Potential Ecological Effects of Chemically Dispersed and Biodegraded Oils

FINAL REPORT

Mytilus edulis © K. Hiscock
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Corophium volutator © T.Galloway

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Contents Page

List of Abbreviations

ANS	Alaskan North Slope crude oil
BTEX	Benzene, Toluene, Ethylbenzene and Xylenes
C9527	Corexit 9527 dispersant
DCM	Dichloromethane
DEFRA	Department for Environment, Food and Rural Affairs
DTI	Department of Trade and Industry
DWAF	Dispersed water accommodated fraction
FB	Forties Blend crude oil
GC-MS	Gas Chromatography-Mass Spectrometry
MCA	Maritime and Coastguard Agency
MMS	Minerals Management Service
PAH	Polycyclic aromatic hydrocarbons
ppm	parts per million
SD25	Superdispersant-25
SOP	Standard Operating Procedure
TLC-FID	Thin Layer Chromatography-Flame Ionisation Detection
UVF	Ultraviolet Fluorescence Spectroscopy
WAF	Water accommodated fraction

Foreword

We are very grateful to Newcastle University for access to Iatroscan TLC-FID facilities; especially to Mr Ian Harrison for technical assistance.

We would like to thank Paul Waines from the School of Biological Sciences, University of Plymouth for his assistance and technical help with the microbiology experiments.

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The interdisciplinary background which allowed this work to be successfully completed was nurtured by the scientific interest in our work shown by the late Dr Peter Donkin.

Executive Summary

The Maritime & Coastguard Agency Counter Pollution Branch is responsible for responding to oil pollution occurring in the United Kingdom (U.K.) Pollution Control Zone. In sponsoring the present research, the MCA, along with the Department for Environment, Food and Rural Affairs) and the Department of Trade and Industry, required a quantitative assessment of the ecological risks of chemically dispersing oils in waters around the U.K. The Minerals Management Service (United States (U.S.) Department of the Interior) required a similar assessment for U.S. waters. Forties Blend crude oil (FB) in conjunction with Superdispersant-25 (SD-25) was used for modelling the effects of possible U.K. spill scenarios and Alaskan North Slope (ANS) crude oil was used in conjunction with Corexit 9527 (C-9527) dispersant, to simulate possible U.S. oil spill scenarios.

The assessments were made at the University of Plymouth, U.K., and the effects of dispersing oil were evaluated utilising environmentally relevant end points involving measurements of the feeding rates, growth and reproduction of two representative marine organisms. The mussel, *Mytilus edulis*, was used to determine any detrimental effects of oil in the water column, and the amphipod, *Corophium volutator*, to measure the risk posed by oil trapped in sediments. Rigorous attention was paid to quality control in all of the experiments and chemical analyses of all water, sediment and mussel tissue were carried out using standardised methods.

Firstly, a standardised method for lightly weathering the oils to simulate 2h spillage on the sea surface was developed, and these lightly weathered oils were used for all of the subsequent experiments.

A quantitative assessment of the rates of biodegradation of the major and minor components of each oil was made at 8 and 15˚C, with and without each of the relevant dispersants. No significant enhancement of biodegradation of the whole oils was observed when dispersants were used and it was noted that the major losses over the study period were due to evaporation.

Mussels were exposed to oil and dispersed oil under standard test conditions and also under conditions thought more likely to mimic environmental concentrations and loadings following an oil spill. The organisms were exposed for 48h and then placed in clean water to emulate the dilution of dispersed oil after a spill in the environment, and to measure the ability of the mussels to recover. Mussels were affected after 48h to a greater extent by dispersed oil. However, in most cases, during the recovery period they recovered to the same extent as those exposed to oil. These results suggest that inter-tidal mussels exposed to all but the highest tested concentrations of dispersed ANS oil and which have been treated with appropriate

concentrations of the licensed dispersants, would recover if successive tides further diluted the oil mixtures.

The amphipods were exposed both to oil in water and via sediments spiked with oil or dispersed oil, at concentrations similar to those measured at real oil spill sites. No significant acute mortality was noted during these exposures. A standardised method was developed for the life cycle test and the effects on the whole life cycle were then measured by determining the numbers of survivors, their growth rates and reproductive success. Chronic oil exposure tests showed ecologically relevant end points were affected by some environmentally realistic concentrations of dispersed and non-dispersed oil even when these did not cause acute toxicity. At the end of the life cycle, dispersant SD-25 did not affect the impact of FB oil but addition of the dispersant C-9527 caused an impact when used to disperse ANS oil. Analysis of sediments showed that when the oils became biodegraded during the experiments (e.g. FB oil) the organisms began to recover. The worst impacts were noted for ANS oil with C-9527 at the same nominal loadings as FB oil with SD-25. Chemical analysis revealed that the treatment of ANS with C-9527 resulted in higher concentrations of ANS oil in the sediments at the same nominal loadings as dispersed FB oil.

The experiments with mussels and amphipods were replicated using oil and dispersed oil which had been spiked with bacteria and allowed to biodegrade for a period of time. No significant differences were found for either organism between dispersed non-biodegraded oil and dispersed oil biodegraded in the water column.

Overall, the experiments conducted showed that although chemically dispersed oil may initially impact mussels and amphipods to a greater extent than would untreated oil, the organisms are mostly able to recover to the same extent as control organisms or to those exposed to oil alone. The exception to this were some exposures of C-9527 dispersed water-accommodated fractions of ANS oil to mussels and amphipods where dispersion led to the highest concentrations of oil in the water and sediments.

Thus, in scenarios where successive tides further dilute these dispersant-treated oils, a net environmental benefit may be maintained and the goal of dispersant use achieved.

1. Background

Oil spill response methods seek to minimise the damage (both environmental and socio-economic) that might otherwise be caused by an oil spill. Each response aims to achieve a net environmental benefit. The use of oil spill dispersants as an appropriate oil spill response method in some circumstances is based on the assessment that dispersing oil into the sea is likely to be less ecologically damaging than allowing oil to move into shallow water or to impact the shoreline. Whilst it is known that dispersing oil carries some risk of causing effects on marine organisms by transiently raising the concentration of some acutely toxic oil components, such as BTEX in the water column, most studies have indicated that this is unlikely to be a major factor in the majority of spills, since the BTEX evaporate rapidly, either from the oil slick before it can be dispersed, or from the water column after dispersion of the oil.

Critics of dispersant use also suggest that dispersing the oil may cause longer term effects if the dispersed oil enters shallow water or becomes incorporated into sediments. This criticism has been countered to date by suggestions that since dispersed oils are diluted into a large volume of water column at sea, the majority of dispersed oil would be more rapidly biodegraded at sea by naturally occurring organisms. Thus the amount of oil available for example for incorporation into sediments would be less when dispersants are applied. The rate of biodegradation of dispersed oil at sea is expected to be faster than that of non-dispersed oil by virtue of the vastly increased surface area of oil which is available for colonisation by micro-organisms. Additionally it has been suggested that some dispersant components may act as extra nutrients for the micro-organisms and that the rapid biodegradation of these surfactants may then enhance the rate of biodegradation of dispersed oil. Evidence certainly suggests that the biodegradation of some oil components occurs within days of a spill and oil droplets produced when dispersants are used have a hydrophilic ‘coating’ that reduces them from adhering to suspended sediments. Therefore sedimentation of chemically dispersed oil is expected to be less likely than sedimentation of physically dispersed oil. However, this will depend on the precise circumstances of the oil spill and biodegradation of some oil components is slow and a recalcitrant residue that is not biodegradable may eventually become incorporated into bottom sediments. Also, the volume of oil that biodegrades, or rate of biodegradation is not the only pertinent factor. A large proportion of the components in oils are not considered toxicologically important; therefore it is the biodegradation of the toxicologically important compounds (or the production of toxic compounds during the biodegradation process) and the concentration and availability of these that perhaps is of most importance in terms of ecological effects.

The aim of the present study was to conduct an assessment of the potential ecological effects of chemically dispersed and biodegraded oils to common shoreline organisms living in shallow water or in sediments. The mussel, *Mytilus edulis* and the amphipod, *Corophium volutator* were used for the assessment since these are widespread organisms which occupy the relevant

ecological niches and are well recognised species used in regulatory tests in Europe and the U.S.A.

2. Objectives and Milestones

The objectives of the project as defined in the Specification to Tender were to:

Provide a quantitative assessment of the rate of biodegradation of selected major (saturates, aromatics, resins and asphaltene) and minor (2, 3, 4 and 5 ring PAHs) oil components under a number of conditions found in waters around the UK.

Provide a quantitative assessment of the potential toxic effects of chemically dispersed oil and the way that this changes as the oil is biodegraded.

Relate the chemical composition of an oil (and the way that this changes with biodegradation) to the observed toxic effects (if any) so that the findings of the study might be extrapolated to other oils.

Provide a quantitative measure of the ecological risk posed by chemically dispersed oil trapped in sediments to benthic biota at different stages in their life-cycle.

These objectives were addressed by fulfilling the following milestones[1]:

Milestone 1: Provision of a quantitative assessment of the rate of biodegradation of the major and minor oil components under a range of conditions found in waters around the UK.

Milestone 2: Provision of a quantitative assessment of the potential toxic effects of chemically dispersed oil and the way that this changed as the oil was biodegraded

Milestone 3: Relating the chemical composition of each oil (and the way this changed with biodegradation) to the observed toxic effects.

Milestone 4: Provision of a quantitative measure of the ecological risk posed by chemically dispersed oil trapped in sediments to benthic biota at different stages of their life cycle.

[1] The 'Extent To Which Milestones Were Met' is described in the Conclusions section page 45.

3. Methods

3.1 Artificial weathering of oil:

The oils were artificially weathered in order to simulate evaporative losses that typically occur during the first 2 – 3 h at sea following a spill; this represents the earliest optimal time that dispersants may be deployed at sea (MCA, personal communication). The details of this method are described in a SOP presented in the Appendix 1. Briefly, approximately 1000 mL of oil was placed in a 2000 mL container on a top pan balance in a fume hood with a controlled airstream and the oil allowed to evaporate. The weight of the oil at the start of the experiment was noted. Triplicate density measurements were taken to calculate the initial volume of oil. Density measurements were made by weighing known volumes of the oil at the beginning, half way through, and at the end of the experiment. Density measurements were compared to published literature (Blenkinsopp *et al*., 1996). The weight of the oil was noted over the duration of the experiment and adjusted to volume using the density measurements. Temperature (18-20° C) and air flow (~0.5 m/sec) through the fumehood was noted during the experiment. The weathering process was halted after 24h. The whole experiment was carried out three times to investigate whether the method was reproducible. These 'weathered' oils were used to produce the starting materials for all experiments. Once 'weathered' the oil was stored in completely filled dark containers in a 'fridge (4°C) until use.

3.2 Assessment of biodegradation:

The two oils with and without their respective dispersants were proffered to the common aerobic bacterium, *Pseudomonas fluorescens* (Texaco) in aqueous solution in the laboratory at 8 and 15 °C. All of the biodegradation studies were carried out using methods based on the aerobic shake flask method. Four experiments were carried out:

1. FB and FB + SD-25 at 15°C
2. Forties FB and FB + SD-25 at 8 °C
3. ANS and ANS + C- 9527 at 15 °C
4. ANS and ANS + C- 9527 at 8 °C

All work was carried out using recognised aseptic techniques. All glassware was sterilised in advance by autoclave (121 °C, 20 min) or dry heat (160 °C, 60 min).

4.2.1 Bacterial Culture:
Pseudomonas fluorescens (strain Texaco), a gram negative rod bacterium, was the micro-organism used for the biodegradation experiments. The bacterium was originally isolated from a metal working fluid waste by Beech and Gaylarde (1989). A pure culture of the bacterium *Pseudomonas fluorescens* (Texaco) was grown in a nutrient broth (13g OXOID in 1 L de-

ionised water) for 24 h at 15 °C. The bacteria were removed from the carbon nutrient source prior to use by centrifugation in phosphate buffer. The residual pellet was then re-suspended in phosphate buffer solution and this process was repeated three times. A minimal salts solution was prepared by dissolving the following salts (w/v); 0.5% NH_4Cl, 0.3% K_2PO_4, 0.2% Na_2SO_4, 0.1% NH_4NO_3, 0.1% KH_2PO_4 and 0.01% $MgSO_4.7H_20$ in 1000 mL de-ionised water and sterilised by autoclave (121 °C).

3.2.2 Batch Experiments:

The biodegradation experiments were based on previous methods employed by Robson (1987); Gough (1989) and Robson and Rowland (1989). For each experiment 10mL of the sterilised minimal salts solution was added to 70 conical flasks (25 mL). This number of flasks allowed for duplicate samples to be taken at each sampling point. The oils were added as 100 μL of a 50 $mgmL^{-1}$ solution in hexane. The dispersants were added at a ratio of 1:10. Lastly 0.5 mL bacterium inoculum was added to half of the flasks (the other half acted as abiotic controls), and the flasks sealed with sterile non-absorbent cotton wool. Incubation (up to 100d) was performed aerobically on a shaking water bath using a dip chiller to maintain the required temperatures, and covered with foil to prevent any action caused by ambient sunlight or artificial light. Flasks containing the substrates but no bacteria were incubated under the same conditions to monitor abiological losses. Procedural blanks were prepared by adding minimal salts and bacteria but no oil or dispersant. A further four biotic cultures (duplicate oil and oil+dispersant) were set aside and used solely to monitor bacterial viability. Samples were taken at 0h, 24h, 3d, 7d, 14d, 21d, 28d, then at approximately 50d and 100d of incubation.

3.2.3 Extraction and Analysis:

Upon sampling 100 μL of internal standards (deuterated (d-)naphthalene, d-phenanthrene, d-pyrene and d-tetracosane) in acetone were injected into each conical flask immediately prior to extraction. Dichloromethane (10mL) was added to each flask and left to stand to kill the bacteria (5 min). This also provided the medium for hydrocarbon extraction. The flask contents were transferred to glassware suitable for centrifugation and samples were centrifuged at 2500 rpm for 20 min to produce a dichloromethane extract layer. The dichloromethane layer was removed using a Pasteur pipette and run through a small column containing a cotton wool plug (to remove any bacteria) into a round bottom flask containing anhydrous sodium sulphate. This process was repeated twice more and the glassware rinsed with the DCM aliquots. After extraction and drying (anhydrous sodium sulphate) extracts were evaporated using micro Kuderna-Danish apparatus to avoid the loss of low molecular weight compounds to ca. 1mL and analysed using cryogenically cooled GC-MS.

3.2.4 Monitoring of Bacterial Culture:

The viability of the bacteria was confirmed throughout the course of the experiment so that any changes could be reliably attributed to biodegradation. Nutrient agar (27g OXOID, 2 g agar) was dissolved in 1 litre of de-ionised water and heated in a steam bath for 1h and sterilised by autoclave. This medium was melted in a steamer (60 min) and allowed to cool in water bath

before pouring 95mL into 3 inch Petri dishes. After setting, the agar the plates were stored under refrigeration.
On each day of sampling four biotic (2 oil, 2 oil + dispersant) and 2 abiotic vessels were tested for viability and bacterial contamination. Using sterile techniques, a solution sample was streaked onto a nutrient agar plate. This was incubated for 24 h at 37 °C and any evidence of growth taken as evidence of viability. Digital photographs of the agar plates were recorded.
An estimate of bacterial population was carried out using the drop count method developed by Miles Misra (1938). Serial dilutions (10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) of the cultures from the viability vessels were prepared in phosphate buffer. From each dilution 25 μL of solution was delivered to a nutrient agar plate in the form of a small drop or lens. Two drops from each dilution were placed on a single plate and allowed to dry. The plate was then inverted and incubated at 37 °C for 24 h. After incubation the colonies present in the drops were counted. The dilutions that produced between 10-20 colonies were used to calculate the number of bacteria in the original flasks. The bacterial population in the culture flasks was measured directly after the cultures had been inoculated and then again at each sampling time.

