri dish (diameter: 90 mm). The sample was then placed under a binocular microscope at a magnification of 200×. The number of clusters encountered in a known number of fields was recorded. With knowledge of the number of clusters, the sample volume, the surface area of the field and the number of fields counted, it was possible to express the cluster concentration as the number of clusters per ml.

This method was used during the first experiment, however due to movements of the clusters in the water and the depth of water it was very difficult to enumerate the clusters in a large volume of water. Therefore cluster enumeration was based on the number of clusters encountered while assessing the oil droplets distributions in the chamber slide.

2.3.3 Estimation of the Hydrocarbon-degrading and Dispersant-degrading Microbial Populations by the Most Probable Number (MPN) Technique

The total number of hydrocarbon-degrading bacteria in each microcosm at specific time points in the experiments was determined using the MPN technique as described by Croft *et al.* (1995), using weathered ANS crude oil as carbon source. In addition, for the dispersant test, the number of bacteria able to grow on the dispersant as the carbon source was determined by the same method, using diluted dispersant as carbon source (same concentration of dispersant as in the microcosms). The dispersant contains a proprietary mixture of compounds, therefore the MPN technique quantified growth on one or more of these compounds. Reduction of INT was used to assess growth in each well (Croft *et al*., 1995) after incubation for three weeks at 8ºC. The samples were processed immediately after removal from the microcosms.

2.3.4 Estimation of the Oil Biodegradation Rate by Radiorespirometry

Samples were processed for radiorespirometry within 24 hours of arrival at the University of Alaska Fairbanks (UAF). Radiorespirometry testing was carried out to assess the mineralization potential of the microbial community for the hydrocarbons hexadecane (all three replicate experiments), phenanthrene (replicate experiments one and two), and 2-methyl-naphthalene (replicate experiment three). For radiorespirometry radiolabeled (^{14}C) hydrocarbon substrates were provided to replicate samples and evolution of $^{14}CO_2$ was measured by established techniques (Brown *et al.*, 1991). Previously sterilised, 40 ml septum vials (I-Chem Research, Hayward, CA) containing 10 ml sample were used as microcosms. After the microcosms were constructed, 50 µl of a 2 g l^{-1} solution (in acetone) of radiolabeled hydrocarbon was added by syringe to each vial through the septum. The resulting initial concentration of added hydrocarbon was then 100 µg per vial (10 µg ml^{-1} culture broth; radioactivity *ca.* 50,000 dpm). Substrates used (Sigma Chemical Co., St. Louis, MO) included the alkane hexadecane (1-^{14}C-labeled), and the polynuclear aromatic hydrocarbons 2-methyl-naphthalene (8-^{14}C-labeled) and phenanthrene (9-^{14}C-labeled). Each treatment was replicated 5 fold (except where noted), and controls were used to check for abiotic $^{14}CO_2$ evolution. Vials were incubated at 10°C for 120 hours (except where noted), killed by adding 1 ml 10N NaOH to stop respiration, then reacidified with 1.5 ml concentrated HCl. Radiolabeled carbon was then collected into a CO_2 absorbing scintillation cocktail and assayed as previously described (Brown *et al.*, 1991).

2.3.5 Total Petroleum Hydrocarbon Content and Oil Chemistry Analysis

The samples for oil chemistry were taken into clean glass jars and stored at - 20ºC prior to analysis. The Extractable Organic Matter (EOM), GC derived alkanes (C_{11} to C_{35}), Total Petroleum Hydrocarbon (TPH), Total Resolved Hydrocarbons (TRH) were determined on 50 ml samples at 6 time points. In addition to the determinations carried out for the 50 ml samples, samples from the end of the experiment (1200 ml split in 2 bottles containing each 600 ml) were also analysed for GC-MS derived selected aromatics and hopane quantification. All the oil chemistry analyses were carried out by the Fossil Fuels and Environmental Geochemistry division of the University of Newcastle Upon Tyne.

Seawater samples were extracted using dichloromethane (DCM). The seawater samples were

extracted three times in sequence to recover as much hydrocarbon as possible, each time using 30 ml portions of solvent. The bottles were rinsed using a mix of DCM and methanol. The solvent and seawater were shaken in a separating funnel and the lower organic solvent layer removed. For the samples from the end of experiment the two 600 ml bottles were extracted separately and the extracts then combined. The combined extracts were reduced in volume using rotary evaporation (at<37^{o}C) and then dried using anhydrous sodium sulphate. An aliquot of the extract was analysed gravimetrically after removal of solvent under a stream of dry nitrogen to determine the total extractable organic matter (EOM). A further aliquot of extract was fractionated into a hydrocarbon fraction (aliphatic and aromatic hydrocarbons) by column chromatography on silica gel 60 (Merck, U.K.) using successive elutions of light petroleum spirit (B.P. 40-60°C; Distol grade, Fisher U.K.) and light petroleum/dichloromethane (4:6 v/v) respectively. The hydrocarbon fractions were analysed by gas chromatography (GC) using flame ionisation detection (Hewlett Packard 5890) and gas chromatography with mass spectrometry detection (HP 5890-5972 MSD). GC-FID data acquisition and processing was carried out using a LabSystems Atlas data system. GC-MS data acquisition and processing were done on the HP Chemstation on the HP5972. Quantification of the fractions was achieved by addition of the following internal standards (i) heptadecylcyclohexane (ICN Biomedical Inc.) for the GC-FID analysis of the aliphatic hydrocarbons, (iii) dodecylperhydroanthracene (gift from British Petroleum plc) for the GC-MS analysis of the hopanes and (iv) naphthalene-d_8 phenanthrene-d_{10}, chrysene-d12 and perylene-d12 (Supelco Ltd.) for the GC-MS analysis of the aromatic hydrocarbons. The total GC detectable hydrocarbon values (or Total Petroleum Hydrocarbons (TPH)) were obtained by measuring the total areas in the chromatograms (excluding those of the solvent peaks) and, after deduction of the areas of the added standards and blank analyses, comparing these with those of the areas of the known added internal standards. The Total GC Resolved petroleum hydrocarbon (TGCR) values were obtained by comparing the areas of the added internal standard peaks with the areas of all the resolved peaks detected by the Atlas data system, operated under standardised conditions.

2.3.6 Estimation of the Toxicity by Microtox® Analysis

Samples for toxicity measurements were immediately frozen (-20 °C) for analysis at the University of Alaska Fairbanks (UAF). Toxicity was assessed using the Microtox® assay. Microtox® is a commercially available bioassay system that is based on inhibition of luminescence of the bacterium, *Vibrio fischeri.* Samples were run using the acute toxicity basic test protocol or in some cases due to low toxicity, the 81.9% Basic Test Protocol for the Microtox® system (Azur Environmental, Carlsbad, CA). All reagents were obtained from Azur Environmental and were stored and used as indicated in the test protocol. Phenol (a well-characterised toxicant) standards were run with each new batch of reagent for quality assurance that our test system was set up optimally. In every case, the results obtained fell within the range published for phenol.

3 Results and Discussion

3.1 OIL DROPLET DISTRIBUTION

In all experiments, the controls presented a pattern of oil dispersion characterised by high numbers of oil droplets at the start of the experiment that decreased with time (Figure 3). The total number of oil droplets were depleted by 30 to 53% at the end of the experiments. The oil droplet size distribution in all the experiments showed that large oil droplets present at the start of the experiment tended to disappear with time. These observations are consistent with the hypothesis that large oil droplets re-coalesced with the oil slick. However, the precise number of oil droplets produced varied from experiment to experiment, indicating that even in 15 litre microcosms operated under identical conditions there was considerable variability in droplet number. However a consistent pattern of droplet distribution was seen.

These findings were completely different from that observed with weathered Forties crude oil at 15^{o}C with mercuric chloride and Corexit 9500 in natural seawater under the same experimental conditions (Swannell & Daniel, 1999). Forties was readily dispersed into small droplets that remained for 28 days at a concentration equivalent to the maximum detection limit using the chamber slide technique ($5x10^{6}$ per ml). The difference between the experiments could be a reflection of both the different oil type (ANS is less dispersable and the oil may form a less stable oil-in-water emulsion than Forties in the presence of Corexit 9500), and the temperature. The lower temperature may reduce the stability of the dispersed phase and reduce the effectiveness of the dispersant.

In all experiments, the dispersant tests presented the same pattern of oil dispersion as the controls characterised by high numbers of oil droplets at the start of the experiment that decreased with time (Figure 4). However, the decrease in oil droplet number was sharper in the case of the dispersant tests with 86 to 95% depletion in the total number of oil droplets at the end of the experiments, an observation which may reflect the biodegradation of the oil droplets, by indigenous hydrocarbon-degraders over time. Again, the number of oil droplets produced varied from experiment to experiment but to a lesser extent than observed with the controls. The oil droplet size distribution in all the experiment was similar to that observed with the controls.

