LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

BIOREMEDIATION OF UNRESOLVED COMPLEX MIXTURES IN MARINE OIL SPILLS: AN ANALYTICAL PERSPECTIVE

By

Evin Patrick McGovern

A thesis submitted to the University of Dublin (Trinity College)
for the degree of Doctor of Philosophy

December 1999

I agree that the library may lend
or copy this thesis on request.

Evin

Evin McGovern

Trinity College Dublin 1999

Acknowledgements

This project was part funded by EU DG XI Environment, Nuclear Safety and Civil Protection as project B4-3040/95/928/jnb/C4. The project was carried out at the Marine Institute Fisheries Research Centre (FRC) laboratories in Abbotstown.

I wish to express my gratitude to all those at FRC who contributed to the project during its course, and especially to Teresa O'Shea for her excellent technical assistance over the course of the project, to Brendan McHugh for his technical assistance during phase I of the project, and to Conor Duffy for his assistance.

As with many multidisciplinary projects, the success of this project was only possible due to advice and collaboration with other experts. Dr. Barry Kiely's team at Bioresearch Ireland, University College Cork carried out the microbiological testing and inocula preparation at their laboratories under contract from the Marine Institute. I am very grateful to Barry for his expert advice on the microbial aspects of the project and enjoyed renewing our collaboration in this field after many years.

I wish to thank my supervisors in Trinity College, Dr. Jim Wilson and Prof. Brian McMurry for their assistance and support throughout the project.

Many thanks to Eugene Nixon for his support for the proposal and his help in carrying out the project, particularly with regard to overcoming the administrative difficulties encountered. I am grateful to the Marine Institute for their sponsorship of this project and support for my registration for a doctorate.

Other people to whom thanks are due include those who supplied oil: Statoil Norway (Thure Ingebriksted), Shell Ireland (PJ Cummins), Chevron UK (Seamus O'Connor), Texaco UK. Thanks to those who gave advice and assistance directly or indirectly, especially at the outset of the project – Dr. Per Wrang, Dr. Jose Biscaya, Dr. Steve Grigson, Dr. John O'Brien, Dr. Alan Dobson and anybody I may have overlooked.

Finally, I am indebted to my wife, Ailve, for her patience over the entire project, but most particularly during the lengthy and antisocial period of thesis preparation.

For my wife Ailve,

and in memory of my parents.

BIOREMEDIATION OF UNRESOLVED COMPLEX MIXTURES IN MARINE OIL SPILLS: AN ANALYTICAL PERSPECTIVE

Table of Contents

Chapter 1. Background

1.3 Petroleum hydrocarbons in the marine environment

1.4 Bioremediation of oil spills

1.5.2 Spectroscopic techniques

1.5.3 Chromatography

1.5.4 Mass spectrometry (MS)

Chapter 2. Analytical Development and Characterisation of Oils (PHASE I A)

Chapter 3. Inocula Development and Evaluation (PHASE 1B)

Chapter 4. Design of Bioremediation Microcosm Trials (PHASE II)

Chapter 5. Bioremediation Trial Results - Microcosm Design

Chapter 6. Bioremediation Trial Results II – Chemical Assessment of Biodegradation

6.2 Gravimetric assessment

6.3 Chromatography

Chapter 7. Oil Spill Identification and Fingerprinting Techniques

Chapter 8. General Discussion and Conclusions

Appendices

A. Analytical methodology

List of Tables

List of Figures

Summary

Unresolved complex mixture (UCM) is the fraction of some petroleums that cannot be resolved by conventional gas chromatography and is characteristic of biodegraded petroleum oils and some refined products such as lubricating oils. This study aimed to investigate the effects of environmental biodegradation, and more specifically shoreline bioremediation, on the chemistry of UCM. A number of wet chemical and instrumental analytical methodologies, including gravimetry, urea adduction, spectroscopy, chromatography and mass spectrometry, were evaluated for their suitability to monitor UCM compositional changes during the course of biodegradation trials. Six UCM rich oils were also characterised using these methods, three crude oils and three lubricating oils. In parallel, an investigation of microbial degradation of UCM in solution was carried out in the laboratory. Using respirometry trials, undefined consortia of natural marine micro-organisms enriched on UCM demonstrated an enhanced ability to metabolise UCM. The pooled crude enriched consortium also performed considerably better at metabolising UCM when compared with a cocktail derived from the apparently dominant individual colonies (not identified) isolated from the crude consortium using standard techniques. Initial investigations of UCM biodegradation using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) were carried out at this stage. An inoculum based on the undefined consortium was prepared for testing in microcosm trials.

Microcosms were designed that provided a controlled environment with a basic simulation of shoreline conditions, including a simple tidal cycle, to assess the effect of bioremediation treatments for the decontamination of UCM contaminated sediments. Treatments included nutrient supplementation (T3) only, inoculation with the enriched consortium (T1), and augmentation with rapidly grown micro-organisms isolated from the sediment used as a base for contamination (T2). Both T1 and T2 were also nutrient supplemented. A seawater control (C1) and poison control (C2) were also employed. Two sets of microcosms using different contaminating oils were set up. The two oils used were environmentally weathered Forties crude oil (~90% UCM) and Alba crude oil, a reservoir biodegraded crude. A comprehensive monitoring programme was undertaken. The microcosms proved a very effective tool for assessing biodegradation. Results indicated that, despite the enhanced capacity of the T1 inoculum for UCM biodegradation exhibited in respirometry trials, bioaugmentation had no appreciable effect on biodegradation of UCM or any UCM component monitored, compared with the nutrient only treatments. Nutrient treatment, *biostimulation*, had a discernible effect, the extent of which differed for various parameters monitored. A simple model $[A]_t = [A]_0.e^{-kt}$ was used to compare biodegradation rates over the course of the microcosm trials (473 days) for GC and gravimetric results. A modest enhancement for total oil and UCM biodegradation was observed for nutrient supplemented microcosms, but a marked acceleration of PAH biodegradation was evident in these microcosms. Compositional changes were also more advanced in nutrient treated microcosms than seawater control units. Results suggested that biodegradation proceeded along intuitive lines, with lower molecular weight UCM material being more degradable. PAH biodegradation rates increased with decreasing ring number and alkyl substitution. In the aliphatic fraction, acyclic components were more biodegradable than cyclic and recalcitrance increased with increasing condensed ring number. The saturate fraction was more biodegradable than the aromatic fraction with the resin fraction most recalcitrant. Washout was a notable feature for Alba microcosms.

This study enabled the comparison of a range analytical tools and techniques for monitoring UCM biodegradation, such as the *n*-octadecane:phytane ratio which was observed to be only useful for monitoring the early stages of biodegradation. A methodology developed for oil spill fingerprinting purposes was adapted for long term GC-MS monitoring of biodegradation and proved very effective, overcoming some of the inherent difficulties in using GC-MS for this purpose. The biomarker compound 17α, 21β-C30-hopane has been used as a conserved internal marker in recent years for assessing biodegradation of hydrocarbons. For Alba nutrient supplemented microcosms, this was seen to be substantially biodegraded and would thus lead to an underestimation of biodegradation and bioremediation. C28 (20R) triaromatic sterane was chosen as a preferred alternative as it was abundant, resolvable by GC-MS and considerably more resistant to biodegradation than C30-hopane. The use of relative ion abundance ratios was also developed as a tool for gaining an insight into bulk compositional change in UCM and this proved very useful. Finally, an investigation into the effect of severe biodegradation on a range of oil spill source identification parameters and patterns was carried out, and the results are presented in the thesis.

Abbreviations and Terms

AMAP	Arctic Monitoring and Assessment Programme
amu	Atomic mass unit
ASTM	American Society for Testing and Materials
ATR	Attenuated total reflectance
C	Carbon
C30-hopane	17α(H), 21α(H)hopane (C30)
CFC	Chlorofluorcarbon
CnD (n=1,2,3)	Substituted dibenzothiophenes (methyl-, dimethyl- and trimethyl- for n= 1,2 3 respectively)
CnP (n=1,2,3)	Substituted phenanthrenes (methyl-, dimethyl- and trimethyl- for n= 1,2 3 respectively)
Cx-benzene	Alkylbenzene. (alkyl substitution of x carbons)
Cx-cyclohexane	Alkylcyclohexane. (alkyl substitution of x carbons)
CI	Confidence interval
CI (MS)	Chemical ionisation
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane {1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane}
DO	Dissolved oxygen
EI	Electron ionisation
Ex. λ	Excitation wavelength
Em. λ	Emission wavelength
EU	European Union
FIA	Fluorescent indicator adsorption
FID	Flame ionisation detection
FT	Fourier transform
GC	Gas chromatography
GC-MS	Mass spectrometry
GEM	Genetically engineered micro-organism
GESAMP	Joint Group of Experts on the Scientific Aspects of Marine Pollution
H	Hydrogen
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IMO	International Maritime Organisation
IR	Infra-red
MI FRC	Marine Institute Fisheries Research Centre
MS	Mass spectrometry
MTBE	Methyl-*tert*-butyl ether
MW	Molecular weight
m/z	Mass:charge ratio
N	Nitrogen
N_2	Molecular nitrogen
NETAC	National Environmental Technology Applications Corporation
NFBC	National Food Biotechnology Centre
NMR	Nuclear magnetic resonance
nm	Nanometre
NSO	Nitrogen, sulfur, oxygen
NRC	National Research Council (USA)
O_2	Molecular oxygen

P	Phosphorus
PAC	Amino-cyano (HPLC column)
PAH	Polynuclear aromatic hydrocarbon
PCA	Plate count agar
PCB	Polychlorinated biphenyl
PCE	Perchloroethylene
Pr	Pristane
Phy	Phytane
PTFE	Polytetrafluorethylene
PSD	Particle size distribution
Psu	Practical salinity unit
Rf	Retention factor
RI	Refractive Index
RIC	Reconstructed ion chromatograph
RSD	Relative standard deviation
SARA	Saturates, aromatics, resins, asphaltenes
sd	Standard deviation
SEC	Size exclusion chromatography
SEEC	*Sea Empress* Evaluation Committee
SIR	Selected ion recording
TAS4	(20R) Ethylcholestane
TDB	Tridecylbenzene
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TPH	Total petroleum hydrocarbon
Tm	17α(H)-22,29,30-trisnorhopane (C27)
Ts	18α(H)-22,29,30-trisnorhopane (C27)
UCC	University College Cork
UCM	Unresolved complex mixture
USEPA	United States Environmental Protection Agency
UV	Ultrviolet
UVF	Ultraviolet/visible fluorescence
v/v	Volume to volume
WWDW	Wet weight/ dry weight
w/w	Weight to weight

1. BACKGROUND

1.1 BACKGROUND

Shoreline bioremediation of oil spills involves intervention by man to accelerate biodegradation of the spilt oil, through optimisation of conditions that may otherwise be rate limiting. Often this simply involves the addition of nitrogen and phosphorus, as natural background environmental concentrations of these elements may limit biodegradation rates. However, in some cases it may entail more complex manipulations. The potential of bioremediation in enhancing biodegradation of oil spills has been demonstrated in the laboratory and the field.

Crude oil and most petroleum products are very complex mixtures of organic chemicals. In general, preferential biodegradation of simpler substrates in petroleum, in particular *n*-alkanes, occurs before biodegradation of more complex molecular structures. This results in a pronounced 'unresolved complex mixture' (UCM), which is a mixture of cyclic and branched hydrocarbons that is unresolvable by capillary gas chromatography analysis with conventional detection techniques. Should clean up goals be defined in terms of concentration or even percentage removal of contamination, removal of the UCM may be critical to achieving desired clean-up objectives. UCM is especially pronounced in weathered crude oils, some fuel oils and also in reservoir biodegraded crude oils. Also, the bulk of petroleum based lubricating oils consists of aliphatic UCM.

This project aimed to ascertain whether there is a role to play for bioremediation in clean-up of shores contaminated with petroleum containing significant UCM. This was to be achieved by investigating a number of bioremediation strategies and evaluating the effect on UCM removal. Broadly, the strategies investigated were *biostimulation* (supplementation with nutrients) and *bioaugmentation* (addition of seed microbial cultures). Although there has been little independent evidence to support the effectiveness of bioaugmentation, it was considered useful to investigate the role of addition of specialist cultures with a demonstrated capacity for UCM degradation. A further objective was to gain an insight into the alteration of UCM during bioremediation, ascertain what component groups were most resistant to biodegradation, and to study biodegradation of specific compounds in the UCM. The study was also intended to give an indication of the limitation of petroleum hydrocarbon bioremediation as a technology in terms of substrate recalcitrance, i.e. which UCM substrates offer greatest resistance to biodegradation.

There are many analytical methods for determining the biodegradation of oil and selection of methods for this purpose undoubtedly has a bearing on defining whether a clean-up operation has been successful for not. This study enabled a comparison of a number of analytical approaches and an evaluation of the suitability of methods for measuring oil spill biodegradation. Furthermore, an insight into how severe biodegradation effects patterns and parameters that are commonly used in fingerprinting and source identification of oil spills was an indirect benefit from the project.

1.2 PETROLEUM HYDROCARBONS AND UNRESOLVED COMPLEX MIXTURES (UCM)

Crude oil is a highly complex mixture of organic compounds, in which hydrocarbons predominate. It is formed, subsurface, at elevated temperature and pressure and derives from biological material (often marine algae) (Tissot & Welte 1978, Brooks 1983). Transformation into crude oil involves a variety of processes such as maturation (thermal alteration), migration to reservoir (chromatographic effect) and changes in the reservoir (primarily biodegradation and water washing). Individual crude oils differ from each other depending on the initial material from which they are derived and the processes involved in this derivation. Most crude oils are similar in their constituents and can be differentiated based on the relative proportions of certain components. Thus, oils formed in the same basin are more likely to exhibit similarities (Wrang & Adamsen 1990a).

Crude oil composition can broadly be classed into normal paraffins, isoparaffins, naphthenes, aromatics, resins, and asphaltenes (Butt 1986). Normal paraffins are *n*-alkanes; a homologous series of the simplest straight chain hydrocarbons. They usually account for 10-20% of the oil but can account for up to 60%. Normally the lower *n*-alkanes predominate. Isoparaffins are branched alkanes, and in particular isoprenoids such as pristane and phytane are important constituents. *n*-Alkanes and to some extent isoprenoids are the components responsible for the typical gas chromatographic trace of environmental crude oil samples. Naphthenes are cyclic aliphatic compounds based on cyclohexane and cyclopentane units, often existing as condensed structures.

The aromatic component of crude oil consists primarily of substituted benzenes or fused ring benzenoids (polyaromatic hydrocarbons, PAH) such as naphthalenes and phenanthrenes. Alkylated PAH compounds are more prevalent than parent PAH structures. High molecular weight fractions usually contain significant naphthenoaromatics, and these are particularly abundant in immature crude oils. The polar fraction contains compounds that incorporate nitrogen, sulfur or oxygen (NSO) in their molecular structure. Sulfur is the most abundant element in crude oil after carbon and hydrogen (Tissot & Welte 1978). Thiophene derivatives are generally abundant in high sulfur crude oils. Asphaltenes are high molecular weight NSO-compounds with complex structures that are defined by their insolubility in *n*-hexane.

Crude oil is often divided into four classes of compounds i.e. saturates, aromatics, resins and asphaltenes, (SARA), (*Table 1.1*). A typical North Sea crude oil is light and contains over 60% saturates, primarily *n*-alkanes.

Saturates	**Aromatics**	**Resins**	**Asphaltenes**
Straight chain hydrocarbons *i.e. n-Alkanes*	Monocyclic and condensed ring (polycyclic aromatic hydrocarbons - PAH)	Nitrogen, oxygen and sulfur containing compounds	High molecular weight components (insoluble in hexane)
Branched alkanes *e.g. isoprenoids, 'T-branched' alkanes*	Substituted mono and polycyclic aromatics		
Cyclic alkanes *e.g. steranes, hopanes, alkylcyclohexanes*			

Table 1.1. The chemistry of crude oil

Biodegraded oils analysed by gas chromatography, (GC), often exhibit a pronounced unresolved 'hump', after biodegradation of simple resolved compounds, (*n*-alkanes). This is the unresolved complex mixture, (UCM), and consists of myriad compounds at levels that cannot be resolved by conventional GC. There can be many structural isomers for most hydrocarbons of the same molecular weight and isomeric possibilities generally increase with increasing molecular weight. This results in complex mixtures that cannot be resolved by gas chromatography. Examples of molecular structures of petroleum compounds are presented in figure 1.1. The UCM comprises of cyclic compounds, and there is also evidence that 'T-branched' alkanes to be a significant contributor (Gough & Rowland 1990). Cyclic aliphatic compounds in crude oils include alkyl substituted cyclohexanes and polycyclic compounds such as steranes and cyclic triterpanes. Aromatic compounds in crude oil include alkyl benzenes, which may have linear or branched substitutions and are often multi-substituted, and substituted polyaromatic hydrocarbons (PAH). Parent PAH are not prevalent in crude oil and their occurrence in the environment is generally associated with fossil fuel combustion products or coal tars/ creosotes.

Killops and Al-Juboori (1990) used a number of analytical techniques for characterisation of UCM. They concluded that approximately 10% seems to be typical for the amount of aromatic carbon. Aromatic components are primarily alkyl benzenoid compounds. Terminal alkyl chains of up to *ca.* C19 are present. Cycloalkanes form an important component and probably comprise of more monocycloalkanes than fused polycyclics. 'T-branched' alkanes have been suggested as a significant component of UCM (Gough & Rowland 1990, 1991). The biodegradability of model compounds, proposed as typical of UCM molecular structures, was examined and these compounds were found to be recalcitrant (Killops & Al-Juboori 1990, Gough *et al.* 1992). UCM is a significant part of reservoir biodegraded crude oils, petroleum based lubricating oils, some fuel oils, and often environmentally weathered oils. As well as aromatic and aliphatic multi-ring compounds, some compounds may have both cycloparaffin and aromatic rings. Although multi-ring aromatics generally exist as condensed rings, more than one ring system may exist for multi-ring aliphatics (Melpolder *et al.* 1956, Hood & O'Neal 1959).

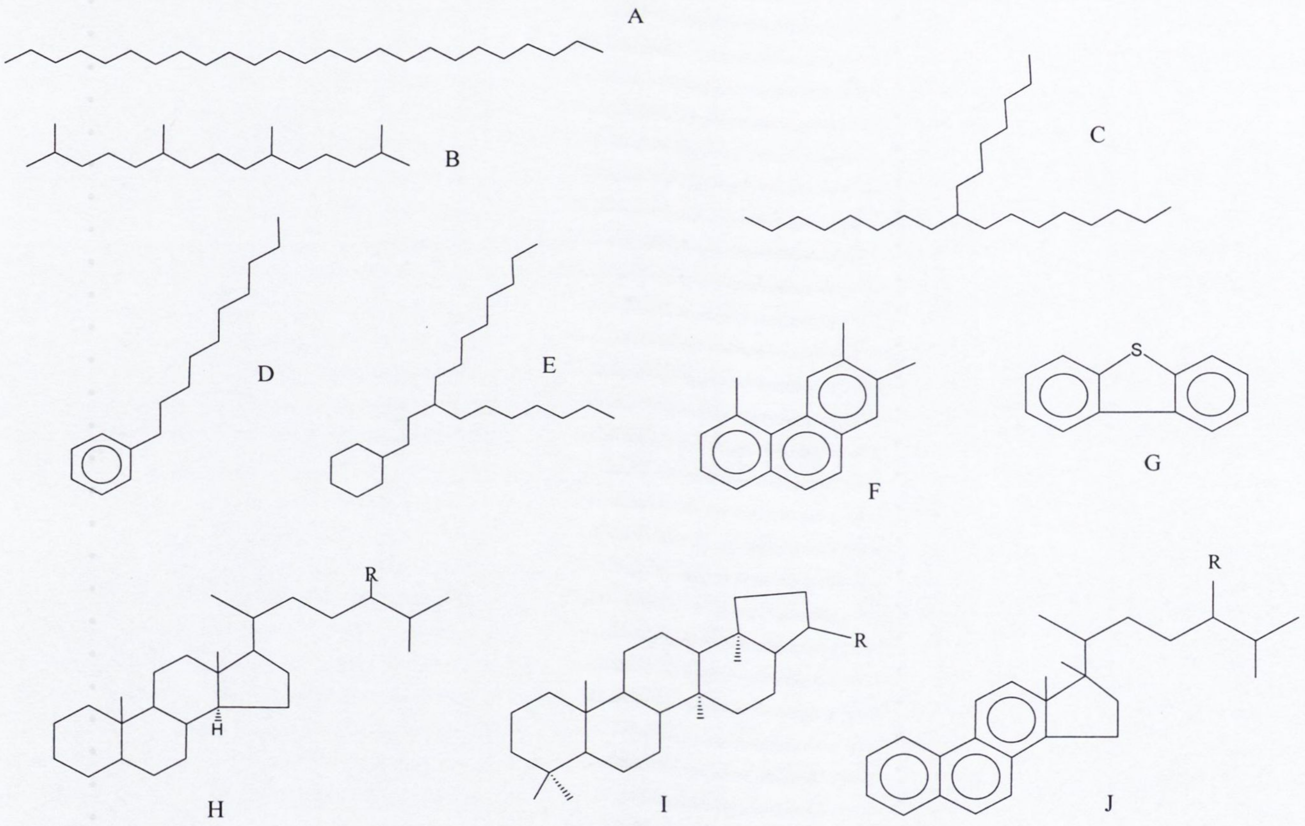


Fig. 1.1: Molecular structures of example crude oil compounds. A) C25 n-alkane [pentacosane], B) Pristane [2,6,10,14 tetramethyl pentadecane], C) a 'T-branched' alkane [9-heptylheptadecane] D) alkylbenzene [n-decylbenzene], E) T-branched alkyl-cyclohexane [8-(2-cyclohexylethyl)-hexadecane], F) a C3-phenanthrene [2,3,5-phenanthrene], G) dibenzothiophene H) steranes, I) hopanes, J) triaromatic steranes

1.3 PETROLEUM HYDROCARBONS IN THE MARINE ENVIRONMENT

1.3.1 Inputs and sources

There is a range of mechanisms by which petroleum hydrocarbons enter the marine environment. The GESAMP report no. 50 (IMO 1993) on the ' impact of oil and related chemicals and wastes on the marine environment', reviewed estimates of global input of petroleum hydrocarbons into the oceans. The main source indicated was urban run-off and discharges, followed by operational discharges from tankers at sea and then accidents from tankers at sea. Other sources included natural seeps, losses from non-tanker shipping, atmospheric deposition, and coastal refinery loss. While the figures quoted are probably outdated (most recent data 1981), they suggest a decrease in petroleum hydrocarbon pollution of the world's oceans, due to international measures taken to reduce hydrocarbon inputs. Despite improvements in preventative measures however, the occurrence of major oil spills due to tanker accidents will always be a risk while oil is transported in bulk by sea. The catastrophic effects of major oil spills on the marine and coastal environment is evident and usually results in public outcry for rapid counter measures to be taken (Miller 1990, Maki 1991, Lord Donaldson's Inquiry 1994, SEEC 1998). As well as severe damage to marine ecosystems and wildlife, such as seabirds, impacts on fisheries and tourist amenities can have direct economic effects. Some of the more infamous marine oil spills are listed below:

Torrey Canyon (Scilly Isles, UK 1967) ~100,000 tonnes
Amoco Cadiz (Brittany, France 1978) ~220,000 tonnes
Ixtoc I blow out (Gulf of Mexico 1979) ~350,000 tonnes
Exxon Valdez (Alaska, 1989) ~ 37,000 tonnes
Gulf war spill, (Persian Gulf, 1991) ~800,000 – 1,000,000 tonnes
Braer, (Shetland Islands, UK, 1993), ~ 84,700 tonnes crude & 1,600 tonnes bunker fuel
Sea Empress, (Milford Haven, UK, 1996). ~ 72,000 tonnes crude & 420 tonnes bunker.

1.3.2 Physical and chemical fate of oil spills

After an oil is spilled at sea its physical and chemical characteristics begin to alter rapidly by processes collectively known as weathering (Butt 1986, Wrang & Adamsen 1990b). Lighter components are evaporated and within 48 hours components with a boiling point of less than that of tetradecane (*n*C14) can be completely removed. Furthermore, polar compounds can be dispersed in the water column, and photo-oxidation can degrade certain compounds (Garret *et al.*

1998). Oil spreads on the water surface forming slicks. Wave action may aid in dispersing the oil, or a water-in-oil emulsion known as 'chocolate mousse' may be formed. Mousse is stable and persistent in the environment. Biodegradation of oil is a slower process (weeks to months after a spill) and depends on factors such as availability of nutrients. The *n*-alkanes are amenable to degradation and are removed in the early stages of biological weathering but the UCM is more resistant due to more complex chemical structures of its components. There are many factors that influence the rate of biodegradation and these will be considered later in this chapter. Substantial physical weathering and wave action can result in the formation of tar balls. These present a small surface area to volume ratio with more weathered exteriors and are therefore slow to biodegrade.

After the Sea Empress oil spill in Milford Haven 1996, it was estimated that approximately 40% of the oil evaporated, 52% dispersed into the water column and 5-7% was stranded on the shoreline. A year later less than 1% of oil remained on the shoreline; the rest having been removed, biodegraded or washed back to sea (SEEC 1998).

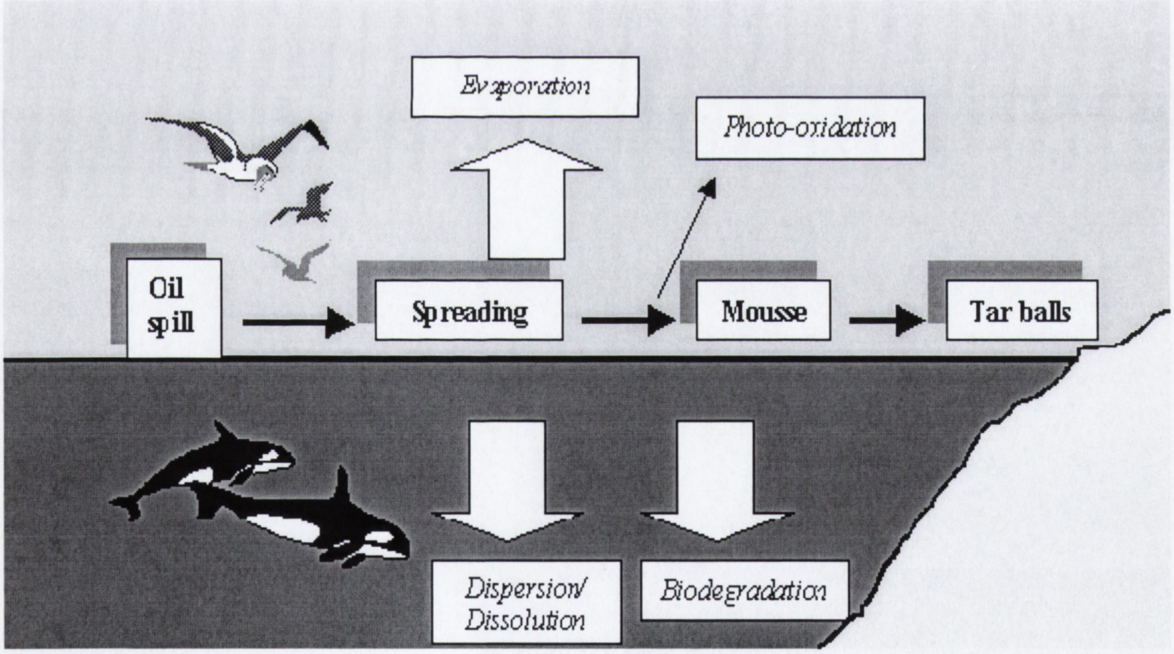


Fig. 1.2 Simple schematic of the primary weathering effects on spilt oil

Fusey and Oudot (1984) investigated the relative influence of physical removal and biodegradation in the removal of hydrocarbons from shoreline sediments and concluded that the stage of biodegradation after experimental oilings was directly related to the residual concentration of the oil in the sediments. Oil was found to persist at depths of 25-50cm on intermittently exposed coarse-grained gravel beaches in Prince William Sound 8 years after the *Exxon Valdez* spill, under the protective cover of cobble/boulder armour (Hayes & Michel 1999, Michel & Hayes, 1999).

1.3.3 Toxicity of petroleum hydrocarbons and impacts of marine oil spills

The effects of major oil spills on marine flora and fauna have been well documented and can cause immediate death of organisms or delayed death by sublethal effects. Massive bird kills occurred after *the Torrey Canyon* and *Amoco Cadiz* spills (IMO 1993). In 1978 the *Amoco Cadiz* lost 220,000 tonnes of crude oil off Brittany. Insignificant effects on fin-fish were observed but aquaculture was damaged (NRC 1985). By 1985, the concentration of the weathered *Amoco Cadiz* in sediments was small when compared to other hydrocarbon inputs. This was due to the numerous more recent hydrocarbon inputs due to the high level of shipping activity (Page *et al.* 1988). Oyster growing areas remained polluted with aromatic hydrocarbons seven years after the spill and other detrimental effects on marine biota were noted seven to eight years after the spill (Berthou *et al.* 1987, Dauvin 1987). The *Exxon Valdez* spill caused major mortalities of sea otters and sea birds, including bald eagles. Clams mussels and finfish were also contaminated (Maki 1991). The huge crude oil release into the Persian Gulf during the Gulf War in 1991, caused extensive environmental damage and one year after the spill most severely impacted areas were found to be halophyte marsh/algal mat complexes and mudflats at the head of sheltered bays. Heavy oiling of burrows was also observed (Hayes *et al.* 1993). Subtidal sediments showed that contamination largely consisted of UCM by 1992 and that levels of contamination had decreased by approximately 50%. The reduction in the 1992-1993 period was much less (Readman *et al.* 1996). Biological effect studies have indicated that the recovery of the subtidal region after major oil spills is generally rapid (Lee & Page 1997).

The effect of oiling of environmentally sensitive ecosystems such as mangrove swamps, coral reefs, and mudflats has also been investigated. The severe damage of oil contamination in mangrove habitat and coral reef has been described. (Burns & Knap 1989)

The *Sea Empress* spill resulted in closures of fisheries, within a designated area, for up to 8 months for some shellfish species. Although significant contamination of fin-fish was not detected, shellfish did show contamination. Negative 'scope for growth' and modulations in cellmediated immunity were recorded after the spill. The bird species most effected were razorbills, guillemots and especially the common scoter. (SEEC 1998)

The initial impact of oil spills on marine ecosystems is related to the physical effects, such as coating and ingestion by marine wildlife and smothering of sediments by oil slicks. The toxic effects of oil have been primarily attributed to the aromatic component. The toxicity of monoaromatics and PAH has been considerably researched and there is growing evidence of mutagenic and carcinogenic effects of PAH (NRC 1985). While higher molecular weight PAH may have a greater inherent toxicity, the greater solubility of lower molecular weight components may increase their uptake and relative effect. Aliphatic UCM does not appear to have significant toxicological effects, UCM oxidation products have been shown to have an increased toxicity on *Mytilis edulis* probably due to increased solubility (Thomas *et al.* 1995)

1.3.4 Clean-up of marine oil spills

In the event of a major oil spill a wide variety of technologies and control agents can be employed to clean-up oil spills or at least to mitigate the effects (Westermeyer 1991, IMO 1993, Lunel *et al.* 1995). Initial efforts usually involve attempts to contain and recover oil or/and to disperse the oil while still at sea. Deployment of booms and skimmers to corral oil at sea is commonly used but such methods are of limited effectiveness, particularly in bad weather. There has been much debate about the use of dispersants, regarding their effectiveness as well as their environmental impact. With modern oil spill dispersants, toxicological impacts of dispersant application are generally due to the effects of dispersed oil rather than dispersants themselves (Singer *et al.* 1993, Burridge 1995). Other approaches include burning, sinking, use of recovery agents and use of emulsion preventers and breakers.

On the shoreline, approaches include removal and disposal elsewhere of contaminated material, hot and cold water washing, use of surfactant or solvent based washing agents, and biodegradation enhancement (bioremediation). In 1991 the *Exxon Valdez* grounded in Prince William Sound in Alaska spilling nearly 11 million gallons of Prudhoe Bay crude oil in an ecologically sensitive area. A massive clean-up operation was launched. Many of the available techniques were used as part of the *Exxon Valdez* spill response. This afforded the opportunity to carry out a major assessment of the clean-up operation and the immediate and long-term effects of the oil spill (Miller 1990, Kelso & Kendziorel 1991).

The appropriate choice of clean-up technologies, or combinations of technologies, depends on the circumstances, such as type of oil, environmental factors, type of location effected (e.g. nature reserve, amenity beach, etc.), and necessity to protect/deflect pollution from a sensitive area. On shorelines, techniques such as power washing can drive oil into the subsurface where it may be slower to degrade, or remove oil from the upper-intertidal zones to mid- and sub-intertidal areas, where it may cause greater damage. (Houghton *et al.* 1991) In some circumstances, the best approach may be "the do-nothing option". The treatment may cause more damage than the oil itself, and hydrocarbon degrading micro-organisms are ubiquitous and will naturally biodegrade oil in time (Atlas 1984).

Bioremediation as a clean-up technology involves accelerating biodegradation rates (Atlas & Atlas 1991, Atlas 1996). It has the advantage of being a technique which is perceived as a natural mechanism for oil removal from the environment, and that it is a relatively 'soft', i.e. low intrusion approach, with minimal physical manipulation of the shoreline. Thus, it may be more suitable for ecologically sensitive environments than more aggressive approaches. It results in the eventual transformation of the pollutants to relatively innocuous substances (carbon dioxide and water) as opposed to removal or relocation in the environment. There are certain instances where bioremediation may not be suitable; a quick clean-up may be required, (amenity beaches for example), or it may be difficult to overcome the rate limiting factor. Furthermore, it can be difficult to predict how effective bioremediation will be for a given spill situation (Hoff 1993). While the technology may be relatively inexpensive, the cost of monitoring over an extended time period may be prohibitive.

1.4 BIOREMEDIATION OF OIL SPILLS

1.4.1 Application and case studies

Bioremediation has been proposed as a technology for clean-up of many organic contaminants in a variety of environments. Land-farming was one of the earliest forms of biotreatment and was used for disposal of waste oils and sludges at refineries, and involved nutrient addition and tilling of contaminated soil (Morgan & Watkinson 1989, Atlas & Bartha 1992). More recently soil bioremediation has involved using more engineered systems such as soil banking or composting. Soil banking utilises a greater degree of engineering and containment, and along with nutrient addition, other strategies to optimise biodegradation are employed such as aeration/oxygenation, amendment of soil structure, surfactant additions, irrigation and pH control (Wilson & Jones 1993). As well as treatment of hydrocarbons, investigations into the role of bioremediation in PCB, pesticide, explosive and metal decontamination have been carried out (Kaplan 1992, Summers 1992, Worne & Fortune 1993). Bioremedial treatment of ground water can involve pump and bioreactor treatment. Much effort has also been put into developing *in situ* bioremediation techniques for ground water and soil (Morgan & Watkinson 1989, Wilson & Jones 1993, Ellis & Gorder 1997). A wide range of commercial products are available that claim to assist bioremediation and many are based on the use of microbial seed cultures.

Bioremediation has also been used to treat marine oil spills (Swannell *et al.* 1995) and was used as one of the shoreline clean-up techniques following the *Exxon Valdez* spill. Oleophilic, (Inipol EAP22), slow release (Customblen), and soluble inorganic fertilisers were applied on a number of beaches (Pritchard & Costa 1991, Prince 1997). Fertiliser levels were adjudged not likely to present toxic effects to marine fish or invertebrates. An increase in hexadecane and phenanthrene mineralisation activities was demonstrated for fertilised as opposed to non-fertilised sites on three beaches (Lindstrom *et al.* 1991). Ratios of total GC detectable hydrocarbons (TGCDHC), total resolved hydrocarbons (TRHC: *n*-alkanes + pristane & phytane) and sum of selected PAH (TPAH) to C30-hopane was used to assess biodegradation. Results indicated significant enhancement of biodegradation rates correlated to the amount of nitrogen delivered per unit of oil (Prince *et al.* 1994, Bragg 1996). On one beach the rate enhancement was approximately five-fold.

Venosa *et al.* reported on the bioremediation of an experimental oil spill on the shoreline of Delaware Bay, USA (Venosa *et al.* 1996). This experiment was designed on a randomised block basis to provide statistically sound evidence for the efficacy of bioremedial treatments. Treatments included nutrient supplementation and nutrient/ microbial inocula addition, and were assessed with reference to untreated control plots. Although substantial biodegradation was observed for the untreated plots, a statistically significant increase in biodegradation rates of total target alkanes:hopane ratios (Σ (*n*C10- *n*C35):hopane) and total aromatic:hopane (ΣPAH:hopane) was observed for the treated plots. No significant difference was detected between the nutrient only and nutrient/inocula treatments.

In 1996 the Sea Empress grounded off Milford Haven releasing 72,000 tons of Forties blend crude oil and approximately 420 tons of bunker fuel. An investigation of bioremediation for treating a mixture of the oils was carried out on a gravel beach at Bullwell bay. Also using a randomised block experiment and hydrocarbon:hopane ratios, it was found that nutrient amended plots were biodegraded by, on average, 37% more than untreated plots. There was no evidence that bioremediation of the sediments increased oil toxicity (Swannell *et al.* 1999).

A shoreline study in the Bay of Brest, France, measured biodegradation rates for total oil, aliphatics, cycloalkanes and aromatics in a bioremediation trial using slow release fertiliser. Norhopane rather than hopane was used as conserved internal marker. High background degradation was observed with no significant improvement for treated plots. This was attributed to the high background nutrient levels (Oudot *et al.* 1998).

The necessity for operational guidelines has been recognised (Swannell *et al.* 1996, Lee 1998, Lee & de Mora 1999), to assist in

- making the decision to bioremediate or not,
- choice of bioremediation product,
- designing monitoring strategies,
- consideration of toxicity and health factors.

1.4.2 Microbial biodegradation of petroleum hydrocarbons

The ability of many microorganisms to utilise hydrocarbons as sole carbon sources was identified as early as 1946 (Zobell 1946). The microflora of the marine environment includes bacteria, microalgae, protozoa, actinomycetes, yeasts, fungi and virus'. The ability to metabolise hydrocarbon is common in marine bacteria and is spread through a wide variety of genera. The genera most frequently described was listed by Floodgate as *Pseudomonas, Achromobacter, Flavobacterium, Acinetobacter, Vibrio, Bacillus, Arthrobcater, Nocardia, Corynebacterium, and Micrococcus* (Floodgate 1984). Hydrocarbonoclastic bacterial numbers have been linked to previous exposure to hydrocarbons (Zobell 1969, Atlas 1981). This is important in determining the rapidity of degradation of subsequent hydrocarbon inputs (Hoff 1993). Bacteria and yeasts apparently are the predominant degraders of hydrocarbons in the marine environment and filamentous fungi do not appear to play as significant a role as for hydrocarbon degradation in soil (Floodgate 1984, Atlas & Bartha 1992). There is some evidence that plasmids may play a role in hydrocarbon biodegradation, as there is an increase in proportion of bacterial populations containing hydrocarbon utilising plasmids (Hoff 1993, Atlas 1995).

There has been much attention on the possible role of white rot fungi in remediation of soil contaminated with persistent organics such as high molecular weight PAH (e.g. gas works sites). White rot fungi have received a lot of research interest as they produce non-specific extracellular ligninolytic enzymes (Lamar 1992). As well as degradation of lignin, these fungi have shown the ability to degrade persistent organics such as DDT, PAH and PCBs (Field *et al.* 1992, Colombo *et al.* 1996). As the enzymes are extracellular they may prove to be useful in the bioremediation of insoluble compounds, when bioavailability is a limiting factor for other microorganisms.

In general the alkanes degrade more readily than aromatics with *n*-alkanes being most biodegradable. The resistance to aliphatic biodegradation increases with degree of branching and cycloalkanes are slow to biodegrade (Atlas 1981). Oudot (1984) used capillary GC and direct MS analysis to determine the relative biodegradability of BAL petroleum (Arabian light crude) in quasi-continuous culture using an enriched mixed marine microbial inoculum. He concluded that the *n*- and *iso*-alkanes exhibited most degradation, followed by 6-, 1-, 5- and 2-ring alkanes, sulfur aromatics and monoaromatics. 3- and 4- ring alkanes and 2- and 3- ring aromatics were moderately susceptible, while 4-ring aromatics and biomarkers were resistant. He concluded the most resistant to be 5-ring aromatics, resins and asphaltenes. Gough and Rowland proposed T-branched alkanes as significant contributors to the UCM and demonstrated the recalcitrance of model branched UCM alkanes (Gough & Rowland 1990, Gough *et al.* 1992). The refractory nature of the larger condensed cycloalkanes has made them useful in fingerprinting severely weathered oils and enabled use of C30 hopane as a conserved internal marker. However, biodegradation of steranes in laboratory conditions has been shown to occur (Chosson *et al.* 1991). The route of attack on structures with long alkyl chains on ring structure is of interest and

C2-C7 alkyl benzenes have been shown in one study to be attacked through the ring structure by a *Pseudomonas* sp.(Smith & Ratledge 1989). It is probable that different populations are responsible for degrading hydrocarbon classes or that they are at least incapable of biodegrading structurally different compounds simultaneously (Prince 1993)

Aerobic biodegradation is undoubtedly the primary mechanism for the biodegradation of hydrocarbons. However, hydrocarbons can also be degraded in anaerobic conditions and this has received research interest due to the potential application in oxygen depleted environments, such as *in situ* treatment of groundwater. Sulfate reducing bacteria and nitrate reducing bacteria have shown an ability to degrade hydrocarbons. The biodegradation of up to 4-ring PAH under denitrifying conditions has been observed for environmental isolates (McNally *et al.* 1998). Rates of anaerobic degradation are generally considerably slower than aerobic biodegradation rates.

1.4.3 Metabolic pathways

Much effort has been spent on the elucidation of biodegradation pathways. For *n*-alkanes, the primary aerobic pathway involves mono-terminal oxidation to the primary alcohol, aldehyde and finally the carboxylic acid. Carboxylic acid biodegradation takes place by β-oxidation with subsequent formation of a fatty acid, two carbon units shorter, and eventual liberation of CO_2. Diterminal (omega) and subterminal oxidation have been reported but are less important mechanisms. Branched chain molecules, such as isoprenoids, undergo omega oxidation and the greater the methyl substitution the more resistant they are to biodegradation (Atlas 1981, Singer & Finnerty 1984).

Cycloalkanes are a major component of crude oil but are quite resistant to biodegradation. Co-oxidation appears to play an important part in cycloparaffin degradation and alkyl substitution often increases the susceptibility to biodegradation. The presence of another substrate is sometimes necessary to enable biodegradation of cyclic compounds by certain micro-organisms (Perry 1979). Although some micro-organisms can not utilise cyclic hydrocarbons as a sole source they may be able to oxidise the compound to a ketone or alcohol, which is a substrate for other microorganisms (Beam & Perry 1974, Atlas 1981, Perry 1984).

Bacterial degradation of aromatics usually involves hydroxylation to form *cis*-dihydrodiols with subsequent formation of catechols and ring-cleavage. Fungi tend to produce *trans*-diol intermediates (Cerniglia 1984, Rosenberg *et al.* 1992, Atlas 1995). Examples of isoprenoid and aromatic degradation pathways are given in figures 1.3 and 1.4 respectively.

Pristane (2, 6, 10, 14 tetramethylpentadecane)
↓
2, 6, 10, 14 tetramethylpentadecanoic acid

(ϖ-oxidation)
↓
2, 6, 10, 14 tetramethylpentadecanedioic acid
↓
2, 6, 10 trimethyltridecanedioic acid
↓
2, 6, 10 trimethylundecanedioic acid
↓
2, 6 dimethylnonanedioic acid
↓
2, 6 dimethylheptanedioic acid
↓
2 methylpentanedioic acid

(β-oxidation)
↓
4, 9, 12 trimethyltridecanoic acid
↓
2, 6, 10 trimethylundecanoic acid

Fig. 1.3: *Pathway for pristane oxidation by* Brevibacterium erythrogenes *(adapted from Singer & Finnerty 1984 in* Petroleum Microbiology *ed. Atlas)*

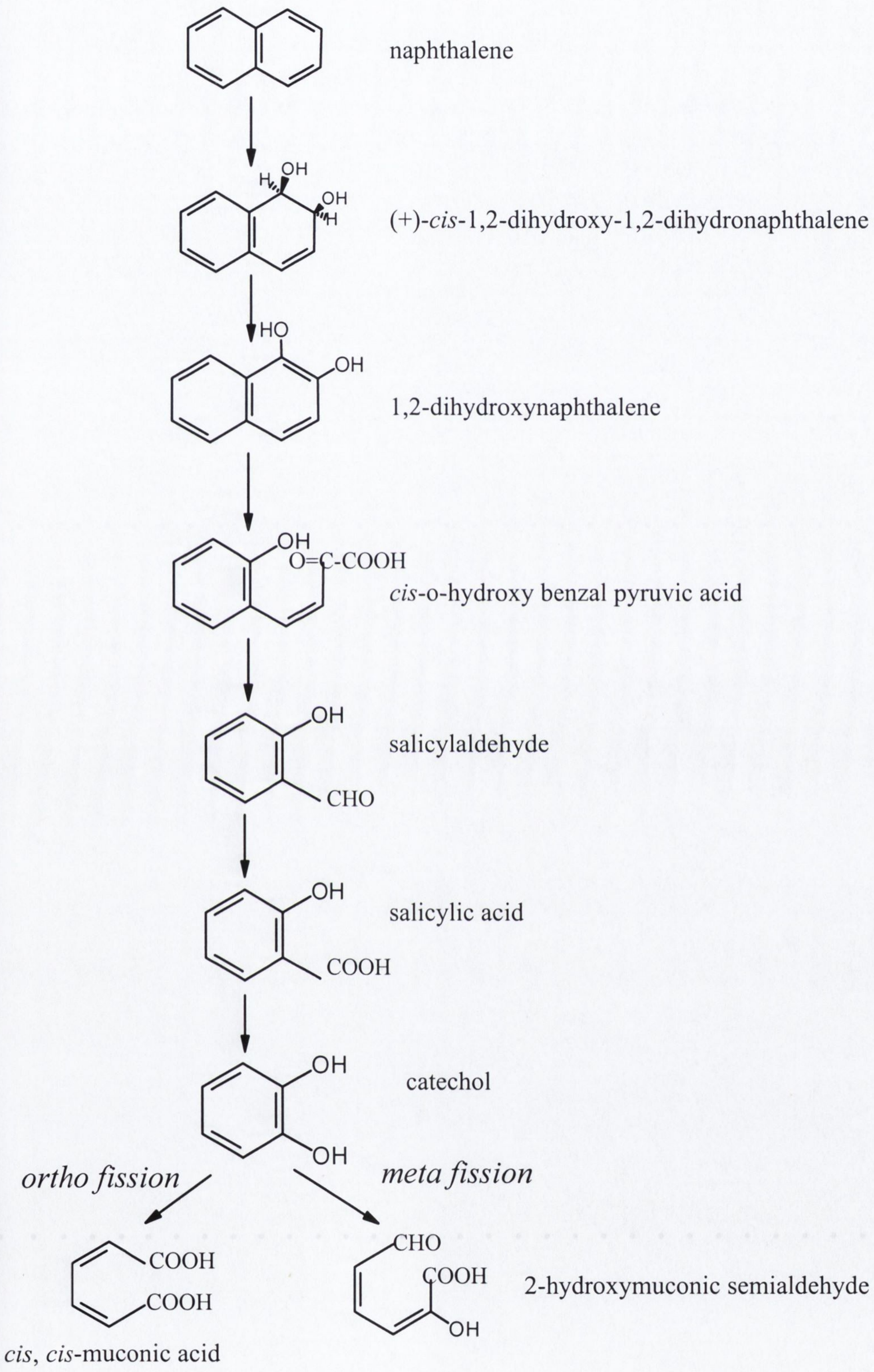


Fig. 1.4 : Pathway *for the bacterial oxidation of naphthalene*
(adapted from Cerniglia 1984 in Petroleum Microbiology *ed. Atlas)*

1.4.4 Factors affecting, and optimisation of hydrocarbon biodegradation

There are many factors that affect and control the environmental rates and these have been extensively reviewed (Morgan & Watkinson 1989, Leahy & Colwell 1990, Atlas & Bartha 1992, Prince 1993, Atlas 1995, Head 1998, Swannell *et al.* 1996, Prince 1997).

1.4.4.1 Nutrients

Metabolism of hydrocarbons requires the availability of certain nutrients, especially nitrogen (N) and phosphate (P) for incorporation into biomass. After an oil spill, the organic carbon source is abundant and nutrient availability may then become rate limiting (Prince 1993). Most bioremediation treatments involve some form of nutrient addition. Shoreline and microcosm nutrient supplementation strategies have included application of soluble inorganic nutrients (Venosa *et al.* 1996, Swannell *et al.* 1999), oleophilic fertilisers (Inipol EAP22™ (Swannell *et al.* 1995, Bragg *et al.* 1996)) and slow release fertilisers (Swannell *et al.* 1999) (Customblen™ (Bragg *et al.* 1996), Max-Bac™(Swannell *et al.* 1995, Oudot *et al.* 1998)). In many instances the application of nutrients did enhance biodegradation rates and slow release, dissolved, and oleophilic fertiliser applications have all been shown to be successful. Economic considerations are therefore likely to be important when selecting the fertiliser for use. In some studies the substantial biodegradation of the control sites due to background nutrients is commented on (Venosa *et al.* 1996, Oudot *et al.* 1998). Oudot *et al.* suggested that if background level of N in the interstitial pore water of the sediment is ≥ 100 μmoles litre^{-1} then bioremediation through fertilisation may be of limited use. Recently it has been suggested that both the absolute amount of inorganic nutrient and the ratio of N:P can effect hydrocarbon degradation rates (Smith *et al.* 1998, Head & Swannell 1999).

Lithium nitrate (Wrenn *et al.* 1997a, 1997b) has been used as a conserved tracer to assess the effect of beach hydraulic dynamics in the intertidal zone on dissolved nutrient concentrations and distributions. Movement of the tracer vertically through the sediment subsurface and horizontally in a seaward direction from the bioremediation zone was observed. Rapid washout of tracer from the bioremediation zone suggested that, in this case, frequent application of dissolved nutrients (24 hourly) would be required to maintain elevated nutrient concentrations, and thus by implication for continued stimulation of biodegradation. Use of tracers such as lithium provides a useful technique for evaluating the role of shoreline physical dynamics on nutrient application during bioremediation thereby assisting the design of an effective nutrient addition programme.

1.4.4.2 Oxygen

As shoreline bioremediation relies on aerobic metabolism, oxygen is critical to the biodegradation rates. Dissolved oxygen concentrations in water are very low (~8mg/l) and therefore could rapidly become limiting in subsurface sediments. Wave action can cause supersaturation of water and oxygen is less likely to be limiting in high- energy beaches. Waterlogged sediments will be oxygen depleted. It should be noted that subsurface water levels will probably not subside with receding tide levels but will form a gradient to open water level dependent on sediment permeability (Wrenn *et al.* 1997a). Adequate sediment drainage to enable O_2 diffusion to hydrocarbon degrading microbes is therefore essential for effective bioremediation (Atlas & Bartha 1992). Tidal cycles facilitate air exchange in intertidal shoreline sediments with good drainage. The low sediment permeability of mudflats means subsurface O_2 is limiting and nutrient addition alone is unlikely to be sufficient for effective bioremediation in these environments.

Unsaturated subsurface soil is normally aerobic, but O_2 utilisation may exhaust supplies faster than they can be replenished by diffusion, while oxygen is often limiting during *in situ* bioremediation of groundwater. Physical techniques to improve aeration include manipulation (raising and lowering) of the ground water table, alteration of soil characteristics using fillers (in soil banking), pumping to improve drainage, and bioventing. Chemical oxygenation through use of hydrogen peroxide (H_2O_2) has also been investigated (Morgan & Watkinson 1992).

1.4.4.3 Seeding

The introduction of cultures into the spill environment is referred to as *bioaugmentation,* or 'seeding', or inoculation (Pritchard 1992). Many of the products commercially available and marketed for bioremediation include microbial formulations. While hydrocarbon degrading microrganisms are ubiquitous in the environment there is a broad range of hydrocarbon structural heterogeneity and some hydrocarbons are resistant to biodegradation. Seeding may introduce a broader range of enzymatic capability than present in the indigenous population. Furthermore, an adaptive period is necessary for the indigenous microbial population so there may be an advantage in adding pre-adapted culture that have demonstrated hydrocarbon degrading ability. While it is theoretically promising, 'seeding' has not been convincingly demonstrated in the field as significantly affecting biodegradation rates. Some commercial products also contain dispersants and/or nutrients and it can be difficult to discern whether it is the introduced microorganisms, as opposed to other additives, that have enhanced biodegradation. After the *Exxon Valdez* spill, 10 products were screened for efficacy in stimulating oil biodegradation, using a two-tier system. After laboratory tests only two products were selected for field trials (Anon. 1991a, Venosa *et al.* 1991). Neither product demonstrated enhanced biodegradation when compared with nutrient application alone in field trials (Venosa *et al.* 1992). In the Delaware Bay study, a mixed consortium isolated from the experimental beach was grown on the same crude oil as that used to contaminate the beach as sole carbon source (Venosa *et al.* 1996). This was used as an inoculum but did not enhance biodegradation relative to the nutrient only plots.

There are potentially many factors that may limit the ability of introduced cultures to survive and proliferate in the environment (Pritchard 1992). Cultures that have been substantially laboratory cultured or that were initially isolated from a different environment may not be viable; a minimum inoculum concentration may be required; competition may occur from indigenous micro-organisms and predation. Environmental influences are paramount and it was shown that the hydrocarbon mineralisation potential in Prince William Sound after *Exxon Valdez* was not related to substrate concentration but other factors such as intensity of physical mixing experienced, treatments received and the availability of alternative carbon sources.

The use of genetically engineered microorganisms (GEMs) has also been proposed (de Lorenzo 1992). The first patent for a recombinant micro-organism was issued to Chakrabarty and General Electric for a hydrocarbon degrading bacteria (Atlas 1992). However, the potential use of genetically modified micro-organisms is controversial due to considerations such as safety, control, and ethical issues. Therefore restrictions remain on environmental release (Atlas & Bartha 1992). Furthermore, the concept of using genetically engineered 'superbugs' to bioremediate highly complex mixtures, such as petroleum hydrocarbons, is suspect (Lethbridge *et al.* 1994).

Generally, there are few details available with most commercial products, but it is probable that many are based on cocktails of individual isolates that demonstrate hydrocarbon mineralisation potential on single hydrocarbon substrates in the laboratory. Thus, the complexity and synergistic effects present in the environment when a range of microbes interact to degrade a range of compounds may not be incorporated. This could be particularly important for biodegradation of cyclic compounds where co-oxidation has been demonstrated to be an important factor. Many of the vendors claims for microbial based bioremediation products remain to be independently verified.

1.4.4.4 Physical state of the oil and bioavailability

The availability of hydrocarbon substrate has been recognised as a key element that may limit biodegradation in the environment (Head 1998). Biodegradation takes place at oil interfaces or on dissolved hydrocarbons. As hydrocarbon solubility is very low, the biodegradation on true dissolved compounds is relatively low. Therefore a high surface area to volume ratio of oil is necessary. Mechanisms of hydrocarbon uptake are via dissolved phase hydrocarbons, direct contact with oil surface, or surfactant solubilised hydrocarbon (Floodgate 1984, Singer & Finnerty 1984). An area that has attracted much attention in recent years is the role of biosurfactants, particularly rhamnolipids, in solubilising and enabling hydrocarbon transport into the cell (Singh & Desai 1988). Rhamnolipids have been shown to enhance biodegradation of octadecane (Zhang & Miller 1992) and to increase solubilisation and removal of soil sorbed phenanthrene from soil columns (Noordman *et al.* 1998). A glycolipid was shown to be more effective than Tween-80 for solubilisation of naphthalene and methyl-substituted naphthalenes from crude oil. It was also shown to be less toxic (Kanga *et al.* 1997). In some cases the use of synthetic surfactants has been shown to enhance biodegradation (Prince 1993, Ducreux *et al.* 1994). Liposome encapsulation of *n*C18 and *n*C36 was also shown to enhance microbial uptake (Miller & Bartha 1989).

If surfactant addition is to be used, the possibility of inhibition of biodegradation due to introduction of competitive substrate should be considered and careful choice of surfactant is required.

1.4.4.5 Other factors – pH, temperature, competitive substrates

Seawater is naturally well buffered and seawater pH enables high rates of oil biodegradation. Generally metabolism rates decrease with decreasing temperature and often biodegradation will be substantially reduced during winter. Furthermore, physical effects on the oil such as increased viscosity may reduce degradation. However, hydrocarbon degrading microorganisms have been isolated for a wide temperature range and oil biodegradation has been observed in polar climates (Sexstone & Atlas 1977, Kennicutt 1990, AMAP 1998).

Obligate hydrocarbonoclastic bacteria are rare and most hydrocarbon degraders can metabolise other substances. If other substrates are available they may be preferentially degraded rather than oil, or alternatively, may actually induce hydrocarbon biodegradation, possibly through biosurfactant production (Zhang & Miller 1992, Prince 1993, Al-Hadrami *et al.* 1996). Bioremediation product formulations are being investigate that include nutrients, alternative carbon sources, surfactants etc. (Kjeilen 1997).

1.5 ANALYSIS OF PETROLEUM HYDROCARBONS

Methods of analysis of hydrocarbons, and in particular UCM, were reviewed, with a view to selecting and evaluating suitable methods for development and use in monitoring bioremediation trials.

1.5.1 Fractionation techniques

There are numerous techniques that can be applied to hydrocarbons and petroleum oils to separate into subgroups or classes. These techniques may lead to direct analytical information pertaining to the subgroups or be used as preparative fractionation for further analysis (Philp 1994).

1.5.1.1 Column and thin layer chromatography

The fluorescent indicator adsorption (FIA) method {ASTM D1319-98} (ASTM 1998) is the petroleum industry standard for hydrocarbon class (group/type) determination. This involves use of silica gel in a specially designed glass column, and separates hydrocarbons into saturates, olefins and aromatics. The method is not suitable for dark coloured samples. Alumina and silica gel are widely used to achieve separation into saturates, aromatics, resins, and asphaltenes (SARA). Separation of olefins is not required for crude oils as they are present in insignificant proportions in crude oils (Tissot & Welte 1978). *Thin layer chromatography (TLC)* can also be used for class separation (Harvey *et al.* 1984) and can be used with FID detection to directly quantify petroleum subgroups (Selucky 1985). Killops and Al-Juboori (1990) used silica gel (Merck 60G, 0.4mm, pre-eluted for 24h with dichloromethane prior to re-activation). Bands were isolated corresponding to aliphatics (Rf>tridecylbenzene), monoaromatics (tridecylbenzene $\geq$ Rf $>$ naphthalene) and polyaromatics (naphthalene $\geq$ Rf $>$ 0) by reference to appropriate standards applied to channels at both edges of the TLC plate. Standards were visualised under UV(254nm)after aerosol application of Rhodamine 6G in methanol. Quantitation was achieved in relation to internal standards (squalane, tridecylbenzene, anthracene) added to each fraction, each at a level of 2µg/mg total hydrocarbons. Gough and Rowland (1990) used argentation TLC in conjunction with column chromatography and triple urea adductions to isolate aliphatic UCM.

1.5.1.2 Molecular sieve and urea and thiourea adductions

Urea adduction (Baron 1961) and 5Å molecular sieve (O'Connor et. al 1962, Murphy 1969) are both used in separating *n*-paraffins from crude oils. Both can form channel complexes that can accommodate straight chain alkane molecules but exclude branched and cyclic molecules due to the 5Å internal diameter crystal lattice. Urea adduction involves a urea clathrate forming around the molecule whereas in a molecular sieve the cavity already exists. A comparison of 5Å molecular sieve with urea adduction (Chukwuemeka & Nwobodo 1994) found urea adduction to include a broader range of adductable *n*-paraffins and also indicated that urea was more efficient. Killops and Al-Juboori (1990) used urea adduction in studying UCM composition. Using reference compounds (eicosane, anthracene, trideyclbenzene, pyrene and squalane) they found that while *n*-eicosane was completely urea adducted as expected, all other reference compounds were also adducted to some extent. They quantified UCM relative to non-adducted squalane (assuming that the same quantities of non-adductable squalane and UCM were retained in the adduct fraction). This suggested that 25% of UCM was adducted suggesting a significant presence of components containing a terminal *n*-alkyl group attached to another structure. In the same paper, thiourea adduction was also employed. This has a crystal lattice of 7Å and should retain branched acyclic components, cycloalkanes and benzenoid compounds with suitable alkyl chains. *n*-Alkanes and some of the larger cyclic components are reported not to be retained but the author found all reference components were retained to some degree.

Silicalite has also been used to remove branched and cyclic fractions from the *n*-alkane fraction (West *et al.* 1990).

1.5.2 Spectroscopic techniques

1.5.2.1 Infrared spectroscopy (IR)

US EPA method 418.1 (US EPA 1983) for measuring total petroleum hydrocarbons (TPH) in water (and soil - EPA 418.1 modified) is based on IR analysis of the hydrocarbon extract in a CFC 113 solution. The aliphatic C-H stretch at ~2930cm^{-1} is quantified against the same peak for a reference solution. This may be a reference oil sample or a mixture of reference compounds in solution. However, the unavailability of CFC as a solvent is likely to make this method redundant. A similar ASTM standard test method for oil and grease and petroleum hydrocarbons in water also employs CFC 113 as the solvent (ASTM 1996). ASTM method D3141-80 (ASTM 1981) uses IR spectroscopy for comparison of waterborne petroleum oils and includes guidelines for interpreting weathering of the oil. FT-IR has been used to qualitatively analyse UCM as a film on a sodium chloride cell (Killops & Al-Juboori 1990).

Comparing %IR/Gravimetric for a number of oils measured by EPA method 418.1 using a single calibration oil illustrates the bias inherent in such a method (Douglas *et al.* 1992). Oils, such as lube oil, with a higher proportion of saturates to total oil than the calibration oil are positively biased, (%IR/Gravimetric > 100%), and oils such as gasoline, with a lower proportion of saturates than the calibration oil are negatively biased, (%IR/Gravimetric < 100%). Measuring the IR absorbance of the oil directly using a fixed path transmission cell or attenuated total reflectance (ATR) can eliminate solvent background absorbance.

1.5.2.2 Fluorescence/Luminescence spectroscopy

Fluorescence spectroscopy has been used for measurement of hydrocarbons in the marine environment (Law *et al.* 1988) using fixed excitation and emission wavelengths and measuring the emission intensity against a crude oil, such as Ekofisk, or a polyaromatic hydrocarbon mixture standard curve. Synchronous scanning fluorescence and fixed excitation scanned emission spectra enables fingerprinting of petroleums (Pharr *et al.* 1992, Shadle *et al.* 1994). However, total fluorescence spectroscopy gives a much more detailed fingerprint, usually displayed as contour plots (Butt 1986). Aliphatic components do not fluoresce and this must be taken into account if using fluorescence for quantification of oil samples, as bias may be introduced if a comparable standard is not used. Mason (1987) has obtained total fluorescence spectra for oil contamination in mussels and also obtained total fluorescence spectra for individual PAH compounds. This enabled information to be gained on the aromatic composition of the oil based on the contour plot, and also preparation of a suitable standard mixture of PAH that produced a similar contour plot.

1.5.2.3 NMR

Killops and Al-Juboori have used FT-^{1}H NMR and FT-^{13}C NMR in the analysis of UCM. Although some useful structural information was obtained for UCM composition, the complexity of UCM made interpretation difficult (Killops & Al-Juboori 1990).

1.5.3 Chromatography

1.5.3.1 Gas chromatography

Gas chromatography with flame ionisation detection is a well-established technique for the analysis of hydrocarbons and oil samples. There are many capillary columns available suitable for crude oil analysis resolving *n*-alkanes, and pristane and phytane, and recently the advent of high temperature columns has enabled detailed investigations of high molecular weight hydrocarbons present in oils, waxes, bitumens and source rocks (Philp 1994). GC is regularly used in estimating the boiling range of both crude petroleums and refined products (ASTM 1984, Yancey et al. 1994). The term 'unresolved complex mixture' refers to the `hump' of unresolved or poorly resolved components in GC-FID traces. The presence of UCM in GC analysis of environmental samples has been considered to provide a better indication of petrogenic contamination than resolved compounds, as there is a greater chance of resolved compounds having a recent biogenic origin (Volkman *et al.* 1992). ASTM prescribe a method for comparison of waterborne petroleum oils by gas chromatography (ASTM 1991b).

1.5.3.2 HPLC

Fractionation of oils by high performance liquid chromatography (HPLC) has proved useful in separating petroleum into component classes. Separation of petroleum into saturates, aromatics, resins and asphaltenes (SARA) has been achieved by initially deasphalting the sample and then performing HPLC analysis using silica, alumina, amino, cyano or aminocyano columns, or various combinations of these columns (Lundanes & Greibrokk 1994). Two columns are commonly used, often amino or cyano as the first column and silica, amino, cyano or amino-cyano as the second. Usually backflushing of the resins from the first column is employed, and often backflushing of the second column is used to determine the aromatics. Grizzle and Sablotny (1986) used two amino columns in series, with hexane to elute saturates, and hexane:methylene chloride gradient to elute aromatics, with separation according to ring number. Polar compounds were eluted by backflushing with 90% methylene chloride in hexane. Detection was achieved with a refractive index (RI) detector and ultraviolet (UV) detector. Pearson and Gharfeh (1986), used a cyano and an amino-cyano column and two backflush valves to achieve separation. Hexane eluted saturates before the aromatics were backflushed from the second column with hexane and then polar compounds eluted from the first column with MTBE. A flame ionisation detector was used. Shadle *et.al.* (1994), used a cyano and a silica column in series to analyse shale oils and crude oils using hexane:methylene chloride gradients and eluting polar compounds in a forward direction. Killops and Readman (1985), used two PAC (amino-cyano) columns in series to analyse the aromatic fraction of sediment extracts. Adsorption of water and irreversible binding of heteroatoms on silica can cause reproducibility problems.

1.5.4 Mass spectrometry (MS)

1.5.4.1 Gas chromatography-mass spectrometry (GC-MS)

GC-MS in positive electron ionisation mode is widely used for petroleum analysis. GC-MS analysis of oils and kerogens is particularly useful in geochemical studies for assessing potential of oil bearing source rocks, correlation of oils and sources, studies of depositional environment, assessment of maturation, extent of biodegradation, and other processes (Peters & Moldowan 1993, Requejo *et al.* 1996). These techniques have also been adapted as the preferred methodology in identifying the source in oil spill incidents (Albaiges & Albrecht 1979, Anon. 1991b, ASTM 1995). The power of GC-MS lies in the use of mass spectrometry as a selective detector to obtain gas chromatographic 'fingerprints' for various groups of components that would be occluded in oil by more concentrated components when analysed by more conventional methods. There are two basic component groups that are used for this purpose (Wrang & Adamsen 1990a, 1990b).

(i) *Substituted aromatics* - Selected ion recording (SIR) of a suitable molecular mass can allow a chromatogram to be obtained for a group of structural substituted aromatic isomers e.g. methyl phenanthrenes at m/z 192. As the chemical and structural properties of such isomers are very similar, the isomer 'pattern' as depicted by a GC-MS trace is relatively resistant to weathering.

(ii) *Biomarkers* - The biomarkers are 'molecular fossils' that can be directly related to a biological precursor compound from their molecular structure, for example cyclic alkanes such as steranes and hopanes. Although biomarker groups tend to have a number of homologous compounds of differing molecular weights, they often have common structural attributes that can be detected as fragments using GC-MS in EI+ mode. Furthermore, stereoisomers of compounds are often present adding to the complexity of the patterns. The resulting chromatogram is referred to as a 'mass fragmentogram'. The term mass

chromatogram will be subsequently used in this report to denote a chromatogram for a given mass-charge ratio, including mass fragmentograms. The aliphatic biomarker mass chromatogram patterns are relatively resistant to weathering, as the compounds are resistant to the primary weathering processes (i.e. evaporation, biodegradation and dissolution).

Standard test procedures such as NORDTEST use diagnostic ions based alkyl substituted 2,3 and 4 ring PAH and dibenzothiophene (e.g. m/z 192, 212, 226 for C1-, C2- and C3-phenanthrenes) and biomarker fragments (e.g. m/z 217 steranes and m/z 191 pentacyclic triterpanes). Other ions that have a diagnostic use include m/z 57, 71, 85 etc for acyclic alkanes, m/z 55 for alkenes and cycloalkanes, m/z 83 for alkylcyclohexanes and m/z 95 for naphthenes (Simoneit 1986). Multivariate analysis (principal component analysis) was used to compare sterane and triterpane data after artificial weathering of crude oils from the Cook and Brent formations in the same well (no. 9) at the Gullfaks field (Brakstad & Grahl-Nielsen 1988). The steranes were unsuitable for identification of weathered oils after four months, but the pentacyclic triterpanes, and in particular the demethylated triterpanes, were deemed a good diagnostic tool for oil identification throughout the course of experimental weathering (1 year).

Prince *et al.* (Prince *et al.* 1994), used the resistance of pentacyclic triterpanes to weathering to suggest the use of 17α(H),21β(H)-hopane (C30-hopane) as a conserved internal marker for estimating the biodegradation of crude oil. As quantification of total crude oil biodegradation in the field is extremely difficult, the use of an internal marker is an invaluable tool for measuring the reduction of the biodegradable components relative to the conserved marker. From biodegradation experiments, Prince *et al.* concluded that C30-hopane was neither biodegraded or generated during the biodegradation of crude oil fractions on time scales relevant to the estimation of oil spill cleansing. More recently in the absence of C30-hopane, the use of norhopane has been used as conserved internal standard (Oudot *et al.* 1998).

The use of 17α(H),21β(H)-hopane as an conserved internal standard was further developed during the EUROCRUDE project, (Anon. 1995). This was a six laboratory collaboration funded by the EU LIFE programme to develop an analytical, (GC-MS), database of crude oils that are produced and transported in European waters, and to evaluate and develop statistical methods and computer software for comparing and matching fingerprints with the database. When spilt oils are compared with suspect sources by GC-MS, the samples are run on the same machine within the same sample batch. Thus, the primary problem faced was to treat the data in such a way that samples analysed on different instruments (or indeed instrument types e.g. quadrupole or magnetic sector instruments), or at different times could be compared. This was achieved firstly by using a Brent crude oil as a reference, for quality assurance of data. Analysis of spilt crude oil samples for comparison with the database involves analysing the reference Brent crude oil by GC-MS, followed by the sample twice, and then again the reference oil. Up to 56 peaks can be used and the integration data for the sample and reference oils are entered into the model for comparison with the database. Four normalisation routines can be used but the most effective for reducing interlaboratory and long term variability is triple normalisation. It involves normalisation against C30-hopane for both samples and reference oils. The hopane normalised samples are averaged and further normalised with the bracketing hopane normalised reference samples.

GC-MS analysis of biomarkers and aromatics has also proved a useful tool in investigations of chronic hydrocarbon contamination of the marine environment, and for reconciling the source (Volkman *et al.* 1992). Tricyclic and pentacyclic terpanes have been considered as indicators of the origin of diffuse lubricating oil contamination in plankton and sediment (Bieger *et al.* 1996). GC-MS analysis of biomarkers and aromatics has also been used in tracking oil spillages and in assessing the environmental effect of such spillages in cases such as the *Sivand* spill, Humber Estuary *1983* (Jones *et al.* 1986), *Exxon Valdez,* Alaska 1989 (Krahn *et al.* 1992, Bence *et al.* 1996), The Gulf War spill, Kuwait *1991* (Sauer *et al.* 1993), and the *Braer* spill, Shetland *1993* (Wolff *et al.* 1993, Glegg & Rowland 1996).

1.5.4.2 Direct sample introduction mass spectrometry

A current ASTM method is available for estimation for 0-6 ring saturates and monoaromatics in non-olefinic saturate petroleum fractions of average carbon number between 16 – 32 (ASTM 1991b). This is based on the fragment peak method devised by Hood and O'Neal (1959). A similar ASTM method enables aromatic type analysis for non-olefinic aromatic fractions (Robinson & Cook 1969, ASTM 1991c).

In their study of UCM Killops and Al-Juboori (1990) used probe MS and used ion abundance of major fragments in the UCM spectrum in their interpretation of UCM composition.

1.5.4.3 Chemical ionisation mass spectrometry (CI-MS)

CI-MS has been less frequently applied to the characterisation of crude oils and heavy oils. CI mass spectra, using N_2O as a chemical ionisation reagent gas, for total crude oils from a range of fields within four geographical areas was tested as a potentially useful fingerprinting tool for distinguishing the geographical origin of the oil (Burke *et al.* 1982). Principal component analysis and discriminant analysis demonstrate the clear differentiation in the oil composition for the diagnostic ions chosen. The ions used as diagnostic ions were of $m/z \geq 180$ to exclude volatile components and $m/z \leq 259$. CI-GC-MS has also been used for analysis of prefractionated heavy oil contaminated soil sample extracts (Pollard *et al.* 1994), although few details are supplied.

1.5.4.4 Chromic acid oxidation

Interpretation of the products of UCM oxidation has proved useful in providing an insight into the composition of the UCM and indeed surprisingly resolved chromatograms can be obtained. Usually chromium (VI) oxidation is used, and in particular CrO_3, (chromium trioxide). A general review of chromium oxidations in organic chemistry is available (Cainelli & Cardillo 1984). Chromium oxidation of saturated hydrocarbons is not especially selective and considerable second stage oxidation does occur. Thus complex mixtures of reaction products are produced. The C-H attacked in chromic oxidation depends on the attached structures. Methyl groups are relatively resistant to oxidation and are eventually oxidised to acetic acid while tertiary hydrocarbons are much more prone to attack and this sensitivity is further increased with a phenyl group attached.

Although oxidation of relatively simple hydrocarbons can give rise to complex mixtures of product, oxidation of the UCM can produce surprisingly resolved chromatograms as there is a limited class of structures that are oxidised and similar compounds tend to be oxidised to the same products. Chromic acid oxidation has been used to study the UCM composition (Gough & Rowland 1990, 1991) and has been proposed as a potential mechanism for fingerprinting oils (Revill *et al.* 1992).