3.2.5 Determination of saturates, aromatics, resins and asphaltenes:
This was carried out using an Iatroscan instrument which is a TLC-FID system that performs quantitative analysis by the detection of zones separated on a thin layer chromarod using a hydrogen flame ionisation detector. This technique was chosen over traditional column chromatography as it offered a reproducible, high throughput, low cost, standard method which provided easy quantitation using standard chromatography software.
After analysis by GC-MS the extracted samples (in 1 mL DCM) were spotted onto the chromarods by means of a micro-syringe (3 μL). On each rack a standard North Sea Oil sample was spotted in duplicate leaving 8 rods for duplicate analysis of four other samples. The rods in their holder were placed in the development tanks and subjected to solvent development to effect sample preparation. To allow separation of the sample into saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes, three development tanks were prepared with 100 mL of solvent in each tank. Tank 1 contained hexane and the chromarods were eluted to 100 %, tank 2 contained toluene and elution was to 60% and tank 3 contained DCM:methanol (95:5) and run to 30%. Drying times between tanks 1, 2, and 3 were 3 and 6 min respectively, followed by 1.5 min in an oven at 60 °C after tank 3 just prior to scanning of the chromarods. The rack of 10 rods was inserted into the Iatroscan and examined with FID detection. Blank analyses were carried out twice a day at the beginning and end of a block of analyses. A mix of squalene, anthracene and undecanol was also examined twice a day to monitor performance of the chromatography system, and a North Sea oil sample was examined on each rack so as to allow inter-rack comparison of samples.

4.3 Ecotoxicology with *Mytilus edulis*:

3.3.1 Collection and maintenance of organisms during acclimation:

Mussels were collected from a 'clean' site, Port Quin, Cornwall (Ordnance Survey Grid reference: SW 971 806). To limit potential geographical, age and size variations, specimens of a similar size were always collected from the same locality. Collection was performed with minimal damage to the byssal thread attachment and specimens were immediately transported back to the aquarium. Mussels were placed in clean glass tanks in 20 L of 10 µm-filtered seawater in a temperature-controlled aquarium 15^{o}C (± 3^{o}C), for a minimum of 1 week to allow the animals to acclimatise.

3.3.2 Preparation of Water-Accommodated-Fractions (WAFs) and chemically dispersed WAFs (DWAFs):

Water accommodated oil fractions (WAFs) and dispersed water accommodated oil fractions (DWAFs) were prepared as described in Ali *et al*., 1995. Exposure preparations were made in 10 L Pyrex glass aspirator jars (diameter 23.4 cm, surface area 430.05 mm^2), placed on magnetic stirrers, in a temperature-controlled room (15 ^{o}C ± 1 ^{o}C). Initially 99:1 and 9: 1 oil:water ratios with a 25: 1 dispersant ratio were used. Further experiments took into account CROSERF recommendations and were based on producing environmentally realistic levels of oil and dispersed oil (Swannell, 1998). Aspirators were filled with water and sealed. Oil and dispersant was added *via* a syringe and sampling was carried out by applying a gentle stream of nitrogen to the top of the oil/seawater surface through the oil addition needle. The tubing already in place meant that the oil water interface was not disturbed during sampling. The number of particles present in various size categories was counted using a Beckman Z2 Coulter Particle Count and Size Analyser. Aliquots (100 mL) of WAF or DWAF were extracted into dichloromethane (DCM) for quantification of hydrocarbon concentrations by UVF analysis.

3.3.3 Exposure Set Up:

Groups of 9 mussels (35-40mm) were exposed to 10L of seawater/WAF/DWAF in aerated glass tanks. In the first set of experiments (using loading ratios) mussel feeding rate and biomarkers were carried out after 7d exposure. In the main experiments, animals were fed an algal culture of *Isochrysis galbana* for the duration of the exposure period in order to ensure that their valves remained open and the animals were actively filtering. Mussel feeding rate was measured after 48h then the animals were placed in clean water to mimic tidal flushing and depuration into clean water. After 7d, with water changes every 48h, feeding rate was measured again and toxicological 'biomarker' measurements were carried out in selected experiments. In replicate experiments further tanks were set up so that half the mussels could be sacrificed at 48h so that body burden measurements could also be made.

3.3.4 Feeding rate Measurement:

For the purpose of measuring filtering rate the animals were transferred from the exposure vessel into individual containers containing a known amount of seawater. The animals were allowed an acclimatisation period of 30 minutes to open their valves and resume pumping prior to the addition of algae. Algal culture (volume pre-determined to give a cell concentration of 12, 000 -15,000

cells/0.5mL) was then added to each beaker and the water gently stirred by use of magnetic stirrer to ensure even distribution of algae within the beaker. An aliquot (20mL) of medium was taken from each beaker and the cell count determined in triplicate per aliquot using a Coulter Counter set to measure particles greater then 3µm in diameter. A further aliquot was taken after 15 minutes and the decline in cell concentration over the time period calculated.

3.3.5 Biomarker Measurements:

Haemolymph extraction was performed by inserting scissors into the shell above the bysall threads to prise the shell open and to drain any excess mantle fluid. A 1 mL syringe with a 21 gauge needle was inserted into the posterior abductor muscle and the haemolymph slowly withdrawn. Following needle removal, haemolymph was expelled into a siliconised Eppendorf tube and held on ice for Neutral Red and phagocytosis assays. The Neutral Red cell viability and phagocytosis assays were adapted from original protocols by Babich and Borenfreunde (1990) and Pipe and Lowe (1995).

Cell viability was assessed by measuring the uptake and rate of release of Neutral Red dye. Haemolymph (50 µL in duplicate) from each mussel was pipetted into a pre-poly-l-lysined microtitre plate covered with a sealer and placed in the fridge for 50 min to allow cell adherence to the plate. Following 50 min all haemolymph was discarded from the plate and 200 µL of 0.004 % Neutral Red solution was added. The plate was covered and left in the dark at room temperature for 3 h. All liquid was removed from the plate and cells were washed once with phosphate buffer solution. Acetic acid/ethanol reagent (200 µL) was then added to lyse the cells and to release the neutral red dye. The plate was placed on a plate shaker for 5 min at 600 rpm, then covered and placed in the dark for 10 min. After 10 min the absorption of visible light by each well plate was read on a spectrophotometer at 550 nm.

Immune response was measured by a phagocytosis assay. Haemolymph (50 µL in duplicate) was placed in each well along with an additional 2 wells as negative controls, placed on a plate shaker at 600 rpm for 5 min then transferred to the fridge for 50 min. At 50 min 100 µL of Bakers formol calcium was added to the negative control cells and left for 10 min at room temperature. At 60 min all liquid was removed from the plate and 50 µL of shaken zymosan solution (5×10^8 particles per mL^{-1}) was added to each well. The plate was covered and placed in the fridge for 30 min. At 30 min, 100 µL of Bakers formol calcium was added to preserve the cells, the plate covered and left for 10 min. Following this the plate was transferred onto a plate shaker for 5 min at 700 rpm to separate the supernantant which was then removed. All cells were carefully washed with 100 µL phosphate buffer solution and placed back onto the plate shaker for 5 min. Supernatant was removed and 100 µL phosphate buffer solution added. In addition 100 µL of zymosan standards (in duplicate) were added to the plate. Following a further 5 min on the plate shaker (700 rpm), 100 µL of acetic acid/ethanol reagent was added to all wells to lyse the cells. After 10 min the plate was shaken for 1 min at 200 rpm then read on a plate reader at 550 nm.

3.3.6 Chemical Analysis:

Water samples (100 mL) of WAFs and DWAFs were taken at 0 and 48h exposure and extracted into DCM (3 × 25 mL + 25 mL rinse of separating

funnel) for quantification of hydrocarbon concentrations by UVF. Fluorescence values were compared to a standard curve derived from known concentrations of oil dissolved in DCM. Samples were then carefully concentrated to 1mL by rotary evaporation followed by micro Kuderna Danish evaporation to minimise loss of volatiles, and analysed by cryogenic GC-MS. Frozen mussel tissue was allowed to defrost at room temperature, dissected over ice and homogenised. The tissue samples were extracted using an alkaline saponification method described by Kelly *et al.* (2000). Internal standards were added to approximately 30 g of wet tissue which were digested with potassium hydroxide pellets (20 g) and methanol (100mL) under reflux (2 hr). When cool, the digests were filtered through solvent-rinsed filter papers (Whatman 113v) into 250 mL separating funnels. The filtrates were twice extracted with *n*-pentane (2 × 50 mL) and the combined extract dried with anhydrous sodium sulphate, which was then transferred to a 100 mL volumetric flask and made up to volume. Fluorescence values were compared to a standard curve derived from the oil dissolved in *n*-pentane. Samples were then carefully concentrated to 1mL by rotary evaporation followed by micro Kuderna Danish evaporation to minimise loss of volatiles, and analysed by GC-MS.

3.4 Ecotoxicology with *Corophium volutator*:

3.4.1 Collection and maintenance of organisms during acclimation:
C. volutator and sediment were collected from an intertidal area of the Avon estuary near Aveton Gifford, south Devon UK (Ordnance Survey Grid reference: SX 683 467). Neonate amphipods were collected and separated from adults by sieving the upper 5 cm of sediment (neonates passed through a 500 µm sieve but were retained on a 300 µm sieve) and transported back to the laboratory within one hour, where they were placed in 5 L culture tanks lined with field-collected sieved (<300 µm) sediment. The tanks were filled with filtered seawater (25 ± 1 psu) which was aerated and maintained at 15 ± 1 °C with a 12:12 h light/dark cycle. The animals were fed weekly and the water replaced 24 h after feeding. Amphipods were maintained under the above conditions for a minimum of 7d after removal from the field to acclimate them to experimental conditions. Water quality measurements: dissolved oxygen, temperature, pH and salinity were measured prior to water changes.

3.4.2 Reference toxicity test:
In order to test that the selected *C. volutator* population is representative of the *C. volutator* populations in general in terms of sensitivity to standard toxicants, the test organisms were exposed to a known toxicant, cadmium chloride ($CdCl_2$) using a method described by Ciarelli *et al*., (1997) and their sensitivity compared with published data. In brief, static aqueous tests of 72 h exposure were performed in the absence of sediment. Twenty adult amphipods were placed in nominal $CdCl_2$ concentrations ranging from 0 to 14.0 mg L^{-1}, two replicates per treatment, with a salinity of 31 psu and gentle aeration *via* a glass Pasteur pipette. The organisms were monitored daily throughout the experiment and the number surviving and deceased recorded

after 72 h. A 72 h LC_{50} value was derived using the trimmed Spearman-Kärber method and compared with literature values.

3.4.3 Preparation of Water-Accommodated-Fractions (WAFs) and chemically dispersed WAFs (DWAFs):
The apparatus was similar to that described by Ali *et al*., (1995). In brief, 25 mL of crude oil was slowly vortex mixed with 2475 mL of 25 psu seawater at a ratio of 1:99 for 24 h within a 5 L Pyrex bottle then left to re-equilibrate for one hour. DWAF was produced as above but with the addition of dispersant with an oil:dispersant ratio of 25:1 premixed with the oil. After mixing, the solution of WAF or DWAF was carefully siphoned off under low nitrogen pressure. As it was desired that the WAF should contain only the soluble component of the oil, the number of particles present in various size categories was counted using a Beckman Z2 Coulter Particle Count and Size Analyser and compared with that of seawater. WAFs were considered acceptable if particle counts were <5× that of seawater. Aliquots (100 mL) of WAF or DWAF were extracted into dichloromethane for quantification of hydrocarbon concentrations by UVF analysis.

3.4.4 Spiking of sediments:
Aliquots of 160 mL of sieved sediment were placed in wide neck glass 500 mL bottles (Schott). Aliquots of 320 mL WAF, DWAF, dispersant and 25 psu seawater were added to the bottles and shaken at 15 °C for 3.5 h at 200 rpm on an orbital shaker. The slurry from each bottle was transferred to 2 L Pyrex beakers, allowed to settle for 16 h then the supernatant poured off and the beakers refilled to the 1200 mL mark with 25 psu seawater. Direct spiking of oil was performed *via* micro-litre syringes into 160 mL aliquots of sieved wet sediment within 2 L Pyrex beakers. Although inefficient in terms of transfer of oil to sediment, this method was used in preference to using carrier solvents and/or spiking into dry sediment, as recommended by Roddie & Thain (2001), as such processes cannot occur in the environment and may result in different proportions of oil components being incorporated into the sediment than would occur naturally. The oil and sediment was homogenised with 50 mL of 25 psu seawater for 60 s using a motorised hand blender to produce a slurry. The blender was rinsed into the vessel with an additional 50 mL of 25 psu seawater. The slurry was left to settle for 24 h, the supernatant poured off and the beakers refilled to the 1200 mL mark with 25 psu seawater. All beakers were left to settle for a minimum of 20 h with gentle aeration before the addition of the amphipods. An additional replicate for each treatment was produced for chemical analyses.

3.4.5 Acute sediment toxicity tests:
Acute sediment tests were based on standard 10d sediment toxicity tests (ASTM, 2000; Roddie and Thain, 2001; USEPA, 1994). A slight alteration to the standard protocol was made in order to give greater consistency with the chronic tests i.e. a 12:12 h light:dark regime was imposed in preference to continuous light. Adult *C. volutator* (size range 4 – 7 mm, n = 20) were exposed to nominal oil spikes of 220, 440 and 880 µg g^{-1} dry wt. and 100 % WAF and DWAF spiked sediment exposures with dispersant-only (spiking concentration 400 mg L^{-1} dispersant) and negative controls (3 replicates per

exposure treatment and a total of 6 control replicates). The animals were not fed during the test. At the end of the test the sediment was gently sieved (300 µm) and the number of alive, dead and missing amphipods in each vessel recorded. Water quality measurements were recorded prior to commencement of the test, on day 5 or 6, and at the end of the test.

3.4.6 Chronic sediment toxicity tests:

Chronic tests were based on the acute 10-day sediment test (Roddie and Thain, 2001) and the USEPA (2001) amphipod chronic sediment test. Neonate *C. volutator* (mean length 1.4 mm, SD 0.31 mm; mean weight 0.1 mg) were sorted from stock by carefully passing organisms through a 500 µm sieve to remove larger organisms. The sediment was then carefully re-sieved (300 µm) to collect the test organisms. Amphipods (n = 30) were transferred to 25 mL beakers *via* plastic Pasteur pipettes and then randomly allocated to exposure vessels. Two litre Pyrex beakers were used as exposure vessels as these provide a greater sediment surface area than the standard one litre beakers. Nominal sediment concentrations were 0**,** 110, 220 and 440 µg oil g^{-1} dry weight sediment. Control treatments were spiked with deionised water. Nine replicates for each of seven treatments were used to provide three replicates per treatment for evaluation after 28d and the remaining six replicates for evaluation upon termination of the test. An additional set of control replicates was used to give a total of 72 exposure vessels. The animals were fed weekly with 2 drops of standard aquarium invertebrate food and the overlying water 80 % replaced 24 h after feeding. Water quality measurements were measured before addition of amphipods and prior to water exchanges during the test.