For the natural dispersion tests, the oil slick emulsified to different extents in each experiment, a greater degree of variability than observed previously with Forties crude oil at 15^{o}C (Daniel & Swannell, 1998). This resulted in three different patterns of oil dispersion (Figure 5). In experiment 1 the oil was only slightly emulsified and the pattern of oil dispersion was characterised by the appearance of a low number of oil droplets by day 7, increasing to a maximum on day 24 and then decreasing up to the end of the experiment. The oil droplets size distribution showed that low numbers of large droplets were present on Day 7 and 24. During experiment 2 the oil slick emulsified to the extent of forming a “mat” of oil on the surface of the water. Possibly as a result of this, the onset of oil dispersion was delayed until day 10 and no significant increase in oil droplet numbers was observed up to day 17. By day 21 the oil droplet numbers started to increase and, in contrast to that observed in experiment 1, continued to increase until the end of the experiment. The oil droplet size distribution suggested a slow increase of numbers of large oil droplets with time. In the case

of the last experiment, the oil slick emulsified to the same extent as in experiment 2 before forming a semi-submerged “ball” of oil and water reducing the surface area of the oil slick. Consequently, this experiment presented the lowest total number of oil droplets. As for the first experiment, oil dispersion began on day 7. The general pattern was similar to that observed during the first experiment but the time scale was offset with the number of oil droplets reaching a maximum earlier (day 17 instead of 24) and starting to decrease earlier. The oil droplet size distribution showed steadily increasing number of large oil droplets, that started to decrease after peaking on day 17. Generally, a higher number of oil droplets were noted in these microcosms with ANS in 35 days than was seen in the microcosms amended with Forties crude oil in 28 days (Daniel & Swannell, 1998).

In summary, most dispersion was seen in the presence of dispersants, although the pattern in the control suggested that ANS was: (a) not as dispersible as Forties crude oil under similar conditions (except 7^{o}C warmer), and (b) the oil-in-water emulsion was not as stable with ANS and Corexit 9500 as it was with Forties and Corexit 9500. In natural seawater, containing ANS and Corexit 9500 there was a consistent observation that oil droplet number was decreasing more rapidly that in the control. This observation is consistent with the suggestion that indigenous hydrocarbon-degrading bacteria were biodegrading ANS. Natural processes (i.e. in the absence of dispersant) did result in oil dispersion during the experiment, albeit at a much lower level than that observed with dispersant. This observation was consistent with previous work conducted with Forties and Gullfaks crude oil at 15^{o}C (Daniel & Swannell, 1998).

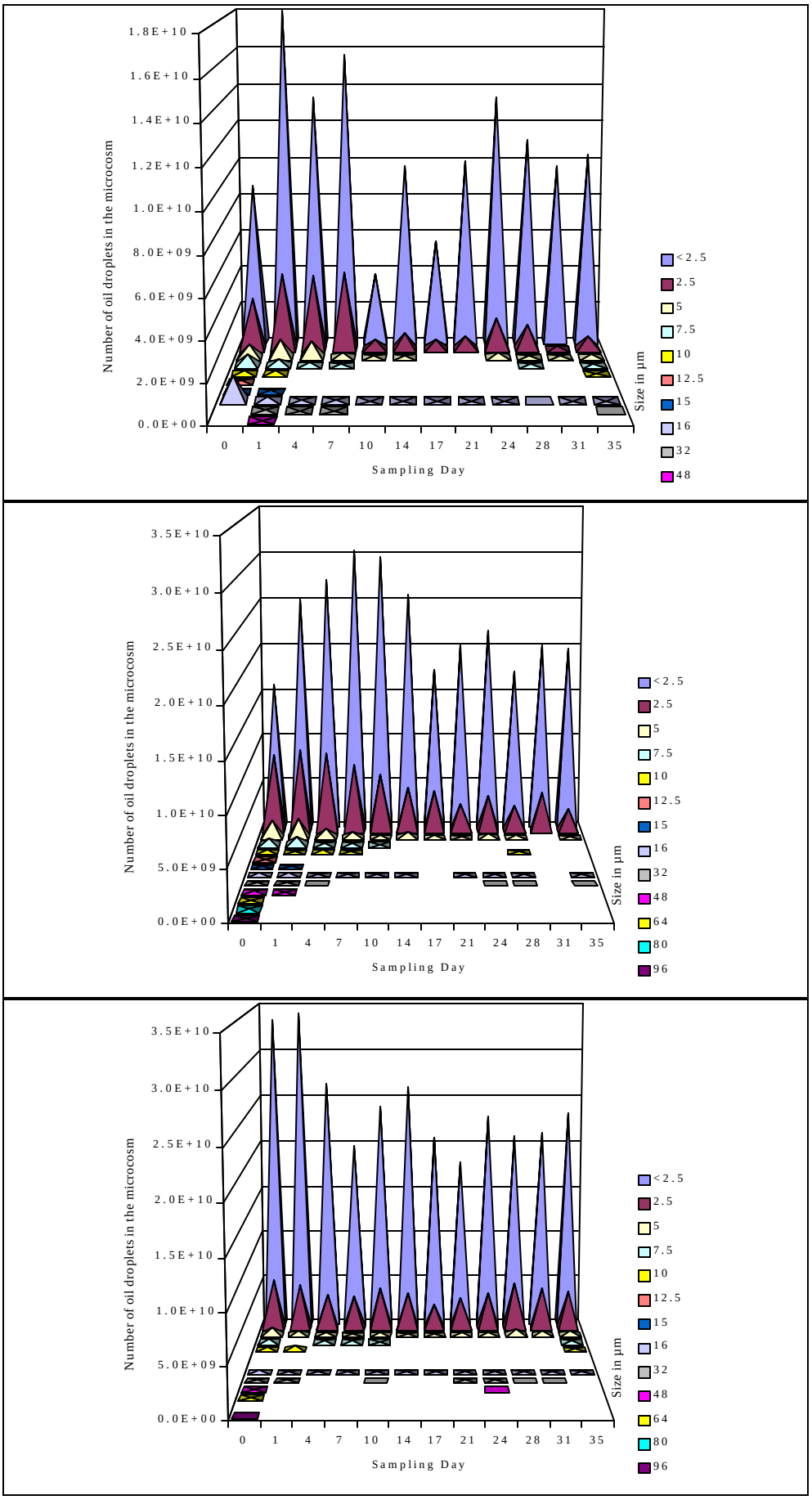


Figure 3. Oil droplet distribution in the control in experiments 1, 2 and 3 (respectively from top to bottom)

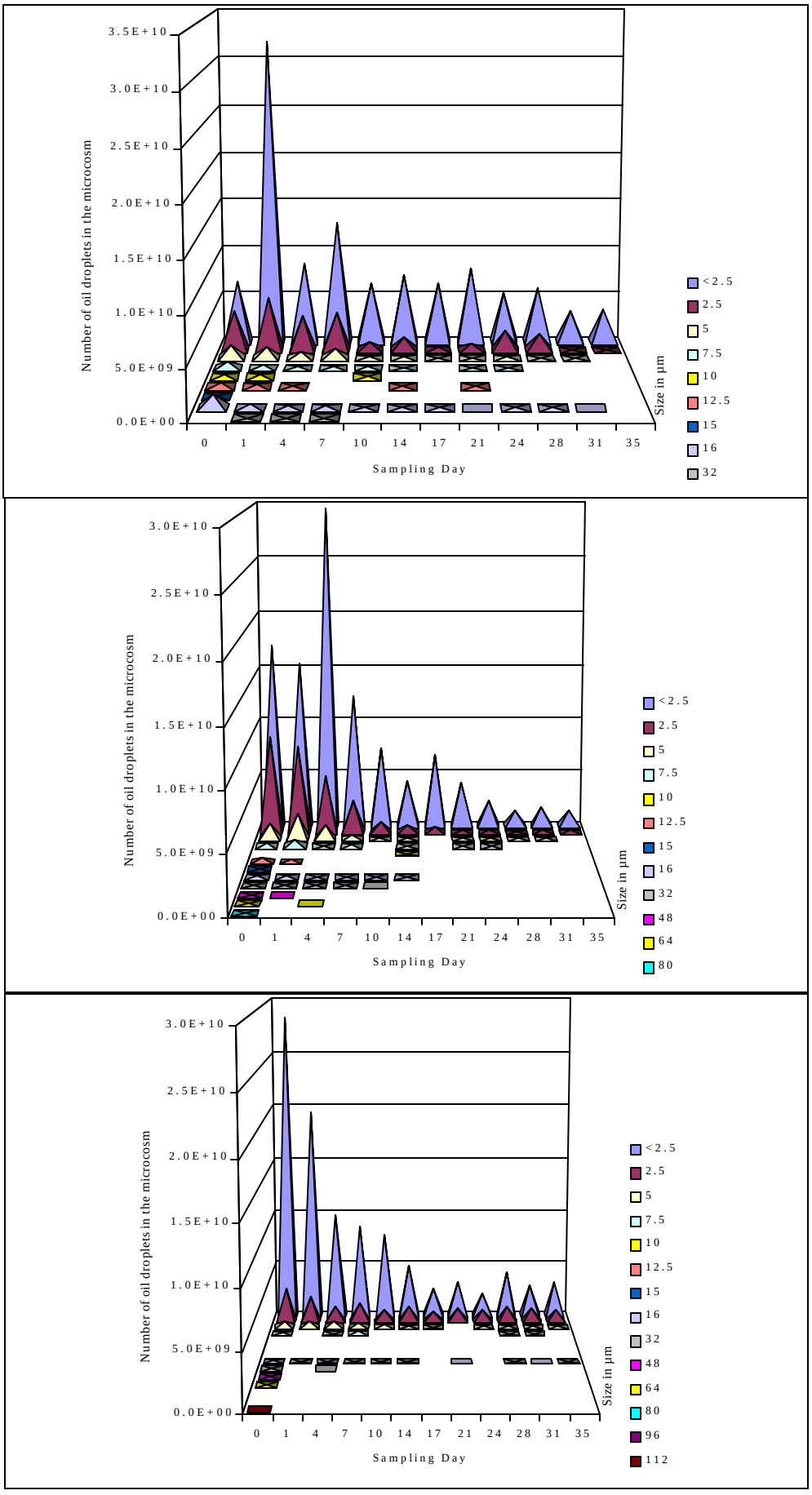


Figure 4. Oil droplet distribution in the dispersant test in experiments 1, 2 and 3 (respectively from top to bottom)