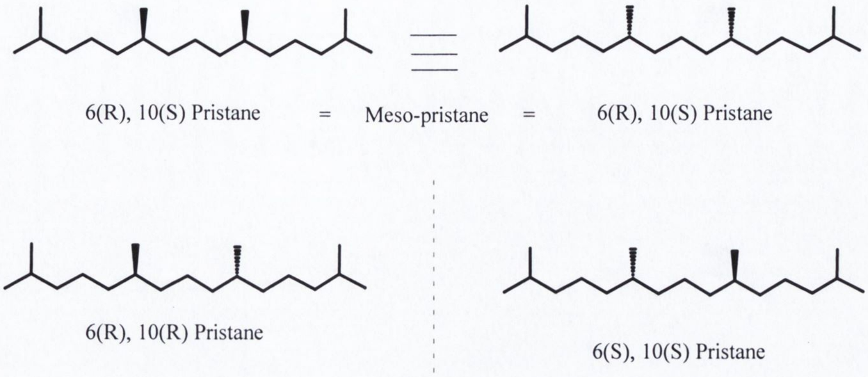
1.5.4.5 Stereochemistry and nomenclature of petroleum hydrocarbons

Some understanding of the structural configurations of petroleum hydrocarbon molecules in three-dimensional space is required when interpreting GC-MS analysis of biomarkers

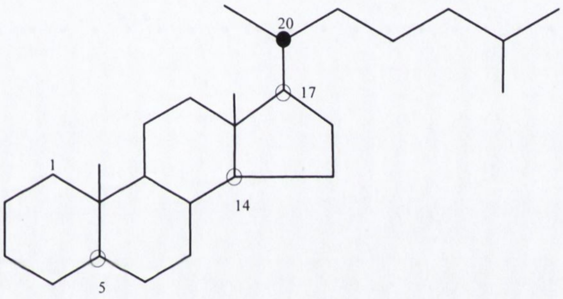
(MacKenzie 1984, Peters & Moldowan 1993). Asymmetric or "*chiral*" carbons, that is carbons with four differing substituents, are common in petroleum hydrocarbons. Two forms of such molecules, known as enantiomers, may exist, each being a mirror image of the other. The two possible configurations around an asymmetric carbon are called "R" and "S" in conventional nomenclature. If more than one asymmetric centre is present, inversion of all the asymmetric centres results in *enantiomers*. However, if less than all the asymmetric centres are inverted the different molecular configurations are referred to as *diastereomers* or *epimers*. As an example pristane contains two asymmetric carbons (fig. 1.5 a). The 6R, 10R and 6S, 10S configuration are mirror images and thus are enantiomers. However, note that the 6S, 10R and the 6R, 10S configurations can be superimposed on one another and they are therefore identical. These configurations are known as meso-pristane and their relationship with 6R, 10R or 6S, 10S pristane is diastereomeric.

A property of enantiomers in solution is that they exhibit optical activity, i.e. the extent and direction of rotation of plane polarised light is equal and opposite for each enantiomer. The physical and chemical properties of enantiomers are very similar, and their chromatographic separation requires special chiral stationary phases. However, diastereomers can have different chemical and physical properties and can generally be separated on conventional stationary phases. Asymmetric configurations in ring systems are distinguished using the nomenclature α and β. Subtituents that are above the plane of the molecule are designated β and those below α. As an example, 5α(H), 14α(H), 17α(H), 20R cholestane, [denoted αααR], indicates that hydrogens at the 5, 14 and 17 positions of the ring structure face below the plane, and that the acyclic asymmetric carbon in the 20 position has an R configuration (fig 1.5 b&c).

Abiotic production of chiral molecules will usually result in a *racemic* (50:50) mix of enantiomers. In biological production of asymmetric molecules, usually only one configuration occurs. The fact that most petroleums are optically active is further evidence of the biological origin of petroleum. The stereochemical configurations present in biological precursor molecules are not necessarily stable during petroleum formation and stereoisomerisation can produce different isomeric patterns in petroleum. The αααR configuration described above is the natural biological configuration of sterane precursors. During petroleum formation stereoisomerisation of the biological configurations results in other configurations occurring in petroleum contributing to, for example the complex sterane pattern observed in GC-MS analysis.

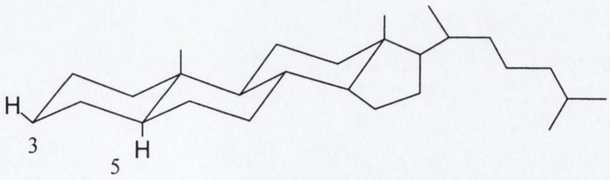


a) Stereoisomeric configurations of pristane



b) 5α(H), 14α(H), 17α(H), 20R cholestane

Solid circles are used to represent β configurations on asymmetric ring carbons and R configurations on acyclic asymmetric configurations. Hollow circles represent the equivalent α and S configurations.



c) 3-d representation of cholestane showing the 5α (H) and the 3β(H) (adapted from Peters and Moldowan 1993).

Fig. 1.5: Stereochemistry and nomenclature of petroleum hydrocarbons.

1.6 OBJECTIVES OF THE PROJECT

There are many factors involved in successfully bioremediating oils spills on the shoreline. The preparation of bioremediation 'guidelines' to aid personnel engaged in an oil spill clean-up has been proposed by researchers in this field (Swannell *et al.* 1996, Lee 1998, Lee & de Mora, 1999). This would assist a clean-up team to decide whether bioremediation is an appropriate tool for a given set of circumstances, and to effectively implement the technology. This project provides additional information to the current knowledge base that may prove useful in the preparation of guidelines.

The objectives of the project were to

- Achieve an understanding into the alteration of the UCM during bioremediation.
- Establish any order of biodegradation for certain compound groups characterised by molecular structural attributes.
- Assess whether bioaugmentation of the microbial population with laboratory enriched cultures from a spill site can enable enhanced biodegradation rates of UCM.
- Determine whether specifically selected microbial cultures may have a role to play in bioremediation of UCM.
- Determine if bioremediation is a suitable technique for treating oil spills with high UCM content such as lubricating oils and some heavily weathered oils.
- Achieve an insight as to whether recalcitrance of UCM components can be attributed to physical parameters (bioavailability) or biological limitations (metabolism).
- Comparison of analytical tools for measuring and assessing biodegradation of petroleum hydrocarbons.
- Insight into the effect of severe biodegradation on patterns and parameters used in fingerprinting and source identification of oil spills.

1.7 OUTLINE OF THE WORK PROGRAMME

The project was divided into two phases. Phase I included a comprehensive literature survey. This was followed by analytical method development to select, develop, and become familiar with methods for UCM analysis, and determine the information set returned by each method. A number of UCM-rich oils were selected and characterised using these methods. These were a physically weathered North Sea crude oil, two reservoir biodegraded North Sea crude oils and three lubricating oils. In parallel with this work, a number of marine sedimentary microbial consortia were selectively enriched on UCM. The capacity of these consortia for UCM biodegradation was evaluated and inocula for the subsequent microcosm trials prepared. GC and GC-MS analysis of the substrate oil was carried out to determine changes to the composition.

Phase II involved microcosm trials using a simple 'tidal simulation' system. Two oils were selected for the trials and bioremediation strategies involving bioaugmentation and biostimulation were tested along with a seawater control and a poison control. This system was designed to be as representative of field conditions as possible, but retain greater control of the system parameters than is possible in the field. A comprehensive analytical monitoring programme, based on the methods developed in phase I, was used to measure and assess biodegradation. An analytical quality assurance programme supported this. Figure 1.6 outlines the project schedule. The microcosm trials were run for 473 days.

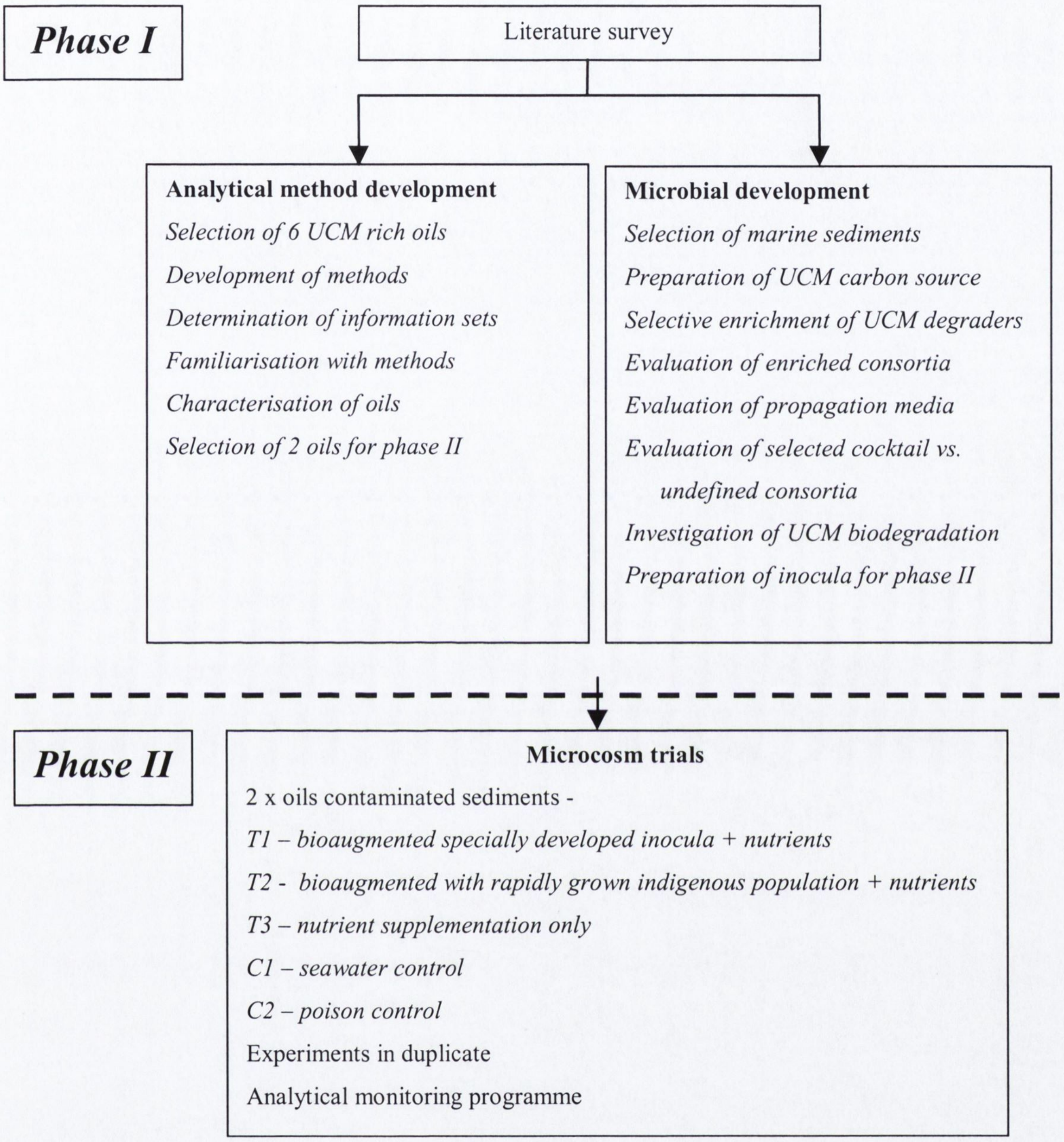


Fig. 1.6: Overview of the project

2. ANALYTICAL DEVELOPMENT AND CHARACTERISATION OF OILS

(PHASE I A)

2.1 PURPOSE

The purpose of this section of the project was:

(i) To develop and refine analytical techniques suitable for analysis of UCM constituent components and suitable for monitoring of biodegradation during bioremediation trials (Phase II).

(ii) Familiarisation with analysis of oil using these techniques.

(iii) To determine information-sets/descriptors returned by these methods.

(iv) To assess the performance of these methods.

(v) To characterise 6 petroleum oils using these methods.

2.2 SELECTION & ACQUISITION OF OILS FOR PHASE I OF PROJECT

6 oils for which UCM is a substantial feature were obtained for this phase of the project and the details of these oils are listed below. Characterisation of these oils by different analytical methods served as an aid in developing these analytical techniques for use in assessing UCM biodegradation in phase II.

2.2.1 Forties crude oil

In March 1996 approximately 200 miles of the south eastern Irish coastline was affected by oiling. Detailed analysis by fluorescence spectroscopy and GC-MS at the Marine Institute Fisheries Research Centre demonstrated that this oil matched the cargo spilled from the *Sea Empress* off Milford Haven (UK), one month earlier (McGovern & Nixon 1996). The oil was a Forties blend crude oil which is a light North Sea crude. Samples of oil were taken for this project from the beach at Kilmore Quay (Co. Wexford) which had been heavily oiled. The oil was black and very viscous, often in the form of small pellets. It had been extensively weathered by evaporation and dissolution but there was no evidence of biological degradation and *n*-alkanes above tridecane (*n*C13) were still present. Weathering processes had resulted in the relative proportion of the UCM being greatly increased in the oil that came ashore with respect to a sample of fresh Forties crude. A gas chromatography trace of the weathered oil is given, (fig. 2.1). *(Fresh Forties blend crude oil was supplied courtesy of Texaco, UK)*

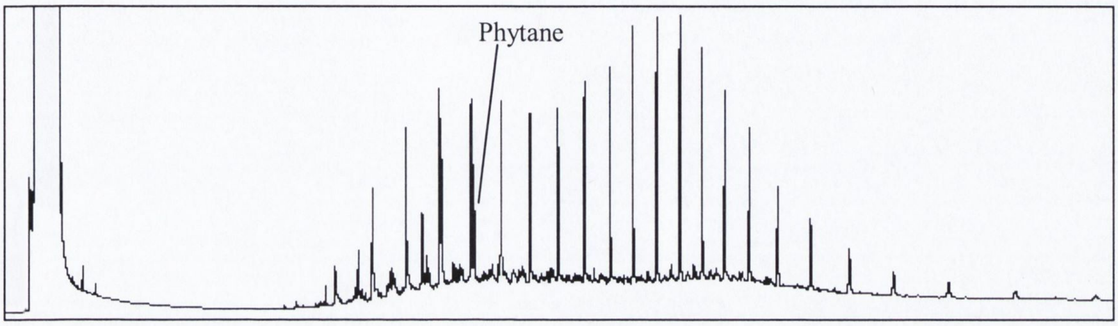


Fig. 2.1: Gas chromatogram of weathered Forties crude oil

2.2.2 Gullfaks crude oil

Gullfaks crude oil is also a light North Sea crude oil. In January 1993 the *Braer* foundered off the Shetland islands releasing its entire cargo of 84,700 tonnes of Gullfaks crude oil (SEEC 1998). Gullfaks is a reservoir-biodegraded crude oil (Horstad *et al.* 1991). Substantial biodegradation has taken place *in situ* within the reservoir over geological time. Thus, the *n*-alkanes have been removed prior to abstraction of oil from the reservoir. Gullfaks, therefore, serves as a good model UCM. Although Gullfaks does contain many resolved peaks they are primarily low molecular weight compounds that will be lost to the atmosphere in a spill situation. A gas chromatography trace of a Gullfaks sample is given, (fig. 2.2).
(Stabilised Gullfaks A was supplied courtesy of Statoil, Norway)

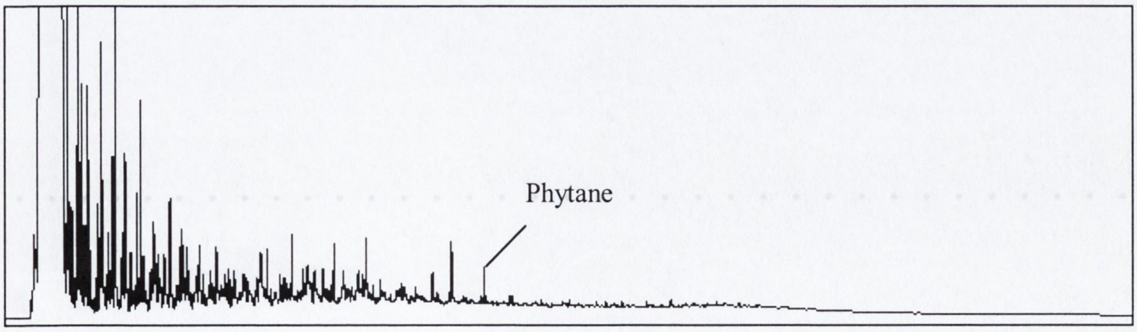


Fig. 2.2: Gas chromatogram of Gullfaks reservoir biodegraded crude oil

2.2.3 Alba crude oil

Alba crude oil is also a reservoir degraded North Sea crude oil. It is heavier than Gullfaks and contains less resolved components. *n*-Alkanes have been completely removed *in situ* prior to abstraction. Alba also serves as a very good model UCM. A gas chromatography trace of an Alba sample is given, (fig 2.3).

(Alba was supplied courtesy of Chevron, UK)

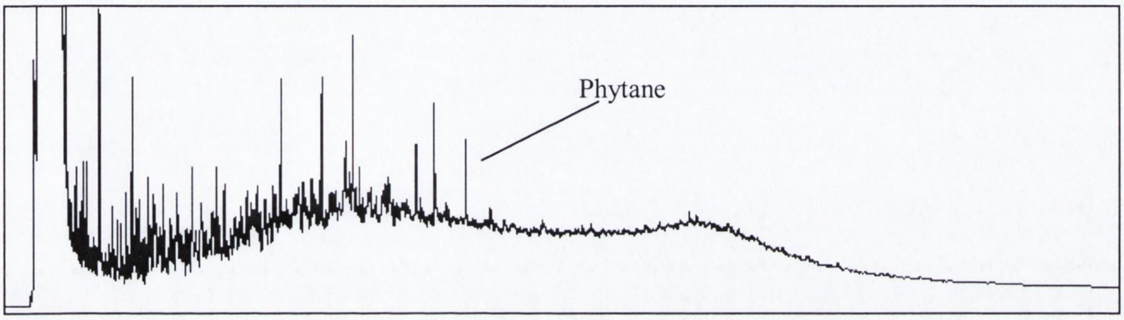
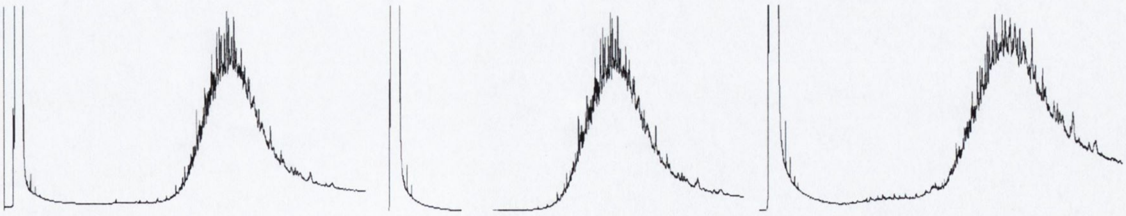


Fig. 2.3: Gas chromatogram of Alba reservoir biodegraded crude oil

2.2.4 Lubricating oils

Three petroleum based lubricating oils were obtained. Lubricating oils are refined products that are primarily aliphatic in content and contain little or no *n*-alkanes. They usually are of a narrow distillation band of relatively high molecular weight hydrocarbons. The three oils used were marine, hydraulic and engine lubricating oils.

(All three lubricating oils were supplied courtesy of Shell Ireland)



a) Engine lubricating oil b) Hydraulic lubricating oil c) Marine lubricating oil

Fig. 2.4: Gas chromatograms of lubricating oils

2.3 ANALYTICAL METHODS

For the purpose of monitoring the alteration of component groups and individual compounds during the bioremediation microcosm trials (phase II), it was necessary to develop methods to adequately measure parameters or 'descriptors' for these components. Absolute measurement of components is unnecessary in many instances, particularly as some of the parameters are defined by the analytical methods, (e.g. asphaltenes are defined as the non-hexane soluble fraction). Relative quantification of parameters indicative of certain components can enable assessment by comparison to other components within the oil, and then compared with the control sample and initial sample t_{zero} to detect trends and comparative changes.

Analytical methods are described in appendix A

2.3.1 Gravimetric

The gravimetric assessment of petroleum oils involved a simple dissolution into solvent, removal of water using anhydrous sodium sulfate, and molecular sulfur using activated copper. *n*-Hexane and dichloromethane (DCM) were used as solvents in separate experiments. Hexane does not dissolve asphaltenes while DCM dissolves a broader spectrum of petroleum components. Solutions were dried under nitrogen to obtain a gravimetric result. This is a measure of the proportion of oil recovered after removal of sulfur, water, asphaltenes, detritus and volatile components. Non-soluble material was also dried and weighed. GC analysis was carried out on the reconstituted dried extract. The methodology is described in more detail in appendix A.3. The percentage of each oil recovered gravimetrically after hexane extraction is given in table 2.1.

	Forties crude	***Alba crude***	***Gullfaks crude***	***Marine lube***	***Engine lube***	***Hydraulic lube***
Mean	52.3%	90.2%	73.9%	96.5%	93.9%	93.7%
Standard deviation	3.7	3.2	2.5	2.3	3.7	4.9
Number	7	13	10	10	7	10

Table 2.1: Gravimetic recovery of oil after hexane extraction/dissolution

The asphaltene residues were estimated gravimetrically as 30% (Forties), 3% (Alba), 1% (Gullfaks), 1% (marine lube), 1% (engine lube) and 1%(hydraulic lube). The presence of detritus (i.e. sediment and other insoluble material incorporated in the oil after it was spilt) may exagerate the actual asphaltene values for the Forties oil. The values for the oils indicate that the lubricating oils comprise predominantly of hexane soluble material with low volatile content, asphaltenes and water as indeed does the Alba crude oil. The 24% of Gullfaks crude not recovered is due to water content and loss of the volatile fraction. The 48% of Forties not recovered was due to the high water content, asphaltenes and debris, such as sand, incorporated into the oil after it was spilt.

A number of silica/alumina gel and solvent combinations were investigated for fractionation of the oils into saturates, aromatics and resins/polars. GC-MS and GC-FID on aliquots of eluant was used to ascertain the aliphatic – aromatic – polar cutoff points. The final selected method (described in appendix A.4) was capable of separating the steranes and pentacyclic triterpanes into the saturates portion and aromatics of a molecular weight greater than methylnaphthalenes into the aromatic fraction. Fractionation and gravimetric assessment of the oils yielded the percentage breakdown given in table 2.2, expressed as a percentage of the hexane extracted oil.

	% Saturates	*% Aromatics*	*% Polars*	*% Retained*
Gullfaks	63.6	30.95	3.90	1.51
Alba	52.7	39.36	2.22	5.73
Forties	59.6	30.90	3.19	6.35
Hydraulic	83.3	8.53	0.94	7.25
Marine	76.1	17.27	2.41	4.19
Engine	82.9	10.58	0.76	5.73

Table 2.2: Fractionation of oils expressed as a percentage of hexane extracted oil

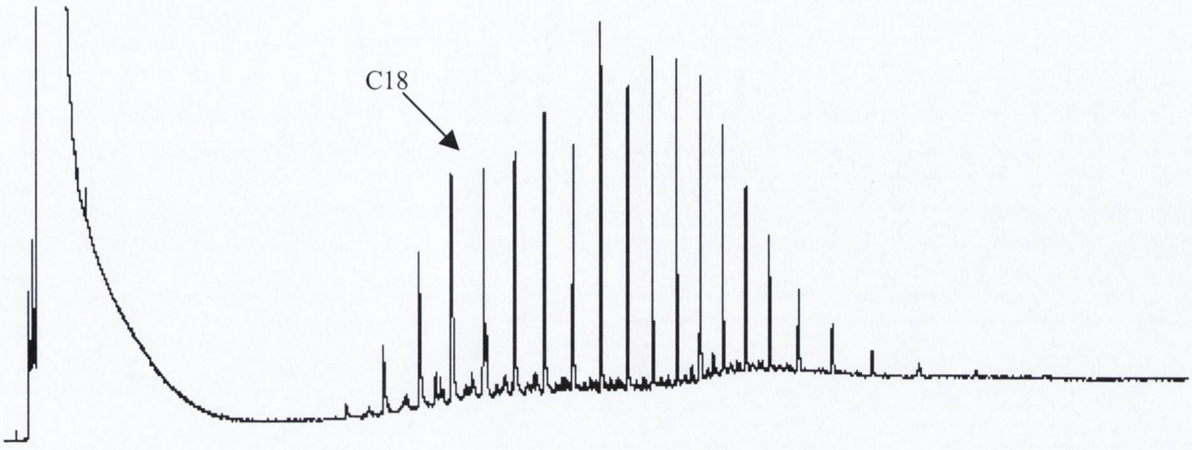
2.3.2 Urea and thiourea adduction

Urea forms a crystal lattice with an internal diameter of 5Å. *n*-Alkyl chains or long *n*-alkyl side chains can be adducted into this lattice but branched components are excluded. Similarly thiourea has a larger lattice (7Å) and is reported to retain cycloalkanes (not tri- and tetracyclics), acyclic alkanes, substituted benzenes but not *n*-alkanes (Killops & Al-Juboori 1990, Nwadinigwe & Nwobodo 1994). The shape of alkyl groups strongly influences adduction. Urea and thiourea adduction experiments on UCM samples containing squalane, tridecylbenzene (TDB) and docosane as internal standards were undertaken (methodology appendix A.5 and A.6). GC analysis of adducts and non-adducts demonstrated squalane and TDB to be retained to some extent in the adduct, as previously reported (Killops & Al-Juboori 1990). The percentage of internal standards that were urea adducted were docosane 96% (SD=4.9), squalane 13% (SD=13) and TDB 53% (SD=18.2) for n=22 (experiments with all of the oils). The percentage of internal standards thiourea adducted was docosane 26.8% (SD=18.5), squalane 39% (SD=31.4) and TDB 24% (SD=10.3) for n=21. The percentage of UCM adducted was calculated based on the total area as measured by GC for adduct and non-adduct. GC analysis shows adducted material to be composed of *n*-alkanes and some UCM over the entire molecular weight range while the bulk of the UCM and resolved isoprenoids are observed in the non-adduct fraction (fig 2.5).

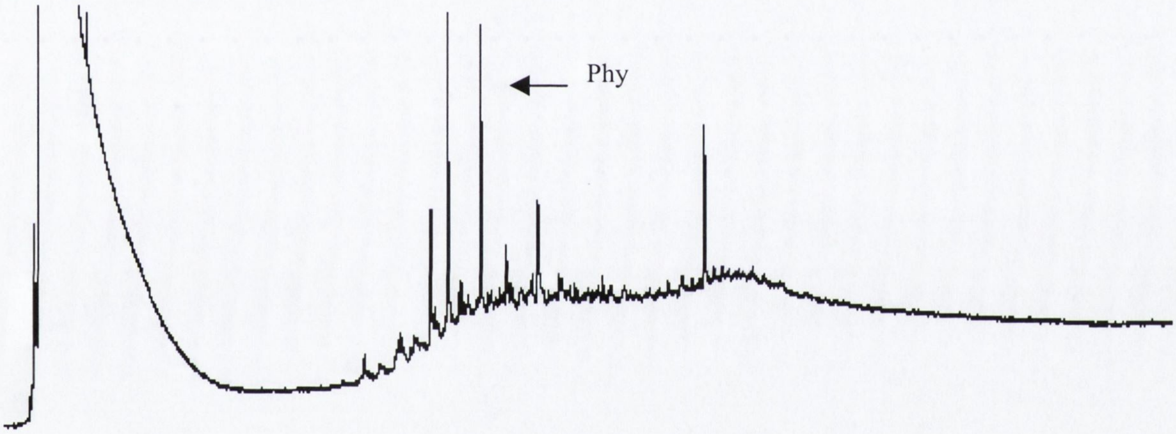
	Urea			**Thiourea**		
Oil	n	% Adducted (mean)	Standard deviation	n	% Adducted (mean)	Standard deviation
Forties	5	32	1.2	3	19	8.6
Alba	2	31	-	3	16	9.2
Gullfaks	2	33	-	4	24	8.7
Hydraulic lube	5	16	6.6	4	23	4.5
Engine lube	2	35	-	4	25	7.0
Marine lube	5	29	13.4	4	29	5.9

Table 2.3: % urea adducted and thiourea adducted material from oils

The technique does not exhibit good reproducibility and care must be taken not to draw many conclusions. All of the oils exhibit a similar proportion of urea adducted material with the exception of the hydraulic lube. This would suggest that they have similar degrees of *n*-alkyl substitution. Also the distribution of squalane and TDB suggest that a significant proportion of the UCM may be due to alkyl benzenes and cycloalkanes with relatively large *n*-alkyl chains. However, it might be expected that the Forties crude oil would exhibit a greater percentage of material adducted than the other oils due to the presence of *n*-alkanes. This was not observed. Also, mass spectrometry suggests that the hydraulic oil is similar in composition to the other lubricating oils making the low percentage included difficult to understand.



Forties crude adducted fraction



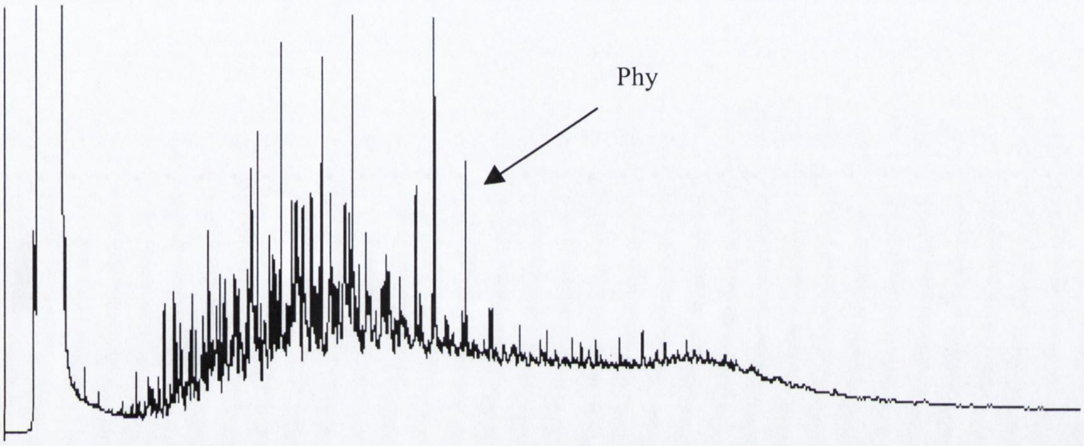
Forties crude urea non-adducted fraction

Fig. 2.5: GC traces of adduct and non-adduct fractions

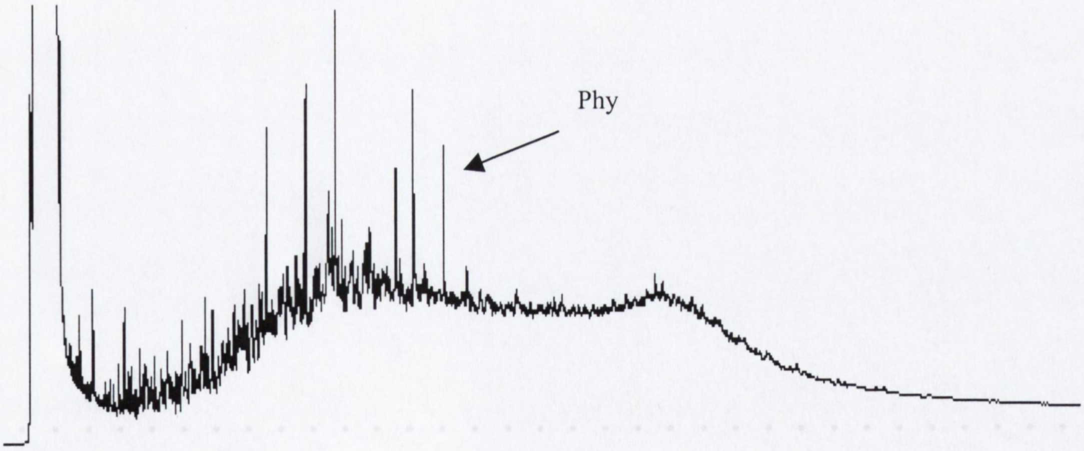
The thiourea adductions would suggest more complex structures than isoprenoids and simple cyclic structures. However, the poor reproducibility of the internal standards distribution and the large standard deviations make the recoveries questionable. Therefore, urea and thiourea adduction were not pursued further as an analytical method for assessing changes in the relative structural composition during the bioremediation trials.

2.3.3 Gas chromatography (GC)

The term 'unresolved complex mixture' (UCM), in effect, derives from gas chromatography analysis of petroleum oils. GC, and in particular capillary GC, enables the separation of complex mixtures of organics based primarily on the boiling point distribution but also based on the chemistry of the components. The analytical methods used for GC analysis of these oils are given in appendix A7. Chromatograms of oil samples dissolved in hexane are displayed in figures 2.1-2.4, and chromatograms of dried oil extracts, displayed in figure 2.6, represent the oils after loss of the lower boiling point components for Gullfaks and Alba crude oils. The dried oils were prepared as described in appendix A.3, i.e. oil was dissolved and centrifuged in hexane and the supernatant dried under a gentle nitrogen stream after removal of water and molecular sulfur. There is no appreciable difference between the chromatograms of the original weathered Forties crude and lubricating oils and the dried samples of the same oils.



Gullfaks (dry oil chromatogram)



Alba (dry oil chromatogram)

Fig. 2.6: Gas chromatograms for nitrogen dried oils (appendix A.3 for methodology)

The Forties crude oil is a weathered oil and lower molecular weight components have been removed, (<*n*C14). Comparison with fresh Forties crude oil indicates that biodegradation has been minimal, with heptadecane:pristane and octadecane:phytane ratios unchanged. A decrease in these ratios is a commonly used indicator of biodegradation as *n*-alkanes are more biodegradable than the isoprenoids. Descriptive data for oils are given in table 2.4. Also included in the table are the upper and lower UCM boundaries of dried oils, in terms of reference *n*-alkane retention. The bimodal distribution of dried crude oil UCM is evident, although most apparent for Forties oil, which also exhibits a bimodal n-alkane distribution (*n*C17 & *n*C24). This is not untypical of weathered oils.

Sample	**Upper and lower UCM boundaries**	**Max. UCM**	**Pristane:Phytane ratio**
Forties crude oil	*n*C13-*n*C35	*n*C20&*n*C30	1.54
Alba crude oil	*n*C13-*n*C35	*n*C19&*n*C30	1.22
Gullfaks crude oil	*n*C13-*n*C35	*n*C19&*n*C30	1.98
Marine lube oil	*n*C20-*n*C36	*n*C30	1.39
Engine lube oil	*n*C13-*n*C35	*n*C25	0.75
Hydraulic lube oil	*n*C13-*n*C35	*n*C26	0.63

Table 2.4: GC parameters. UCM description in terms of n-alkane retention

A good regression ($R^2 = 0.995$), for UCM quantification based on total area versus concentration, was calculated from a 7 point standard curve between 3 and 300 mg/g for Forties crude oil in hexane. This demonstrated the linearity across a wide concentration range.

To quantitatively ascertain whether the UCM profile is changing, the UCM chromatogram can be sub-divided into defined sections and the areas measured. Division of UCM into measurable *n*-alkane to *n*-alkane +1 segments based on the retention times of *n*-alkanes in a standard oil analysed with each sample batch enabled the UCM to be described in quantitative terms based on boiling point distribution. An example bar chart from such an exercise is given in figure 2.7 and although resembling the UCM profile it does give a quantitative picture of the boiling point/molecular weight distribution of the oils that is suitable for quantitatively assessing the change in profile of UCM with biodegradation.

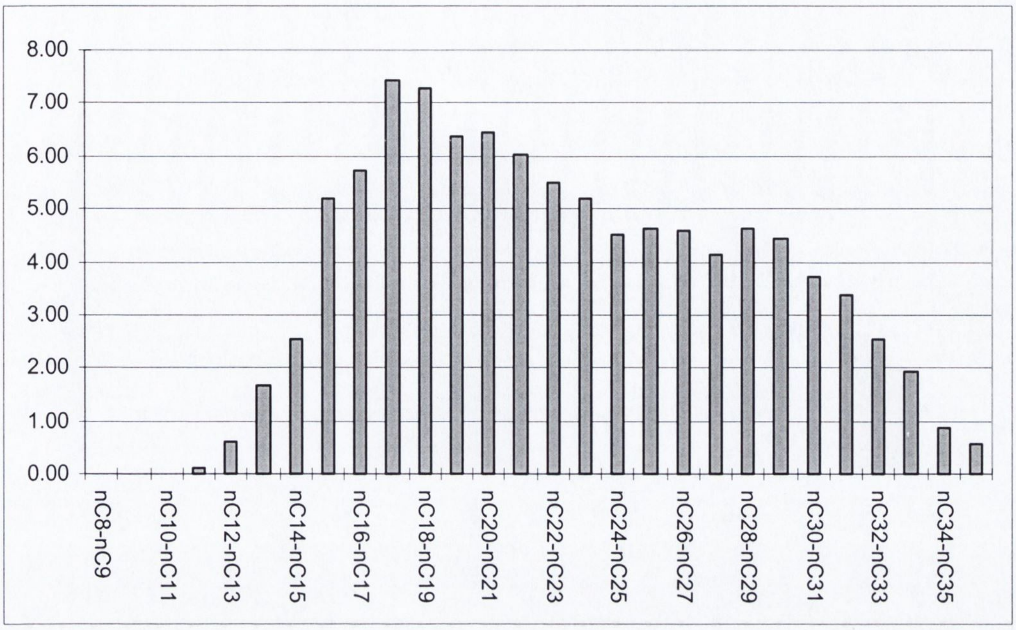


Fig. 2.7: UCM area 'segments' between n-alkanes (Peak ends) as percentage of the total area. Measured by GC-FID for Forties crude oil{Baseline subtracted & area excluding n-alkanes}

2.3.4 Spectroscopic

Fluorescence (UVF) spectroscopy

Fluorescence spectroscopy has been used as a method for measurement of total hydrocarbon contamination in the environment and also for fingerprinting of petroleum oils (Butt 1986, Law *et al.* 1988, Ehrhardt *et al.* 1991). Fixed wavelength (excitation and emission), synchronous scanning and total fluorescence spectroscopy have been employed for oil analysis.

Aromatic compounds strongly fluoresce at excitation wavelengths in the ultra-violet /visible spectrum while the aliphatic components of oil do not. Therefore, fluorescence spectroscopy of petroleum oil is based on the aromatic fraction of the oil. Thus, a requirement for the accurate use of fluorescence spectroscopy in quantification of hydrocarbons in environmental samples is that the reference oil should have a similar chemistry to the analyte oil, especially in terms of aromatic concentration and composition.

Total fluorescence spectra of the oils were obtained using a computer linked Perkin Elmer LS30 instrument and displayed as contour plots (Methodology: appendix A.9). Total fluorescence spectra were also obtained for a number of aromatic hydrocarbons to determine how the number of condensed aromatic rings and degree of substitution of rings effected the region of maximum excitation/emission. A BASIC™ programme was written to control the LS30 spectrometer and collect data through an RS232 interface. Data acquisition was acquired by automatic sequential scans through the basic programme. This involves fixing excitation wavelengths and acquiring emission intensities while scanning emission wavelengths. After each scan the excitation wavelength is automatically increased by a fixed interval and a scan of emission wavelengths again carried out, albeit using a higher initial emission wavelength. This was continued until the maximum excitation wavelength was reached. The combined scans are collected as one ASCII text file organised as three columns of data; excitation wavelength, emission wavelength and emission intensity. Surfer™, (*Golden Software* – an off-the-shelf software package) was used to plot contours. Considerable effort was undertaken to demonstrate the validity of the above data acquisition process. This was achieved by comparison of emission intensities of a sample at a number of fixed excitation and emission wavelengths, for direct measurements from the instrument and equivalent datapoints as recorded in the ASCII files acquired. Also fixed excitation/scanned emission spectra were visually compared for direct instrument printouts and the same data as recorded on the ASCII files and plotted using EXCEL. The results indicated that data collected in ASCII format was a valid representation of the spectrometer output.

The total fluorescence spectra for the 6 oil samples are given in figure 2.8. and selected aromatic compounds in figure 2.9. A gradual increase in the excitation and emission wavelengths that correspond to emission intensity maxima for compounds with increasing ring number is evident. The 5-ring perylene exhibiting a noticeably higher excitation and emission wavelength for the intensity maximum than the 4-ring chrysene. The three crude oils demonstrate similar patterns as do the three lubricating oils, but the lubricating oils are markedly different from the crude oil spectra. Each of the three crude oils has a maxima at approximately Exλ = 230nm and Emλ = 338-342nm with a shoulder peak at Exλ = 270nm and Emλ = 360-370nm. Comparison with the spectra for the individual compounds suggest that the bulk of the emission is produced by substituted 2- and 3-ring aromatic compounds. The ratio of the intensity of the two maxima (i.e. the maxima and the shoulder peak) is similar for each oil, suggesting a similar distribution of 2- and 3-ring aromatics.

The spectra for the lubricating oils exhibited the main maxima at Exλ = 200nm and Emλ = 290 – 295 nm, and smaller maxima at Exλ = 240 nm, Emλ = 338-340nm.The latter would suggest 2-ring aromatics and the larger maxima is probably due to monoaromatic components. There is no evidence of significant 3- ring or higher aromatics in the lubricating oils although it would appear that that the marine lubricating oil has a higher aromatic content than the engine or hydraulic lubricating oils.

Fixed wavelength fluorescence spectroscopy was carried out using Alba as a calibrant. The wavelengths selected for this purpose corresponded to the maxima observed for the total fluorescence spectra of the crude oils. 7-point standard curves for emission intensity vs. Alba crude oil concentration for Exλ = 230 nm, Emλ = 340 nm and Exλ = 270 nm, Emλ = 365 nm show good linearity with regressions of 0.9999 in the concentration range 0.1 – 7.0 μg/g *n*-hexane. It is noted that the former wavelength produces a standard curve largely based on the presence of 2- ring aromatics while the latter is probably based primarily on 3-ring aromatics.

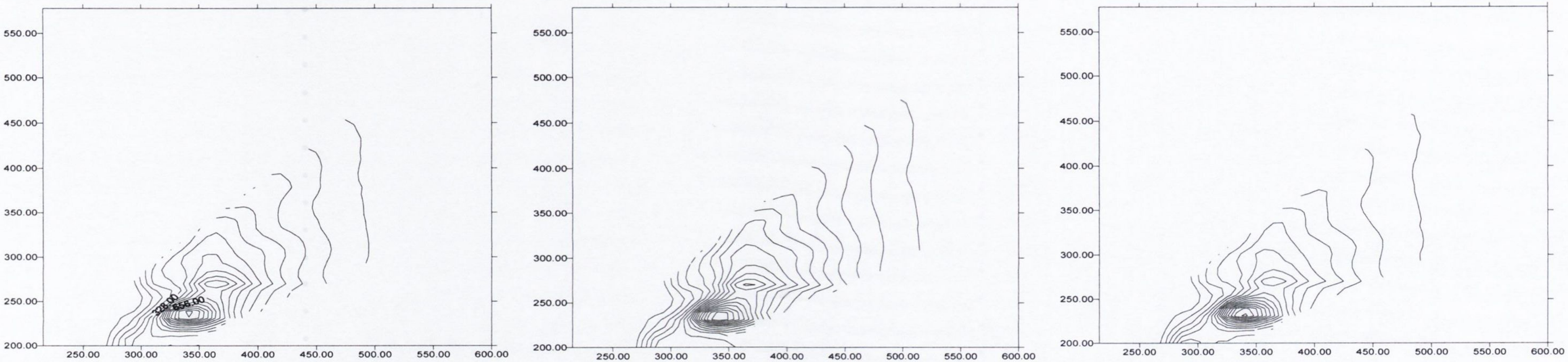
Quantitatively analysing the other oils at these wavelengths, using Alba as calibrant indicated very low concentrations of 2- and 3-ring aromatics in hydraulic and engine lube oils, with slightly higher levels in marine lube oil. The levels of 2- and 3-ring aromatics in Forties oil were about half that in Alba and Gullfaks had similar levels of 2-ring but less 3-ring aromatics than Alba.

A simple method for assessing the change in the aromatic composition of the oils over time is to measure the ratio of emission intensities for the total fluorescence maxima, (Exλ = 230 nm, Emλ = 340 nm and Exλ = 270 nm, Emλ = 365 nm). This is given in table 2.5. This ratio is an indication of the 2- ring : 3-/4- ring prevalence in an oil. Experiments showed that this ratio is independent of the concentration of the oil sample within the linear range. These ratios also compared well to ratios taken from total luminescence contour plots at the given wavelengths.

Oil	**Ratio (I270 / I230)**	
Alba	0.825 (0.014)	n=7
Forties	0.715 (0.021)	n=5
Gullfaks	0.569 (0.012)	n=5
Marine	0.558 (0.020)	n=5
Hydraulic	0.133 (0.010)	n=7
Engine	0.162 (0.011)	n=6

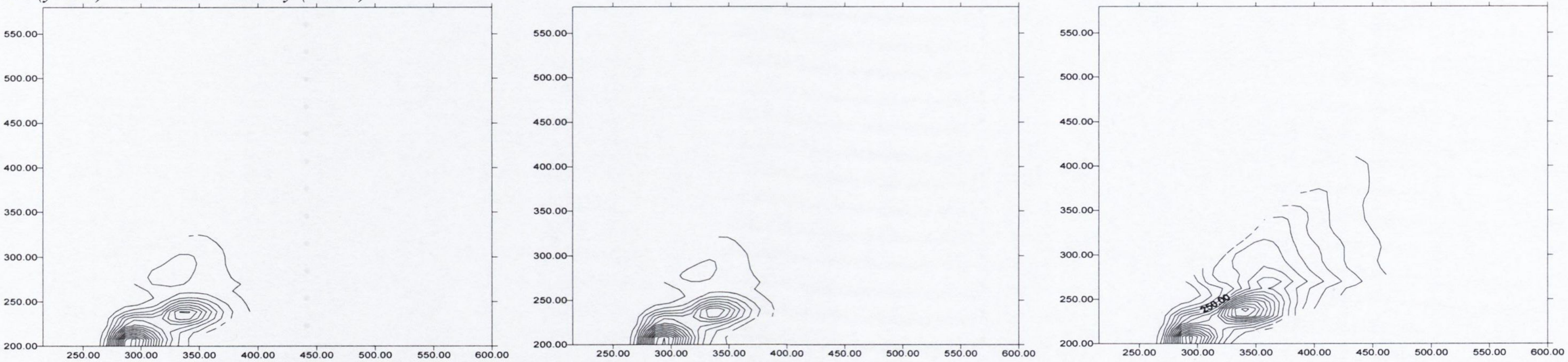
Table 2.5: Ratio of fluorescence intensity at Exλ=270nm, Emλ=340nm and Exλ=230nm, Emλ= 365nm –expressed as mean & (standard deviation) of the ratio

For phase II analysis, the intended use of fluorescence spectroscopy, based on these tests, was to obtain contour plots as descriptors of aromatic profile. The ratios of the intensity of the two maxima (for Exλ = 230 nm, Emλ = 340 nm and Exλ = 270 nm, Emλ = 365 nm) were to be recorded as an indication of the change of the relative concentrations of 2- to 3-\4- ring aromatics in the system.



Forties Alba Gullfaks

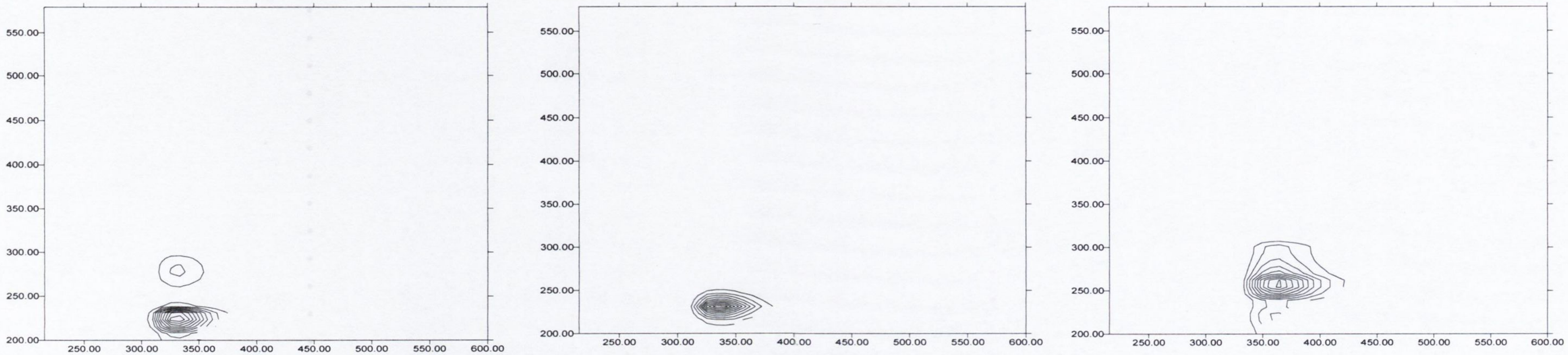
Total fluorescence spectra of three crude oils. Contour plots are normalised to display 16 contour line levels. Plot emission wavelength nm (x-axis) vs. excitation wavelength nm (y-axis) vs emission intensity (z-axis)



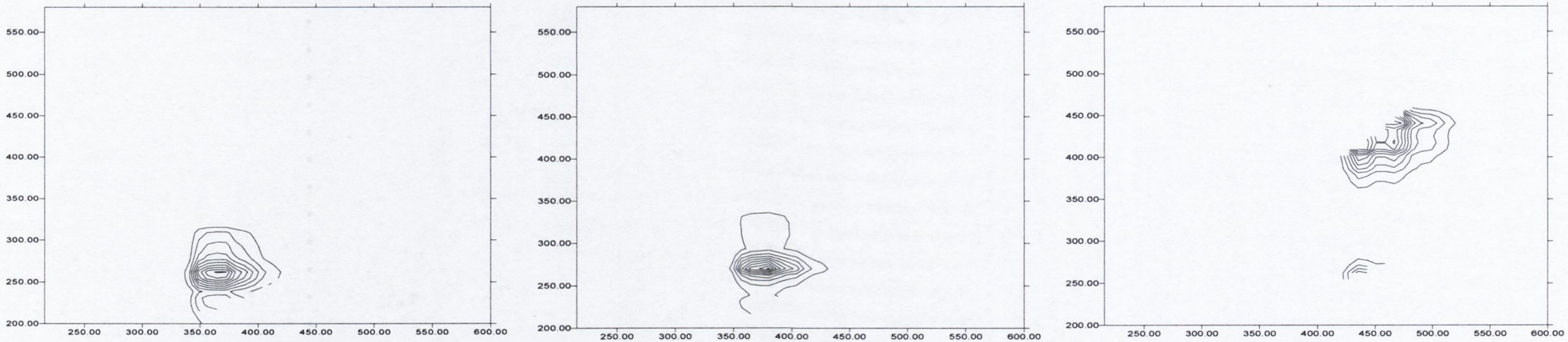
Engine lube Hydraulic lube Marine lube

Total fluorescence spectra of three lubricating oils. (Plot details as for crude oils)

Fig. 2.8: Total fluorescence spectra for 6 oils



Naphthalene Dimethylnaphthalene Phenanthrene



Dimethylphenanthrene Chrysene Perylene

Fig. 2.9: Total fluorescence spectra of 6 PAH compounds. Contour plots are normalised to display 16 contour line levels. Plot emission wavelength nm (x-axis) vs. excitation wavelength nm (y-axis) vs emission intensity (z-axis)

Infrared (IR) spectroscopy

Quantification of petroleum hydrocarbons by IR spectroscopy is similar in principle to quantification by fluorescence spectroscopy, except that the quantification is based on the aliphatic portion of the oil rather than the aromatic portion. IR spectroscopy provides a common standard method for determination of petroleum hydrocarbons in the environment and several national and international agencies recommend a standard method based on IR (USEPA 1983). An aliphatic carbon – hydrogen bond (C-H) stretch results in the absorbance of infra-red radiation at 2930cm^{-1} . Since most oils consist primarily of aliphatic hydrocarbons the aliphatic C-H is the predominant molecular bond. The absorption of IR radiation obeys the Beer-Lambert law and thus can be linearly related to concentration of the analyte and also to the path length. Thus, measuring absorbance for C-H stretch and comparison against a standard curve, prepared from a suitable reference oil or mixture of compounds, enables quantification based predominantly on the aliphatic component of the oil. A prerequisite is that solvents used must contain no C-H bonds.

The ASTM 'Standard Test Method for Oil and Grease and Petroleum Hydrocarbons in Water', (method 3921 – 96) (ASTM 1996), was assessed as a basis for an IR quantification. As with other standard IR based hydrocarbon quantifications the recommended solvent is CFC 113, a solvent no longer readily available. A suitable alternative solvent was necessary for this analysis. Perchloroethylene, (tetrachloroethane, PCE), was tested as an alternative and it was found to absorb to some extent at the ~3000cm^{-1} region. With a double beam instrument and using a reference cell, this could be adequately blanked. Oil samples were diluted in PCE and anhydrous sodium sulfate used to remove water. Silica gel was used to remove polar components which may cause interference. Serial dilutions of Alba were prepared in PCE to assess the linear working range of the technique on the instrument. The solutions were placed in 10 mm quartz cuvettes and the absorbance scanned from 3300cm^{-1} to 2700cm^{-1} (over the range of aliphatic C-H stretch absorbance). The peak height at 2960cm^{-1} was measured and a linear regression of R^2=0.985 was determined for a 5 point standard curve in the concentration range of 4 – 101 ug.g^{-1} PCE. Based on the literature study, a number of other wavelengths in the same region were considered, i.e. 2960cm^{-1}, 2880cm^{-1}, 2860cm^{-1}. The 2960cm^{-1} was the most sensitive and best corresponded to aliphatic stretch. The response of the other five oils relative to Alba highlights the bias due to the different proportions of aliphatic components, (table 2.6). However, this bias is not as great as for the fluorescence method as the aliphatic content of the oils accounts for a significantly higher proportion than the aromatic content. The lowest response when related to Alba, is for Forties oil but this may be due to the higher water content and the presence of detritus.

Absorbance cm^{-1}

Oil	**2960**	**2930**	**2880**	**2860**	**2960 +2930 +2860**
Gullfaks	1.03 (.15) n=5	0.94(.12) n=5	0.95(.13) n=5	0.95(.12) n=5	0.94(.13) n=5
Forties	0.49(.06) n=5	0.75 (.10) n=5	0.69(.12) n=5	0.82(.13) n=5	0.66(.10) n=5
Marine	0.93(.11) n=3	1.05(.18) n=3	0.90(.15) n=3	1.11(.16)n=3	1.00(.20) n=3
Hydraulic	0.94(.16) n=4	1.04(.11) n=4	1.01(.22) n=4	1.13(.10) n=4	1.02(.12) n=4
Engine	0.93 (.07) n=4	1.01(.06) n=4	1.01(.15) n=4	1.22(.14) n=4	0.96(.06) n=4

Table 2.6 : IR response of oils relative to Alba crude oil

Mean (Standard deviation) n=sample number

The 'Standard Test Method for Comparison of Waterborne Crude Oils by Infrared Spectroscopy' (ASTM method D3414 – 80) (ASTM 1991a), was used as the basis for a qualitative examination of the oils to discern differences. Droplets of oil in solvent solutions were allowed to air dry onto NaCl windows and a full scan, $4000cm^{-1}$ – $600cm^{-1}$, was recorded to computer using an analogue to digital converter (12 bit resolution). The main peaks when examined (excluding the $\sim 3000cm^{-1}$ region) were at $\sim 1377cm^{-1}$, $1458cm^{-1}$, and smaller peaks at $1604cm^{-1}$, $1708cm^{-1}$, and peaks in the 'oil fingerprint' region of $700cm^{-1}$ to $900cm^{-1}$. The $1708cm^{-1}$ carbonyl peak may increase with oxidative weathering. However, there are few reproducible spectral features that distinguish the oils and give an insight into their different compositions.

2.3.5 High performance liquid chromatography (HPLC)

Normal phase HPLC methods have been used to class fractionate petroleum hydrocarbons into saturates, aromatics, resins and asphaltenes, (SARA), and often for further fractionation of aromatic fractions based on ring size (Lundanes & Greibrokk 1994). Asphaltenes are measured gravimetrically prior to HPLC. The method developed in this experiment used an amino (NH_2) and a cyano (CN) column in series with a back-flushing configuration. *n*-Hexane followed by an *n*-hexane:DCM gradient was used as the mobile phase to elute the saturate and aromatic fractions and back flushing with 100% DCM was used to elute the polar compounds (methodology appendix A.8).

A suitable detector was not available for direct quantification of saturates. Using UV detection at 265nm, a range of aromatic standards was injected to ascertain the elution order of different ring number compounds. Substituted compounds elute prior to the parent aromatics. Perylene was used as an internal standard to enable a relative quantification approximately based on ring number and an Alba crude oil sample was run with each batch of samples. Oil sample extracts were dried under N_2 and reconstituted prior to analysis. The chromatograms were divided into segments based very approximately on ring number, using retention time of reference parent PAH compounds as the point of division between segments. Measurement of the reduction of a 'ring-number' component over the period of the bioremediation trials can be achieved by relating the values to a reference oil or internal standard. Table 2.7 presents the aromatic distribution based on ring number as a percentage of equivalent Alba oil concentration. This does not relate to absolute concentrations as different compounds have different detector responses.

	Mono-aromatics	*Di-aromatics*	*Tri-aromatics*	*>Tri-aromatics*	*Resins*	*Total*
Gullfaks	0.948	1.015	0.990	0.756	0.685	0.890
Forties	0.432	0.642	0.908	0.785	0.688	0.792
Engine	0.852	0.271	0.039	0.016	0.014	0.113
Hydraulic	0.879	0.269	0.38	0.007	0.002	0.111
Marine	1.638	0.435	0.095	0.075	0.073	0.226

Table 2.7: Aromatic component distribution for oil samples relative to Alba reference oil

A 5-point phenanthrene standard curve based on peak area within the range of 16-399 $\mu g.g^{-1}$ *n*-hexane gave a linear regression coefficient of 0.9991 and a 4-point naphthalene standard curve within the range of 44-391 $\mu g.g^{-1}$ *n*-hexane gave a linear regression coefficient of 0.9999.

The aromatic content of the lubricating oils was shown to be predominantly monoaromatic. The crude oils show primarily three and four ring components, although this is to some extent a function of differing detector sensitivities to different components. The aromatic distribution is similar for the lubricating oils.

The role of HPLC in phase II was to monitor the biodegradation of aromatic UCM in the bioremediation trials.

2.3.6 Mass Spectrometry

Mass spectrometric analysis was carried out on a VG (Micromass) TRIO 2000 quadrupole instrument. Solid insertion probe analysis was used in positive electron ionisation mode (EI+) and gas chromatography – mass spectrometry (GC-MS) was carried out in positive electron (EI+) and chemical ionisation (CI+) modes. Data acquisition in total ion current scan (TIC) mode and selected ion recording (SIR) mode was used.

Solid insertion probe analysis was carried out on all of the oils in full scan mode to obtain mass spectra of the oils. However, practical difficulties resulted in poor quality spectra. The combination of separation and molecular structural data obtained using gas chromatography-mass spectrometry analysis in EI+ and also in CI+ modes provided a powerful technique for quantitative and qualitative analysis of UCM components.

GC-MS (TIC) chromatograms are similar to those obtained using GC-FID but structural information is obtained for resolved peaks. EI+ spectra produce generic spectra for many compounds such as alkanes, due to extensive fragmentation. The combination of retention time, EI+ spectra and methane-CI+ spectra (which is a softer technique and induces less fragmentation than EI+) enabled tentative peak assignations (appendix B).

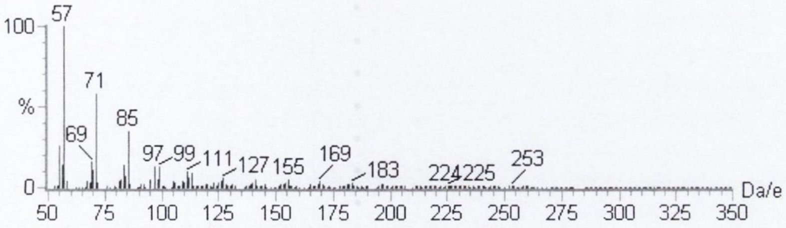
Total spectra were obtained for these oils by combining the spectra for the total UCM and subtracting a combined baseline spectrum from the same chromatogram (fig 2.10). *n*-Alkanes and isoalkanes (*i*-alkanes*)* fragment readily with electron ionisation and reveal a similar fragmentation pattern (Rose & Johnstone 1982, Smith & Busch 1999). The primary fragments are the series C_nH_{2n+1} (i.e. 57, 71, 85 etc.) which dominate their spectra. Cycloalkane spectra are similar to those of *n*-alkanes/*i*-alkanes but the principal fragment series corresponds to $C_nH_{2n+1-2r}$ where r=number of rings, (i.e. 55, 69, 83, 97 for monocyclic alkanes) and the molecular ions are more abundant. The cyclohexane fragmentation pattern is similar to that obtained for mono-alkenes but although often present in oils containing cracked products, alkenes are not significantly present in crude oils (Tissot & Welte 1978, Wrang & Adamsen 1990a).

The total oil spectra show fragments indicative of the relative composition of the oils. Fragments associated with *n*-alkanes/*i*-alkanes (m/z 57, 71, 85, 99…), substituted cyclohexanes (m/z 83), substituted methylcyclohexanes (m/z 97), dicyclic alkanes (m/z137), substituted benzenes (m/z 91) and substituted methylbenzenes (m/z 105) are apparent at varying abundances in each oil (Killops & Al-Juboori 1990, Peters & Moldowan 1993). Figure 2.11 shows ratios of the primary peaks from the series enabling comparisons of the relative hydrocarbon class composition between the different oils. These ratios are not measurements of the actual proportion of component classes, such as alkanes:cyclohexanes. However, they serve as indicators, and changes in the proportions of component classes will be reflected as changes in ion abundance ratios.

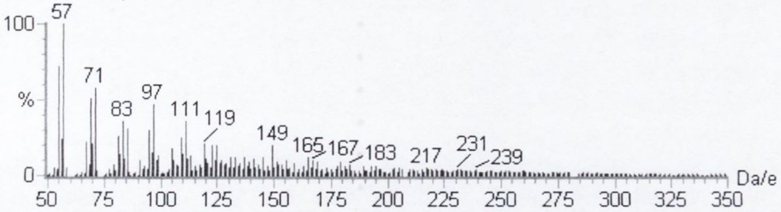
With the exception of the weathered Forties crude oil the total spectra for the oils all reveal a significant proportion of monocyclic alkanes and the m/z 97 abundance suggests substituted methylcyclohexanes to be a significant component of all the UCMs. The spectra of the weathered Forties crude oil is dominated by the C_nH_{2n+2} series, indicative of the *n*-alkanes present. However, the spectra of the biodegraded Forties oil after removal of the *n*-alkanes is typical of the other crude oils although the degree of cyclicisation in the UCM appears to be less than for Alba or Gullfaks. The Gullfaks crude oil also contains a greater relative proportion of alkylated benzenes and methylbenzenes as demonstrated by the m/z 91 and 105 ion abundances. It is believed that monocyclic compounds in petroleum often contain methyl groups on the ring as well as a longer alkyl substitution (Hood & O'Neal 1959). 141 and 155 m/z fragments, evident in Alba and especially Gullfaks, are indicative of 2-ring aromatics (substituted naphthalenes). Fragments indicative of 2-ring saturated components are also evident.

The three lubricating oils are all very similar in composition in terms of cycloalkanes: *n*-/*i*-alkanes. It should be noted that alkenes may be present in the lubricating oils as they are refined products and may contain some cracked product. The ratios of m/z 83:97 are similar for all suggesting they have a similar proportion of cyclohexane to methylcyclohexane based components. The spectra indicate the presence of substituted cyclohexanes and methylcyclohexanes, and bicyclic saturates. However, fragments due to aromatics, including alkylbenzenes are not a significant contributor to the spectrum.

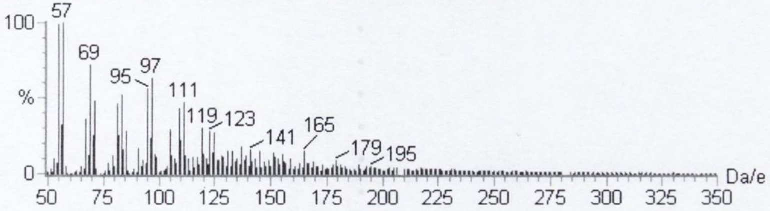
For ratios calculated for Alba crude oil, 9 repeat analyses gave relative standard deviations of between 1.4% (69/71) and 13.1% (97/83) as presented in table 2.8.



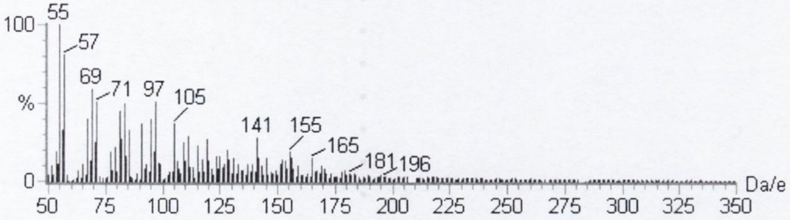
a) Weathered Forties crude oil mass spectrum



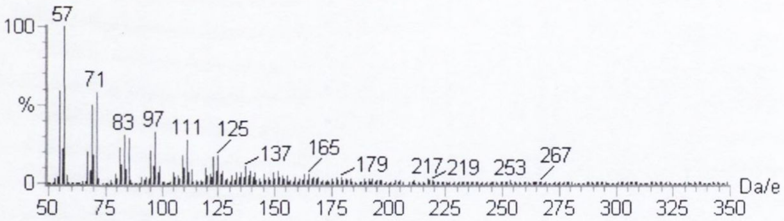
b) Biodegraded Forties crude oil mass spectrum



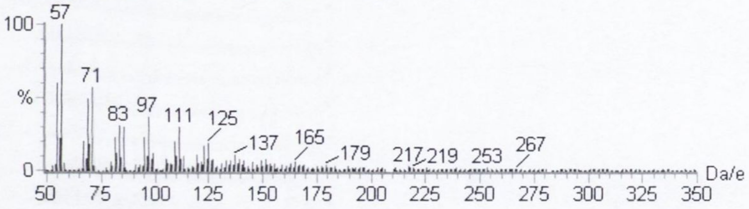
c) Alba crude oil mass spectrum



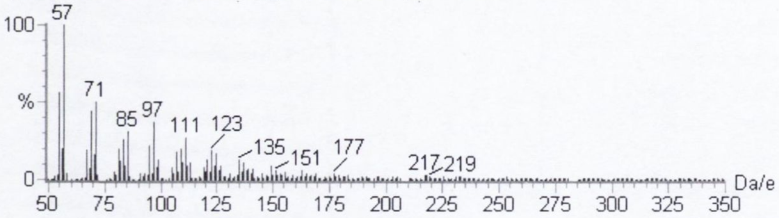
d) Gullfaks crude oil mass spectrum



e) Engine lubricating oil mass spectrum



f) Hydraulic lubricating oil mass spectrum



g) Marine lubricating oil mass spectrum

Fig. 2.10: Mass spectra for crude oils and lubricating oils (Combined spectra over entire UCM and combined baseline subtracted)

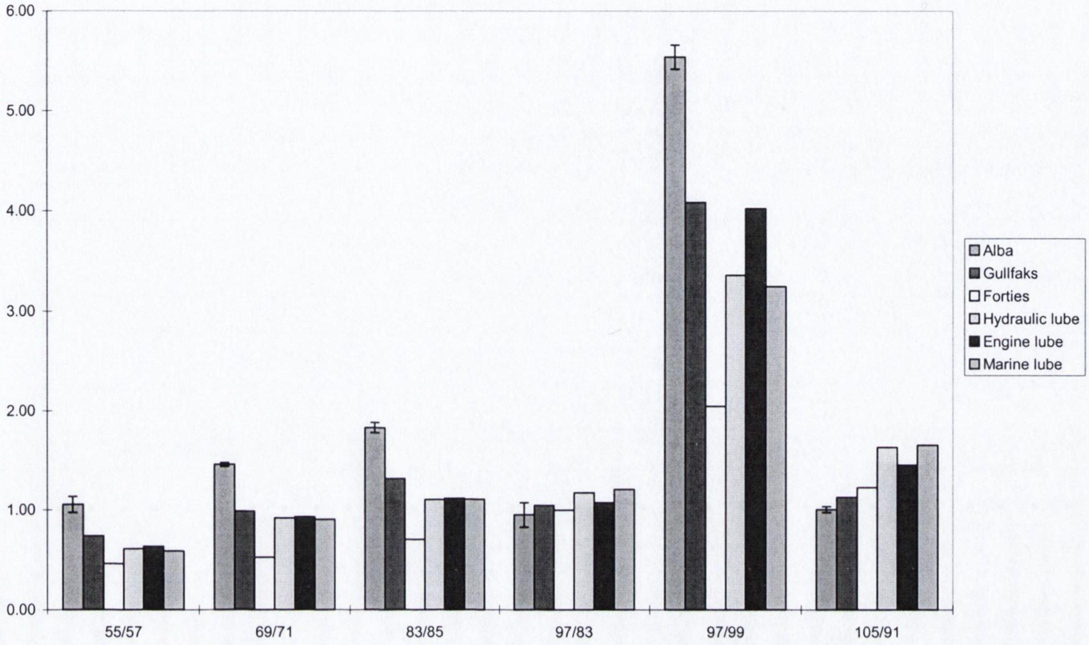


Fig. 2.11: Ion abundance ratios from EI+ mass spectra of dried oils (all results for a mean of 2 samples except Alba which is for a mean of 9 samples. Error bars for Alba represent ±1 standard deviation)

	Ion abundance ratios mz/mz								
	55/57	**69/71**	***83/85***	**97/83**	**99/97**	**95/97**	**105/91**	**99/91**	**99/105**
ALBA1	1.00	1.45	1.79	1.03	0.18	0.96	1.09	0.59	1.01
ALBA2	0.99	1.46	1.79	1.08	0.18	0.95	1.10	0.61	1.01
ALBA3	0.99	1.46	1.78	1.06	0.18	0.96	1.09	0.60	0.93
ALBA4	1.00	1.47	1.79	1.07	0.18	0.96	1.10	0.60	0.97
ALBA5	1.07	1.45	1.83	0.94	0.18	0.98	0.98	0.54	1.02
ALBA6	1.07	1.50	1.92	0.76	0.18	1.02	0.91	0.47	0.77
ALBA7	1.15	1.43	1.86	0.86	0.18	1.00	0.91	0.49	0.94
ALBA8	1.17	1.45	1.88	0.82	0.17	1.02	0.86	0.45	0.87
ALBA9	1.18	1.44	1.89	0.84	0.17	1.02	0.88	0.46	0.89
Average	*1.07*	*1.46*	*1.84*	*0.94*	*0.18*	*0.98*	*0.99*	*0.53*	*0.93*
Std dev	*0.08*	*0.02*	*0.05*	*0.12*	*0.00*	*0.03*	*0.10*	*0.07*	*0.08*
RSD	*7.6%*	*1.4%*	*2.8%*	*13.1%*	*2.1%*	*2.9%*	*10.5%*	*12.3%*	*8.8%*

Table 2.8 : Total ion abundance ratios for repeated alba oil analysis by GC-MS.

ASTM D2786-91 (ASTM 1991b) provides a method for quantifying the composition of petroleum saturates and monoaromatics from mass spectra fragmentation patterns, using the method devised by Hood and O'Neal (1959). This method employs inverse matrices to calculate the percentage of 0-6 ring saturates and monoaromatics. Using combined spectra for the lubricating oils and applying these matrices suggests the lubricating oil composition given in table 2.9, assuming an average molecular size ~C24 and applying a deisotoping procedure to account for the effect of heavy isotopes. Results presented are the mean of two GC-MS analyses in the same sequence, although the duplicate analyses agree very well. These calculations are based on matrix tables assuming that isoalkanes are the dominant acyclic components and using an average carbon number of 24 for hydraulic and engine oils and 28 for the marine oil. This information was estimated from GC data. There is in fact little difference between the estimation of aliphatic composition for these three oils.

	Marine	**Hydraulic**	**Engine**
Isoalkanes	30%	31%	31%
1-ring	20%	20%	20%
2-ring	15%	15%	14%
3-ring	12%	9%	10%
4-ring	10%	12%	13%
5-ring	2%	2%	2%
6-ring	1%	1%	1%
monoaromatics	11%	9%	9%

Table 2.9: Composition of the lubricating oils calculated from mass spectra (based on ASTM method D2786-91)

An alternative method of examining the oil to identify components within the UCM, is to select certain ions indicative of given components and plot a mass chromatogram for these ions. Alba mass chromatograms are given in appendix B and homologous series of substituted cyclohexanes, substituted methyl cyclohexanes, substituted methylbenzenes are apparent. The m/z 83 homologous series at suggest linear alkylcylcohexanes while the m/z 97 shows an homologous series of peak groups, suggestive of different positions of methylation in substituted methylcyclohexanes. The tentative peak assignations are given on the basis of retention times and EI+ and CI+ spectra.

Using techniques based on oil fingerprinting (Anon. 1991a, Peters & Moldowan 1993, ASTM 1995). GC-MS with selected ion recording was used to examine two distinct sets of compounds within the UCM. The distribution of substituted polyaromatic compounds was analysed for all oil samples by selecting the molecular ions and plotting mass chromatograms, (Alba oil – appendix B). The three crude oils contained significant substituted naphthalenes, phenanthrenes and dibenzothiophenes. The lubricating oils as expected contained only trace quantities.

The other group of components used in GC-MS fingerprinting techniques are often referred to as biomarkers as they have a clear structural relationship to the biological molecule from which they derive. The isoprenoids could be classed as biomarkers, but for this purpose the larger cycloalkane based structures, such as steranes and pentacyclic triterpanes, were used. Steranes yield a common molecular fragment at m/z 217 in EI+ MS and a chromatogram or 'fragmentogram' at m/z 217is useful for sterane analysis. Fragmentograms were also obtained for monoaromatic steranes (m/z 253), triaromatic steranes (m/z 231), hopanes (m/z 191), methyl hopanes (m/z 205), and norhopanes (m/z 177). Selected fragmentograms for Alba are displayed in appendix B.

The biomarker patterns are similar for the crude oils. This is not surprising as all three crude oils have a North Sea origin. Triaromatic steranes were not detected in the lubricating oils but the steranes and pentacyclic triterpanes were particularly abundant.

Biomarkers exhibit a relative biological recalcitrance. Due to this, 17 α(H), 21β(H)-hopane (C30-hopane) has been used as a conserved internal standard in the assessment of biodegradation (Prince *et al.* 1994b). However, even with this normalisation technique, when samples such as biodegraded oil and control/ reference, or spilled oil and suspect source, are to be compared they must be analysed in the same batch due to the inherent variability of GC-MS. The LIFE funded EUROCRUDE project developed a technique for comparing spilled crude oils with a database of GC-MS data (Anon. 1995). This enables biomarker and PAH data to be compared with oils collected at different times, on different instruments, and in different laboratories. This technique involves analysing an oil sample in duplicate bracketed by a reference crude oil, (in this case Brent crude). The sample data (peak areas for up to 56 biomarkers and substituted PAH) are normalised against the C30-hopane and then against the C30-hopane normalised reference oil before comparison with the database. The database contains data collected and normalised in a similar format, i.e. Brent crude bracketed and C30-hopane normalised. To ascertain the effectiveness of this technique the Forties, Gullfaks and Alba crude oils were analysed using the EUROCRUDE system. The technique of reference oil bracketing improved the comparability of data acquired in different batches.

2.3.7 Other methods evaluated

Column chromatography fractionation of the oils using Sephadex LH-20 gel proved unsuitable with high variability. Silica gel thin layer chromatography also proved unsuitable.