After 28d exposure, the amphipods from three replicates of each treatment were counted, collectively weighed to 0.1 mg (amphipods were carefully blotted on absorbent paper to remove excess water) and individually measured to the nearest 0.1 mm (excluding antennae) under a dissecting microscope. The test was terminated when reproduction was apparent in all replicates of the control treatment. Survivorship, wet weight and length of organisms were recorded. The numbers of mature adult (≥5.0 mm), sub-adult (<5.0 mm), gravid females and neonates were also counted. Although the majority of neonates could be detected from their movement and separated from the debris for enumeration, the separation from the debris of the remaining organisms was facilitated by the addition of 70 % isopropanol plus few drops of Rose Bengal solution (ca 1g L^{-1}). All neonates were preserved in the 70 % isopropanol /Rose Bengal solution for recounting for quality assurance (QA) purposes.

3.4.7 Chemical analyses of water and sediment:

Water samples aliquots (100 mL) of WAF or DWAF were extracted into DCM (3 × 25 mL + 25 mL rinse of separating funnel) for quantification of hydrocarbon concentrations by UVF analysis. Fluorescence values were compared to a standard curve derived from oil dissolved in DCM. Samples were then carefully concentrated to 1mL by rotary evaporation followed by micro Kurderna Danish to minimise loss of volatiles and analysed by GC-MS. Frozen sediment samples were allowed to defrost at room temperature then mixed with a stainless steel spatula. The dry weight percentage of each

sample was determined by weighing subsamples (×2) in pre-weighed foil dishes then re-weighing after drying at 105 °C for 16 h. The sediment samples were extracted using an alkaline saponification method described by Kelly *et al*., (2000). In brief internal standards were added to approximately 50 g of wet sediment samples and digested with potassium hydroxide pellets (5 g) and methanol (100mL), under reflux for 2 hours. When cool, the digests were filtered through solvent-rinsed filter papers (Whatman 113v) into 250 mL separating funnels. The filtrates were twice extracted with *n*-pentane (2 × 50 mL) and the combined extract dried with anhydrous sodium sulphate, which were then transferred to 100 mL volumetrics and made up to volume. Fluorescence values were compared to a standard curve derived from the oil dissolved in *n*-pentane. Samples were then carefully concentrated to 1ml by rotary evaporation followed by micro Kurderna Danish to minimise loss of volatiles and analysed by GCMS.

3.5 Biodegradation of Oil for Toxicity Tests:

WAFs and DWAFs were prepared as normal for toxicity tests with *Mytilus edulis* and *Corophium volutator*, and inoculated with a mixed bacterial culture representative of those found in seawater at a hydrocarbon contaminated site. Pure cultures of each bacterium were grown in nutrient broth at a suitable temperature to achieve growth in 24-48h. The cultures were then placed in a cold room (4˚C) to minimise growth whilst an estimate of the bacterial population was carried out using the Miles Misra method (*ca* 10^7 mL^{-1}). The bacteria were then re-suspended in phosphate buffer solution and made up to the same concentrations as those reported in chronically impacted oil spill sites and shipping lanes and spiked into WAFs and DWAFs to simulate environmental biodegradation. The vessels were left for two weeks and then exposure to the organisms took place as previously described. All work was carried out using aseptic techniques and procedures described in section 3.2

4. Results and Observations

4.1 Oil Weathering:

Evaporative weathering experiments with FB and ANS crude oils in a laboratory fume hood (0.5 m sec^{-1} airflow, 18-20°C) for 24h yielded approximately 28% and 20% volume losses respectively. Excellent reproducibility was shown in triplicate experiments. The evaporative losses were comparable with those from both experimental oil spills at sea and from real spills and provided a reproducible substrate for the toxicity and biodegradation experiments described below. Detailed results are presented in Appendix 2.

4.2 Oil Biodegradation:

The viability of the bacteria used was monitored throughout the experiment. At every stage samples were taken and incubated overnight. In each case bacterial growth was visually evident confirming that the bacteria were viable throughout the experiments. To check no biodegradation was occurring in abiotic controls, plates were streaked with samples from abiotic vessels. No growth was evident, confirming that no biodegradation was occurring in these vessels. The Miles Misra method for determining the number of bacteria in each vessel gave variable results throughout the experiments. However, in all experiments higher bacterial counts were found in dispersed treatments.

At the end of the experiments there was no observed difference in the gas chromatograms of FB oil without dispersant at either temperature (8 and 15°C). There was no observed increase in biodegradation of the oil treated with SD-25 at 8°C, but there was an observable reduction of *n*-alkanes when compared to the internal standard (d-*n*-tetracosane) at 15 °C when SD-25 was used. This was supported by measurements of the ratios of selected individual hydrocarbons. For example, the ratio of *n*-heptadecane to pristane decreased from 2 to 0.9 in the oil-only treatment over the course of the experiment at 15°C, but decreased from 2 to 0.3 in the oil with dispersant treatment at 15°C. Also a 20 % relative increase in the unresolved complex mixture (UCM) of the dispersed treatment was noted. For the experiments with ANS, no reproducible increase in biodegradation was noted with increase in temperature or with C-9527 dispersant.

For FB crude oil no significant differences in total saturates, aromatics, resins and asphaltenes as determined by TLC-FID, were found at either temperature or with dispersant use, even though some of the individual *n*-alkanes were visibly reduced at 15 °C as noted above, whilst for ANS oil a small reduction in the aromatic hydrocarbons was noted in the biotic treatments with and without dispersant at the higher temperatures. However, no differences were

observed in individual aromatic components when examined by GC-MS. For example, distributions of methylphenanthrene isomers remained unchanged, when changes in these isomers have previously been used to indicate biodegradation in oil samples (Rowland *et al*., 1986). Detailed results are presented in Appendix 3.

5.3. Toxicity Tests with *Mytilus edulis*:

(a) Scoping experiments

In initial tests at high oil:water loadings (Figure 1), and nominal dispersant concentrations of 50-400 μg mL^{-1} (ppm) mussel feeding rate was reduced (i.e. a toxic response observed) in all exposures to dispersed oil (DWAF) but only at the highest exposures to non-dispersed oil (WAF).

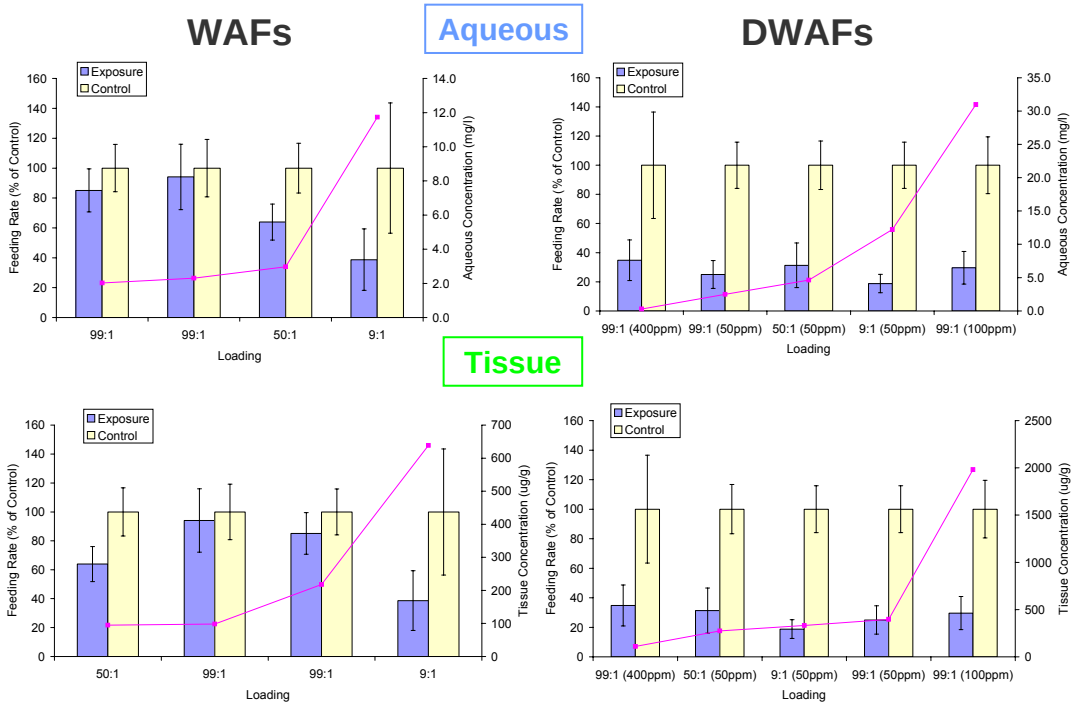


Figure 1. Effects of the exposure of mussels (*M. edulis;* n=12) to non-dispersed (WAF) and dispersed (DWAF; Superdispersant 25) weathered Forties Blend crude oil for 7d at different, but generally high, oil:water loadings. Histograms show the effects on the feeding rates of the treated mussels (blue) compared with untreated control organisms (yellow); a decrease in feeding rate indicating a toxic effect. Also shown (pink line emphasises discrete points) are the concentrations of hydrocarbons actually measured in both the water ('aqueous') and the mussel body tissues ('tissue'). The latter measurements obviate the need to rely on nominal oil:water loadings and overcome the difficulties in the production of consistent, reproducible WAF and DWAF fractions. The data indicate that whilst only the highest oil:water loading (9:1) produced a toxic effect for the non-dispersed oil, all loadings caused a toxic effect when the oil was dispersed and when both oil and dispersant was present. Later experiments clarified the individual contributions of oil and dispersant to this effect. Loadings figures in parentheses refer to the nominal concentrations of dispersant (50-400 ppm).

For non-dispersed oil, mussel feeding rate was related both to the aqueous concentrations of oil and to the accumulated hydrocarbon body burdens.

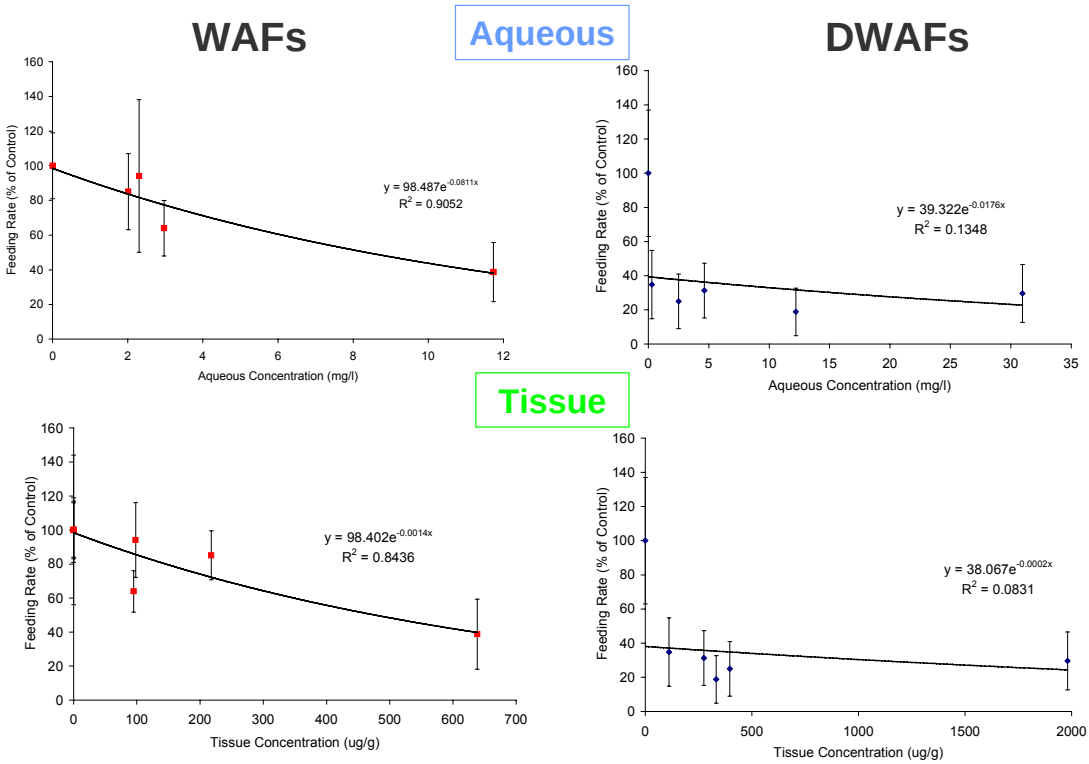


Figure 2. The relationship (upper graphs) between mussel feeding rate and the aqueous hydrocarbon concentrations ('aqueous') of mussels exposed to water accommodated weathered Forties Blend crude oil (WAF) alone or weathered Forties Blend crude oil dispersed with Superdispersant-25 (DWAF). Also shown (lower graphs) is the relationship between the hydrocarbon body burdens of mussels exposed to Forties Blend crude oil (WAF) alone or to weathered Forties Blend crude oil dispersed with Superdispersant-25 (DWAF).
Increasing concentrations of oil in the water and accumulated by the mussels caused an increasing toxic effect (decreasing feeding rate) but for dispersed oil the effects were pronounced even at low concentrations of oil, suggesting perhaps that the dispersant was also producing a toxic effect at these loadings since the amount of oil present was perhaps unlikely to produce such an effect.

A similar response was observed for the retention of Neutral Red dye, *i.e*. as exposure concentrations of non-dispersed oil in the water and accumulated by the mussels, increased, the feeding rate and dye retention decreased in a linear fashion (Figure 3). The latter indicates an increased cell lysis and is the expected toxic response. However, when mussels were exposed to dispersed oil, this relationship was less clear.

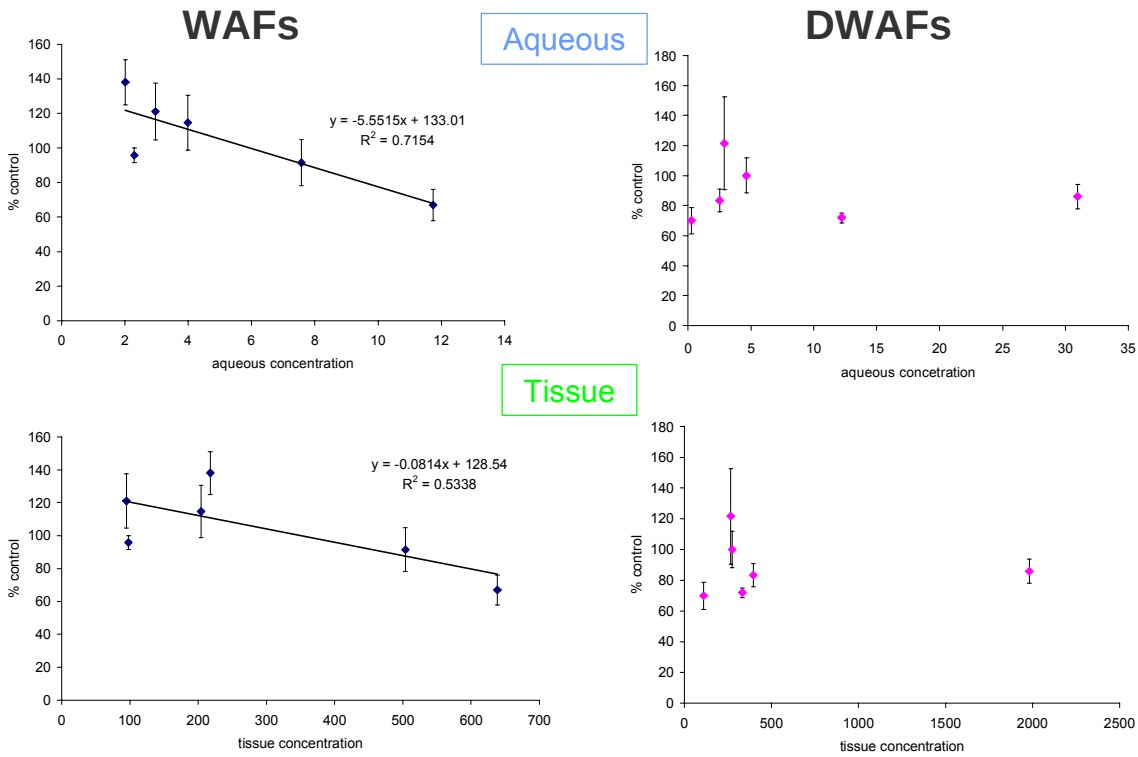


Figure 3. The relationship between the retention of Neutral Red dye by mussel cells and the aqueous concentrations and hydrocarbon body burdens of mussels (n=12) exposed to Forties Blend crude oil (WAF) or Forties Blend crude oil dispersed with Superdispersant-25 (DWAF). Decreases in the retention of dye indicate an increased cell lysis or 'leakiness' which is the expected toxic response.