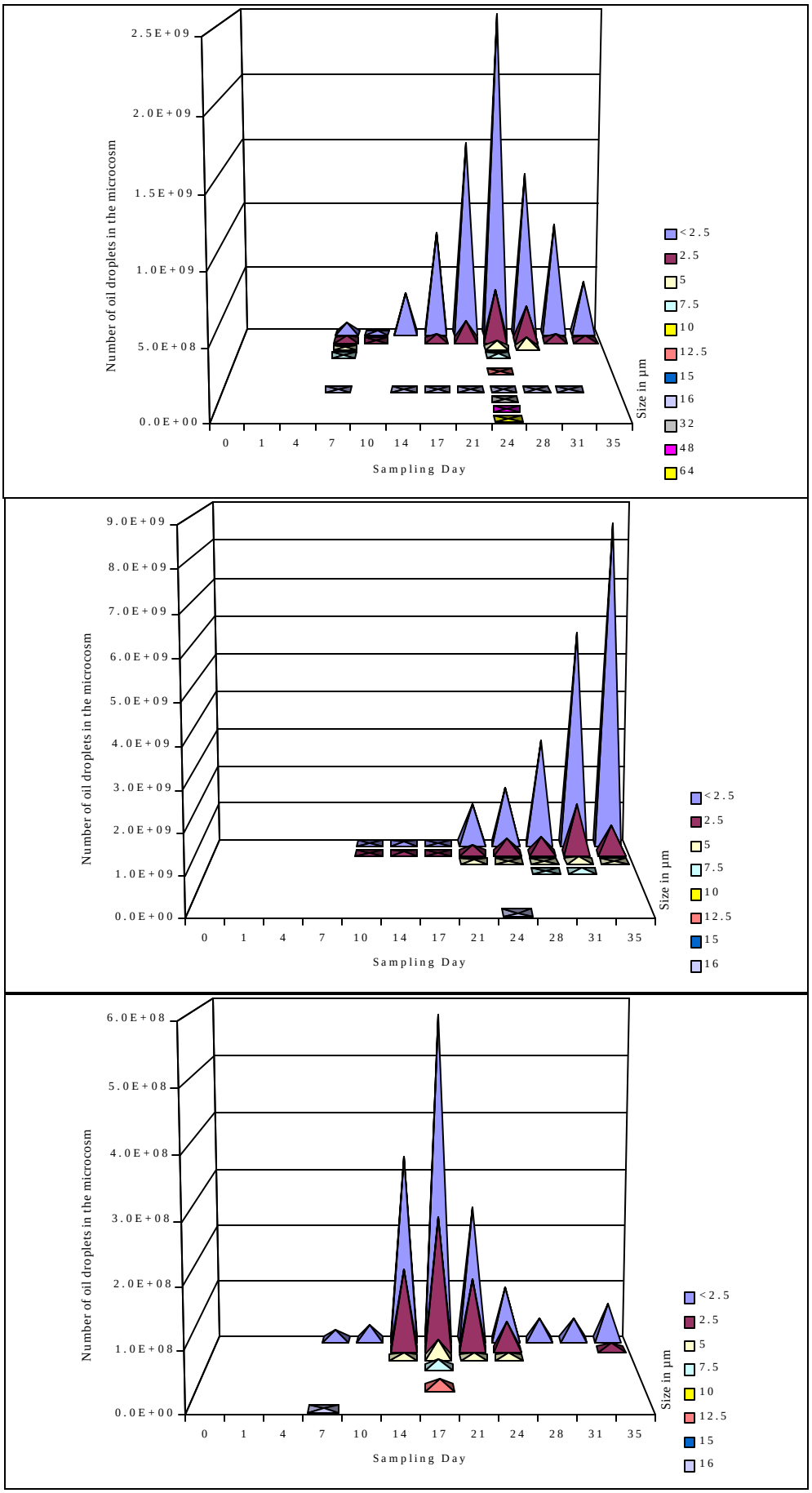


Figure 5. Oil droplet distribution in the natural dispersion test in experiments 1, 2 and 3 (respectively from top to bottom)

3.2 MICROBIAL POPULATIONS

No hydrocarbon-degrading bacteria were detected at any time in any of the controls, as expected. For the dispersant tests there was an initial increase in the hydrocarbon-degrading population in all of the experiments (Figure 6). After this initial increase, the hydrocarbon-degrading population stabilised over time. There are a few differences from experiment to experiment. It should be pointed out that by day 4 in all three dispersant test clusters of oil droplets and bacteria were forming in the water column. These structures were observed by microscopy and were observed to contain high numbers of bacteria (Plate 1). The size of these clusters ranged from 10 µm to 1-2 mm, a size at which the larger clusters could not be sampled for bacterial enumeration. It is therefore likely that after day 10, a large proportion of the hydrocarbon-degrading population was associated with oil droplets in the clusters and was not enumerated. This was likely to have led to an underestimation of the hydrocarbon-degrading population in the microcosms over time that could explain the high variations between experiments.

During the microscopic enumeration of the oil droplets, an assessment of the microbial colonisation of the oil droplets was completed. In the microcosms treated with dispersant, examination showed that by day 4 at least 40 % of the oil droplets were colonised by 1 or more bacteria. The percentage of colonised bacteria increased to approximately 80% by day 17 and stayed at that level for the remainder of the experiments. Thus, in the presence of dispersant the oil droplets rapidly became colonised, and clusters of bacteria and a number of oil droplets formed. This pattern has been observed previously with Gullfaks and Forties crude oil(Daniel & Swannell, 1998) and in these cases resulted in extensive biodegradation of oil.

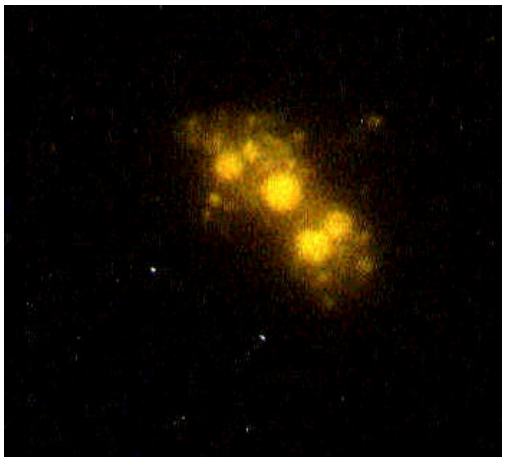


Plate 1. Cluster of oil droplet and bacteria under epifluorescence at a magnification of ×400, the biggest oil droplet diameter is 5 µm

In the case of the natural dispersion test, an initial increase (between day 0 and 4) in the hydrocarbon-degrading population occurred in all the experiments (Figure 6). After the initial increase the general trend seemed to be a steady increase with time (excepting experiment 3). Variation between experiments could be explained by the formation of clusters. The clusters were observed in each experiment shortly after the onset of oil dispersion (Day 7-10). The percentage of oil droplets colonised at the onset of oil dispersion was approximately 60% in

all experiments and increased rapidly to approximately 80%, a level of colonisation sustained to the end of the experiment. Again, the oil droplet colonisation results show that the indigenous microbial populations are interacting closely with the oil and presumably biodegrading it.

The average maximum growth rates of the hydrocarbon-degrading population in the microcosms containing dispersants was $2.4x10^4$ cells/ml/day (unbiased standard deviation= $5.7x10^3$ cells/ml/day). This was one fifth of the rate seen with Forties and Corexit 9500 at 15^oC, although the population sizes were a similar order of magnitude in both experiments (Swannell & Daniel, 1999). In the absence of dispersant, fairly similar patterns of growth were seen, which contrasts with previous work. With Forties, slow growth and small population sizes were seen in the absence of dispersant (Swannell & Daniel, 1999). In contrast, with ANS, similar maximum growth rates were observed with and without dispersant, and this may reflect the higher number of oil droplets found with ANS in the absence of the dispersant, than was found with Forties.

An estimation of the size of the dispersant-degrading population was also carried out for the dispersant tests (Figure 7). During experiment 1 there were generally less dispersant-degraders present than hydrocarbon-degraders. During experiments 2 and 3 there were more dispersant-degraders present than hydrocarbon-degraders. In each experiment a microbial population was detected that was able to use the dispersant as the sole carbon source for growth.