An in-depth literature review indicated that chromic acid oxidation would be unsuitable for monitoring over the course of the bioremediation trials due to the complexity of products and the complexity of target UCM.

2.4 CONCLUSIONS

Six UCM-rich oils were acquired and characterised. They were also used for developing, evaluating, and familiarising with analytical methods for use in the bioremediation trials (phase II). UCM composition of three crude oils were similar with isoalkanes, saturated mono- and polycyclic, and mono- and polyaromatics present. Forties weathered oil consisted of *n*-alkanes as well as a broad UCM. The crude oil UCMs exhibited a bimodal distribution when analysed by GC. The Alba oil contained proportionally more higher molecular weight aromatics than the other oils. Lubricating oils, though containing saturated cyclic components, contained little aromatics and that was predominantly monoaromatics.

Alba and weathered Forties crude oils were selected for use as target oils in phase II of the project. Alba presents a broad range of UCM components of varied polarity and molecular weight. Weathered Forties crude contains *n*-alkanes as well as a substantial UCM. The biodegradation of UCM in crude oils, one with and one without *n*-alkanes present, was considered to be of interest.

A number of analytical methods refined in this phase of the project were applied in subsequent monitoring of the biodegradation trials. In particular GC, GC-MS (EI+ TIC and SIR), spectroscopy, gravimetry and HPLC methods were used, based on the evaluations carried out. Urea and thiourea adductions, TLC, size exclusion chromatography, chemical ionisation mass spectrometry, chromic acid oxidation were not used in phase II.

3. INOCULA DEVELOPMENT AND EVALUATION (PHASE 1B)

3.1 BACKGROUND

This section of the project was carried out in parallel with characterisation of the oils /analytical method development. Respirometry trials, microbiological testing and preparation of the inocula was carried out by Dr. Barry Kiely's team under contract at BioResearch Ireland - National Food Biotechnology Centre (NFBC), University College Cork where the necessary equipment and facilities are available. Analytical chemistry was undertaken at the Marine Institute's Fisheries Research Centre laboratories.

Objectives

The objectives of this part of the project were:

1. To develop a consortium of selected marine micro-organisms which can demonstrate UCM biodegradative capacity.
2. To produce an acclimated inoculum based on this microbial consortia for use in the bioremediation trials (Treatment 1: see phase II).
3. To produce an inoculum based on the indigenous population from a marine sediment to act as a 'booster' of indigenous microbes during the bioremediation trials (Treatment 2: see phase II).

These objectives were achieved as follows:

- Culture microbial consortia from marine sediments that have been exposed to hydrocarbons.
- Selective enrichment of the consortia on UCM.
- Preliminary evaluation of the capacity of consortia and individual microbial strains isolated from the consortia for UCM biodegradation by respirometry.
- Identification of a suitable rapid growth media for production of the inocula for use in bioremediation trials.
- Production of developed inocula in sufficient quantity for bioremediation trials *(Treatment 1).*

- Production of non-specific ‘booster’ microbial population from natural marine sediment microflora for use in bioremediation trials (*Treatment 2*).
- Preliminary investigation of the effect of biodegradation on UCM in laboratory flask conditions.

3.2 ENRICHMENT OF MICROBIAL CONSORTIA FROM MARINE SEDIMENTS

The possibility of selecting and using a commercial microbial based bioremediation product as an inocula for bioremediation was considered. Many are readily available on the market, particularly for hydrocarbon bioremediation. It was considered that these products are almost exclusively developed for application based on total oils and thus are most likely to contain an abundance of *n*-alkane degraders. The abundance of strains in these products with an ability to degrade more complex chemical structures typical of UCM was questionable. It was therefore decided that development of a marine microbial ‘cocktail’, by selective enrichment on UCM as a sole carbon source, was more appropriate.

Sediment samples were obtained from the industrialised Cork Harbour area, and specifically from sites where local activities, such as boating, were expected to result in chronic exposure to hydrocarbons. Thus, these are sites where it would be most likely to isolate hydrocarbon degrading bacteria.

Forties crude oil was used as UCM substrate for the enrichment of the consortia. As this oil contained significant *n*-alkanes it was not immediately suitable. Therefore, n-alkanes were removed from the Forties crude oil by treatment with known *n*-alkane degraders in minimal media in shake flasks. GC-MS analysis demonstrated the complete removal of *n*-alkanes but remaining UCM (fig. 3.1). The residual oil was sterilised for use as an enrichment substrate. Further GC-MS analysis of the UCM showed that other relatively simple components had also been substantially biodegraded. Examination of a chromatogram for m/z 83 demonstrated that a homologous series of compounds, presumably linear alkylcyclohexanes had been removed. The removal of a series of peaks, presumably alkylbenzenes, is also evident from the 91m/z trace. Therefore the ‘Forties UCM’ used for selective enrichment and for evaluation of microbial consortia consisted of structurally complex components that would be relatively slow to degrade in the natural environment.

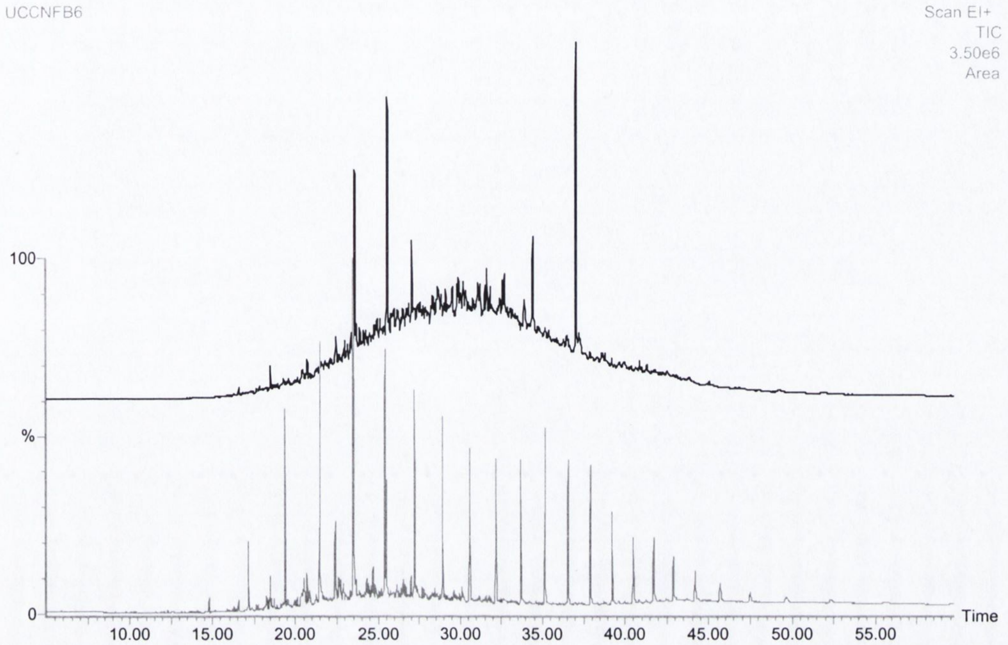


*Fig. 3.1: Weathered Forties crude oil GC-MS (TIC) trace (t=0) (*bottom*), and same oil after treatment with n-alkane degraders for 24 days (*top*). This t=24 day biodegraded oil was used as the UCM carbon source for the enrichment procedures. The late eluting peak in this chromatogram is a phthalate ester co-extracted from the sample collection vessel during analysis.*

Sediments were added to sterilised UCM/Bushnell-Haas (Anon. 1991a, Appendix C) sea water media in flasks and shaken for ten weeks at 28°C. Six sediment samples were enriched in this manner, (flasks 1,2,4,5,6,7). A control sample flask containing sediment and minimal media but no UCM substrate was also set up using the same sediment as in flask 7, (flask 7 control). Bacterial enumeration of the flasks was undertaken by pour-plates, using plate count agar (PCA) prepared with sea water. Bacterial numbers in the range 1.3×10^6 - 1.6×10^7 were obtained for UCM exposed flasks and for flask 7 control (no UCM) a count in the order of 1.1×10^6 was obtained. This suggests growth of bacteria capable of metabolising UCM. It should be noted that it is recognised that a large percentage of environmental microbes may not proliferate on plate count media (Amann *et al.* 1995).

Samples were taken from the flasks for evaluation using respirometry trials, but the enrichments were continued during these evaluations.

3.3 EVALUATION OF ENRICHED CULTURES

Degradation/growth trials were conducted in a ten chamber electrolytic respirometer, and growth assessed by measuring oxygen consumed by the growing biomass with UCM as the sole carbon source in Bushnell-Haas seawater minimal media. Incubation was at 30°C, although it was recognised that this may not be the optimal temperature for many enviromental microorganisms.

GC-MS TIC and SIR analysis and GC-FID was carried out on the UCM at the end of respiration tests using methodologies developed in phase I A of this project, and described in appendix A (GC-MS data is presented in appendix D.1). For SIR analysis total PAH group areas were used (e.g. total area for C2-phenanthrenes) or selected peaks. Mass chromatograms in appendix B show peaks quantitatively assessed using GC-MS SIR.

3.3.1 Determination of suitable propagation medium for crude inoculum

A sample of crude enrichment broth (flask 7) was grown overnight on three substrates; seawater containing glucose, succinate and LB broth. A representative sample from each medium was washed and used as an inoculum for growth tests on UCM. Using respirometry, oxygen consumption rate was measured to assess if these growth substrates were detrimental to UCM degradation rates. The results of this experiment are shown in figure 3.3.

The results indicate that the use of glucose does not negatively affect, and may in fact stimulate, subsequent respiration of the consortium on UCM when compared to crude preparations from flasks 5 and 7. LB broth does not inhibit respiration but succinate appears to inhibit the respiration rate. Glucose was therefore chosen as the preferred propagation substrate.

GC-FID and GC-MS analysis was carried out on UCM from the abiotic control and the glucose grown samples from the respirometry trials. The lighter end of the chromatogram was reduced, and pristane and phytane and other isoprenoids were completely removed in the sample compared with the control. Furthermore, ion abundance ratios from total mass spectra (see section 2.3.6. Appendix A.11) of the samples showed an increase in the ratios of 55/57, 69/71, 83/85, 97/99. This indicates a decrease in the relative abundance of *i*-alkanes to cycloalkanes and methylcycloalkanes. The 105/99 and 91/99 ratios also increase indicating a similar decrease in the *i*-alkane to substituted benzene compounds. A shift in the 83/95 ratio is indicative of preferential biodegradation of cyclohexanes with respect to bicycloalkanes. An Alba sample was run as a reference and compared very well to previously calculated ion abundance ratio data.

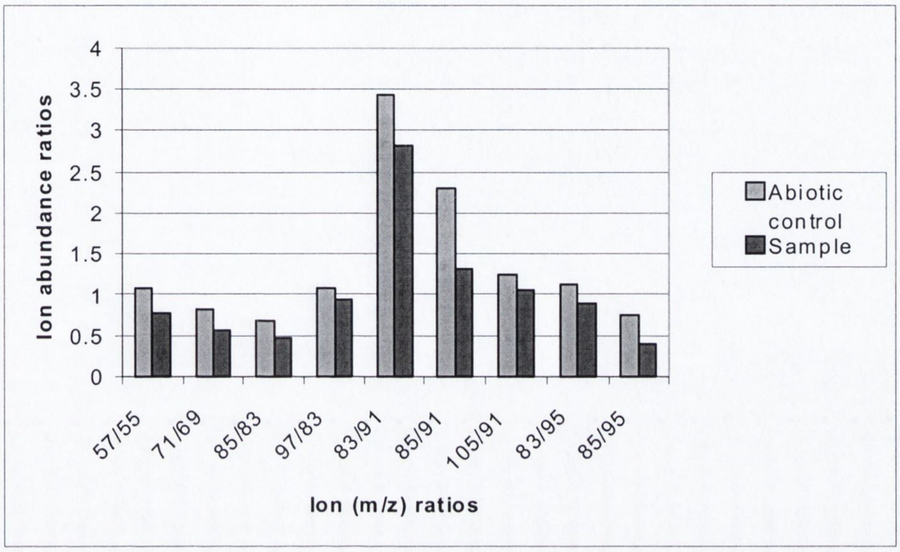


Fig. 3.2: Respirometry trial - sample and control
Ion abundance ratios from mass spectra of UCM after 2 weeks, for sample subjected to enriched consortia and for an abiotic control

Peak areas for individual aromatics and biomarker compounds were normalised against C30-hopane peak area as determined by GC-MS analysis (see section 2.3.6 Appendix A.11). There was no evidence of significant reduction of any of the compounds in the UCM sample compared with the control. The 12-day length of the trial may not have been sufficient to allow extensive biodegradation of these components to occur.

3.3.2 Comparison of enrichments on UCM as sole carbon source

Each crude enrichment from flasks 1,2,4,5,6 and 7 (labelled EF 1, EF 2 etc.) was grown overnight on glucose media and inoculated into respirometer flasks containing seawater media and 'Forties UCM'. An abiotic control containing no inoculum was also set up. Also, a sample from the control flask (sediment from site 7 treated as other flasks but not exposed to UCM – labelled CF 7) was inoculated. The results of this experiment are represented in figure 3.4

Interpretation of results was made difficult, as three flasks leaked during the course of the six week trial. Samples 6 and 7 leaked after 130 hours incubation and the abiotic control after 512 hours. The abiotic control however had shown no activity to this point. However, of the remaining enrichment flasks all samples indicated that the microbial consortia present were capable of significant respiration of UCM components. There appears to be only minor differences in the relative rates achieved between samples, possibly due to population differences.

As a control, the consortia in sample 7 (CF 7) which had NOT had previous exposure to UCM did not show any significant oxygen consumption until 640 hours of incubation, when compared with the test samples, and even then the activity was relatively minor. This suggested significant degradation of UCM by unacclimated populations may not occur until 3-4 weeks.

Total counts for each flask were as follows:

Flask	Count (CFU/ml)
EF1	2.8×10^6
EF2	8×10^4
EF4	2×10^6
EF5	$< 10^4$
EF7 test	1.1×10^6
EF7 control	3.6×10^5

The ion abundance ratios from combined mass spectra for the first half of the UCM chromatogram are displayed in figure 3.5. The results concur with the previous section, indicating that the *i*-alkanes are being biodegraded at a faster rate than the substituted cyclohexanes and the substituted benzenes. This also suggests that substituted cyclohexanes and benzenes are degraded at similar rates to their methylated counterparts. EF6 does not show this trend but respirometry for this flask showed no activity before the flask leaked, and continued to show no activity afterwards. The slight change in the ion abundances ratios for the control sample not previously exposed to UCM, is in agreement with the comparative extent of respiration for that sample. In general, the degree of alteration of ion abundance ratios is in accordance with the extent of activity for each sample as measured by respirometry.

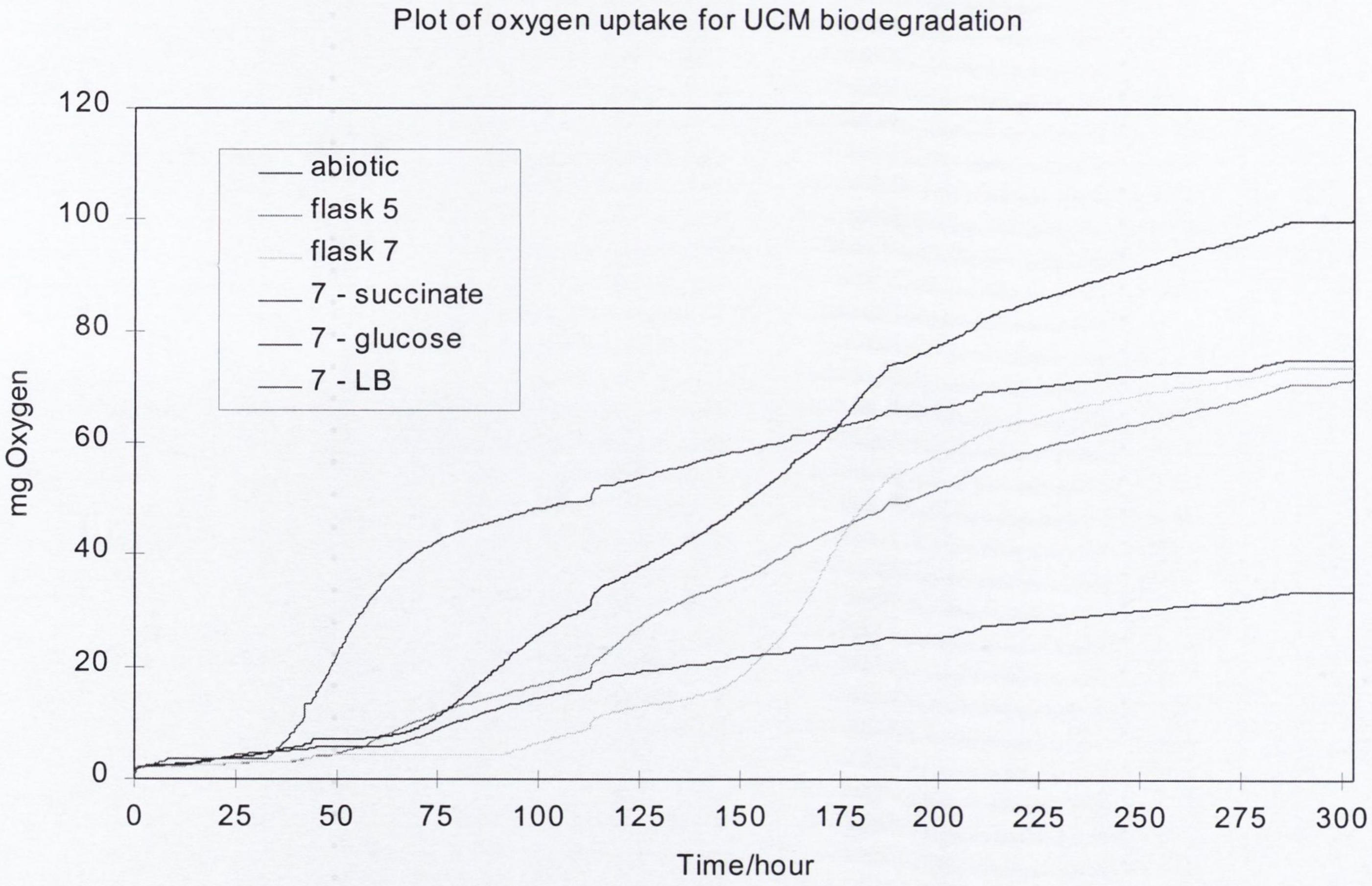


Fig. 3.3: Evaluation of rapid propagation media on subsequent capacity of enriched consortia to biodegrade UCM

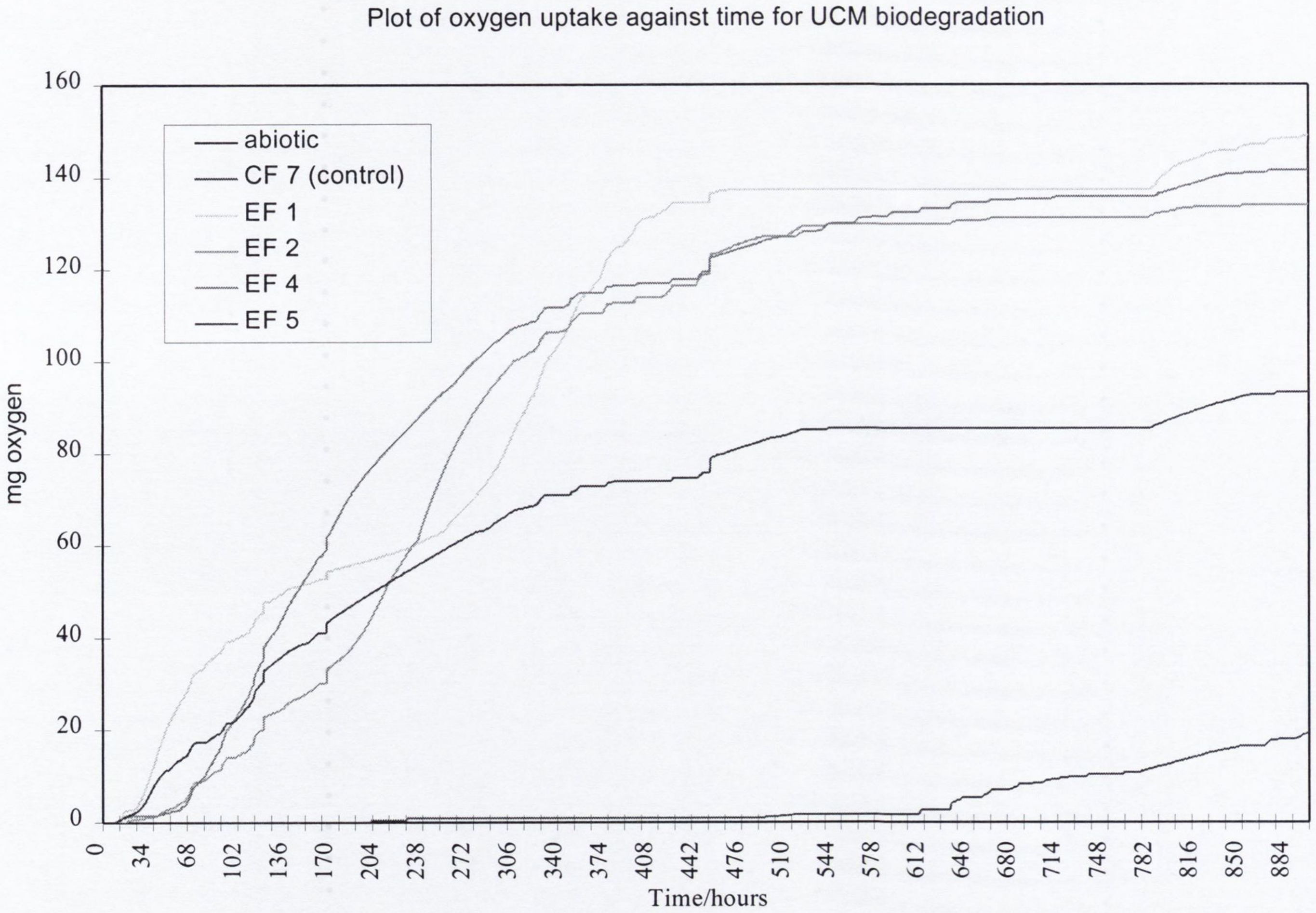


Fig. 3.4: Respirometry on UCM by enriched (EF) and non-enriched (CF) microbial consortia. (Abiotic control plotted until leak at 512 hours. To this point abiotic control had shown ~0 mg O_2 *consumption).*

Selected ion (SIR) GC-MS shows the relative reduction of components within the UCM. These analyses also indicate that EF1 and EF2 show the greatest reduction for selected substituted methylbenzenes and naphthenes, but also for substituted PAH and biomarkers. The significant amount of GC-MS data is reduced by presenting summed component data (e.g. Σ C2-phenanthrenes) to C30-hopane ratios and plotting these values for the test as a percentage of the equivalent value for the abiotic control (fig. 3.6). The actual percentage reduction of components may be even greater than indicated as it is possible that the C30-hopane concentration may also have been partially reduced in the samples with greatest activity (see Chapter 6.5.2).

The analytical evidence supports the respirometry trials, which indicate that the basic marine environmental consortia tested are capable of degrading UCM at a slow rate, with some weeks necessary for acclimation. Consortia enriched on UCM demonstrate a substantially greater capacity for UCM metabolism and degrade a range of complex UCM structures. These consortia were considered suitable for preparation of an inoculum for use in the bioremediation trials as they exhibited an inherent UCM metabolic advantage over unadapted cultures of marine micro-organisms.

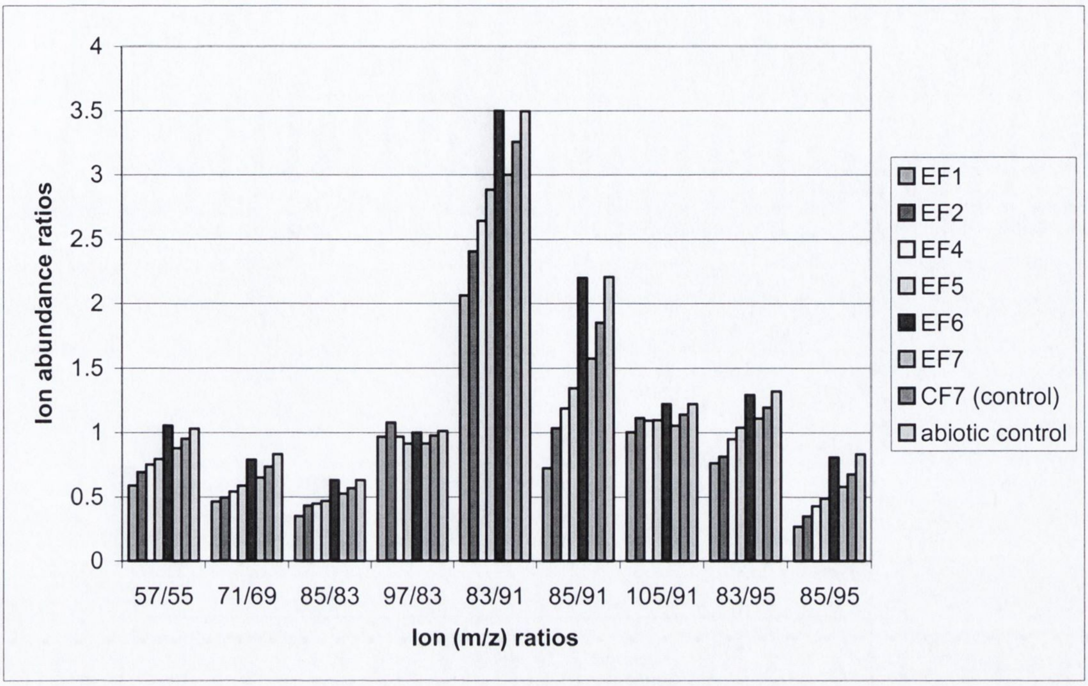


Fig. 3.5: Respirometry trials for enrichment flasks - Ion abundance ratios from Forties UCM mass spectra after 6 weeks

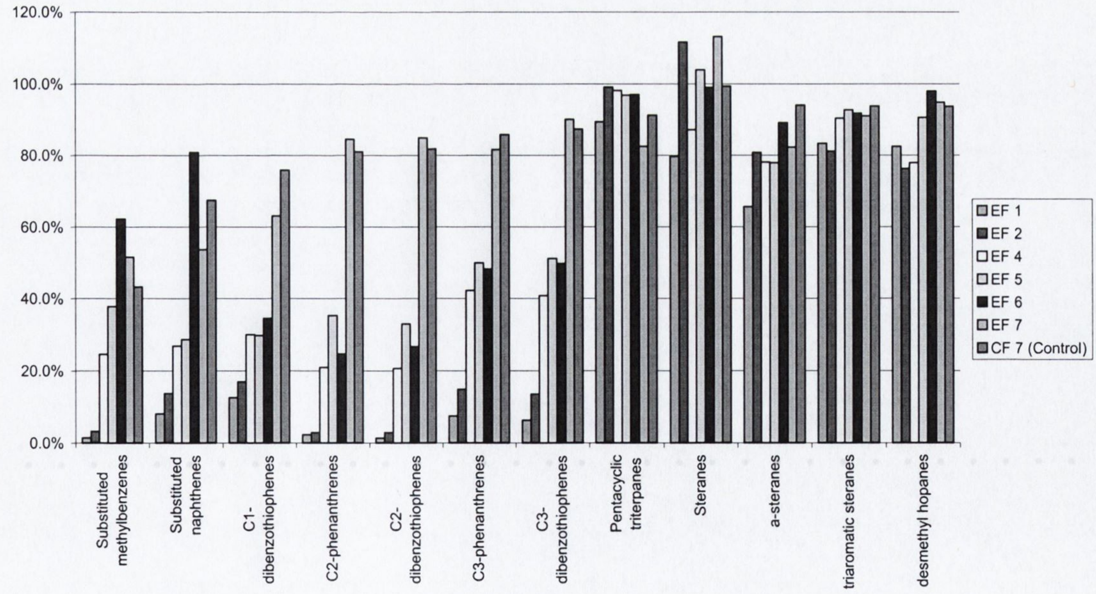


Fig. 3.6: Reduction in UCM component groups during respirometry tests for consortia efficacy on Forties UCM. GC-MS component group:C30-hopane ratios expressed as a percentage of component group:C30-hopane ratios for abiotic control sample

3.3.3 Efficiency of isolated strains vs crude enrichment broths

A number of visually identified representative colonies isolated on PCA from the 7 enrichment flasks were grown in minimal plate count (PC) broths overnight. A cell suspension of each culture was pooled and subjected to respirometry trials and compared with a sample crude enrichment broth, pooled from the enrichment flasks, (the inoculum chosen for the *bioaugmentation* treatment in the subsequent bioremediation microcosm trials – phase II).

Respirometry vessels were set up as shown in table 3.1

Test	**Carbon source**
UCC11 -abiotic control	UCM
UCC12 - pooled crude consortium pre-enriched on UCM (EF1-7)	None
UCC13- pooled crude consortium pre-enriched on UCM (EF1-7)	UCM
UCC14- cocktail of representative isolated cultures	UCM
UCC15- population from sediment used for bioremediation trials (T2)	UCM
UCC16- Control population – no pre-exposure to UCM (CF7)	UCM

Table 3.1: Setup of respirometry trials for evaluating efficiency of isolated strains vs crude enrichment broths

Based on relative oxygen consumption, results indicated that the selected culture inoculum was not as efficient as crude enrichments when grown on 'Alba UCM' as sole carbon source. As indicated in figure 3.7, the crude enrichment showed substantial biodegradation, while the pooled PCA plate isolated cultures showed poor biodegradative capacity. This may be due to the loss of efficient UCM degraders in the isolation steps, particularly bearing in mind the inability of many environmental bacteria for proliferation on PCA. Also co-metabolism may play a role in UCM degradation and synergistic influences may be lost in isolation. No attempt was made to speciate the bacterial cultures. Interestingly, the control sample (CF 7) that had not been previously exposed to UCM showed a significant ability to metabolise UCM, conflicting with the results observed for the previous experiment, (fig. 3.4). This may be due to the use of 'Alba UCM' as sole carbon source in this respirometry test, as opposed to 'Forties UCM' in the previous test, which has been substantially physically weathered, and also has been shown to have many of the relatively simpler substrates, such as linear cycloalkanes, removed prior to use. Thus, the Forties UCM presents a mixture comprising of more refractory substrates.

The booster population used in the microcosm trials in phase II consisted of a population grown overnight on glucose with 'Alba UCM' from a sample of sediment to be used in the microcosm trials. The respiration observed was less than that for the enriched population.

The selected GC-MS total ion chromatograms for extracts of samples taken at the end of the respirometry experiment are displayed in figure 3.8, and give a useful indication of the alteration of UCM. Clearly, lower molecular weight components, including resolved isoprenoids, are degraded preferentially. However, biodegradation does occur across the molecular weight range. GC-MS spectra combined over the molecular weight entire range.

Again, changes in ion abundance ratios (fig. 3.9), based on the total oil combined spectra, accord with the extent of respiration observed, (e.g. UCC13 – crude pre-enriched consortium-exhibited maximum respiration and maximum change to relative ion abundance ratios). Alba reference oil samples used to bracket the GC-MS analysis sequence demonstrate the reproducibility of this technique for this sequence and the abiotic control agrees well with the reference sample. The results are in agreement with that observed from GC-MS analysis from the other respirometry trials. However, more extensive changes appears to have occurred in this experiment, and respirometry indicates greater O_2 uptake. In this test, the results suggest that methylated cycloalkanes and methylated benzenes are more amenable to biodegradation than their non-methylated counterparts based on the m/z 97/83 and 105/91 ion abundance ratios.

GC-MS SIR (fig. 3.10) indicated that complete removal of C1-C3 phenanthrenes and C1-C3 dibenzothiophenes occurred for UCC13. Biodegradation occurred preferentially for lower ring number compounds but partially biodegraded components indicate that there may be changes in isomer patterns. Dibenzothiophenes were more resistant than phenanthrenes. None of the treatments showed substantial change in the pentacyclic triterpane pattern. Analysis of a series of peaks for the m/z 137 chromatogram, possibly due to substituted aliphatic bicyclic compounds indicate removal of these components in all samples, bar the abiotic control. Sterane patterns have been affected by biodegradation, as is indicated by the mass chromatograms in fig 3.11. In particular the two initial peaks, attributed to (20S) and (20R) 13β, 17α-diacholestane, labelled S1 and S2 respectively in figure 3.11a, have been almost completely removed in UCC13. Changes in PAH isomer and biomarker patterns have implications in oil spill fingerprinting/ source identification techniques (see chapter 7).

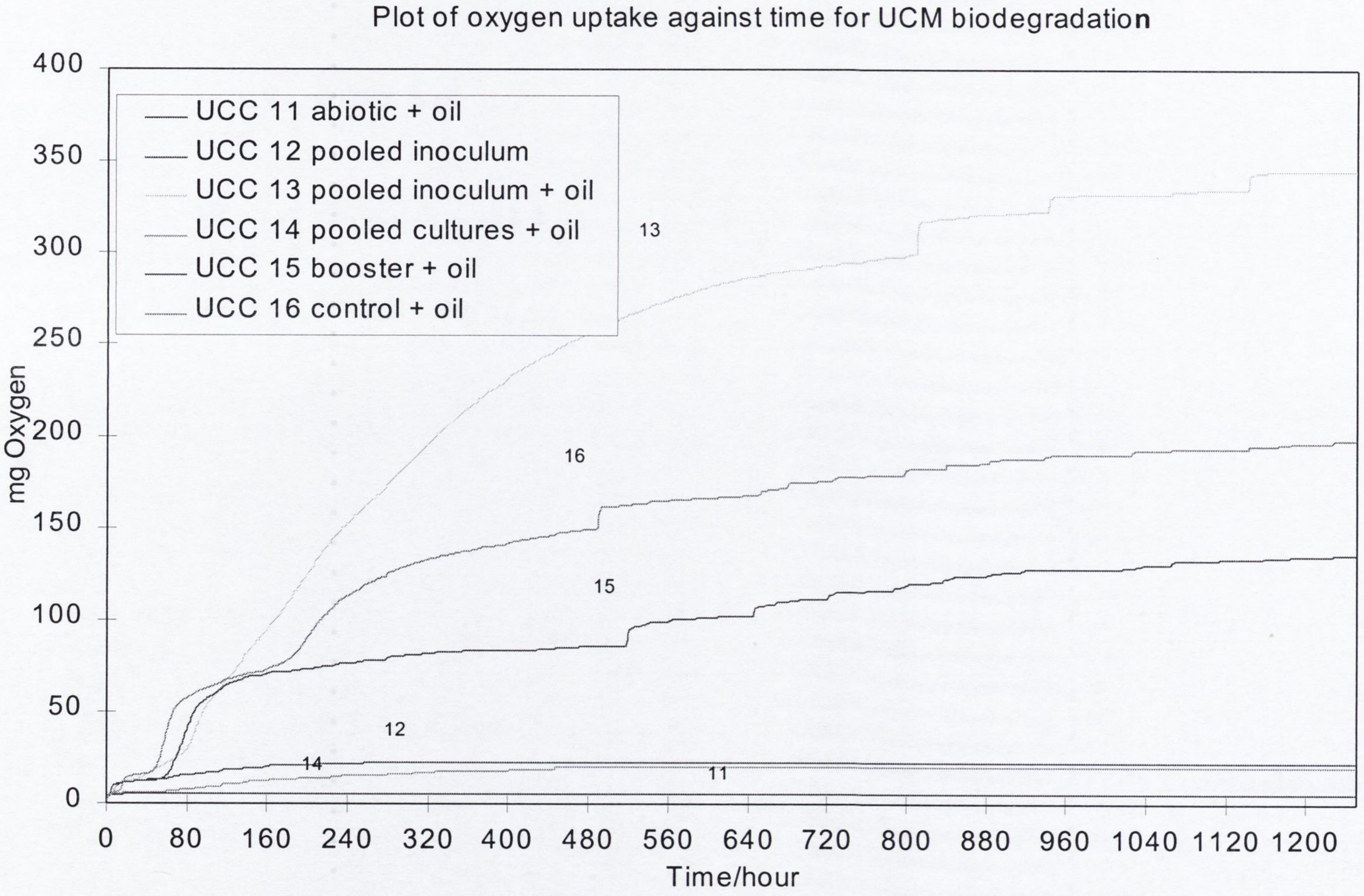


Fig. 3.7: Respirometry – comparison of pooled selection of micro-organisms(14) and crude enriched (13) and unenriched (16) consortia

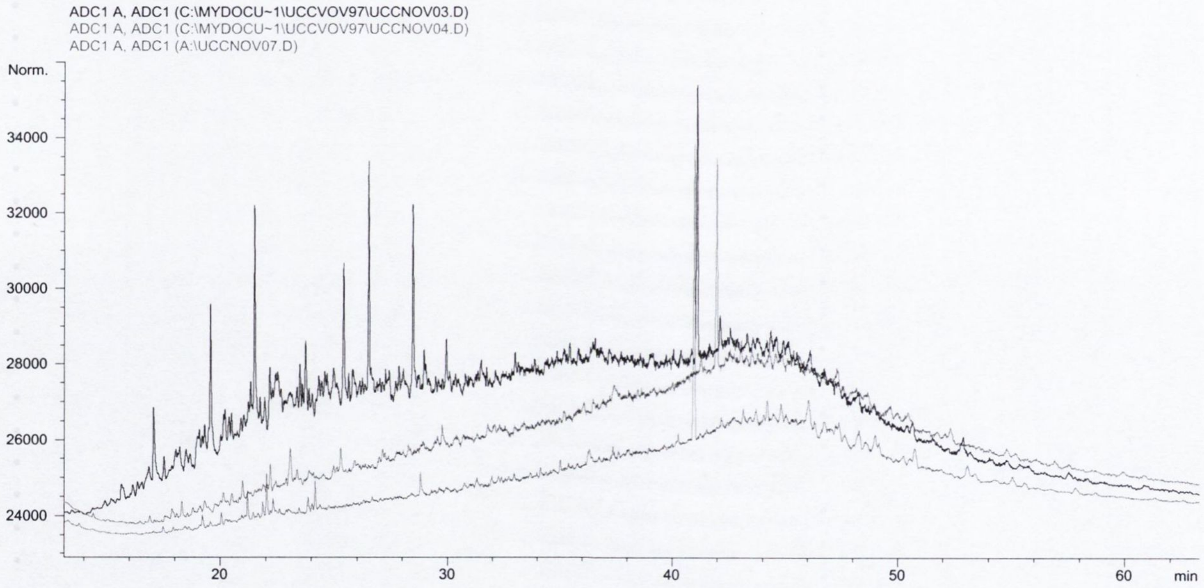


Fig. 3.8: GC chromatograms of extracts from respirometry trial.

Blue= abiotic control (UCC 11),

Green= treated with CF 7 consortium (not enriched UCC 16),

Red – Treated with enriched consortium (UCC 13)

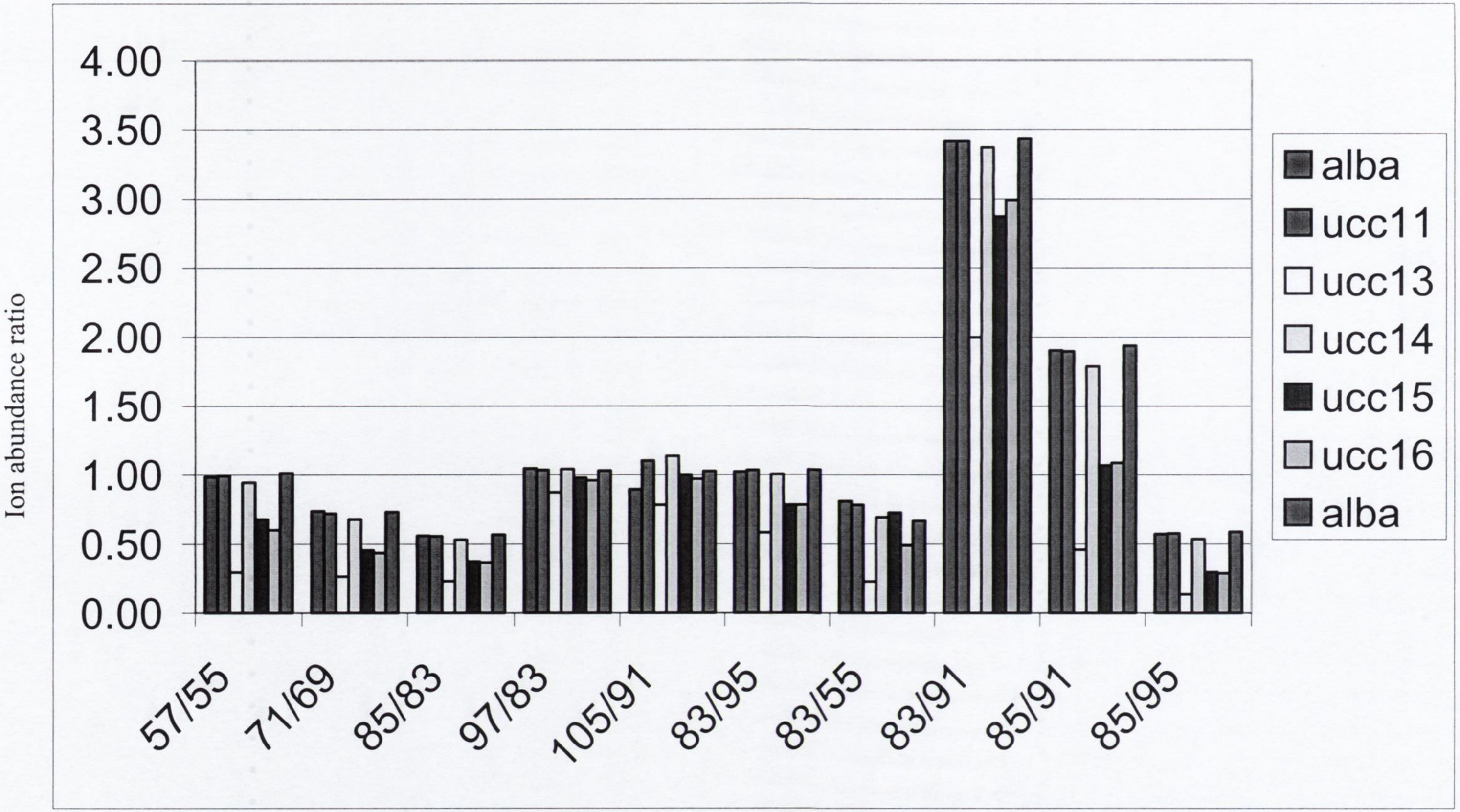


Fig. 3.9: GC-MS ion abundance ratios for total oils from respirometry trials. Alba reference oil samples were used to bracket the analytical batch sequence

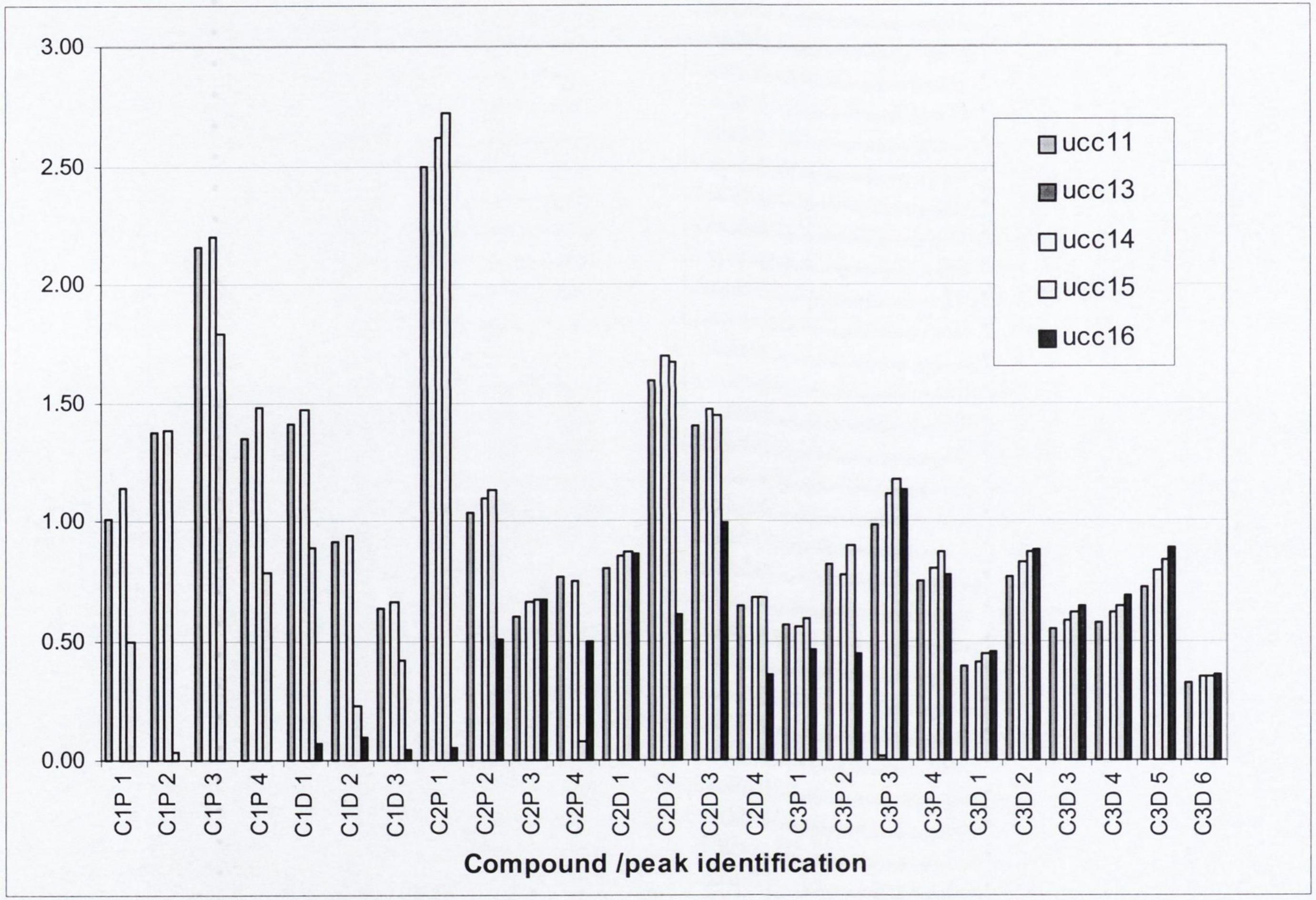


Fig. 3.10: *Biodegradation of aromatic components in respirometry test flasks. Complete removal of these aromatic components has occurred for UCC 13. Degradation expressed normalised to the refractory internal marker C30-hopane. (Peak assignations are given in appendix B)*

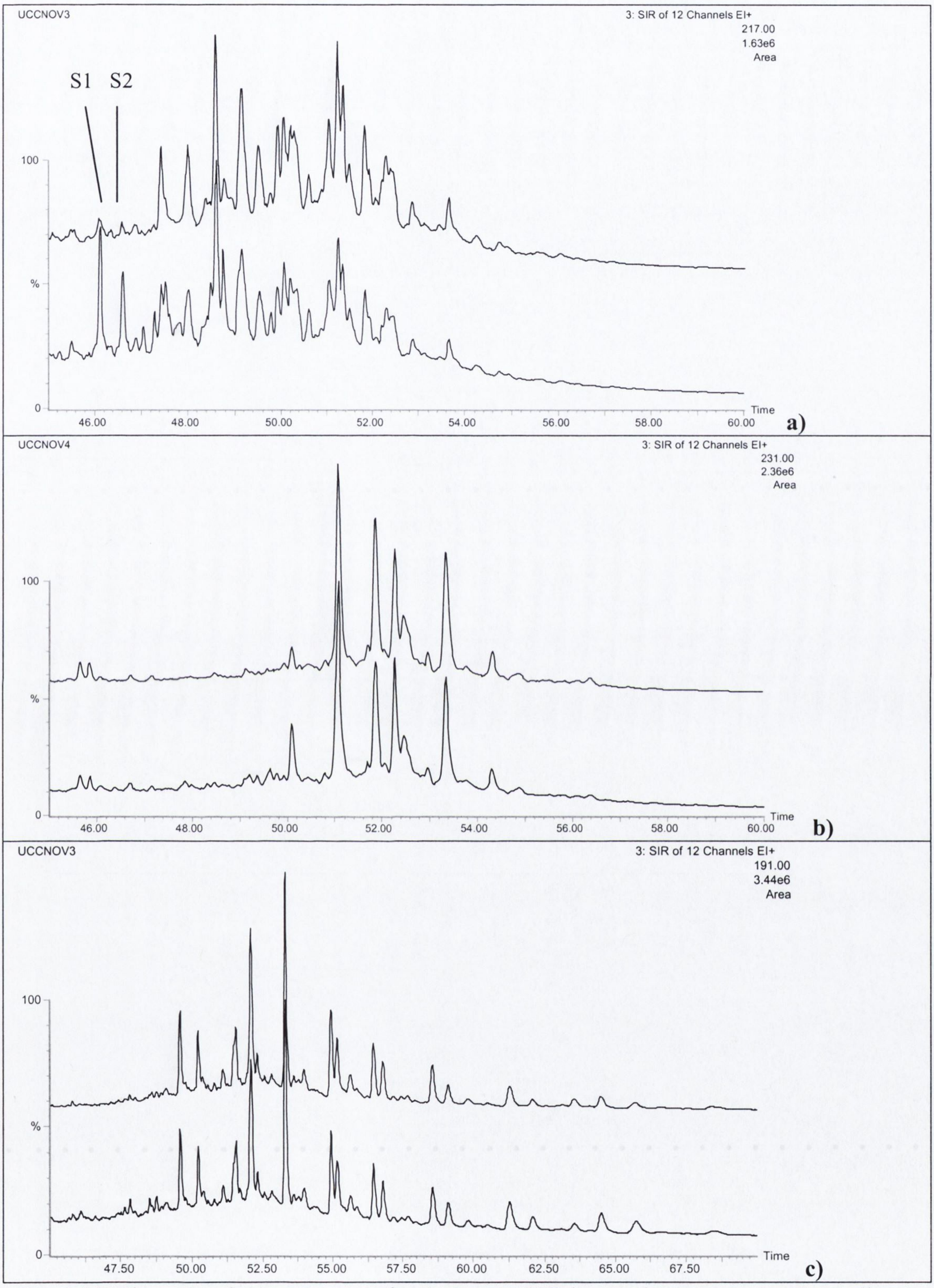


Fig. 3.11: Biomarker fragmentograms for a) steranes b)Triaromatic steranes c) pentacyclic triterpanes – for each the upper trace represents sample UCC 13 (biodegraded UCM) and the lower UCC 11 (abiotic control)

3.4 PREPARATION OF INOCULA FOR MICROCOSM TRIALS –PHASE II

The specialised consortia, (microcosm T1), was prepared from the pooled broths from flasks 1, 2, 4, 5 and 7. The booster population, (microcosm T2), was prepared from the sediment being used for the microcosm trials. Each of these inocula was grown separately overnight in a Bushnell-Haas/Glucose media and also in the presence of UCM (Alba crude oil). The resulting suspensions were used as an inoculum for the microcosm trials.

3.5 CONCLUSIONS

Laboratory results indicate that the crude consortium, pre-enriched on UCM, has the capability to biodegrade UCM. There is a significant acclimation period for crude consortia that have not been previously exposed to UCM. It is probable that this acclimation period depends on the chemical composition of the UCM.

A cocktail of predominant microbial colonies, isolated using simple PCA plate culture techniques, demonstrated a very poor ability to metabolise UCM compared with the crude pre-enriched consortia. To rapidly propagate the consortia with minimal effect on the UCM degrading capability glucose was selected as a suitable medium based on respirometry trials.

The results suggest that a crude environmental consortium of micro-organisms, pre-enriched on UCM, may have a role in biotreatment of slowly degradable hydrocarbons and UCM, assuming that metabolism is the rate limiting factor. However, this is dependent on whether the performance of micro-organisms in the laboratory is transferred to the field, i.e. if they can effectively proliferate, compete with indigenous population and actively metabolise the target substrate.

GC and GC-MS have been demonstrated to be useful for assessing the changes in UCM with biodegradation. Lower molecular weight hydrocarbons are degraded preferentially but biodegradation occurred across the entire UCM molecular weight range of the crude oils. The branched components were degraded more rapidly than the cyclic components and alkylcyclohexanes more rapidly than alkylbenzenes. The greater the number of rings in the molecular structure, the less the extent of biodegradation observed, both for aromatic and aliphatic components. Methylcyclohexanes and methylbenzenes may be more amenable to biodegradation than their non-methylated counterparts. Biomarkers are resistant to biodegradation but there are changes to the sterane pattern with severe biodegradation, and the reduction of the two C27-diacholestane peaks is particularly evident.

4. DESIGN OF BIOREMEDIATION MICROCOSM TRIALS (PHASE II)

4.1 BACKGROUND

Microcosms were designed to assess bioremediation strategies for UCM biodegradation, and in particular *bioaugmentation* and *biostimulation* strategies were compared with control microcosms. Laboratory studies in phase I of this project (see chapter 3) demonstrated the resistance of UCM to biodegradation, and also the capability of an acclimated enriched consortium to greatly enhance biodegradation. Crude microbial consortia were considered more likely to be effective than commercial 'cocktails' of individual micro-organisms as 'seed'. Therefore, the consortium developed in phase I was used as 'seed' inoculum for the microcosm trials. Two UCM rich crude oils were selected as test oils, based on phase I analysis. A range of analytical methodologies was employed to assess the alteration of UCM composition. This was an effort to establish the UCM components that are most resistant to biodegradation, and thus the components that limit the effectiveness of bioremediation as a clean-up technique.

4.2 EXPERIMENTAL DESIGN OF BIOREMEDIATION TRIALS

4.2.1 Design

The following treatments were evaluated:

Treatments

- T1 : Bioaugmented with a specially prepared inocula/microbial consortia (see chapter 3.4) + indigenous population + nutrients
- T2 : Bioaugmented with booster population (see chapter 3.4) + indigenous population + nutrients
- T3 : Indigenous population + nutrients

Controls

- C1 : Sea water control
- C2 : Poison control

The treatments were designed to ascertain whether inoculation with a specialised microbial consortia with a laboratory demonstrated capacity for UCM degradation (T1), or increased numbers of non-target selected indigenous species prepared from sediment to be used in the microcosm tests (T2), exhibit enhanced biodegradation over the indigenous microflora population supplemented with nutrients (T3). The seawater control (C1) represents the untreated (i.e. natural) situation with an indigenous population present but not artificially supplemented with nutrients. Microbial growth is inhibited with mercuric chloride as a poison control, (C2), and any reduction in sediment hydrocarbon concentration from this system is due to abiotic factors.

Each experiment was performed in duplicate and for two oils. A total of in 20 experimental units resulted, i.e. ((3 x treatments + 2 controls) x 2) x 2 oils.

Treatment	***T1***	***T2***	***T3***	***C1***	***C2***
Microbial inocula that exhibits capacity for UCM biodegradation	✓				
Booster population inocula		✓			
Indigenous population	✓	✓	✓	✓	✓
Nutrient addition	✓	✓	✓		
Oil/UCM contaminated sediment	✓	✓	✓	✓	✓
Sea water	✓	✓	✓	✓	✓
$HgCl_2$ poison					✓

Table 4.1: Bioremediation trials experimental design

4.2.2 Selection of oils for trials

The selection of crude oils for the microcosm trials was based on the characterisation carried out in phase I. Forties weathered crude oil and Alba crude oil were chosen. The crude oils contain more aromatic compounds and a wider molecular weight range than the lubricating oils, thus presenting a broader diversity of UCM components for study. Alba, as a relatively heavy reservoir biodegraded crude oil, provides a very good model UCM. Although the Forties crude oil does contain a significant amount of *n*-alkanes, it was chosen as the presence of *n*-alkanes may influence the degradation of more recalcitrant UCM compounds either positively or negatively. Furthermore, the presence of relatively simple substrates, such as *n*-alkanes, is useful for comparative purposes with experiments where they are not present.

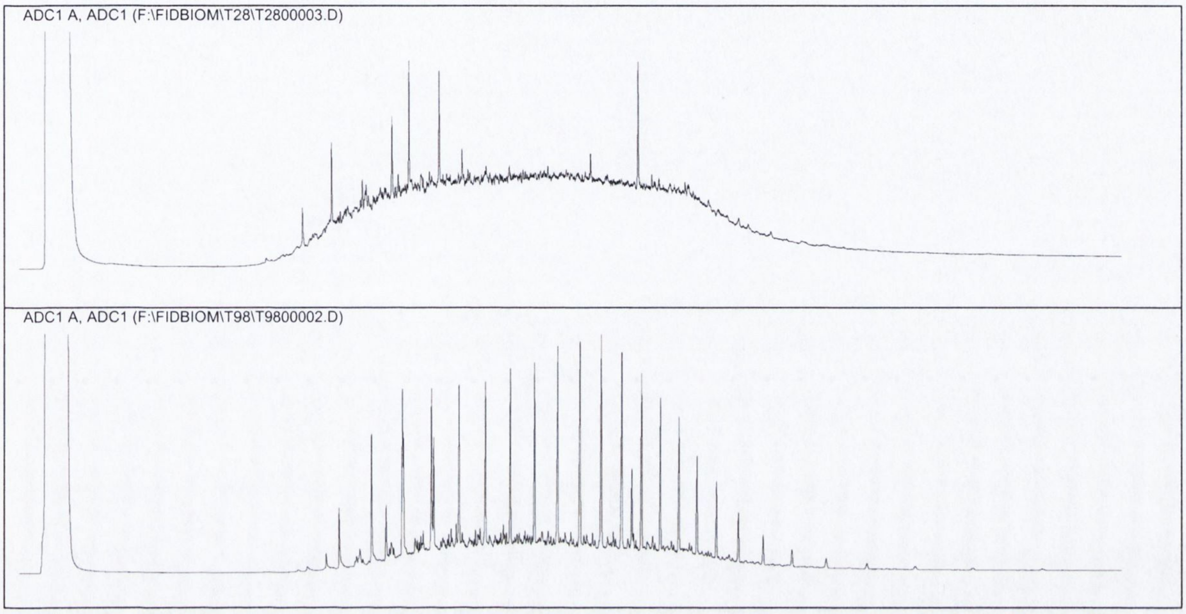


Fig. 4.1: GC chromatograms of the initial oil extract from sediment (t=0 samples). Top=Alba (At=0), and bottom= Forties (Ft=0).

4.2.3 Nomenclature

In referring to a given microcosm and microcosm sample the labels T1, T2, T3, C1 and C2 are used to denote the treatments and controls as outlined in table 4.1. The letters F and A are used to denote Forties and Alba oil respectively and the numerals i and ii used to distinguish between duplicate experiments. Thus FiiT3 denotes the microcosm test (duplicate no. 2), containing Forties contaminated sediment with nutrient addition but no bioaugmentation. Samples taken for analysis use this labeling system followed by the code 't = number' indicating the number of days after the initiation of the trials that the sample was taken. If the Roman numeral representing the duplicate test unit is not used, the code refers to both duplicate or a mean value for the duplicate test units, e.g. AT3 = mean of AiT3 and AiiT3. If '(t=number)' follows 't=0' this refers to the time zero sample analysed as a reference sample along with samples taken at t=number, e.g. At=0 (t=280) is the reference samples analysed with the t=280 day batch of microcosm samples.

4.3 MICROCOSM UNIT DESIGN

Much consideration was given to the design of the bioremediation units and a simple 'tidal simulation' system was developed. This involves recirculation of seawater/nutrient using a fill and discharge system on a 12-hour cycle.

4.3.1 Spiked sediment

A coarse intertidal coastal sediment was obtained from Howth in the Dublin Bay region. Particles >2mm and <250μm were removed. A particle size distribution test was carried out on the resulting sediment. Sediment was contaminated with oil in pentane (general-purpose reagent grade, Labscan) carrier solvent. To ensure that bacterial populations in the sediment were only minimally affected by the contamination process, a fraction of sediment was contaminated and diluted with fresh sediment after drying. Oil was dissolved in *n*-pentane and applied to 15kg of sediment and mixed thoroughly. The solvent was evaporated from the sediment over a 5 day period, assisted by regular mixing and spreading and an electric fan. The contaminated sediment was diluted with a further 46kg of uncontaminated sediment and thoroughly mixed. This process was carried out for both Forties and Alba oils and the calculated final concentration of oil in sediment was 3.1% w/w for Forties and 4.1% for Alba, not accounting for loss due to evaporation and non-pentane soluble material. T=zero samples were taken at this point and contaminated sediment not used in the test was kept in cold storage for use as analytical reference samples throughout the trials.

4.3.2 Microcosm Unit Design

Approximately 7kg of sediment was supported in a 250μm nylon square mesh 'sock'. This was supported by a durable plastic "oyster mesh" (wide mesh) basket resting upon a cobble bed within a 25 litre vessel. This vessel stood above a 25 litre reservoir containing seawater (with nutrients if required). The vessel was filled with seawater/nutrients media from the reservoir by means a 24-channel peristaltic pump (Ismatec BVP) operating on a timer. At a specified level in the vessel, the seawater was discharged into the reservoir by siphon. The siphon system was constructed with tubing (Tygon R3603, 2.4 mm i.d.) that exited the vessel through a cable gland. A wider diameter tubing (Tygon R3603, 4 mm i.d.) was used for much of the siphon length within the vessel to reduce capillary action and thus avoid discharge prior to the set level. Ismatec 3.18

mm i.d. (Tygon R3607) tubing was used for delivering nutrients from reservoir to the microcosm vessel. for The periodicity was set by control of pump flow rates, timer settings (pump was on for 7 hours out of each 12 hour cycle) and tubing dimensions to achieve a 12-hour cycle period. The approximate flow into the vessel was in the region of 50 mls/min with an approximate flow out of 90 mls/min. The advantages of such a system is that it ensures adequate aeration and exposure to nutrients and is entirely self-contained. Furthermore, it is a simple representation of a tidal system, although in the field the tide does not recirculate and exhibits more complex cycles than represented by this design.

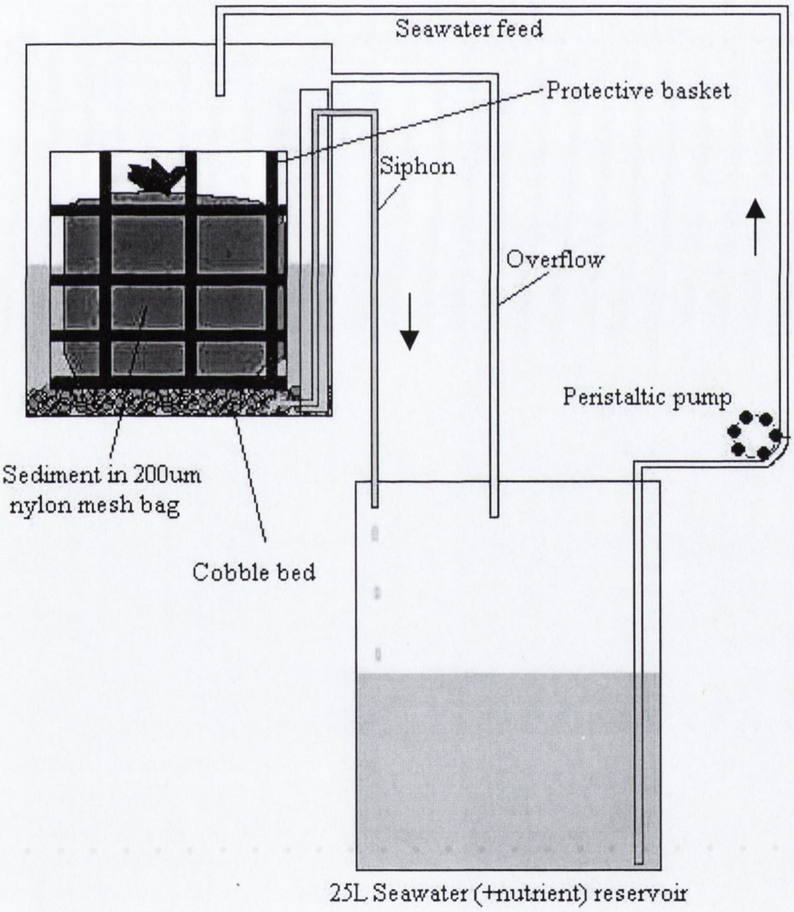


Fig.4.2: Bioremediation trials: Microcosm unit design

4.3.3 Seawater/ nutrients

Seawater was obtained from the Dublin bay region at the same location as the sediments. This seawater was used alone for the seawater control (C1) experiments, and was used as solution for the three nutrient addition treatments (T1, T2 and T3). The nutrient formulation used was Bushnell-Haas in line with US EPA/NETAC guidelines for screening bioremediation products for use in marine oil spill situations (Anon. 1991a, appendix C). The poison control was prepared by addition of $HgCl_2$ to seawater to 1% strength. Seawater/nutrients for each reservoir was changed at approximately 8 week intervals with the exception of the seawater in the poison control reservoirs. A small amount of seawater was retained in each reservoir at each change.

4.3.4 Inoculation of T1 and T2 tests

The inocula for T1 and T2 were prepared as previously described. 2l of each inocula was available and 500 mls of the specially developed consortia was gradually added to each T1 test (AiT1, AiiT1, FiT1, FiiT1) in the microcosm units with constant mixing. The T2 tests were similarly inoculated. Care was taken through the course of the trials not to cross contaminate the trials. As no evidence of enhancement of biodegradation in inoculated trials was evident in the initial stages of the experiment (chapter 6) the tests were reinoculated at t=281 days.

4.3.5 Location

The microcosms were set up in an ‘portacabin’. The room was kept in the dark to ensure algal growth did not cause competition for nutrients and foul the siphon system.

4.3.6 Temperature

This experiment was scheduled to run through winter months. To ensure low temperatures did not effectively limit degradation, and due to the limited time frame of the trials, nighttime temperatures were elevated using an electric fan. Maximum and minimum temperatures were recorded daily at three positions in the room to demonstrate that temperatures were constant throughout the room.

Fig. 4.3: *Photograph of the experimental setup, showing microcosm units (vessels- higher tier and reservoirs -lower tier) and 24 channel peristaltic pump*

4.4 MONITORING OF MICROCOSMS AND OIL BIODEGRADATION

4.4.1 Assessment & testing of the units

A number of parameters were measured and assessed during the microcosm trials. pH of the reservoir solutions was recorded on a number of occasions using an Orion pH meter calibrated in the range pH 7 – 9. Measurements of pH, salinity, dissolved oxygen (DO), temperature of the reservoir solution was recorded using a Minisonde Multiprobe. Nutrient levels were analysed using a Brann & Luebbe TRAACs 2000 system and salinity using a Portasal salinometer.

Wet weight: dry weight determinations of the drained sediments (just prior to a fill cycle) were carried out by weighing before and after oven drying for 24 hours at 120°C.

Microcosm reservoirs were monitored for evaporation of solution and, if necessary, topped with laboratory grade water to their initial volume to avoid increases in salinity. This was rarely required.

4.4.2 Respirometry trials to assess UCM biodegradation potential of microcosm microbial populations

Soil samples were taken on t=280 days and washes from the duplicate samples were combined and respirometry trials carried out to assess the capability of the sediment population for degradation of UCM. This was intended to see did the treated tests, and in particular the inoculated tests, exhibit a greater capability for UCM biodegradation. The respirometry method was as described earlier (phase I), using 1g of Alba as the UCM substrate in each test.

4.4.3 Sampling for biodegradation monitoring

Each sediment was mixed in the nylon mesh bag by pummeling at regular intervals to ensure homogeneity. Each test unit had its own sampling equipment to ensure cross contamination did not occur. Samples were taken with PTFE spatulas into solvent rinsed glass museum jars.

4.4.4 Chemical monitoring of oil biodegradation

The monitoring programme involved regular sampling and chemical analysis of sediments for assessment of UCM biodegradation. Based on phase I of this project, the following monitoring programme was implemented to ascertain changes in bulk parameters or individual components throughout the bioremediation trials, (table 4.2).

The full battery of analytical tests was not applied at each sampling point for practical reasons. A large amount of data was generated and the measured parameters and reference parameters stored as raw data (e.g. peak areas) in a purpose designed Microsoft Access™ database system. Data analysis was performed primarily using Microsoft Excel™ and SPSS™ as well as specialist scientific software packages.

Analytical methodology used is described in detail in appendices A1-11. Gravimetric, gas chromatography and gas chromatography-mass spectrometry data are presented in appendices D2-5.

4.5 QUALITY ASSURANCE

Microcosm tests were set up in duplicates. The microcosms were attended daily (working days) and a comprehensive log book maintained.

Archived Forties and Alba t=0 samples were used as reference materials throughout the course of the experiment, enabling an evaluation of analytical variability.

Alba crude oil was also used as a reference oil in some instances and in particular was used in GC-MS analysis. The performance of the reference oil can be used to assess the variability of these tests over the period of the trials. Also for selected ion GC-MS analysis, selected biomarker compounds were employed as a conserved internal standard.

The raw data stored in the Access database was screened using Excel to check for outliers. Outliers were few and when checked and could generally be attributed to errors in data input/ transfer.

Analysis	*Analysis detail*	*Information set*	*Sampling interval (days)*	*Quality assurance*
Gravimetric	(i) Extraction (ii) Silica/alumina fractionation and weight determination	Total oil Saturates/aromatics/polars breakdown	0, 28, 56, 98 140, 203, 280, 350, 473 0, 28, 56, 98 140, 473	Alba reference oil run with each batch
GC-FID	(i) Total UCM quantification (ii) UCM segment quantification based total area between *n*-alkanes	Total oil/ucm UCM profile - whether it is reduced across the ucm or lower molcular weight materials removed initially	0, 28, 56, 98 140, 203, 280, 350, 473 0, 28, 56, 98 140, 203, 280, 350, 473	Forties t=0/ Alba t=0 reference samples
Spectroscopy	(i) Infra-red scan of oils (ii) NMR spectroscopy oils (i) Total luminescence spectroscopy scans	Other changes e.g. increase in carbonyl content Total oil based on aliphatic content Change in aromatic profile Ratio of 2- ring: 3-ring aromatic	0, 473 0, 473 0, 203, 407	Forties t=0/ Alba t=0 reference samples Forties t=0/ Alba t=0 reference samples Forties t=0/ Alba t=0 reference samples
GC-MS EI+	(i) Full scan mode (ii) Selected ion monitoring	Total oil mass spectra indicating Relative changes in component classes e.g. cyclics, alkanes, substituted benzenes Biodegradation of many individual components i.e. cycloalkanes, naphthenes, alkylbenzenes, substituted PAH, steranes, hopanes	0, 56, 203, 473 0, 28, 280, 473	Samples run in duplicate bracketed by Alba reference oil. Forties t=0/ Alba t=0 reference samples Samples run in duplicate bracketed by Alba reference oil. Normalised vs. C30 hopane and Alba. Forties t=0/ Alba t=0 reference samples
HPLC	(i) Dual column with reverse flow switching	Aromatic content based on ring number & polar fraction	0, 280	Alba reference oil Perylene internal standard Forties t=0/ Alba t=0 reference samples

Table 4.2:Hydrocarbon monitoring programme for bioremediation microcosm trials

5. BIOREMEDIATION TRIALS RESULTS – MICROCOSM DESIGN

5.1 CONTAMINATION OF SEDIMENT

By using pentane as carrier solvent for contaminating the sediment, non-pentane soluble components present in the oils such as asphaltenes were not transferred to the sediment. Recoveries calculated from gravimetric analysis indicates that due to non-pentane solubility, a significant proportion of oil components were not transferred to the sediment, particularly for the Forties oil (see gravimetric results, chapter 6.2.1). This can be attributed to non-soluble oil components (asphaltenes), debris and water contained in the oil. As the oil was well mixed into 25% of the total sediment in carrier solvent, and the dried contaminated sediment thoroughly mixed into uncontaminated sediment (i.e. ¼ dilution), the oil should be homogeneously distributed (chapter 6.2.1). This method was used to ensure that even contamination of sediment would be achieved but that the indigenous sedimentary microbial population would not be exposed to the carrier solvent which may potentially be inhibitory.

5.2 SEAWATER CYCLE

Based on average flow rates (in & out), pump on/off times, and available vessel volume, the theoretical cycle periodicity is graphed in figure 5.1. The periodicity of 12 hours was chosen to approximate field conditions. However, the mechanism does not yield the complex sinusoidal graphs that are associated with true tidal cycles. The cycle height is 30 cm and the average submerged time for the sediments is 6.3 hours per cycle so the sediment is exposed to atmosphere for an average of 47% of the cycle time (34% for bottom sediment and 61% for top).

In a shoreline application of bioremediation where oil contamination is treated *in situ*, the position of the oil on the shore in relation to the tide lines is important, as the length of time sediment is submerged may vary greatly. This will influence factors such as aeration and nutrient exposure.

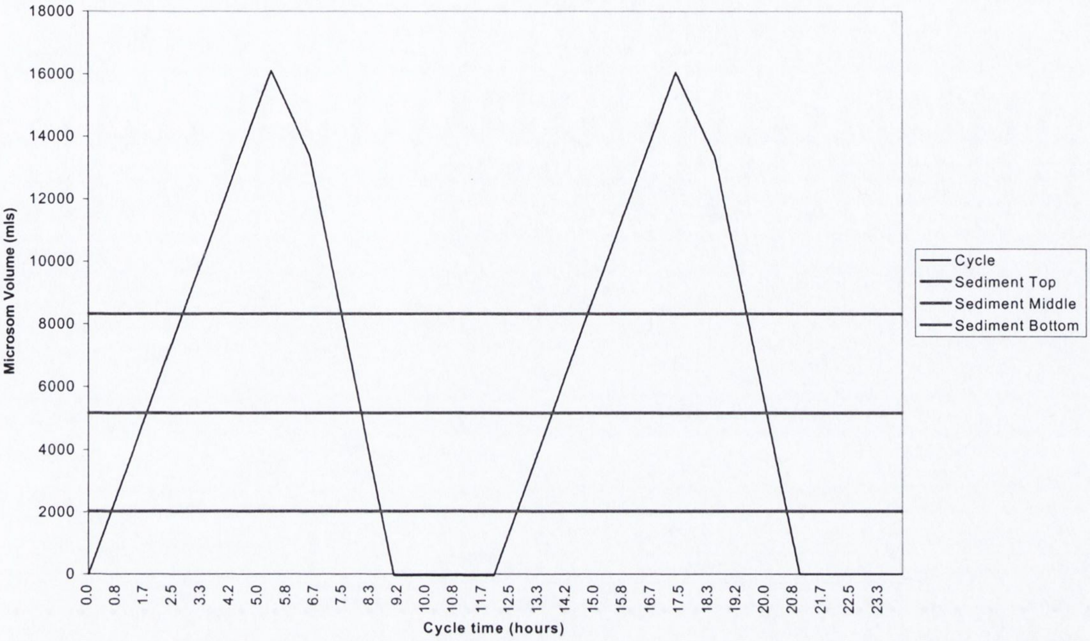


Fig. 5.1: *Approximate microcosm seawater cycle periods (break in slope due to pump off period)*

5.3 TEMPERATURE

Daily monitoring of temperatures at three different locations in the room indicated temperatures were constant throughout the experimental space. Temperature details are given in table 5.1 and figure 5.2. An electric fan was used to elevate nighttime air temperatures to ensure that degradation continued during the winter months. Air temperature fluctuations during the course of the experiment can be considered typical of a field situation.