The phagocytosis bioassay is a well-accepted measure of autoimmune function. The autoimmune response of mussels exposed to FB oil or to oil dispersed in water with SD-25 (Figure 4) was typical of that expected from a toxicological effect with autoimmune induction at low exposure levels followed by a reduction at higher levels of exposure.

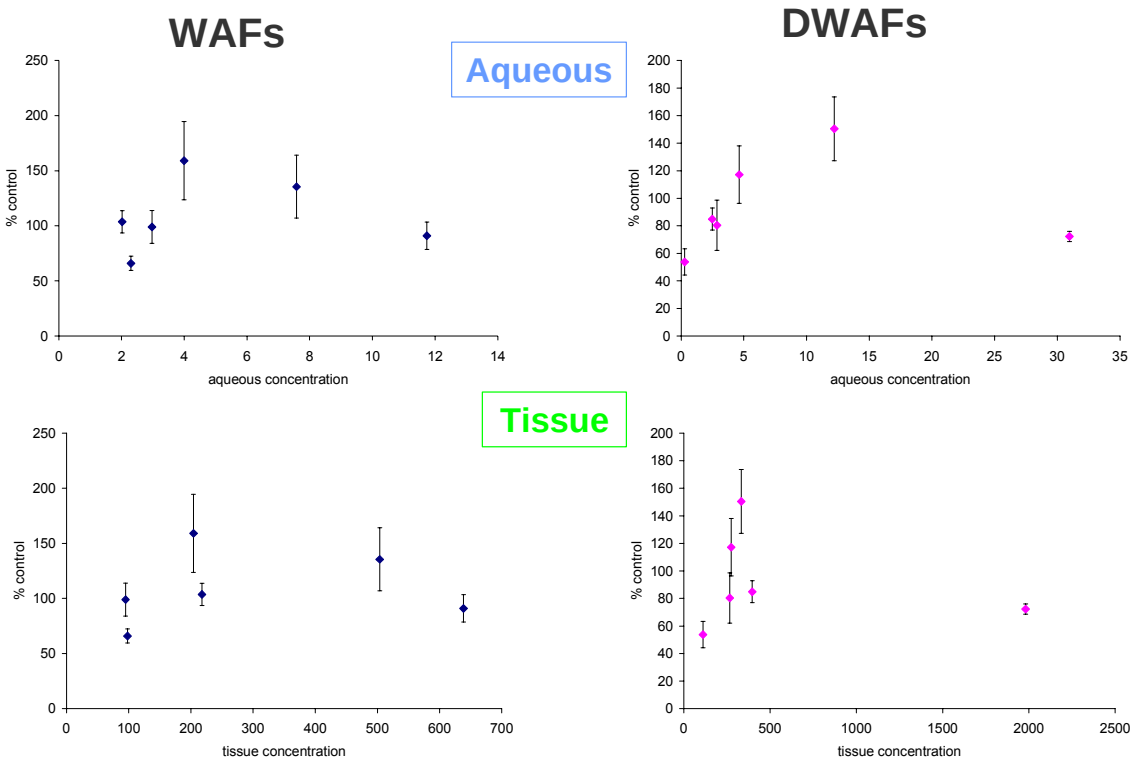


Figure 4. The relationship between phagocytosis (immune function) and the aqueous hydrocarbon concentrations ('aqueous') of mussels exposed to water accommodated weathered Forties Blend crude oil (WAF) alone or weathered Forties Blend crude oil dispersed with Superdispersant-25 (DWAF). Also shown (lower graphs) is the relationship between pahgocytosis and the hydrocarbon body burdens of mussels exposed to Forties Blend crude oil (WAF) alone or to weathered Forties Blend crude oil dispersed with Superdispersant-25 (DWAF). An increase in auto immune response followed but a reduction- as observed in both WAF and DWAF-exposed organisms, is a typical toxicological response.

Figure 5 shows that the relationship between the concentrations of FB oil in the water used in the mussel exposure experiments and the concentrations of FB oil in the tissues of the exposed mussels, was linear. However, since this was true for the experiments conducted with both non-dispersed (WAF) and dispersed oil (DWAF), the observed apparent toxicity of the DWAF (e.g. Figure 1) could not be due to the oil. Rather, this suggested that the SD-25 was causing a toxic effect. Experiments were therefore conducted with SD-25 alone (in the absence of oil).

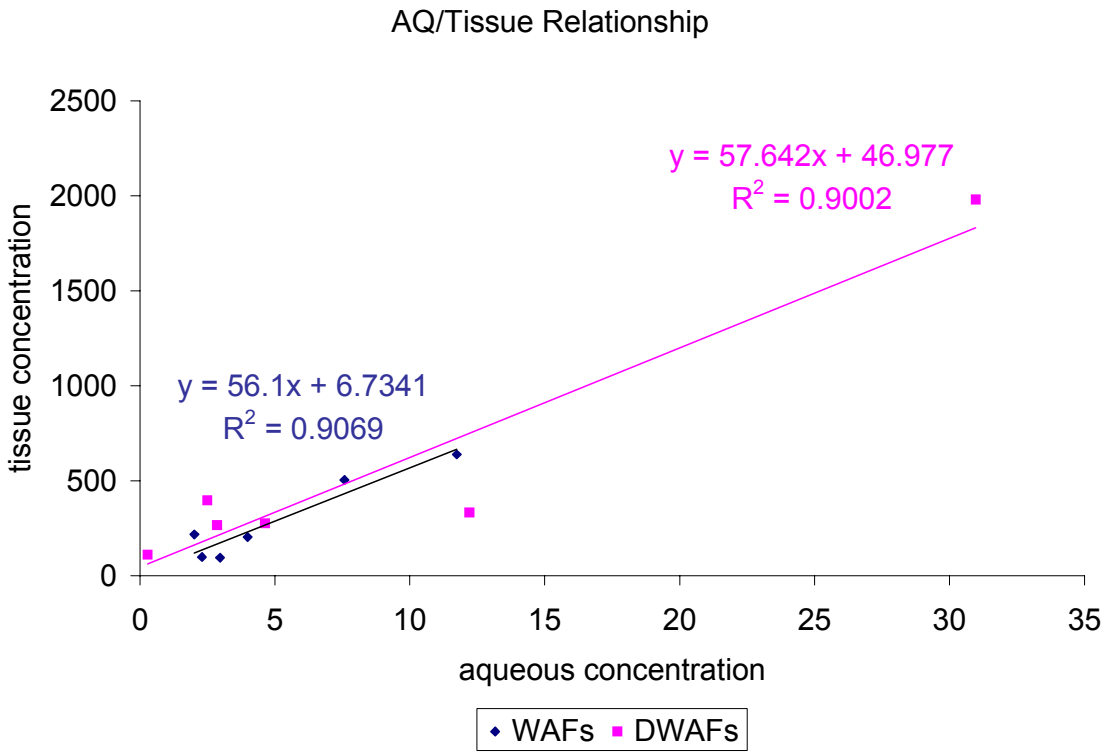


Figure 5. The relationship between aqueous concentration and accumulated body burdens for mussels exposed to Forties crude oil and Superdispersant-25.

When SD-25 was tested alone at the concentrations used in the treatments with dispersed oil (e.g. Figure 1) it did indeed cause a significant reduction in mussel feeding rate (Figure 6).

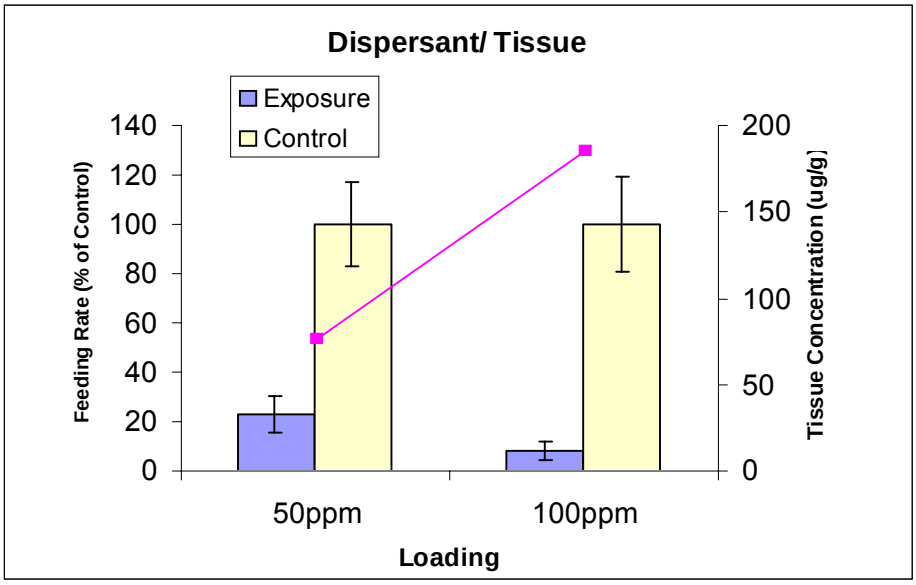


Figure 6. Feeding rate of mussels (n=12) unexposed (controls) or exposed to Superdispersant-25 at concentrations used in treatments with dispersed Forties Blend oil under similar conditions (*cf* Figure 1). Clearly, given these results, the reduction in feeding rates observed in the experiments with DWAF shown in Figure 1 can be attributed to the effects of the dispersant rather than of the dispersed oil. Concentrations (loadings) of dispersant shown are nominal and represent the lower concentrations used in the scoping experiments with dispersed oil (cf Figure 1).

(b) Exposures of mussels to environmentally realistic concentrations of oils

In the remainder of the toxicity experiments with mussels, more environmentally realistic oil and dispersant loadings and exposure concentrations were used. A 'spiked recovery' regime was also investigated as described in the experimental section, whereby mussels exposed to toxicants for 2d were allowed a further 5d recovery period in clean water.

Upon exposure to environmentally realistic concentrations (nominally 10-15 ppm) of either SD 25 or C-9527 dispersant, followed by a further 5d recovery period, mussel feeding rate was not significantly reduced compared with unexposed controls (Figure 7). The highest concentration of C-9527 did cause a reduction in feeding rate after 48h compared with controls, but the feeding rate was restored to that of controls after a further 5d in clean water.

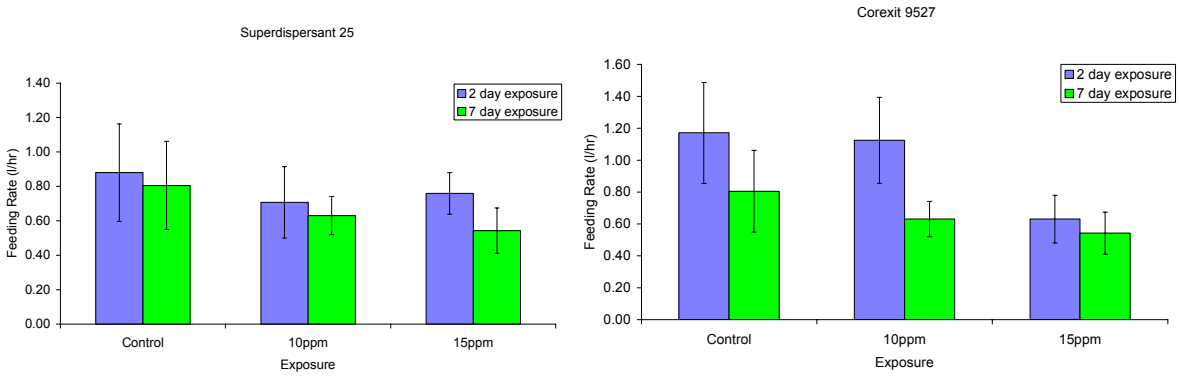


Figure 7. Effects on mussel feeding rate of exposure to the dispersants, Superdispersant 25 and Corexit 9527 at environmentally realistic concentrations (highest measured concentration 13ppm) for 48h followed by 5d in clean water (n=9). Only the highest concentration of C-9527 caused a reduction in feeding rate after 48h compared with controls, but the feeding rate was restored to that of controls after a further 5d in clean water

Figure 8 shows the effects on feeding rate of mussels exposed to FB oil with and without SD-25. Mussel feeding rate was reduced after 48h when exposed to the oil and to a greater extent to the dispersed oil (since the dispersant increased the concentrations of oil in the water, as illustrated by the right hand y axis of the histograms in Figure 8. After the recovery period in clean water the feeding rate of the mussels improved for both and a greater recovery was noted for the mussels exposed to the dispersed oil. Two of the experiments (exposures 4, 5) showed very high variability due to mussel spawning in the exposure vessels.