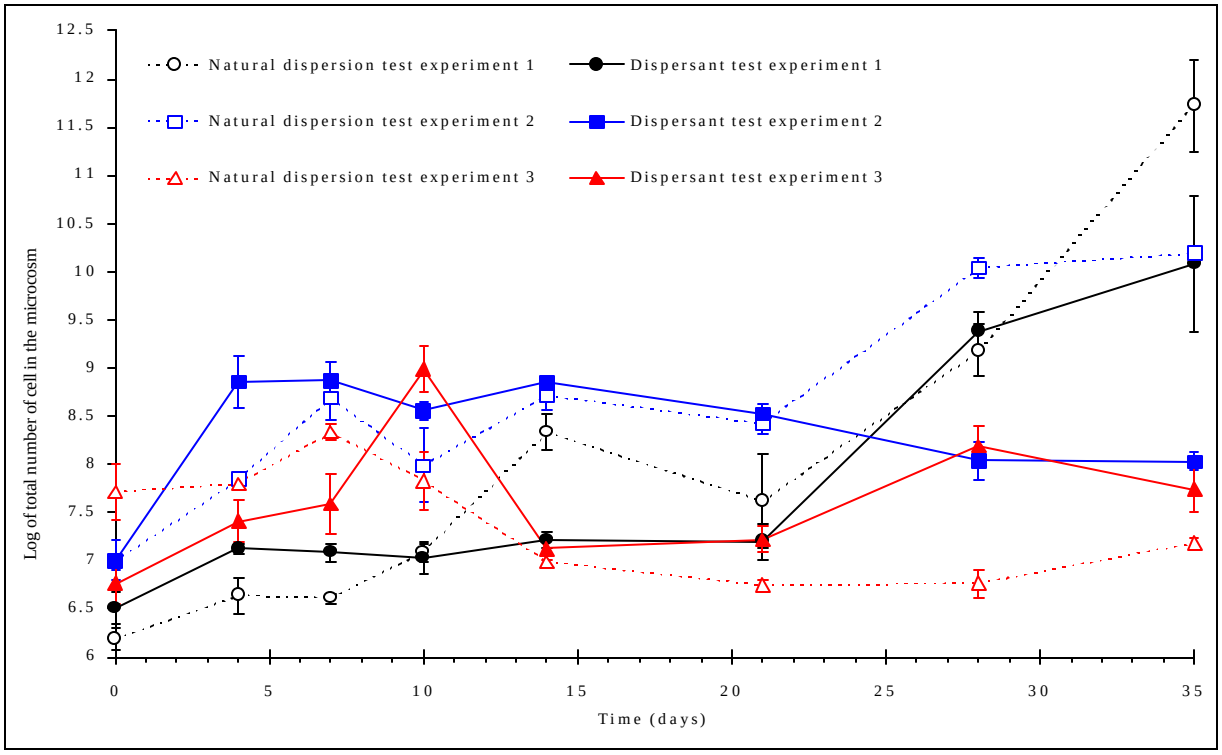


Figure 6. Hydrocarbon-degrading population in the biologically active microcosms (error bars represent the standard error of the mean)

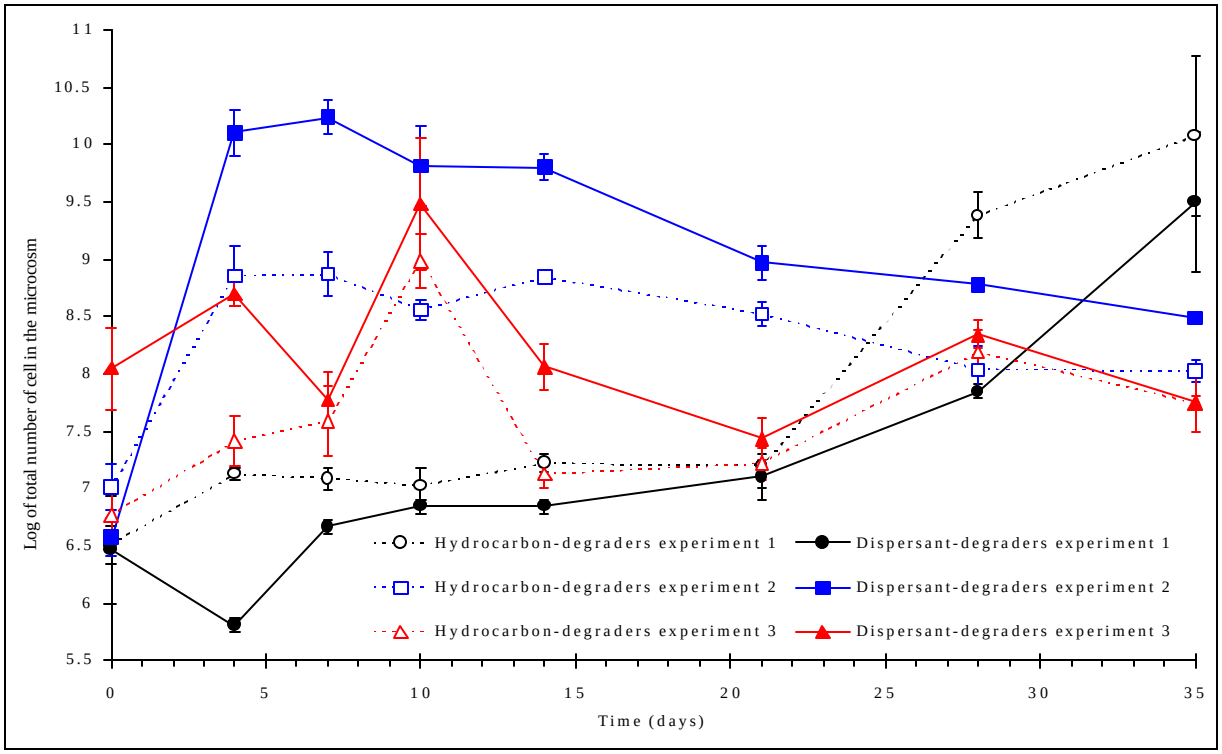


Figure 7. Hydrocarbon and dispersant-degrading population in the dispersant tests
(error bars represent the standard error of the mean)

In summary, oil degraders proliferated in all biologically-active microcosms. In the microcosms amended with dispersant, the population rapidly colonised (within 4 days) the oil droplets and large clusters of bacteria and oil were formed. Maximum microbial growth rates were approximately one fifth of those observed with Forties at 15°C, presumably reflecting slower growth on oil at the lower temperature of 8°C, an observation made by many workers in the field (Leahy & Colwell, 1990). In the absence of the dispersant, the onset of colonisation was delayed, although microbial growth rates and population sizes were greater than had been observed previously with Forties crude oil (Daneil & Swannell, 1998). This difference probably reflects the greater natural dispersion seen with ANS at 8°C, than was seen with Forties at 15°C. In the dispersant treated microcosms a substantial amount of micro-organisms were capable of biodegrading Corexit 9500, an observation consistent with other workers (Varadaraj *et al.* 1995; Swannell & Daniel, 1999).

3.3 MICROTOX

All the tests performed indicated that the toxic effect was too low to calculate an EC_{50}, which is the concentration of the toxic compound causing the inhibition of bioluminescence of 50% of the test bacteria. Thus there was no evidence that dispersing the oil was increasing the toxicity of the oil substantially. Generally dispersed oil at the levels noted in this experiment, did not present a toxic threat to the test organism.

3.4 RADIORESPIROMETRY

For both experiments 1 and 2 the hydrocarbon-degrading population and the hexadecane mineralization (utilisation of hexadecane as a carbon source leading to the formation of

carbon dioxide and water) results for the dispersant tests show the same pattern over time (Figure 8). In experiment 3, the two sets of data present the same trends but showed more discrepancies than the other experiments. The results suggest that alkane biodegradation was occurring in the microcosms amended with dispersant and that the rate of degradation was dependent on the number of hydrocarbon-degrading bacteria present in the water column.

In the case of the natural dispersion tests (Figure 9), the hexadecane mineralization broadly follows the same pattern as the hydrocarbon-degrading population for experiments 1 and 2 except for day 35 where the mineralization drops severely while the hydrocarbon-degrading population continues to rise. During experiment 3 the pattern of hexadecane mineralization over time was close to that observed in the 2 other experiments however, the hydrocarbon-degrading population was different from that observed previously.

Higher rates of hexadecane mineralization were observed in the dispersant test than in the natural dispersion tests suggesting that the dispersant promoted the biodegradation of oil. Maximum rates of hexadecane mineralization ranged from 10-40 µg/ml/day with dispersant, whereas without dispersant the rates ranged from 3.2-7.5 µg/ml/day.

The rate of mineralization of phenanthrene was also determined for experiments 1 and 2, and the rate of mineralization of 2-methyl-naphthalene was determined for experiment 3. For both dispersant and natural dispersion tests the rates recorded were very low. These data indicate that, at least under the test conditions, aromatic compounds may not be readily degraded by these microbial populations.