	Average (°C)	Standard Deviation
Daily minimum temperatures	17.2	20%
Daily maximum temperatures	23.6	15%
Daily temp. differences	6.3	34%

Table 5.1: *Microcosm trials air temperatures – descriptive statistics*

Temperature limits microbial metabolism and at low temperatures, slower biodegradation is to be expected. Hydrocarbon biodegradation has generally found to be optimal at 30-40°C (Bossert & Bartha 1984) but at lower temperatures environmental populations with lower optimal temperatures may be selected (Morgan & Watkinson 1989). Temperature may also affect biodegradation by affecting individual hydrocarbon solubility in water and influencing fluidity of the hydrocarbon bulk. The lower the viscosity, the greater the ease of spreading, thus presenting a greater surface area to micro-organisms for biodegradation. In reality, it is difficult to predict the affect of temperature on selection of microbial populations in the environment. At best, a knowledge of the general effect of temperature can probably assist in the decision making process of whether to bioremediate or not.

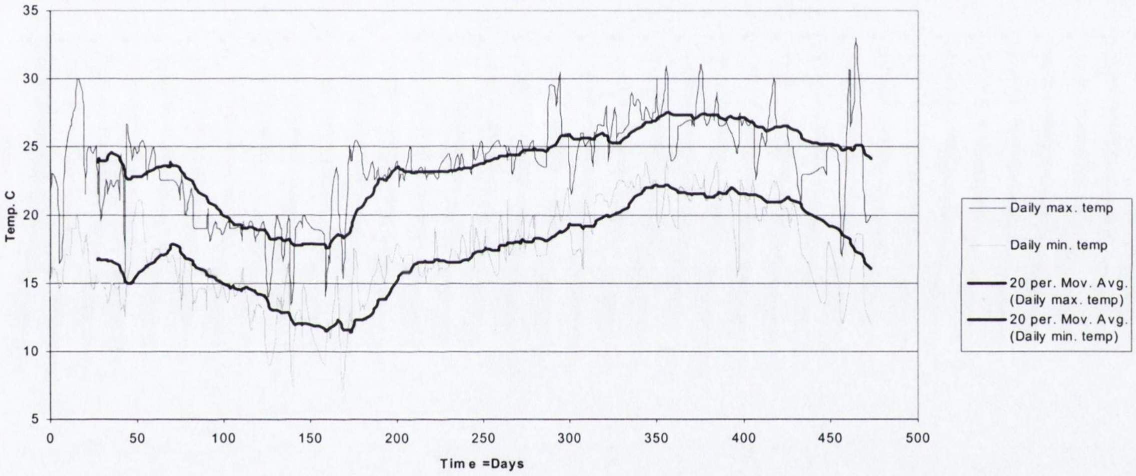


Fig. 5.2: Air temperature fluctuations over the course of the experiment. Daily maximum and minimum temperatures are plotted and general trends depicted using moving averages.

5.4 NUTRIENTS

Much literature has indicated that nutrients are a key limiting factor in shoreline biodegradation of petroleum hydrocarbons (Floodgate 1984). Many fertiliser formulations have been used to supplement nitrogen and phosphous levels (Swannell *et al.* 1996). The use of oleophilic fertilisers such as Inipol EAP22™ (Elf Aquitane) and slow release fertilisers such as Customblen™ (Pritchard 1991), and Max-Bac™ (Oudot *et al.* 1998) and applications of nutrient solutions have been investigated (Venosa *et al.* 1996).

In the course of this experiment real seawater was used as opposed to artificial seawater as the intention of the project was to simulate field conditions as much as possible. Thus, the C1 seawater control contains a natural level of nutrients and T1-T3 microcosms contain seawater supplemented with a nutrient formulation. The seawater was collected from the Dublin bay area and salinity analysis indicated that the water did not contain significant freshwater. Limited analysis indicated that the stock seawater contained relatively high nutrient levels. In stock seawater, total oxidised nitrogen was ~32.4µmol/l which was effectively entirely attributable to nitrate. Silicate levels were measured at ~7.4µmol/l and ortho-phosphate at ~1.8µmol/l. These results are not untypical of Dublin Bay area and total oxidised N is below the winter median for the inner bay/estuary (Anon. 1999). The design in this experiment involved recirculation of seawater with a change approximately every eight weeks, unlike a field situation, which would receive a fresh supply of background nutrients at each tidal cycle.

It is difficult to predict when nutrient concentrations may be limiting for a given situation. Most estimates of optimal N and P are based on batch studies of oil biodegradation in solution (Atlas 1981, Floodgate 1984). Much research has shown an increase in biodegradation in solution, on shorelines (Bragg *et al.* 1996, Swannell *et al.* 1996, Venosa *et al.* 1996) and in soils (Bossert & Bartha 1984, Morgan & Watkinson 1989) with nutrient supplementation, indicating that in these situations it was limiting. LePetit and N'Guyen (1976) estimated that the phosphorus concentration required for optimum growth was $2 - 8 \times 10^{-4}$M in seawater when salinity was close to the oceanic mean and 1.5×10^{-3} M at lower salinities. Other studies have shown nitrogen requirements in the range of 3.2 – 11 mg/l and phosphorus of 0.6 – 2 mg/l (Floodgate 1984). Optimum C/N ratios in soils have been reported from 9:1 to 200:1 (Morgan & Watkinson 1989). Floodgate (1979) proposed the concept of a nitrogen demand, analogous to a biochemical oxygen demand. Based on data for natural Irish Sea populations challenged with Kuwait crude at 14°C, a nitrogen demand of ~4nmoleN/ng oil was proposed. If this was to hold true for the microcosm units in this experiment, it would suggest severe nitrogen limitation in the C1 units, with only minimal degradation occurring. However, significant biodegradation was shown in C1 units over the course of the experiment as presented later in the report. A number of shoreline bioremediation studies have found high background levels of oil biodegradation without nutrient supplementation. For a study in France, background N concentrations of 70 – 280 μM resulted in high rates of biodegradation for a topped light Arabian crude oil, and nutrient addition did not significantly increase the rates (Oudot *et al.* 1998). Venosa *et al.* (1996) also recorded high biodegradation rates for a Nigerian light crude oil in an experimental spill in Delaware Bay with background nitrate of ~60 μM. Nutrient addition resulted in a relatively minor but significant increase in biodegradation. Nutrient addition did significantly enhance biodegradation during the *Sea Empress* spill bioremediation trials (Swannell *et al.* 1999) but background nutrient levels were lower 25-184 μg/l total inorganic nitrogen (~1.8-13.1μM).

A study of oil biodegradation in seawater after the gulf war spill, reported that the otherwise rapid degradation of aromatic compounds was inhibited by addition of nutrients while saturate degradation, as measured by C17:pristane ratios, was enhanced (Fayad & Everton 1995).

The bioremediation programme undertaken after the *Exxon Valdez* spill involved treating shorelines with Inipol EAP 22 and Customblem fertilisers. Bragg *et al.* (1994) concluded that biodegradation rates depended mainly on the concentration of nitrogen within the shoreline, the oil loading, and the extent to which natural biodegradation had already taken place.

The nutrient formulation used for supplementation for theT1-T3 microcosms was a modified Bushnell-Haas described in the NETAC/US EPA guidelines for ‘Oil spill bioremediation products testing’ (Anon. 1991a). This formulation includes many essential minerals as well as N and P and is the same media used in respirometry trials in phase I. This has been demonstrated to be an effective media for supporting metabolism of hydrocarbons in the laboratory.

Algal growth could have resulted in competition for nutrients in the microcosms and was avoided by maintaining the experiment in a darkened environment. Algal growth was not observed during the course of the trials.

5.5 SEDIMENT CHARACTERISTICS

Prior to the experiment the sediment was sieved to a range of >250μm and < 2mm. A particle size distribution (PSD) shows that the sediment particle size is completely within this range and well distributed within the range (fig. 5.3 & table 5.2).

BS Sieve Size (mm)	% Passing
5	100
2.36	100
1.18	85
0.6	62
0.425	39
0.3	11
0.212	0
0.15	0
0.075	0

Table 5.2: Particle size distribution sediment

Fig. 5.3: Particle size distribution of the microcosm sediment. Percentage passing through sieve.

Hexane soxhlet extractable material in uncontaminated sediment was negligible, (<0.03% w/w). The sediment density was measured at 1.6kg/l and the pore size was estimated, by displacement, as 39% of the total sediment volume. Some simple experiments were carried out to investigate water holding and drainage characteristics of the sediment. The resistances to gravity assisted passage of water through Alba contaminated and uncontaminated sediment held in a Buchner funnel (~2 cm depth, 7.5 cm diameter) were similar for both seawater and freshwater. Wet weight/dry weight (WWDW) determinations indicated that water drained very rapidly from contaminated sediment to equilibrium, and residual water (13% v/v, 34% pore volume) was retained over a 24-hour period.

The test results have implications for aeration/oxygenation of the sediment as well as exchange of seawater within the sediment. Contaminated sediment, despite the presence of hydrophobic hydrocarbons did not offer a noticeably greater resistance to seawater penetration. The sediment drainage reaches a certain level rapidly but further drainage will not occur within a single microcosm cycle period.

Water was observed to flow easily through the mesh bags containing the sediments at all stages of the trials. Removing sediment cores to the centre of the sediment bulk contained in the mesh bags during fill and empty cycles allowed visual observation of the water penetration within the bulk sediment. As the water level rose in the surrounding vessel the water level rose in the drilled sediment well at the same level. During the empty cycle the water clearly drained with the lowering of the external water level. This indicates that water penetration into the bulk sediment during the fill cycle and subsequent water drainage occurred effectively, enabling efficient air and water exchange.

A WWDW determination was carried out on sediment samples taken from the centre of the sediment mesh bags for each experiment at t=350. The samples were taken toward the end of a ‘low tide’ period. Results are tabulated in table 5.3 and indicate that the water retention is generally lower for microcosm sediments than observed for the previous (buchner funnel) tests. Approximately 77% of the pore volume is available for air transfer during ‘low tide’ periods.

Microbial activity takes place on dissolved phase hydrocarbons or at the oil – water/air interface, unless the interface is due to a water in oil emulsion thus limiting oxygen. The surface area:volume ratio of the bulk hydrocarbon is important to maximise biodegradation. As oil was well mixed into sediment, it can be assumed that the oil presents a high surface area per gram of sediment for microbial activity to take place.

Duplicate		***Alba***	***Forties***
I	*T1*	24.3%	31.2%
	T2	21.5%	23.8%
	T3	23.6%	27.6%
	C1	26.3%	23.2%
	C2	19.5%	21.0%
II	*T1*	28.6%	24.4%
	T2	19.0%	23.5%
	T3	28.2%	20.6%
	C1	19.9%	19.0%
	C2	20.8%	15.5%
	Average	*23.2%*	*23.0%*
	Std. Dev.	*3.6%*	*4.4%*

Table 5.3: *Moisture content of drained microcosm sediments at t=350 as % volume of pore space*

5.6 WATER PARAMETERS

The above results indicate effective seawater penetration into bulk sediment, and subsequent drainage and air replacement, suggesting that oxygen levels within the sediment were probably representative of near ideal field conditions. Dissolved oxygen, pH, salinity, water temperature and specific conductivity values for microcosm seawater just prior to water changes are given in table 5.4. Measurements of dissolved oxygen levels in the seawater reservoirs prior to water/nutrient changes indicated that levels were quite low. The low levels of dissolved oxygen in the microcosms would not be typical of a field situation, (which would experience a constant supply of new seawater and wave action).

Seawater is a well-buffered media. pH values for the microcosms seawater on 4 different occasions just before a water change are listed in table 5.4 and in general the treated microcosms (T1-T3) are slightly lower in pH than the controls, possibly due to microbial activity in the treated microcosms. Aerobic hydrocarbon metabolism usually involves the production of acids such as carboxylic acids that may result in lowering of pH. However, as the pH for all samples is in the range 6-8 it should not hinder biodegradation.

Time (days)	*t=27*	*t=54*	*t=386*				*t=467*			
Sample	**pH**	**pH**	**Temp (°C)**	**DO mg/l**	**Salinity**	**PH**	**Temp (°C)**	**DO mg/l**	**Salinity**	**pH**
AiT1	7.25	7.12	23.0	3.2	33.4	6.97	17.8	5.0	33.7	7.63
AiT2	7.21	7.11	22.9	3.0	33.7	6.96	18.1	4.4	34.2	7.73
AiT3	7.22	7.06	23.1	3.5	33.2	6.92	17.9	4.6	33.5	7.68
AiC1	7.81	7.48	23.1	3.5	33.8	7.44	17.8	5.6	33.9	8.2
AiC2	8.06	7.83	22.8	5.7	36.3	7.94	16.6	-	37.5	8.33
FiT1	7.47	7.24	22.6	2.8	33.2	6.84	17.9	5.1	33.5	7.33
FiT2	7.44	7.2	22.6	3.1	33.3	7.00	17.9	4.7	33.6	7.62
FiT3	7.44	7.18	22.6	1.5	33.3	6.88	17.9	3.8	33.6	7.64
FiC1	7.86	7.45	23.3	3.6	33.8	7.35	17.8	5.4	34.1	8.02
FiC2	8.03	7.75	23.0	5.7	36.2	7.97	17.2	-	36.3	8.34
AiiT1	7.32	7.08	23.0	3.0	33.3	6.91	17.6	5.3	33.3	7.64
AiiT2	7.1	6.91	22.9	3.5	33.2	6.91	17.4	5.1	33.4	7.61
AiiT3	7.37	7.1	23.9	2.6	33.3	6.91	17.5	5.2	33.4	7.71
AiiC1	7.85	7.58	23.0	3.7	33.7	7.57	17.3	5.4	33.8	8.23
AiiC2	8.01	7.76	22.7	5.6	39.9	7.91	17.3	-	39.9	8.24
FiiT1	7.42	7.14	22.6	3.3	33.1	6.91	17.3	5.1	33.2	7.6
FiiT2	7.46	7.22	22.6	3.8	33.3	6.93	17.3	4.6	33.6	7.73
FiiT3	7.42	6.84	22.6	3.8	32.8	6.91	17.2	4.8	33.0	7.6
FiiC1	7.83	7.6	23.3	4.1	33.8	7.67	17.5	5.2	34.2	8.21
FiiC2	7.93	7.79	23.0	5.7	35.3	7.93	17.1	-	38.8	8.38
Average			*22.9*				*17.4*			
Std. Dev.			*0.3*				*0.8*			

Table 5.4: Seawater parameters in microcosms

5.7 OXYGEN

Oxygen is required for aerobic biodegradation to take place and is often a limiting factor within sediments. There are three sources of oxygen in bioremediation: water dissolved, air, and oxygen incorporated in larger molecules, such as polar petroleum compounds. Oxygen incorporated in petroleum molecules is not a significant factor in aerobic bioremediation of crude oil. The measured microcosm seawater oxygen content was low and obviously was not being replenished in the bulk water at a rate equivalent to microbial use. However, oxygen solubility in seawater is low and oxygen content in saturated bulk seawater would still be a limiting factor based on the following calculations. Oxygen has a low solubility in water (~8mg/l) and it has been calculated that oxidation of 1 litre of hydrocarbon will exhaust the dissolved oxygen (DO) of 385,000-400,000 litres of water (Atlas & Bartha 1992). A similar calculation can be carried out for this experiment. Assuming, that each tidal flush contained saturated DO of 8mg O_2 / litre and knowing a sediment pore volume of 39%, this implies a DO input of 1.95mg O_2 kg sediment^{-1} .cycle^{-1}. Using a stoichiometric oxygen requirement based on the following formula,

$$(\text{-}CH_2\text{-}) + 1\tfrac{1}{2}\,O_2 = CO_2 + H_2O$$

and knowing the oil concentrations used in this experiment, and assuming each tidal cycle resulted in total utilisation of the saturated O_2, it can be calculated to require a minimum of 37 and 71 years to achieve total biodegradation of the Forties and Alba oils in the microcosms respectively, using bulk water dissolved oxygen alone.

During the empty cycle, the atmospheric oxygen introduced into the pore space by water displacement results in an input of approximately 56mg O_2 kg sediment^{-1}.cycle^{-1}. Further replenishment of pore space gaseous O_2 will also take place during an 'empty cycle' by diffusion as O_2 is utilised by microorganisms.

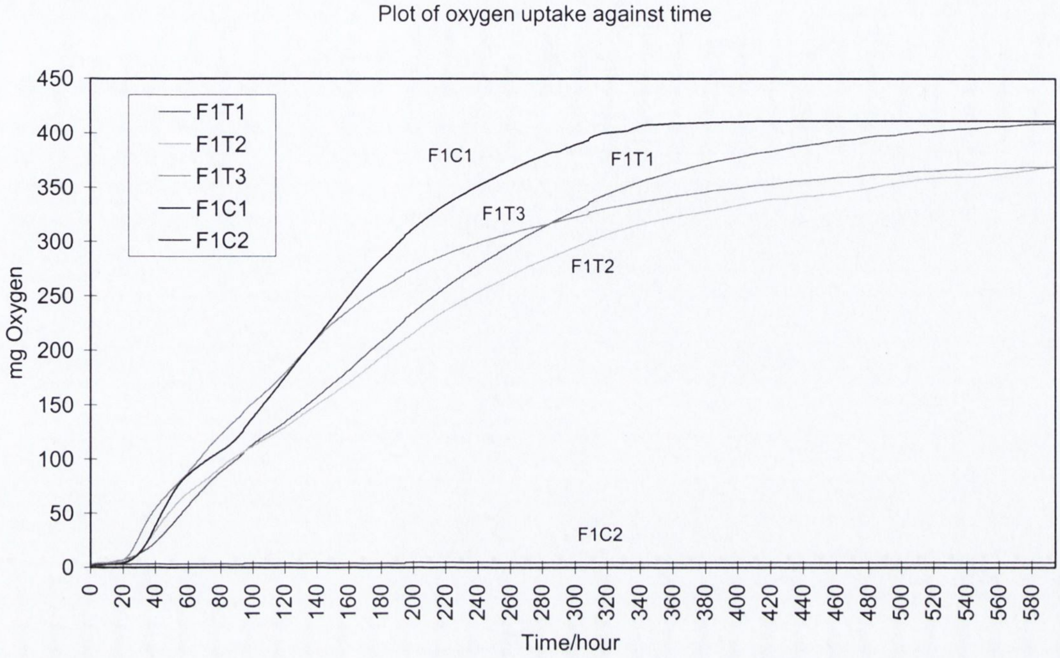
The results and calculations indicate that oxygen was unlikely to be a limiting factor during the course of this experiment, especially considering the complex nature of the substrate used, for which rapid biodegradation is unlikely. This trial was meant to simulate field conditions, and subsurface sediment contamination in field situations will, more often than not, have a sediment structure where oxygen limitation is much greater. Introduction of gaseous O_2 into the pore space is the major route of oxygen acquisition, with bulk seawater DO being relatively unimportant.

This illustrates one of the advantages of a shoreline cycle as opposed to soil bioremediation. If the sediment drains efficiently with ebbing tide, the falling water level will draw air into the interstitial sediment space. Soil bioremediation relies on oxygen diffusion and forced aeration and/or oxygenation through oxidising agents such as hydrogen peroxide. The use of bulking agents mixed in with soil or sediment can improve drainage and oxygen transfer through soil/sediment. Although, tidal flushing is an important mechanism for aeration of subsurface sediments, it is important to consider sediment structure and tidal dynamics when proposing bioremediation of a shoreline with subsurface contamination.

5.8 MICROBIOLOGY

The main focus of this project was on the alteration of UCM chemistry during bioremediation. Consequently, only limited microbiological testing of the microcosms was carried out.

Respirometry trials were carried out on sediment washes from samples taken from the microcosms on day t=280. This was intended to establish whether, after this period, the microbial population in the inoculated treatments, T1 and T2, exhibited greater potential for UCM biodegradation than the uninoculated treatment T3 and the seawater control. Duplicate sediment washings were pooled and used as inocula for the respirometry, with Alba UCM as the sole carbon source. The results, shown in figure 5.4, indicate that there was no microbiological activity in the poison controls (C2). For Forties microcosms, the C1 sample showed the maximum respiration with the T1 having a slower initial rate. For the Alba the T1 exhibited the greatest initial respiration rate with T2 showing slightly higher total respiration over the entire experiment time. T3 respiration leveled off at ~200 hours. Significant differences between the treatments T1-T3 and C1 are not evident, suggesting that the initial inocula either did not establish sufficiently to achieve enhanced UCM biodegradation in the T1 microcosms or that in the intervening period, the indigenous population had ‘caught up’ with the added inocula.



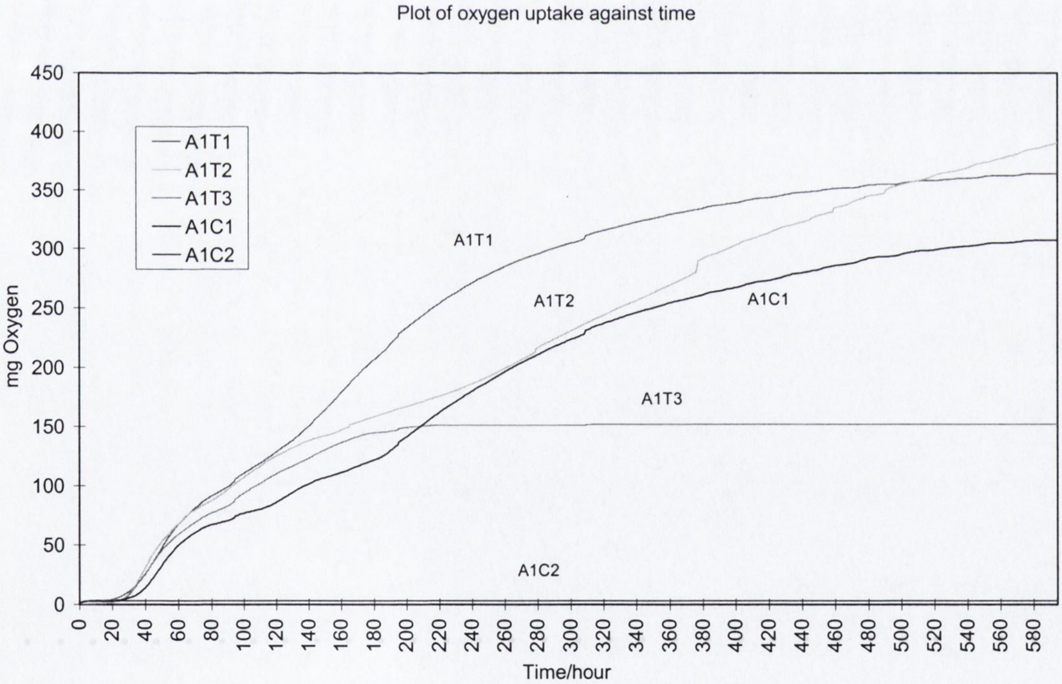


Fig. 5.4: Respirometry trials on microcosm sediments washes (sampled at t=280 days) Top = Forties trials, bottom = Alba trials. Duplicate microcosm sediment washes were pooled for respirometry.

5.9 MICROCOSM AND FIELD SITUATION: A COMPARISON

This pilot study was carried out using microcosms rather than field trials and the following points should be taken into consideration:

- All field situations are unique and for bioremediation there is a very complex array of environmental factors involved. No field trial can definitively demonstrate that the technology will work in another field situation.
- Field trials are by their nature heterogeneous, while greater homogeneity can be achieved in a microcosm system. This ensures that control experiments are representative of the treated experiments. In this case, it can be demonstrated that microcosm PSD, water exchange cycles, initial contamination levels and distribution are near identical for each experimental unit for a given oil type. This would be extremely difficult to achieve in a field situation.
- Much greater sampling and analysis is required in a field trial to control variability. Considering the large analytical workload for this project, this would be impracticable.
- The control and measurement of many parameters is possible in a microcosm study that would be difficult if not impossible in a field trial.

In attempting to assess different bioremediation treatments and controls the following sources of variability limit the ability of a monitoring programme to discern significant differences between treatments.

- *Analytical variability* – variability inherent in measurement of a parameter
- *Sample variability* – a measure of the representativity of a sample for the entire population (treatment plot).
- *Treatment variability* – variability between replicate treatments

In field situations, sample variability is typically high due to the inherent heterogeneity of oil contaminated sediment. Techniques such as pooling samples and analysing replicates from a given treatment plot may be used to reduce this variability, but this can greatly increase the analytical workload. The microcosm design used for this experiment ensured initial homogeneity of sediment contamination by mixing of the total sediment prior to sampling, thus minimising sample error. Furthermore, the ability to control other parameters (such as seawater exposure, sediment characteristics etc.) reduces the variability inherent between replicate treatments. It is impossible to exert the same degree of control of these factors in a field situation.

The reasons outlined above illustrate the importance for using microcosm systems to evaluate and understand bioremediation and the processes involved. This does not preclude the necessity of carrying out trials and evaluations to demonstrate the efficacy of bioremediation in the field.

6. BIOREMEDIATION TRIAL RESULTS II CHEMICAL ASSESSMENT OF BIODEGRADATION

6.1 VISUAL

Oil was washed from sediments and adhered to the walls of the reservoirs. However, the oiled reservoir walls were most evident for the C2 tests, and for the C1 tests to a lesser extent. Much less oiling occurred for the T1, T2 and T3 tests suggesting enhanced biodegradation of the washed oil in treatments to which nutrients have been added. This pattern was evident for the duplicate Alba and Forties experimental sets.

6.2 GRAVIMETRIC ASSESSMENT

6.2.1 Extractable matter

Four randomly selected samples from the Alba and Forties spiked sediment were analysed initially to demonstrate the homogeneity of the sediments before use in the microcosms. The method is given in appendix A1 and data in appendix D2. Averaged gravimetric results for these samples were used as t=0 values. Alba and Forties contaminated sediment was also stored for use as a reference material throughout the trials for assessment of analytical uncertainties. The descriptive statistics for the gravimetric results for the reference sediment are given in table 6.1.

	Alba t=0	***Forties t=0***	***Alba t=0 Total reference***	***Forties t=0 Total reference***
Mean mg/g	29.6	16.6	30.9	16.0
Std. Dev.	2.7	0.2	2.6	1.1
RSD	9.2%	1.0%	8.4%	6.9%
n	4	4	13	10

Table 6.1: *Gravimetric results for t=0 reference sediment. Column 1& 2 present statistics based on four initial t=0 samples. Columns 3 & 4 present statistics for the total t=0 reference samples analysed over the course of the trials.*

The results indicate a higher degree of uncertainty in gravimetric determination of Alba compared with Forties contaminated sediment. For the initial t=0 sample this is largely attributable to one result of the four. Over the course of the experiment, the long-term uncertainty is largely attributable to batch-to-batch analytical differences, as the results for t=140 and 203 are somewhat lower than anticipated for all samples at these times.

Gravimetric analysis exhibited a steady decrease in oil concentration in microcosm sediments over the course of the trials. Gravimetric analysis of all sediments was carried out at t=0, 28, 56, 98, 140, 203, 280, 350, 407 and 473 days, (appendix A). The percentage reduction from the t=0 samples (i.e. based on all t=0 measurements) is given for all samples at the final sampling point (t=473) in table 6.2

	Alba			***Forties***		
	I	***II***	***Mean***	***I***	***II***	***Mean***
T1	50.7%	47.7%	49.2%	68.1%	65.5%	66.8%
T2	54.1%	49.7%	51.9%	66.5%	64.0%	65.3%
T3	47.8%	39.4%	43.6%	71.9%	67.6%	69.8%
C1	41.2%	39.7%	40.4%	58.3%	63.8%	61.0%
C2	63.2%	60.2%	61.7%	15.5%	23.5%	19.5%

Table 6.2: Percentage reduction of oil concentrations in microcosms at t=473 days

The trends observed at t=473 were typical of all sampling periods. The results indicate that the duplicate microcosms behaved similarly, and this was observed throughout the experiment. For the Forties sediment, slightly more oil was removed from the treated sediments than for the seawater control (C1). Approximately 20% of oil was lost from the poison control sediments, probably due to sediment washing. Due to the higher concentration of Alba, compared with Forties, in the initial sediments, more oil was removed in those sediments, although this is still generally less than 50% of the oil. Again the treated samples (T1-T3) show slightly more oil removal than the seawater control samples (C1) but, interestingly the poison control shows greater oil removal. Although the Forties oil was actually weathered by water washing at sea before use in this experiment, it is unlikely that solubilisation of components in the Alba compared to the Forties is responsible for such a substantial concentration reduction in the poison control, as both oils are pentane soluble fractions. Presumably, the removal of oil in both Alba C2 test units is due to water washing of the oil from the sediments, but why it is greater in the poison controls than the other units is not clear, although possible explanations are discussed later in this thesis. Respirometry studies showed no biological activity in C2 sediments. The absence of the *n*-paraffin waxes in Alba oil results in a lower viscosity than the Forties and thus it is probably more amenable to washing from the sediment. As a pentane soluble UCM it may behave in a similar manner to a hydrocarbon based lubricating oil, (which consist primarily of aliphatic UCM – see phase I). For both Alba and Forties contaminated sediments, there is no evidence from the gravimetric results that addition of micro-organisms have enhanced biodegradation.

There was clear batch-to-batch analytical variation for gravimetric results and this can be corrected by normalising with the results for the Alba t=0 reference samples that were analysed at each sampling period. This reduces the RSD for the other reference material (Forties t=0) from 8.0% to 5.4% for the time series. In general the duplicate experiments compare well. The Alba reference normalised graphs are given in figures 6.1 a&b.

A simple kinetic model is used to estimate the rate of biodegradation of hydrocarbons and to compare the treatments. Venosa used first order rate model to determine biodegradation rate constants for bioremediation of an experimental spill in Delaware Bay (Venosa *et al.* 1996). His model used the washout rate of C30-hopane in determining the rate of oil removed solely by biodegradation. In this case first order integrated rate equations (eq. 6.1) are used to compare rate constants based on the complete dataset (both duplicates) for each treatment.

$$d[A]_t/dt = -k[A]_t \qquad \text{(eq. 6.1)}$$

$[A]_0$ is the initial concentration of oil, $[A]_t$ is the concentration at time=t and –k is the rate constant. The integrated rate equation (eq. 6.2) can be solved using regression statistics.

$$[A]_t = [A]_0 e^{(-kt)} \qquad \text{(eq. 6.2)}$$

The transformed version (eq. 6.3) yields the equation for a straight line which can also be solved using linear regression.

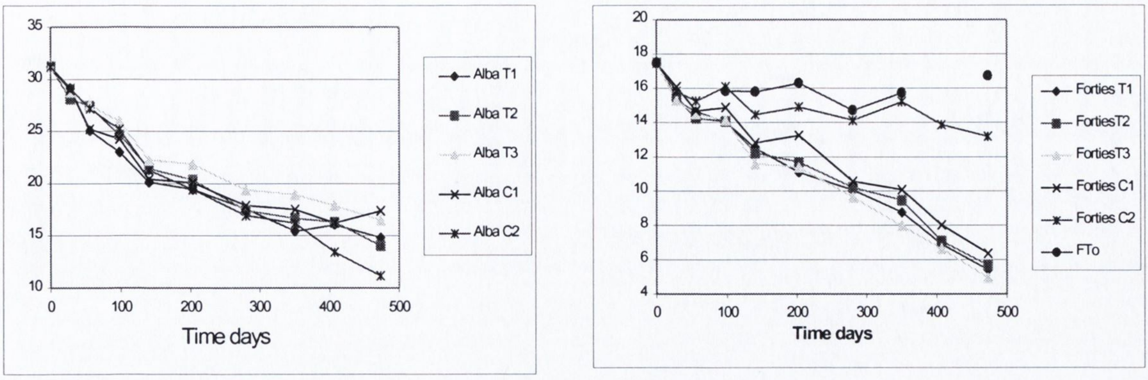
$$\ln[A]_t = -kt + \ln[A]_0 \qquad \text{(eq. 6.3)}$$

The calculated rate constants and regression statistics were calculated with SPSS software, using Levenberg-Marquadt non- linear regression. It should be noted that this is a simple model that does not account for the growth of microbial population, the complexity of the substrate, the effect of nutrient concentration, temperature fluctuation and other environmental factors. The results are given in table 6.3.

Forties	***k (day^{-1})***	***±95% CI***	***R^2***
T1	2.24×10^{-3}	0.16×10^{-3}	0.965
T2	2.14×10^{-3}	0.18×10^{-3}	0.948
T3	2.39×10^{-3}	0.18×10^{-3}	0.964
C1	1.84×10^{-3}	0.16×10^{-3}	0.945
C2	6.36×10^{-4}	1.37×10^{-4}	0.318
Alba			
T1	2.01×10^{-3}	0.25×10^{-3}	0.856
T2	1.88×10^{-3}	0.20×10^{-3}	0.918
T3	1.55×10^{-3}	0.21×10^{-3}	0.840
C1	1.80×10^{-3}	0.28×10^{-3}	0.774
C2	2.16×10^{-3}	0.13×10^{-3}	0.976

Table 6.3: Estimated rate constants (k), 95% confidence intervals, and coefficients of determination (R^2) for non-linear solutions of gravimetric biodegradation using the model given in equation 6.2.

The coefficient of determination (R^2) value is a measure of how well the data fits the simple biodegradation model. R^2 values suggest a reasonably good fit for Forties but less so for Alba. The duplicates demonstrate good agreement throughout the course of the experiment, with the exception of AT3. AiiT3 exhibited a lower biodegradation rate than other nutrient supplemented microcosms and this is reflected in the lower k value estimated for AT3. The Forties results indicate a higher biodegradation rate for the nutrient treated samples (T1-T3) as opposed to the C1sample and that biodegradation is the primary mechanism of removal. The wash out rate of the Alba oil as indicated for the C2 experiment is even greater than the removal (washout + biodegradation) for the other Alba experiments (T1-C1).



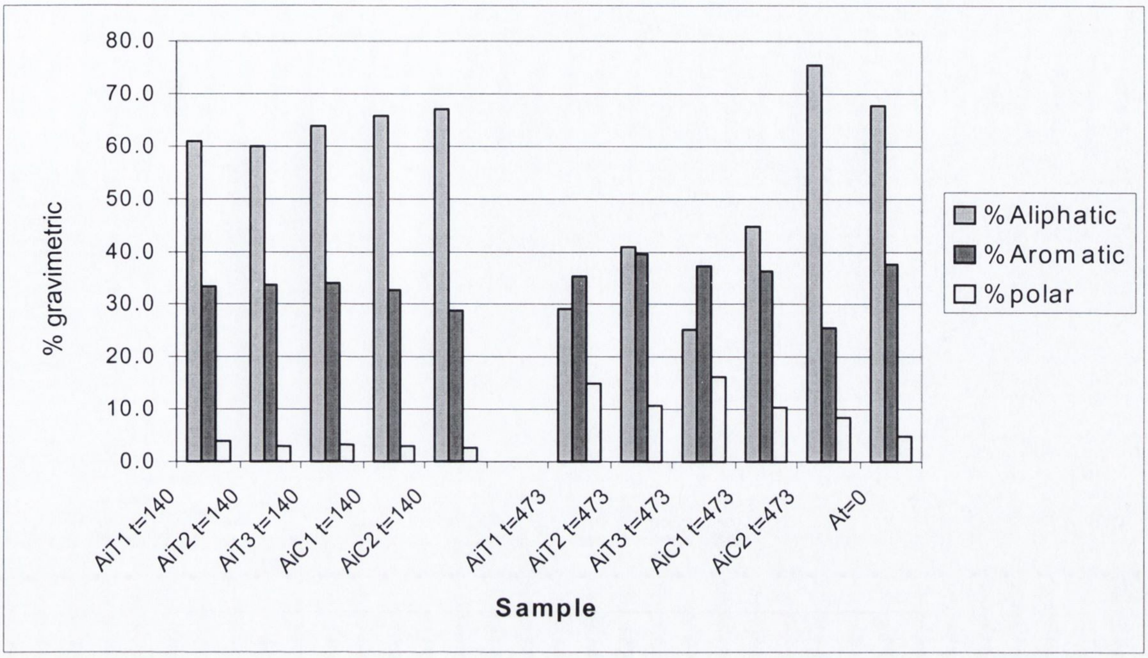
a) Alba (duplicate mean) sediment concentrations

b) Forties (duplicate mean) sediment concentrations

Fig. 6.1: Gravimetric reduction of hydrocarbon in microcosms (normalised to Alba reference samples) [y-axes: concentration mg oil/g sediment - normalised to t=0 for each batch using Alba t=0 reference oil samples]

6.2.2 Fractionation

Alumina and silica gel column chromatography was used to achieve aliphatic, aromatic and polar class fractionation for t=0, 28, 140, and 473. For Alba and Forties microcosms, fractionation shows that biodegradation of the aliphatic UCM to be substantially greater than the aromatic component, and that the polar fraction is most resistant, when compared to the poison control and t=zero samples. This is illustrated in figure 6.2. This is in agreement with many previous observations (Oudot 1984, Oudot *et al.* 1998). The t=473 experiments suggest that a greater reduction in the aliphatic proportion has occurred in the nutrient supplemented trials compared with C1 microcosms.



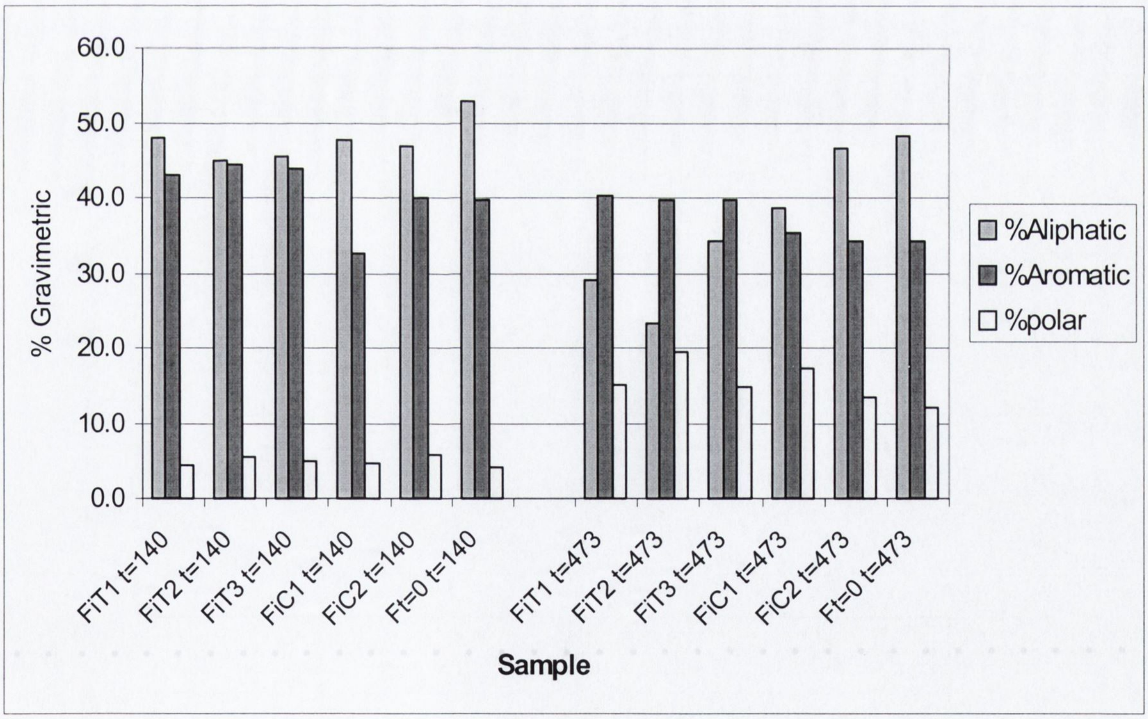


Fig. 6.2: *Gravimetric results expressed as % aliphatic, aromatic and polar components for Alba (i) (top) and Forties (i) (bottom) microcosms at t=0, 140 and 473 days.*

6.3 CHROMATOGRAPHY

6.3.1 Gas chromatography

Gas chromatography with flame ionisation detection (GC-FID) was used to quantify and assess biodegradation of resolved *n*-alkanes and isoprenoids as well as UCM biodegradation. The methodology is described in appendix A.7 and raw data is presented in appendix D3. For the purpose of this project the UCM is regarded as hexane soluble oil, excluding *n*-alkanes. Thus, the Alba oil is considered to consist of UCM only. For Forties contaminated sediment, using an *n*-alkane standard to quantify *n*-alkanes and heptamethylnonane as a UCM model compound for quantifying UCM, it is estimated that in the region of 90% of the oil is UCM, by weight. This illustrates the importance of considering UCM degradation in bioremediation experiments as monitoring of *n*-alkane degradation alone may severely overestimate total oil biodegradation.

n-Heptadecane (*n*C17) –pristane (Pr) ratios are frequently used as indicators of biodegradation as they are resolvable by GC, and as pristane (2,6,10,14 tetramethylpentadecane - an isoprenoidal C19 alkane) is more resistant to biodegradation than the straight chain *n*C17. The *n*C18:phytane (a C20 isoprenoid) ratio can be similarly used. Examining the *n*C17:pr and *n*C18:phy ratios over the course of the microcosm trials for mean results from the duplicate Forties oil units, shows a rapid decrease in both ratios for T1, T2, T3 and C1 until t=140 days. This remained stable before increasing again as the rate of biodegradation of pristane and phytane became greater than that of residual *n*-alkanes. This was not evident for the C2 poison controls indicating that no biodegradation had taken place for these experiments. These results clearly demonstrates the limitations of using these ratios for assessing biodegradation and such indicators are only suitable for monitoring the early stages of biodegradation of crude oil. The *n*C17:Pr and *n*C18:Phy ratio reductions are initially greater for the treated samples as opposed to C1 samples and the subsequent increase is less. This suggests enhanced degradation for the nutrient treated microcosms but no differences for inoculated microcosms.

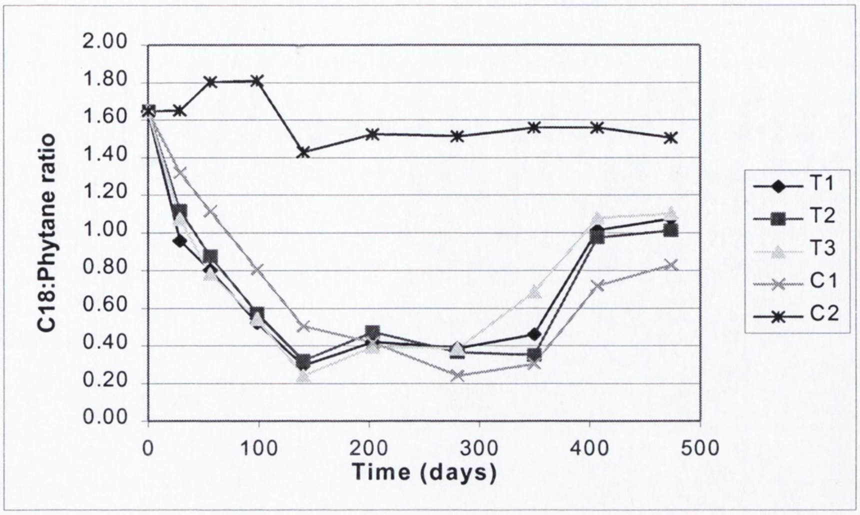


Fig. 6.3: *Forties C18:phytane ratios vs. time for microcosms (mean of duplicate units)*

Quantification of oil components using GC was carried out by reference to the same components in Forties t=0 reference samples that were analysed at intervals in each batch of GC analyses. By bracketing with reference samples, *n*-alkanes, pristane and phytane , UCM 'segments' and total UCM were quantified relative to the same components in the reference sample. The segments of UCM were defined as the UCM section eluting between two consecutive *n*-alkanes, not including the *n*-alkane area, and background subtracting to correct for baseline drift. (i.e. UCM (*n*C23-C27) is the relative quantity of UCM eluting in the interval between *n*C16 to *n*C20 but subtracting all *n*-alkane contributions).

The Forties contaminated sediment showed clear biodegradation of *n*-alkanes and isoprenoids based on measured relative concentrations of components, *n*-alkane:isoprenoid ratios and changes in profile of the gas chromatograms. UCM was also substantially biodegraded. Biodegradation took place across the entire molecular weight/boiling point range of the oil. For Forties microcosms, using the model described in equation 6.2, the rate constants and 95% confidence intervals were calculated using non linear regression for total hydrocarbon and selected components.

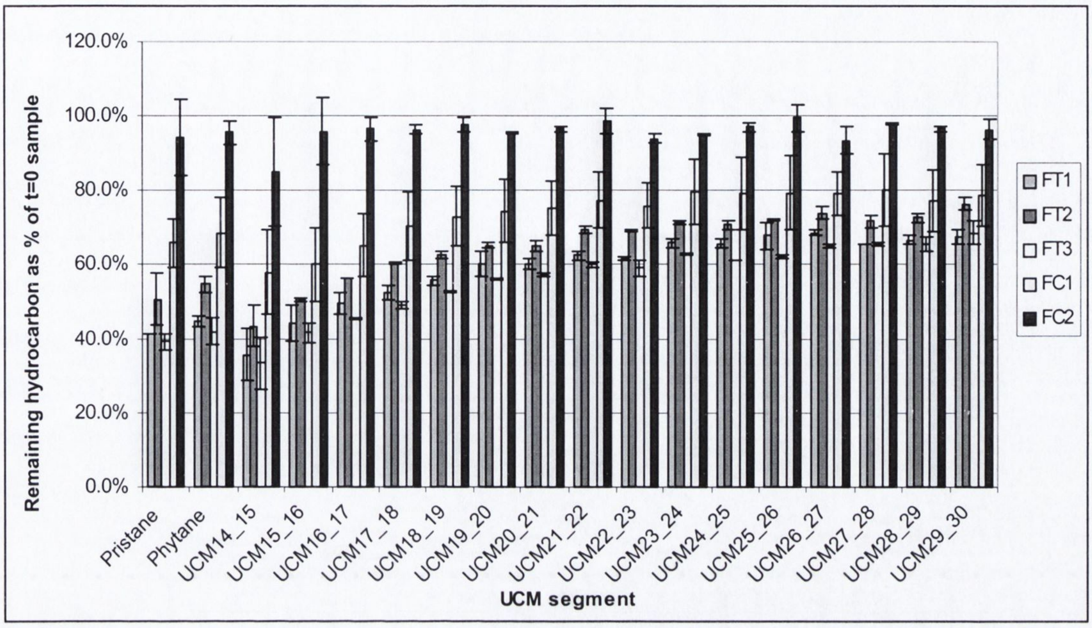
	FT1		***FT2***		***FT3***		***FC1***		***FC2***	
	K $x10^{-2}.day^{-1}$	R^2	***K*** $x10^{-2}.day^{-1}$	R^2	***K*** $x10^{-2}.day^{-1}$	R^2	***K*** $x10^{-2}.day^{-1}$	R^2	***K*** $x10^{-2}.day^{-1}$	R^2
nC15	1.39 ± 0.29	0.873	1.30 ± 0.33	0.786	1.51 ± 0.36	0.834	0.81 ± 0.11	0.950	0.01 ± 0.03	0.032
nC20	1.09 ± 0.23	0.823	0.90 ± 0.18	0.840	1.14 ± 0.24	0.859	0.63 ± 0.05	0.983	<0.01 ± 0.01	0.046
nC25	0.74 ± 0.11	0.940	0.69 ± 0.11	0.927	0.83 ± 0.12	0.946	0.50 ± 0.11	0.875	0.02 ± 0.02	0.001
nC30	0.48 ± 0.13	0.856	0.44 ± 0.11	0.862	0.52 ± 0.13	0.875	0.40 ± 0.19	0.744	0.03 ± 0.05	0.299
nC35	0.30 ± 0.10	0.768	0.24 ± 0.19	0.687	0.31 ± 0.10	0.793	0.26 ± 0.09	0.733	<0.01 ± 0.04	0.011
Phytane	0.28 ± 0.08	0.815	0.24 ± 0.08	0.744	0.31 ± 0.08	0.839	0.21 ± 0.07	0.723	0.02 ± 0.02	0.072
UCM (nC16-20)	0.28 ± 0.03	0.894	0.24 ± 0.05	0.825	0.30 ± 0.04	0.931	0.21 ± 0.04	0.853	0.02 ± 0.03	0.000
UCM (nC23-27)	0.19 ± 0.04	0.843	0.17 ± 0.06	0.709	0.20 ± 0.04	0.878	0.13 ± 0.04	0.749	0.02 ± 0.03	0.070
UCM (nC30-34)	0.14 ± 0.03	0.787	0.13 ± 0.05	0.672	0.16 ± 0.03	0.837	0.11 ± 0.04	0.640	0.02 ± 0.03	0.159
Total n-alkanes	0.67 ± 0.11	0.977	0.63 ± 0.08	0.951	0.73 ± 0.10	0.952	0.46 ± 0.07	0.952	<0.01 ± 0.03	0.002
Total UCM	0.23 ± 0.04	0.886	0.20 ± 0.05	0.768	0.25 ± 0.04	0.896	0.17 ± 0.04	0.798	0.02 ± 0.03	0.093
Total HC (GC)	0.28 ± 0.03	0.936	0.27 ± 0.06	0.790	0.31 ± 0.03	0.934	0.21 ± 0.04	0.871	0.02 ± 0.02	0.053

Table 6.4: Biodegradation rate constants of Forties oil components with 95% confidence intervals and coefficients of determination (R^2) determined from model $[A]_t = [A]_0e^{-kt}$ {HC= total hydrocarbon as determined by GC}

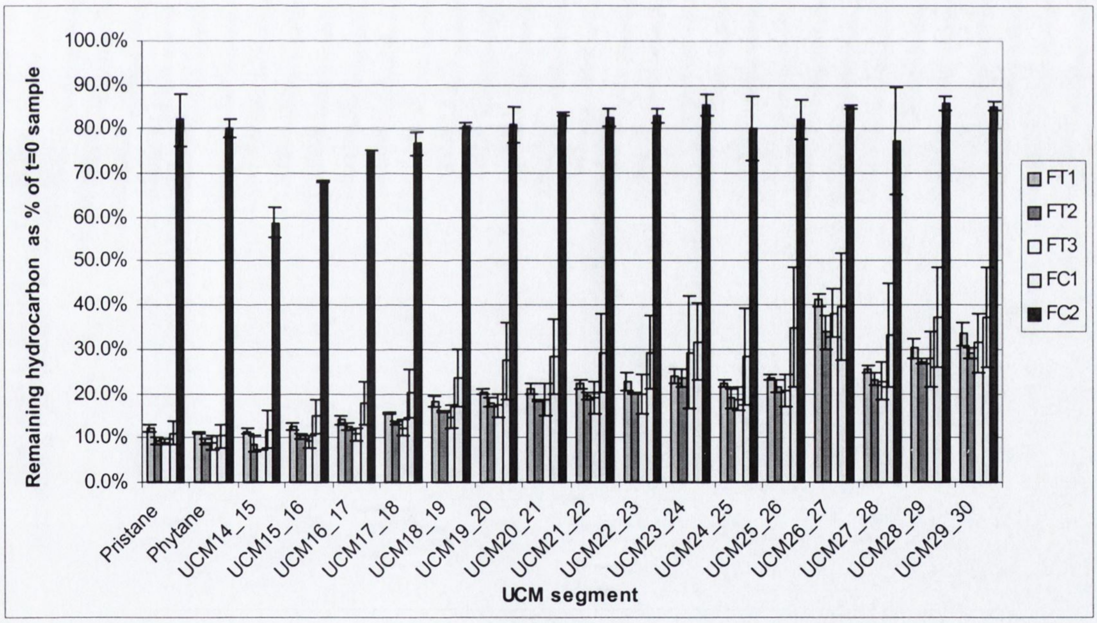
Although the rate constants for total hydrocarbon biodegradation calculated from GC data are slightly higher than those calculated from gravimetric data, in general they agree well. There is no apparent difference between FT1-FT3 but all of the components in T1-T3 show an increase in the rate constant with respect to FC1. The magnitude of this increase is less for slower degrading *n*-alkanes. For example, an increase of biodegradation rate constant of 1.86 fold is observed for *n*C15 in FT3 compared with FC1 while an increase of 1.17 fold is observed for *n*C35. These are similar to the observations of Venosa, (1996) although the rate constants for equivalent compounds observed by Venosa were higher in the Delaware bay field study, (e.g. *n*C15 untreated k=0.03 day^{-1}). As the relative increase in rate constant for nutrient amended treatments is larger for the more degradable components, it is clear that if bioremediation treatments are assessed by measuring biodegradation of simple substrate such as *n*-alkanes, the effect of nutrient addition on total remediation may be overestimated. Conversely, these results also suggest that if a more biodegradable oil had been used for these experiments, the effect of nutrient supplementation on biodegradation rates would have been more pronounced.

Ranking the order of biodegradation for the selected components reveals *n*C15 > *n*C20 > *n*C25 > *n*C30 > *n*C35 ~ phytane ~ UCM(*n*C16-20) > UCM(*n*C23-27) >UCM(*n*C30-33). It is interesting to note that phytane has a similar rate constant to the UCM(*n*C16-20) as phytane elutes in the centre of this section of UCM.

The biodegradation of UCM at t=280 and 473 days is pictured in figure 6.4 clearly showing a biodegradation preference for low molecular weight similar to that observed for *n*-alkanes. There has been substantial biodegradation across the UCM molecular weight range at 473 days and there is no longer a significant difference between T1-T3 treatments and C1. However, as seen in figure 6.4a, an enhancement in UCM biodegradation for nutrient supplemented microcosms is apparent earlier in the experiment and this is most apparent for lower molecular weight substrate. The reduction of UCM in fuel oil and preferential degradation of lower molecular weight hydrocarbons has been reported in soil microcosms (Chainneau *et al.* 1995).



a) 280 days



b) t=473 days

Fig. 6.4: UCM biodegradation for Forties UCM segments and isoprenoids at t=280 days (top) and 473 days (bottom). Bars indicate the average value for the duplicate microcosms and y-error bars indicate the value for each individual unit. {e.g. UCM 19_20 = UCM eluting within the n-C19 and n-C20 alkane retention interval}

Figure 6.5 shows the biodegradation of oil components for FC1 and FT3. A greater difference for *n*-alkanes is evident between treated and non treated test units, than for more complex and slower degrading UCM. However, the treatment and control converge with time. There is little biodegradation of residual *n*-alkanes during the latter stages of monitoring and this may have been due to unavailability of residual compounds to biodegradation.

As has been discussed previously, the Alba UCM was substantially washed from sediment over the course of the trial. It has been proposed earlier in this report that the physical properties of the Alba UCM make it amenable to water washing due to the lack of *n*-alkane waxes. It must be considered that washing of the UCM may have played at least a partial role for the Forties UCM removal during the course of bioremediation.

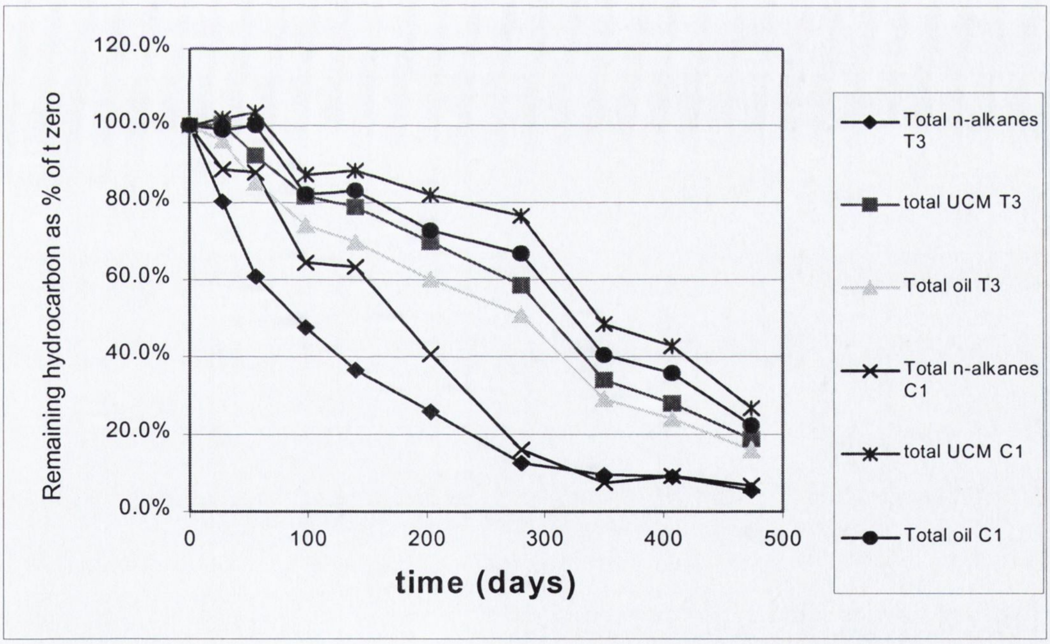


Fig. 6.5: A comparison of the biodegradation of total n-alkanes, total UCM and total hydrocarbon for FC1 and FT3. (average of duplicate experiments)

For the Alba oil experiments, washing of the Alba from the poison control was evident, as also observed by gravimetric analysis. However, the GC profiles of the AT1-T3 and AC1 experiments clearly show the occurrence of biodegradation. The UCM profile of AC2 remains intact throughout the experiment as indicated in figure 6.6. For other microcosms, the lower molecular weight preference for UCM segments was observed, as for Forties UCM, and the biodegradation of UCM was more advanced for the treated experiments than the seawater control with little difference between the treated experiments, with the exception of one of the T3 experiments. The extent of washing appears to have been even greater in the poison controls than for the other units. This may have been due to microbial uptake of oil, or simply the effect of microbial growth on sediment structure in the other units. GC-MS analysis of biomarkers, detailed in section 6.5.2 of this thesis, provides further evidence that the effect of washing was much greater in AC2 than other Alba microcosms. As for the Forties microcosms, rate constants were calculated to enable comparisons in biodegradation rates between AT1-T3 and AC1. The calculated biodegradation rates indicate a significant but relatively minor increase in UCM biodegradation for AT1 and AT2 with respect to AT3. The extent of biodegradation was considerably less for one of the T3 tests (AiT3) throughout the experiment than for the other five nutrient supplemented microcosms, i.e. duplicate AT1 and AT2 and AiiT3. The reason for this is not clear. The biodegradation rates calculated for Alba are noticeably higher than for equivalent components in the Forties microcosms. There are many possible reasons for this, such as the effect of water washing of Alba oil; the effect of 'sparing' of UCM components in the presence of more readily degradable *n*-alkanes for Forties microcosms; effect of different initial oil concentrations for Forties oil and Alba; different microbial populations selected due to different oil composition; or differences in bioavailability due to different oil chemistry. As evidenced for Forties microcosms, the biodegradation rate of UCM components decreases as molecular weight increases. Comparing these results with those obtained for gravimetric analysis indicates similar estimates of rate constants, albeit slightly higher values using GC measurement.

Figures 6.7 - 6.9 are a representation of the degradation of different components with time for both oils. These illustrate differences in biodegradation rates for different components, and greater biodegradation in nutrient treated microcosms, particularly in the earlier part of the project for relatively degradable components. Curve shapes indicate that while the exponential model fitted may be reasonable for the readily degradable substrates in the oil, it does not provide a good model for less degradable components, due to the effect of 'sparing'. For example, in the graph for the C20 *n*-alkane, more rapid biodegradation proceeds from the outset, while for *n*C30 and *n*C35, initial biodegradation is slow but accelerates after ~100 days.

Relative standard deviations of At=0 samples determined over the course of the monitoring programme were as follows: UCM(*n*C16-C20) 13.6%, UCM(*n*C23-27) 12.6%, UCM(*n*C30-34) 12.6%, Total UCM 12.0% for n=11.

	AT1		**AT2**		**AT3**		**AC1**		**AC2**	
	K $x10^{-2}.day^{-1}$	**R^2**	**K $x10^{-2}.day^{-1}$**	**R^2**	**K $x10^{-2}.day^{-1}$**	**R^2**	**K $x10^{-2}.day^{-1}$**	**R^2**	**K $x10^{-2}.day^{-1}$**	**R^2**
Phytane	0.51 ± 0.05	0.911	0.48 ± 0.08	0.091	0.41 ± 0.08	0.898	0.37 ± 0.06	0.898	0.28 ± 0.04	0.957
UCM (nC16-20)	0.41 ± 0.06	0.882	0.42 ± 0.05	0.908	0.33 ± 0.05	0.868	0.30 ± 0.05	0.803	0.28 ± 0.04	0.825
UCM (n23-27)	0.35 ± 0.05	0.850	0.34 ± 0.04	0.918	0.29 ± 0.04	0.859	0.27 ± 0.05	0.734	0.28 ± 0.05	0.733
UCM (n30-34)	0.24 ± 0.04	0.815	0.25 ± 0.04	0.815	0.24 ± 0.05	0.762	0.20 ± 0.05	0.502	0.24 ± 0.05	0.865
Total UCM	0.26 ± 0.03	0.952	0.26 ± 0.03	0.933	0.21 ± 0.04	0.868	0.18 ± 0.03	0.865	0.18 ± 0.02	0.914

Table 6.5: Biodegradation rate constants of Alba oil components with 95% confidence intervals and coefficients of determination (R^2) *determined from the model:* $[A]_t = [A]_0e^{-kt}$

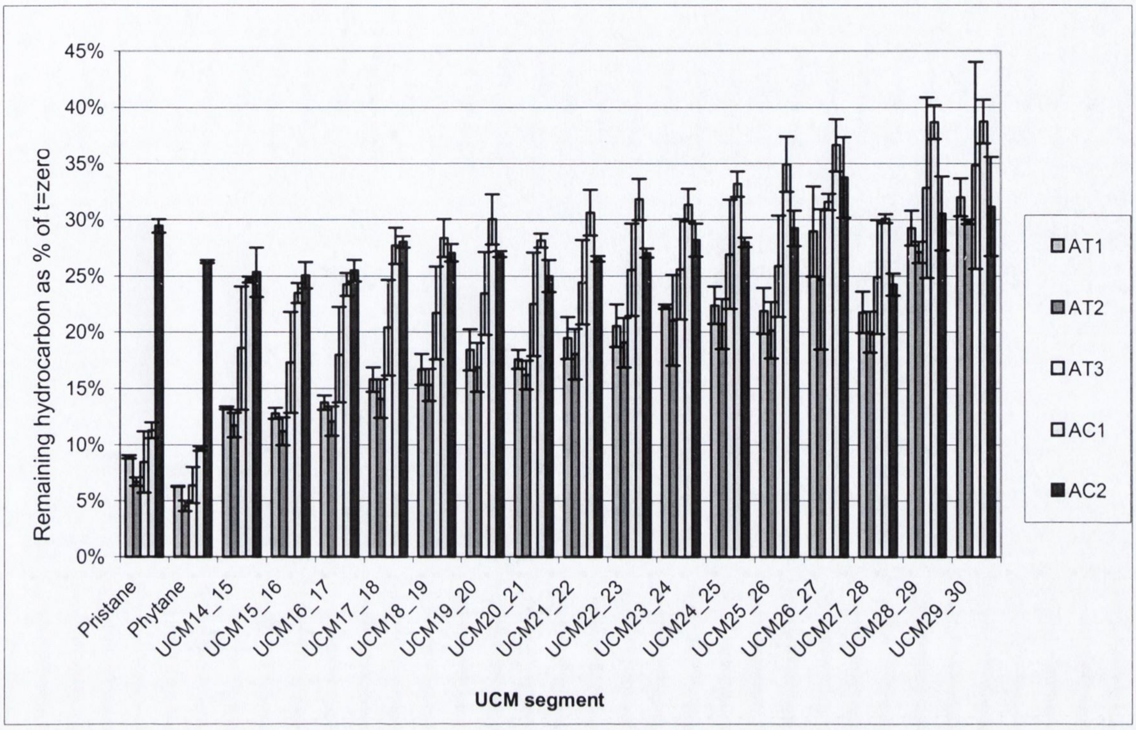


Fig. 6.6: *UCM biodegradation for Alba UCM segments and isoprenoids at t=473 days. Bars indicate the average value for the duplicate experiment and y-error bars indicate the value for each individual microcosm. {e.g. UCM 19_20 = UCM eluting within the nC19 and nC20 alkane retention interval}*

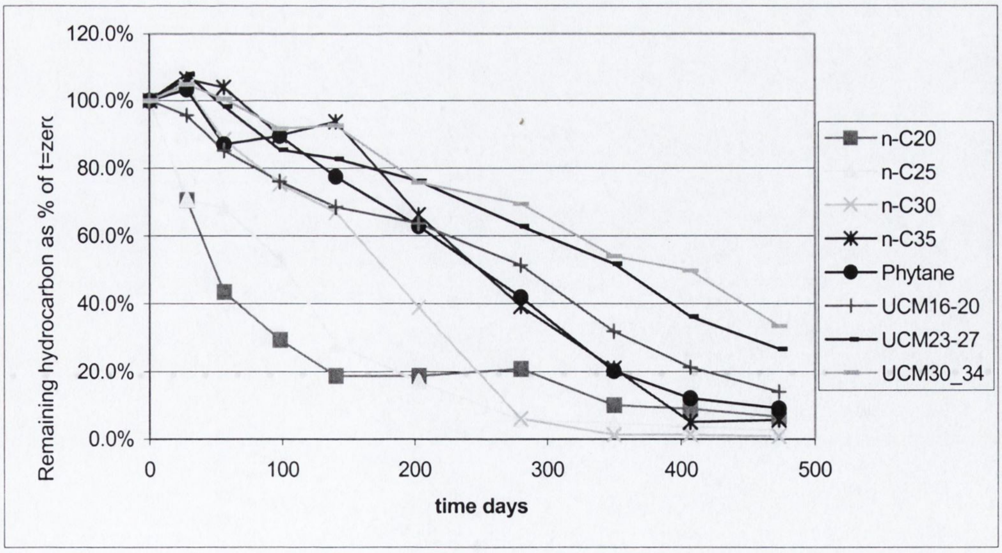
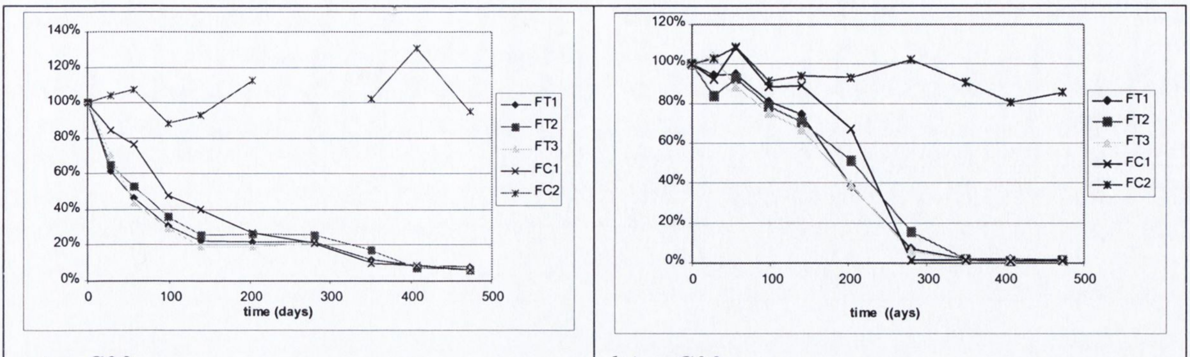
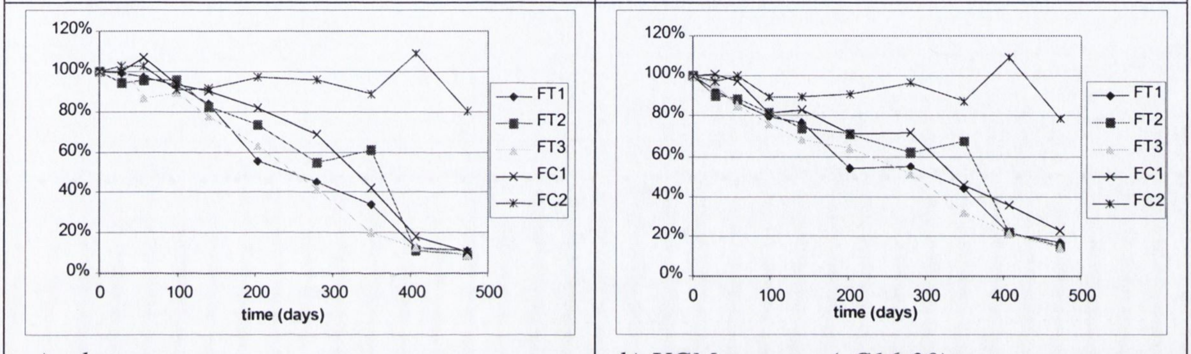


Fig. 6.7: *The biodegradation of different oil components (Forties oil) for T3 treatment*



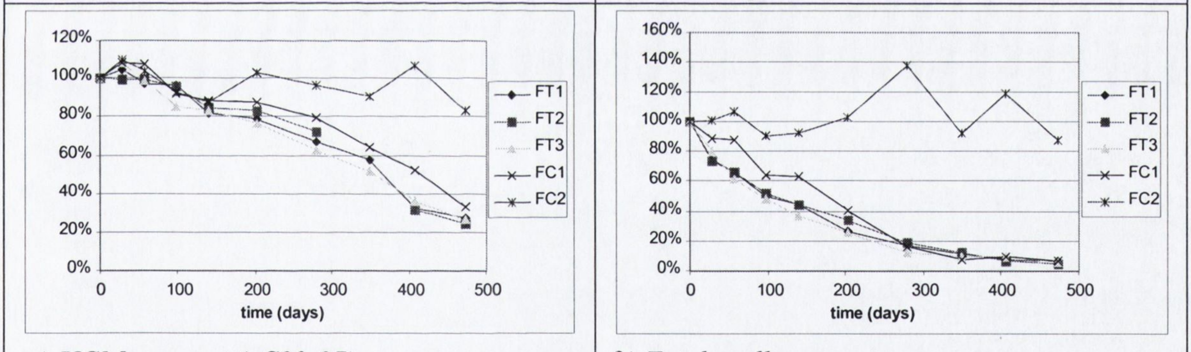
a.) n-C20

b.) n-C30



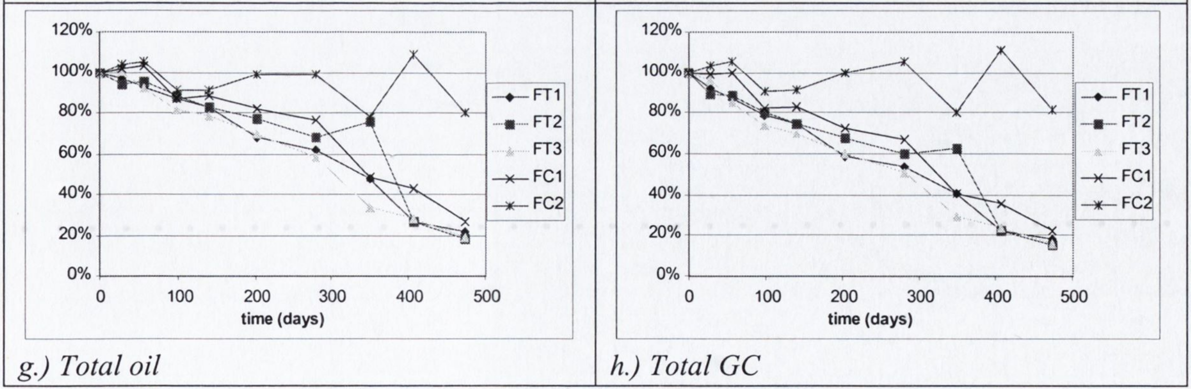
c.) phytane

d.) UCM segment (nC16-20)



e.) UCM segment (nC23-27)

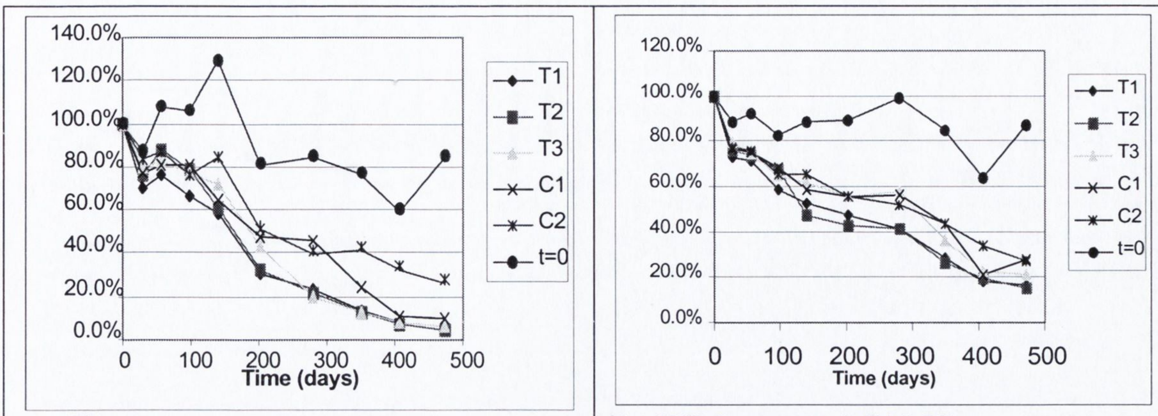
f.) Total n-alkanes



g.) Total oil

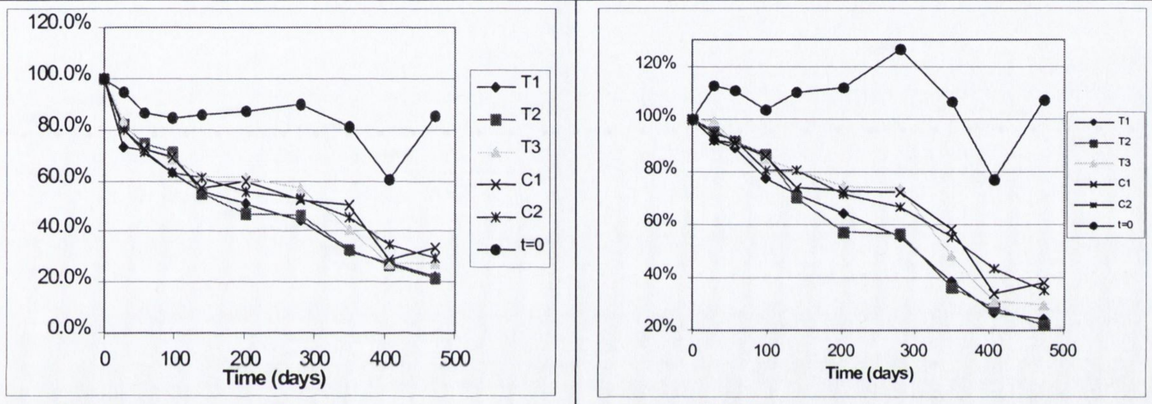
h.) Total GC

Fig. 6.8: Plots representing biodegradation of oil components in Forties microcosms (average results for duplicate microcosms). Y-axes presents % hydrocarbon remaining with respect to t=0 samples.



a.) Phytane

b) UCM segment (nC16-20)



c) UCM segment (nC23-27)

d.) Total UCM

Fig. 6.9: Plots representing removal of oil components in Alba microcosms (average results for duplicate microcosms). Y-axes are % hydrocarbon remaining with respect to t=0 samples.