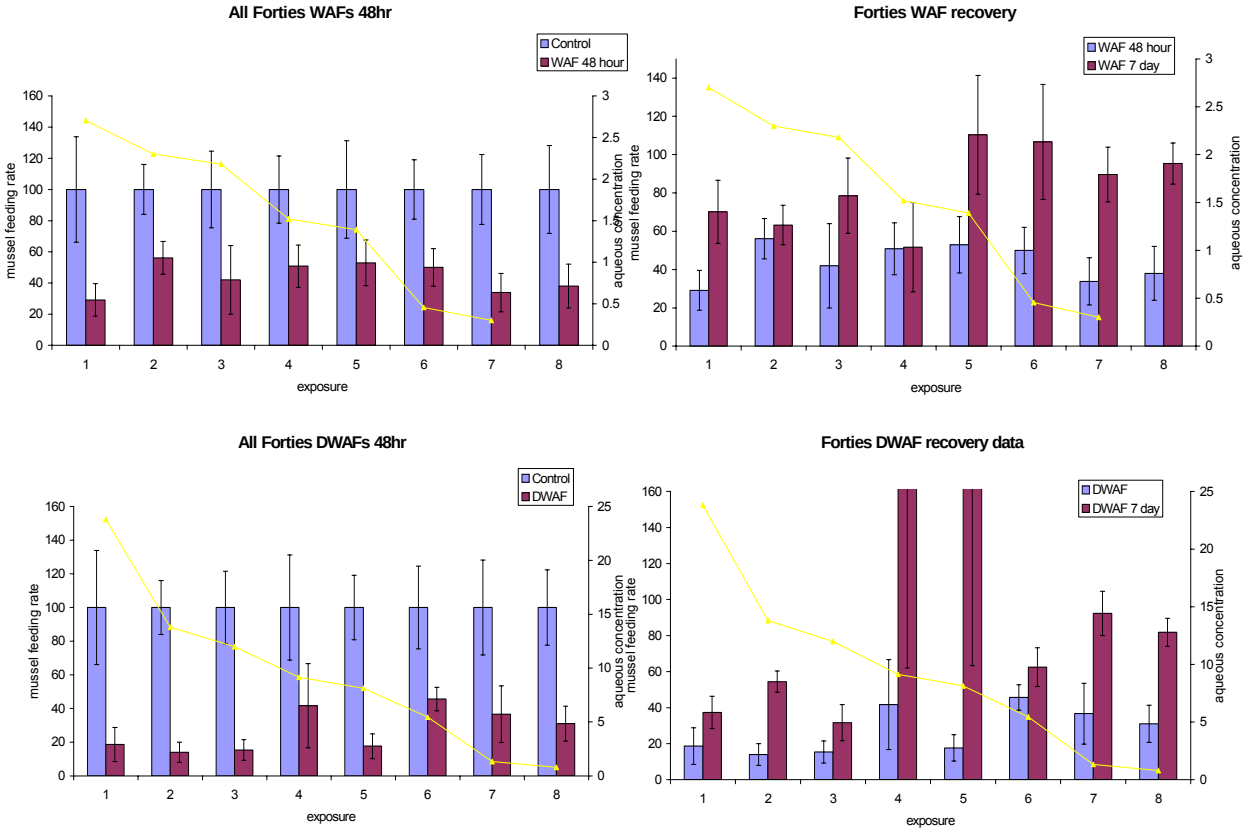


Figure 8. Effects on mussel feeding rate to the exposure of mussels (*M. edulis;* n=9) to non-dispersed (WAF; upper graphs) and dispersed (DWAF; Superdispersant 25; lower) weathered Forties Blend crude oil for 2d and after a further 5d in clean water. Histograms show the effects on the feeding rates of the treated mussels (red) compared with untreated control organisms (blue); a decrease in feeding rate indicating a toxic effect. Also shown (yellow line emphasises discrete points) are the concentrations of hydrocarbons actually measured in the water. The data indicate that whilst 48h exposures to WAF or DWAF produced toxic effects at all concentrations (ca 0.25-25 mg L^{-1}), after 5d in clean water, mussels recovered from the exposures to at least the same extent as control organisms. The oil:water loadings in these experiments resulted from stirring 3.5 g oil on the surface of 10L of seawater as advocated previously (Swannell *et al*., 1998; *cf* Singer *et al*., 2001) for the production of environmentally realistic concentrations of DWAF. Exposures 1-8 reflect successive dilutions of these WAFS/DWAFs as shown by the falling actual measured concentrations of oil (yellow line). Aqueous concentrations are in mg L^{-1}.

Figure 9 shows the feeding rate of mussels exposed to ANS with and without C-9527 dispersant. Mussel feeding rate was reduced after 48h when mussels were exposed to the oil (WAF) and to a much greater extent when mussels were exposed to dispersed oil (DWAF). At the highest aqueous exposure significant mortality was observed. After the 5d recovery period the mussels increased their feeding rate.

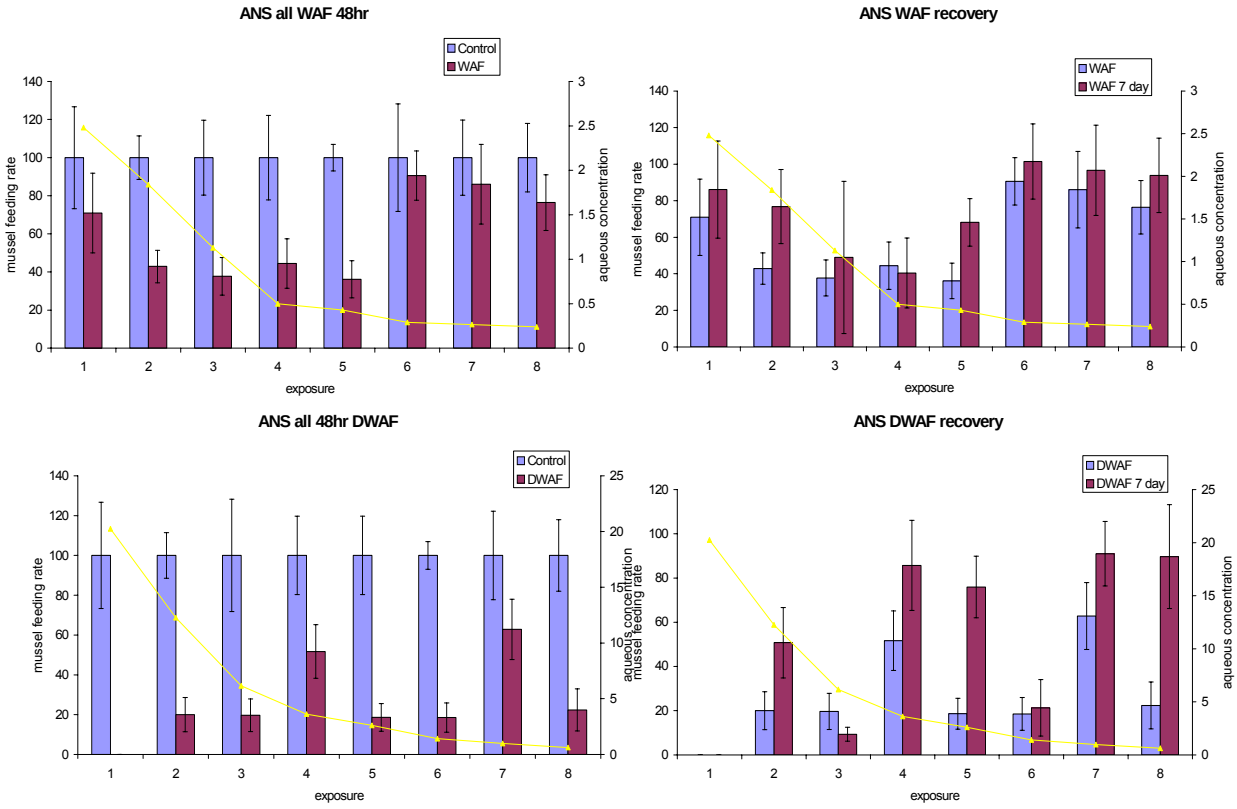


Figure 9. Effects on mussel feeding rate to the exposure of mussels (*M. edulis;* n=9) to non-dispersed (WAF; upper graphs) and dispersed (DWAF; Corexit 9527; lower) weathered Alaska North Slope crude oil for 2d and after a further 5d in clean water. Histograms show the effects on the feeding rates of the treated mussels (red) compared with untreated control organisms (blue); a decrease in feeding rate indicating a toxic effect. Also shown (yellow line emphasises discrete points) are the concentrations of hydrocarbons actually measured in the water. The data indicate that whilst 48h exposures to WAF or DWAF produced toxic effects at all concentrations (ca 0.25-25 mg L^{-1}), after 5d in clean water, mussels recovered from the exposures to at least the same extent as control organisms, except at the highest concentration where the organisms died after 2d (exposure 1). Aqueous concentrations are in mg L^{-1}.

The data in Figures 8 and 9 were also plotted against aqueous exposure concentrations which for both oils were in the range of 0-3 mg L^{-1} and for dispersed oil 0-25 mg L^{-1}. It would appear that although highly comparable exposures took place, the ANS oil did not impact mussel feeding rate as much as the FB oil, whilst the dispersed ANS impacted the mussels more than the dispersed FB oil. In general, the higher initial aqueous exposures caused the greatest reductions in feeding rate and these organisms recovered less well.

Concentrations in the tissues revealed that recovery was related to depuration of oil from the tissues. The mussels exposed to dispersed oil all had higher concentrations of oil in their tissues compared to mussels exposed to oil alone after 48h. After recovery in clean water the amount of oil in the tissues decreased with a concurrent increase in feeding rate which was very dramatic for dispersed exposures. Mussels exposed to FB oil had higher concentrations of oil in their tissues than mussels exposed to ANS oil, and this was also true of the dispersed oils, although no consistent pattern was observed.

At environmentally realistic concentrations, no effects measurable *via* toxicological biomarker end points were evident, even at the highest aqueous and tissue concentrations where significant reductions in feeding rate were observed.

(c) Exposure of mussels to biodegraded oils

Mussels exposed to biodegraded oils showed a very similar pattern to those exposed to the 'weathered' oils (section b above).

Figure 10A shows that at the highest oil:water loading, mussel feeding rate was significantly reduced by both non-dispersed ((WAF) and dispersed (DWAF) biodegraded weathered FB oil (blue bars) but after being placed in clean water for a further 5d, the mussels were able to depurate the toxicants and to recover to the same extent as untreated control mussels (pink bars). With a further 50% dilution of oil in water, the same scenario was observed. Thus similar toxic responses were observed for the biodegraded oils and the undegraded weathered oils (Figure 10B).

The same toxicological response was observed for biodegraded ANS oil compared to undegraded oil (Figure 11A and B).

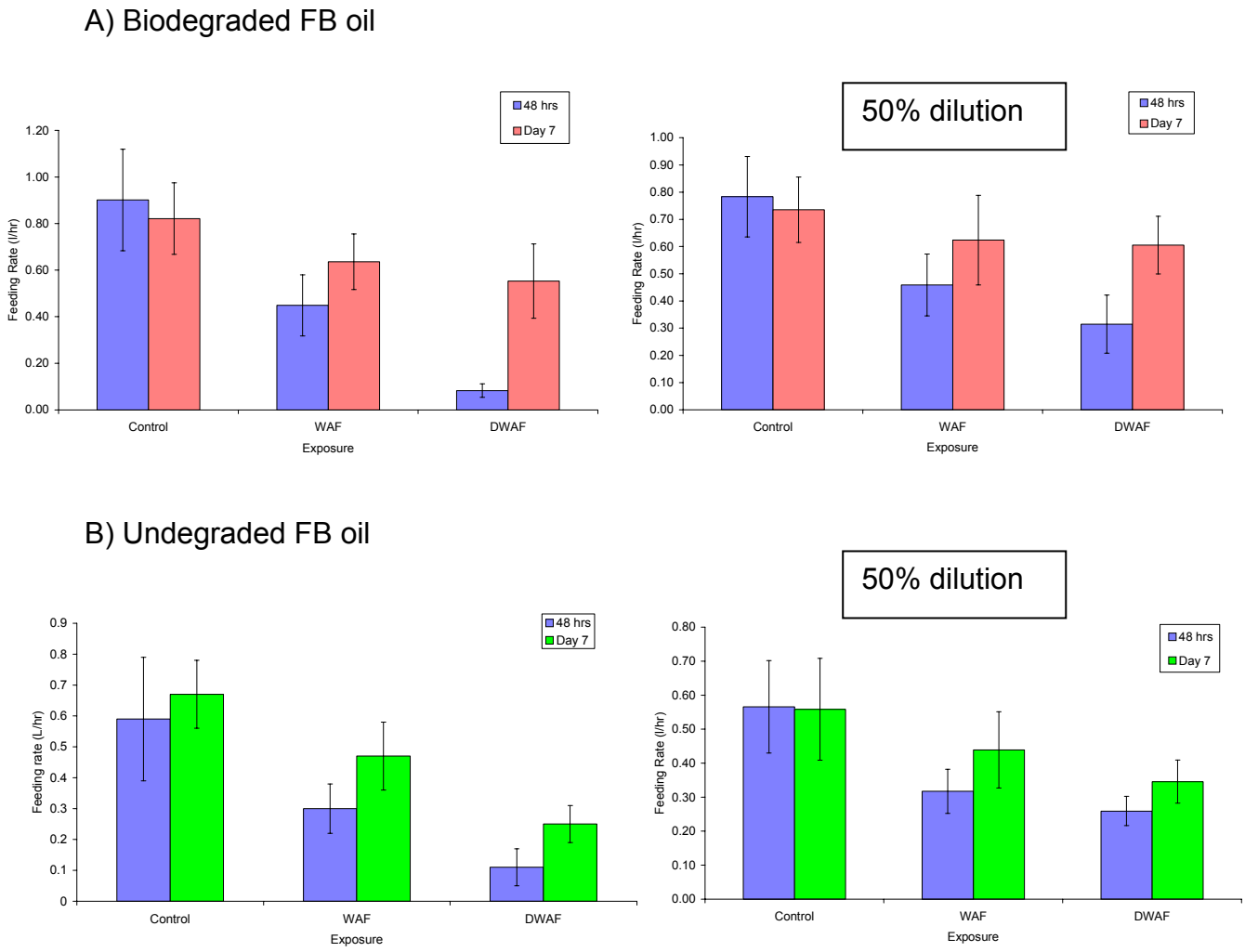
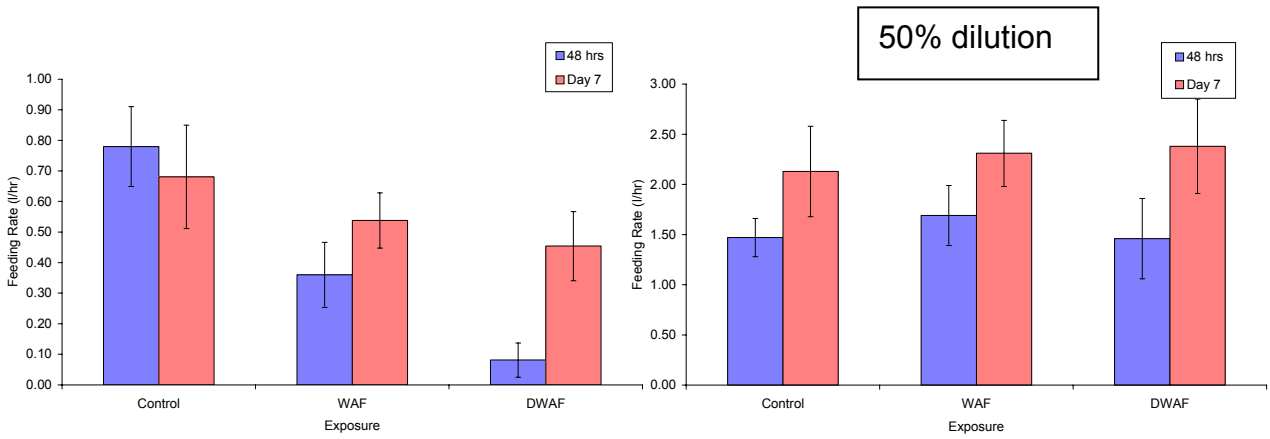


Figure 10. Effects on mussel feeding rate to the exposure of mussels at two oil:water loadings (*M. edulis*; n=9, mean ± 2 standard errors) to non-dispersed (WAF) and dispersed (DWAF; SD-25) weathered and biodegraded Forties Blend crude oil for 2d and after a further 5d in clean water (A) compared to the effects on mussel feeding rate of the exposure of mussels to non-dispersed (WAF) and dispersed (DWAF; SD-25) weathered Forties Blend crude oil for 2d and after a further 5d in clean water (B) at the same oil:water loadings. The data indicate that whilst 48h exposures to WAF or DWAF produced from biodegraded oil resulted in toxic effects, after 5d in clean water, mussels recovered from the exposures to at least the same extent as control organisms and effects were similar to those with non-biodegraded oil.