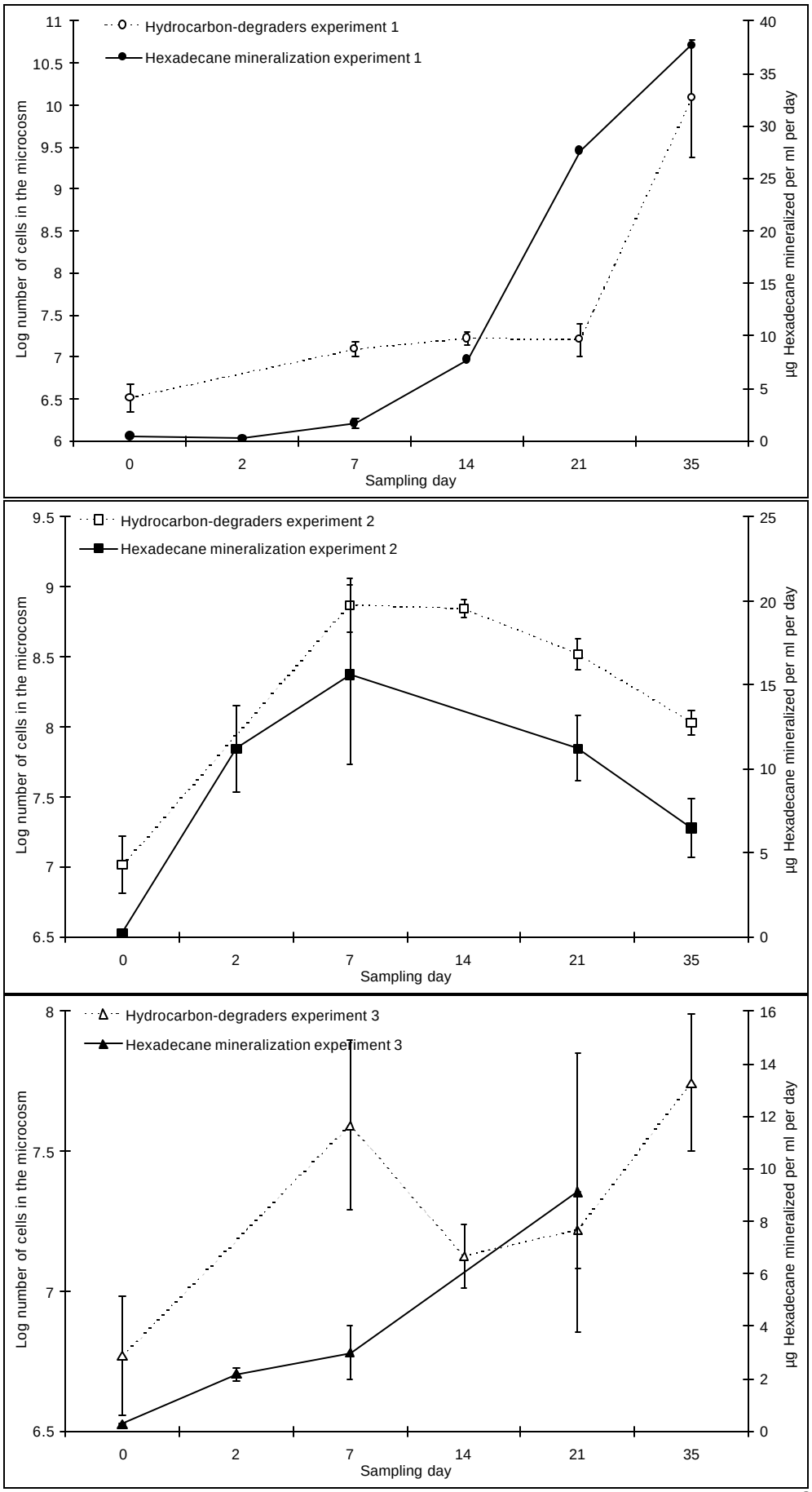


Figure 8. Hydrocarbon-degrading population and hexadecane mineralization for the dispersant tests in experiments 1, 2 and 3 (respectively from top to bottom)

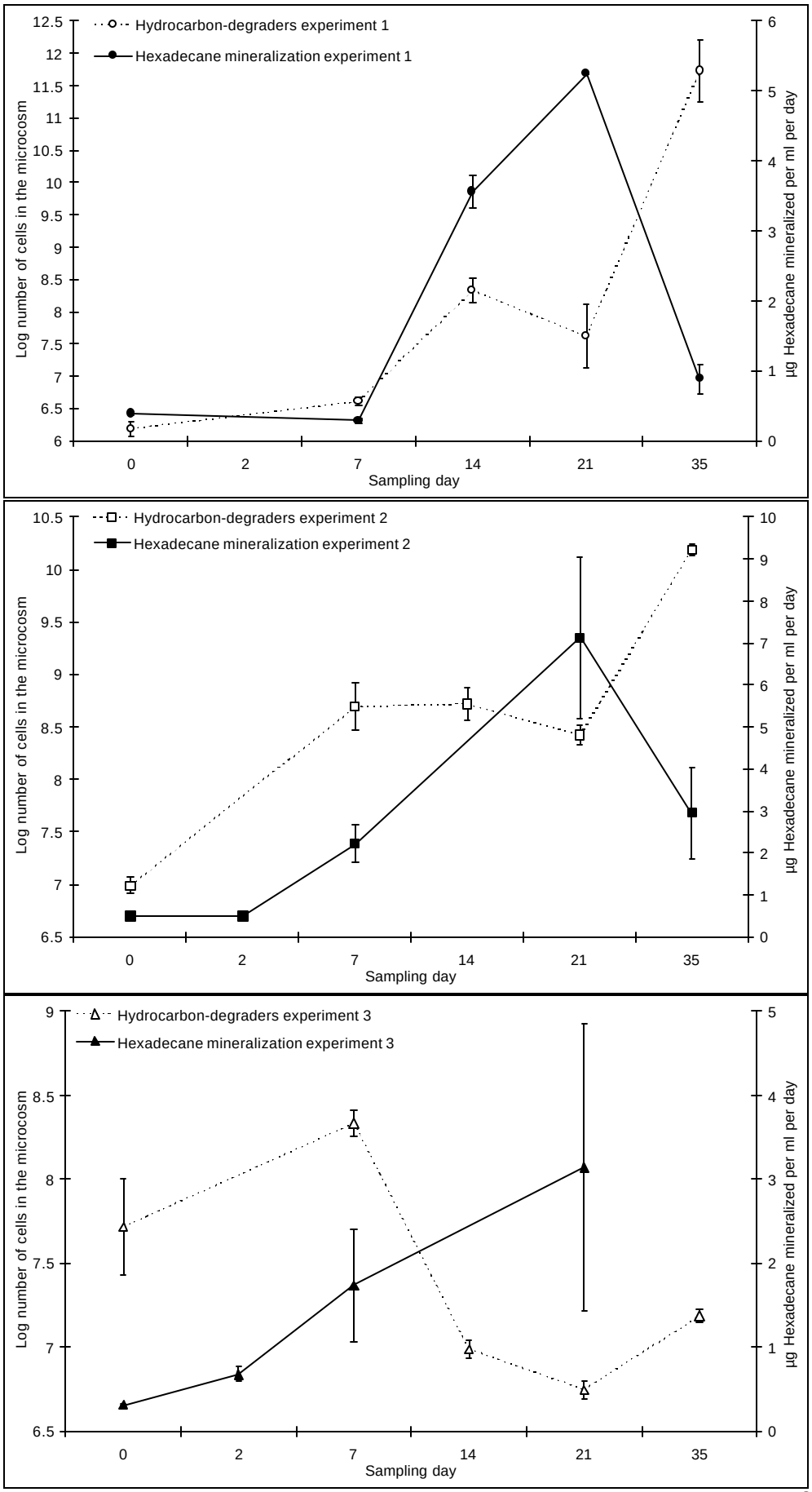


Figure 9. Hydrocarbon-degrading population and hexadecane mineralization for the natural dispersion tests in experiments 1, 2 and 3 (respectively from top to bottom)

3.5 OIL CHEMISTRY

There was a good correlation between the patterns of the volume of dispersed oil calculated from the oil droplet distribution and the TPH (Total Petroleum Hydrocarbon) for the controls and the dispersant tests in all the experiments (Figure 10). This indicated that the oil droplet distribution was qualitatively an accurate measure of oil dispersion (although the measure was not accurate quantitatively as noted previously (Daniel & Swannell, 1998)). However, in the case of the natural dispersion tests (Figure 10) there was a lesser correlation between the two sets of data suggesting that at low levels of oil dispersion the oil droplet distribution method had a poor resolution.

The results for TPH, TRH (Total Resolvable Hydrocarbons) and Sum of the C_{11} to C_{35} *n*-alkanes from the three experiments were averaged for each treatment (Figure 11), except for the natural dispersion tests where the triplicates were too different from each other to be sensibly combined. For TPH, TRH and the sum of the C_{11} to C_{35} *n*-alkanes, the control and dispersant test averages showed logarithmic patterns of reduction over time. The correlation factors and slopes of the trendlines are presented in Table 2 below:

	Correlation factor	Slope
Averaged TPH between day 2 and 28 for the control	0.90	-42477
Averaged TPH between day 2 and 28 for the dispersant test	0.93	-66601
Averaged TRH between day 2 and 28 for the control	0.67	-18458
Averaged TRH between day 2 and 28 for the dispersant test	0.84	-23075
Averaged sum of the C_{11} to C_{35} *n*-alkanes between day 2 and 28 for the control	0.99	-2893
Averaged sum of the C_{11} to C_{35} *n*-alkanes between day 2 and 28 for the dispersant test	0.90	-4289.6

Table 2. Correlation factors and slopes of the trendlines.

The trendlines calculations do not include day 0 because it is likely that the sharp decrease in TPH, TRH and the sum of the C_{11} to C_{35} *n*-alkanes from day 0 to day 2 was due to physical processes only (re-coalescence to the oil slick). The first evidence of oil droplet colonisation in any of the dispersant tests was on day 1. Day 35 was not included in the calculations because the analysis was carried out on much larger samples at this time (1200 ml against 50 ml).

The data showed that on average the slope is lower in the controls than in the microcosms treated with dispersant only. These data suggest that biodegradation was occurring in the microcosms treated with dispersant leading to a greater removal rate of oil. However, the increases were relatively small against a high background of physical removal (i.e. oil recoalescence, resurfacing and evaporation) of oil from the water column of the control. In previous work, these physical processes were so slow as to be undetectable (Swannell & Daniel, 1999). It should be noted however that in the case of TRH the correlation factors are low, consequently the results are more uncertain.

Figure 11, confirmed that generally speaking, the difference in oil concentration between control and dispersant test was small. Only for the sum of the C_{11} to C_{35} *n*-alkanes were the differences between the controls and dispersant tests on average greater than the standard errors of the mean. This suggests that the difference, albeit limited, was significant.

In the case of the natural dispersion tests, no clear evidence of oil biodegradation could be obtained from the TPH, TRH or sum of the C_{11} to C_{35} *n*-alkanes over time simply because of the low concentrations of oil dispersed (Figure 12).

To assess the extent of biodegradation at the end of the experiments (day 35), the concentrations of selected biodegradable fractions of oil were compared with the non-degradable biomarker 17α, 21β hopane. The results were expressed as the ratio of TPH, TRH, sum of the C_{11} to C_{35} *n*-alkanes, and the sum of selected aromatics (naphthalene, methylnaphthalenes, dimethylnaphthalenes, trimethylnaphthalenes, dibenzothiophene, methyldibenzothiophenes, dimethyldibenzothiophenes, phenathrene, methylphenanthrenes and dimethylphenanthrenes) to hopane and averaged by treatments Figure 13). In the case of the dispersant tests, the results from experiment 1 were discarded as the values of the ratios were clearly spurious (ratios above that observed for both controls and original oil). This was probably due to an error in the hopane analysis by GC/MS. Therefore the remaining two dispersant tests are presented separately in Figure 13.