6.3.2 High performance liquid chromatography

HPLC methodology was based on commonly used analytical procedures for petroleum fractionation. This employed an amino- and a cyano- column in series and aromatic fractions were quantified based on ring number, and back flushing was used to quantify polar compounds. This analysis was carried out at times = 0, 28 and 280. The method involved quantification of selected area divisions of a complex chromatogram. The divisions were based on the elution of PAH standards of different ring numbers, as investigated in phase I. Perylene was used as an internal standard and the divisions of chromatograms were carried out relative to perylene retention. Forties oil was used as a standard and bracketed approximately every five samples. Saturates were not quantified. Quantification of the area divisions was carried out using perylene and relative to the bracketed standard oil. Despite the use of these techniques, the method proved variable with time, and small variances in retention created large errors in quantification of the chromatogram divisions. Factors such as temperature fluctuations (a column oven was not used) and poor control of normal phase solvent delivery may have contributed to the variation in retention times.

The effect of biodegradation on aromatic UCM at t=280 days is presented in graphical form in figures 6.10 a & b. It is difficult to draw conclusions based on the HPLC results. The Alba microcosms indicate a general removal of all the aromatic components, including in the poison control, presumably due to washing, as previously discussed. A change in profile for AT1-C1 compared with AC2 and At=0 suggests preferential degradation for lower ring number components, particularly mono-and diaromatics. The Forties microcosms suggest a more pronounced biodegradation of monoaromatics.

There was a notable difference between results from duplicate microcosms for both oils. As this HPLC method was time consuming and not robust, HPLC analysis was not carried out on final samples from the project.

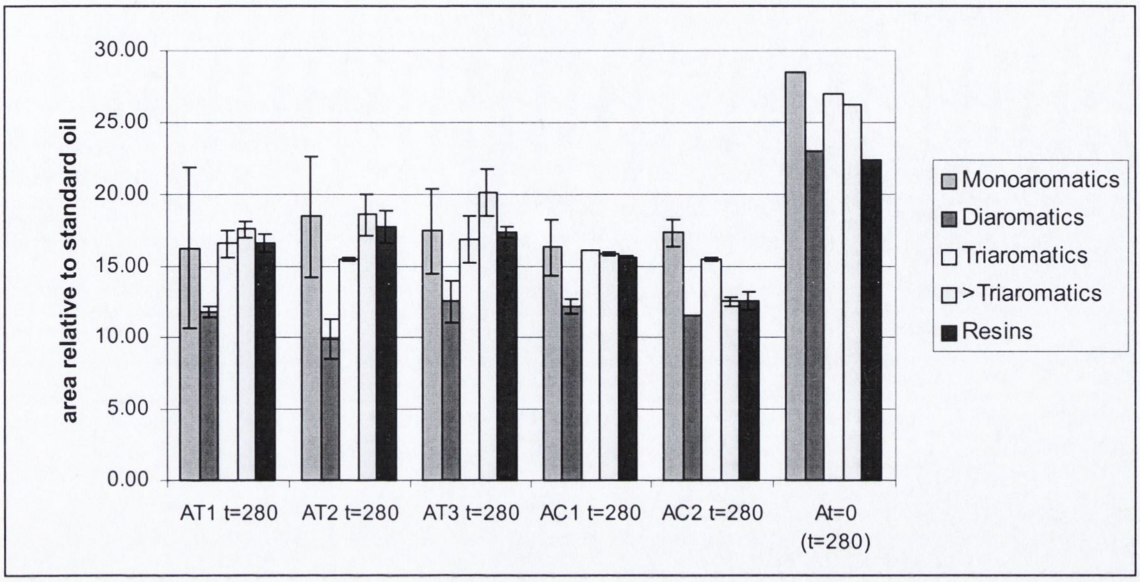


Fig. 6.10 a): *Alba aromatic biodegradation at t=280 days. Mean result for duplicate microcosms. Error bars denote results for individual microcosms.*

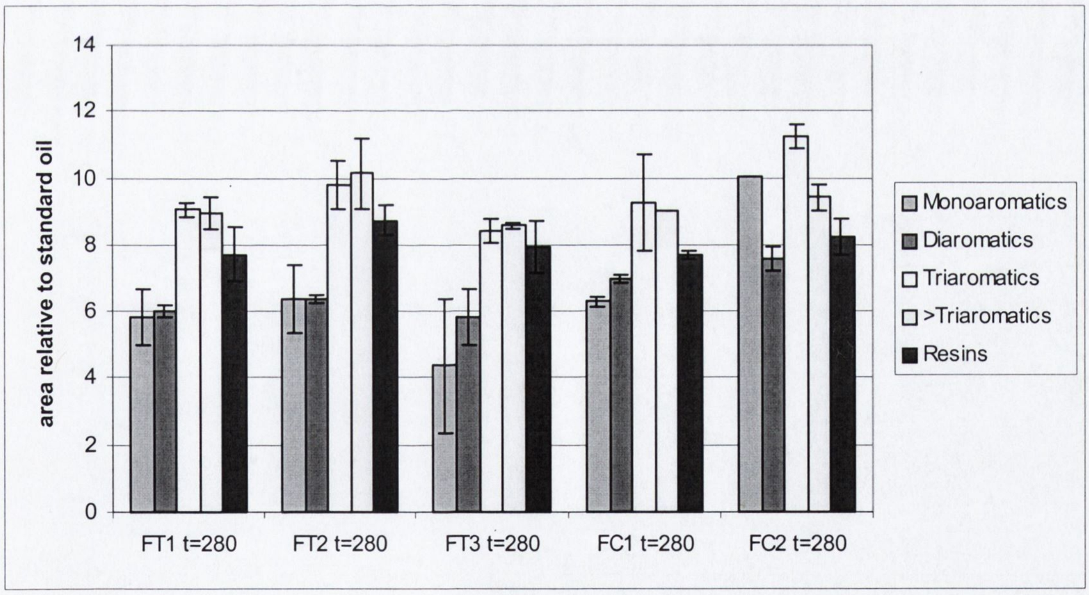


Fig. 6.10 b): *Forties aromatic biodegradation at t=280 days. Mean result for duplicate microcosms. Error bars denote results for individual microcosms.*

6.4 SPECTROSCOPY

6.4.1 Fluorescence spectroscopy

Total fluorescence spectroscopy was used to assess the changes in the aromatic profile of the oil with biodegradation. Excitation/emission maxima for the crude oils were identified in phase 1 of this project. An emission intensity maximum at excitation wavelength of 230nm and emission wavelength of 340nm for both oils corresponds largely to two ring aromatic components. A shoulder of exλ ~ 265nm and emλ~365nm can be largely attributed to three ring aromatic components, and therefore the ratio of intensity of these peaks gives an indication of the relative biodegradation of these components. The ratios of the maxima intensities (fig. 6.11) suggest that the 2-ring components degrade more rapidly than the 3-ring aromatics. However, the reproducibility for this measurement was not good. These results further illustrate the caution necessary in using fluorescence spectroscopy to determine biodegradation of hydrocarbons as not only is the technique selective for aromatic components but the choice of wavelength is selective for particular aromatic components. Biodegradation of these components may not reflect total biodegradation of oil or even of total aromatics/PAH. The ratios also suggested that for both Alba and Forties oil, biodegradation is more advanced for the nutrient treatments than for the seawater control.

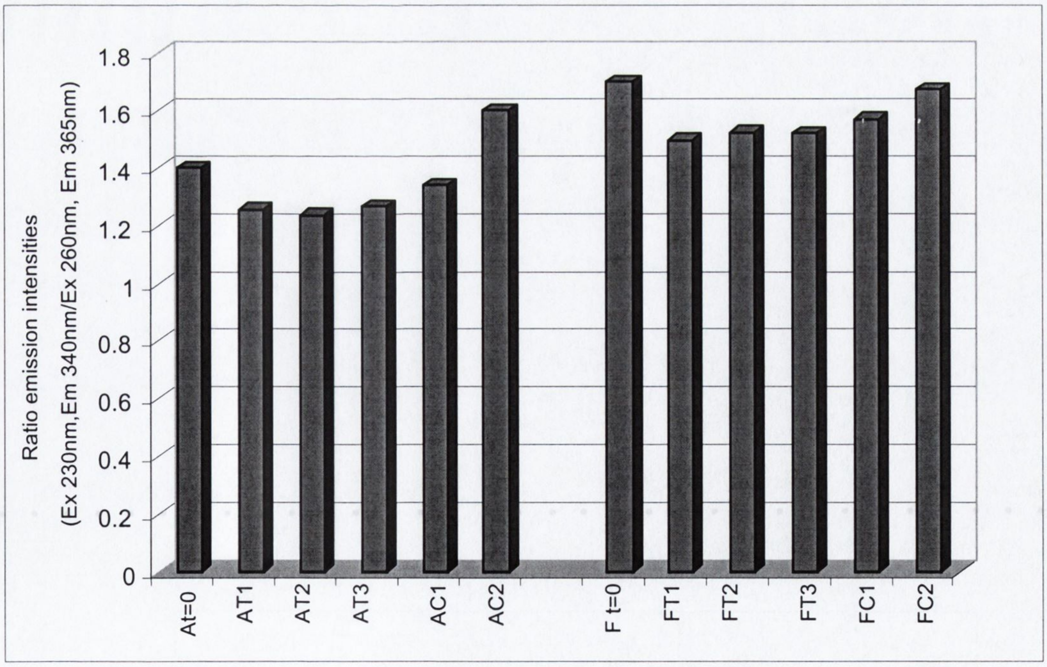


Fig. 6.11: *Fluorescence spectroscopy: ratios of emission intensities for ex.λ 230nm, em.λ = 340nm and ex.λ=270nm, em.λ=360nm for microcosms at t=407 days*

6.4.2 Infra red and Nuclear magnetic resonance spectroscopy

IR spectroscopy was not used during the assessment due to instrumental and methodological difficulties. FT-^{1}H-NMR and FT-^{13}C-NMR was carried out on two test samples at t=473. However, the complexity of the samples meant that results were difficult to interpret, with little useful structural information. These analyses were not pursued further.

6.5 MASS SPECTROMETRY

6.5.1 Gas chromatography-mass spectrometry - total ion chromatogram (TIC) analysis

The usefulness of molecular structural information in mass spectra for evaluating the biodegradation of UCM components was demonstrated in phase I of this project. In monitoring of the bioremediation trials, total ion chromatograms were obtained for microcosm samples at a number of sampling times. Analysis was performed in duplicate and bracketed with Alba as a reference oil using the sequence: reference , sample, sample, reference (Appendix A11). Rawdata is available in appendix D4. Complete spectra were obtained by averaging spectra across the entire chromatogram, and baseline subtracting to account for column bleed. Ratios of selected ion abundances were used to examine the changes in UCM composition due to selective biodegradation of components with different structural attributes. To correct for variability in fragmentation patterns for different analytical batches, over the time frame of the experiment, ratios were normalised to equivalent ratios for bracketed reference oil samples, i.e. for each ion abundance ratio, the sum of that ratio for both analyses of a sample was divided by the sum of the equivalent ion abundance ratio for the bracketing reference oil samples.

Ion abundance ratios do not infer absolute differences in biodegradation rates for different components, as part of the abundance for low molecular weight ion fragments can be attributed to other components, aside from the component of interest. For example, a cyclohexane with a large *n*-alkyl substitution will exhibit a fragmentation pattern based on the ring and the alkyl substitution. Also, a contribution to the abundance of a fragment may result from the heavy isotopic abundance of fragments with slightly lower monoisotopic mass. Notwithstanding, this method does provide very useful information on the relative biodegradability of major components of crude oil.

The results indicate significant differences in biodegradation rates for different compound classes. For Alba, (fig. 6.12), the m/z ratios attributed to the acyclic and cyclic component series, C_nH_{2n+1} (m/z 57, 71, 85, 99 amu etc.) with respect to C_nH_{2n-1} (m/z 55, 69, 83, 97 amu etc.) respectively, demonstrate a shift towards increasing cyclicity of the oils. This is illustrated by the reduction of the 71/69 amu ratios and the 85/83 ratios and indicates that the bulk of branched acyclic saturates are more amenable to biodegradation than the cyclic saturate component of crude oil in field simulated conditions. Other ratios examined such as the 83/95 and 85/95 suggest considerably more rapid biodegradation of the acyclics and monocyclic saturates when compared to bicyclic components. M/z 97 is a common fragment of substituted methylcyclohexanes while m/z 83 is usually attributed to substituted cyclohexanes. A comparison of these ions suggests that substituted methylcyclohexanes may be slightly more amenable to biodegradation than their non-methylated counterparts. Results for substituted methylbenzenes (m/z 105) and substituted benzenes (m/z 91) suggest a similar preference for the methylated species. The 83/91 ratio also suggests a substantially greater biodegradation for saturated monocyclics, compared with aromatic monocyclics. Trends are not obvious over the course of the trials for relative ion abundances that employ m/z105. The reason for this is unclear.

It is apparent that there is no change over the course of the trial in the ratios for AC2, which also compares very well for Alba t=0 reference sample over the course of monitoring. This indicates that the composition of Alba UCM was not altered in the poison control microcosm, although it was substantially washed from the sediment over the course of the trial. Thus, the physical process of washing rather than dissolution is the most probable mechanism of removal of oil from AC2 sediment.

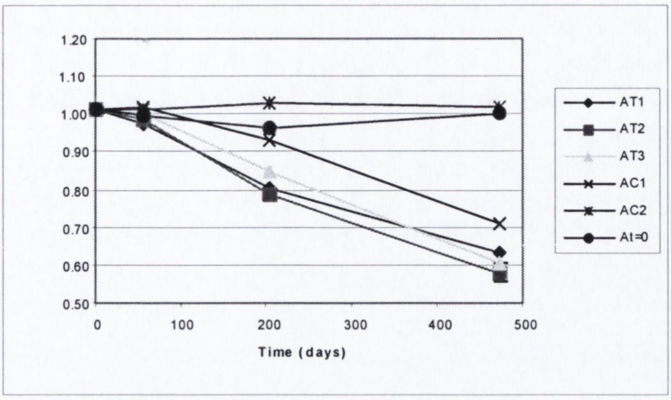
Where differences in the rate of degradation were detected, the T1-T3 results tended to cluster indicating no difference between treatments. Changes in ratios for C1 microcosms were less marked. This provides further evidence that nutrient addition stimulated the rate of biodegradation of UCM in the microcosms but that inoculation had no appreciable effect. Results for duplicate microcosms compared very favourably.

GC-MS TIC analysis was not as useful for evaluating biodegradation of Forties UCM, (fig. 6.13), as the C_nH_{2n+1} series includes an input from *n*-alkanes, by definition a non-UCM component. Therefore, ratios based on fragments from the C_nH_{2n+1} series reflect the input of *n*-alkanes as well as *i*-alkanes. However, similar biodegradation preferences as those observed for Alba microcosms were evident. Differences between treatments for the Forties microcosms were more difficult to discern. Although all treatments, with the exception of the poison controls, exhibited greater biodegradation of acyclics compared with monocyclics, nutrient addition did not seem to effect the trend. However, a notable difference in biodegradation of acyclics and monocyclics compared with bicyclics was apparent at t=203. This was no longer evident at t=473. The observations on relative biodegradability observed for Alba microcosms were again evident, suggesting *n/i*-alkanes> cycloalkanes, monocycloalkanes> bicycloalkanes, Cx-methylbenzenes > Cx-benzenes, Cx-methylcyclohexanes> Cx-cyclohexanes, Cx-cyclohexanes>Cx-benzenes.

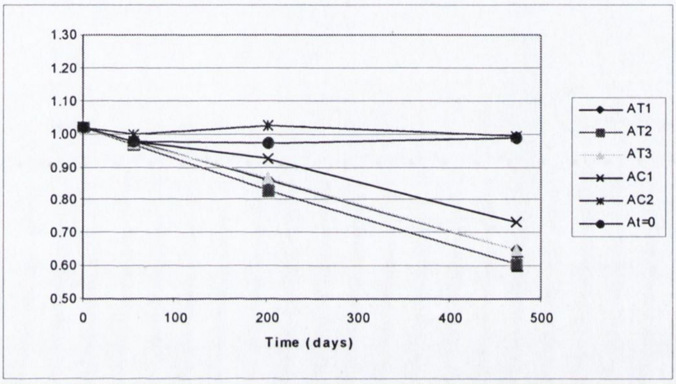
A visual examination of TIC mass spectra obtained by averaging spectra for both front and latter portions of selected Alba UCM chromatograms, (t=473), indicates similar changes to the spectra for the two divisions of the chromatograms when compared to the equivalent spectra for the AC2 microcosms. This suggests that preferential biodegradation of different components, as described above, occurs generally across the entire UCM molecular weight range.

As illustrated above, ion abundance ratios from combined GC-MS spectra proved to be a very useful tool for the study of relative compositional changes in bulk UCM. The use of a reference oil for normalisation provided very good reproducibility and reliability over the course of monitoring.

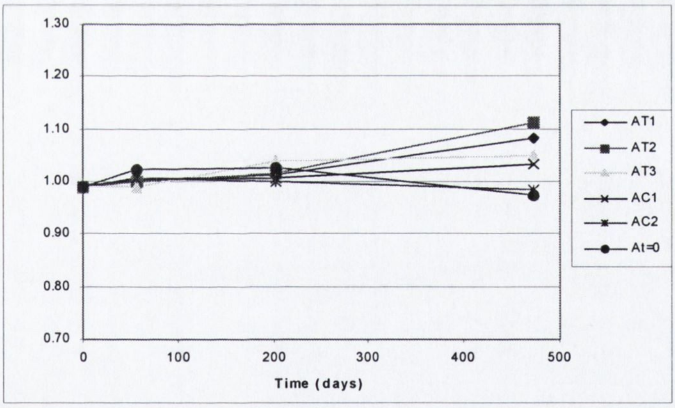
a) 71/69
~ *i*-alkanes/
cyclics



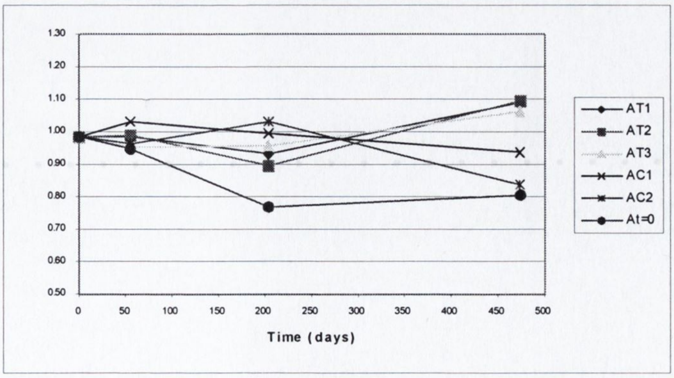
b) 85/83
~ *i*-alkanes/
Cx-
cycloahexanes



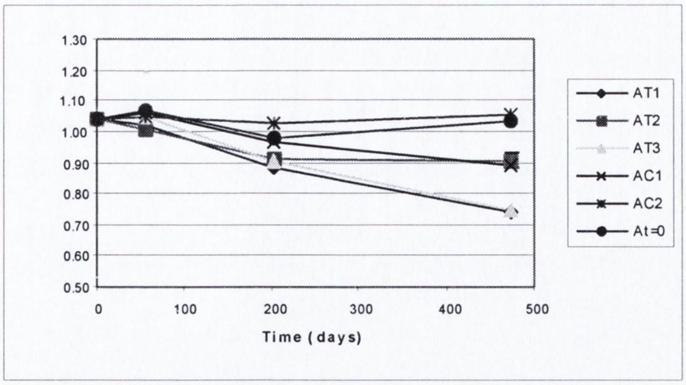
c) 83/97
~ Cx-
cyclohexanes
/Cx-methyl-
cyclohexanes



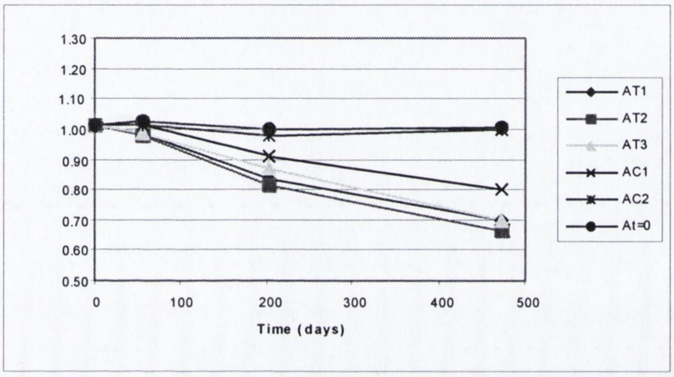
d) 91/105
~Cx-benzenes/
Cx-methyl-
benzenes



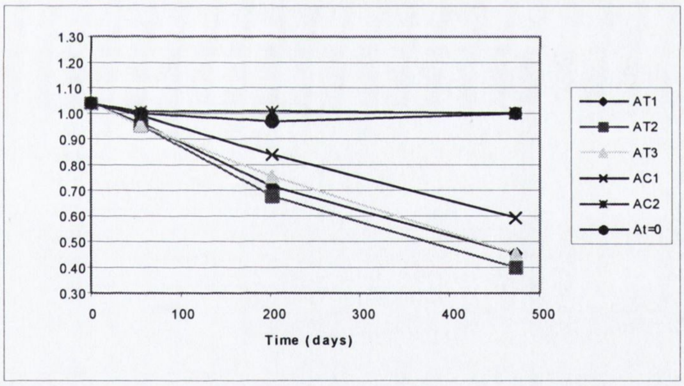
e) 83/91
~ Cx-cyclohexanes
/Cx-benzenes



f) 83/95
~ Cx-cyclohexanes
/bicyclic
alkanes



g) 85/95
~ *i*-alkanes
/bicyclic
alkanes



h) 97/105
~ Cx-methyl-cyclohexanes/
Cx-methyl-benzenes

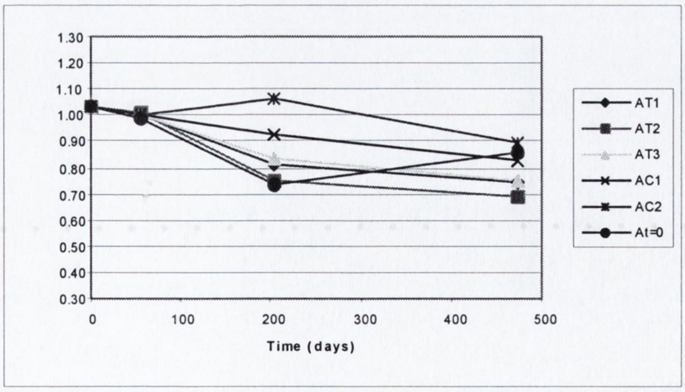
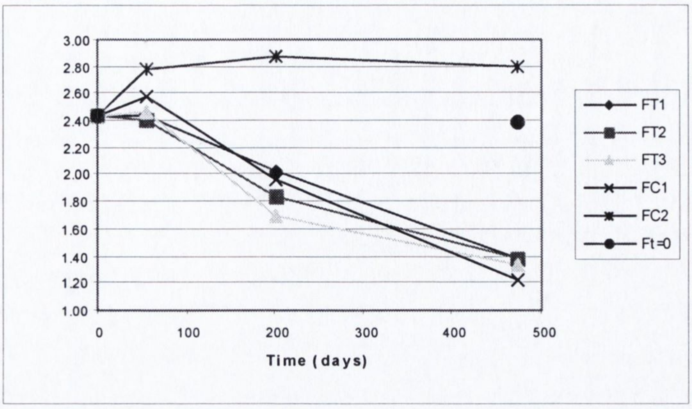
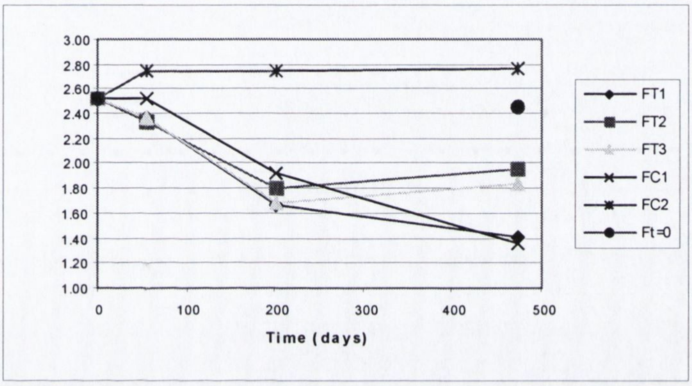


Fig. 6.12: Ion abundance ratio trends for Alba microcosms. Mean of duplicate microcosms. [y-axes: Total ion abundance (intensity) ratios.]

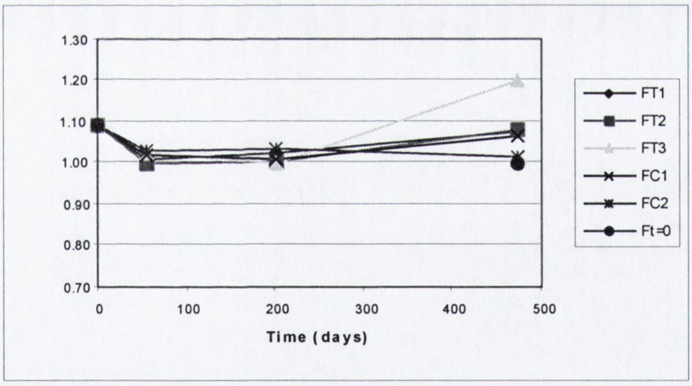
d) 71/69
~ *i/n*-alkanes
/cyclics



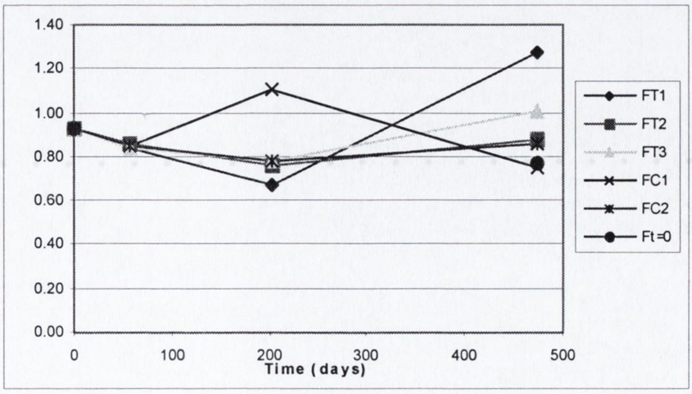
e) 85/83
~ *i/n*-alkanes/
Cx-
cyclohexanes



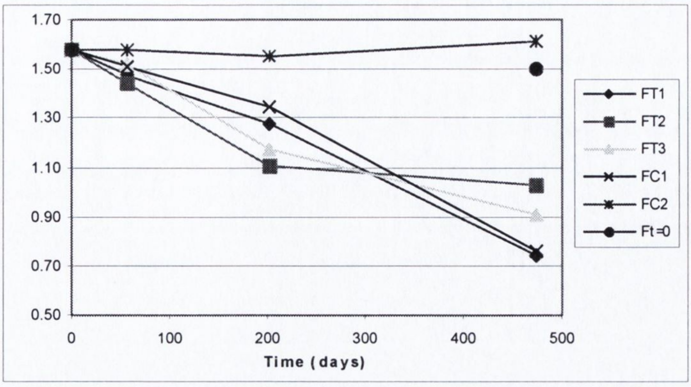
f) 83/97
~ Cx-
cyclohexanes/
Cx-methyl-
cyclohexanes



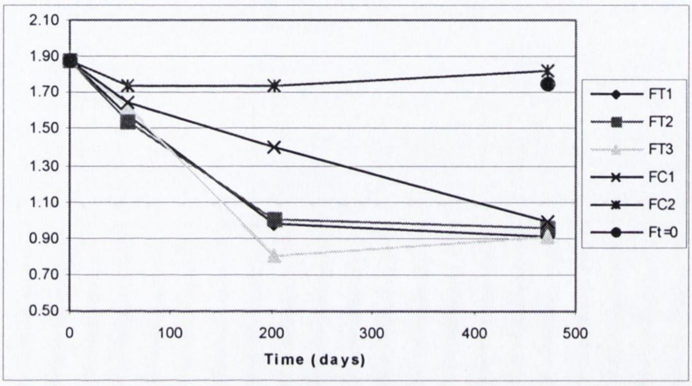
d) 91/105
~Cx-benzenes/
Cx-
Methyl-
benzenes



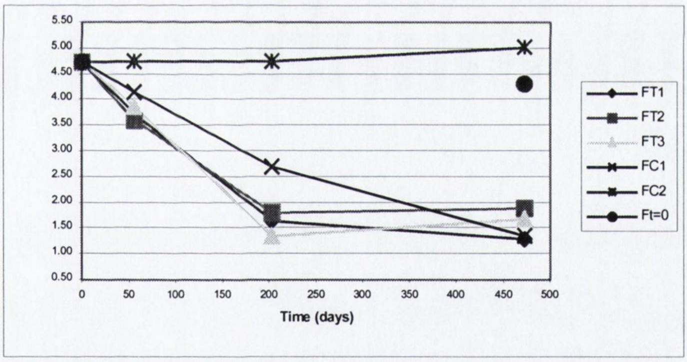
e) 83/91
~ Cx-
cyclohexanes
/Cx-benzenes



f) 83/95
~ Cx-
cyclohexanes
/bicyclic
alkanes



g) 85/95
~isoalkanes
/bicyclic
alkanes



h) 97/105
~ Cx-
methyl-
cyclohexanes/
Cx-
Methyl-
benzenes

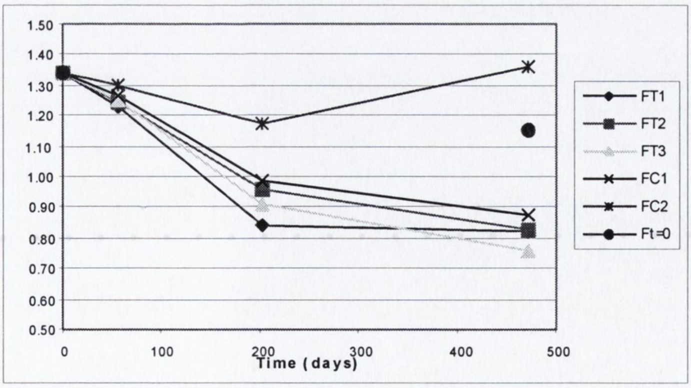


Fig. 6.13: *Ion abundance ratio trends for Forties microcosms. Mean of duplicate microcosms. [y-axes: Total ion abundance (intensity) ratios.]*

ASTM method 2786-91 (reapproved 1996) (ASTM 1991b) provides a method for estimating the composition of the saturate fraction of petroleum from the mass spectrum. Saturate fractions of Alba (Ai) sediment extracts at t=473 days were isolated using column chromatography and this method applied to determine the effect of biodegradation on components, including isoalkanes, naphthenes classified based on ring number, and monoaromatics. Deisotoped ion abundances were used and calculations based on the sum of specified fragment ion abundances and inverse matrices selected for an average carbon number. In this case an average carbon number of 24 was selected and the appropriate inverse matrix that assumed *i*-alkanes were the dominant 0-ring saturates employed. Results are expressed as volume percent. The estimated composition data are presented in table 6.6 and figure 6.14. These results suggest that 0-,1- and 2- ring alkanes are the main contributor to the saturate fraction but that 3-, 4-ring alkanes are also significant contributors. 5-ring alkanes are only minor contributors to Alba and 6-ring components were not detectable. Monoaromatics are also significant components but there is a discrepancy between the At=0 and AiC2. It should be noted that while non-condensed rings are not a feature of the aromatic component of petroleum, non-condensed naphthenes may contribute significantly to the saturate fraction. An example would be two cyclohexyl groups connected by an alkyl chain. Such a compound would be detected as part of the 1-ring saturates using this method (Hood & O'Neal 1959).

The composition of the saturate fraction of Forties oil was estimated as 39%, 20%, 13% 10%, 10% 1%, 0% for 0-6 ring saturates respectively and 8% for monoaromatics for Ft=0. For FC2 at t=473, the same breakdown was calculated at 39%, 19%,12%, 8%, 9%,0%, 0% (0-6 ring saturates) and 13% monoaromatics. Other Forties microcosms saturate fractions were not analysed.

Changes in the saturate profile indicate a similar extent of biodegradation of AiT1 and AiT3, (AiT2 was not analysed). AiC1 has not undergone the same extent of biodegradation as the nutrient treated microcosms. The composition of AiC2 is very similar to At=0 despite washout. Although 0-3 ring saturates and monoaromatics have been substantially degraded in AiT1 and AiT3, 4- and 5- ring saturates have not. The order of biodegradation proceeds according to ring number, with lower number of condensed rings being more amenable to biodegradation. A previous observation using this method of analysis for measuring naphthene biodegradation in crude oil in quasi-continuous culture suggested an order of 0>6>5>1>4>2>3 rings (Oudot 1984).

GC-MS TIC may be useful in characterising oil with significant UCM prior to application of a remediation technique. Combined spectra may be used to estimate proportions of different component classes including naphthene composition in target oil and chromatographic data and reconstructed ion chromatography can also provide useful information on composition. This information could then be used for assessing the biodegradability of the oil and a subsequent evaluation of the suitability of bioremediation as a clean-up tool.

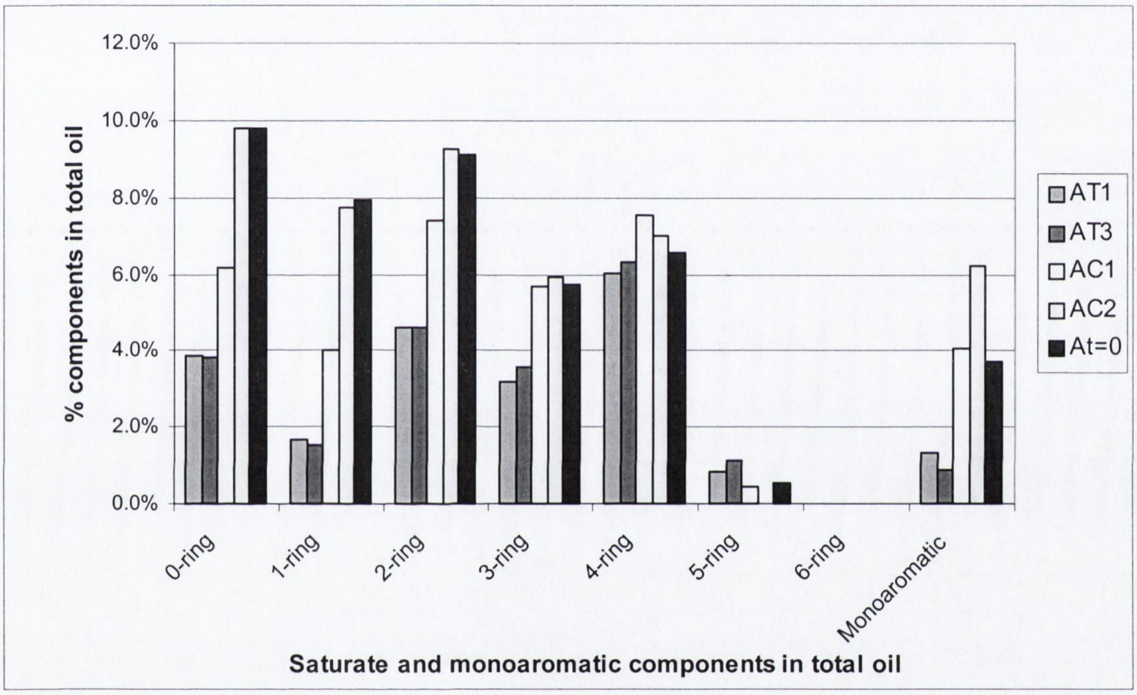


Fig. 6.14: *Saturate and monoaromatic composition in Alba microcosm sediments at t=473 days, expressed as percentage of total petroleum extracts.*

	AiT1	***AiT3***	***AiC1***	***AiC2***	***At=0***
Expressed as volume percent of saturate fraction					
0-ring	18%	18%	17%	21%	23%
1-ring	8%	7%	11%	17%	18%
2-ring	21%	21%	21%	20%	21%
3-ring	15%	16%	16%	13%	13%
4-ring	28%	29%	21%	15%	15%
5-ring	4%	5%	1%	0%	1%
6-ring	0%	0%	0%	0%	0%
Monoaromatic	6%	4%	12%	14%	9%
% Saturate fraction by weight	*21.5%*	*21.8%*	*35.3%*	*45.9%*	*43.4%*
Expressed as percent of total oil					
0-ring	3.9%	3.8%	6.2%	9.8%	9.8%
1-ring	1.6%	1.5%	4.0%	7.7%	7.9%
2-ring	4.6%	4.6%	7.4%	9.2%	9.1%
3-ring	3.2%	3.6%	5.7%	5.9%	5.8%
4-ring	6.0%	6.3%	7.5%	7.0%	6.6%
5-ring	0.8%	1.1%	0.4%	0%	0.5%
6-ring	0%	0%	0%	0%	0%
Monoaromatic	1.3%	0.9%	4.1%	6.2%	3.7%

Table 6.6: Composition of saturate fraction of Alba microcosm sediment at t=473 days as calculated by mass spectrometry fragment peak analysis (ASTM 1991b).

6.5.2 Gas Chromatography- Mass Spectrometry (Individual components - Selected ion analysis)

GC-MS analysis was used to monitor biodegradation of substituted PAH and aliphatic components, including the recalcitrant 'biomarkers' at t= 0, 203 and 473 days. The analytical methodology was based on the EUROCRUDE system, (Anon. 1995), as refined in phase I of this project for the purpose of monitoring the bioremediation trials (appendix A.11).

In GC-MS measurement of biodegradation, 17α, 21β-hopane (C30-hopane [P6 – appendix B.X]) is commonly used as a conserved internal marker, which compensates for errors such as differences in sample heterogeneity, extraction efficiency and instrument variability. Previous studies have shown insignificant biodegradation of this compound relative to total hydrocarbon, and as it is usually abundant in crude oils, and can be resolved by GC-MS, it has proven useful in assessing biodegradation of other oil components (Swannell *et al.* 1995, Bragg *et al.* 1996, Head 1998). Removal of C30-hopane has been attributed to washing, enabling calculation of true biodegradation (Venosa *et al.* 1996).

GC-MS is commonly used to compare and match oil spill fingerprints. For this purpose, samples must be analysed not only on the same instrument but in the same batch, as differences in source conditions can cause substantial differences in mass spectra and therefore fingerprints. The EUROCRUDE system was designed to compare GC-MS analysed spilt oil samples with a database of GC-MS information on oils collected and analysed at different times, by different laboratories, on different instruments. The system involved analysing each sample in duplicate, bracketed by a reference oil (Brent crude oil). As well as standardisation of each peak against the equivalent peak in the reference oil samples, the option of normalisation against C30-hopane as an internal standard was preferred.

When using GC-MS to carry out long term monitoring of hydrocarbon biodegradation, some form of external standardisation is required, along with normalisation to C30-hopane. As described above, the strategy used for monitoring of biodegradation in the microcosms was adapted from the EUROCRUDE design, but used Alba crude oil in place of Brent as the reference oil. An examination of ratios of selected analyte peak areas to C30-hopane peak areas, (m/z 191 chromatogram), from this experiment indicated that analytical variation was extremely high over the course of the monitoring trials. This was most obvious for the reference Alba crude oil, which provided the largest single sample dataset. The data presented below demonstrates that the use of the reference oil as an external standard, in conjunction with use of a conserved internal marker, provided an extremely useful methodology for measuring biodegradation over the course of a lengthy time period, compensating for analytical variability.

GC-MS data from this experiment were examined to check the presumption that C30-hopane (P6) was not biodegraded during the course of the trials, thus acting as a true *conserved* internal marker. It has already been shown that substantial washing of Alba UCM from sediment occurred. This infers significant C30-hopane removal due to washing. To assess the behaviour of C30-hopane in the trials, the mean peak area for C30-hopane of duplicate microcosms was normalised against the mean C30-hopane peak areas of bracketing reference samples and corrected for dilution and amount of sediment extracted. The results are plotted in figure 6.15.

The percentage removal of C30-hopane at 473 days is presented in table 6.7. The value for AC2 indicated washout of 66%, a figure close to the gravimetric measurement of total oil removed. However, less removal of C30-hopane in AC1 microcosms suggest that less washout occurred in microcosms with biological activity. This also agrees with the evidence from GC and gravimetric analysis that less oil had been washed from these sediments. The AT1-T3 microcosms demonstrate high removal of C30-hopane, suggesting substantial biodegradation of this compound. This is supported by examination of the m/z 191 chromatograms. The pattern for pentacyclic triterpanes is not significantly altered for the AC1 and AC2 microcosms, but is substantially changed for T1-T3 treatments. Results for Forties microcosms are also in agreement with GC and gravimetric results, in that washout was not a substantial feature for the poison control and thus presumably also not a feature for other microcosms. Forties microcosm results also suggested greater biodegradation of C30-hopane in nutrient treated microcosms. Estimated removal of C30-hopane from microcosms is presented in figure 6.15 and table 6.7. It should be noted that the values given in table 6.7 are mean values for duplicate microcosms and that the apparently lower biodegradation rates for AT3 and FT2 reflects that one of each these duplicates exhibited significantly lower biodegradation than other nutrient treated microcosms.

Results demonstrate that if C30-hopane is used for normalisation purposes in this case, its biodegradation will result in an underestimation of the extent of oil/oil component biodegradation. Furthermore, as removal of C30-hopane is greatest for the T1-T3 microcosms, the difference between nutrient treated and non-treated test units will also be underestimated, hence underestimating bioremediation. Plotting summed biomarkers groups (e.g. sum of pentacyclic triterpanes GC-MS m/z 191 areas) shows an increase in all summed biomarker patterns with respect to C30-hopane for nutrient treated microcosms (fig. 6.16). This suggests that not only is C30-hopane degraded, but it is one of the most rapidly degraded compounds in the biomarker suites examined.

Clearly C30-hopane is not a good internal marker for estimation of hydrocarbon biodegradation for the oils used in this experiment. Therefore, other candidate compounds were investigated as potential conserved internal markers.17α, 21β-30-norhopane (C29-norhopane [P4 appendix B. X]) has also been used as a conserved marker in bioremediation studies (Oudot *et al.* 1998). In this instance, it exhibited less removal than C30-hopane, but there was evidence that biodegradation did occur for this component also. P1 (18α(H),-22,29,30- trisnorhopane [Ts]) exhibited greater biological recalcitrance, but the lower abundance of this compound resulted in higher analytical variability and it proved of limited use for normalisation. Similarly, a number of steranes (such as S6: C29 (20S)13β, 17α, diasterane) were examined as they exhibited greater resistance than C30-hopane and other steranes. However, these also exhibited higher analytical variability due to incomplete resolution. The primary criteria for selection of a conserved internal standard, apart from conservation, were the analytical considerations of abundance and chromatographic resolution. Of the biomarker components monitored over the course of this experiment, those that best fitted these criteria were the triaromatic steranes. The most abundant of these was the GC-MS peak TAS1 [appendix B. XIII] (20R cholestane/ 20S ergostane) but this was apparently the most susceptible to biodegradation of the triaromatic steranes monitored, (see chapter 7). The C28-triaromatic sterane, (TAS4 ~20R stigmastane [~ ethylcholestane]), was tested as a potential conserved internal standard. The removal of TAS4 is presented in figure 6.17 and table 6.7. For Alba microcosms it is apparent that less biodegradation has occurred for TAS 4 than C30-hopane. Plotting the ratios of C30-hopane:TAS4 also provides evidence that TAS4 is a more suitable internal marker than C30-hopane for monitoring Alba. It is notable that at 203 days greater reduction of TAS4 than C30-hopane is apparent. The results are not as clear for Forties microcosms. At 473 days, P6 removal was greater than that for TAS4 for microcosms where most biodegradation has occurred (FT1 and FT3). However, TAS4 removal is greater for other microcosms. It is probable that abiotic removal of triaromatic steranes exceeded that of C30-hopane due to greater water solubility. However, TAS4 is considerably more resistant to biodegradation and thus is a more suitable internal marker for monitoring advanced stages of biodegradation. Based on this, it was decided to use TAS4 for internal normalisation for assessing biodegradation of Alba components. Biodegradation of Forties oil components was measured using C30-hopane.

Alba	**AT1** (n=2)	**AT2** (n=2)	**AT3** (n=2)	**AC1** (n=2)	**AC2** (n=2)	**At=0** (n=1)
P6 (C30-hopane)	85%	84%	69%	38%	66%	19%
TAS4	57%	55%	41%	36%	68%	15%
P6/TAS4	64%	65%	47%	2%	-13%	3%
Forties	**FT1** (n=2)	**FT2** (n=2)	**FT3** (n=2)	**FC1** (n=2)	**FC2** (n=2)	**Ft=0** (n=1)
P6 (C30-hopane)	60%	33%	64%	17%	11%	3%
TAS4	53%	58%	38%	38%	22%	-2%
P6/TAS4	11%	-50%	20%	-36%	-21%	-12%

Table 6.7: Percentage reduction in sediment concentrations of C30-hopane (P6), 20R-ethylcholestane (triaromatic sterane TAS4) and P6/TAS4 ratio at t=473 days wrt. t=0 sediments. Mean of duplicate microcosms.

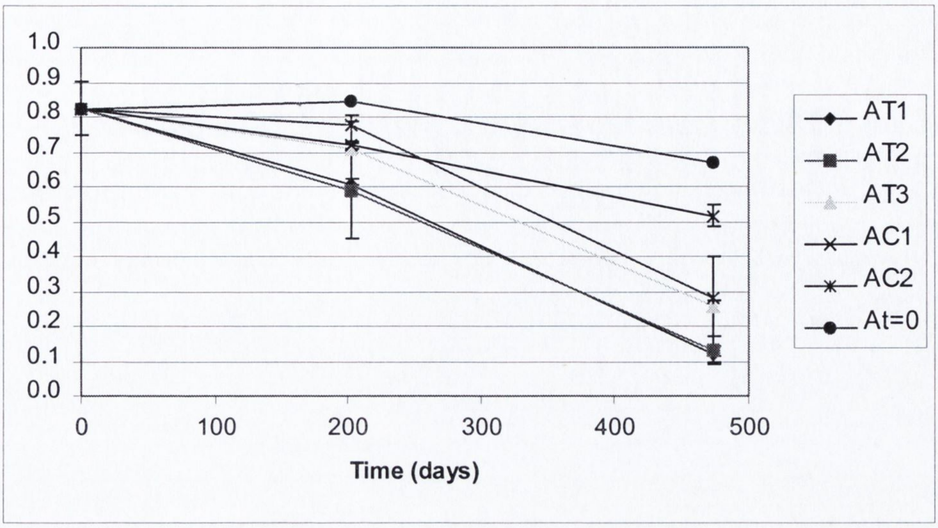
A full description of the GC-MS technique including the combined external (reference oil) and internal normalisation procedure is given in appendix A.11. For Alba microcosms the biodegradation of PAH components was measured against time using this procedure. The sums of individual groups of substituted phenanthrenes (C1P, C2P, C3P), and dibenzothiophenes (C1D, C2D, C3D) are plotted against time to compare the microcosm treatments. The peaks used for quantification are given in appendix B. The use of the reference oil compensates for differences in MS source conditions and peak integration differences, (particularly for partially resolved peaks), at different sampling times.

The plots of substituted-PAH revealed extensive biodegradation in the microcosms (Fig. 6.18). The extent of biodegradation was related to the extent of substitution; the less substituted species being biodegraded more rapidly, and almost completely removed at t=203 days. For Alba microcosms, at t=203 days there is a marked difference between the extent of biodegradation for C1P, C2P, C1D and C2D for the nutrient treated microcosms compared with the C1 microcosms.

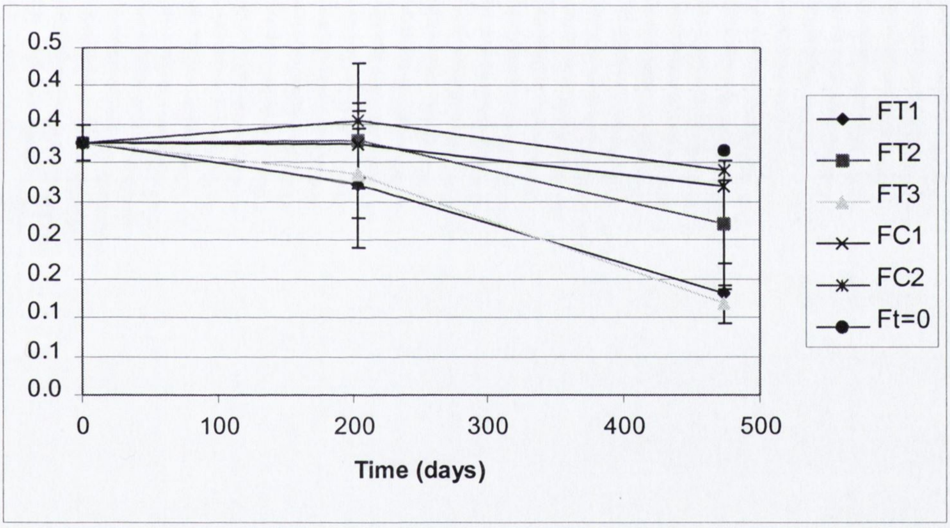
The difference is not evident at t=473 days. The difference for C3P and C3D is still evident at t=473. Very close agreement is evident for the three nutrient treated microcosms.

The extent of biodegradation for individual peaks, (as labelled in appendix B), relative to TAS4 and reference oil, for treated Alba microcosms at t=473 days and t=0 samples are plotted for phenanthrenes, dibenzothiophenes and biomarkers in figure 6.19. Plots for Forties microcosms based on C30-hopane normalisation are presented in figure 6.20. The plots use the mean values for all three nutrient supplemented microcosms at t=473 days (i.e. 6 microcosms), as no discernible differences are apparent for these treatments, and the mean of t=0 analysis at t=0, 203 and 473 days, (4 analyses). Three ring PAH have almost been completely degraded at this time for both Forties and Alba oil. For Alba microcosms, the decrease in many of the biomarker components with respect to t=0 samples not only indicates partial biodegradation of these compounds, but also that TAS4 is one of the most biologically refractory biomarkers. Examination of data also suggests that most steranes and many pentacyclic triterpanes are apparently more resistant to biodegradation than C30-hopane.

Further investigation of GC-MS data using reconstructed ion chromatography, (RIC), for Alba microcosm samples reveal biodegradation of other components. Although not quantified, homologous series of alkylcyclohexanes (m/z=82/83) were reduced relative to C30-hopane at t=203. Homologous series at m/z 105 (presumably due to alkyl methylbenzenes) were also partially degraded at t=203 with lower members of the series being preferentially degraded. The m/z 95 hump associated with naphthenes (Simoneit 1986) has also been reduced with respect to C30-hopane at t=280.



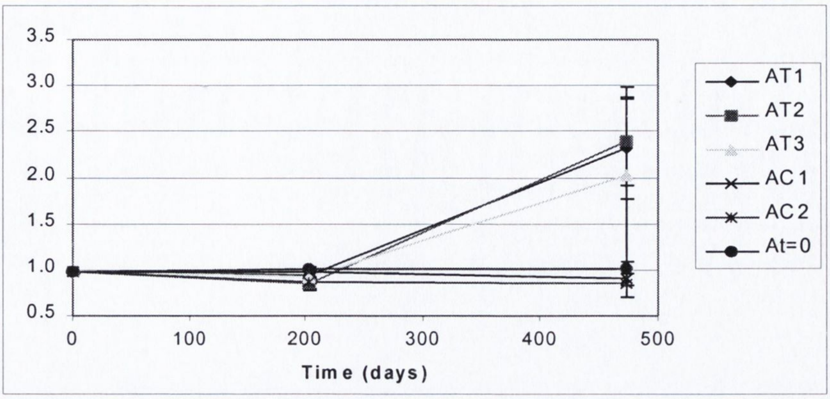
a): C30 hopane removal from Alba microcosms



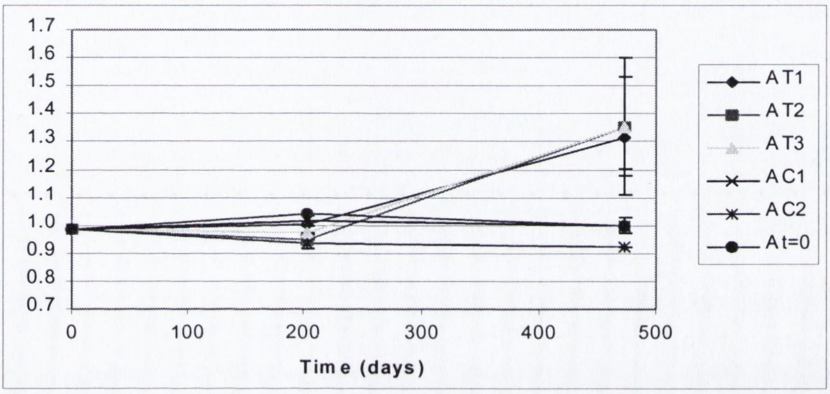
b): C30 hopane removal from Forties microcosms

Fig. 6.15: C30 hopane (P6) removal from microcosms - mean of duplicate treatments (error bars represent individual microcosms). Sample C30-hopane areas normalised against C30-hopane in Alba reference oil.

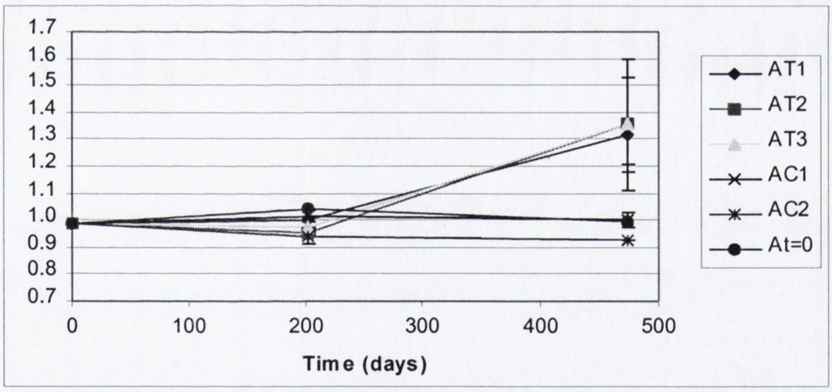
a)
Steranes
Σ 15 peaks



b)
Pentacyclic
triterpanes
Σ 16 peaks



c)
Triaromatic
steranes
Σ 4 peaks



d)
β-steranes
Σ 4 peaks

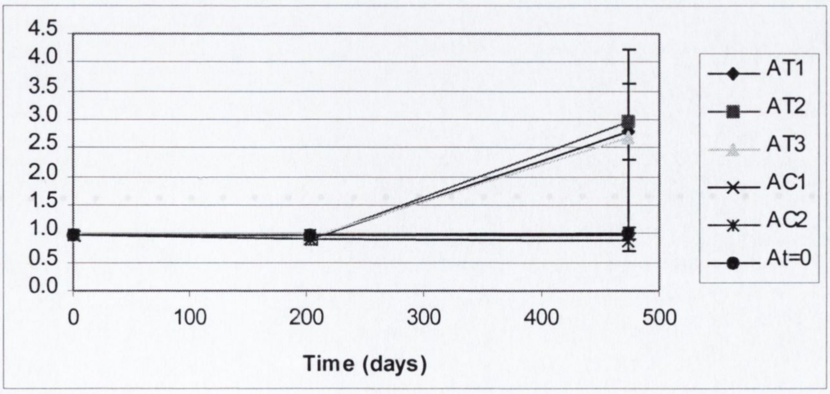
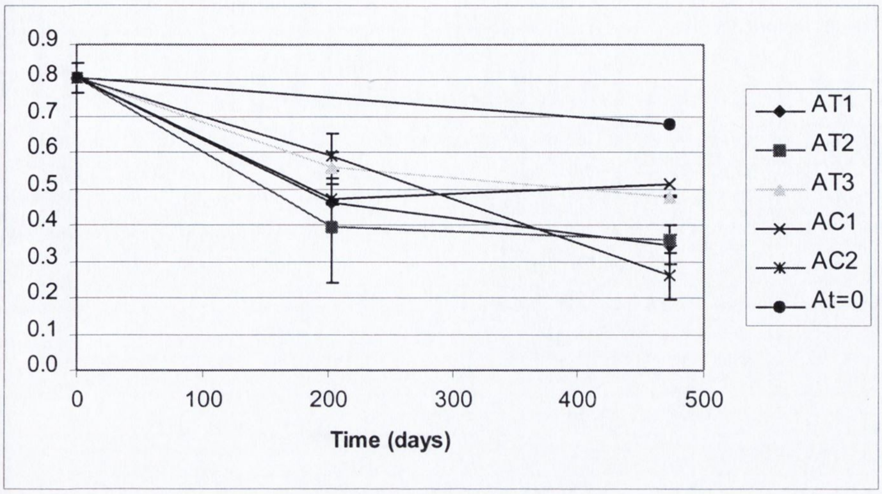
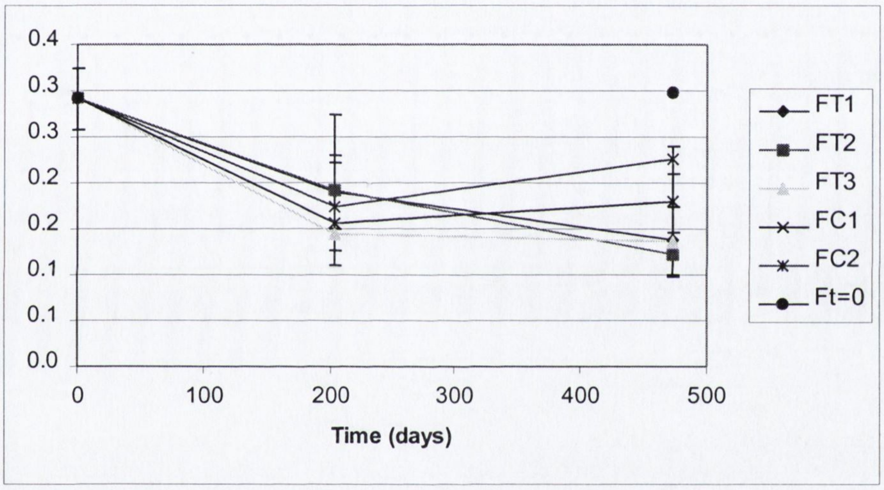


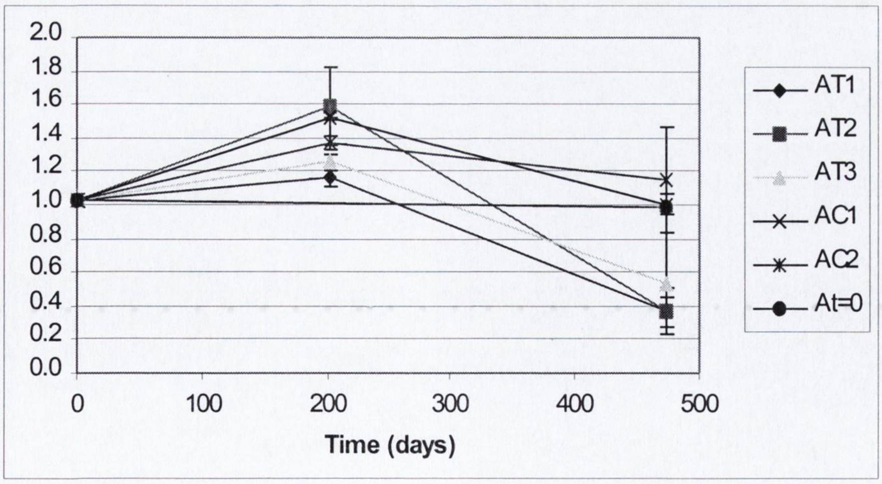
Fig. 6.16 a-d): Removal of biomarker parameters from microcosm sediment relative to P6 (C30-hopane) and normalised to reference oil. Mean values for duplicate microcosms with error bars denoting individual microcosms.



a): TAS4 removal from Alba microcosms



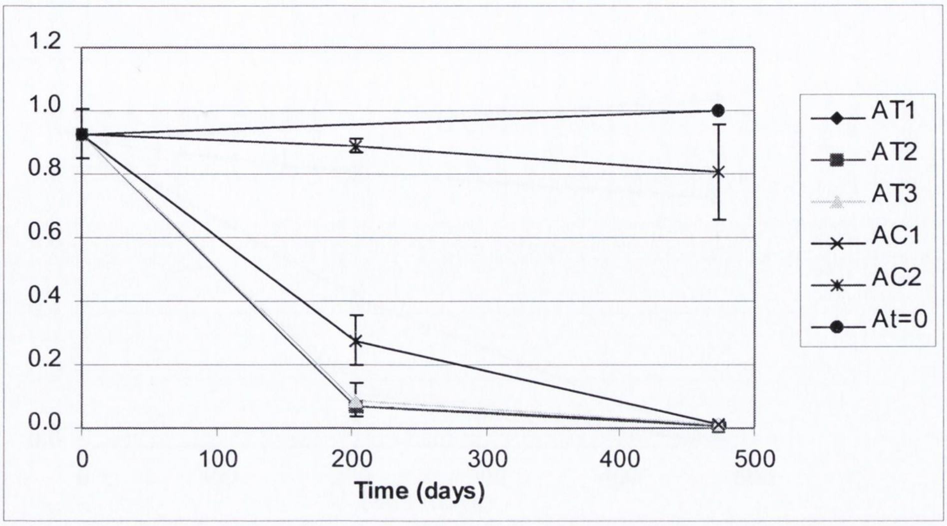
b): TAS4 removal from Forties microcosms



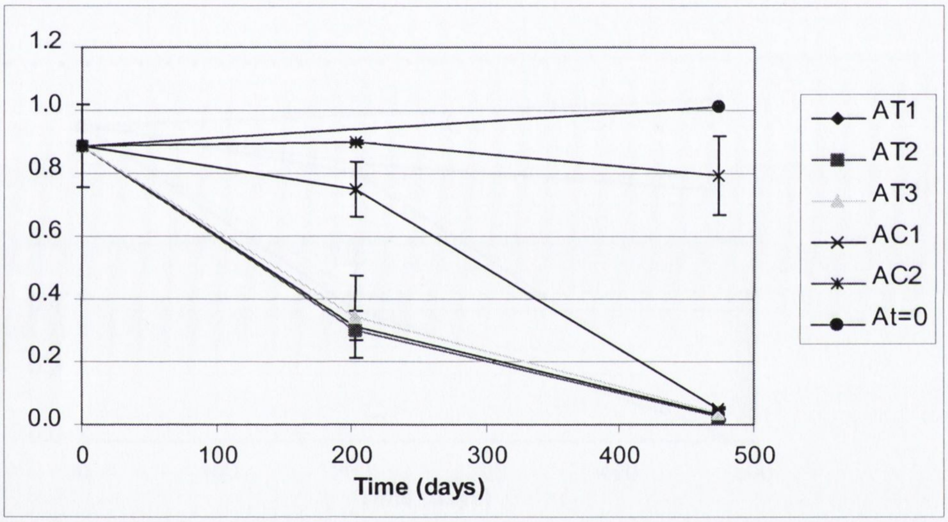
c): P6/TAS4 in Alba microcosms

Fig. 6.17: *Removal of triaromatic sterane (TAS4 -20R ethylcholestane) from microcosms normalised to Alba reference oil.*

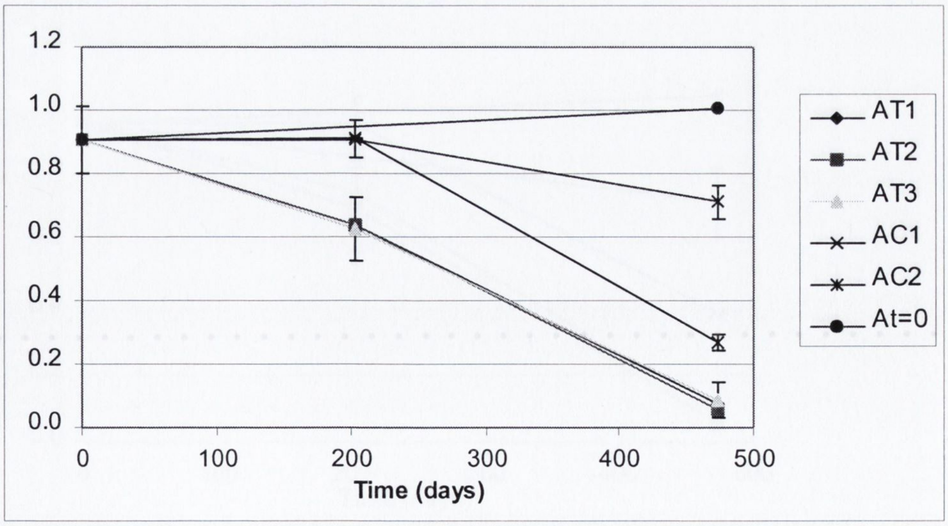
a) C1P
Σ 4 peaks



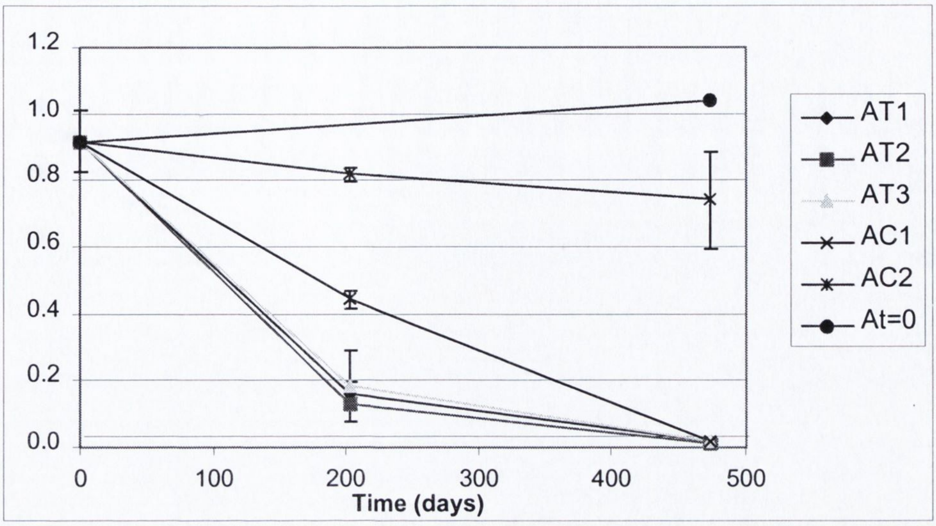
b) C2P
Σ 4 peaks



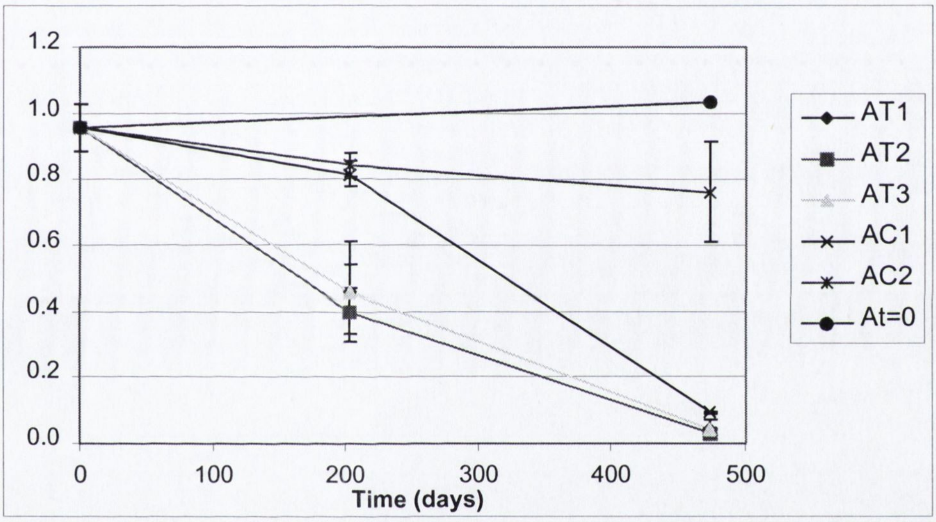
c) C3P
Σ 4 peaks



d) C1D
Σ 3 peaks



e)C2D
Σ 4 peaks



f)C3D
Σ 6 peaks

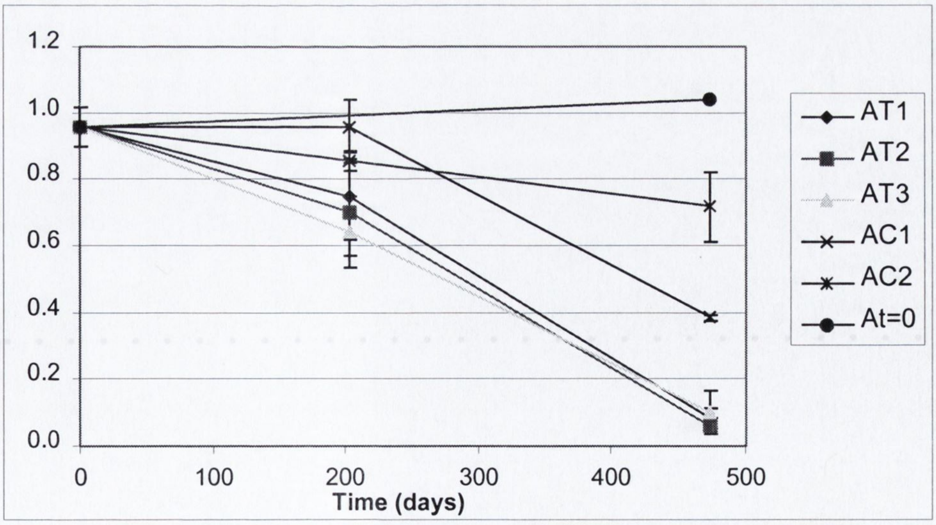
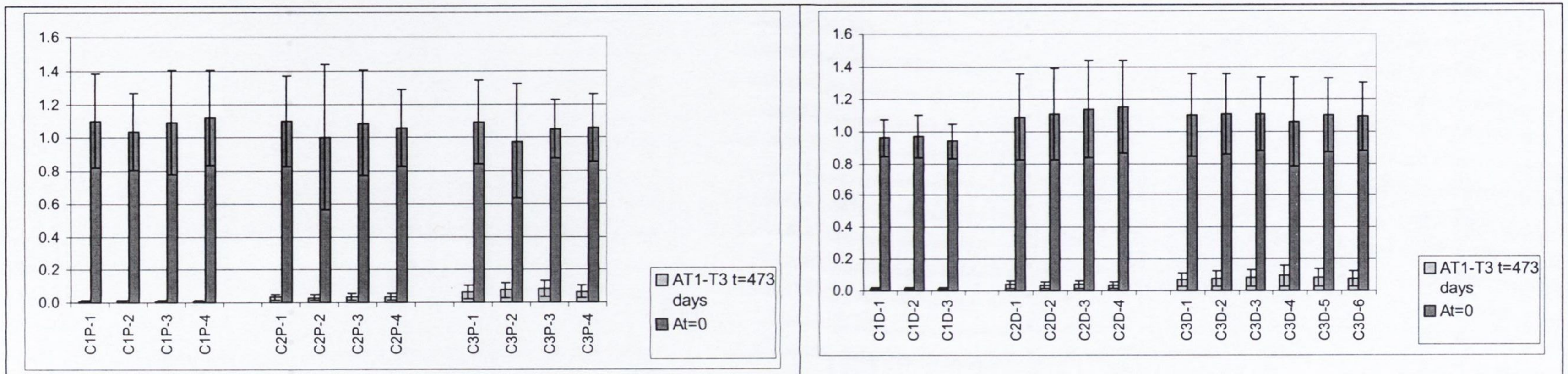
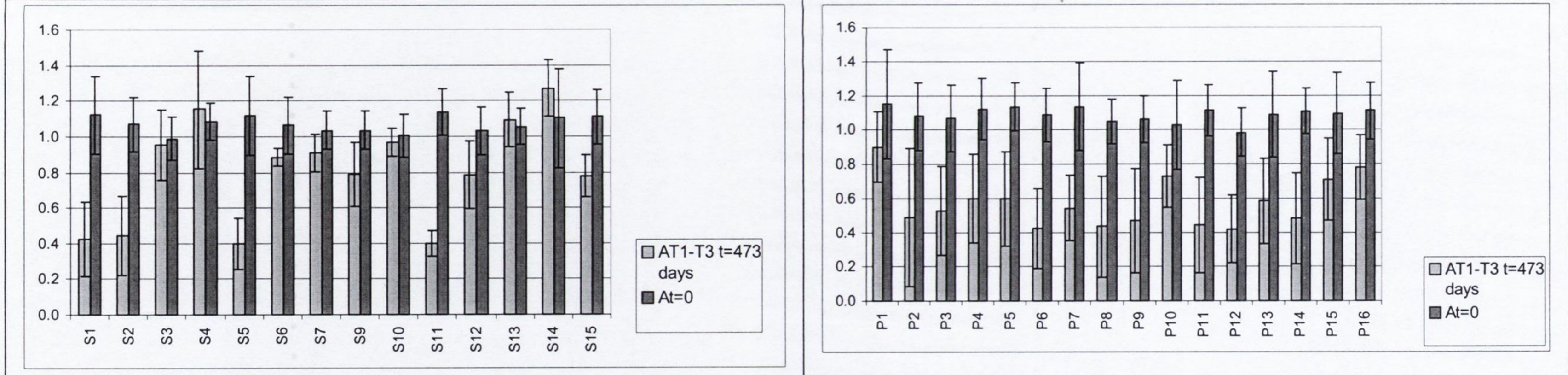


Fig. 6.18 a-f): Biodegradation of C1-, C2-, and C3- phenanthrenes and dibenzothiophenes normalised to TAS4 and reference oil for mean of duplicate Alba microcosms. Error bars denote individual microcosm values.



a) Substituted phenanthrenes

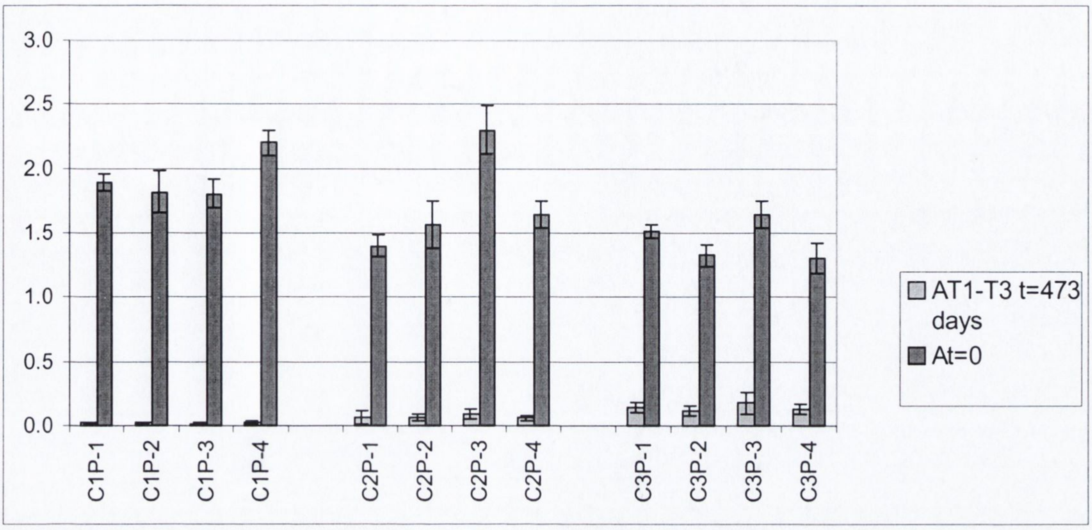
b) Substituted dibenzothiophenes



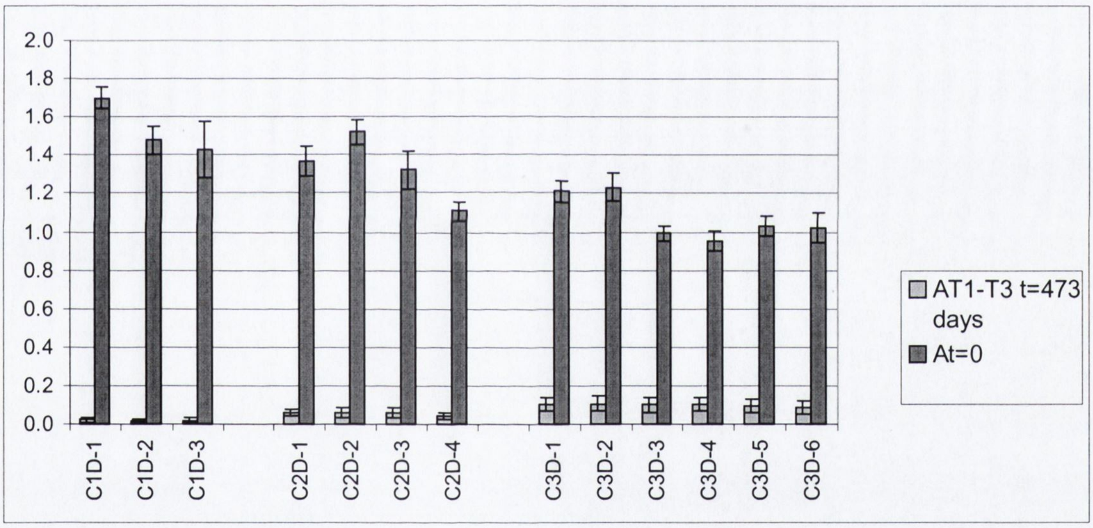
c) Steranes

d) Pentacyclic triterpanes

Fig. 6.19: Biodegradation of individual parameters for treated microcosms at t=473 days compared with t=0 samples. Mean for AT1-AT3 (n=6, t=473) and t=0 (analysed at t=0, 203 and 473 days, n=4). Mean values = (parameter/TAS4)sample: (parameter/TAS4)reference sample. Error bars = 1xStandard deviation



a) Substituted phenanthrenes



b) Substituted dibenzothiophenes

Fig. 6.20: Biodegradation of substituted PAH in Forties oil for treated microcosms at t=473 days. Mean for FT1-FT3. (n=6, t=473) and t=0 (analysed at t=0, 203, 473 days, n=3). Mean values = (parameter/C30-hopane)sample: (parameter/C30-hopane)reference sample. Error bars = 1xStandard deviation

7. OIL SPILL SOURCE IDENTIFICATION AND FINGERPRINTING TECHNIQUES

7.1 BACKGROUND

Analytical results from the bioremediation trials, and in particular GC-MS results, provide useful information for establishing the effect of severe biodegradation on analytical data commonly used for correlation or differentiation of oil spills with suspect sources. Many analytical techniques have been applied for the purpose of identifying or confirming the source of spilt oil (ASTM 1981, Butt 1986, ASTM 1991a, Anon. 1991b, ASTM 1995, McGovern 1998). Other related purposes are temporal and spatial tracking of oil in the environment, and distinguishing the burden of oil due to a given source from other petroleum inputs and background hydrocarbon levels. In the case of major spills issues of compensation and liability can mean that there are often huge financial stakes dependent on the outcome of chemical analysis. Data collected during this experiment for the Alba microcosms was used to compare commonly used oil spill identification techniques and assess the robustness and limitations of these techniques when applied to severely weathered oil.