A) Biodegraded ANS oil



B) Undegraded ANS oil

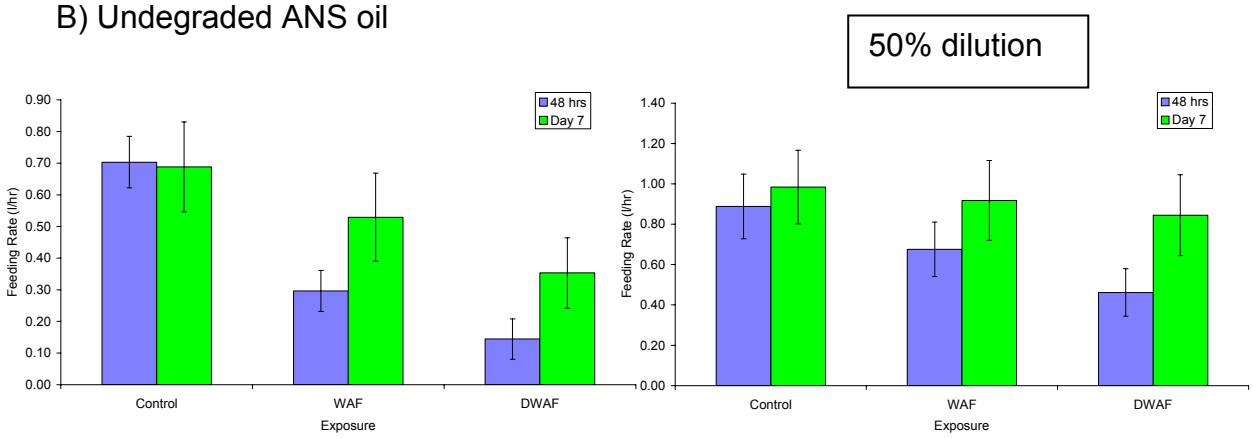


Figure 11. Effects on mussel feeding rate to the exposure of mussels at two oil:water loadings (*M. edulis*; n=9, mean ± 2 standard errors) to non-dispersed (WAF) and dispersed (DWAF; C-9527) weathered and biodegraded Alaska North Slope crude oil for 2d and after a further 5d in clean water (A) compared to the effects on mussel feeding rate of the exposure of mussels to non-dispersed (WAF) and dispersed (DWAF; C-9527) weathered Alaska North Slope crude oil for 2d and after a further 5d in clean water (B) at the same oil:water loadings. The data indicate that whilst 48h exposures to WAF or DWAF produced from biodegraded oil resulted in toxic effects, after 5d in clean water, mussels recovered from the exposures to at least the same extent as control organisms and effects were similar to those with non-biodegraded oil.

4.4 Toxicity Tests with *Corophium volutator*:

(a) Acute toxicity.

The major aim of the following bioassays with *C. volutator* was to conduct a long term life-cycle assessment of the effects of the oils and dispersants. However, it was deemed appropriate first to examine the acute toxicity of the dispersants and the oils in short term exposures.

When *Corophium volutator* was exposed to the dispersants under an aqueous exposure regime, both SD-25 and C-9527 were found to be of low to moderate toxicity. SD-25 was slightly less toxic than C-9527 and the amphipods were more able to recover following sub lethal exposure to SD-25. The results of the dispersant tests, including data for tests on additional species have been published (Scarlett *et al.*, 2005) and this publication is presented in Appendix 4.

In acute sediment exposures, no significant effects were observed with FB oil using WAF and DWAF spiked sediment or using whole oil spiked sediment at nominal concentrations up to 440 μg g^{-1} dry weight sediment. For the ANS oil, no effect was elicited by the whole oil spiked sediment up to a nominal concentration of 440 μg g^{-1} dry weight. Some mortality was recorded within the DWAF spiked exposure but this was not significantly different to the C- 9527 control.

(b) Chronic toxicity of non-biodegraded oils

Figure 12 shows the results from the chronic sediment tests with *Corophium volutator* with FB crude oil and SD-25 at 28, 75 and 110d. Survivorship was only significantly affected at the highest nominal spike concentration of oil (440 μg g^{-1} oil per dry weight sediment) whilst growth rate was affected for up to 70d by both the addition of WAF, DWAF and whole oil to the sediments. However, no significant difference was observed between the survivorship or growth rates of amphipods living in WAF and DWAF spiked sediments compared to controls, by the end of the test (110d). Surviving organisms recovered and showed no significant differences in reproduction (Figure 12 bottom). Sediment analysis showed the oil to have biodegraded within the sediment during the lifecycle of the amphipods, suggesting that as the concentration of oil in the sediment decreased, the organisms were able to recover.

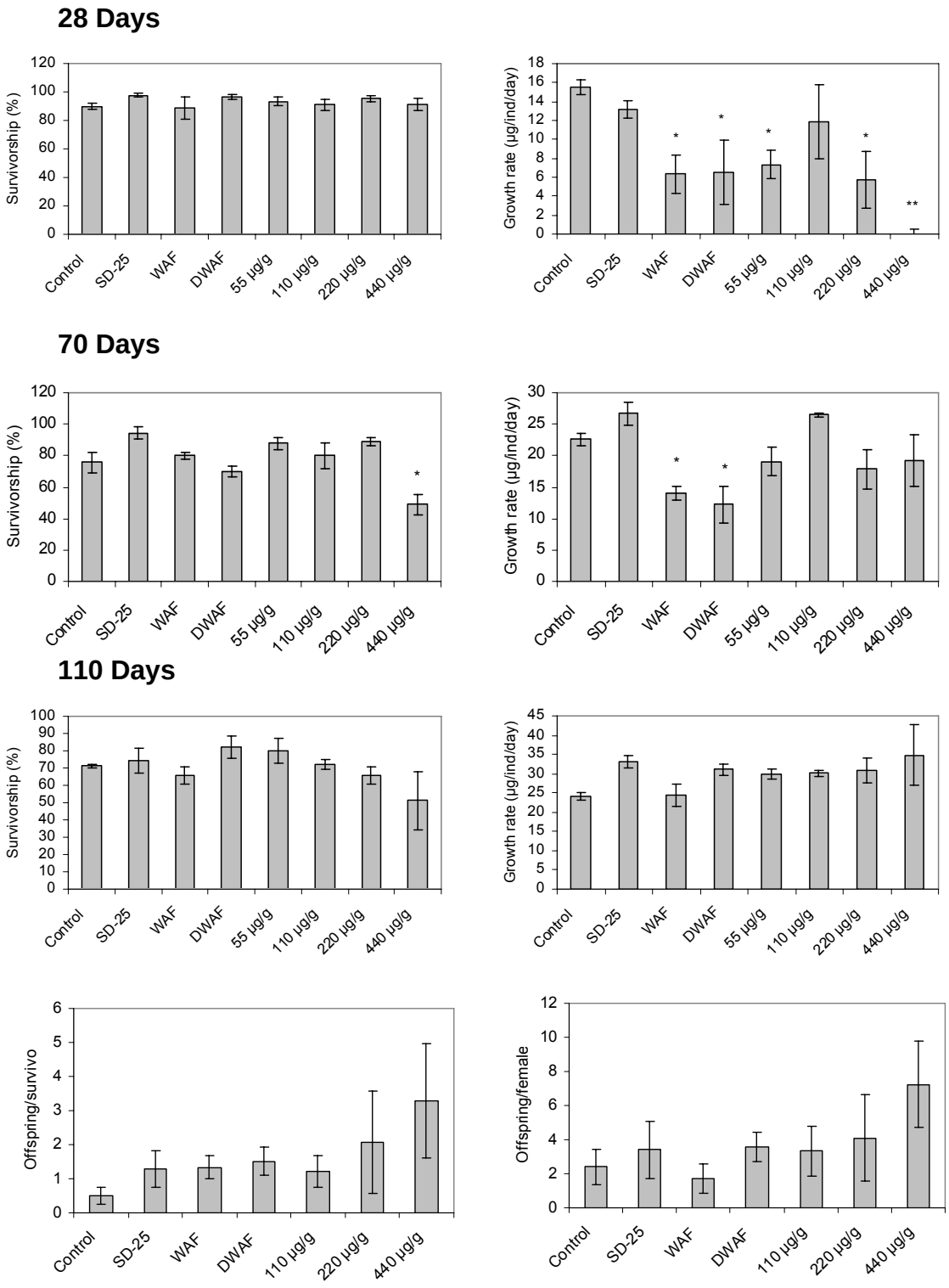


Figure 12. Survivorship and growth rates of the amphipod *Corophium volutator* after exposure to Superdispersant-25, or weathered Forties Blend crude oil, either as a water accommodated fraction (WAF), a dispersed WAF (SD-25) or added as whole oil to sediments at 110-440 µg g^{-1} after 28 , 75 and 110d . Whilst toxic effects on growth rate were observed at intermediate points, by the end of the life cycle, no significant effects on growth rate or survivorship were apparent except at the highest concentration of oil, where survivorship was effected. The effects on reproduction throughout the life cycle (110d) are also shown (bottom histograms) in terms of the ratios of offspring to survivors and offspring per female.

Figure 13 shows the results of ANS oil and C-9527 dispersant on aspects of the life cycle of the amphipod. Significant mortality occurred only within DWAF-exposed organisms, whilst growth rate was affected by all oil treatments except the WAF spiked sediment. A significant difference between DWAF and WAF exposed organisms was noted and reproduction was affected by all oil treatments except the WAF spiked sediment. Sediment analysis showed some biodegradation but substantial concentrations of oil remained in the sediment, probably accounting for the effects observed.

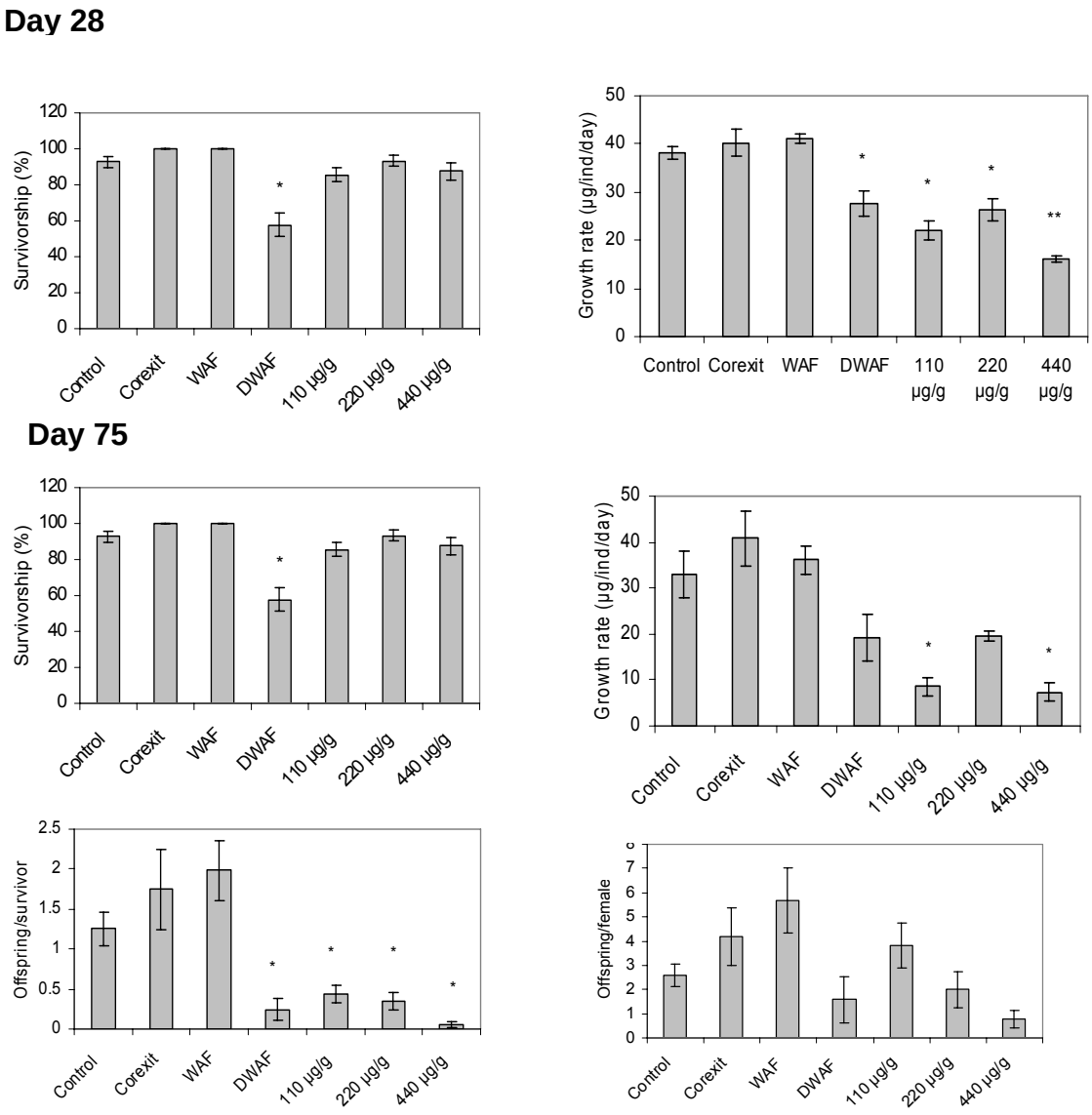


Figure 13. Survivorship, growth rates of the amphipod *Corophium volutator* after exposure to Corexit 9527, or weathered Alaska North Slope crude oil, either as a water accommodated fraction (WAF), a dispersed WAF (C-9527) or added as whole oil to sediments at 110-440 µg g^{-1} after 28 (top) and 75d (middle). Toxic effects on survivorship and growth rate were observed at intermediate points and at the end of the life cycle. Also shown (bottom histograms) are the effects on reproduction after 75d. Again, DWAF affected reproduction more than did WAF. The effects are thought to be due to the higher concentrations of dispersed ANS oil in the DWAF–treated sediment.

Figure 14 shows a comparison of the relationship between growth rate and sediment concentrations determined by UVF for the first 28d period of the life cycle assessment of *Corophium volutator* for both oils. A dramatic difference between the oils can be observed. For FB oil the WAF and the DWAF spiked sediments result in similar concentrations of oil (20-35 μg g^{-1} dry weight) and do not elicit a strong effect on growth rate, whilst for ANS oil the spiking of sediment with DWAF results in a much higher concentration of oil (*ca* 125 μg g^{-1} dry weight) than the WAF (< 10 μg g^{-1} dry weight) and in turn a much lower growth rate than produced by exposure to WAF.

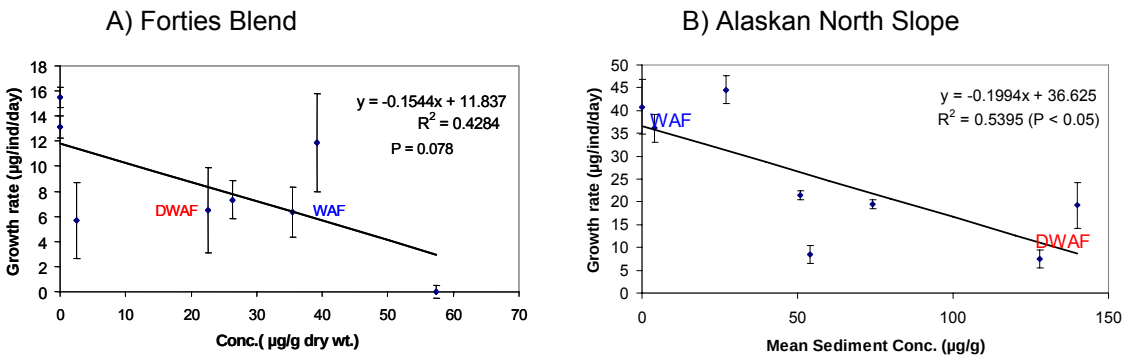


Figure 14. Relationship between growth rate and sediment concentrations for the first 28 d period of the life cycle assessment of *Corophium volutator* for dispersed (DWAF) and non-dispersed (WAF) Forties Blend and Alaska North Slope oils with Superdispersant-25 and Corexit 9527 dispersants respectively. Also shown are the effects of growth rate on exposures to the oils spiked directly into sediments. Whilst the correlations between concentrations and growth rate are poor overall, the figures serve to illustrate the very different effects of C-9527 dispersed ANS oil compared to non-dispersed ANS oil, whereas the effects of SD-25 dispersed FB oil compared to non-dispersed FB oil are not very different.