For the TPH and the *n*-alkanes, the ratio to hopane was less in the biologically-active dispersant-amended microcosms in comparison to the control. There was no effect on aromatic biodegradation, an observation consistent with the radiorespirometry results for aromatics. An interesting observation from the detailed oil chemistry analysis was the degree of weathering of ANS that occurred in the control. This may be a result of the surface and re-dispersion of oil droplets throughout the experiment in the controls. Certainly in all the microcosms, oil remained on the surface of the seawater for the entire experiment. However, there is only qualitative evidence of enhanced biodegradation in the biologically active microcoms in comparison to the controls.

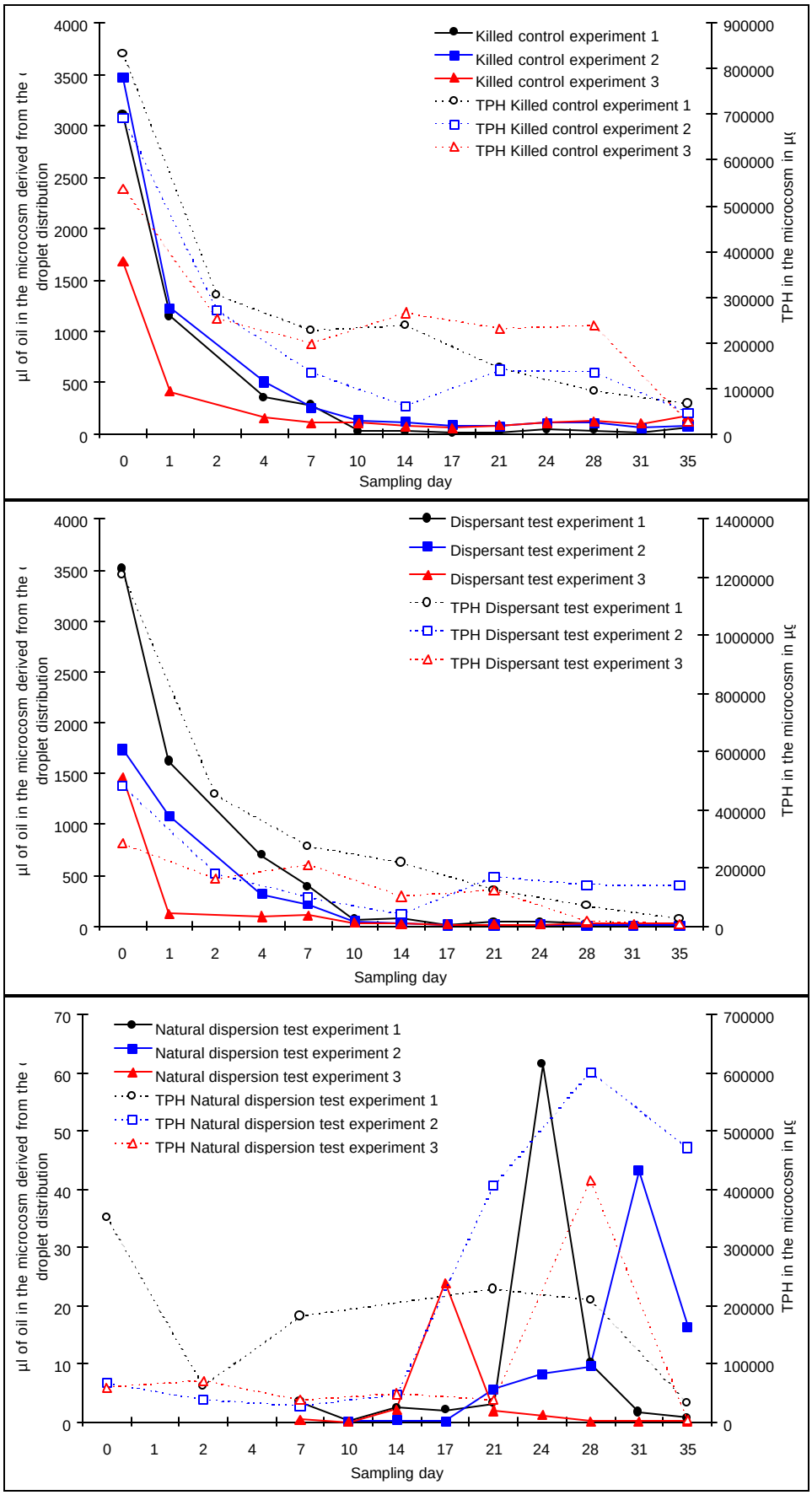


Figure 10. Volume of oil derived from the oil droplet distribution over time versus TPH over time in all experiments (controls, dispersant tests and natural dispersion tests respectively from top to bottom)

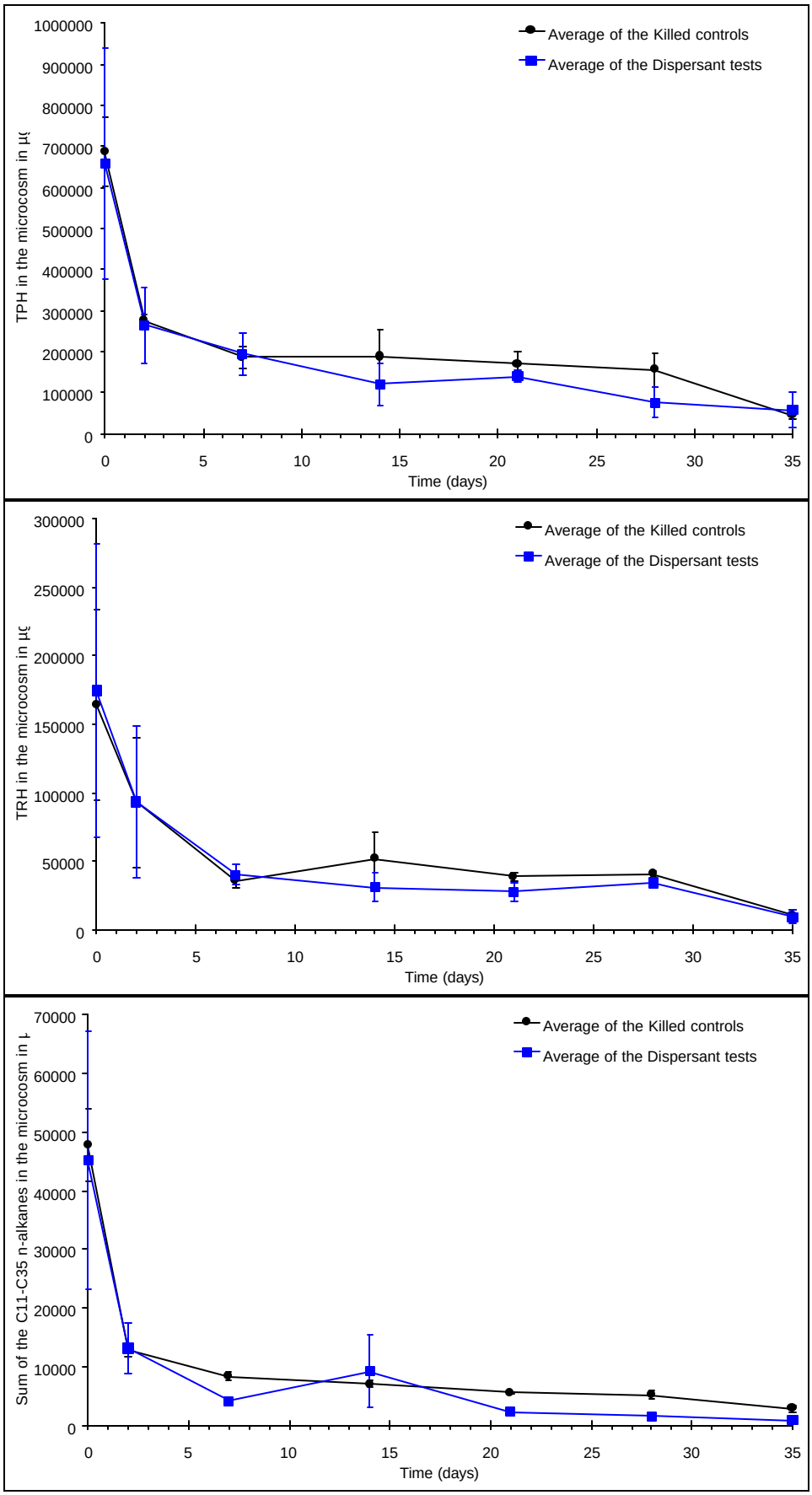


Figure 11. Average of the three experiment results for the controls and dispersant tests for TPH, TRH and the sum of the C_{11} to C_{35} *n*-alkanes (respectively from top to bottom)