Chemical analysis techniques used for oil spill identification include infra-red spectroscopy, synchronous scanning and total fluorescence spectroscopy, measurement of nickel and vanadium, gas chromatography and gas-chromatography-mass spectrometry. Often combinations of techniques are used, such as capillary GC with flame ionisation detection as an initial screening method followed by comprehensive GC-MS analysis for confirmation. Expert interpretation of results is required based on matching a range of parameters or indeed patterns or fingerprints. Two fundamental requirements of an oil identification analytical programme is that:

a) it should have a high power of differentiation between different oils

b) it should be insensitive to weathering or at least it should be possible to account for the effects of weathering during interpretation.

The power of differentiation is the inherent ability of an analytical methodology to distinguish between different oil or oil product samples. This may be simple in many cases. For instance if comparing a fresh diesel sample with a heavy fuel oil, most analytical techniques will be able to distinguish the samples easily. In this case the difference in the boiling range and general composition will be obvious using GC analysis. However, often it is difficult to differentiate oils and even crude oils and heavy bunker oils can be difficult to tell apart (Dahlmann 1994). When oils appear similar using given analytical techniques, it is difficult to decide whether they match or not. The given technique may not have the ability to distinguish between similar oils, bearing in mind that crude oils are complex mixtures of hydrocarbons the composition of which can be very similar for oils from many sources. Furthermore, even if minor differences between spill and suspect source sample are evident, this may be due to analytical variablility. This is further complicated as sometimes spilt oil, for example from ship's bilge, can be a mixture of oil from a number of sources. It is impossible to categorically define the specificity of a particular method; that is to say that it can distinguish a particular oil or petroleum product from all other oils and petroleum products in existence. For this reason, samples of all possible suspect sources, (including mixtures such as bilge if relevant), are generally analysed along with spill oil samples, and usually in the same batch to reduce analytical error. The object is that all potential suspects will be eliminated with the exception of the sample representing the polluting oil. This can then be declared a match and provide strong evidence in presenting a legal case. Other supporting evidence can include information on possible sources, prevalent wind speed/direction and tide/currents (often presented using retrospective modelling), and estimated quantity spilt. Obviously, the greater the power of differentiation of the analysis the more effective the power of elimination of suspects and therefore the greater the ability to identify the culprit.

Another problem when matching spilt oil with suspect source samples is that weathering can have a rapid and marked effect on the integrity of the sample. This needs to be accounted for in interpretation of analytical results. Physical changes such as slick formation, emulsification, dispersion or formation of tar balls can occur. The primary causes of weathering that effect oil composition, and thus fingerprinting, are evaporation, dissolution into the water column and biodegradation. Evaporation generally removes volatile hydrocarbons (<*n*C14) very rapidly and up to 40% of a typical light crude oils may be lost within 24 hours. Dissolution preferentially removes more polar components of the oil. Thus the aromatic compounds are typically more amenable to dissolution than aliphatics. Biodegradation is a slower process, and will usually only become an issue in correlating spill samples weeks to months after a spill. However, the effect of biodegradation on spill patterns and parameters is less predictable than for physical weathering. Oil spill identification guidelines generally do not provide assistance for interpreting and comparing oils that have undergone substantial biodegradation.

After a major oil spill it can be difficult to determine when oil contamination in a given area has returned to background, or pre-spill norms, especially as there is rarely significant or comparable pre-spill monitoring data available. This can make it difficult to assess the extent of environmental damage, and in turn have implications for the determination of reparation due. These issues presented difficulties after the infamous *Exxon Valdez* spill in Prince William Sound, Alaska in 1989. Organic geochemistry was widely used after the *Exxon Valdez* oil spill to distinguish Prudhoe Bay crude oil from multiple other sources, including diesel and diesel soot, pyrogenic hydrocarbons, natural oil seeps, and California (Monterey) tars (Bence *et al.* 1996).

When using parameters (such as component ratios, e.g. pristane:phytane) or patterns to distinguish spilt oil from other sources, a knowledge of the effect of biodegradation on these parameters/patterns is necessary to fully use such data. Biodegradation of spilt oil can make correlation with its source more difficult. If a correlation is found for an environmentally weathered oil and a fresh suspect source sample, examination of the extent of weathering, including biodegradation, on certain parameters and patterns, may assist in roughly estimating the length of time the oil has been exposed to weathering, and therefore when it was spilt.

7.2 PRISTANE:PHYTANE RATIO

Gas chromatography with flame ionisation is commonly used in source identification of oil spills. ASTM (1991a) provide a standard method, and other standard methods such as Nordtest (Anon. 1991b) use capillary GC as a screening tool. GC provides a profile of hydrocarbon composition primarily based on boiling point distribution. GC traces can be very susceptible to weathering depending on the oil sample, and for a light crude oil evaporation of the lighter fraction and dissolution of aromatics will rapidly alter the trace. The remaining profile is based primarily on the *n*-alkanes, the isoprenoids such as pristane, phytane and farnesane and the UCM. These components have very limited power for distinguishing different oils. Furthermore the *n*-alkanes are probably the oil component most susceptible to biodegradation. As already discussed (see chapter 6) C17:pristane and C18:phytane ratios are useful indicators of biodegradation, although there are limitations as has been demonstrated earlier (chapter 6.3.1). Pristane and phytane are more resistant to biodegradation and their ratio is often used as simple source indicators. Pristane and phytane ratios from the Alba microcosms were used to establish the robustness of these values over the course of long term monitoring and the effect of severe biodegradation on the ratio. The results given in table 7.1 are means for the duplicate microcosms and summary statistics. All the pristane:phytane ratios were plotted against time for the course of the experiment and are presented in figure 7.1. The mean of all available data (AT1, AT2, AT3, AC1, AC2, At=0 reference) and two standard deviations are presented. Also the mean of AC2 and At=0 reference samples (i.e. those samples for which no biodegradation has occurred) is presented with two standard deviations. Clearly, while there is some analytical variability during monitoring, the ratios exhibit good robustness and are relatively insensitive to severe biodegradation. Only at 473 days is there a significant change in the ratios for the most severely biodegraded oils, i.e. in the nutrient supplemented microcosms with the exception of AiT3. AiT3 has already been shown to have undergone less degradation than the other 5 nutrient supplemented microcosms, (AiT1, AiiT1, AiT2, AiiT2, AiiT3). Two standard deviations for these 5 samples are also depicted on the graph. Interestingly, the ratios increase suggesting phytane (*i*C20) to be more degraded than pristane (*i*C19).

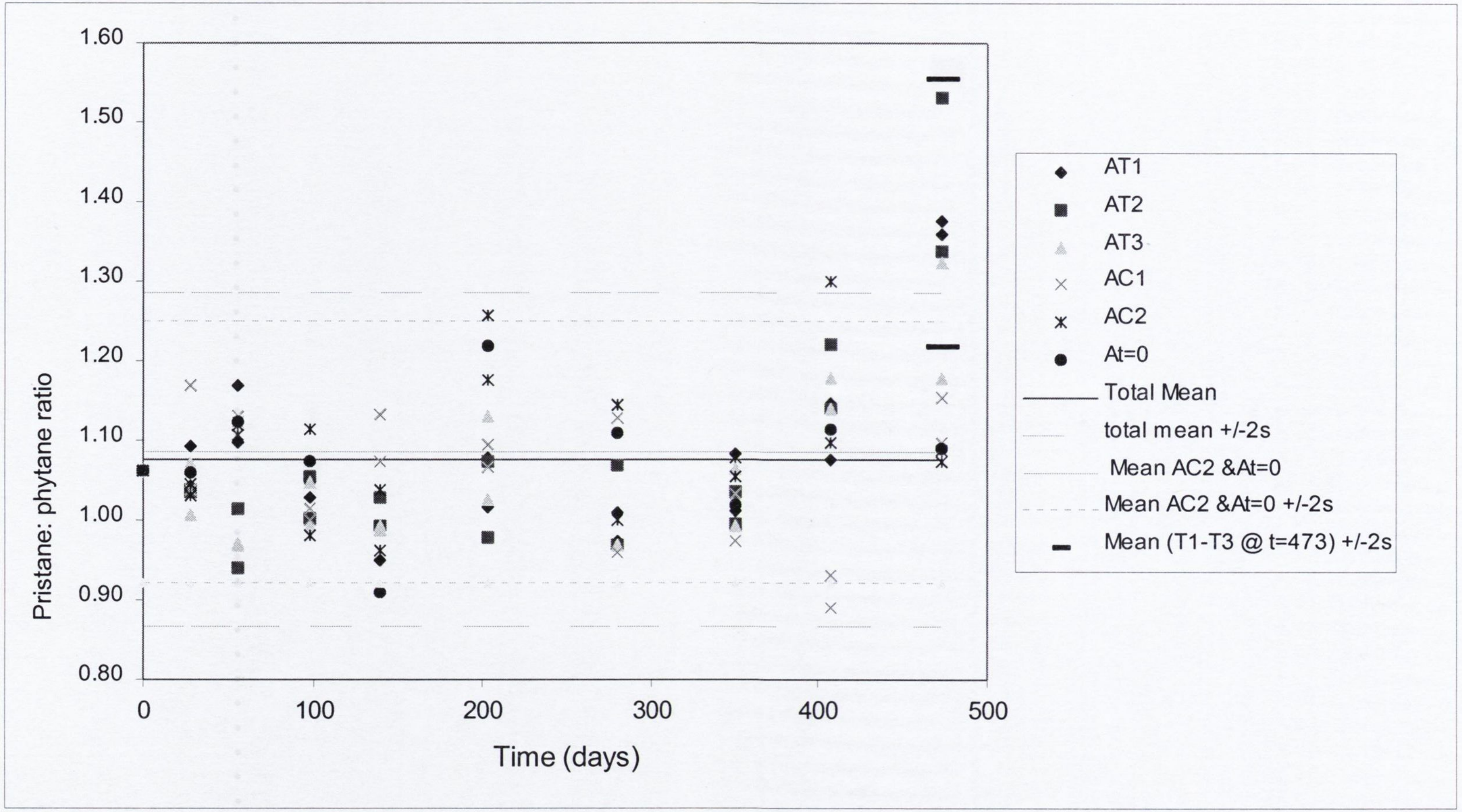


Fig. 7.1: Pristane:phytane ratios for Alba microcosms during the course of the monitoring trials. The mean of all data and the mean of AC2 & At=0 data are presented with ± 2 x standard deviations. For 5 most degraded oils at t=473 days, (AT1 and AT2 i&ii, AiiT3), ± 2 x standard deviations are also presented.

Time (days)	*AT1*	*AT2*	*AT3*	*AC1*	*AC2*	*At=0*
0	1.06	1.06	1.06	1.06	1.06	1.06
28	1.07	1.04	1.04	1.10	1.04	1.06
56	1.13	0.98	0.97	1.13	1.11	1.12
98	1.04	1.03	1.02	1.01	1.05	1.07
140	0.97	1.01	0.99	1.10	1.00	0.91
203	1.05	1.03	1.08	1.08	1.22	1.22
280	0.99	1.02	0.97	1.04	1.07	1.11
350	1.05	1.01	1.03	1.00	1.07	1.02
407	1.11	1.18	1.16	0.91	1.20	1.11
473	1.37	1.44	1.25	1.13	1.08	1.09
Mean	*1.09*	*1.08*	*1.06*	*1.06*	*1.09*	*1.08*
2 x Standard Deviation	*0.30*	*0.30*	*0.20*	*0.16*	*0.17*	*0.16*
Number (n)	*18*	*18*	*17*	*18*	*18*	*10*

Table 7.1: Pristane:phytane ratios for Alba microcosms as measured by GC-FID (Peak area) during microcosm trials. Values are for mean of duplicate microcosms and summary statistics for all available samples.

7.3 TOTAL FLUORESCENCE SPECTROSCOPY

Spectroscopic analysis used in oil spill fingerprinting usually employs infra-red (IR) or fluorescence spectroscopy (UVF). IR spectroscopy provides a relatively low power of differentiation. Synchronous fluorescence spectroscopy and total fluorescence spectroscopy offers greater specificity and is based on the aromatic fraction of the oil. In chapter 2 total fluorescence spectra are given for 3 crude oils and 3 lubricating oils (figure 2.8). The substantially different aromatic composition between the lubricating oils and crude oils makes their differentiation obvious. However, the three North Sea crudes have similar bulk aromatic compositions and the fingerprints are quite similar. In figure 7.2 the total fluorescence spectra for Alba microcosm extracts are given comparing samples that have been substantially biodegraded with oil that has only been exposed to mild abiotic weathering (AC2) and a sample of the initial contaminated sediment (At=0). The analytical method used is described in appendix A.9. Athough all 6 oils yield similar spectra, there is a clear difference in the relative intensity of the maximum value for the samples that are most biodegraded compared with AC2 and At=0. The maximum peak (at

~Ex. λ =230nm and Em.λ = 340 nm) is less pronounced and this is presumably due to the preferential reduction in 2 ring aromatics with respect to higher ring number aromatics. This effect is most apparent for the nutrient treated microcosms. Bearing in mind the lack of specificity exhibited by this technique for distinguishing between three similar north sea crude oils (Gullfaks, Alba and weathered Forties crude oils), and the effect biodegradation has on the plots, total fluorescence spectroscopy is limited in its application for source identification of spilt crude oils. Unless two oils for comparison have a substantially different aromatic composition, it may not be possible to differentiate them. The possibility of alteration of the aromatic composition and its subsequent effect on the total fluorescence spectrum must be taken into account when comparing and interpreting spectra. Although total fluorescence spectroscopy may be useful as a first tier screening tool in an oil spill identification programme, capillary GC will generally prove a much more effective alternative, offering a much more detailed dataset and hence much greater specificity.

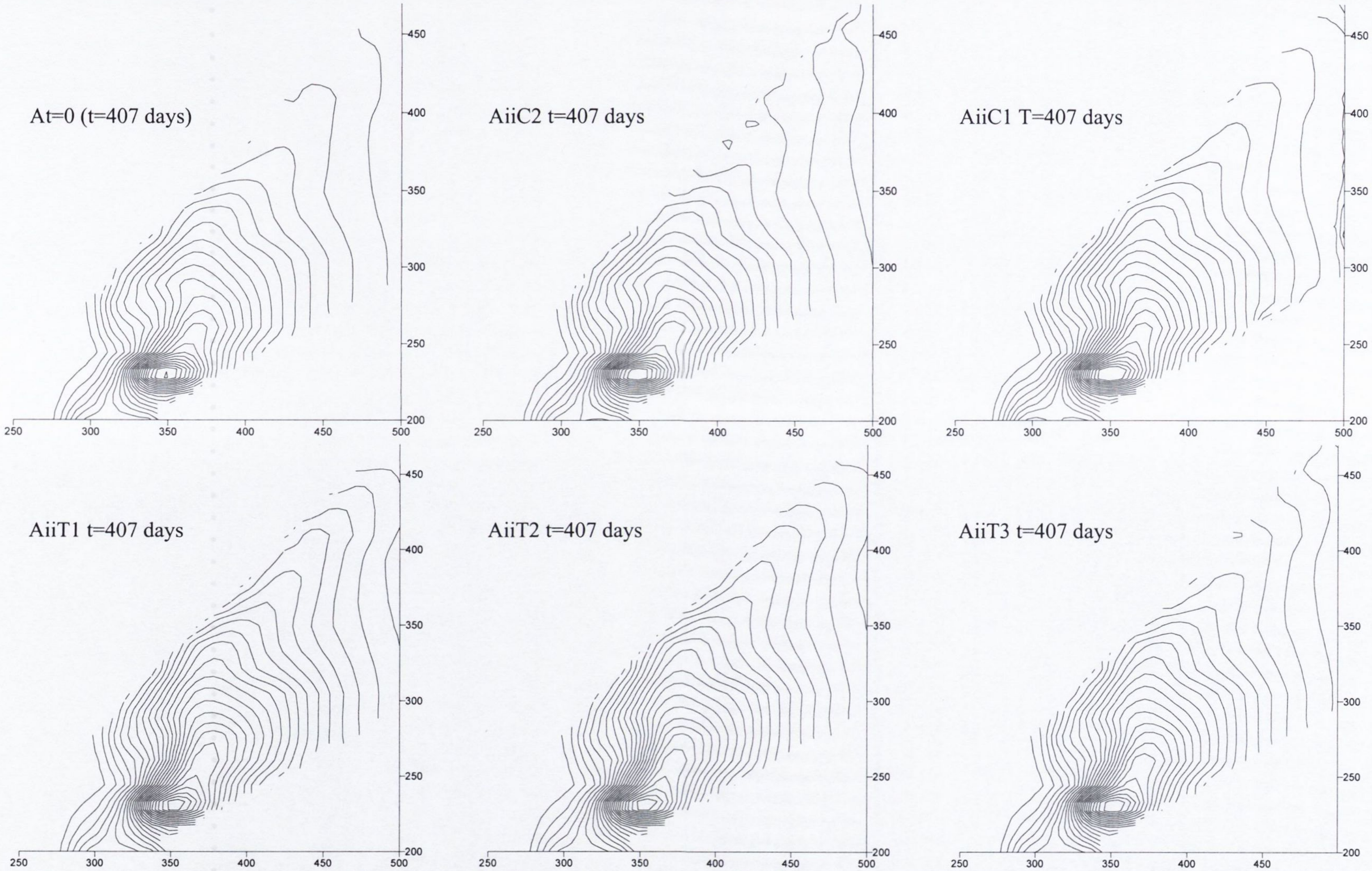


Fig. 7.2: total fluorescence spectra of Alba oil samples from microcosms at t=407 days. Y axes = excitation wavelength (nm). X-axes = emission wavelength (nm). Contours represent emission intensity normalised to maximum intensity.

7.4 PAH PATTERNS AND RATIOS

In recent years GC-MS fingerprinting has been the preferred tool for confirmation of oil spill source identification. GC-MS chromatograms of alkyl-PAH and dibenzothiophene groups, (including heterocyclic dibenzothiophenes) and biomarkers probably provide the most powerful tool in correlation of spilt oil with suspect source oils (Albaiges & Albrecht 1979, Boehm *et al.* 1997, Kaplan *et al.* 1997). The similarity in physico-chemical properties of individual isomers of substituted PAH, for example the C2-phenanthrenes, means that individual compounds in the group are similarly susceptible to weathering. Therefore, the isomer patterns are resistant to physical weathering. Biomarkers are resistant to physical and biological weathering and thus patterns of steranes, pentacyclic steranes, triaromatic steranes are also commonly used in fingerprinting. As for other oil spill source identification techniques, knowledge of the effects of weathering, especially biodegradation, is necessary for interpretation of GC-MS fingerprints. This is particularly true if trying to track oil contamination over a prolonged time period, or identify a contamination source temporally or spatially distant from the contamination event. A major advantage of GC-MS is that many mass chromatograms can be obtained during one analysis, and although many of the differences between different oils can be relatively subtle, the large amount of information provides a very high power of differentiation between oils.

GC-MS methodology used in this experiment is in line with the widely used Nordtest method (Anon. 1991b) and the EUROCRUDE system (Anon. 1995). PAH patterns for Alba microcosms were examined at t=203 and t=473 days to assess changes to oil fingerprints (fig. 7.3). Indeed the Nordtest method is currently (1999) being considered for adoption as a European (EN) Standard.

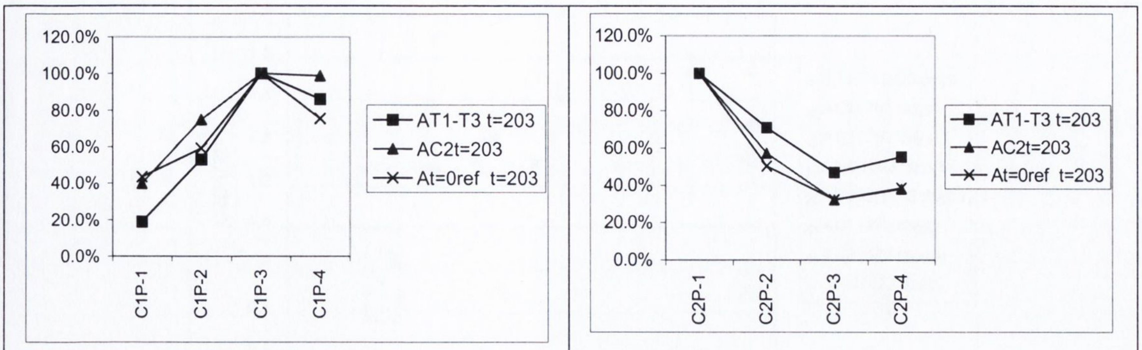
Although alkyl-PAH patterns (mass chromatograms for selected isomer groups) are resistant to physical weathering due to the similar physico-chemical properties of the isomers, they are unlikely to be resistant to biological weathering due to selective biodegradation of different molecular steric configurations. Using peaks identified in appendix B, differences in PAH patterns are evident for AT1-T3(mean) when compared with At=0 and AC2 samples at t=203 days. The first two peaks of C1-phenanthrene (C1P) trace (corresponding to 3-, and 2-methylphenanthrene) are reduced with respect to the second doublet (1- and 9- methyl-phenanthrenes) for the biodegraded oils, although there is a small discrepancy between the non-biodegraded oils. The four selected C2P peaks were increased relative to C2P-1 in biodegraded oil. The C3P trace however showed no apparent difference between biodegraded and non-biodegraded oil at this stage. Changes to substituted dibenzothiophene patterns were also observed for biodegraded oil. A reduction in C1D-1 and C1D-3, (4-, and 1-methyl dibenzothiophene), relative to C1D-2, (3+2 methyl dibenzothiophene) was evident. C2D-1, C2D-2 and C2D-4 have been reduced relative to C2D-3. Differences are also evident for the C3D trace although there is a discrepancy between the AC2 and the At=0. The alteration of C1P patterns, due to selective biodegradation, is similar to that observed by Bayona *et al.* (1986) for C1P during biodegradation experiments for an Arabian light crude oil residue in sea water, with a bacterial isolate (*Pseudomonas* sp.). However, these observations differ from Bayona's for C1D pattern changes. They observed more rapid degradation of 2/3-methyldibenzothiophenes.

Interestingly, C1P, C2P, C1D patterns show no obvious differences between biodegraded oil and non-biodegraded oil at t=473 days, and the C2D patterns show a lesser difference than at t=203. C3P and C3D patterns are different at this time. This suggests that as biological weathering proceeds, the higher molecular weight components are more robust for fingerprinting purposes. A possible explanation of the close match of severely biodegraded oil C1P, C2P and C1D patterns with those of non-biodegraded oil, is that these hydrocarbons in the biodegraded oil remain only as the fraction that is not bioavailable. In essence, they may have been incorporated in what is a 'subsample' of the original oil, which is excluded from biodegradation. This suggests that the non-bioavailable fraction was sequestered early in the experiment.

Ratios of PAH, such as C2D/C2P and C3D/ C3P, are often used in source allocation techniques for spilt hydrocarbons (Sauer *et al.* 1993, Douglas *et al.* 1996, Boehm *et al.* 1997). The relative abundance of dibenzothiophenes can be extremely variable in crude oils primarily due to differences in source material during oil formation. In carbonate oils (generated from algal and bacterial organic matter in the absence of terrestrial organic matter) the dibenzothiophenes can account for up to 41.7% of total PAH while in paralic oils (enriched in higher plant organic matter) it can be <2.1% (Requejo *et al.* 1996). C2D/C2P and C3D/C3P ratios proved especially useful in distinguishing *Exxon Valdez* oil from background sources 5 years after the spill (Bence *et al.* 1996). Michel & Hayes (1999) used C2P/C3P vs C2D/C3D plots to categorise oil into moderate weathering, advanced weathering and extreme weathering eight years after the *Exxon Valdez* spill. Scatter plots using C3D/C3P as a source indicator and C3D/C3C (C3-chrysene) as a biodegradation indicator have demonstrated good environmental stability of these ratios and the ability to easily distinguish between three different crude oils, (Alaska North Slope, North Sea, Iranian), each exhibiting a wide range of weathering, from different sources after a number of spills in different geographical locations (Douglas *et al.* 1996).

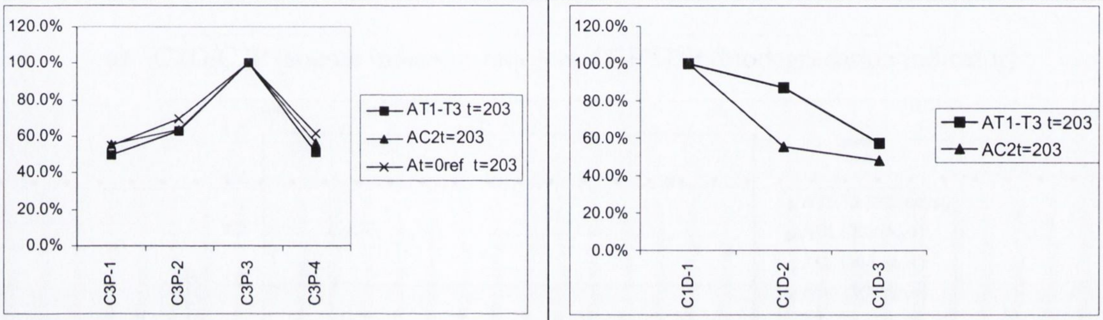
The effect of severe biodegradation on C2D/C3P and C3D/C3P ratios for Alba crude oil in the microcosms was investigated to further establish the stability of these ratios. Data was examined using scatterplots of substituted dibenzothiophenes/substituted phenanthrenes as source indicators. 4-ring PAH such as chrysenes were not monitored during the course of these trials and therefore C3D/C3C based biodegradation indicators could not be applied. Biodegradation rates for PAH tend to be reduced with increasing ring number and increasing alkylation. C3D is degraded more rapidly than C3C and therefore a marked decrease in this ratio is indicative of weathering. As increased alkylation tends to reduce biodegradation rates, ratios of C2:C3 substituted compounds for the same parent PAH can also serve as biodegradation indicators, albeit less sensitive than ratios of components with different ring numbers. This is illustrated in section 6.5.2.

As GC-MS data was collected using Alba reference oil bracketing each sample analysed in duplicate, the reference oil data provided an indication of the analytical robustness of these ratios. The mean C3D/C3P ratio for reference oil at t=203 days was 0.87 (min. = 0.81, max 1.02, sd = 0.5) and at t=473 days the mean was 0.94 (min. = 0.73, max = 1.28, sd = 0.19). A closer inspection of the data revealed that this variation is largely accounted for by instrumental batch to batch variations. For this reason it was decided to employ normalisation of the two averaged sample ratios to the bracketing reference sample values (appendix A.11) similar to the procedure carried out in section 6.5.1. As well as normalising for differences in instrument tuning and differences in source and analyser condition, this improves integration of total areas by having a standard for direct comparison. Scatterplots of C3D/C3P vs. C3P/C2P and C2D/C2P vs. C3P/C2P for Alba microcosms are given in figure 7.4 a&b. These demonstrate clustering of similar samples. AC2 and At=0 for both 203 days and 473 days are clustered. This suggests a good stability of these ratios to water washing. AT1-T3 (t=203 day) samples are also clustered with an increase in both source indicator and biodegradation ratio. Both AC1 t=473 days samples indicate the highest source and biodegradation ratio, these ratios being considerably higher than for the more degraded AT1-T3 (=473 day) samples. Both C2D/C2P and C3D/C3P ratios show the same trend although it is most apparent for C2D/C2P which comprises of more degradable components. The results suggest that the ratios are not stable with severe biodegradation as C2P and C3P are degraded slightly more rapidly than C2D and C3D respectively. However, as biodegradation proceeds the ratios (both source indicator and biodegradation indicators regress towards their initial values. This may be due to the biodegradation ‘curves’, as rates of degradation of the slower degrading component exceeds that of the residual more rapidly degraded component, and/or an increase in the relative contribution of the non bioavailable fraction of hydrocarbon. Figure 7.4c is a scatterplot of both source indicators to give a two-dimensional source indicator plot. C3D/C2D can be used as an alternative biodegradation indicator to C3P/C2P. The results presented indicate that the differences in biodegradation rate between alkylated dibenzothiophenes and their equivalent alkylated phenanthrenes will affect the stability of the source indicator ratios examined and this provides a clear indication as to the limitations for these ratios. However, as the relative abundance of dibenzothiophenes can be very large in different crude oils, these ratios may still have important application for distinguishing between oil sources even after severe biodegradation, if the difference between inherent source oil indicator ratios is greater than the variation that occurs due to biological weathering.



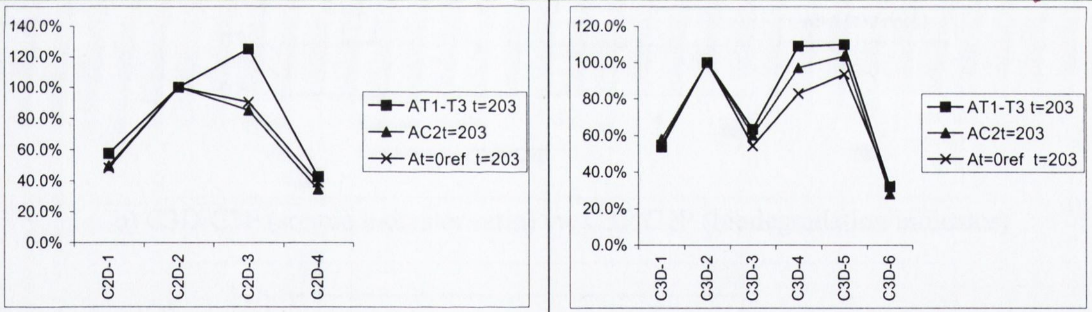
a) C1-Phenanthrenes

b) C2-Phenanthrenes



C3-phenanthrenes

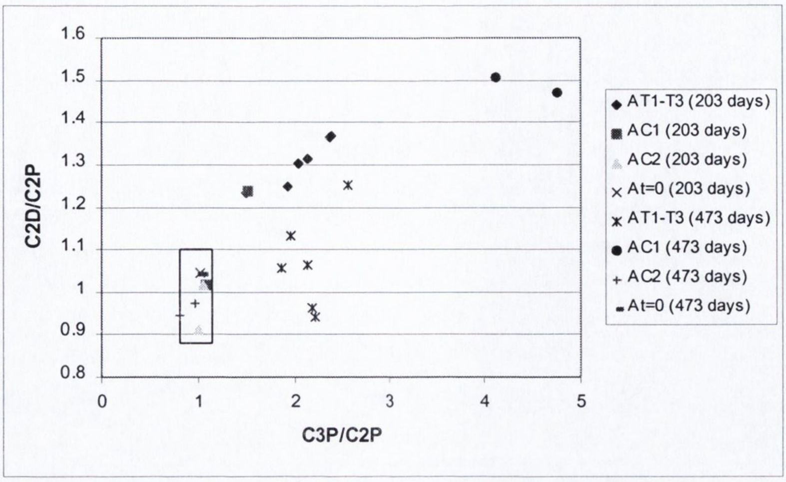
C1-dibenzothiophenes



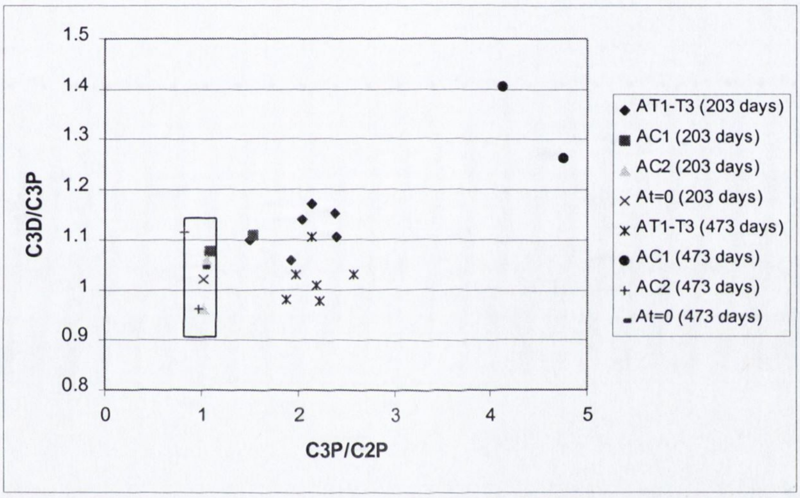
C2-dibenzothiophenes

C3-dibenzothiophenes

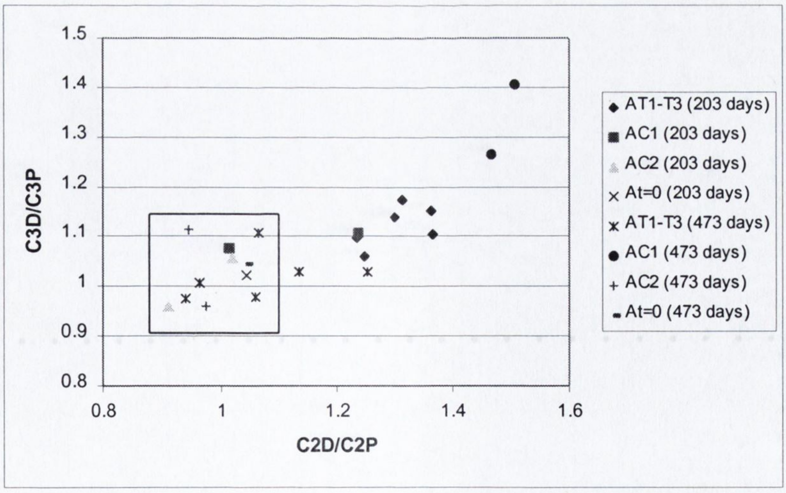
Fig. 7.3: Alteration of GC-MS substituted-PAH patterns based on selected peak areas for biodegraded oil, (mean AT1-T3), and non-biodegraded oil, (AC2 and At=0), at t=203 days



a) C2D/C2P (source indicator ratio) vs. C3P/C2P (biodegradation indicator)



b) C3D/C3P (source indicator ratio) vs. C3P/C2P (biodegradation indicator)



c) C3D/C3P vs. C2D/C2P (both source indicator ratios)

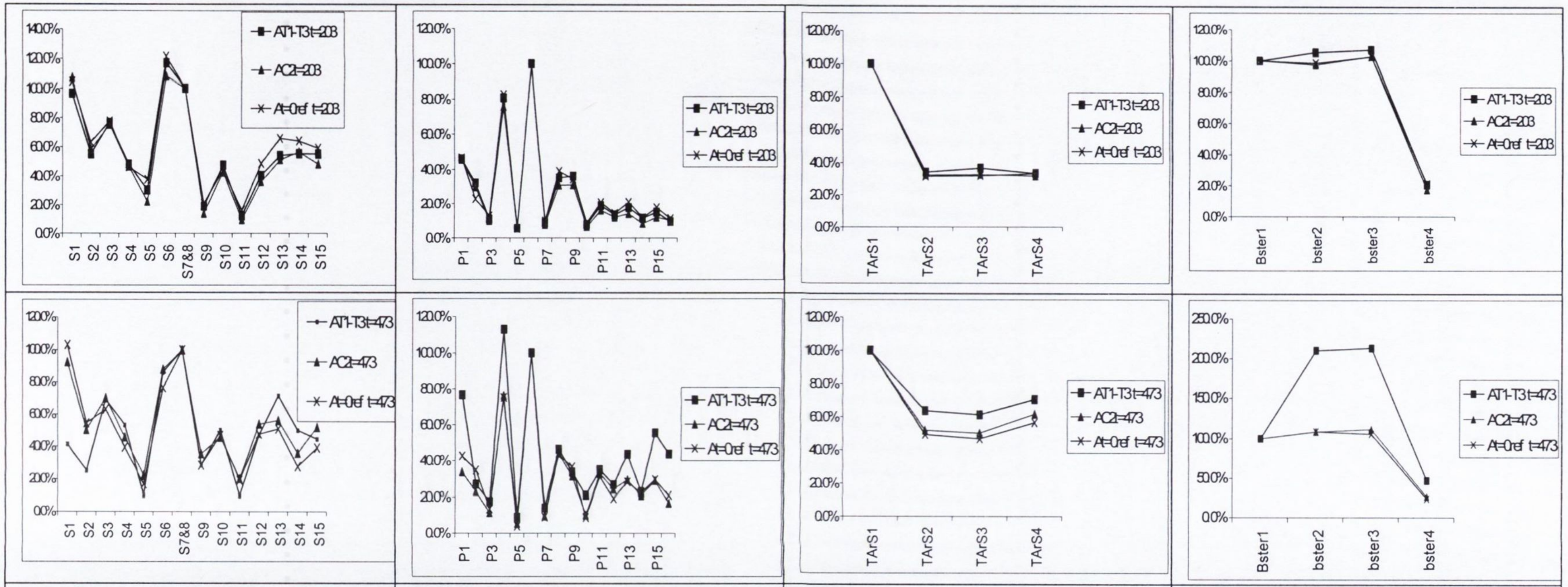
Fig. 7.4: Effect of severe biodegradation on C2D/C2P and C3D/C3P PAH source indicator ratios. {Boxes indicate 2 standard deviations for each parameter for all At=0 and AC2 samples (i.e. non biodegraded samples)}

7.5 BIOMARKER PATTERNS

The biomarkers patterns, calculated as for the PAH patterns, are unaltered at t=203 days but there are substantial effects on the patterns for severely biodegraded oils at t=473 days (fig. 7.5). Biodegraded sterane and hopane patterns have been demonstrated in-reservoir for certain biodegraded oils (MacKenzie 1984). Isomerisation of the natural biological configuration for sterane precursors of 5α(H), 14α(H), 17α(H), 20R, *(αααR),* results in an increasing amounts of other configurations in crude oil (Peters & Moldowan 1993). Laboratory produced studies have agreed with geological observations for sterane biodegradation sequences of αααR>αββR>αββS>αααS and C27>C28>C29 (Chosson *et. al* 1991) although αααR>αααS>αββR>αββS has also been described (Peters & Moldowan 1993). Refer to section 1.4.4.5 for a description of biomarker stereochemistry and nomenclature.

Examination of the AiiT2 t=473 mass chromatogram for steranes (m/z 217 & m/z 218) shows reduction of C27-diasteranes but C28-diasteranes and C29-diasteranes do not show the same reduction and are quite pronounced in the sterane pattern in the biodegraded oil (fig. 7.6. b&c). C27αααS & C27αααR appear to have been biodegraded. Also C27αββR & S have been partially degraded with respect to C28 and C29 αββ (R+S). Partial, biodegradation of αααC28 and αααC29 is evident when compared with αββ C28 and C29 and the R epimer is further degraded than the S.

Biodegradation has also affected the hopane pattern at t=473 days (Fig. 7.6 a). An increase in the relative abundance of a peak corresponding with 22, 29, 30 trisnorhopane (P1) suggests that many of the pentacyclic triterpanes may be partially biodegraded. Moretanes are also seen to be more resistant than hopanes. Differences in patterns are also evident for other biomarkers such as triaromatic steranes and demethylated hopanes.



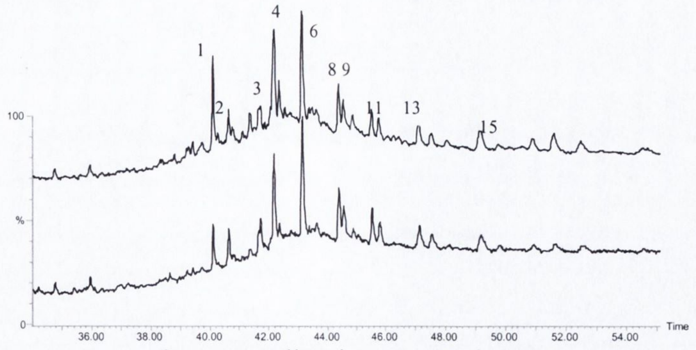
a) Steranes (m/z 217)

b) Pentacyclic triterpanes (m/z 191)

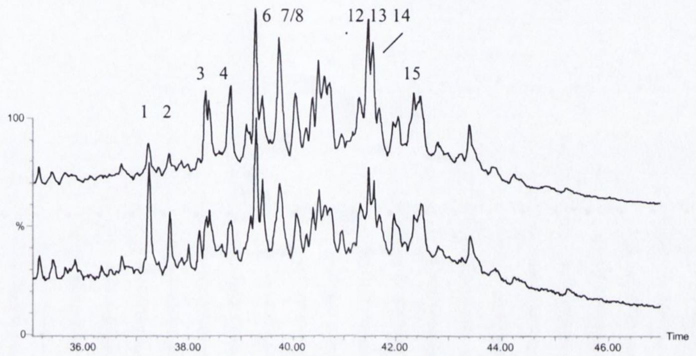
c) Triaromatic steranes (m/z 231)

d) β-steranes (m/z 218)

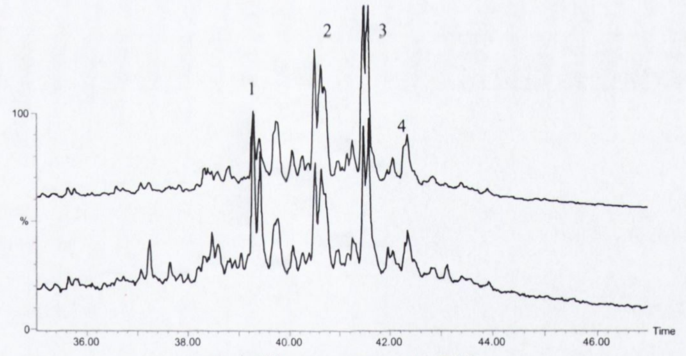
Fig. 7.5: Alteration of biomarker GC-MS patterns based on selected peak areas for biodegraded oil (mean AT1-T3), and non-biodegraded oil (AC2 and At=0) at t=203 days upper, and t=473 days lower



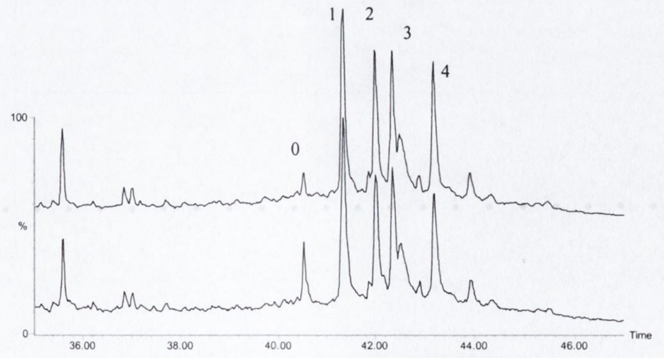
a) pentacyclic triterpanes m/z 191



b) steranes m/z 217



c) 14β steranes m/z 218



d) triaromatic steranes m/z 231

Fig. 7.6: Biomarker patterns at t=473 days for AiiT2 (upper) and AiiC2 (lower) (for peak identifications –see table in appendix B, further peak assignations are given in appendices BX-BXIII)

7.6 BIOMARKER INDICES

Component indices used as maturity and biodegradation markers in geochemical studies have been adapted for use in hydrocarbon source allocation studies (Hostettler *et al.* 1992, Killops & Howell 1988, Aboul-Kassim & Simoneit 1995). Apart from the pristane:phytane ratios already examined, four GC-MS determined biomarker ratios that are commonly used as geochemical maturity indices were examined in this study to assess their environmental stability with severe biodegradation. Two hopane based indices were calculated; the trisnorhopane ratio or Ts/Tm (= P1/P2 appendix B.X) and the C31-homohopane 22S/(22S + 22R) ratio (= P8/P9 appendix B.X). Two sterane based indices were also calculated; C29 $\alpha\alpha\alpha$ ethylcholestane 20S/(20S+20R) and C29 (S+R) ethylcholestane $\alpha\beta\beta(\alpha\beta\beta+\alpha\alpha\alpha)$. These are presented in table 7.2 as means of the data available for the Alba microcosms along with standard deviations. Only the Ts/Tm ratio has been significantly altered. Again, examination of data showed quite high batch to batch variations in calculation of the other indices and no change in the indices could be detected. Variation in chromatographic resolution between batches is one of the reasons for increased error in these ratios as a significant portion of column must be removed each time the EI source is removed from the instrument for cleaning or maintenance. This makes integration data difficult to compare for different batches. Using the bracketing Alba reference oil samples normalises for this error and the results calculated by this method (appendix A.11) are also presented in table 7.2. This reduced the batch to batch error between the poison controls and AT0 samples and therefore gives a clearer picture of the effect of biodegradation on these indices. The increase in the Ts/Tm ratio is still apparent but the C31 homohopane 22S/(22S+22R) ratio and the C29 sterane 20S/(20S+20R) ratio do not appear to have been affected even by severe biodegradation. However, an increase for the C29 sterane $\beta\beta/(\beta\beta+\alpha\alpha)$ index for AT1-T3 (t=473 days) suggests that this ratio was somewhat affected by severe biodegradation. This agrees with the observed mass chromatogram pattern changes suggesting that the $\alpha\alpha\alpha$ configurations were further degraded than the $\alpha\beta\beta$ at t=473 days.

In conclusion, nutrient supplemented microcosms demonstrated significant alteration of oil source identification patterns and parameters, and the patterns/parameters were considerably less affected for seawater control microcosms. This was true for both Alba and Forties microcosms and indicates the more advanced state of biodegradation in the treated microcosms.

Sample	*Time (days)*	*n*	*Ts/Tm*		*C31 homo-hopane 22S/(22S+22R)*		*C29 sterane 20S/(20S+20R)*		*C29-(S+R) sterane ββ/(ββ+αα)*	
			ratio	***SD***	***ratio***	***SD***	***Ratio***	***SD***	***ratio***	***SD***
At=0	0	4	1.5	0.1	0.60	0.01	0.26	0.01	0.44	0.01
AT1-T3	203	12*	1.4	0.1	0.49	0.04	0.13	0.03	0.53	0.05
AC2	203	4*	1.5	0.1	0.50	0.03	0.11	0.01	0.56	0.04
At=0 ref.	203	2	1.5	-	0.53	-	0.15	-	0.55	-
AT1-T3	473	12*	3.2	1.3	0.58	0.43	0.11	0.02	0.57	0.03
AC2	473	4*	1.5	0.2	0.58	0.19	0.19	0.08	0.47	0.02
At=0 ref.	473	2	1.2	-	0.54	-	0.22	-	0.48	-
Alba reference oil normalised indices below										
At=0	0	2[§]	1.0	-	0.50	-	0.48	-	0.49	-
AT1-T3	203	6	0.9	0.2	0.50	0.02	0.49	0.02	0.47	0.03
AC2	203	2	1.0	-	0.52	-	0.48	-	0.48	-
At=0 ref.	203	1	1.2	-	0.49	-	0.48	-	0.52	-
AT1-T3	473	6	2.4	1.0	0.49	0.03	0.50	0.03	0.61	0.03
AC2	473	2	1.3	-	0.50	-	0.49	-	0.52	-
At=0 ref.	473	1	1.0	-	0.49	-	0.50	-	0.51	-

Table 7.2: Mean values for biomarker indices and standard deviations for nutrient supplemented and poison control samples from Alba microcosms and for t=0 samples.

(Values in upper table are not normalised with Alba reference oil and values in the lower table are normalised with Alba reference oil)

** Mean of duplicate microcosms (e.g 6 microcosms for T1-T3) analysed twice*

[§] Calculated from sequence described in appendix A.11.

8. GENERAL DISCUSSION AND CONCLUSIONS

There are many factors involved in successfully bioremediating an oiled shoreline. An understanding of UCM biodegradation is important for effective use of bioremediation as a technology. Many petroleum contaminants will contain significant UCM and, in general, at least partial degradation of UCM may be required to achieve clean-up, depending on the criteria used for assessing clean-up efficacy. The preparation of 'bioremediation guidelines' for use in the event of an oil spill has been proposed (Swannell *et al.* 1996). Such guidelines would aid personnel engaged in a clean-up operation to

a) decide whether bioremediation is an appropriate tool for a given situation
b) design and implement a bioremediation strategy and subsequent monitoring programme.

This project evaluated treatment strategies for bioremediation of UCM-rich oils. Also, the bioremediation trials and monitoring programme provided information relating to the role of oil composition, analytical aspects of monitoring, and other environmental factors in enhancing oil biodegradation. This information augments the current pool of knowledge, on which to develop 'bioremediation guidelines'.

8.1 MICROCOSM DESIGN

The microcosm design proved effective for a pilot scale evaluation of shoreline bioremediation of UCM-rich oils. There are obvious deficiencies in the microcosm design for simulation of a shoreline environment, as the microcosms are not a true representation of a field application. Recirculation of seawater was used, as a constant supply was not available. Also there is an absence of physical factors, such as wave action. For the purpose of investigating the role of specific factors, the microcosm design offered a number of advantages over a field trial, such as

- control of variables (such as particle size, water/air exposure) which can be designed to suit the needs of the study
- exclusion of external variables of no special interest to the study

- homogeneity of contamination, and therefore better sample representativity and reduction of total variability
- reduced sampling and analysis required
- ease of measurement and monitoring of parameters
- release of oil into the environment, which may be difficult to control even at pilot scale, is not required
- a poison control microcosm can be incorporated to investigate physical and physico-chemical aspects on oil removal
- practical considerations e.g. access and security

Two different oils were used in the experimental design. Alba contaminated sediment contained UCM only and Forties contaminated sediment contained approximately 90% UCM but also some *n*-alkanes. The Alba UCM included approximately 9.8% 0-ring, 7.9% 1-ring, 9.1% 2-ring, 5.8% 3-ring and 6.6% 4-ring saturates as estimated by mass spectrometry. 5- and 6- ring naphthenes were very minor contributors to UCM. Forties oil contained greater quantities of 0- and 1-ring saturates. Experiments were set-up in duplicate and the general agreement of replicate microcosms illustrated the usefulness of the microcosm design. However, these considerations do not preclude the need for field trials to demonstrate the efficacy of bioremediation. This microcosm design does offer a useful and relatively simple tool for further investigations of shoreline bioremediation/biodegradation.

Coarse sediment was used in the microcosms, this exhibited good drainage characteristics, ensuring adequate exposure to oxygen and seawater/nutrients. Sediment characteristics and tidal dynamics are important factors influencing shoreline oil biodegradation, if oil has, or is likely to permeate below the sediment surface, and these factors require prior consideration when designing/selecting bioremediation treatments.

8.2 BIOAUGMENTATION

Hydrocarbon degrading bacteria are ubiquitous in the marine environment and will be naturally selected after an oil spill due to the abundance of substrate. The necessity of introducing micro-organisms is questionable, and there is little evidence to suggest that inoculation directly enhances hydrocarbon biodegradation in the field, beyond secondary effects through nutrients or surfactants incorporated in the inocula (Atlas 1995).

In Phase I, enhanced UCM biodegradation was demonstrated in laboratory conditions, using a crude marine environmental consortium that had been previously enriched on UCM carbon source. This suggests that for complex substrates, as presented by UCM, there may be a significant period of adaptation of an indigenous population following a spill. Theoretically, seeding with a population that does not require a lengthy adaptive phase could accelerate UCM biodegradation in the field. For this project, an undefined consortium was considered more appropriate than a product based on isolated single cultures as 'seed', as the latter approach may lose synergistic effects that could be beneficial for degradation of complex substrates present in UCM. Also, common isolation techniques may not isolate the most efficient microbial strains for UCM biodegradation from environmental samples (Amman 1991). Respirometry trials indicated that a cocktail of primary colonies isolated from the selectively enriched undefined consortium performed very poorly when compared with crude undefined environmental consortia, for UCM biodegradation.

The selectively enriched consortium was used as an inoculum for the bioremediation microcosm trials (T1 treatments). For comparison, a second consortium was rapidly grown from a sediment (microcosm sediment prior to artificial contamination) using a simple carbohydrate substrate in the presence of UCM (T2 treatment). Both T1 and T2 included nutrient supplementation. T3 microcosms were nutrient supplemented but no 'seed' population was added. Chemical monitoring of microcosms indicated that inoculation had no appreciable effect on biodegradation rates of Alba UCM or Forties weathered crude oil. Respirometry trials on sediment sampled at t=280 days indicated that the microbial populations in the microcosms exhibited similar capacity for UCM biodegradation. Therefore, while the inocula exhibited a superior performance in laboratory respirometry tests, this was not transferred to field simulated conditions. Possible reasons for this are:

- Inocula were not viable or did not establish sufficiently to have a discernible effect relative to the individual populations.
- Inocula did not retain, or activate UCM degrading potential that had been demonstrated in the laboratory
- Indigenous population rapidly adapted to available substrate.

Laboratory trials show the potential for improving the technology of bioremediation through enhancing metabolic capacity by seeding. As an approach, this could be beneficial for heavy oils or UCM-rich oils, that are naturally slow to degrade, and where indigenous populations may have a significant adaptive period. However, the transfer of laboratory demonstrated potential to the field is difficult. Greater understanding of the factors that contribute to introduced micro-organisms viability, proliferation and hydrocarbon degrading activity in the open shoreline environment is required before bioaugmentation can become a credible bioremediation strategy.

8.3 BIOSTIMULATION

Nutrient supplementation has been convincingly shown to accelerate hydrocarbon biodegradation in many instances (Bragg *et al.* 1996, Swannell *et al.* 1996, Venosa *et al.* 1996). In this study, three microcosms, (T1 - T3), including the inoculated microcosms, were supplemented with a dissolved modified Bushnell-Haas media. As has already been discussed, inoculation had no appreciable effects. Therefore, T1, T2 and T3 microcosms can be considered as replicates, in that they all received the same nutrient load. Chemical monitoring indicated that, although biodegradation rates were quite slow, nutrient supplementation did significantly accelerate rates of biodegradation. GC analysis indicated that the mean biodegradation rate constant for total hydrocarbon in FT1-T3 was $(2.9 \pm 0.4) \times 10^{-3}$ day^{-1} [$(2.3 \pm 0.4) \times 10^{-3}$ day^{-1} UCM, $(6.8 \pm 0.1) \times 10^{-3}$ day^{-1} *n*-alkanes] and the rate constant for the non-nutrient supplemented microcosms, FC1, was $(2.1 \pm 0.4) \times 10^{-3}$ day^{-1} [$(1.7 \pm 0.4) \times 10^{-3}$ day^{-1} UCM, $(4.6 \pm 0.7) \times 10^{-3}$ day^{-1} *n*-alkanes]. Rate constants calculated from gravimetric analysis for total hydrocarbon biodegradation were $(2.3 \pm 0.2) \times 10^{-3}$day^{-1} for FT1-T3, and $(1.8 \pm 0.2) \times 10^{-3}$day^{-1} for FC1. Although, these differences are relatively small, they are notable. Different components of the oil exhibited different rate constants.

The results for Alba microcosms were more difficult to interpret, as physical washing from sediment was an important factor in oil removal. However, GC-MS analysis of biomarkers indicated that this was greater for AC2 microcosms than for other Alba microcosm units, and indeed total removal of hydrocarbon was greatest for AC2 units. Presumably microbial activity retained petroleum hydrocarbon in the sediment either by uptake or adherence and retention, or by alteration of sedimentary characteristics. Gravimetric analysis demonstrated little difference in the biodegradation of UCM in Alba T1-T3 microcosms, compared with AC1 microcosms. Gas chromatography analysis suggested a modest increase in biodegradation for nutrient supplemented microcosms in comparison with AC1 microcosms. The mean rate constant for UCM (~total hydrocarbon) biodegradation in AT1-T3 was (2.4 ± 0.3) x 10^{-3}.day^{-1} and in AC1 (1.8 ± 0.3) x 10^{-3}.day^{-1}. Rate constants for the equivalent UCM components were higher for Alba microcosms than for Forties microcosms. Although there are many possible reasons for this, it may have been due to at least partial abiotic removal from the sediments.

The slightly higher values for rate constants obtained for GC as opposed to gravimetric analysis may be a function of the lower biodegradability and analytical response of the resin fraction of the oil. The resin fraction is relatively resistant to biodegradation, and also will not contribute to flame ionisation detection, proportionately to its mass, compared with saturates and aromatics. Mean hydrocarbon removals of 67% and 61% were determined by gravimetric analysis and 84% and 78% by GC analysis for FT1-T3 and FC1 microcosms respectively at t=473 days.

When examining the rate constants for different *n*-alkanes, the higher the rate constant for intrinsic biodegradation (C1), the greater the magnitude of effect for nutrient supplementation, e.g. a mean 1.73 fold increase in k for *n*C15 and 1.09 fold increase for *n*C35. However, this was not evident for phytane or UCM segments. Mean increases in k of 1.47 fold (total *n*-alkanes), 1.37 fold (total hydrocarbon) and 1.33 fold (total UCM) were observed for biostimulated Forties oil microcosms. This suggests that for oils primarily containing simple substrates, such as *n*-alkanes, biostimulation may be most effective for those oils with highest intrinsic biodegradation rates. The mean increase in the estimated rate constant for UCM biodegradation in nutrient supplemented Alba microcosms with respect to AC1 microcosms was also 1.33.

That greater biodegradation occurred in nutrient supplemented Alba microcosms is supported by results of other analytical techniques which showed substantial differences in oil composition between treated and non-treated microcosms. GC profiles for samples taken at t=473 days are typical of biodegraded UCM and there is a significant difference at this sampling time between biostimulated microcosms and seawater controls.

Other indicators such as C18: phytane ratios and GC-MS measurement of individual aromatic components suggested increased rates of removal for biostimulated microcosms. Investigation of changes in bulk properties indicative of biodegradation, such as fractionation, aromatic composition based on ring number, and mass spectral data, indicated that bulk compositional changes were more advanced for T1-T3 microcosms, than C1 microcosms.

GC-MS analysis of PAH biodegradation best illustrated the difference between nutrient supplemented microcosms and intrinsic biodegradation. C1- and C2-phenanthrenes and C1- and C2-dibenzothiophenes levels were substantially lower in T1-T3 than C1 at t=203 days, but levels had converged at t=473 days. For Alba microcosms, C1P exhibited 92% biodegradation for AT1-T3 compared with 70% for AC1 at t=203 days and C2P exhibited 64% biodegradation in AT1-T3 and 16% for AC1. Similar results, albeit with slightly less biodegradation, were evident for C1D and C2D, (e.g. C2D AT1-T3 ~54%, AC1~ 15% at t=203 days). At 203 days, no biodegradation of C3P and C3D was evident for AC1, but ~30% and ~27% respectively for AT1-AT3. A notable difference between levels of C3D and C3P in treated and non-treated microcosms remained evident at t=473 days (e.g. C3D - AT1-T3 ~ 92%, AC1~60%).

Although nutrient addition clearly stimulated oil/UCM biodegradation, by the end of the experiment, as residual levels of Forties oil contamination were approached, there was less difference between total oil concentrations and composition in the treated microcosms and seawater controls, compared with midway through the experiment, (percentage oil removed FT1-T3=84%, FC1=78% at t= 473 days; FT1-T3= 45%, FC1=33% at t= 203 days). The difference between treated and non treated at t=473 days was more pronounced for Alba, (percentage oil removed AT1-T3=75%, AC1=62% at t= 473 days; AT1-T3= 35%, AC1=27% at t= 203 days).

For both Forties and Alba T1-T3 microcosms, GCMS biomarker fingerprints exhibited alterations in the sterane, pentacyclic triterpane, and triaromatic sterane patterns. These alterations are associated with advanced biodegradation. This was not observed for the C1 and C2 microcosms.

The oil composition on sediment was not altered for AC2 microcosms, and indicated washout, rather than dissolution or evaporation, was the primary mechanism for removal. Washing of oil from FC2 sediments was considerably less. This may be attributable to the physical properties of the Alba crude oil, which may be expected to behave like a lubricating oil, due to the absence of *n*-paraffins. For AC1 removal of the recalcitrant compounds C30 –hopane and TAS4 was considerably less than AC2, suggesting that washout may not have been as important a removal mechanism as biodegradation in the other Alba microcosms. Washing was not a major factor in Forties oil removal and little biomarker removal was observed for FC2 tests and for FC1 tests. With over 75% of TPH (GC) removed, only ~3% of C30-hopane was removed. Visual examination for T1-T3 treatments suggested that leached oil was more rapidly degraded in the reservoirs than in C1 reservoirs.

Duplicate microcosms behaved similarly, with the exception of AT3 where one replicate demonstrated lower biodegradation and UCM degrading potential as measured by respirometry. Clustering of monitoring data was evident for T1, T2 and T3 microcosms for both oils and illustrated the efficacy of the microcosms for these investigations.

Two key factors that influence the rate of hydrocarbon biodegradation are oil composition and nutrient levels. Background nutrient concentrations in the C1 microcosms enabled intrinsic biodegradation to take place, with relatively minor enhancement in total hydrocarbon biodegradation by biostimulation. For substituted phenanthrenes and dibenzothiophenes a much greater increase in removal rate was indicated. The enhancement observed in these trials would probably not justify using bioremediation for clean-up of a real spill of similar oils with similar exposure to background nutrient levels. However, should background levels of nutrients be low, the enhancement factor would be larger, and bioremediation would be a more suitable option. Therefore, it is vital to carry out an evaluation of background nutrient levels and oil characterisation, along with an assessment of other factors such as temperature, tidal dynamics etc., prior to a decision about the applicability of bioremediation in a given case. Based on the results of this experiment, even if nutrient addition does not greatly increase the rate of removal of total hydrocarbons, a much greater increase in biodegradation rates of certain target components, such as PAH may be achieved. As ecotoxicological effects are particularly associated with the PAH fraction of crude oil, this may be sufficient to justify bioremediation through nutrient supplementation. For a spill of UCM-rich oil, such as lubricating oil, the potential for washout must be considered prior to choosing a clean-up strategy.

8.4 CHANGES TO OIL COMPOSITION DURING BIOREMEDIATION TRIALS

UCM was biodegraded and degradation occurred across the entire molecular weight range used in this experiment (GC *n*-alkane boundaries *n*C14 – *n*C35), but lower molecular weight UCM was degraded more rapidly than higher molecular weight material. For weathered Forties oil, which contained approximately 10% *n*-alkanes, UCM was degraded simultaneously with *n*-alkanes, albeit at a slower rate. Aliphatic UCM was degraded more rapidly than aromatic UCM, with resins being most recalcitrant. Asphaltenes were not a significant component of the contaminating oils.

Fluorescence spectroscopy and HPLC indicated that aromatics were degraded preferentially according to ring number. Resistance of alkyl substituted aromatics increased markedly with increasing substitution. Homologous series of alkylbenzenes and alkylcyclohexanes identified by GC-MS were rapidly removed. GCMS analysis indicated that acyclic UCM components were removed more rapidly than naphthenes and that naphthenes were less effectively degraded with increasing condensed ring number. Alkyl cyclohexanes were degraded slightly more rapidly than alkylbenzenes. Results also suggested that Cx-methylated benzenes and Cx-methylcyclohexanes were less resistant to biodegradation than their non-methylated counterparts. At an advanced stage of biodegradation, degradation of some 4- and 5- ring polycyclic compounds was evident. At t=203 days substituted phenanthrenes and dibenzothiophenes had been largely degraded.

In considering bioremediation the target oil composition must be considered, but also the physical state of the oil. Sediment in this experiment was artificially contaminated, such that the oil presented a relatively high surface area for biodegradation. Heavily weathered oils may form tar balls, severely limiting degradation. However, an *n*-alkane deficient oil may not have the same tendency as *n*-alkane rich oil, to form tar balls, and therefore bioavailability may be greater.

A residual level of the most biodegradable components is probably due to unavailability of a fraction of oil for biodegradation, possibly due to sediment binding or inaccessability to micro-organisms. In this instance it is estimated that about 1% of the oil may be unavailable for biodegradation, based on residual levels of readily degradable substrates.

8.5 ANALYTICAL ASPECTS AND MONITORING OF BIOREMEDIATION

The use of different analytical techniques for monitoring oil biodegradation over the course of the trials allowed comparisons of analytical approaches to bioremediation monitoring to be made. Biodegradation studies have employed absolute measurement techniques, such as concentrations of oil or individual compounds, and relative techniques, such as determination of oil or oil components relative to a conserved internal marker.

The selection of analytical methodologies and monitoring tools are of critical importance in bioremediation. Whether a particular bioremediation treatment is adjudged 'successful' may depend on the monitoring tools used. The criteria for evaluation of treated and non-treated sites are also important. For instance, should target values/concentrations be used as decontamination goals, as is often the case with contaminated land, or should treatments be evaluated in terms of biodegradation rate enhancement. While the latter is preferable, it has implications for the cost of monitoring required. Results for this experiment suggest that, even if bioremediation enhances biodegradation rates, a strict clean-up target based on total petroleum hydrocarbon (TPH) may not necessarily be achieved sooner.

GC-FID exhibits a bias for assessments of total oil biodegradation, when compared with gravimetry. GC analysis indicated greater oil removal, presumably as polar materials are not determined proportionately by GC. (FT1-FT3 removal: 84% - GC, 67% - gravimetry; AT1-T3: 75% - GC, 48% - gravimetry). Other absolute measurement techniques such as fluorescence and infrared spectroscopy are based on particular oil components, (aromatic and aliphatic components respectively for fluorescence and infra-red), and biodegradation of these components may not reflect the biodegradation of the total oil. This bias is inherently greater for fluorescence spectroscopy as the aromatic fraction represents a smaller proportion of the oil, and choice of wavelength is further selective for specific aromatic components.

While absolute measurements were possible for microcosms, the heterogeneity of hydrocarbon contamination in 'real world' scenarios presents problems for the use of such techniques. To avoid this, conserved internal markers are commonly used. As isoprenoids are more resistant to biodegradation than *n*-alkanes, C17:pristane and C18:phytane ratios are commonly used indicators of biodegradation, and have the advantage of being independent of concentration and sample heterogeneity. During the bioremediation trials, severe limitations were apparent with C18:phytane ratios as a monitoring tool. A decrease in the ratio was only evident early in the experiment, while in the latter stages, the rate of isoprenoid biodegradation exceeded that of residual *n*-alkanes, and ratios increased again substantially.

The GC-MS analytical scheme devised for this project was adapted from the EUROCRUDE design (Anon. 1995), and employed external normalisation to a bracketing reference oil and internal normalisation to a conserved internal marker. This system proved very effective for monitoring biodegradation over a long period as it compensates for sample variability and also the analytical variability that is inherent in GC-MS analysis.

In recent years most investigators have used C30-hopane as a conserved internal marker (Swannell *et al.* 1995, Bragg *et al.* 1996, Venosa *et al.*1996, Head 1998). This compound is a pentacylic triterpane and is often considered to be resistant to weathering, including biodegradation. In this experiment, partial biodegradation of C30-hopane was evident for treated microcosms at t=473, especially for the Alba oil. This conclusion is supported by significant changes to biomarker patterns. GC-MS data indicated that not only was this compound biodegraded but that it was one of the most degradable of the biomarker compounds examined. Although, many other biomarker parameters were effected to a lesser degree than C30-hopane, another pentacyclic triterpane or sterane was not identified as a suitable alternative. The criteria for selection of an internal marker are conservation, abundance and chromatographic resolution. The triaromatic steranes were observed to be among the more biologically recalcitrant biomarkers in the oils and they also fulfilled the criteria of abundance and resolution in the two crude oils. However, due to the aromatic nature of these compounds they are likely to be more water-soluble and therefore more prone to abiotic weathering. Thus they are not ideal internal markers. The C28-(20R) triaromatic sterane (20R ethylcholestane/stigmastane ~ TAS4 [appendix BX.III]) was selected as the most appropriate triaromatic sterane for investigation. While these results indicate that there are limitations in using C30-hopane as an internal marker, this is dependent on the biodegradability for total oil and target monitoring components, relative to the C30-hopane. In nutrient treated Alba microcosms, with only 48% of the oil removed as determined gravimetrically, (75% by GC), C30-hopane had been significantly biodegraded. 79% of C30-hopane had been removed by a combination of washing and biodegradation, and normalisation to TAS4 suggested that this was primarily due to biodegradation (~59%). GC-MS analysis suggests that partial biodegradation of TAS4 in Alba may have occurred, but not of the order experienced for C30-hopane. In fact TAS4 was observed to be one of the most refractory of the biomarkers monitored. C30-hopane removal was also apparent in Forties microcosms (T1-T3) but not FC1 or FC2. However, preferential removal of TAS4 with respect to C30-hopane, probably due to abiotic factors, was greater in some Forties microcosms. In summary, selection of a conserved internal marker for GC-MS analysis depends on circumstances. Both TAS4 and C30-hopane were abundant and easily resolved in the oils used for this experiment. However, C30-hopane exhibited better abiotic conservation characteristics, (i.e. lower water solubility), and TAS4 exhibited better biological conservation. For monitoring of the Alba trials C30-hopane depletion through biodegradation was the greater, so TAS4 was selected as internal marker. This was less clear for Forties oil so C30-hopane was used for evaluation of biodegradation for Forties microcosms.

An appropriate use of conserved internal markers in a bioremediation/ biodegradation monitoring programme would be to incorporate both triaromatic steranes, pentacyclic triterpanes and any other candidate marker compounds into the analytical programme. The data can be evaluated to select which is most appropriate when data has been collected. There is an argument that the polyaromatic TAS4 would be a more suitable internal normaliser than polycyclic alkanes, such as C30-hopane, for determination of true biodegradation of PAH, as its physicochemical characteristics would be more similar to PAH. Another approach may be the use of TAS4 in GC-MS analysis of saturate and aromatic fractions. Providing an appropriate cut is made, C30-hopane could be used as an internal marker for assessment of aliphatic biodegradation and TAS4 for aromatics. Whatever the approach, it is essential that verification of the conservation of proposed internal markers is carried out before assessment of GC-MS data.

A qualitative or semi-quantitative approach to advanced bioremediation assessment would be to define stages of biodegradation, as previously described. Biodegradation of biomarkers, and biomarker indices, can then be used as indicators of advanced biodegradation, similar to C17:pristane ratios in the early stages of biodegradation.

A bioremediation monitoring strategy should be based on a number of parameters, such as TPH, PAH, alkanes. GC-MS offers the most useful monitoring tool as total hydrocarbons and individual parameters can be measured, including conserved internal markers. Use of m/z 57 has been proposed for quantifying total oil (Reddy & Quinn 1999), but this would be inappropriate for biodegraded oils as it does not account for selective biodegradation of aliphatic UCM components.

Ion abundance ratios also proved a relatively simple and reliable tool for investigating compositional changes in bulk UCM with biodegradation over a lengthy time series, when used with bracketed reference oil. Mass spectrometry should be used to determine bulk UCM composition, prior to using bioremediation to clean-up a UCM-rich oil spill.

8.6 OIL SPILL FINGERPRINTING

Oil spill source identification techniques usually involve a two-tier system. Screening by GC, total fluorescence or another technique is followed by confirmatory GC-MS analysis. Total fluorescence spectroscopy provides poor specificity and thus limited power for distinguishing different oil sources. Furthermore, preferential biodegradation of lower molecular weight PAH alters the spectrum. As a first choice screening tool capillary gas chromatography offers a better alternative, providing much more detailed information.