(c) Acute and chronic toxicity of biodegraded oils

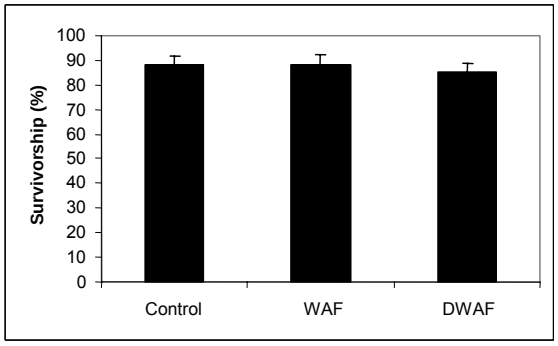
The same acute and life cycle tests were carried out with biodegraded FB oil with and without SD-25 and with biodegraded ANS oil with and without C-9527. For the acute ten day exposures no significant differences in survivorship were found for either of the biodegraded oils or the dispersed biodegraded oil.

Figure 15 shows a comparison of the results from the life cycle assessments, in terms of survivorship and reproduction, at day 28 for biodegraded weathered FB oil with and without SD-25 and for biodegraded weathered ANS oil with and without C-9527.

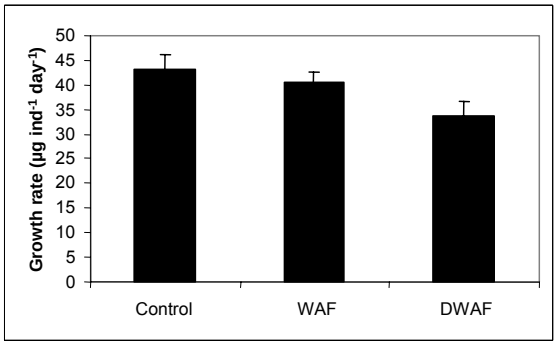
For the FB oil and SD-25 dispersant, survivorship was unaffected at 28d and although the growth rate of organisms exposed to DWAF was reduced, this was not statistically significant (P=0.05). For the ANS oil and C-9527 dispersant, although survivorship and growth rate appeared to be slightly depressed for the DWAF spiked sediments, again this was not significantly different from the result obtained for WAF (P=0.05), although it was different to the control. Apart from the exposure to dispersed ANS oil, concentrations of oil in the sediments were hardly elevated above control concentrations.

FB oil and SD-25

Survivorship

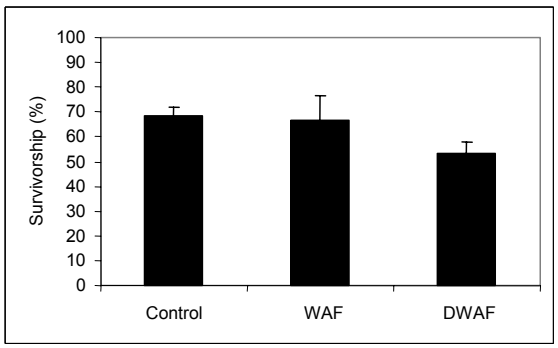


Growth

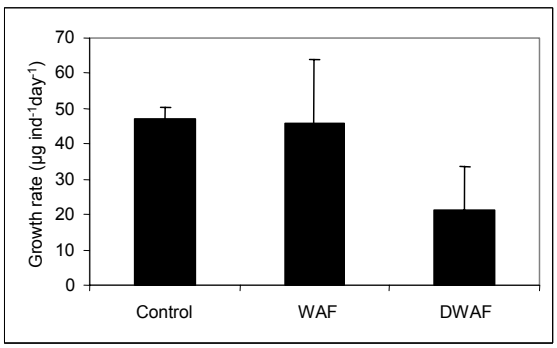


ANS oil and C-9527

Survivorship



Growth



5. Discussion

5.1 Oil Weathering:

One of the most important initial processes affecting crude oils spilled at sea is evaporation. This is especially important for 'light' oils with high API gravities. During the *Sea Empress* spill for example, between 35 % and 45 % of the volume of Forties crude oil had evaporated 9 to 37h after the spill, and about 20-30% evaporation of Alaskan North Slope crude oil was observed after the *Exxon Valdez* incident (SEEEC, 1998). Sea trials were conducted in September 1997 where fairly large quantities of Forties and Alaskan North Slope were intentionally spilled at sea (Lewis *et al*., 1998). The Forties crude oil lost 30 % of the original volume in 2h (compared to 41 % volume in 24h) and the ANS crude oil lost 19 % of original volume in 2h (compared to 32.5% in 24h). During other experimental spills of crude oil it has been noted that oils typically lose 20-30% by weight after about 1h and 40-50 % by weight after several days depending on composition and environmental conditions. (Walker *et al.*, 1992; Buchanan & Harford, 1998).

Previous studies have employed a range of conditions for artificially weathering crude oil. Blenkinsopp *et al*., (1996) reported 10% evaporative weight loss of Alaskan North Slope in a fumehood in 24 hours. Daniel and Swannell (1998) performed oil weathering by maintaining a known volume of Forties crude oil at 50 °C until the volume had decreased to 25 % of the initial value. This procedure accelerated the evaporation of volatile components of the oil known to inhibit the biodegradation of oil due to toxic effects on microorganisms (Floodgate, 1984; Swannell *et al*., 1994). Later, the same authors distilled Forties crude at 250 °C to remove volatile components of the oil to produce a weathered oil said to be similar to that observed at sea after 24-48h. Davies *et al*., (2001) also weathered ANS crude oil by distillation at 250°C to simulate the natural process of evaporation of the lighter fractions of the oil that are toxic to bacteria.

It was agreed at a meeting at the University of Plymouth with the MCA (12th November 2002) that for experimental purposes in the present programme, oils would be weathered in a fumehood by gentle airflow to approximately 75% of the initial volumes in order to simulate a spill of oil for about 2h at sea, under 'typical' North Sea conditions. The target response time of the MCA to an oil spill at sea is 2h or less. It was agreed that distillation of the oil would be avoided (*cf* Swannell *et al*., 1994; Davies *et al*., 2001) as this might represent a more aggressive and long term weathering effect and would probably produce oils atypical of those likely to be encountered following spills of fresh crude oil into the marine environment.

The laboratory weathering procedure developed and presented herein as a SOP (Appendix 1) was reproducible and produced lightly weathered oils which were then used in all further experiments. Judging from the comparison of the volume losses with those of the same oils in real and experimental spills, these laboratory weathered oils were quite representative of oils spilled at sea for 2h under conditions similar to those encountered in experimental

spills of the same oils (*cf* (Walker *et al.*, 1992; Buchanan & Harford, 1998) although detailed compositional analysis such as those conducted herein (Appendix 2) have rarely been conducted on the products of experimental spills so far as we are aware.

5.2 Oil Biodegradation:

The evidence from a multitude of previous studies has shown that it is implausibly expensive and logistically demanding to conduct sufficient biodegradation experiments in replicate to allow all of the environmental variables which may have an effect on oil degradation to be modelled (e.g. Leahy & Colwell 1990; Lindstrom & Braddock 2002; Literathy *et al.*, 1989; Siron *et al.*, 1995; Stewart *et al*.,1989).

A thorough review of the literature was made in the present study, which confirmed this. A limited and feasible number of experiments were therefore planned in order to address the effects of two temperatures and dispersant addition on the biodegradation of lightly weathered FB and ANS oils in the presence and absence of SD-25 or C-9527 dispersants respectively. No nutrient addition was used to enhance degradation, a single species of bacterium was used, only lightly weathered oils were studied and analysis of 'whole' oil rather than the simply the floating/WAF oil was made.

With the above caveats in mind, it was considered that the results of the biodegradation experiments with lightly weathered FB and ANS oils showed that, compared to evaporative weathering losses, biodegradation had only minor effects on the whole oils whatever the temperature, oil or dispersant combination.

Although at the higher temperature (15°C) there was evidence for some increased biodegradation of some of the components of FB oil identifiable by GC-MS when SD-25 was used to disperse the oil, (similar to a previous study but with a different dispersant; Swannell and Daniel, 1999) this effect was not reflected in the oil as a whole as determined by TLC-FID of the saturates, aromatics, resins and asphaltenes.

In contrast, whilst there was no evidence for the increased biodegradation of the components of ANS oil resolvable by GC-MS when oil was dispersed with C-9527 and incubated with bacterial inoculum at the higher temperature, there was evidence of a reduction of the total aromatic hydrocarbons assayed by TLC-FID in both dispersed and non-dispersed oil treatments at this temperature.

Although, overall and in so far as they can be compared, the biodegradation results appear to support previous findings (Swannell and Daniel, 1999; Davies *et al*., 2001) that biodegradation of some oil components was increased for FB oil using dispersant at 15 °C and that biodegradation of ANS (but in this study using C-9527 dispersant rather than that used previously) at 8 °C was not increased, biodegradation of these lightly weathered oils

appeared to be negligible when compared to abiotic evaporative losses, under the conditions used herein.

The low extent of biodegradation may have been due to the low nutrient status of the vessels, to the use of a single bacterial species or to the fact that the whole oil in the vessels was analysed rather than just the fraction in the water phase. Iatroscan TLC-FID has previously been used by Cavanagh *et al*., (1995), Goto *et al*., (1994) and Watson *et al*., (2002) to monitor 'whole' oil degradation. These authors showed that there was a reduction in the proportion of saturated and aromatic hydrocarbons after biodegradation but that the amounts of asphaltenes remained fairly constant. However Watson *et al*., (2002) also reported that sample inoculated with seawater only, displayed negligible biodegradation of the hydrocarbons and most hydrocarbon losses could be accounted for by evaporation (by comparison with killed controls). These samples were thought to be nutrient limited and the results were also consistent with those of Fedorak and Westlake (1981) who also reported limited biodegradation of crude oil with no nutrient addition.

In the present study higher bacterial counts were found at both temperatures for both oils when dispersants were present. This suggests that perhaps the bacteria were able to utilise the dispersants and that measurement of increased bacterial activity alone is not enough to conclude that enhanced biodegradation of oils has occurred.

The work so far suggests there may be some seasonal implications of using oil spill dispersants based on the premise of increased biodegradation in terms of temperature and nutrients, as no increase in biodegradation was observed for either oil with dispersant at the lower temperature of 8 °C. In the short term, biodegradation is only important for oil in the water phase and not the whole oil, so in the assessment of toxicity (discussed below) the water phase was used. In the longer term, at 15 °C, dispersant use appeared to increase the biodegradation of the resolved aliphatic compounds from the Forties oil, although these are not thought to play a major role in the toxicity of the oil. The aromatic compounds as a whole, which are thought to be toxicologically important, were reduced compared to abiotic controls, in the Alaskan North Slope oil, for both dispersed and non-dispersed samples.

5.3 Toxicity Tests with *Mytilus edulis*:

Mytilus edulis was chosen as a species representative of coastal and estuarine regions as the mussel has a widespread geographic range, and is a sessile filter feeder which has been used as a 'bioindicator' in many studies including the worldwide 'Mussel Watch' program,. The 'Scope for Growth' measurement has been used to assess the health of the environment and as mussel feeding rate makes up an important component of this measurement, the results from the toxicity tests are of direct ecological relevance (e.g. Widdows & Donkin 1989; Widdows *et al.*,1995).

In initial scoping studies using laboratory methods for producing standardised WAFs and DWAFs and high oil:water:dispersant loadings, a good linear relationship was found between reduced mussel feeding rate and a reduction in Neutral Red dye retention (a measure of cellular integrity) and the aqueous and tissue concentrations for all WAF exposed organisms. No relationship was found for the DWAF exposed organisms; both feeding rate and Neutral Red assay values were depressed even at measured concentrations similar to those used in WAF exposures. Thus it was deemed more likely that the dispersant was responsible for causing the toxic effects observed. Indeed, upon exposure to dispersant only at the same concentrations used in the oil plus dispersant scoping assays, mussel feeding rate was severely reduced, suggesting the dispersant has indeed caused the toxic effects- possibly by disruption of membranes although this would require further investigation to prove.

Upon exposure to more environmentally realistic concentrations of oil and dispersed oil, mussel feeding rate was reduced after 48h but recovered after a further 5d in clean seawater. This was the general response observed for both oils and both dispersed oils except at the highest concentration of dispersed ANS oil where the mussels died.

However some differences between the two oils, both non-dispersed and dispersed, were observed. In general FB oil affected feeding rate slightly more than did ANS oil after 48h, but recovery was broadly the same after 5d.

Dispersed ANS oil reduced feeding rate to a somewhat greater extent and recovery was not as pronounced in some mussel exposures as were recoveries of mussels exposed to dispersed FB oil. We sought an explanation for this.

For the same mixing regimes the concentrations of the two oils in the WAF and DWAFs were very similar and were over exactly the same concentration range. Also the particle size distribution was very comparable over a range of particle sizes, with the WAFs for both oils having a much lower concentration of particles compared to the DWAFs for both oils. Although dispersed ANS oil contained more hydrocarbons than did dispersed FB oil, the concentrations were not very different. ANS oil contains a higher percentage contribution of aromatic components than does FB oil which contains a much greater proportion of aliphatic (saturates) components (as measured by TLC-FID) and generally speaking, aromatic components are thought to be more toxicologically important. It would appear from the toxicology results that at the same aqueous concentration FB oil WAF contained more bioavailable toxicologically important components than did ANS oil WAF. In contrast, when C-9527 dispersant was used, ANS oil DWAF apparently contained more bioavailable toxicologically important components than FB oil WAF. This was also supported by analysis of the tissue burdens which showed that mussels exposed to FB oil only (WAF) contained higher concentrations of hydrocarbons than ANS oil only (WAF), accounting for the increased effect but mussels exposed to the dispersed oils also contained high concentrations of hydrocarbons.

From the body burdens it would appear that, in general, mussels exposed to DWAFs can depurate toxicants to a greater extent than mussels exposed to WAFs. Thus overall recoveries of feeding rate in mussels exposed to WAF or DWAF were broadly similar.

Mussels exposed to biodegraded oils showed very similar results to those exposed to non-biodegraded oils and mussels exposed to dispersed biodegraded oils showed very similar results to those exposed to non-biodegraded dispersed oils.

It appears that, unsurprisingly, the concentrations of oil in the water column and accumulated in mussel tissues are the most important factors governing the toxicity of chemically dispersed and biodegraded oils. If dispersion of an oil spill occurs following application of the dispersants and oils studied herein mussels appear mostly to be able to recover from the impacts (except at the highest concentrations of dispersed ANS oil) so long as they are washed by successive tides of clean seawater. Under such conditions the use of chemical dispersants to treat oil spills well offshore at appropriate recommended concentrations may have a net environmental benefit.