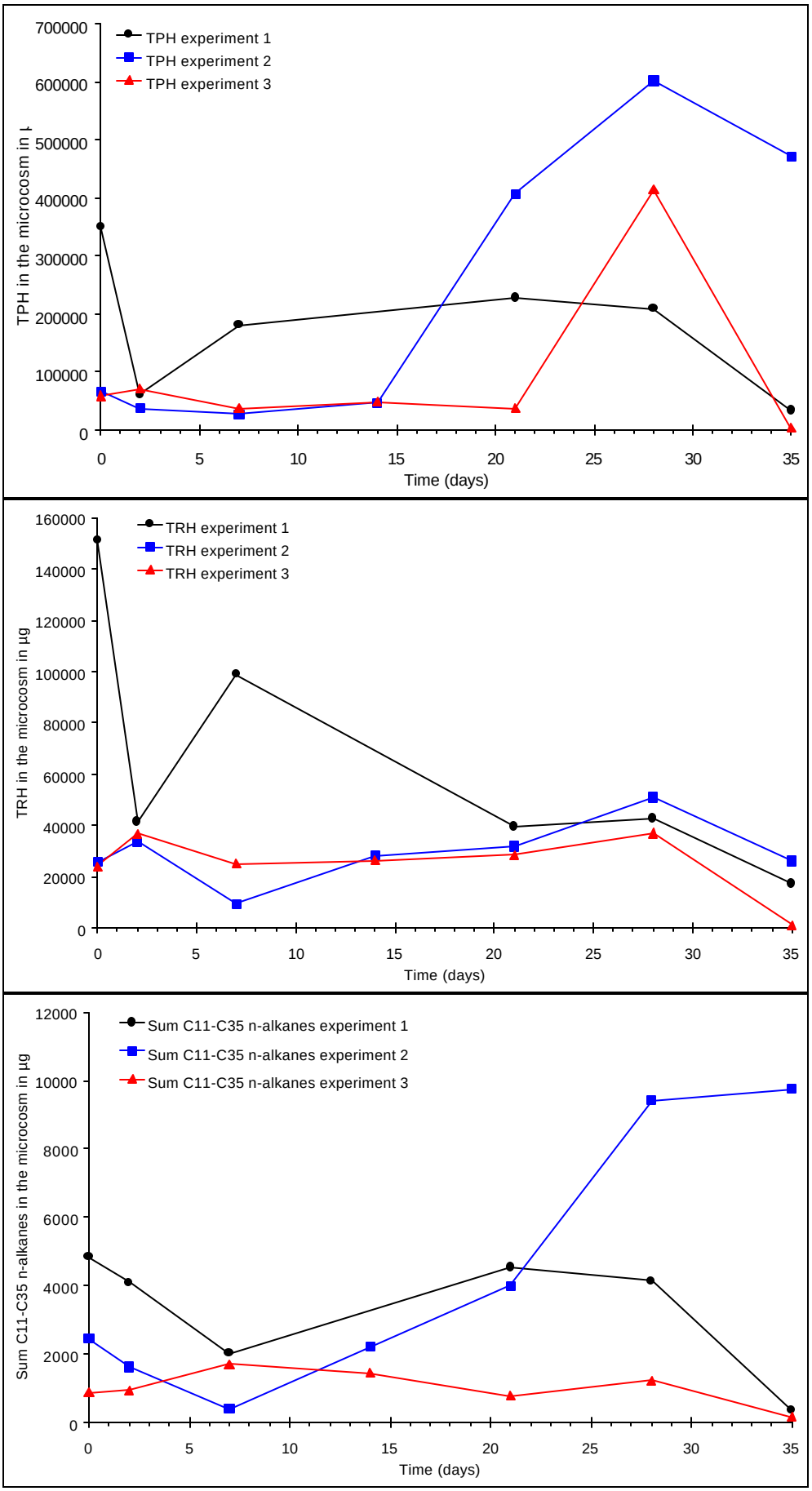
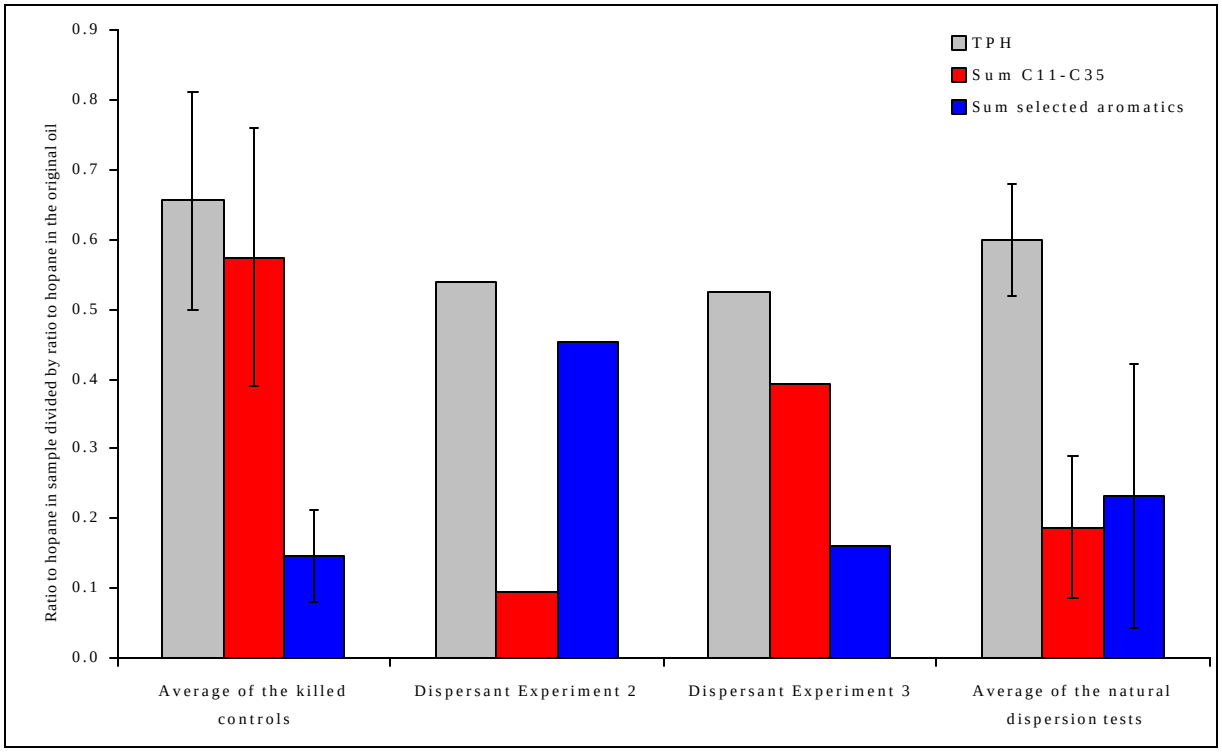


Figure 12. Natural dispersion tests results for TPH, TRH and Sum of the C_{11} to C_{35} *n*-alkanes over time (respectively from top to bottom)

Figure 13. Ratio of TPH, sum of the C_{11} to C_{35} *n*-alkanes and the sum of selected aromatics to hopane to original oil averaged by treatment at the end of the experiments (error bars represent the standard deviation)



4 Discussion

Overall the experimentation produced results which were different from those observed with Forties weathered crude oil at 15°C. Fundamentally there was evidence that at 8°C the addition of dispersants stimulated microbial colonisation of dispersed oil droplets and promoted microbial decomposition of components of the oil. However, the effect was small in comparison to previous observations and this may be related to oil type and the lower temperature. The results are considered in more detail below.

In the controls and dispersant tests, oil droplet number decreased over time, however the change in the control was small in comparison to the dispersant tests. Previously with Forties crude oil, the oil droplet number remained high and the distribution was largely unchanged over the course of the experiment (Swannell & Daniel, 1999). In the dispersant tests, oil droplets were rapidly colonised by bacteria, and clusters of oil droplets and bacteria trapped in a gel-like matrix were observed to form. These "clusters" have been noted in previous experiments (Swannell & Daniel, 1999). With time, the size of the clusters increased and they sank to the bottom of the microcosms, presumably because of a decrease in their buoyancy due to oil biodegradation. This process and microbial decomposition of oil probably caused the sharp decrease of the number of oil droplets over time in the dispersant tests in comparison to the controls. In the tanks unamended with dispersant, only relatively small numbers of oil droplets were recorded, and oil dispersion was only seen 7 days after the start of the experiment. Those droplets that were seen were colonised with bacteria, suggesting that even in the absence of dispersant micro-organisms rapidly colonise physically-dispersed oil droplets. Thus the results from the analysis of the oil droplet distribution suggests that the dispersant addition promotes the rapid formation of an oil in water emulsion, and that in the presence of micro-organisms, these droplets colonise rapidly forming clusters of oil droplets and bacteria (presumably as a result of microbial growth). The sharp decrease in droplet number in the dispersant amended, biologically-active microcosms is consistent with the biodegradation of oil and the sinking of residual oil in clusters. Dispersion did occur in the absence of dispersant but at a much slower rate and to much more limited extent. Micro-organisms were however involved in colonising dispersed droplets even without dispersants.

The number of hydrocarbon degrading bacteria in the biologically-active microcosms tended to increase after the addition of dispersant. However the rate of growth was about 5 fold slower than that observed previously at 15°C with Forties crude oil, possibly due to the lower temperature and the different oil type. Towards the end of the experiment, the numbers tended to decline, possibly as a result of the settling of clusters. Surprisingly, the growth of hydrocarbon-degraders was relatively rapid in the absence of dispersant, and higher numbers were achieved in these microcosms. This contrasts sharply with previous observations (Swannell & Daniel, 1999) and is difficult to explain, although we may speculate that overall biomass levels were probably higher in the dispersant treated microcosms, given the amount of material that had settled onto the base of the microcosms.

The indigenous microbial population could clearly biodegrade the dispersant, an observation consistent with previous work (Varadaraj *et al.*, 1995; Swannell & Daniel, 1999). These results suggest that Corexit 9500 does not have a toxic effect on the growth of the naturally-

occurring bacteria under the conditions of the experiment. This observation is supported by the fact that the Microtox analyses showed no evidence of toxicity to marine bacteria.