Pristane:phytane ratios were observed to be resistant to biodegradation for Alba oil over the course of the experiment although an increase in these ratios was observed for nutrient treated microcosms at the end of the experiment.

The stability of dibenzothiophene based PAH ratios (C3D:C3P and C2D:C2P) with advanced biodegradation and the implications for the use as source allocation indicators was examined. The results suggested that biodegradation did affect the ratios. However, depending on the individual circumstances in which they are used, this may or may curtail their usefulness as source indicators. The global variability of dibenzothiophenes in crude oils and oil products is high and in a particular situation, the change of ratio induced by biodegradation may not be significant in comparison to the difference in ratio attributable to different sources of contamination.

The data collected in this experiment afforded an opportunity to evaluate the robustness of GC-MS fingerprinting patterns in severely biodegraded oil. Alba microcosm samples were used for this purpose. The patterns are commonly used as ‘signatures’ in source identification and allocation. Alkyl-PAH derivatives, including dibenzothiophenes, and biomarkers were examined. At t=203 days, changes in biodegraded PAH patterns were observed, presumably through selective biodegradation of individual isomers. Higher molecular weight PAH and biomarker patterns, which had been transformed to a lesser extent, were less affected. At t=473 days, the biomarker patterns for steranes, hopanes, and triaromatic steranes and C3D, C3P were substantially effected. PAH that had demonstrated significant pattern change at t=203 days, reverted to the original pattern, despite being present at residual levels. This may be due to a residual fraction remaining that is not bioavailable having been sequestered early in the experiment. Therefore, the residual oil would be representative of the original oil. The sterane biodegradation sequence agreed with previous geochemical observations suggesting that the lower molecular weight compounds are least recalcitrant, that the $\alpha\alpha\alpha$ configuration is more degradable than $\alpha\beta\beta$ and that S configurations are more resistant than R.

Four commonly used maturity indices that have also been used for source allocation studies were assessed to discern their resistance to biodegradation. The Ts/Tm index was significantly affected in the most severely degraded samples at t=473 days. Results also suggested an increase in the C29 (S+R) ethylcholestane $\beta\beta/(\beta\beta+\alpha\alpha)$ ratio. There was no evidence of severe biodegradation effecting the C31-homohopane 22S/(22S+22R) index or the C29 ethylcholestane 20S/(20S+20R) index.

Comparing parameters and indices from different sample batches analysed at different times proved difficult as a high analytical batch to batch variability was present. Using the GC-MS analysis sequence described in appendix A.11 adapted from the EUROCRUDE system reduced this variability as evidenced from comparing reference and control samples. If source allocation/identification analysis is to be carried out by GC-MS and cannot be completed in one batch, it is recommended that this system is used. Although it increases the number of samples for analysis, it enables compensation for error in source and analyser conditions and chromatographic resolution and peak integration.

Knowledge of the changes that occur in GC-MS fingerprints of environmentally biodegraded petroleum can be incorporated into oil spill fingerprinting guidelines, and should reduce the possibility of declaring an incorrect non-match. In particular certain changes in biomarker patterns or indices, as observed in this project, may be attributable to biodegradation. The extent of biodegradation of other components that would naturally precede biomarker biodegradation should also be taken into account.

8.7 OVERALL FINDINGS AND RECOMMENDATIONS

Although only a simple representation of field conditions, the microcosm design proved an effective mechanism for studying bioremediation in a controlled field simulated environment. This was illustrated by the generally good agreement of replicate experimental units.

Despite the promise of laboratory respirometry studies, bioaugmentation did not enhance biodegradation of UCM or other oil components during bioremediation trials. Respirometry trials results had suggested that a significant adaptive phase is required prior to UCM biodegradation in the laboratory by environmental micro-organisms, and that complex consortia may be more effective than cocktails of individually selected micro-organisms for degrading the range of complex molecular structures present in UCM. However, the specialist inocula developed did not transfer this capability to the microcosm trials.

Substantial washout of oil occurred for the Alba microcosms and this was greatest for the poison control microcosms. Presumably, Alba crude oil is amenable to washout due to the absence of *n*-paraffins, resulting in physical properties akin to lubricating oils. Lower washout rates in other Alba microcosms may have been due to microbial growth modifying sediment characteristics and/or uptake of hydrocarbon by micro-organisms. Washout for Forties microcosms was relatively minor. Washout of oil may lead to increased contamination of subsurface sediments and potentially slower biological removal from the environment. Bioremediation may aid in fixing hydrocarbons in the upper sediment layers by increasing microbial uptake or modifying sediment characteristics. Alternatively physical removal of surface contamination may be preferred to avoid subsequent leaching into substrata.

Rate constants calculated from GC and gravimetric data showed increases in biodegradation for total hydrocarbon, UCM and *n*-alkanes. In general the increases were relatively modest. Rate constants were calculated using the model $[A]_t = [A]_0e^{(-kt)}$. Despite the simplicity of the model it proved useful for assessing biodegradation. GC-MS analysis revealed a marked increase in biodegradation of substituted PAH in nutrient augmented microcosms. Also UCM compositional changes were more advanced for nutrient supplemented microcosms with respect to sea water control microcosms, providing further evidence for enhanced biodegradation with nutrient treatment. Compositional changes were observed to be mostly as might be intuitively expected with biodegradation rates in the following order: saturates>aromatics>polars. For saturate compounds, the higher the number of rings the greater the recalcitrance. Recalcitrance of aromatic compounds increased with ring number and alkyl substitution. While nutrient addition produced only a minor increase in total oil biodegradation rates, it is probable that this increase would have been greater if the background nutrient levels were lower. Prior to bioremediation of UCM rich oil, a detailed assessment of oil composition and background nutrient levels is required. Bioremediation may be an appropriate clean-up tool if background nutrient levels are low and UCM does not consist of a high level of polycyclic alkanes or resins, or if removal of simpler substrates such as PAH is the main priority.

The importance of designing an appropriate monitoring strategy for evaluating bioremediation and the necessity of understanding the inherent advantages and limitations of different analytical approaches was evident from this project. Analytical techniques with different bases can produce very different pictures of petroleum biodegradation despite measuring ostensibly similar parameters. (For example, measurement of "total oil" biodegradation as measured by gas chromatography, gravimetric analysis or spectroscopy). Depending on the assessment/evaluation criteria, this may be critical in judging whether clean-up goals are attained and thus the success of a bioremediation clean-up operation. The relative merits and problems associated with different analytical techniques used for bioremediation monitoring were discussed in the relevant sections of this thesis. The limitations of using *n*-alkane /isoprenoid ratios, such as *n*C18:phytane, for monitoring biodegradation were demonstrated and these should only be used for monitoring the early stages of petroleum biodegradation. Any bioremediation monitoring strategy should consider the analytical programme and assessment/evaluation criteria in conjunction. An awareness of the scope of analytical methods used in monitoring is vital, and monitoring tools should be appropriate with respect to operational goals/targets. The target parameters should be carefully selected. While total oil removal may not be greatly enhanced, biodegradation of specific components, such as PAH, may be considerably accelerated.

GC-MS proved to be a very useful technique for assessing changes to bulk UCM composition, as well as the investigation of biodegradation of individual compounds. However, both 17α, 21β – hopane (C30-hopane) and C29-norhopane, which have been commonly employed as conserved internal markers, were partially biodegraded. An investigation into suitable alternatives indicated that the triaromatic steranes, and in particular C28 (20R) triaromatic sterane (20R ethylcholestane ~TAS4 appendix B.XIII), were appropriate and fulfilled the criteria of abundance, resolution and biological recalcitrance. A potential disadvantage of TAS4 is that it may be slightly more prone to abiotic weathering, as it is likely to be more water soluble than pentacyclic triterpanes, such as C30-hopane. For monitoring of bioremediation operations, the selection of which compound to use depends on circumstances but if C30-hopane degradation is likely, TAS4 would provide a more appropriate alternative. Therefore, C28 (20R) triaromatic sterane (TAS4), should be considered for inclusion in any GC-MS monitoring of oil biodegradation/ bioremediation as a potential conserved internal marker. If C30-hopane is to be used as such, the premise that it is not biodegraded over the course of monitoring must be verified. TAS4 should be given particular consideration as an alternative conserved internal marker to C30-hopane for heavy oils and UCM rich oils.

Comparing GC-MS analysis between batches requires some form of external standardisation. In this project, the oil spill fingerprinting methodology designed as part of the EUROCRUDE system was employed and proved to be a very effective tool for long term monitoring of biodegradation of hydrocarbons by GC-MS. This technique should also be considered for long term monitoring of hydrocarbon bioremediation. A system was devised that employed ion abundance ratios derived from total ion chromatograms for assessment of bulk changes to the UCM with biodegradation. This provided a useful mechanism for acquiring semi-quantitative data on UCM compositional changes over a lengthy time series. Changes to biomarker parameters (e.g. Ts/Tm ratio, removal of C27 diasteranes) have potential as semi-quantitative indicators of advanced biodegradation of petroleum, similar to the use of *n*C18:phytane in the early stages of biodegradation.

In conclusion, bioremediation of UCM-rich oil is realistic in certain situations, but there are a number of key factors to consider prior to treatment:

- Enhanced biodegradation rates may be achieved by addition of nutrient if background nutrient levels are low.
- Biodegradation rates will still be relatively slow, so clean-up may require a lengthy time period.
- It is critical to consider the composition of the oil prior to treatment, as different components offer different levels of resistance to biodegradation. For instance, if contaminating oil consists of very high levels of polycyclic saturates or polar compounds, bioremediation may not be suitable. Comprehensive characterisation of the spilt oil should be carried out in advance.

- The potential for washout of UCM-only oil should be considered. This may be a more important factor than biodegradation for oil removal. Alternatively, oil may be washed deeper into sediments where other factors, aside from nutrient levels, (such as oxygen availability), may limit biodegradation/bioremediation.
- Although there is reason to suspect that bioaugmentation with microbes specifically adapted for biodegradation of complex UCM hydrocarbons, may enhance UCM biodegradation, there is little evidence at present to support this as a realistic treatment technique.
- Careful consideration of the monitoring programme is important. Clear definition of clean-up goals is required. It must be remembered that the choice of analytical methodology may be critical in whether clean-up is adjudged to be 'successful'. TAS4 should be included as an analyte in GC-MS monitoring as C30-hopane may be biodegraded.

The effect of severe biodegradation on GC-MS oil spill fingerprints and parameters was also examined. Alteration of GC-MS PAH and biomarker patterns were evident during the course of the trials. The effects of biodegradation on PAH source indicator (substituted dibenzothiophenes to substituted phenanthrene ratios (CxD/CxP)), pristane: phytane ratios and four biomarker indices were investigated and the robustness of these parameters with respect to advanced biodegradation assessed. The information presented may be useful in preparing guidelines for interpretation of oil spill fingerprints and parameters, particularly with regard to assessing whether differences in fingerprints/parameters for compared samples could be due to severe biological weathering, or do in fact reflect a difference in source.

8.8 FURTHER RESEARCH

Further basic research is required into the colonisation of introduced 'seed' cultures in the environment. Further understanding of issues such as viability, inocula concentration, competition for nutrients, and predation is necessary. Laboratory results suggest that for UCM hydrocarbons and other resistant substrates, it is probable that if 'seed' cultures could establish themselves, actively biodegrading target compounds, biodegradation rates could be enhanced. However, at present there is very little evidence to support the use of inoculation in the treatment of hydrocarbon contamination, even when a prolonged adaptation period of the indigenous population is required.

Research into the effect of background nutrient concentration may assist in the decision making process regarding the application of bioremedial technologies. In particular, this can aid decisions regarding the potential enhancement of biodegradation in a given spill situation, and thus the cost-effectiveness of bioremediation as a clean-up tool.

Research into biodegradation of polar compounds and resins is warranted as these components exhibit the greatest resistance to biodegradation.

9. REFERENCES

Aboul-Kassim, T.A., Simoneit, B.R.T. (1995). "Petroleum hydrocarbon fingerprinting and sediment transport assessed by molecular biomarker and multivariate statistical analyses in the eastern harbour of Alexandria, Egypt". *Marine Pollution Bulletin* **30**, 63-73.

Albaiges, J., Albrecht, P. (1979). “Fingerprinting marine pollutant hydrocarbons by computerised gas chromatography-mass spectrometry," *International Journal of Environmental & Analytical Chemistry* **6**, 171-190.

Al-Hadhrami,M.N.; Lappin-Scott H.M.; Fisher P.J. (1996). “Effects of the addition of organic carbon sources on bacterial respiration and *n*-alkane biodegradation of Omani crude oil”. *Marine Pollution Bulletin* **32**, 351-357

Amann, R.L., Ludwig, W., Schleiffer, K-H. (1995). “Phylogenetic identification and *in-situ* detection of individual microbial cells without cultivation". *Microbiological Reviews* **59**, 143-169.

AMAP (1998). “AMAP assessment report: Arctic pollution issues. Arctic Monitoring and Assessment Programme”. ISBN 82-7655-061-4.

Anon. (1991a). “Oil spill bioremediation products testing protocol methods manual” Prepared for US Environmental Protection Agency Bioremediation Action Committee. National Environmental Technology Applications Corporation. NETAC.

Anon. (1991b). “Oil spill identification. Nordtest method”. NT CHEM 001 Edition2.

Anon. (1995). “European crude oil identification system”. (Project Eurocrude). Final Report to EU DGXI *LIFE project (LIFE 92-1/INT/00).*

Anon. (1999). “Screening of Irish coastal waters for eutrophication. Common procedure for the identification of eutrophication status of the maritime area of the Oslo and Paris Commissions” In preparation for presentation to OSPAR Assessment and Monitoring Committee 1999.

ASTM (1981). “Standard test methods for comparison of waterborne petroleum oils by Infrared Spectroscopy”. American Society for Testing and Materials. *ASTM D3414 - 80* (reapproved 1985 reaffirmed 1986).

ASTM (1984). “Standard test method for boiling range distribution of petroleum fractions by gas chromatography”. American Society for Testing and Materials. *ASTM D 2887 –84.*

ASTM (1985). “Proposed test method for boiling range distribution of crude petroleums by gas fractions”. American Society for Testing and Materials. *ASTM D-2 proposal P 167.*

ASTM (1991a). “Standard test methods for comparison of waterborne petroleum oils by gas chromatography”. American Society for Testing and Materials. *ASTM D 3328 – 90.*

ASTM (1991b). “Standard test method for hydrocarbon types analysis of gas-oil saturates fractions by high ionising voltage mass spectrometry”. American Society for Testing and Materials. *ASTM D2786-91*

ASTM (1991c). “Standard test method for aromatic types analysis of gas-oil aromatic fractions by high ionising voltage mass spectrometry”. American Society for Testing and Materials. *ASTM D3239-91.*

ASTM (1995). “Standard practice for oil spill source identification by gas-chromatography and positive ion electron impact low resolution mass spectrometry”. American Society for Testing and Materials. *ASTM D5739-95.*

ASTM (1996). “Standard Test Method for Oil and Grease and Petroleum Hydrocarbons in Water”. American Society for Testing and Materials. *ASTM D 3921-96.*

ASTM (1998). “Standard test method for hydrocarbon types in liquid petroleum products by fluorescent indicator adsorption”. American Society for Testing and Materials. *ASTM D1319-98.*

Atlas, R.M. (1984) “Petroleum microbiology”. Macmillan, New York. ISBN 0-02-949000-6.

Atlas, R.M.; Bartha R. (1992). “Hydrocarbon biodegradation and oil spill bioremediation”. *Advances in Microbial Ecology* **12**, 287-338.

Atlas, R.M. (1992). “Oil spills: regulation and biotechnology”. *Current Opinion in Biotechnology.* **3**, 220-233.

Atlas, R.M. (1995). “Petroleum Biodegradation and Oil Spill Bioremediation”. *Marine Pollution Bulletin* **31,** 178-182.

Atlas, R.M. (1981). “Microbial degradation of petroleum hydrocarbons : an environmental perspective". *Microbiological Reviews* **45**, 180-209.

Atlas, R.M. (1996). “Slick solutions” *Chemistry in Britain* **May 96**, 42-44.

Atlas, R.M. Atlas, M.C. (1991). “Biodegradation of oil and bioremediation of oil spills*”*. *Current Opinion in Biotechnology* **2**, 440-443.

Baron, M. (1961). “Analytical applications of inclusion compounds”. *Physical Methods in Chemical Analysis*. Ed. Berl W.G. **IV**, pp.226-230. Academic Press, New York.

Bayona, J.M, Albaiges, J., Solanas, A.M., Pares, R., Garrigues, P., Ewald, M., (1986). “Selective aerobic degradation of methyl-substituted polycyclic aromatic hydrocarbons in petroleum by pure microbial cultures”. *Fate of Hydrocarbons in the Environment: An Analytical Approach* pp. 193-208. Ed. Albaiges, J.; Frei, R.W. Gordon and Breach Science Publishers, S.A.

Beam, H.W., Perry, J.J. (1974) “Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism”. *Journal of General Microbiology* **82**, 163-169.

Bence, A.E., Kvenvolden, K.A., Kennicutt II, M.C., (1996). “Organic Geochemistry applied to environmental assessments of Prince William Sound, Alaska, after the *Exxon Valdez* oil spill – a review”. *Organic Geochemistry,* **24**, 7-42.

Berthou, F.G., Balouet, G., Bodennec, G., Marchand, M. (1987). “The occurrence of hydrocarbons and histopathological abnormalities in oysters for seven years following the wreck of the *Amoco Cadiz* in Brittany, France”. *Marine Environmental Research*, **23**, 103-133.

Bieger, T., Hellou, J., Abrajano, T.A. (1996). “Petroleum biomarkers as tracers of lubricating oil contamination”. *Marine Pollution Bulletin* **32**, 270-274.

Boehm, P.D., Douglas, G.S., Burns, W.A., Mankiewicz,P.J., Page, D.S., Bence, A.E. (1997). “Application of petroleum hydrocarbon chemical fingerprinting and allocation techniques after the *Exxon Valdez* oil spill”. *Marine Pollution Bulletin* **34**, 599-613.

Bossert, I., Bartha. R. (1984). “The fate of petroleum hydrocarbons in soil ecosystems”. *Petroleum Microbiology* pp.435-473. Ed. Atlas, R.M. Macmillan, New York.

Bragg, J.R., Prince, R.C., Harner, E.J., Atlas, R.M. (1996). “Effectiveness of bioremediation for the *Exxon Valdez* oil spill”. *Nature* **368**, 413-418.

Brakstad, F., Grahl-Nielsen, O. (1988). “Identification of weathered oils”. *Marine Pollution Bulletin* **19**, 319-324.

Brooks, J. (1983). “Petroleum geochemistry and fossil fuel exploration” *Chemistry in Britain* **May 83**, 390-399

Burke, P., Jennings, K.R., Morgan, R.P., Gilchris, C.A. (1982). “Identification of crude oils by selective chemical ionisation mass spectrometry”. *Analytical Chemistry* **54**, 1304-1306.

Burns, K.A., Knap, A.H. (1989). “The *Bahia las Minas* oil spill. Hydrocarbon uptake by reef building corals”. *Marine Pollution Bulletin* **20**, (8), 432-441.

Burridge, T.R., Shir, M.A. (1995). “The comparative effects of oil dispersants and oil dispersant conjugates on the germination of marine macroalga *Phyllospora comosa* (Fucales: phaeophyta*)”*. *Marine Pollution Bulletin* **31**, 446-452.

Butt J.A., ed. (1986). *Characterisation of spilled oil samples - Purpose, sampling, analysis and interpretation*. Institute of Petroleum Marine Environment Committee. John Wiley & Sons.

Cainelli, G., Cardillo, G. (1984). *Chromium oxidations in organic chemistry*. Springer Verlag ISBN 3-540-12834-4

Cerniglia, C.E. (1984). "Microbial transformation of aromatic hydrocarbons". *Petroleum Microbiology* pp. 99-128. Ed. Atlas, R.M. Macmillan, New York.

Cerniglia, C.E. (1992). "Biodegradation of polycyclic aromatic hydrocarbons". *Biodegradation* **2**, 351.

Chaineau, C. H., Morel, J., Oudot, J. (1995). "Microbial degradation in soil microcosms of fuel oil hydrocarbons from drill cuttings". *Environmental Science & Technology* **29**, 1619-1621.

Chosson, P., Lanau, C., Connan, J., Dessort, D. (1991). "Biodegradation of refractory hydrocarbon biomarkers from petroleum under laboratory conditions". *Nature* **351**, 640-642.

Chukwuemeka, N.A., Nwobodo, I.F. (1994). "Analysis of *n*-paraffins in light crudes: molecular sieve and urea adduction techniques revisited". *Fuel* **73**, 779-782.

Colombo, J.C., Cabello, M., Arambarri, A.M. (1996). "Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and lignolytic fungi". *Environmental Pollution* **94**, 355-362

Dahlmann, G. (1994). "On the differentiation between crude oil and bunker oil (or sludge) residues". (Criteria for differentiating crude oil and bunker oil – unpublished)

Dauvin, J.C. (1987). "Evolution à long terme (1978-1986) des populations d'amphipodes des sables fins de Pierre Noire (Baie de Morlaix, Manche Occidentale) apres la catastrophe de l'*Amoco Cadiz*". *Marine Environmental Research* **21**, 247-273.

de Lorenzo,V. (1992). “Genetic engineering Strategies for Environmental applications”. *Current Opinions in Biotechnology*. **3**, 227-231.

Douglas, G.S., Bence, A.E., Prince, R.C., McMillen, S.J., Butler, E.L. (1996). “Environmental stability of selected petroleum hydrocarbon source and weathering ratios”. *Environmental Science & Technology* **30**, 2332-2339.

Douglas, G.S., McCarthy, K.J., Dahlen, D.T., Seavey,J.A., Steinhauser, W.G., Prince, R.C., Elmendorf, D.L. (1992). “The use of hydrocarbon analyses for environmental assessment and remediation”. *Journal of Soil Contamination* **1**, 197-216.

Ducreux, J., Ballerini, D., Bocard, C. (1994) “The role of surfactants in enhanced *in-situ* bioremediation”. *Hydrocarbon Remediation* pp. 237-243. Eds. Hinchee, R.E., Alleman, B.C., Hoeppel, R.E., Miller, R.N. Lewis Publishers, Boca Raton, Ann Arbour, London, Toyko.

Ehrhardt, M., Klungsoyr, J., Law, R.J. (1991). “Hydrocarbons: Review of methods for analysis in sea water, biota, and sediments”. *Techniques in Marine Environmental Sciences no. 12*. International Council for the Exploration of the Sea. Copenhagen. ISBN 0903-2606.

Ellis, B., Gorder, K. (1997). “Intrinsic bioremediation: an economic option for cleaning up contaminated land”. *Chemistry & Industry*, **Feb. 97**, 95-98.

Fayad, N.M., Everton, E. (1995). “A unique biodegradation pattern of the oil spilled during the 1991 Gulf War”. *Marine Pollution Bulletin* **30**, 239-246.

Field, J.A., de Jong, E.C., Gumersindo, F., de Bont, J.A.M. (1992). “Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi". *Applied & Environmental Microbiology* **58**, 2219-2226.

Floodgate, G.D. (1979). “Nutrient limitation”. *Proceedings of workshop on microbial degradation of pollutants in marine environments* pp. 107-118. Eds. A.W.Bourquin and P.H Pritchard,. EPA-66019-79-012. Environmental Protection Agency, Gulf Breeze, Florida.

Floodgate, G.D. (1984). "The fate of petroleum hydrocarbons in marine ecosystems". *Petroleum Microbiology* pp. 355-397. Ed. Atlas, R.M., Macmillan, New York.

Foght, J.M., Fedorak, P.M., Westlake, D.W.S., (1990). "Mineralization of (super(14)C)hexadecane and (super(14)C)phenanthrene in crude oil". *Canadian Journal of Microbiology* **36**, 169-175.

Fusey, P., Oudot, J. (1984). "Relative influence of physical removal and biodegradation in the depuration of petroleum-contaminated seashore sediments". *Marine Pollution Bulletin* **15**, 136-141.

Garrett, R..M., Pickering, I.J., Haith, C.E., Price, R.C. (1998). "Photo-oxidation of crude oils". *Environmental Science & Technology* **32**, 3719-3723.

Glegg, G A, Rowland, S J (1996). "The *Braer* oil spill - hydrocarbon concentrations in intertidal organisms,". *Marine Pollution Bulletin* **32**, 486-492.

Gough, M.A., Rhead, M.M., Rowland, S.J. (1992). "Biodegradation studies of unresolved complex mixtures of hydrocarbons; model UCM hydrocarbons and the aliphatic UCM". *Organic Geochemistry* **18**, 17-22.

Gough, M.A., Rowland, S.J. (1990). "Characterisation of unresolved complex mixtures of hydrocarbons in petroleum". *Nature*, **344**, 648-650.

Gough, M.A., Rowland, S.J. (1991). "Characterisation of UCM of hydrocarbons from lubricating feed stocks". *Energy Fuels* **5**, 869-874.

Grizzle, P.L, Sablotny, D.M. (1986). "Automated liquid chromatographic compound class group-type separation of crude oils and bitumens using chemically bonded aminosilane". *Analytical Chemistry* **58**, 2389-2396.

Harvey, T.G., Matheson, T.W., Pratt, K.C. (1984). "Chemical class separation of organics in shale oil by thin-layer chromatography". *Analytical Chemistry* **56**, 1277-1281.

Hayes, M.O., Michel, J. (1999). “Factors determining the long term persistence of *Exxon Valdez* oil in gravel beaches”. *Marine Pollution Bulletin* **38**, 92-101.

Hayes, M.O., Michel, J., Montello, T.M., Aurand, D.V., Al-Mansi, A.M., Al-Moamen, A.H., Sauer, T.C., Thayer, G.W. (1993). “Distribution and weathering of shoreline oil one year after the Gulf War oil spill”. *Marine Pollution Bulletin* **27**, 135-142

Head, I.M. (1998). “Bioremediation: towards a credible technology”. *Microbiology* **144**, 599-608.

Head, I.M., Swannell, R.P.J. (1999). “Bioremediation of petroleum hydrocarbon contaminants in marine habitats”. *Current Opinion in Biotechnology* **10**, 234-239

Hoff, R.Z. (1993). “Bioremediation: An overview of its development and use for oil spill cleanup”. *Marine Pollution Bulletin* **26**, 476-481.

Hood, A., O'Neal, M.J. (1959). “Status of application of mass spectrometry to heavy oil analysis”. *Advances in Mass Spectrometry* **1**, 175-192.

Horstad, I., Larter, S.R., Mills, N. (1992). “A quantitative model of biological petroleum degradation within the Brent Group reservoir in the Gullfaks Field, Norwegian North Sea”. 15. Int. Meet. of the European Assoc. of Organic Geochemists, Manchester (UK), 16-20 Sep 1991. Advances in Organic Geochemistry 1991. Part 1. Advances-and-Applications-in-Energy-and-the-Natural-Environment. Ed.Manning, D.A.C., **19**, 107-117.

Hostettler, F.D., Rapp, J.B., Kvenolden, K.A. (1992). “Use of geochemical biomarkers in bottom sediment to track oil from a spill, San Francisco Bay, California”. *Marine Pollution Bulletin* **24**, 15-20.

Houghton, J.P., Lees, D.C., Ebert, T.A. (1991). “Evaluation of the condition of intertidal and shallow subtidal biota in Prince William Sound following the *Exxon Valdez* oil spill and subsequent shoreline treatment”. NOAA, National Ocean Service, Hazardous Materials Response Branch. Report no. HMRG 91-1.

IMO (1993). IMO/FAO/UNESCO/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP) (1993). *Impact of Oil and Related Chemicals and Wastes on the Marine Environment*. IMO, London.

Jones, D.M., Rowland, S.J., Douglas, A.G., Howells, S. (1986). “An examination of the fate of Nigerian Crude oil in surface sediments of the Humber estuary by gas chromatography and gas chromatography- mass spectrometry”. *International Journal of Environmental Analytical Chemistry* **24**, 227-247.

Kanga, S. A., Bonner, J.S., Page, C.A., Mills, M.A., Autenrieth, R.L. (1997). “Solubilisation of naphthalene and methyl-substituted naphthalenes from crude oil using biosurfactants”. *Environmental Science & Technology* **31**, 556-561.

Kaplan, D.L., (1992). “Biological degradation of explosives and chemical agents”. *Current Opinions in Biotechnology* **3**, 253-260.

Kaplan, I.R., Galperin, Y., Lu, S.T., Lee, R.P. (1997). “Forensic environmental geochemistry: differentiation of fuel-types, their sources and release time”. Organic Geochemistry 27, (5/6), 289-317

Kelso. D.D., Kendziorel, M., (1991). “Alaska's response to the *Exxon Valdez* oil spill”. *Environmental Science & Technology* **25**, 16-23.

Kennicutt, M.C. (1990). “Oil Spillage in Antarctica”. *Environmental Science & Technology* 24, 620-624.

Killops, S.D., Al-Juboori, A.H.A. (1990). “Characterisation of unresolved complex mixture (UCM) in the gas chromatograms of biodegraded petroleums”. *Organic Geochemistry,* **15**, 147-160.

Killops, S.D., Howell, V.J. (1988). “Sources and distribution of hydrocarbons in Bridgwater Bay (Severn Estuary, U.K.) intertidal surface sediments”. *Estuarine, Coastal & Shelf Science*, **27**, 327-261.

Killops, S.D., Readman, J.W. (1985). “HPLC fractionation and GC-MS determination of Aromatic hydrocarbons from oils and sediments”. *Organic Geochemistry*, **8**, 247-257.

Kjeilen, G., (1997). “ Summary and conclusions from BIOREN subprojects”. EAM 97/004. EU assisted project. Elf Petroluem Norge. Stavanger 6/4/1998.

Krahn, M.M., Burrows, D.G., Ylitalo,G.M., Brown, D.W., Wigren, C.A, Collier, T.K., Chan,S-L., Varanasi, U. (1992). “Mass spectrometric analysis for aromatic compounds in bile of fish sampled after the *Exxon Valdez* oil spill”. *Environmental Science & Technology*, **26**, 116-126.

Lamar, R.T. (1992). “The role of fungal lignin-degrading enzymes in xenobiotic degradation”. *Current Opinion in Biotechnology*, **3**, 261-266.

Law, R J., Fileman, T. W., Portmann, J. E. (1988). "Aquatic environment protection: analytical methods. Methods of analysis for hydrocarbons in marine and other samples". *Ministry of Agriculture, Fisheries and Food Directorate of Fisheries Research (UK).*

Leahy, J.G., Colwell, R.R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiological Review* **54**, 305-315.

Lee, K. (1998). “Accelerating natural processes of oil spill remediation”. *Paper presented to 86th statutory meeting of the International Council for the Exploration of the Sea (ICES) – 1998 Annual Science Conference, Cascais, Portugal. September 16-19, 1998.* Publication # CM 1998/V:11.

Lee, K., De Mora, S. (1999). “*In situ* bioremediation strategies for oiled shoreline environments” *Environmental Technology* **20**, 783-794.

Lee, R.L., Page, D.S., (1997). “Petroleum hydrocarbons and their effects in subtidal regions after major oil spills”. *Marine Pollution Bulletin*, **34**, 928-940.

LePetit, J., N'Guyen, M.H. (1976). "Phosphorus requirements of bacteria metabolising hydrocarbons in the sea”. *Canadian Journal Microbiology* **22**, 1364-1373.

Lethbridge, G., Vits, H.J.J., Watkinson, R.J. (1994). "*Exxon Valdez* and bioremediation" *Nature* **371**, 97

Lindstrom, J.E., Prince, R.C.; Clark, J.C., Grossman, M.J., Yeager, T.R., Braddock, J.F., Brown, E. J. (1991). "Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the t/v *Exxon Valdez* oil spill". *Applied & Environmental Microbiology*, **57**, 2514-2522.

Lord Donaldson's Inquiry into the prevention of pollution from merchant shipping. (1994). "Safer Ships, Cleaner Seas". HMSO, London.

Lundanes, E., Greibrokk, T. (1994). "Separation of fuels, heavy fractions and crude oils into compound classes: A review". *Journal of High Resolution Chromatography* **17**, 197-202.

Lunel, T., Swannell, R., Rusin, J., Wood, P., Bailey, N., Halliwell, C., Davies, L., Sommerville, M., Dobbie, A., Mitchell, D., McDonagh, M., Lee, K. (1995). "Monitoring the effectiveness of response operations during the *Sea Empress* incident: a key component of the successful counter-pollution response". *Spill Science & Technology* **2**, 99-112.

MacKenzie, A.S., (1984). "Applications of biological markers in petroleum geochemistry". *Advances in Petroleum Geochemistry* Vol .1, pp.115-214. Ed. Brooks, J., Waples, D.W.). Academic Press.

Maki, A.W. (1991). "The *Exxon Valdez* oil spill: Initial environmental impact assessment". *Environmental Science & Technology* **25**, 24-29.

Mason, R.P. (1987). "A comparison of fluorescence and GC for the determination of petroleum hydrocarbons in mussels". *Marine Pollution Bulletin* **18**, 528-533.

McGovern, E., Nixon, E. (1996). "Characterisation of beached oil from the Wexford coast". *Final Report OS96_1*, and "Interpretation of results and comments relating to final report OS96_1". Irish Marine Institute Fisheries Research Centre.

McGovern, E. (1998). “ Tracing oil spills at sea”. *Chemistry Review*, **7**, 4-8.

McGovern, E. (1999). “Bioremediation of unresolved complex mixtures in marine oil spills”. *EU project B4-3040/95/928/jnb/C4 Final Report.* Irish Marine Institute Fisheries Research Centre.

McNally, D.L., Mihuelcic, J.R., Lueking, D.R. (1998). “Biodegradation of three- and four-ring polyaromatic hydrocarbons under aerobic and denitrifying conditions”. *Environmental Science & Technology* **32**, 2633-2639.

Melpolder, F.W., Brown, R.A., Washall, T.A., Doherty, W., Headington, C.E., (1956). “Composition of lubricating oil: Use of new separating and spectroscopic methods”. *Analytical Chemistry* **28**, 1936-1945.

Michel, J., Hayes, M.O. (1999) “Weathering patterns of oil residues eight years after the *Exxon Valdez* oil spill”. *Marine Pollution Bulletin* **8**, 855-863.

Miller, S.S. (1990). “In a faraway state”. *Environmental Science & Technology* **24**, 1286-1289.

Miller, R.E., Bartha, R. (1989). “Evidence from liposome encapsulation for transport-limited microbial metabolism of solid alkanes”. *Applied & Environmental Microbiology* **55**, 269-274.

Morgan, P., Watkinson, R. J. (1989). “Hydrocarbon degradation in soils and methods for soil biotreatment”. *CRC Critical Reviews in Biotechnology* **8**, 305-333.

Morgan, P., Watkinson, R.J., (1992). “Factors limiting the supply and efficiency of nutrient and oxygen supplements for the *in situ* biotreatment of contaminated soil and groundwater”. *Water Research* **26**, 73-78.

Murphy, M.T.J. (1969) “Analytical Methods” *Organic Geochemistry* pp.74-88. Ed. Eglinton, G. Murphy, M.T.G., Springer, Berlin.

NRC (1985). “Oil in the sea, inputs, fates and effects” National Research Council, National Academy Press, Washington, DC.

Noordman, W.H., Wei, J., Brusseau, M.L., Janssen, D.B. (1998). “Effects of rhamnolipid biosurfactants on removal of phenanthrene from soil”. *Environmental Science & Technology* **32**, 1806-1812.

O'Connor, J.G., Burrow, F.H., Norris, M.S. (1962). “Determination of normal paraffins in C20 to C32 paraffin waxes by molecular sieve adsorption. Molecular weight distribution by gas liquid chromatography”. *Analytical Chemistry* **34**, 82-85.

Oudot, J. (1984). “Rates of microbial degradation of petroleum components as determined by computerised capillary gas chromatography and computerised mass spectrometry”. *Marine Environmental Research* **13**, 277-302.

Oudot, J., Merlin, F.X., Pinvidic, P. (1998). “Weathering rates of oil components in a bioremediation experiment in estuarine sediments”. *Marine Environmental Research* **45**, 113-125.

Page, D.S., Foster, J.C., Fickett, P.M., Gilfillan, E.S. (1988) “Identification of petroleum sources in an area impacted by the Amoco Cadiz oil spill”. *Marine Pollution Bulletin* **19**, 107-115.

Pearson, D.C., Gharfeh, S.G. (1986). “Automated high performance liquid chromatography determination of hydrocarbon types in crude oil residues using a flame ionization detector”. *Analytical Chemistry* **58**, 307-311

Perry, J.J. (1984). “Microbial metabolism of cyclic alkanes”. *Petroleum Microbiology* pp. 61-97. Ed. Atlas, R.M. Macmillan, New York.

Perry, J.J. (1979). “Microbial cooxidations involving hydrocarbons”. *Microbiological Reviews* **43**, 59-72.

Peters, K.E., Moldowan, J.M. (1993). *The Biomarker Guide: Interpreting molecular fossils in petroleum and ancient sediments*, Ed. Prentice Hall, Englewood Cliffs, New Jersey 07632

Pharr, D.Y., McKenzie, K., Hickman, A.B., (1992). “Fingerprinting petroleum contamination using synchronous scanning fluorescence spectroscopy”. *Ground Water* 30, (**4**), 484-489.

Philp, R.P. (1994) "High temperature gas chromatography for the analysis of fossil fuels: A review" *Journal of High Resolution Chromatography* **17**, 398-406.

Pollard, S.J.T., Kenefick, S.L., Hrudey, S.E., Fuhr, B.J., Holloway, L.R., Rawluck, M. (1994). "A tiered analytical protocol for the characterisation of heavy oil residues at petroleum-contaminated hazardous waste sites" Analysis of soils contaminated with petroleum constituents, pp38-52. Ed. O'Shay,T.; Hoddinott,K.B. ASTM STP 1221, (ASTM publication code number 04-012210-38), Philadelphia, USA.

Prince, R.C. (1997). "Bioremediation of marine oil spills". *Trends in Biotechnology* **15**, 158-160.

Prince, R.C., Clark, J.R., Lindstrom, J.E., Butler E.L., Brown, E.J., Winter, G., Grossman, M.J., Parrish, P.R., Bare, R.E., Braddock, J.F., Steinhauer, W.G., Douglas, G.S., Kennedy, J.M., Barter, P.J., Bragg, J.R., Harner, E.J., Atlas, R.M. (1994a). "Bioremediation of the *Exxon Valdez* oil spill: Monitoring safety and efficacy". *Hydrocarbon Remediation* pp.107-124. Eds. Hinchee, R.E., Alleman, B.C., Hoeppel, R.E., Miller, R.N. Lewis Publishers, Boca Raton, Ann Arbour, London, Toyko.

Prince, R.C., Elmendorf, D.L., Lute, J.R., Hsu, C.S., Haith, C.E., Senius, J.D., Dechert, G.J., Douglas, G.S., Butler, E.L. (1994b). "17a(H), 21(b) - Hopane as a conserved internal marker for estimating the biodegradation of crude oil". *Environmental Science & Technology* **28**, 142-145.

Prince, R.C. (1993). "Petroleum spill bioremediation in marine environments". *Critical Reviews in Microbiology* **19**, 217-242.

Pritchard, P.H., Costa, C.F. (1991). "EPA's Alaska oil spill bioremediation project". *Environmental Science & Technology* **25**, 372-379.

Pritchard, P.H. (1992). "Use of inoculation in bioremediation". *Current Opinions in Biotechnology* **3**, 232-243.

Readman, J. W., Bartocci, J., Tolosa, I. Fowler, S.W., Oregioni, B., Abdulraheem, M.Y. (1996). “Recovery of the coastal marine environment in the Gulf following the 1991 war-related oil spills”. *Marine Pollution Bulletin* **32**, 493-498.

Reddy, C.M., Quinn, J.G., (1999). “GC-MS analysis of total petroleum hydrocarbons and polycyclic aromatic hydrocarbons in seawater samples after the North Cape oil spill”. *Marine Pollution Bulletin* **38**, 126-135.

Requejo, A.G., Sassen, R., McDonald, T, Denoux, G., Kennicutt II, M.C., Brooks, M., (1996). “Polynuclear aromatic hydrocarbons (PAH) as indicators of the source and maturity of crude oil”. *Organic Geochemistry*, **24**, 1017-1033.

Revill, A.T., Carr, M.R., Rowland, S.J. (1992). “Use of oxidative degradation followed by capillary gas chromatography-mass spectrometry and multidimensional analysis to fingerprint unresolved complex mixtures of hydrocarbons”. *Journal of Chromatography* **589** , 281-286.

Robinson, C.J., Cook, G.L. (1969). “Low resolution mass spectrometric determination of aromatic fractions from petroleum”. *Analytical. Chemistry* **41**, 1548-1554.

Rose, M.E., Johnstone, R.A.W. (1982). Mass spectrometry for chemists and biochemists. pp. 203-209, Cambridge University Press, UK.

Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., Ron, E.Z. (1992). “Petroleum bioremediation”. *Biodegradation* **3**, 337-350.

Sauer, T.C., Brown,-J.S., Boehm, P.D., Aurand, D.V., Michel, J., Hayes, M.O. (1993). “Hydrocarbon source identification and weathering characterization of intertidal and subtidal sediments along the Saudi Arabian coast after the Gulf War oil spill”. *Marine Pollution Bulletin* **27**, 117-134.

SEEC (1998). “The environmental impact of the *Sea Empress* oil spill”. *Final Report of the Sea Empress Environmental Committee*. London Stationary Office, ISBN 0-11-702156-3

Selucky, M.L. (1985). “Quantitative class separation of coal liquids using thin layer chromatography with flame ionisation detection”. Lipids **20**, 546.

Sexstone, A.J., Atlas, R.M. (1977). “Response of microbial populations in Arctic Tundra soils to crude oil”. *Canadian Journal of Microbiology* **23**,1327-1333.

Shadle, L.J., Kalkunte, S.S., Webb, D.L. (1994). “Characterisation of shale oils. 1. Analysis of Fischer assay oils and their aromatic fractions using advanced analytical techniques”. *Fuel Process Technology* **37**, 101-120.

Simoneit, B.R.T. (1986). “Application of molecular marker analysis to vehicular exhaust for source recognition”. *Fate of Hydrocarbons in the Environment: An analytical approach.* pp.117-148; ed. Albaiges, J.; Frei, R.W. Gordon and Breach Science Publishers, S.A.

Singer, M.E., Finnerty, W.R. (1984). “Microbial metabolism of straight-chain hydrocarbons and branched alkanes”. *Petroleum Microbiology* pp. 1-59. Ed. Atlas, R.M., Macmillan, New York.

Singer, M.M., George, S., Benner, D., Jacobson, S., Tjeerdema, R.S., Sowby, M.L. (1993). “Comparative toxicity of two oil dispersants to the early life stages of two marine species”. *Environmental Toxicology and Chemistry* **12**, 1855-1863.

Singh, M., Desai J.D. (1988). “Hydrocarbon emulsifying activity of bacterial strains: potential of *Arthrobacter paraffineus*”. *Current Science* **57**, 1307-1308.

Smith, R.M., Busch, K.L. (1999). Understanding mass spectra: A basic approach. John Wiley and Sons, New York.. ISBN 0-471-29704-7.

Smith, V.H., Graham, D.W., Cleland, D.D. (1998). “Application of resource ratio theory to hydrocarbon degradation”. *Environmental Science & Technology*. **32**, 3386-3395.

Smith, M.R., Ratledge, C. (1989). "Catabolism of alkylbenzenes by *Pseudomonas* sp. NCIB 10643”. *Applied Microbiology & Biotechnology* **32**, 68.

Sousa, S.; Duffy, C., Weitz, H., Glover, L.A., Bar, E., Henkler, R., Killham, K. (1998). "Use of a lux-modified bacterial biosensor to identify constraints to bioremediation of BTEX-contaminated sites". *Environmental Toxicology & Chemistry* **16**, 1039-1045.

Stallard, B.R., Garcia, M.J., Kaushik, S. (1996). "Near-IR reflectance spectroscopy for the determination of motor oil contamination in sandy loam". *Applied Spectroscopy* **50**, 334-338.

Sugai, S.F., Lindstrom, J.E., Braddock, J.F. (1997). "Environmental influences on the microbial degradation of *Exxon Valdez* oil on the shorelines of Prince William Sound". *Environmental Science & Technology* **31**, 1564-1572.

Summers, A.O., (1992). "The hard stuff: metals in bioremediation". *Current Opinion in Biotechnology* **3**, 271-276.

Swannell, R.P.J., Croft, B.C., Grant, A.L., Lee, K. (1995). "Evaluation of bioremediation agents in beach microcosms". *Spill Science & Technology* **2**, 151-159.

Swannell, R.P.J.; Lee, K., McDonagh, M. (1996). "Field evaluations of marine oil spill bioremediation". *Microbiological Reviews* **60**, 342-365.

Swannell, R.P.J., Mitchell, D.M., Lethbridge, G., Jones, D., Heath, D., Hagley, M., Jones, M., Petch, S., Milne, R., Croxford, R., Lee, K. (in prep.). "A field demonstration of the efficacy of bioremediation to treat oiled shorelines following the *Sea Empress* spill*"*. *Environmental Technology* **20**, 863-873.

Thomas, K.V., Donkin, P., Rowland, S.J. (1995). "Toxicity enhancement of an aliphatic petrogenic unresolved complex mixture (UCM) by chemical oxidation". *Water Research* **29**, 379-382.

Thomas, T.L., Mays R.L. (1961). "Separations with molecular sieves". *Physical Methods in Chemical Analysis* Vol. IV pp.82-85. Ed. Berl W.G, Academic Press, New York.

Tissot, B.P., Welte, D.H. (1978). "Petroleum formation and occurrence: a new approach to oil and gas exploration". Springer-Verlag, Berlin Heidelberg New York, ISBN 3-540-08698-6.

US EPA (1983). "Total recoverable petroleum hydrocarbons by infra red spectroscopy". Method 418.1. *Methods for the Analysis of Water and Wastes.* United States Environment Protection Agency, Washington D.C.

Venosa, A.D., Haines, J.R. Allen, D.M. (1992). "Efficacy of commercial inocula in enhancing biodegradation of weathered crude oil contaminating a Prince William Sound beach". *Journal of Industrial Microbiology* **10**, 13.

Venosa, A.D., Suidan, M.T., Wrenn, B.A., Strohmeier, K.L., Haines, J.R., Eberhart, B.L.; King, D., Holder, E. (1996). "Bioremediation of an experimental oil spill on the shoreline of Delaware Bay". *Environmental Science & Technology* **30**, 1764-1775

Venosa, A.D., Haines, J.R., Nisamaneepong, W., Govind, R., Pradhan, S., Siddique, B. (1991). "Screening of commercial inocula for efficacy in stimulating oil biodegradation in closed laboratory system". 3rd Annual Symposium of the GCHSRC. *Journal of Hazardous Material* **28**, 131-144.

Volkman, J.K., Holdsworth, D.G., Neill, G.P., Bavor, H.J.,Jr. (1992). "Identification of natural, anthropogenic and petroleum hydrocarbons in aquatic sediments". *Science of the Total Environment* **112**, 203-219.

West, N., Alexander, R., Kagi, R.I. (1990). "The use of silicalite for rapid isolation of branched and cyclic alkane fractions of petroleum". *Organic Geochemistry* **15**, 499-501.

Westermeyer, W.E. (1991). "Oil spill response capabilities in the United States". *Environmental Science & Technology* **25**, 196

Wilson, S.C., Jones, K.C. (1993). "Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review". *Environmental Pollution* **81**, 229-249.

Wolff, G.A., Preston, M.R., Harriman, G., Rowland, S.J. (1993). "Some preliminary observations after the wreck of the oil tanker *Braer* in Shetland". *Marine Pollution Bulletin* **26**, 567-571.

Worne, H.E., Fortune, I. (1993). "Bioremedation of hazardous and toxic wastes". *Proceedings of HMC/Superfund `92* pp. 477-493. HMRCI 13th Annual National Conference.

Wrang, P.; Adamsen, D. (1990a). *Oil identification by GC/MS II: Fingerprintng of biomarkers: General principles and description of method.* The National Environment Research Institute, J. no. 9-7001. Denmark.

Wrang, P.; Adamsen, D. (1990b). *Oil identification by GC/MS III: weathering.* The National Environment Research Institute, J. no. 9-7001. Denmark.

Wrenn, B.A.; Makram, T.S.; Strohmeier, K.L.; Eberhart, B.L.; Wilson, G.J.; Venosa, A.D. (1997a). "Nutrient transport during bioremediation of contaminated beaches: Evaluation with lithium as a conservative tracer". *Water Research* **31**, 515-524.

Wrenn, B.A., Suidan, M.T., Strohmeier, K.L., Loye Eberhart, B., Wilson, G.J., Venosa, A.D., Haines, J.R., Holder, E. (1997b). "Influence of tide and waves on washout of dissolved nutrients from the bioremediation zone of a coarse sand beach: Application in oil spill bioremediation". *Spill Science & Technology* **4**, 99-106.

Yancey, J.A., Kosman, J.J., Grills, J.J., Cavalier, R.C., Irion, J.W. (1994). "Importance of quality control in a gas chromatographic method to characterise crude oils". *Journal of High Resolution Chromatography* **17**, 463-468.

Zhang,Y., Miller, R.M. (1992). "Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid". *Applied & Environmental Microbiology* **58**, 3276-3282.

Zobell, C.E. (1946). "Action of micro-organsims on hydrocarbons". *Bacteriological Review* **10**, 1-49.

Zobell, C.E. (1969). "Microbial modification of oil in the sea". *Proceedings of the joint conference on prevention and control of oil spills*, pp. 317-326.

APPENDIX A

Analytical Methodology

A.1. Extraction of hydrocarbon from contaminated sediment (microcosm samples) and gravimetric analysis

Reagents

Anhydrous sodium sulfate (analytical grade, BDH)
n-Hexane (Analytical grade or Pestiscan, Labscan)
Copper turnings (BDH)

Method

Sediment samples were collected into pre-solvent rinsed museum jars and frozen (<-10°C) until use. Subsamples were taken for analysis and allowed to air dry overnight in a fume cupboard. Approximately 10g of sediment was weighed and mixed with an equal amount of anhydrous sodium sulfate. This was soxhlet extracted with ~ 100 mls *n*-hexane, for 7 hours with *ca*. 2 minute cycle. Activated copper filings (see appendix A.3 for activation procedure) were included in the extract flask to remove molecular sulfur. Hexane was the chosen solvent as sediment was initially contaminated in pentane carrier solvent. While other solvents, such as dichloromethane, may extract a broader range of oil components, an aliphatic solvent was considered suitable as only pentane soluble oil components should have been transferred to the sediment during the contamination process.

The extract was concentrated to less than 10 mls by rotary evaporation (Rotavapor R110 Buchi). This was quantitatively transferred to a preweighed solvent rinsed disposable glass vial and brought to dryness using a gentle nitrogen (zero grade) stream at ~40°C (Techne SC-3 Sample Concentrator). The weight of dried oil was recorded.

A.2. Analytical regime for microcosm experiments

After gravimetric determination of oil samples, samples were reconstituted to 10mls in *n*-hexane (analytical grade or better, Labscan). Exact concentrations were determined by weight. Aliquots were taken for analysis by analytical methods selected for given sampling interval. Analytical methods were as described in appendix A (A.4, A.7, A8, A.9, A.10, A.11). If fractionation was carried out at a given sampling interval, a second sediment sample was extracted (as A.1) and analysed (A.4).

A.3. Oil Dissolution Method & Gravimetric analysis (phase I)

Reagents

n-Hexane (Analytical grade or Pestiscan, Labscan)
Dichloromethane (Analytical grade or Pestiscan, Labscan)
Methanol (Analytical grade or Pestiscan, Labscan)
Anhydrous sodium sulfate (Analytical grade, BDH)
Hydrochloric acid (Analytical grade, BDH)

Method

300 mg of oil was accurately weighed into a preweighed test tube. 5 mls of hexane was added and the solution sonicated for 15 mins. This solution was then centrifuged (Sorvall T6000 Dupont) at 2500 RPM x 10mins to remove sediment and asphaltenes. (DCM was also used as a solvent for comparison).

The supernatant was decanted into a preweighed test tube. The remaining pellet was dried under nitrogen and the percentage asphaltenes determined gravimetrically on a 5-place balance.

Water was removed by passage through a column (for dimensions see appendix A.4) packed with 1-2 ml anhydrous sodium sulfate column. The oil solution was eluted with 5ml hexane followed by 5ml dichloromethane.

To remove molecular sulfur activated copper filings were added to the eluant until no further blackening of the copper was visible. The copper was activated by washing in HCl for 10 mins followed by washing in hexane and storage in methanol prior to use.

Aliquots of oil extract were sometimes taken at this stage for analysis by other techniques. The solution was brought to dryness under a gentle stream of nitrogen (zero grade) at ~40°C (Techne SC-3 sample concentrator) and gravimetrically determined using a 5-place analytical balance, (i.e. the proportion of oil recovered after removal of sulfur, water, asphaltenes, detritus and volatile components). Further analysis of the dried oils was carried by other techniques after appropriate reconstitution in *n*-hexane.

A.4. Fractionation by column chromatography

Reagents

n-Hexane (Analytical grade or Pestiscan, Labscan)
Diethyl ether (Analytical grade or Pestiscan, Labscan)
Methanol (Analytical grade or Pestiscan, Labscan)
Silica gel (Mesh 70-230) (Merck)
Aluminium oxide 90 neutral (Merck)

Method

Silica gel 60 (Mesh 70-230) and aluminium oxide 90 were both activated overnight at 120°C. Custom-made glass columns (20cm height, 1 cm internal diameter, 25ml reservoir, PTFE stopcock – manufactured by AGB, Dublin) were plugged with solvent-rinsed glass wool, rinsed with DCM and then hexane, and then filled with hexane. 6 mls of silica was poured into each column. The silica was allowed to settle for two hours and before 3 ml of alumina was poured on top, the columns were then allowed to settle for a further two hours.

Samples were prepared as described in section A1 or A3. The dried oil was reconstituted in 1ml n-hexane applied to the column by Pasteur pipette. A further 2 x 1ml vial washes with elution solvent were carried out to ensure that complete transferal. The following elution profile was carried out:

Fraction	**Elution solvent**	**Mls**	
1	Hexane	25	*Aliphatic*
2	85% Hexane/15% Diethyl ether	25	*Aromatic A*
3	50% Hexane/50% Diethyl ether	25	*Aromatic B*
4	50% Diethyl ether/50% Methanol.	25	*Polar*
5	Methanol	25	*Post-polar*

Eluants were collected in glass vials and dried under nitrogen (Techne SC-3 Sample Concentrator) for gravimetric determination. Further analysis by GC/GC-MS was sometimes carried out. For aliphatic, aromatic and polar class fractionation, fraction 1 contained saturates, fraction 2 and 3 were pooled for the aromatics and fraction 4 contained resins. The elution profile is based on that described by Law *et al.* 1988.

A.5. Urea adduction

Reagents

n-Hexane (Analytical grade or Pestiscan, Labscan)
Acetone (Analytical grade or Pestiscan, Labscan)
Methanol (Analytical grade or Pestiscan, Labscan)
Water (distilled, deionised)
Urea (Aldrich ACS reagent)
Docosane (from "Aldrich library of chemical standards" – 29,850-6)
Squalane (Sigma)
Tridecylbenzene (Aldrich 99%)

Method

0.5g of urea was ground using mortar and pestle and 2ml of methanol were added to a test tube. This was vortexed and sonicated for 10min to prepare a saturated solution. 100 μl of an internal standard containing 2μg each of docosane, tridecylbenzene (TDB) and squalane was added and the weight accurately recorded. 8mg of dried oil in *n*-hexane was added and then vortexed and heated to 30°C. 0.1ml of acetone was then added. After drying under nitrogen the complex was washed with 4 x 1ml hexane and then 1ml of methanol/hexane 1:1. The pooled wash was collected into a disposable glass vial and dried under nitrogen at ~40°C (Techne SC-3 Sample Concentrator).

The urea complex was then dissolved in 2ml of methanol/water 3:1 and the adducts extracted by liquid-liquid partitioning with 3 x 1ml of hexane and dried under nitrogen. Samples were resuspended in 1ml hexane and analysed by GC-FID (Appendix A.7) to check the ratio of adducts:non-adducts by measurement of total areas.

A.6. Thiourea adduction

Reagent

Thiourea (Aldrich ACS reagent)

Method

The same procedure as in A5 with thiourea replacing urea was carried out with the exception that acetone is not added.

A.7. Gas Chromatography (Flame ionisation Detection) [GC-FID]

Reagents

n-hexane (Analytical grade or better, Labscan)
Squalane (Sigma)

Method

Samples were prepared as described in A1 or A3. Both total and dried oils were analysed. Squalane (2µg/ml final concentration) was added as an internal standard. Microcosm sediment extracts were made up to 10 mls in *n*-hexane. The following GC conditions were used:

Column:	Ultra2 (5% Crosslinked PH ME Siloxane) (Hewlett Packard) 25 M x 0.2 mm x 0.33 µm	
Carrier gas:	Helium (linear velocity approximately 35cm.sec^{-1})	
Temperature programme:	Initial temperature:	50°C
	Initial time:	1 min
	Rate:	6°C/min
	Final temperature:	300°C
	Final time:	33 mins
	Total time:	75.66 mins
Injection port:	300°C	
Detector:	FID	
Detector temp:	300°C	
Detector gases:	H_2 and air (N_2 (oxygen-free) make up gas)	

Data analysis was performed using HP Chem Station. Resolved compounds and UCM total and segment area integrations were performed. Quantification of UCM was made by subtracting a blank solvent chromatogram to compensate for baseline drift. A baseline was drawn from the start to the end of the UCM and the total are obtained. For quantification of UCM segments (area between alkanes), perpendiculars were dropped at retention times corresponding to *n*-alkane peak ends and the area between each segment taken, using the UCM baseline. The area of the *n*-alkane in each segment was also quantified and subtracted from the segment area to give a UCM segment area. When using oils containing no *n*-alkanes, a forties oil sample was run in that batch to ascertain the *n*-alkane peak end retention times.

A.8. HPLC

Reagents

Dichloromethane (HPLC grade, Labscan)

n-Hexane (HPLC grade, Labscan)

Toluene (Analytical grade, Labscan)

Tridecylbenzene (Aldrich 99%)

Naphthalene. fluorene, phenanthrene, fluoranthene, chrysene, perylene ("Aldrich library of chemical standards" –30,027-6)

2,6-dimethylnaphthalene & 3,6 dimethylnaphthalene (Aldrich)

Method

HPLC was used to class fractionate the oils based on aromatic ring number and polar components. Samples were prepared and the asphaltenes measured gravimetrically as described in A1 or A3. For microcosm sediment samples an aliquot of sample (reconstituted in 10 mls *n*-hexane) was accurately diluted by 1/5 for Alba samples and ½ for Forties samples and perylene was added as an internal standard.

HPLC System

A Shimadzu HPLC system was used and employed 2 LC-6A pumps with mixing chamber, a SCL-6B system controller, a SIL-6B injector and a SPD-6AV UV detector. Data collection was carried out with a Shimadzu Chromatopac CR4AX. Back flushing was achieved using a Rheodyne switching valve with pneumatic actuator (Model 5701) and a Rheodyne solenoid valve kit (Model 7163). The detection wavelength was 265nm. An amino (Waters Spherisorb silica -NH_2, 5µm x 25 cm x 4.6mm i.d.) and cyano (Waters Spherisorb silica -CN, 5µm x 25 cm x 4.6mm i.d.) column were used in series.

Mobile Phase/Gradient system

Mobile Phase A = 2% Dichloromethane in Hexane

Mobile Phase B = 100% Dichloromethane

Solvents were membrane filtered, degassed for 20 minutes under vacuum and for a further 15 minutes in a sonic bath.

100% Mobile phase A was maintained for 22 minutes then a linear gradient to 100% B at 25 minutes. The flow was reversed through both columns to the detector at 26 minutes and maintained for 5 minutes. The total flow rate throughout was 1.5ml/min. The instrument was re-equilibrated in mobile phase A for 14 minutes between injections. The sample injection volume was 25µl.

Standards

A standard mixture containing TDB, toluene, naphthalene, 2,6-dimethylnaphthalene, fluorene, phenanthrene, 3,6-dimethylphenanthrene, fluoranthene, chrysene, and perylene, was used to assess elution order of aromatic components and to identify sections for integration based on ring number. Perylene (20µg/g final concentration in *n*-hexane) was added to the diluted oil extracts as an internal standard. Quantification was based on perylene internal standard and bracketed Alba reference oil.

A.9 Fluorescence Spectroscopy

Reagents

n-Hexane (Pestiscan grade, Labscan)

Method

Samples were prepared as described in A1 or A3 for fluorescence analysis using an Perkin Elmer LS30 instrument. The samples were appropriately diluted in *n*-hexane until they were within the linear range of the instrument. A BASIC computer programme via an RS232 interface enabled instrument control and data acquisition for total fluorescence, fixed wavelength and synchronous scans.

Total fluorescence measurements were made using an Emλ-Exλ offset of 10nm in the range Exλ=200-600nm and fluorescence scans were repeated at 10nm intervals. Only every fifth data point was acquired to reduce the file size. (see chapter 2.3.4 for a more complete explanation).

n-Hexane blanks were run under the same conditions as the samples and background subtracted using Microsoft Excel. Fresh blanks samples were analysed daily and the instrument periodically checked throughout the day for contamination of the cell.

Total fluorescence contour plots were carried out using Surfer™ (Golden Software) by Kriging. The region of high emission intensity for *n*-hexane was excluded from data acquisition.

Fixed wavelength contour plots were carried out setting wavelengths on the instrument and directly recording intensities from the LED display.

A.10. Infrared spectroscopy

Reagents

Tetrachlorethylene (Spectroscan, Labscan)

Anhydrous sodium sulfate (Analytical grade, BDH)

Silica Gel 60 (70-230 mesh) (Merck)

Water (Distilled, deionsied)

Method

IR spectra were recorded using a double beam Perkin Elmer 289 IR spectrophotometer. Signals were acquired on the instrument chart recorder but also on computer using 12-bit resolution analogue to digital conversion. (Picolog/ Pico ADC16).

Quantitative analysis

100mg of each oil was dissolved in 10ml tetrachloroethylene (PCE) by sonication and centrifuged (Sorvall T6000 Dupont) at 2500 rpm for 10 minutes. Anhydrous sodium sulfate was added to remove water. These stock solutions were diluted appropriately to bring them within the instrumental linear working range. Silica gel 60 was added to the flasks, to a 0.03mg/ml proportion, with a magnetic pellet and allowed to stir for 10 minutes. The gel was then allowed to settle. The silica gel was activated overnight at 150^0C, cooled and 2% deactivated by the addition of water (2% by weight) prior to use.

The IR spectra for the oil extracts in the $3500cm^{-1}$ to $2500cm^{-1}$ region at a slow scan speed were recorded using quartz cells(10mm) with PCE as reference. Although some absorbance by PCE occurred in the region of interest, it was successfully blanked using the double beam instrument. Peak height was recorded at 3030, 2960, 2930, 2880 and 2860 cm^{-1} . An Alba crude oil standard curve was prepared to assess the working range and the linearity of the peak height to concentration.

Qualitative analysis

IR spectra for recorded for the oils in the range 4000 to 600 cm^{-1} using sodium chloride cell windows. Each oil was applied to the window as a thin film of whole oil or concentrated tetrachoroethylene extract from which the solvent was allowed to evaporate. A blank sodium chloride cell was used as reference. A polystyrene film was used for instrument calibration. A medium slit and a scan time of 30 minutes were used. The absorbance frequencies for each oil were recorded.

A.11. Mass spectrometry

Reagents

n-Hexane (Analytical grade or better, Labscan)

Method

Samples were prepared as described in A.1 or A.3. Microcosm sediment samples were reconstituted with 10 mls hexane.

GC-MS (Electron ionisation)

Mass spectrometry was carried out on a VG Trio 2000 (Micromass) research grade quadrupole instrument linked to a HP5890 series II and HP 7663a autosampler. Data acquisition and analysis employed a PC with Masslynx 2.1 software (Micromass)

1μl of sample was injected.

The MS was tuned using with heptacosa (perfluorotributylamine) on 4 ions (69, 219, 502 and 614 amu) and also mass calibrated with heptacosa. Electron energy was 70eV and source temperature 180°C.

General Instrumental Parameters (GC-MS)

Injection: Splitless (1μl)

Column: DB5-MS, 30m x 0.25mm i.d. x 0.5μm film thickness (J&W Scientific)

Carrier gas: Helium

Temperature Programme: Initial temperature: 50°C

Initial time: 1 minute

Rate: 60°C/minute

Final temperature: 300°C

Final time: 24 minutes

Total time: 67 minutes

Transfer Line: 280°C

Injection Port: 300°C

Selected ion recording (SIR) Acquisition

Many combinations of ions were analysed by selected ion recording. The following table lists the selected ions used indicative of given compound groups. The ions were recorded during specific retention windows. e.g. pentacyclic triterpanes, steranes, triaromatic steranes were generally recorded in the final retention window (after 38 minutes) as they elute late in the chromatogram. The ions recorded are listed in table A.1.

Full scan data acquisition mode

Full scan data was acquired in the range 50-500amu

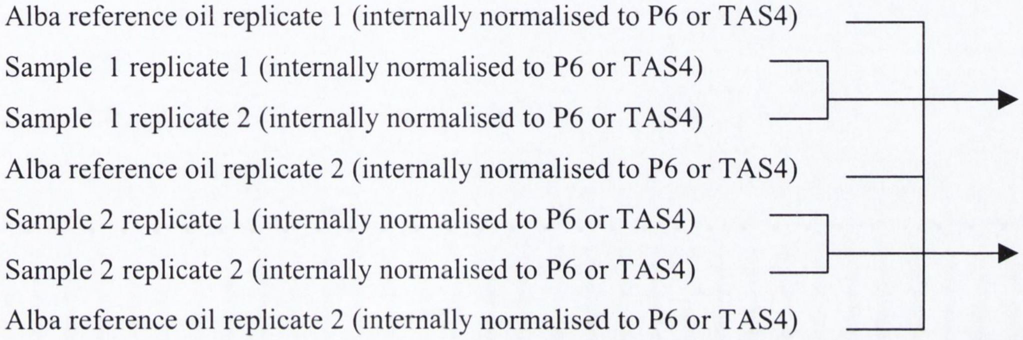
GC-MS (Chemical ionisation)

Methane was used as the reagent gas for analysis in CI+ mode. Tuning was carried out using the reagent gas ions (19 and 29amu) and also using octacosane at 294 and 652 amu. The octacosane was introduced by solid probe. GC conditions for CI-MS were as for GC-MS with EI+. Data acquisition was in full scan mode in the range of 50-500 amu.

Bioremediation monitoring analytical scheme

Samples were analysed in duplicate bracketed with Alba reference oil. For SIR analysis, all peak areas were normalised to C30-hopane (P6 appendix B. X) or TAS4 (triaromatic sterane – appendix B.XIII) and averaged for duplicate samples and then normalised to the similarly treated bracketing reference samples. Solvent blanks were analysed at regular intervals.