5.4 Toxicity Tests with *Corophium volutator*:

C. volutator was chosen as a test organism because it is widespread, occupies a relevant ecological niche and is widely used in regulatory tests in Europe and the U.S.A. However, prior to the present study the organism was used mostly for short-term acute toxicity tests. The work required herein necessitated development of a new long-term life cycle bioassay. The subsequent method (Appendix 3) is likely to be useful for many future toxicological studies.

Experiments with lightly weathered FB oil and SD-25 dispersant showed that over a 110d life cycle, few impacts on growth rates, survivorship or reproduction were observable, although at intermediate points effects were seen. However, by continuing to monitor survivorship of the amphipod following an initial exposure to sediment spiked with weathered dispersed ANS crude oil, sediment that would not normally be considered toxic during a 10-day acute test (mortality ≤20 %) was found to significantly reduce survivorship during the 75d life-cycle of the organism. In addition, it was also observed that non-acutely toxic sediment treated with DWAF could significantly depress growth rates of amphipods leading to significantly reduced fecundity. The concentrations of crude oil within the sediment reported in these studies are consistent with subtidal concentrations following real spills e.g. *Sea Empress* (Nikitik and Robinson, 2003; SEEEC, 1998) and experimental chemically dispersed spills (Boehm *et al*., 1987). Although only one treatment i.e. with the DWAF-spiked sediment, caused significant mortality during the chronic test; all other oil-exposed organisms had reduced growth rates with the exception of the WAF-spiked sediment in which no detectable ANS-derived hydrocarbons was found. This is consistent with C-

9527 dispersing and stabilising the oil as small droplets that then became associated with the sediment. Despite the degradation of the oil within the sediment, the amphipods were not able to recover, leading to reduced fecundity. Had these contaminated sediments been collected from the environment, chemically analysed and subject to the standard acute testing test (Roddie and Thain, 2001), it is nlikely that population level effects would have been observed.

The life cycle study with ANS oil was conducted during the summer (June – August) using neonates produced by amphipods that had over-wintered from the previous year. In their natural environment, *C. volutator* born at the beginning of the summer period can, if conditions are suitable, grow, mature and reproduce in the same year. Individuals born later in the season grow more slowly and over-winter to reproduce the following year (Wilson, 1989). Comparison of the growth rates of control organisms in the ANS life cycle study with those from the study of FB oil initiated the previous autumn suggested that the early season cohort have a greater capacity for growth than the late season cohort, i.e. despite identical laboratory conditions, the neonates in the reported study grew at a greater rate than those in the previous test. Studies conducted at different times of year may therefore not be directly comparable. Sediment contaminants may have a longer period in which they can degrade during the life-cycle of late season neonates, thus allowing the organisms a greater time to recover in the absence of the toxicant. In the FB oil life cycle assessment, recovery was observed for the organisms and this appeared to correlate with decreasing concentrations of the oil in the sediment as measured by UVF and also observed by Gc-MS analysis. It is therefore recommended that chronic sediment tests be initiated in summer using the first generation cohort of neonate *C. volutator*. In terms of oil pollution, it is possible that a spill occurring in early summer will have a greater affect on amphipod growth rates than if it occurred later in the year.

The use of dispersants in the nearshore shallow water environment may result in surface oil being transported to the benthos leading to mortality of sensitive species; this is believed to have occurred during the *Sea Empress* spill off Milford Haven SW Wales in 1996 where it was observed that amphipod populations disappeared following the spill (Nikitik and Robinson, 2003; SEEEC, 1998). In the current study of ANS oil the chemically dispersed spiked oil proved to reach the highest concentrations within the sediment and to have the greatest toxic effect in terms of survivorship. However, survivors of the DWAF exposure had significantly greater mean growth rates than organisms exposed to the highest nominal whole oil-spiked sediment, suggesting that oil-tolerant individuals can prosper following the elimination of more sensitive individuals, thus creating the potential for oil-tolerant populations to develop. If this is the case, natural selection might produce a population of oil-tolerant individuals under circumstances of chronic contamination. In order to investigate subtle population effects, it would be necessary to have larger numbers of replicates to overcome variability within treatments, and to test the second generation of oil-exposed amphipods.

When the experiments were replicated with sediments spiked with biodegraded WAFs and DWAFs the responses were minimal and appeared to correlate with sediment concentrations as these were barely above control values apart from that spiked with dispersed ANS which showed a minimal effect.

6. Conclusions

(a) Milestones

Milestone 1: Provide a quantitative assessment of the rate of biodegradation of the major and minor oil components under a range of conditions found in waters around the UK.

Earlier work funded by the MCA found that dispersants can enhance the rate of biodegradation of dispersed oil (for example, Forties crude (FB) with Corexit 9500 at 15°C). A further study funded by the US Department of the Interior, Mineral Management Service (MMS) found a much slower rate of biodegradation at a lower temperature (Alaska North Slope Crude (ANS) with Corexit 9500 at 8°C). The work in the present study carried forward these earlier studies and explored the rate of biodegradation of each oil at both 8°C and 15°C in terms of the major (saturates, aromatics, resins and asphaltenes) and minor (e.g. PAH) oil components with the dispersants currently authorised for use in the U.K. and U.S. Importantly the oils were subjected to the equivalent of only a 2 hour environmental weathering period (the time window for initial dispersant use). Few previous studies have used such realistically lightly weathered substrates and a reproducible standard protocol was developed based on field trial data.
Biodegradation experiments were conducted in replicate with the aerobic bacterium, *Pseudomonas fluorescens*, for up to 100 days without nutrient supplementation at 8 and 15 °C. FB was examined with and without Superdispersant 25 (SD25) which is currently licensed for UK use, and Alaskan North Slope oil (ANS) was examined with and without Corexit 9527 which is currently licensed for use in the USA. In each case the whole oil residues were examined. A series of abiotic controls were also examined. Examination of the rate of biodegradation of the major compound classes (saturates, aromatics, resins and asphaltenes) was made by Iatroscan (thin layer chromatography-flame ionisation detection) and examination of individual resolved hydrocarbons by cryogenic gas chromatography – mass spectrometry (GC-MS).

Milestone 2: Provide a quantitative assessment of the potential toxic effects of chemically dispersed oil and the way that this changes as the oil is biodegraded

The toxicity of the oils, dispersants and dispersed oils (FB with SD25 and ANS with Corexit 9527) was assessed using *Mytilus edulis* and *Corophium volutator*. Mussels were exposed to water accommodated fractions (WAFs) and dispersed water accommodated fractions (DWAFs) - firstly by a standard method of production by using different loadings, and secondly following a method to produce environmentally realistic exposures. The main method of assessment was by the depression of mussel feeding rate, but cellular responses such as neutral red, phagocytosis and micronuclei were also measured into some experiments. Mussels were exposed for 48hrs, their feeding rate measured, placed in clean water and their feeding rate measured

again at 7 days to mimic the dilution and depuration that would be experienced in environmental scenario. This procedure was duplicated allowing half the organisms to be sacrificed after 48 hours so that chemical analysis could be performed on organisms at both 48 hours and 7 days. WAFs and DWAFs were also incubated with bacteria and the same procedure carried out to observe any differences in toxicity due to biodegradation. Acute and chronic sediment toxicity tests were carried out with Corophium volutator as described for Milestone 4 to assess toxicological effects and again WAFs and DWAFs were incubated with bacteria and the tests repeated to observe the effects of biodegradation on toxicity.

Milestone 3: Relate the chemical composition of an oil (and the way it changes with biodegradation) to the observed toxic effects.

In all experiments chemical analysis by standardised methods was performed on all matrices and at all time points to give maximum chemical verification of results. All WAFs and DWAFs were extracted and analysed both prior to and during assays, and mussel tissue and sediment were extracted at test sampling points. All samples were analysed by ultraviolet fluorescence spectroscopy (UVF) to give a concentration of oil in the matrix analysed, and then concentrated and analysed by gas chromatography- mass spectrometry (GCMS) to look at the range of components present in each sample.

Milestone 4: Provide a quantitative measure of the ecological risk posed by chemically dispersed oil trapped in sediments to benthic biota at different stages of their life cycle.

The amphipod *Corophium volutator* is widespread and important in estuarine food webs and as such been included in numerous pollution studies and is a well established sediment test organism. Standard acute sediment toxicity tests were carried out with aqueous and sediment exposures. However, these do not test key life stage events such as moulting and reproduction, and perturbation of life history parameters in response to toxicant exposure provides a means of highlighting whether a specific life stage is particularly sensitive. Therefore life cycle exposures using Corophium *volutator* were carried out which provide a means of testing lethal and sub lethal effects resulting from long terms laboratory exposures and enable laboratory results to more accurately predict the likely effects of chronic exposure on the survival, growth and reproduction of the test organism in the field.
A laboratory method was designed for determining the chronic toxicity of oils associated with whole sediments. The test was conducted using neonates of the estuarine amphipod *Corophium volutator* at 15°C, salinity 25 psu and a 12h light:12h dark photoperiod. The endpoints were survival and growth after 28 days and survival, growth and reproduction of amphipods upon termination of test i.e. reproduction within all control vessels. The sediment chronic toxicity test was used to investigate the toxicity of sediment spiked with the oils at realistic post spill environmental concentrations as well as water-accommodated-fractions (WAFs) and chemically-dispersed fractions (DWAFs). Nine replicates, each containing 30 amphipods, were initially exposed to seven treatments; from these, three replicates from each

treatment were assessed after 28 days and six replicates upon termination of the test. Sediment oil concentrations were quantified using UVF and GCMS.

(b) Extent to which milestones were met.

- A laboratory Standard Operating Procedure was produced for the simulated weathering of oils at sea.
- Dispersants SD-25 and C-9527did not significantly enhance the biodegradation of FB or ANS oils respectively under the conditions tested – evaporation was the dominant process.
- Dispersants were not toxic to the mussel *Mytilus edulis* at environmentally realistic concentrations produced by dilutions of water-accommodated fractions of FB or ANS oil produced by stirring 3.5g of oil layered on 10L of water.
- Mussels were exposed to oil and dispersed oil to mimic environmental concentrations and loadings. The organisms were exposed for 48h and then placed in clean water for 5d to the effects of successive clean tides. Mussels were affected after 48h to a greater extent by dispersed oil than by non-dispersed oil. However, in most cases, during the recovery period mussels recovered at least to the same extent as those exposed to oil.
- These results suggest that inter-tidal mussels exposed to typical post-spill concentrations of oils which have been treated with appropriate concentrations of these licensed dispersants, would recover if successive tides further diluted the oil mixtures. However, if dispersants were to be applied to oil spills in nearshore/shallow water situations (a practice not currently within UK guidelines), benthic communities may, under certain conditions (i.e. if dilution does not occur) be more adversely affected than if the oil remained on the sea surface.
- A chronic sediment test using *C. volutator* was developed from standard acute tests. A laboratory Standard Operating Procedure was produced.
- Chronic oil exposure tests showed ecologically relevant endpoints to be affected by environmentally realistic concentrations of lightly weathered oils that did not cause acute toxicity
- Addition of dispersant C-9527 caused a greater impact when used in association with ANS oil than use of dispersant SD-25 with FB oil.
- The time of year of spill may affect impacts upon some species i.e. spring-summer > autumn-winter.
- *C. volutator* is a robust intertidal species that may be adapted to ubiquitous PAH contamination. Hence, subtidal species are likely to be more sensitive and therefore a safety margin should be considered when formulating guidelines based on results obtained from this study.
- Recovery from impact should be reasonably rapid (i.e. as soon as oil has degraded sufficiently) as long as source populations exist, but oil-tolerant populations may adapt
- This study only exposed two species and the impact upon other species or upon interaction between species cannot be extrapolated.

- No significant differences were found for both organisms between non-biodegraded and oil biodegraded in the water column.
- All responses were related to the level of oil exposure - therefore as long as adequate dilution in the water column and/or biodegradation in the sediment occurs, recovery from impact may occur.

7. Recommendations

1. Areas of coastal benthos that are particularly biodiverse or contain rare/endangered species should be identified and these areas subject to greater regulation when permission to spray dispersants is sought. Establishing the locations of sensitive areas where damage to the benthic community may arise from the use of dispersants would enable rapid decision making in the event of a nearshore spill

2. The results of this project have shown that although chemically dispersing oil may initially impact these organisms to a greater extent than physically dispersed oil, the organisms are mostly able to recover to the same extent as control organisms if they are exposed to clean water for a recovery period, thus maintaining a net environmental benefit. The project has generated a large amount of additional chemical data extra to the original tender, in the form of water, mussel and sediment extracts (~1000 analyses, and therefore it would be prudent to 'data mine' this large amount of information to provide an insight into what individual components are responsible for the effects observed, and to determine the critical 'cut off' concentrations beyond which recovery would no longer be observed.

3. It is often assumed that chronic toxicity in sediment-dwelling organisms is totally dependent upon the concentration of non-polar organic chemicals within the sediment, but there is a paucity of published data, and there is evidence that polar organics from weathered petroleum products also contribute to the toxicity (e.g. (Neff *et al*., 2000; Zemanek, 1997). In addition, oxidative degradation products in sediment following the *Exxon Valdez* oil spill showed that polar fractions were as toxic as the aromatic fraction *(Wolfe et al*., 1995). It would be useful to know what the relative contribution of the polar fraction is to the chronic toxicity of weathered oil and how the addition of dispersant would alter this contribution.

4. The suggestion that an oil-tolerant population may be created from survivors of oil-exposure is speculation but has serious ecological implications. Indeed, the ubiquitous nature of PAHs in estuarine sediments may have already selected hydrocarbon-tolerant populations of *C. volutator* which would account for their relative insensitivity to oil exposure. It would therefore be useful to test the sensitivity to oil of offspring from survivors of oil exposure.

9. Publications/Presentations

The work reported herein has been presented at quarterly meetings with the funders, and in the scientific and public arena in the form of oral and poster presentations at the British Organic Geochemistry 15th Annual Meeting in Plymouth in July 2003, the 14th European Meeting of the Society of Environmental Toxicology and Chemistry in Prague in April 2004, and the 15th European Meeting of the Society of Environmental Toxicology and Chemistry in Lille in May 2005.

Oral presentations:

Smith, E.L., Scarlett, A., Canty, M., Galloway, T.S., Rowland, S.J. (2005) The role of biodegradation on the toxicity of dispersed oil to estuarine and coastal organisms. 15th European Meeting of the Society of Environmental Toxicology and Chemistry, Lille, May 2005.

Scarlett, A., Galloway, T.S., Canty, M., Smith, E.L., Rowland, S.J. (2005) Comparison Of Sediment Avoidance Behaviour Tests With Acute And Chronic Crude Oil Exposure Tests Using The Marine Amphipod *Corophium volutator* Or Let The Organisms Decide: An Alternative Approach As An Adjunct To Standard Sediment Toxicity Tests. 15th European Meeting of the Society of Environmental Toxicology and Chemistry, Lille, May 2005.

Smith, E.L., Burnett, K., Scarlett, A., Canty, M., Galloway, T.S., Rowland, S.J. (2004) The potential ecological effects of chemically dispersed and biodegraded oil.14th European Meeting of the Society of Environmental Toxicology and Chemistry, Prague, April 2004.

Smith, E.L., Scarlett, A., Canty, M., Galloway, T.S., Rowland, S.J. (2003) The potential ecological effects of chemically dispersed and biodegraded oil. 15th British Organic Geochemistry Annual Meeting, Plymouth, July 2003.

Poster presentations:

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