The rates of hexadecane mineralization demonstrated that oil biodegradation was occurring in the biologically-active microcosms. Maximum rates of hexadecane mineralization ranged from 10-40 µg/ml/day with dispersant, whereas without dispersant the rates ranged from 3.2-7.5 µg/ml/day. Thus, dispersant addition clearly stimulated biodegradation of components of the oil by competent members of the natural population of bacteria. There was some evidence in the dispersant treated microcosms that the rate of degradation was closely related to the size of the hydrocarbon-degrading populations. In the biologically-active microcosms untreated with dispersants the higher populations of hydrocarbon-degrading bacteria did not result in a higher rate of hexadecane mineralization than in the microcosms treated with dispersant. This may simply be a reflection of differences in oil concentration. Much more oil was dispersed in the dispersant amended microcosms, than was observed in the absence of dispersant. Clearly therefore, even though the dispersant-amended microcosms had less bacteria suspended per ml, the fact that there was more oil droplets in the water column meant that a higher rate of mineralisation was observed. This suggests that in the absence of dispersant the bacteria were relatively starved of biodegradable hydrocarbons. The study of changes in oil concentration gave unexpected results. The rate of reduction in oil concentration in the controls was much more significant than was anticipated, presumably because the oil was resurfacing and combining with the surface slick. This may have been a dynamic process; droplets may have combined with the slick and then re-formed as dispersed droplets repeatedly over the course of the experiment. There is evidence for this as the comparison of ratios of oil components to hopane in the control at the start and the end of the experiment shows substantial weathering, even in the absence of a competent oil-degrading microbial population. The weathering was probably the result of evaporation that occurred as oil floated on the surface of the seawater. At a higher temperature with Forties crude oil, less weathering was observed in the control and there was virtually no surface slick present over the course of the experiment (Swannell & Daniel, 1999).

There was some evidence that less oil remained in the biologically-active microcosms amended with dispersant at the end of the experiment, but the effects were not statistically significant. The ratios of oil components to hopane at the end of the experiment indicated that the oil was more degraded at the end of the experiment in the biologically-active microcosms than in the controls. However, in experiments with Forties at 15^{o}C over 28 days the biodegradable components of the oil had been virtually completely removed (Swannell & Daniel, 1999).

Our previous work with Forties crude oil at 15ºC suggested that in addition to increasing the amount of oil present in the water column for biodegradation, Corexit 9500 stimulated the growth of the hydrocarbon-degrading population, leading to increased oil biodegradation in comparison to natural dispersion.

From this work some conclusions can be drawn:

- The mechanisms of the interaction between bacteria and dispersed oil correlates with the findings of previous work. Oil droplets were rapidly colonised by bacteria leading to the formation of clusters of oil droplets and bacteria trapped in a gel-like matrix. With time,

the size of the clusters increased and they sank at the bottom of the microcosms, presumably because of a decrease in buoyancy due to oil biodegradation.

- Oil degraders proliferated in all biologically-active microcosms. Maximum microbial growth rates for the dispersant tests were approximately one fifth of those observed with Forties at 15^{o}C, presumably reflecting slower growth on oil at the lower temperature of 8^{o}C. In the absence of the dispersant, the onset of colonisation was delayed, although microbial growth rates and population sizes were greater than had been observed previously with Forties crude oil. This difference probably reflects the greater natural dispersion seen with ANS at 8^{o}C, than was seen with Forties at 15^{o}C.

- The utilisation of hexadecane as a carbon source leading to the formation of carbon dioxide and water is called hexadecane mineralization. The hexadecane mineralization results suggest that *n*-alkanes biodegradation was occurring in the natural dispersion tests. They also suggest that in presence of dispersant the rate of mineralisation was substantially increased. This observation is supported in part by the oil chemistry analysis, although again, less oil degradation was observed with ANS at 8^{o}C than was found with Forties at 15^{o}C.

In conclusion, there is evidence that dispersant addition promotes the biodegradation of ANS crude oil at 8ºC. However, the effect is much smaller than that observed with a more dispersible and biodegradable oil (Forties) at 15ºC. From the results of the present work, it is not possible to conclude whether this is due to the different nature of the oil or to the effect of temperature or to a combination of both parameters. An important extension of this work would be to run a set of experiments with ANS crude oil at 15ºC, or with Forties crude oil at 8ºC to determine which of the parameters is reducing the effect of dispersant on the biodegradation of oil under simulated marine conditions.

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Appendices

CONTENTS

Appendix 1

Photograph of the completed experimental apparatus during experiment 3

Appendix 2
Timetables of the three experiments

Legend:

ODDS: Small oil droplet distribution
ODDL: Large oil droplet distribution
CLUS: Cluster distribution
MPN: Estimation of the Hydrocarbon-degrading and Dispersant-degrading Microbial Populations by the Most Probable Number (MPN) Technique
TPH: Total Petroleum Hydrocarbon Content
RADIO: Radiorespirometry
X1: Original seawater sample for radiorespirometry
X2: Biologically active microcosms samples for radiorespirometry
µTOX: Estimation of the Toxicity by Microtox® Analysis
OC: Oil chemistry analysis by GC-MS

Experiment 1

Day	Date		ODDS	ODDL	CLUS	MPN	TPH	RADIO	µTOX	OC
0	Mon	3-Apr	X	X	X	X	X	X1	X	X
1	Tue	4-Apr	X	X	X					
2	Wed	5-Apr					X	X2		
3	Thu	6-Apr								
4	Fri	7-Apr	X	X	X	X				
5	Sat	8-Apr								
6	Sun	9-Apr								
7	Mon	10-Apr	X	X	X	X	X	X2	X	
8	Tue	11-Apr								
9	Wed	12-Apr								
10	Thu	13-Apr	X	X	X	X				
11	Fri	14-Apr								
12	Sat	15-Apr								
13	Sun	16-Apr								
14	Mon	17-Apr	X	X	X	X	X	X2	X	
15	Tue	18-Apr								
16	Wed	19-Apr								
17	Thu	20-Apr	X	X	X					
18	Fri	21-Apr								
19	Sat	22-Apr								
20	Sun	23-Apr								
21	Mon	24-Apr	X	X	X	X	X	X2	X	
22	Tue	25-Apr								
23	Wed	26-Apr								
24	Thu	27-Apr	X	X	X					
25	Fri	28-Apr								
26	Sat	29-Apr								
27	Sun	30-Apr								
28	Mon	1-May	X	X	X	X	X	(X2)	(X)	
29	Tue	2-May								
30	Wed	3-May								
31	Thu	4-May	X	X	X					
32	Fri	5-May								
33	Sat	6-May								
34	Sun	7-May								
35	Mon	8-May	X	X	X	X	X	X2	X	X

Experiment 2

Day	Date		ODDS	ODDL	CLUS	MPN	TPH	RADIO	µTOX	OC
0	Thu	1-Jun	X	X	X	X	X	X1	X	X
1	Fri	2-Jun	X	X	X					
2	Sat	3-Jun					X	X2		
3	Sun	4-Jun								
4	Mon	5-Jun	X	X	X	X				
5	Tue	6-Jun								
6	Wed	7-Jun								
7	Thu	8-Jun	X	X	X	X	X	X2	X	
8	Fri	9-Jun								
9	Sat	10-Jun								
10	Sun	11-Jun	X	X	X	X				
11	Mon	12-Jun								
12	Tue	13-Jun								
13	Wed	14-Jun								
14	Thu	15-Jun	X	X	X	X	X	X2	X	
15	Fri	16-Jun								
16	Sat	17-Jun								
17	Sun	18-Jun	X	X	X					
18	Mon	19-Jun								
19	Tue	20-Jun								
20	Wed	21-Jun								
21	Thu	22-Jun	X	X	X	X	X	X2	X	
22	Fri	23-Jun								
23	Sat	24-Jun								
24	Sun	25-Jun	X	X	X					
25	Mon	26-Jun								
26	Tue	27-Jun								
27	Wed	28-Jun								
28	Thu	29-Jun	X	X	X	X	X	(X2)	(X)	
29	Fri	30-Jun								
30	Sat	1-Jul								
31	Sun	2-Jul	X	X	X					
32	Mon	3-Jul								
33	Tue	4-Jul								
34	Wed	5-Jul								
35	Thu	6-Jul	X	X	X	X	X	X2	X	X

Experiment 3

Day	Date		ODDS	ODDL	CLUS	MPN	TPH	RADIO	µTOX	OC
0	Mon	31-Jul	X	X	X	X	X	X1	X	X
1	Tue	1-Aug	X	X	X					
2	Wed	2-Aug					X	X2		
3	Thu	3-Aug								
4	Fri	4-Aug	X	X	X	X				
5	Sat	5-Aug								
6	Sun	6-Aug								
7	Mon	7-Aug	X	X	X	X	X	X2	X	
8	Tue	8-Aug								
9	Wed	9-Aug								
10	Thu	10-Aug	X	X	X	X				
11	Fri	11-Aug								
12	Sat	12-Aug								
13	Sun	13-Aug								
14	Mon	14-Aug	X	X	X	X	X	X2	X	
15	Tue	15-Aug								
16	Wed	16-Aug								
17	Thu	17-Aug	X	X	X					
18	Fri	18-Aug								
19	Sat	19-Aug								
20	Sun	20-Aug								
21	Mon	21-Aug	X	X	X	X	X	X2	X	
22	Tue	22-Aug								
23	Wed	23-Aug								
24	Thu	24-Aug	X	X	X					
25	Fri	25-Aug								
26	Sat	26-Aug								
27	Sun	27-Aug								
28	Mon	28-Aug	X	X	X	X	X	(X2)	(X)	
29	Tue	29-Aug								
30	Wed	30-Aug								
31	Thu	31-Aug	X	X	X					
32	Fri	1-Sep								
33	Sat	2-Sep								
34	Sun	3-Sep								
35	Mon	4-Sep	X	X	X	X	X	X2	X	X