GCMS analysis sequence



Alba reference oil replicate 1 (internally normalised to P6 or TAS4)
Sample 1 replicate 1 (internally normalised to P6 or TAS4)
Sample 1 replicate 2 (internally normalised to P6 or TAS4)
Alba reference oil replicate 2 (internally normalised to P6 or TAS4)
Sample 2 replicate 1 (internally normalised to P6 or TAS4)
Sample 2 replicate 2 (internally normalised to P6 or TAS4)
Alba reference oil replicate 2 (internally normalised to P6 or TAS4)

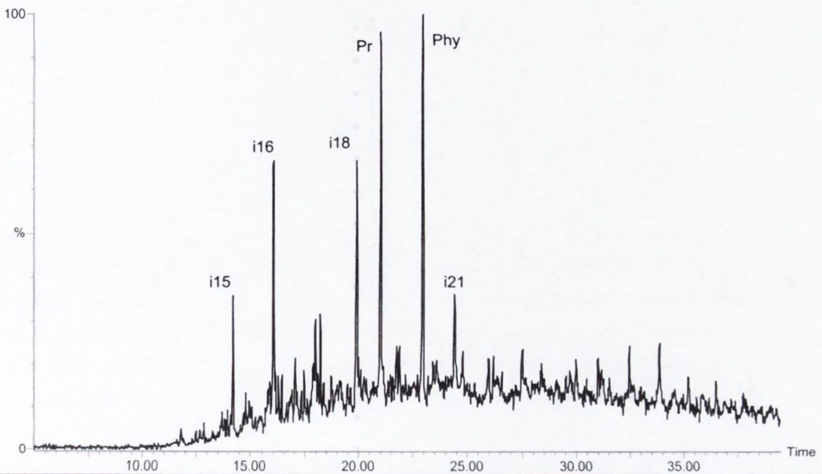
Group	**m/z**
cycloalkanes	
Cx-cycloalkanes	83
alkanes/isoprenoids	85
Cx-benzenes	91
Cx-methyl cylcohexanes	97
Cx-methylbenzenes	105
naphthenes	137
C2-naphthalenes	156
Fluorene	166
C3-naphthalenes	170
isoprenoids	183
C4-naphthalenes	184
C1-phenanthrenes	192
C1-dibenzothiophenes	198
C2-phenanthrenes	206
C2-dibenzothiophenes	212
C1-pyrene	216
C3-phenanthrenes	220
C3-dibenzothiophenes	226
norhopanes	177
pentacyclic triterpanes	191
methyl hopanes	205
steranes	217
14β-steranes	218
monoaromatic steranes	253
Triaromatic steranes	231

Interscan delay = 0.1s

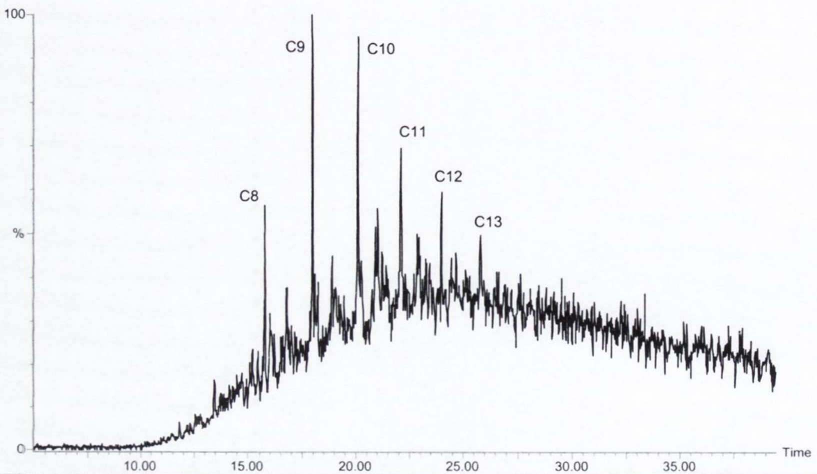
Table A.1: Ions (m/z) recorded in GC-MS SIR mode

APPENDIX B

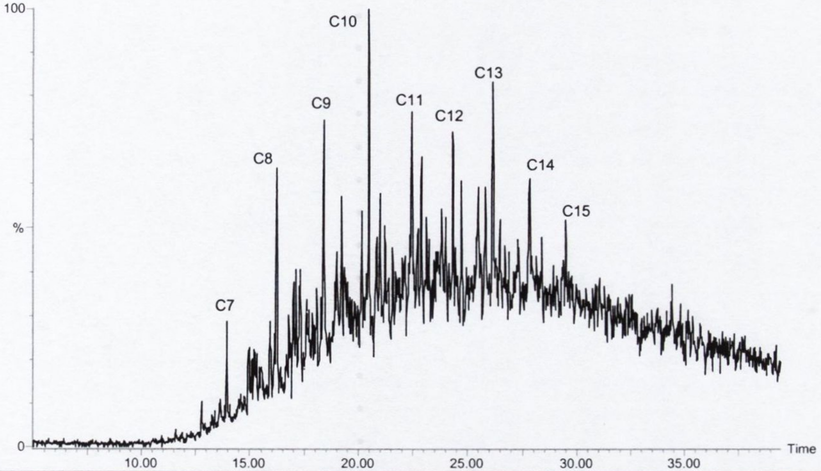
Selected Mass Chromatograms for Alba Oil



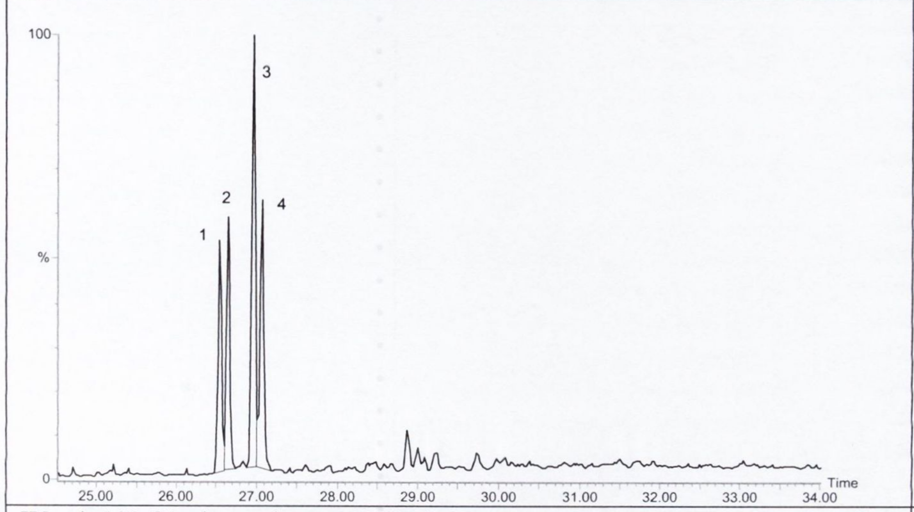
I m/z 85 Isoprenoids – Alba oil



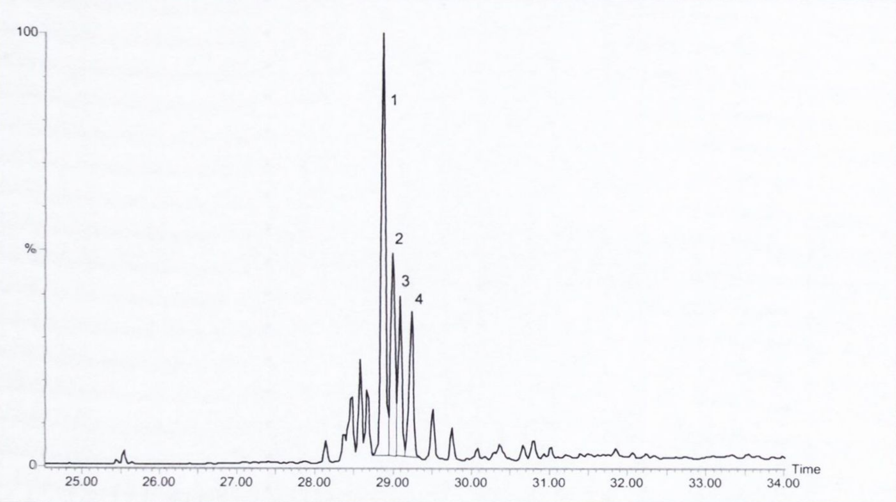
II m/z 83 Alkylcyclochexanes - Alba oil (Cn – n=no. carbons in substituent chain)



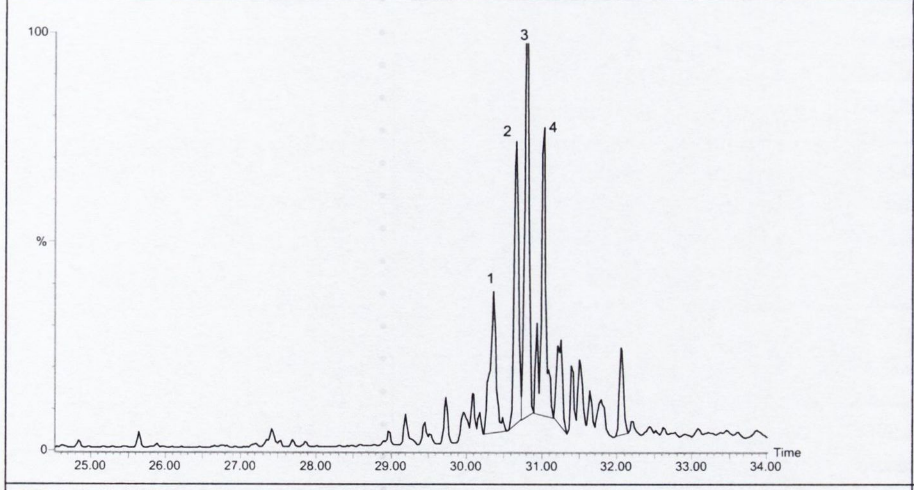
III m/z 105 Alkylmethylbenzenes - Alba oil (Cn – n=no. carbons attached to ring)



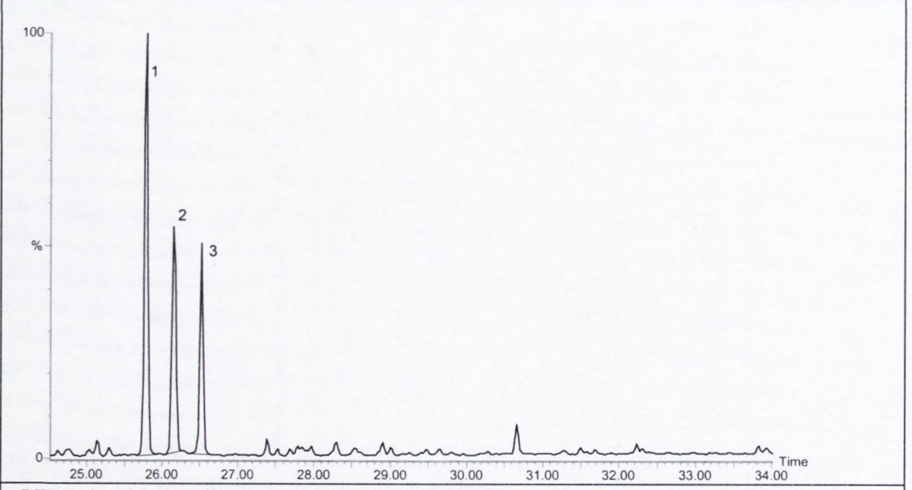
IV m/z 192 C1-phenanthrenes (C1P-n)



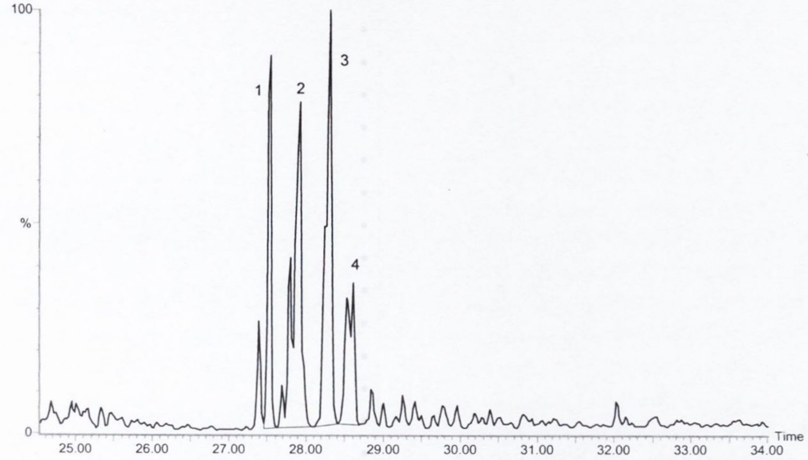
V m/z 203 C2-phenanthrenes (C2P-n)



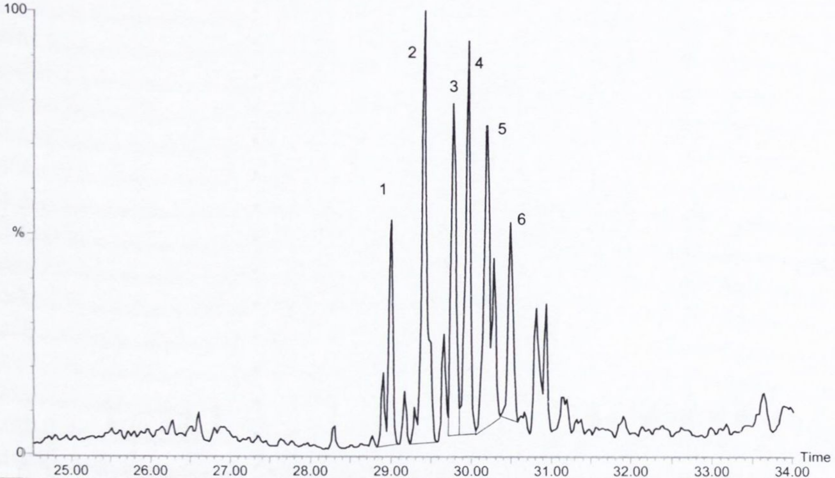
VI m/z 220 C3-phenanthrenes (C3P-n)



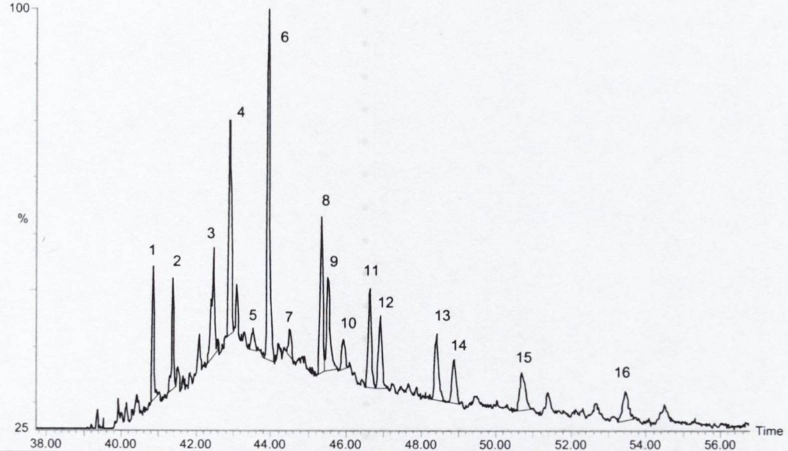
VII m/z 198 C1-dibenzothiophenes (C1D-n)



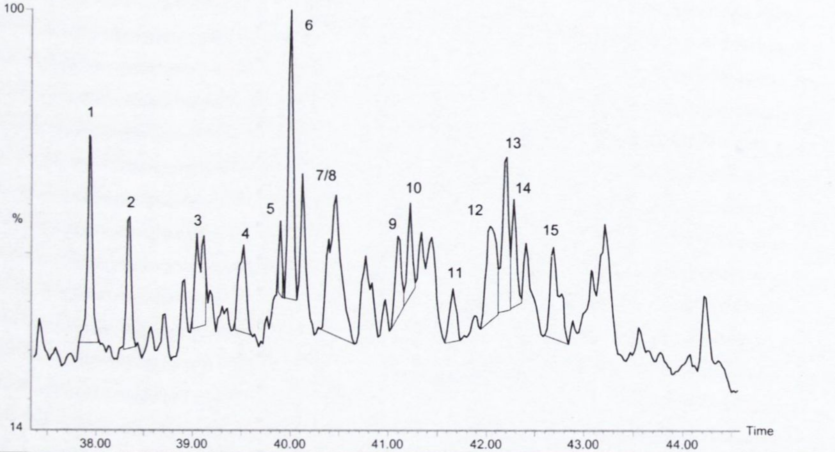
VIII m/z 212 C2-dibenzothiophenes (C2D-n)



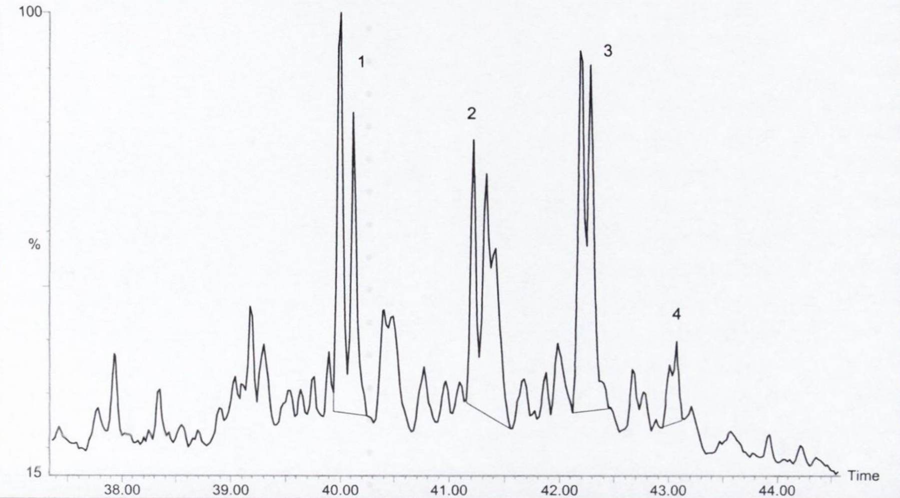
IX m/z 226 C3-dibenzothiophenes (C3D-n)



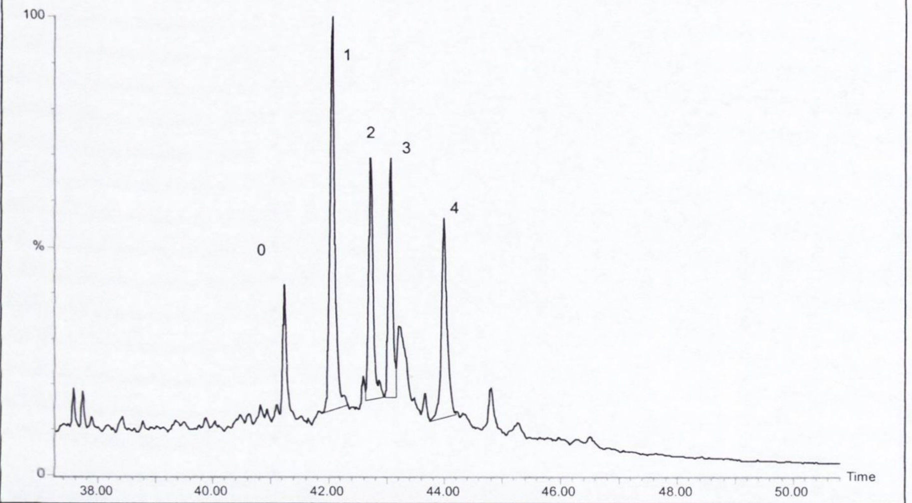
X m/z 191 pentacyclic triterpanes (Pn - see table B.1)



XI m/z 217 steranes (Sn - see table B.1)



XII m/z 218 14β-steranes



XIII m/z 231 triaromatic steranes (TASn – see table B.1)

Peak	**m/z**	***Compound***	**Carbon no**
		Pentacyclic triterpanes m/z 191 (appendix B.X)	
P1	191	18α(H)-22,29,30-trisnorhopane (C27) (Ts)	27
P2	191	17α(H)-22,29,30-trisnorhopane (C27) (Tm)	27
P3	191	17α(H),18α(H),21β(H),28,30 bisnorhopane (C28)	28
P4	191	17α(H), 21β(H)30 norhopane (C29)	29
P5	191	17β(H), 21α(H)30 norhopane (C29) (17β(H) normoretane)	29
P6	191	17α(H), 21α(H)hopane (C30)	30
P7	191	17β(H), 21α(H)hopane (C30) (17β(H) moretane)	30
P8	191	(22S)17α(H), 21β(H)30 homohopane (C31)	31
P9	191	(22R)17α(H), 21β(H)30 homohopane (C31)	31
P10	191	17β (H) homomoretane	
P11	191	(22S)17α(H), 21β(H)30 bishomohopane (C32)	32
P12	191	(22R)17α(H), 21β(H)30 bishomohopane (C32)	32
P13	191	(22S)17α(H), 21β(H)30 trishomohopane (C33)	33
P14	191	(22R)17α(H), 21β(H)30 trishomohopane (C33)	33
P15	191	(S+R)17α(H), 21β(H)30 tetrakishomohopane (C34)	34
P16	191	(S+R)17α(H), 21β(H)30 pentakishomohopane (C35)	35
		Steranes m/z 217 (appendix B.X1)	
S1	217	(20S)13β,17α-diacholestane (C27)	27
S2	217	(20R)13β,17α-diacholestane (C27)	27
S3	217	(20S)13β,17α-24-methyldiacholestane (C28)	28
S4	217	(20R)13β,17α-24-methyldiacholestane (C28)	28
S5	217	(20S)5α,14α,17α-cholestane(C27)	27
S6	217	(20S)13β,17α-diasterane (C29)	29
S7	217	(20R)13β,17α-diasterane (C29)	29
S8	217	(20R)5α,14α,17α-cholestane(C27)	27
S9	217	(20S) 5α,14α,17α-24-methylcholestane(C28)	28
S10	217	(20S+R) 14β,17β-24-methylcholestane(C28)	28
S11	217	(20R) 5α,14α,17α-24-methylcholestane(C28)	28
S12	217	(20S) 5α,14α,17α-24-ethylcholestane(C29)	29
S13	217	(20R)14β,17β-24-ethylcholestane (C29)	29
S14	217	(20S)14β,17β-24-ethylcholestane (C29)	29
S15	217	(20R) 5α,14α,17α-24-ethylcholestane(C29)***	29
		Triaromatic steranes m/z 231 (appendix B.XIII)	
TAS0	231	(20S) Cholestane	26
TAS1	231	(20R) Cholestane/ (20S) Ergostane	26,27
TAS2	231	(20S) Ethylcholestane	28
TAS3	231	(20R) Methylcholestane	27
TAS4	231	(20R) Ethylcholestane	28

Table B.1: Biomarker peak assignations

APPENDIX C

Minimal media used to support growth on UCM

(Reference Anon. 1991a)

Stock solutions: (Prepared in distilled water.).

A: N & P salts: (made up to 1000ml and adjusted to pH 7.2).

KH_2PO_4`	8.50g	(Sigma, min. 99.0%)
K_2HPO_4	21.75g	(Sigma, min. 99.0%)
$Na_2HPO_4.7H_2O$	50.2g	(Sigma ACS reagent)
NH_4Cl	2.50g	(Sigma, min. 99.5%)

B. 22.50g $MgSO_4.7H_20$ (General Purpose Reagent, BDH) in 1000 mls.

C. 27.50g $CaCl_2$ (Merck) in 1000 mls.

D. 0.25g $FeCl_3.6H_20$ (Analar, BDH) in 1000 mls distilled water.

E. Trace elements: (made up to 500 mls).

$MnSO_4.H_2O$	15.1 mg	(Sigma, min 99.0%)
H_3BO_3	28.6 mg	(Analar, BDH)
$ZnSO_4.7H_2O$	21.4 mg	(Sigma, min. 99.0%)
$(NH_4)_6Mo_7O_{24}$	18.4 mg	(Sigma, ACS reagent)
$FeCl_3$	17.9	(General Purpose Reagent, BDH)
EDTA	32.1 mg	(Sigma, min. 99%)

Final Concentrations:

Laboratory based microbiological work (respirometry trials, selective enrichment, preparation of the inocula) were carried out using this nutrient formulation diluted in natural seawater as follows: 20 mls solution A and 2 mls each stock solutions B to E per litre .

For the microcosm trials 400 mls of stock solution A and 40 mls each of stock solutions B to E were added to each reservoir and diluted to 20 litres with seawater at each nutrient change. A small quantity of seawater from the previous batch (~200mls) was retained in each reservoir at each nutrient change.

APPENDIX D
Analytical Data for Respirometry Studies and Microcosm Trials

D.1 GC-MS data from analysis of oils from respirometry studies

Total ion abundance for combined GC-MS spectra for oils from respirometry trials
Determination of suitable propagation medium – (see chapter 3.3.1)

Ion Intensity X 10,000

Sample	*55*	*57*	*69*	*71*	*77*	*82*	*83*	*85*	*91*	*92*	*95*	*97*	*99*	*105*
Abiotic control	11.4	12.2	9.98	8.29	0.979	2.68	7.4	4.96	2.16	0.59	6.52	7.9	1.71	2.69
Sample (glucose medium)	15.6	12.3	14.1	8.07	1.64	3.44	10	4.65	3.54	0.819	11.3	9.44	1.63	3.72
Alba	23.1	20	19.5	13.1	2.41	5.98	14.7	7.7	4.85	1.32	13.9	14.1	2.53	5.48

Total ion abundance for combined GC-MS spectra for oils from respirometry trials
Comparison of enrichments on UCM as sole carbon source – (see chapter 3.3.2)

Ion Intensity X 10,000

Sample	*55*	*57*	*69*	*71*	*77*	*82*	*83*	*85*	*91*	*92*	*95*	*97*	*99*	*105*
EF1	3.89	2.28	2.49	1.15	0.442	0.696	1.85	0.649	0.897	0.211	2.42	1.79	0.258	0.9
EF2	1.93	1.34	1.35	0.666	0.186	0.334	0.909	0.391	0.378	0.0843	1.12	0.98	0.157	0.42
EF4	3.52	2.64	2.91	1.57	0.375	0.701	1.89	0.849	0.715	0.174	1.99	1.83	0.304	0.782
EF5	4.77	3.79	4.01	2.36	0.499	0.988	2.68	1.25	0.93	0.226	2.58	2.45	0.414	1.02
EF6	11.2	11.8	9.93	7.83	0.976	2.7	7.24	4.55	2.07	0.599	5.61	7.24	1.41	2.53
EF7	7.45	6.55	6.57	4.27	0.84	1.86	4.92	2.58	1.64	0.433	4.44	4.5	0.821	1.73
CF7 (control)	14.9	14.2	13.6	10	1.76	4.78	12.9	7.33	3.96	1.09	10.8	12.6	2.29	4.52
abiotic control	13.2	13.6	11.9	9.92	1.33	3.9	10.1	6.38	2.89	0.855	7.66	10.2	1.98	3.53

Summed peak areas for GC-MS SIR analysis of oils from respirometry trials.
Comparison of enrichments on UCM as sole carbon source. (See chapter 3.3.2 – Peaks summed are given in appendix B)

m/z	**Abiotic control**	**EF1**	**EF2**	**EF4**	**EF5**	**EF6**	**EF7**	**CF7**
105 Methylbenzenes	779753	12451	9907	128310	199397	508733	279736	617714
137 naphthenes	143473	12680	7793	25836	27754	121734	53656	177396
198 C1-dibenzothiophenes	43242	5967	2930	8701	8698	15647	18974	60181
206 C2-phenanthrenes	1726788	43844	19724	241945	411937	446218	1016361	2567442
212 C2-dibenzothiophenes	1449428	21835	16511	200600	323440	406040	857215	2174870
220 C3-phenanthrenes	2438593	201256	144407	691034	824339	1234220	1385443	3836365
226 C3-dibenzothiophenes	1459356	101390	78552	399141	505118	762532	914836	2336019
191 Pentacyclic triterpanes	928253	911373	365985	611108	608371	944903	533260	1553525
217 Steranes	217372	190281	96570	127082	152845	225750	171190	395792
218 a-steranes	563337	405847	181070	295028	296865	526830	322457	971292
231 triaromatic steranes	723101	662192	233388	438558	453440	696233	458085	1243637
177 desmethyl hopanes	283793	257261	86166	148349	173879	291660	187366	487588
C30 pent	201807	221787	80325	135448	136598	211862	140539	370162

Total ion abundance ratios for total combined GC-MS spectra for oils from respirometry trials. Comparison of isolated strains vs. crude enrichment broth. (See chapter 3.3.3 – Peaks summed are given in appendix B)

Ion Intensity X 10,000

Sample	***55***	***57***	***69***	***71***	***77***	***82***	***83***	***85***	***91***	***92***	***95***	***97***	***99***	***105***
Alba	0.809	0.797	0.786	0.581	0.0912	0.243	0.618	0.345	0.181	0.0531	0.604	0.647	0.119	0.162
ucc11	0.782	0.778	0.791	0.57	0.0907	0.236	0.594	0.33	0.174	0.0506	0.574	0.615	0.113	0.192
ucc13	0.226	0.0662	0.226	0.0592	0.0387	0.0577	0.142	0.0326	0.071	0.0142	0.244	0.124	0.0126	0.0555
ucc14	0.692	0.654	0.721	0.49	0.0819	0.203	0.526	0.279	0.156	0.0428	0.524	0.549	0.0964	0.177
ucc15	0.727	0.493	0.752	0.34	0.102	0.2	0.534	0.199	0.186	0.0423	0.682	0.523	0.0741	0.186
ucc16	0.487	0.293	0.456	0.198	0.0564	0.111	0.308	0.112	0.103	0.0227	0.394	0.296	0.041	0.1
Alba	0.666	0.675	0.694	0.508	0.0793	0.199	0.505	0.285	0.147	0.0445	0.487	0.521	0.0957	0.151

Peak areas for GC-MS SIR analysis of oils from respirometry trials.
Comparison of isolated strains vs. crude enrichment broth. (See chapter 3.3.3 – Peaks summed are given in appendix B)

	Alba	**ucc11**	**ucc13**	**ucc14**	**ucc15**	**ucc16**	**Alba**
C1P 1	305253	300216	0	296739	181558	0	311500
C1P 2	361692	408720	0	359088	14150	0	400581
C1P 3	589951	641766	0	572435	654004	0	678666
C1P 4	368117	401499	0	384270	284378	0	411966
C1D 1	384210	419759	0	382190	322848	16078	426985
C1D 2	258625	270649	0	244502	84062	23066	289093
C1D 3	174045	189877	0	172866	153456	9287	191472
C2P 1	693618	741076	0	681350	994893	12190	783182
C2P 2	280570	306406	0	284439	412119	118728	315440
C2P 3	1627765	178564	0	171273	243669	158943	181004
C2P 4	201338	228223	0	194531	28892	117568	216371
C2D 1	227214	237195	730	220460	318774	203974	251637
C2D 2	445053	471954	1361	440851	610903	143364	491946
C2D 3	387500	415509	591	381198	526981	235346	431422
C2D 4	177256	191647	849	176336	249057	84680	198634
C3P 1	148857	167027	0	144012	216464	108799	168373
C3P 2	207249	243846	0	200506	326993	104472	250427
C3P 3	295898	292433	4855	289383	427935	268182	337895
C3P 4	203545	222188	0	208585	317616	183357	239750
C3D 1	109854	116244	0	107186	161664	107811	122736
C3D 2	217287	227081	0	214678	316352	207966	245526
C3D 3	155482	161488	0	150362	226475	151466	173628
C3D 4	163532	169654	0	160291	234470	162371	183193
C3D 5	209196	213876	0	205227	305085	210411	231429
C3D 6	90439	95641	0	91079	127582	83476	103206
P1	80677	97934	117431	92272	125574	82492	96865
P2	57732	60043	69910	57010	81832	55388	65863
P3	85027	94041	116376	84922	119632	83157	89175
P4	198609	224080	282183	195636	281925	187712	221643
P5	15320	12748	21483	10832	9107	9648	16723
P6	265048	297103	361759	259952	364992	236596	286428
P7	21725	29489	34014	25681	36519	24678	31128
P8	120926	127589	166738	110969	171479	109090	126661
P9	88167	93394	120140	72673	120781	70200	82979
P10	42516	46364	59097	45843	54501	39744	46051
P11	87073	95259	113760	72925	119608	73260	90774
P12	69100	73388	78628	53719	91704	56072	69884
P13	69385	79093	89942	71220	98289	61146	75247
P14	47434	52865	51576	42820	63202	43047	53650
P15	71263	85845	64776	60344	98871	62218	72521
P16	58830	57570	39091	42685	64077	37265	45705

D.2 Gravimetric analysis results Forties and Alba microcosms samples

Date sampled		Time	Gravim 1 mg/g	Gravim 2 mg/g	Date sampled		Time	Gravim 1 mg/g	Gravim 2 mg/g
28/08/97	FiT1	28	15.48	15.48	28/08/97	AiT1	28	29.30	30.18
25/09/97	FiT1	56	15.42		25/09/97	AiT1	56	26.77	
06/11/97	FiT1	98	14.18		06/11/97	AiT1	98	22.67	
18/12/97	FiT1	140	10.90		18/12/97	AiT1	140	17.94	
19/02/98	FiT1	203	10.03		19/02/98	AiT1	203	16.54	
07/05/98	FiT1	280	11.26		07/05/98	AiT1	280	19.64	
16/07/98	FiT1	350	9.14		16/07/98	AiT1	350	15.93	
11/09/98	FiT1	407	7.07		11/09/98	AiT1	407	16.43	
16/11/98	FiT1	473	5.29		28/08/97	AiT2	28	29.01	28.88
28/08/97	FiT2	28	16.05		25/09/97	AiT2	56	26.54	
25/09/97	FiT2	56	15.07		06/11/97	AiT2	98	24.45	
06/11/97	FiT2	98	14.28		18/12/97	AiT2	140	19.66	
18/12/97	FiT2	140	10.87		19/02/98	AiT2	203	0.00	
19/02/98	FiT2	203	9.57		07/05/98	AiT2	280	19.02	
07/05/98	FiT2	280	10.68		16/07/98	AiT2	350	17.37	
16/07/98	FiT2	350	9.20		11/09/98	AiT2	407	17.02	
11/09/98	FiT2	407	7.20		16/11/98	AiT2	473	13.61	
16/11/98	FiT2	473	5.56		28/08/97	A1T3	28	29.59	29.81
28/08/97	FiT3	28	16.36	15.93	25/09/97	A1T3	56	25.66	
25/09/97	FiT3	56	15.77		06/11/97	A1T3	98	24.93	
06/11/97	FiT3	98	14.78		18/12/97	A1T3	140	18.97	
18/12/97	FiT3	140	10.90		19/02/98	A1T3	203	17.91	
19/02/98	FiT3	203	9.77		07/05/98	A1T3	280	20.36	
07/05/98	FiT3	280	10.25		16/07/98	A1T3	350	18.24	
16/07/98	FiT3	350	7.76		11/09/98	A1T3	407	17.70	
11/09/98	FiT3	407	6.55		16/11/98	A1T3	473	15.48	
16/11/98	FiT3	473	4.67		28/08/97	AiC1	28	29.49	30.10
28/08/97	FiC1	28	16.50	16.14	25/09/97	AiC1	56	26.68	
25/09/97	FiC1	56	15.62		06/11/97	AiC1	98	25.48	
06/11/97	FiC1	98	14.94		18/12/97	AiC1	140	17.22	
18/12/97	FiC1	140	11.42		19/02/98	AiC1	203	16.53	
19/02/98	FiC1	203	11.34		07/05/98	AiC1	280	19.16	
07/05/98	FiC1	280	12.09		16/07/98	AiC1	350	18.06	
16/07/98	FiC1	350	10.70		11/09/98	AiC1	407	16.63	
11/09/98	FiC1	407	8.81		16/11/98	AiC1	473	17.43	
16/11/98	FiC1	473	6.92		28/08/97	AiC2	28	30.35	29.50
28/08/97	FiC2	28	16.46	15.18	25/09/97	AiC2	56	0.00	
25/09/97	FiC2	56	15.97		06/11/97	AiC2	98	25.99	
06/11/97	FiC2	98	16.54		18/12/97	AiC2	140	18.42	
18/12/97	FiC2	140	13.04		19/02/98	AiC2	203	17.19	
19/02/98	FiC2	203	12.47		07/05/98	AiC2	280	19.11	
07/05/98	FiC2	280	15.18		16/07/98	AiC2	350	17.81	
16/07/98	FiC2	350	15.35		11/09/98	AiC2	407	14.17	
11/09/98	FiC2	407	14.62		16/11/98	AiC2	473	10.91	
16/11/98	FiC2	473	14.03		28/08/97	AiiT1	28	29.95	
28/08/97	FiiT1	28	15.54	16.06	25/09/97	AiiT1	56	26.85	
25/09/97	FiiT1	56	15.87		06/11/97	AiiT1	98	24.90	
06/11/97	FiiT1	98	14.85		18/12/97	AiiT1	140	18.54	
18/12/97	FiiT1	140	11.75		19/02/98	AiiT1	203	16.20	
19/02/98	FiiT1	203	8.64		07/05/98	AiiT1	280	18.30	
07/05/98	FiiT1	280	10.47		16/07/98	AiiT1	350	16.80	
16/07/98	FiiT1	350	9.41		11/09/98	AiiT1	407	16.44	
11/09/98	FiiT1	407	7.03		16/11/98	AiiT1	473	15.49	
16/11/98	FiiT1	473	5.72		28/08/97	AiiT2	28	29.24	27.98
28/08/97	FiiT2	28	15.72	15.66	25/09/97	AiiT2	56	31.96	
25/09/97	FiiT2	56	15.38		06/11/97	AiiT2	98	26.52	
06/11/97	FiiT2	98	14.80		18/12/97	AiiT2	140	19.17	
18/12/97	FiiT2	140	11.24		19/02/98	AiiT2	203	17.13	

Date sampled		Time	Gravim 1 mg/g	Gravim 2 mg/g	Date sampled		Time	Gravim 1 mg/g	Gravim 2 mg/g
19/02/98	FiiT2	203	10.11		07/05/98	AiiT2	280	18.63	
07/05/98	FiiT2	280	11.34		16/07/98	AiiT2	350	17.95	
16/07/98	FiiT2	350	10.85		11/09/98	AiiT2	407	16.35	
11/09/98	FiiT2	407	7.29		16/11/98	AiiT2	473	14.91	
16/11/98	FiiT2	473	5.97		28/08/97	AiiT3	28	29.60	28.90
28/08/97	FiiT3	28	15.64	14.70	25/09/97	AiiT3	56	33.15	
25/09/97	FiiT3	56	15.22		06/11/97	AiiT3	98	28.74	
06/11/97	FiiT3	98	14.42		18/12/97	AiiT3	140	21.28	
18/12/97	FiiT3	140	10.08		19/02/98	AiiT3	203	18.92	
19/02/98	FiiT3	203	9.42		07/05/98	AiiT3	280	21.55	
07/05/98	FiiT3	280	10.56		16/07/98	AiiT3	350	21.94	
16/07/98	FiiT3	350	9.10		11/09/98	AiiT3	407	18.89	
11/09/98	FiiT3	407	7.06		16/11/98	AiiT3	473	5.37	
16/11/98	FiiT3	473	5.37		28/08/97	AiiC1	28	29.46	29.67
28/08/97	FiiC1	28	16.47	16.86	25/09/97	AiiC1	56	27.19	
25/09/97	FiiC1	56	15.77		06/11/97	AiiC1	98	24.71	
06/11/97	FiiC1	98	15.82		18/12/97	AiiC1	140	19.72	
18/12/97	FiiC1	140	11.82		19/02/98	AiiC1	203	17.26	
19/02/98	FiiC1	203	10.97		07/05/98	AiiC1	280	19.43	
07/05/98	FiiC1	280	10.71		16/07/98	AiiC1	350	19.14	
16/07/98	FiiC1	350	10.65		11/09/98	AiiC1	407		
11/09/98	FiiC1	407	7.64		16/11/98	AiiC1	473	17.87	
16/11/98	FiiC1	473	6.01		28/08/97	AiiC2	28	29.79	29.82
28/08/97	FiiC2	28	16.52	16.71	25/09/97	AiiC2	56	29.05	
25/09/97	FiiC2	56	16.66		06/11/97	AiiC2	98	26.08	
06/11/97	FiiC2	98	16.74		18/12/97	AiiC2	140	20.06	
18/12/97	FiiC2	140	13.15		19/02/98	AiiC2	203	15.52	
19/02/98	FiiC2	203	12.65		07/05/98	AiiC2	280	17.44	
07/05/98	FiiC2	280	15.30		16/07/98	AiiC2	350	16.53	
16/07/98	FiiC2	350	16.89		11/09/98	AiiC2	407	13.36	
11/09/98	FiiC2	407	13.81		16/11/98	AiiC2	473	11.81	
16/11/98	FiiC2	473	12.69			At0	0	32.33	
	Ft0	0	16.81			At0	0	29.35	
	Ft0	0	16.62			At0	0	30.89	
	Ft0	0	16.55			At0	0	25.98	
	Ft0	0	16.41			At0	28	31.57	32.37
	Ft0	98	16.37			At0	56	33.35	
	Ft0	140	14.31			At0	98	32.25	
	Ft0	203	13.74			At0	140	28.26	
	Ft0	280	15.88			At0	203	26.29	
	Ft0	350	16.69			At0	280	33.69	
	Ft0	473	16.98			At0	350	33.08	
						At0	407	31.95	
						At0	473	31.64	

D.3 Gas chromatography (FID) results for microcosm trials – peak/UCM areas (see chapter 6.3.1)

Forties microcosm analysis *n*-alkane & isoprenoid

	Time	g. sed extract	g. hexane Reconst.	nC14	nC15	nC16	nC17	nC18	nC19	nC20	nC21	nC22	nC23	nC24	nC25	nC26	nC27	nC28	nC29	nC30	nC31	nC32	nC33	nC34	nC35	nC36	nC37	Prist	Phyt
Fi	0	11.013	6.37	1854	19106	41464	46719	49258	48003	49530	51201	59676	68655	79816	73101	73746	62019	51868	46827	35390	27864	20675	14166	9658	7433	5249	3764	40300	29596
Fii	0	9.99	6.2	1231	10746	31644	49617	51017	48868	48619	51237	56578	63156	71430	66765	65731	55496	45679	41565	30558	24754	19547	12331	8480	7094	4759	3757	39516	31288
Ft0	28	10	6.477	1328	16558	34860	45137	44497	45003	44644	45734	51857	58788	68390	94914	63474	54928	46011	42332	31098	24642	20065	12349	9088	7291	4974	3970	35991	26791
FiT1	28	10	6.44	987	10996	18648	20093	23659	26333	25019	28590	35330	42226	52678	48520	52373	47944	40642	34841	28019	20964	16068	11224	7462	6475	4400	3239	37883	27386
FiT2	28	10.02	6.37	1873	7591	18006	23566	25884	26531	27891	29196	35296	42585	52790	50556	51491	43768	38251	33447	23670	21537	17095	10147	6767	5638	4004	2751	32216	25666
FiT3	28	10	6.41	1247	9245	21893	27081	30220	30499	33919	35200	43520	50746	61204	58328	58977	50851	41690	36117	27594	21838	16220	10625	7522	6158	4607	3272	38248	28439
FiC1	28	10.01	6.58	1508	9082	22420	29701	33491	34582	36441	38848	46425	53340	64648	60036	60771	49865	40013	33425	25804	20122	15174	10036	6765	5448	3528	2890	33218	25928
FiC2	28	10.01	6.53	1488	18446	37459	45407	46917	46867	48080	49709	55077	65095	74490	68797	66893	57294	44958	39622	30021	23290	16629	12973	8978	7405	5294	3865	37736	27830
FiiT1	28	10.03	6.68	795	7304	23069	22137	26270	29146	30436	32428	38547	47765	56438	52288	54101	45073	35962	29273	22405	17093	13769	8883	6183	4868	3378	2429	33395	24939
FiiT2	28	9.98	6.36	1698	8791	20827	28421	31159	32263	32346	34686	39728	47910	56747	52308	52874	44093	36414	31518	22434	17662	13431	8857	6320	5189	3640	3018	33287	25369
FiiT3	28	9.94	6.42	1087	6123	19363	25452	29140	30204	30822	33697	38295	50804	56979	53430	54845	46417	37211	32205	28961	20014	17141	10492	7684	6635	4728	4946	34015	26922
FiiC1	28	10	6.57	1230	11573	30274	35892	35593	37427	39545	41964	47737	57963	65791	65136	59941	48546	39200	31378	23293	18188	15014	9519	6885	5836	8466	5025	34801	26453
FiiC2	28	10.05	6.41	1152	11335	34322	43024	43735	46388	46845	49593	56962	64877	75342	68439	65677	50893	10852	34650	25588	19430	15049	10076	7039	5761	3101	3000	31450	27016
Ft0	28	10	6.477	3160	17998	36242	43941	43949	44794	46469	49229	54828	53383	71237	62032	61358	49148	39016	32132	22898	17573	13242	7742	5757	4638	2649	2202	35437	26412
Ft0	56	10.409	6.477	5884	27781	65907	99150	83568	87668	97812	100198	114664	131913	152078	147498	138074	110361	91290	83644	59712	47552	35336	23510	14578	12043	7430	5239	53398	40785
Ft0	56	10.409	6.477	5328	33682	70488	101805	84018	84367	88594	98839	115132	120740	148199	135433	134438	107633	85776	74391	53490	41571	30550	20432	12903	9648	6290	4577	54936	49359
FiT1	56	9.513	6.59	1521	7986	24100	39535	34148	37132	39340	44802	58792	62230	85412	85662	87281	78614	68541	62597	46776	37270	28846	19067	11689	8472	6338	4488	46170	42525
FiT2	56	9.164	6.9	2292	13209	25282	37930	35580	38526	42715	45331	55908	65770	81735	81834	88662	75279	65115	59109	44328	35501	28200	18536	12091	10143	6394	4401	46522	41127
FiT3	56	8.992	4.69	3503	15666	35897	58816	49471	56165	63164	68335	86615	104337	125810	127296	131817	112729	94820	84900	61802	48765	37117	23996	15426	12574	8049	5587	61381	49229
FiC1	56	9.048	6.44	2092	14507	35551	55756	48260	55524	63722	67704	82815	99860	118162	119058	119190	96772	79374	70143	52799	40274	31170	20325	12407	9510	6618	4676	50795	45850
FiC2	56	9.252	6.47	5605	32698	63710	98469	84556	89276	98207	98740	115530	131051	153354	147257	137535	110395	89394	78803	58438	43460	33951	21560	13213	11151	6973	4844	52063	46946
Ft0	56	10.409	6.477	5503	33552	69176	98573	85293	89302	97524	98173	112315	126778	146607	135732	128138	104994	85693	73865	56816	41950	31457	20480	12717	10705	6596	4334	56351	46965
Ft0	56	10.409	6.477	6904	38307	79321	106371	85236	91768	104821	102846	117885	133221	151449	143398	138340	103272	81774	72156	52620	39516	29940	18841	12019	9396	6305	3870	51722	47612
FiiT1	56	9.078	6.47	1524	13582	29251	41673	38336	43215	44878	50897	64035	76094	95651	100557	96852	83002	68032	62042	47103	36310	28266	17925	11544	9325	6152	3896	51832	43680
FiiT2	56	9.144	6.36	3938	14124	28842	43383	36495	12978	49326	51216	60858	73626	85406	93379	90550	77368	65612	58835	44837	34748	27860	19821	12002	9271	6212	3960	46606	41168
FiiT3	56	9.222	6.33	1461	8506	17661	26249	23146	25609	30253	34895	43402	54995	74372	78741	81535	69005	59064	54935	42605	32612	26265	16900	10761	9848	5891	4189	48968	41587
FiiC1	56	8.849	6.62	2019	16178	39418	60975	52961	58904	69264	71477	85867	100790	123581	125174	117352	94617	76573	66621	50215	37885	30100	18375	11889	9466	6161	4210	48491	44997
FiiC2	56	9.445	6.57	3934	25368	60397	93700	80024	83583	95284	92062	105019	122620	141101	133628	126020	100424	79810	70193	49739	40135	29561	19645	13481	10782	6186	4707	50085	44282
Ft0	56	10.409	6.477	7810	47004	82878	125727	100852	106132	116599	119400	137065	154708	179658	158372	148658	121838	95171	84349	60700	45270	34368	22047	12514	11414	7641	3441	57206	56243
Ft0	98	9.889	6.72	5922	35235	69946	106183	90831	97153	106867	102670	120988	140514	162750	159104	161383	118472	95023	86562	64271	48710	35344	22914	14273	10569	7366	4804	58125	48970
Ft0	98	9.889	6.72	5028	42330	76294	112656	95421	101426	111566	111147	129236	147644	163577	163577	151775	120354	94626	81787	59826	45264	33728	21732	12098	10002	6687	4790	61005	52874
FiT1	98	8.603	6.52	2288	5346	15039	21150	20015	22651	25642	29088	36861	44360	71090	71090	72504	69371	57263	54218	42578	33325	26505	17461	11604	9043	6462	5234	48236	40046
FiT2	98	8.766	6.45	2984	9640	19012	27342	22888	27774	34222	35834	44361	51039	76605	76605	77102	68459	66346	57443	43216	35282	28141	18867	11883	10097	6565	6530	51808	44230
FiT3	98	8.954	6.85	3507	11391	21709	31452	25621	30251	34530	40140	48993	60402	86851	86851	88475	76141	64898	59376	44417	35690	27925	17874	11600	9122	5639	4015	51928	43254
FiC1	98	8.982	6.55	4045	14287	30744	40422	33100	39537	48645	52762	63309	80707	112922	112922	111313	94362	78014	69591	53333	41187	30780	20252	12804	10368	7089	5780	54866	46637
FiC2	98	9.8	6.6	8614	34724	76667	105702	86679	94408	105676	104991	120072	136469	154761	154761	145621	115574	91423	79761	59795	44143	33641	20461	15471	10610	8254	6721	55699	48146
Ft0	98	9.889	6.72	8572	42271	82434	105373	84594	93240	107056	103156	119480	134829	157696	156507	154658	113651	91266	79334	60847	45418	34210	21988	14771	10600	8473	5081	60652	46657
Ft0	98	9.889	6.72	8100	37187	74999	106705	86238	95407	107260	104176	118215	135012	152795	147919	136348	106949	84795	72773	53354	39244	30861	18406	12522	9333	6015	3844	57124	48129
FiiT1	98	8.97	6.79	2378	10694	23220	32805	24533	31453	37902	40411	48736	59510	76159	85147	85881	85469	63383	58885	44887	35226	27304	16722	11634	9416	6298	4540	55588	45464
FiiT2	98	8.779	6.42	2630	12334	26272	36499	29655	35924	41525	45790	54395	65941	81578	89421	89840	82837	65435	59094	44356	34914	27762	17542	12383	9283	6114	4361	56075	47118
FiiT3	98	9.021	6.63	1384	6856	16760	20665	19548	21437	25500	28821	37509	44656	55432	67516	64619	58039	52054	46961	37541	29238	23314	14668	10105	7702	5515	3690	47883	39814
FiiC1	98	8.868	6.41	2847	12447	30410	43580	39124	45132	53336	57587	68659	82964	101472	105858	101070	89065	69806	61317	46322	35298	27254	16834	11311	8288	5922	3619	52201	43226
FiiC2	98	9.073	6.46	6667	31397	62173	91973	78796	85273	91521	93623	93623	120706	137457	131954	124488	100786	78633	68579	48632	38271	29513	17573	12668	9040	5937	3877	52190	43301
Ft0	98	9.889	6.72	12320	48935	91413	138853	105251	113196	130039	127044	127044	162269	185164	179312	166145	127945	103964	87556	66836	49556	36911	22917	15026	11599	7555	4511	63123	57783

	Time	g. sed extract	g. hexane Reconst.	nC14	nC15	nC16	nC17	nC18	nC19	nC20	nC21	nC22	nC23	nC24	nC25	nC26	nC27	nC28	nC29	nC30	nC31	nC32	nC33	nC34	nC35	nC36	nC37	Prist	Phyt
Ft0	140	10.158	6.55	1503	24252	52666	57236	55188	63284	76531	70210	88603	97409	115580	121153	113815	101627	85848	82350	70446	5909	50648	35557	25141	19882	12250	9527	56368	28959
Ft0	140	10.158	6.55	5691	34468	66007	88644	76872	81713	91872	94417	105037	120500	144138	144422	142912	122306	104203	100175	79270	64224	49018	29866	18866	13710	8858	5827	50923	39352
FiT1	140	9.193	6.47	1049	6790	12203		11118	13805	14509	17595	22476	29728	40951	47103	53810	57452	54224	57511	50946	42950	35589	23153	15534	11084	7513	4750	39539	27580
FiT2	140	9.177	6.51	320	5091	9264	27342	11882	14360	17612	17821	25568	31811	38477	44069	51357	50548	50280	52783	44658	39895	32837	22786	15624	11546	7513	5085	46388	28256
FiT3	140	9.069	6.49	280	4852	14040	10565	12639	14684	18449	18670	26489	34050	45213	45609	56452	59737	55923	59868	51445	39955	41336	22724	15555	11769	7603	5160	39275	29201
FiC1	140	8.951	6.47	1111	8498	18181	16485	18854	22699	26220	29742	37397	53248	68321	78740	87611	78681	71519	71852	61448	50031	39564	25353	16818	12550	8116	5256	44523	29246
FiC2	140	9.276	6.48	4603	21941	50471	61982	60971	64681	73577	71170	80573	96032	111817	114589	111557	95471	82646	80226	65811	52370	42023	26336	17604	12930	8154	5538	46240	31071
Ft0	140	10.158	6.55	6004	33729	60867	72604	69638	71228	78742	79770	93523	106594	123138	121717	12311	104514	92633	89564	72436	56779	45396	26877	19432	12712	8146	5923	49960	36986
Ft0	140	10.158	6.55	6894	35525	63618	92261	82120	83595	89111	93299	105140	125558	142819	146994	141155	119740	105012	98182	74122	57015	42060	25873	14726	11136	6877	4592	54469	42647
FiiT1	140	8.895	6.52	393	6093	13374	11138	15917	18488	20084	23719	29087	37484	49766	60931	66208	62488	59916	60514	49506	41118	30656	20607	13409	9702	6448	4377	43201	32602
FiiT2	140	9.339	6.5	622	8242	15974	13452	17632	19845	23142	25940	33667	42831	55022	67182	38538	67191	62157	62939	53028	42869	34162	21212	13356	10462	6380	4520	43901	32521
FiiT3	140	8.972	6.42	185	3080	8154		8031	9015	10741	11160	14393	16716	21019	23186	28942	35236	39410	44328	39238	34833	28188	18379	11800	8860	5843	3969	38392	26422
FiiC1	140	8.391	6.46	1226	7544	17794	22297	27278	30570	33961	38884	48327	58314	77866	83364	85540	78386	73360	69578	54342	44040	32809	20860	12840	8377	5646	3700	43825	33292
FiiC2	140	8.957	6.48	4641	19349	48382	65687	63364	66432	73576	75282	85839	98389	113657	116681	114228	95221	82494	77718	62135	47454	35791	21467	12906	9230	5833	3668	47366	35234
Ft0	140	10.158	6.55	8320	36448	67424	89441	78679	78678	89531	89493	103889	119834	139260	140418	141315	119004	102885	96388	74741	55659	41077	25194	15115	11016	6873	4512	55242	41220
Ft0	203	9.922	6.45	9998	36082	55183	77840	65813	77345	80786	87856	101358	106139	142569	129139	127173	113240	93344	83318	63598	49540	33862	18015	13120	7448	4187	2312	62048	49844
Ft0	203	9.922	6.45	9645	32632	51505	72819	64780	71788	78166	77420	91412	100990	129062	121718	117361	106252	84604	72476	54761	38870	27051	13868	9572	5288	3267	1990	57753	47806
FiT1	203	9.293	6.4	2067	7660	10967	16526	15199	15968	18439	17270	19027	18800	64028	22537	25543	27507	24947	27905	21966	18548	14652	8517	6913	4013	2276	1582	34395	32956
FiT2	203	8.836	6.48	1746	6683	9867	13557	13439	13329	14686	13277	16852	14916	18619	20550	22797	25347	24478	24278	20088	15158	12533	7412	5828	3983	2151	1476	31867	30159
FiT3	203	9.184	6.4	1435	55541	9705	12056	11666	12638	14269	12218	15476	13840	16496	18733	18389	19924	19609	20178	18066	15161	11830	8167	6844	3666	2134	1276	35352	32846
FiC1	203	9.323	6.39	2715	10285	14418	21422	18220	18768	19245	20441	25380	24592	68962	45556	50378	54245	48388	47268	37554	28757	20739	11136	7844	4838	2674	1574	43398	39117
FiC2	203	9.481	6.4	9399	30559	54093	72802	67719	71609	77854	75392	87040	91473	140420	110368	106900	93884	77887	65710	46506	34872	24399	14272	9421	5327	3047	1801	51342	44749
Ft0	203	9.922	6.45	9636	34988	54864	74933	67505	73131	78775	79784	93482	104744	134904	124700	122274	109672	89442	76527	57268	41583	30752	16435	11454	7188	4017	2392	60040	52356
Ft0	203	9.922	6.45	10243	38676	55762	75495	67781	73141	80418	78638	94803	106471	141382	124904	128816	107754	86800	76443	52206	37114	26707	13830	10040	5381	2863	1667	64744	50705
FiiT1	203	9.369	6.51	330	1876	3771	7157	7697	11408	13627	10807	14096	13590	53542	19813	21494	22736	22506	21508	18751	15488	12770	7360	6006	4367	2449	1409	16305	20256
FiiT2	203	9.099	6.61	2363	8709	14314	21692	18634	19388	22337	20746	22208	24689	86080	40410	44967	49782	42296	38506	31762	24817	17826	10035	8472	5266	2569	1543	42255	37072
FiiT3	203	9.235	6.48	1429	5850	9734	12886	11648	12774	13131	12784	14335	14067	62931	20262	21506	27258	26886	26343	23045	18584	13645	8519	6568	4602	2311	1466	29252	26844
FiiC1	203	9.223	6.47	1920	7897	11623	17338	14481	18093	20678	17185	19060	19225	63444	35372	39557	45824	43556	40437	33810	27991	18873	12133	9154	5671	3007	1868	42862	39329
FiiC2	203	9.351	6.37	8706	35282	60189	87458	77386	86581	91329	85548	100298	107289	132443	127556	125692	109925	88675	75151	54383	41182	27843	14446	10066	5982	3465	2307	57673	50401
Ft0	280	9.81	6.459	2706	9128	14364	17414	19086	22322	29424	29890	41723	43940	73463	43339	36085	26872	19144	13413	8482	6047	3546	1681	1093	559			31087	30305
Ft0	280	9.81	6.459	2911	9195	15784	20321	20203	24981	29608	33897	44693	49886	80169	50315	42429	30357	20993	14542	8619	4935	2586	1226	753	360			39726	35124
FiT1	280	9.786	6.08	1074	3473	5528	7576	6817	7141	6863	6524	6052	6127	22796	3640	2724	2848	2384	1680	1309	913	669	351	340	155			18073	17741
FiT2	280	9.681	6.74	855	3315	5348	7572	6660	7219	7188	6561	6197	5102	2829	3242	2158	1969	1747	1175	930	776	438	403	382	212			17043	17981
FiT3	280	9.597	6.415	830	2885	4269	6329	5631	6664	6120	5388	4570	5308	3649	2561	2195	1619	1128	439	329	355	475	322	361	197			16850	16156
FiC1	280	10.264	6.519	900	3674	5888	8188	7109	7571	7149	6623	7463	5074	25787	3661	2147	2165	1075	328	120	234	397	179	397	260			31079	29273
FiC2	280	10.531	6.414	5568	23373	41283	55079	57319	59499	60472	57793	61561	62087	83042	57176	48219	36952	24470	17798	10811	6762	4149	1853	1146	828			37418	38518
Ft0	280	9.81	6.459	3185	10500	15859	21505	21327	26726	31775	36958	48294	54253	85972	55719	48524	34438	24378	17611	10844	6758	4206	1842	1193	614			42835	37082
Ft0	280	9.81	6.459	3239	11314	20266	22397	23983	28434	34738	41815	54216	61397	90935	63229	54345	40872	27332	19029	10981	6727	3866	1581	980	674			42327	43397
FiiT1	280	10.07	6.347	622	3192	5820	7998	7376	8029	8158	7294	6684	5411	23854	3273	2208	1981	1271	617	169	348	263	206	314	210			18866	18866
FiiT2	280	10.135	6.512	829	4158	7172	10222	8842	9192	9411	8451	8430	6924	31046	5793	4262	4794	4562	3260	2441	1544	1198	586	387	232			25914	24271
FiiT3	280	9.858	6.544	581	2850	5311	7416	6681	7203	7313	6626	6554	5562	5127	3969	2396	2163	1508	1003	938	800	780	448	350	258			16089	15864
FiiC1	280	10.83	6.373	700	356	5497	7781	6576	7225	7676	6680	7161	5018	24187	5032	2703	2411	1180	562	139	85	248	246	342	278			29141	27461
FiiC2	280	10.039	6.378	4365	21632	44444	63057	61176	62100	63192	61578	67004	66710	85547	60602	53790	40798	27067	19117	11794	7213	4549	1924	1202	620			47375	39753
Ft0	280	9.81	6.459	3406	11734	18146	23612	24024	28711	34871	39883	52096	58899	90186	60849	51432	38653	26026	18578	11417	6914	4067	1869	1181	712			45344	39742
Ft0	350	10.465	6.376	18402	69307	108384	136902	138038	147085	154277	153581	171047	180828	300255	170467	138988	100333	64177	43833	26629	16423	11066	5820	4409	2822	2011	1354	124917	109558
Ft0	350	10.465	6.376	17211	66396	106161	137369	137142	144635	152859	151621	168066	177546	292509	164926	135745	94100	62079	41931	25431	16862	10631	5992	4602	3000	1882	1371	121670	108475

	Time	g. sed extract	g. hexane Reconst.	nC14	nC15	nC16	nC17	nC18	nC19	nC20	nC21	nC22	nC23	nC24	nC25	nC26	nC27	nC28	nC29	nC30	nC31	nC32	nC33	nC34	nC35	nC36	nC37	Prist	Phyt
FiT1	350	9.391	5.294	1166	8057	13570	20360	18820	20355	20155	18878	18094	15382	112549	11006	7101	4816	2732	734	514	98	169	225	563	629	379	493	38806	38685
FiT3	350	10.331	5.193	1972	7742	11925	17029	16163	16356	16104	14766	14595	12657	61204	9364	4624	2943	2350	594	308	148	168	75	530	462	379	603	26054	23484
FiC1	350	9.509	5.276	1363	7496	11895	17539	15326	16812	16490	15785	15745	13759	9120	9318	3566	4185	2116	1013	263	170	84	535	787	751	647	721	51281	54065
FiC2	350	10.436	5.715	11733	61847	116872	169554	173960	171681	176930	176791	187952	186924	204873	163510	141999	96824	61031	40644	24385	15781	9825	5898	4361	2853	2067	1623	126404	110182
Ft0	350	10.465	6.376	16724	65257	104698	136571	138488	146220	149088	150983	165857	172030	282906	159296	128308	89197	57796	38715	22338	14905	9730	5329	4074	2944	2165	1459	130457	107287
Ft0	350	10.465	6.376	17437	66520	106416	139659	140494	147526	153962	158749	174915	183496	307824	173785	141113	98044	65150	45462	28214	18734	12380	6726	4873	3513	2280	1416	132004	107625
FiiT1	350	10.07	5.197	1104	6548	14191	24116	21052	21711	22968	21624	19902	15952	66521	11826	6750	7643	3675	1318	427	185	314	181	611	870	589	504	50964	48132
FiiT2	350	9.913	4.788	2609	12571	23278	34803	31070	36008	35173	31635	32218	26073	30912	22230	11638	8247	4609	1480	650	343	677	800	2011	2272	1677	1556	90307	88832
FiiT3	350	10.598	4.607	1344	8112	17256	25484	24645	25332	26226	24438	23677	20406	110488	12424	7723	7014	3906	1111	578	267	573	415	1142	1500	906	909	37695	35994
FiiC1	350	10.506	5.06	1408	7494	14441	21478	18880	21011	20409	19198	20231	15827	22507	14797	6345	6289	3035	997	521	295	254	93	1164	977	442	519	58880	57534
FiiC2	350	10.919	5.415	11892	63834	125581	187852	187651	197067	202478	200699	213565	219016	273495	203764	162707	111701	73552	49715	30077	19491	13363	7395	5765	4076	2590	1880	139324	121695
Ft0	350	10.465	6.376	21574	74398	122981	164177	157884	161851	178504	179701	202287	210700	344447	194379	151105	103151	67892	43957	26531	17578	11794	7089	5779	3913	2753	1999	146326	124017
Ft0	407	10.047	6.312	27122	91137	149291	186804	181691	187694	208775	206749	230997	255147	277366	246241	203706	151825	97033	67046	40916	25364	15990	8070	5862	4129	2886		167880	140001
Ft0	407	10.047	6.312	30811	101441	155199	205483	201733	200991	232719	23095	267937	276088	312077	265252	216492	15762	97936	66420	40251	24969	15664	7918	5884	3729	2282		185676	153728
FiT1	407	10.998	5.059	2604	10974	17315	24344	23600	23378	22323	21064	20249	17371	91347	9960	6112	3734	2046	797	1459	534	241	126	421	564	305		27113	23726
FiT2	407	10.444	5.498	4264	14436	22986	30992	28808	28960	28755	26071	25196	22561	105924	13038	7614	6296	4389	1055	559	380	402	177	615	381	92		34120	28473
FiT3	407	10.719	5.423	3795	13702	22203	30864	28396	29227	28555	26304	26433	24305	109057	13298	8015	5283	4875	1284	703	674	368	118	635	221	64		32602	26042
FiC1	407	10.619	5.661	4591	14831	22297	30864	27766	29052	28946	27652	27047	23509	119096	14303	8974	8499	5509	931	484	246	410	703	1167	665	285		43167	42533
FiC2	407	11.114	5.361	33414	112982	192416	279817	280116	288886	318574	297376	327792	337857	450104	309281	248143	177206	106827	69329	40134	24671	15547	7970	6056	4014	2597		212334	182845
Ft0	407	10.047	6.312	35907	116596	168962	226171	221928	232398	274116	255538	303322	311566	352755	304900	252066	179213	114976	80244	48962	32027	18121	11019	7686	4940	3291		196401	170093
Ft0	407	10.047	6.312	57675	169211	249237	327416	331871	340260	372409	382403	441933	455799	509118	422119	329510	219744	132375	86217	52661	31438	19317	113809	8374	6020	4064		293698	260409
FiiT1	407	10.857	5.332	4057	16174	31114	46476	43951	45049	45432	42490	42708	35779	33908	19905	10557	7036	5165	1441	794	864	544	297	668	456	322		46978	42462
FiiT2	407	10.784	5.516	1595	8595	15097	21252	20454	19918	19917	20534	18776	16561	10422	8767	4912	4032	2748	1118	482	171	159	107	563	421	279		24653	21842
FiiT3	407	10.936	5.382	2676	12085	24555	37696	35193	36922	36878	34089	33679	30477	157564	16214	9305	5039	4779	1112	781	662	392	259	509	420	326		37538	32956
FiiC1	407	10.921	4.761	3478	14603	26494	37127	35024	36773	36758	35946	36855	32423	157902	17935	10492	8892	5282	1654	576	603	285	251	1004	833	362		46652	44673
FiiC2	407	11.135	4.757	81152	276942	467158	680611	692182	725681	787955	766917	828252	810465	1105490	703066	496857	301534	173823	105599	61205	43345	29660	18153	15269	11679	8485		432964	436214
Ft0	407	10.047	6.312	41626	138781	197621	254322	257346	259500	278018	279471	344803	347412	387050	316095	252145	158800	105151	68526	37909	25353	16458	9403	8051	4964	3883		204035	194377
Ft0	473	9.884	6.176	18374	56001	82722	103486	100513	103468	111382	11381	128767	131404	178607	123299	100022	69579	45443	31774	19890	13395	8748	4744	3474	2264	1509	1032	96563	78586
Ft0	473	9.884	6.176	17671	52152	73783	92805	90826	95034	103620	100185	116966	118473	156941	106915	82870	57655	34281	22075	13140	8521	5408	2554	1919	1410	973	726	85308	70661
FiT1	473	11.149	6.321	1254	5416	8930	12268	11502	11716	11500	10342	9438	8150	33255	4033	2559	1255	1274	253	200	107	105	279	255				13354	10945
FiT2	473	11.32	6.452	1274	4515	7625	10583	10185	10120	9372	8614	7937	6933	42085	3694	2386	1352	1126	140	114	138	52	208	173				11971	9666
FiT3	473	11.496	6.413	691	3307	6631	8933	8159	8603	8276	7967	7804	7332	32610	3718	2609	1014	1084	288	179	161	137	133	308	91			10268	7643
FiC1	473	11.157	6.351	1164	5096	8389	12363	10338	10990	10737	9990	10761	9324	56537	5177	3367	1853	1371	304	257	179	237	262	254	122	367	261	16163	13074
FiC2	473	11.029	6.377	14693	49558	85880	128663	120760	131485	148930	138040	154302	151514	210452	134120	105916	67882	39620	31081	15552	9121	5939	3067	2805	2134	1200	887	101988	81275
Ft0	473	9.884	6.176	27393	82544	116152	154748	143034	149961	176272	164405	182033	191823	255231	172532	136651	92181	55123	37441	19649	11915	7777	4160	3118	2682	1560	1083	129580	112302
Ft0	473	9.884	6.176	31874	88416	130900	168782	167542	169832	202032	189734	225355	225196	303267	210110	166115	115317	70182	46951	28404	18621	14046	5999	4177	2802	2056	1341	147007	123257
FiiT1	473	11.401	6.418	1810	7913	13094	18528	17125	17799	17047	15785	14739	13584	14919	6564	3511	1937	1873	602	323	247	206	78	301	350	265		21484	15530
FiiT2	473	11.323	6.189	981	4788	8211	11848	11275	10757	11585	10441	9094	8048	2445	3727	1764	1685	1497	472	511	387	225	411	273	271	192		14128	11605
FiiT3	473	11.157	6.329	1056	5988	10515	17313	16320	16290	16597	13456	13684	12170	11190	6035	3494	1872	2127	519	214	575	287	191	410	212	280		15704	14358
FiiC1	473	11.19	5.735	1202	5005	8015	11750	10152	10668	10467	9256	8162	8929	62482	4290	2736	2030	1503	358	227	351	289	156	339	608	177		15344	11671
FiiC2	473	11.472	6.624	18483	61862	106067	157405	157376	171783	186934	174719	195177	202399	226666	179830	142211	96720	59926	39404	23427	14114	9306	4638	3425	2394	1890	971	121423	103272
Ft0	473	9.884	6.176	29751	90146	122683	163192	154204	158095	178693	177944	203951	211599	282455	192487	154868	102521	62420	39960	23551	13415	8258	4594	4383	2459	1668	1091	147625	120616

Forties microcosm analysis - ‘UCM segments’

	Time days	Tot. ucm	C14-15	C15-16	C16-17	C17-18	C18-19	C19-20	C20-21	C21-22	C22-23	C23-24	C24-25	C25-26	C26-27	C27-28	C28-29	C29-30	C30-31	C31-32	C32-33	C33-34	C34-35	C35-36	C36-37
Ft0	0	3565949	55704	114280	176582	277183	291997	253145	250108	227440	218848	210588	206311	180041	186860	161916	149815	128099	109227	90584	72575	58335	47540	33043	25011
Ft0	0	3405015	73005	130481	189377	285972	292060	256144	239903	226879	211549	193958	184324	158527	164984	138366	128951	109993	96553	76097	61677	51221	40794	31114	21095
Ft0	28	3049566	45287	101031	163348	255265	263468	248527	220920	198753	189360	172628	144018	144456	137202	121860	110193	92640	80689	61832	49153	38426	27955	20623	15772
FiT1	28	2992608	37816	84039	149447	244004	254105	225860	218098	213701	199036	187833	178615	151366	153833	126393	114038	98285	83299	67288	52409	43467	33537	25860	19325
FiT2	28	2918218	38033	80927	137667	237133	246113	216175	210751	206334	191790	180370	169918	144755	154055	122934	109866	98601	80574	63681	51275	41964	32657	25935	18270
FiT3	28	3116696	45579	85676	165258	256909	269133	234452	228733	221810	205013	194221	185158	161585	157347	129538	117728	97563	85260	69804	54823	42298	32823	26241	18543
FiC1	28	3016305	29477	77057	140382	231992	247292	324291	214743	211706	201933	190420	176052	153282	155896	130497	117359	91932	80924	62710	48796	38761	28420	24386	15251
FiC2	28	3152698	39042	91780	158204	264252	269020	238730	226630	222475	206422	193123	179307	157669	163768	136868	122859	99420	90305	73811	57022	46464	35061	29223	19322
FiiT1	28	2646219	25519	60738	122371	219889	238823	215403	204493	207634	188348	180507	171437	134619	129893	111473	98244	85826	61045	51344	39684	33754	23811	16853	12094
FiiT2	28	2712806	34577	76042	140030	230260	244472	216106	212933	195449	195092	174428	166855	135360	138752	111895	92108	82033	66243	56903	39717	32961	24143	18717	12434
FiiT3	28	2808558	25320	67095	131076	229027	246772	218757	217831	213571	194477	183143	178041	146079	149466	117408	102264	86212	73460	60183	46819	38448	28821	24257	15905
FiiC1	28	2871653	33789	77299	145841	238369	250793	226086	217680	210978	207295	183340	180538	145098	143770	121904	102415	86979	75168	54573	50904	37018	27867	22508	15886
FiiC2	28	2984525	36525	72810	137741	231386	250610	227133	218227	219068	206976	188996	183985	155351	154722	155800	109927	91057	82975	63287	56755	43073	33200	30957	17692
Ft0	28	2940192	55961	107658	151220	248011	259564	229394	215904	211844	208864	182298	174656	150483	141410	114208	106227	79697	72881	57732	48213	36823	25582	23041	13574
Ft0	56	5979352	65939	162144	293136	452673	495685	442612	440100	429557	397358	374000	383089	319355	306465	256548	235581	212265	164615	139511	113068	90038	68210	50468	36171
Ft0	56	6196271	67573	162518	304032	472749	526076	472809	460139	444775	412489	378415	404433	303357	302481	240891	216605	187153	154743	128661	97763	79442	60318	46042	32427
FiT1	56	4946370	36058	109468	216852	365186	407285	380047	369150	365264	349903	329382	319445	266832	262940	216797	195900	175818	137811	117412	88346	73365	57269	42271	27790
FiT2	56	4887355	34364	131463	216737	356048	396863	370256	363949	361915	340574	325614	309084	257150	262488	214519	195747	176402	138409	116233	89477	72580	54923	41772	28967
FiT3	56	6970528	56232	158972	304283	517317	571432	535673	528313	523004	486447	465191	498998	371188	360303	302082	271317	242869	188348	158541	119936	99294	72496	54581	36640
FiC1	56	5417625	33088	105384	235351	400689	457565	407252	419524	409802	385992	362594	339780	291966	296323	243839	212828	196306	147268	127548	97235	78656	59475	43303	32511
FiC2	56	5837504	51374	147055	269770	443352	490794	446917	443373	434004	403861	373793	371564	306484	314754	249577	223367	204917	155221	131849	102971	84930	62500	46843	32319
Ft0	56	5808075	67286	163094	297419	461296	500579	455129	432646	428489	398266	367018	377467	299189	288038	231513	217006	188995	146532	127172	95125	79089	56348	44748	33441
Ft0	56	5853196	69365	158480	297937	472911	514930	465938	457448	440882	409340	380148	389011	295852	280359	236734	207837	181004	141786	118493	91073	73213	56681	40554	30388
FiiT1	56	5083831	56027	132288	211269	379537	422717	388243	386518	371309	351992	336249	365343	269409	272850	212527	194227	160857	140436	109198	87180	70292	51910	39652	28255
FiiT2	56	5100499	58985	134990	211420	378550	449381	379186	379943	367279	347281	349321	351785	269569	274904	212531	196106	162856	142886	109352	86719	70687	52556	39689	28344
FiiT3	56	4875889	36404	103663	188762	354093	406824	367557	360673	359804	345531	325510	364757	255861	273412	217204	191688	167159	132506	109401	87669	70458	51669	39365	29473
FiiC1	56	5338443	50382	128715	223055	410478	457913	407280	402758	396080	376343	354162	376438	277196	282691	231306	204390	177456	139757	113415	89548	72419	53521	40238	29686
FiiC2	56	5592460	67077	149139	226313	449585	479948	421183	420151	409196	384392	363571	299989	285830	286607	238504	310066	184831	143771	121170	94950	75232	55946	42723	31836
Ft0	56	6771142	76166	191468	265307	586137	607456	533685	525901	507283	481491	432529	454247	355749	329604	282715	243198	210471	164094	134475	104279	85373	61580	45116	35083
Ft0	98	6336292	92861	193948	294584	489565	543331	473801	481794	459096	431389	386600	368553	306432	338694	274037	247300	209484	181175	145686	112834	92439	69140	50417	35936
Ft0	98	6674652	87420	193207	341788	526862	569066	614674	510582	481422	452334	427504	373990	319359	341383	261524	246568	211011	168848	142637	105081	89089	62675	50681	35699
FiT1	98	4779802	48878	118646	198109	350579	393908	359588	354712	335989	333288	299545	310079	251588	301389	203687	192398	159422	139228	110746	89690	71506	52841	40109	28140
FiT2	98	5190840	52142	131841	221108	392881	434573	391994	386281	365606	363750	329709	310695	274894	296163	215782	211115	176004	151456	120527	98528	79091	57740	45311	33549
FiT3	98	5015347	55334	132289	232432	382926	418308	382742	379704	364403	342203	304165	290110	261696	280899	220278	197521	171392	147681	112943	94309	72498	50696	41507	36474
FiC1	98	5437818	57860	145774	244585	415959	463181	411064	415109	388869	376922	347877	318559	275882	303739	236522	220952	187181	151111	129721	95758	81309	57958	41114	26882
FiC2	98	6145270	82524	183952	285057	505646	535736	468391	478792	440985	421287	397773	350179	299228	327669	256214	237120	200221	163022	138446	102191	83723	61133	42888	29702
Ft0	98	6128455	80806	170549	317186	503775	540513	486578	468048	441469	423380	392057	329790	297213	319142	250077	234822	196393	159393	135547	106476	81727	63382	44836	33215
Ft0	98	6001202	79974	182455	310878	490934	537106	468373	470007	447027	409311	374189	340132	307413	302381	247352	219392	183835	154735	122783	94844	75328	56493	42295	32177
FiiT1	98	5389724	49746	133861	237785	407135	458526	406282	407957	391548	371876	350090	377224	282876	311041	225851	205751	175858	147576	119181	90681	73807	54298	39071	28151
FiiT2	98	5514100	44923	132139	240464	414503	467593	417200	418612	402358	381975	358532	364876	290711	321879	230963	214298	183353	154056	123386	95749	77610	59075	43616	34191
FiiT3	98	4621129	13737	105024	203880	351122	391730	354485	348194	342444	331661	292145	276442	244810	270937	199860	182753	156249	132999	106688	83196	68610	50416	39602	29524
FiiC1	98	5204840	53575	122951	243012	400678	441999	396861	389968	386343	362241	338850	326196	275530	294600	222714	198827	167657	145372	111874	89009	73593	49731	39498	28884
FiiC2	98	5543755	65157	161553	283872	442867	483047	453090	409093	414437	377347	348853	341611	269490	291712	227458	200482	185757	136554	119297	89275	72570	53142	40845	29135
Ft0	98	7318607	98121	206163	383552	588528	646285	575085	571564	570994	494319	483663	411308	359347	375042	301246	266743	234239	179535	151534	112559	92457	66941	51090	37329
Ft0	140	5476423	64391	122018	188150	371582	395810	348592	351361	334022	319908	313081	312132	264283	273447	244473	241823	222244	256912	194199	159516	150170	118469	93889	71558

	Time days	Tot. ucm	C14-15	C15-16	C16-17	C17-18	C18-19	C19-20	C20-21	C21-22	C22-23	C23-24	C24-25	C25-26	C26-27	C27-28	C28-29	C29-30	C30-31	C31-32	C32-33	C33-34	C34-35	C35-36	C36-37
Ft0	140	6228932	87547	156771	237748	440122	478947	429080	421269	411121	386937	375674	381592	316037	337583	297043	288562	262021	224922	198056	145722	121292	84567	58865	42065
FiT1	140	4331230	49227	84838	153316	281031	308376	294131	296025	271858	271336	249272	252993	219471	249715	199897	205267	189260	168048	150116	117173	103374	72518	55302	37879
FiT2	140	4353710	40601	87872	120209	272089	307671	287784	279755	267829	261094	259290	305467	222902	248154	201062	200328	200740	176107	148275	126555	104313	79580	59557	44656
FiT3	140	4380325	40392	85158	141135	275214	308111	281323	283758	277234	275894	258408	309176	228571	249846	210820	205352	191291	179510	143645	123452	104387	71500	54830	38342
FiC1	140	4555057	48267	83216	171994	299232	328062	313581	287935	274761	278453	262882	315264	227794	257221	223388	222031	207823	192241	152369	129266	104740	67843	50824	30444
FiC2	140	4995604	59412	119745	195836	351107	374836	332552	332547	316226	305441	289074	320556	239021	266887	228051	226448	213299	186147	163790	133507	107322	77235	57986	44624
Ft0	140	5580727	55823	140220	242101	400833	421655	377123	371867	354887	342836	315861	327290	375586	312668	239575	245967	228196	198582	175907	135285	113808	76311	53918	32799
Ft0	140	6158143	71028	168318	273904	456520	484500	439967	432397	417744	398815	369718	378995	313294	358910	278972	280792	252555	206480	172604	123504	101031	62667	43622	27849
FiiT1	140	4604764	46862	92648	160954	305636	341083	318903	324206	302327	295704	281543	264212	247288	262571	229971	215415	204687	172429	144801	111433	90188	63614	47059	32301
FiiT2	140	4752150	47981	103890	165418	313984	356475	314956	336824	314959	296419	291203	255870	272629	291029	228121	223491	213117	173789	151560	114638	91652	65934	47443	35065
FiiT3	140	4137686	39138	81792	127701	242923	285572	276207	262019	271880	251203	257153	305791	224147	234074	207468	201140	188301	165951	141665	108220	81942	62568	43357	32814
FiiC1	140	4607712	50824	111194	169243	316334	339187	329130	318124	309963	311443	302612	235628	273910	236129	215556	216242	198000	161705	141792	106088	86604	61663	41227	30230
FiiC2	140	4931307	66075	130176	186457	365489	403396	351075	338037	334068	329314	320081	250696	279769	243427	227496	226148	203624	166914	144317	107227	86564	59535	39171	27913
Ft0	140	6039912	66116	158299	273979	443449	474087	431413	428236	406032	384586	365080	373824	301673	336805	275968	269377	255854	194743	173623	128105	99121	67846	49080	33664
Ft0	203	5631697	70082	170251	304584	448348	485992	431980	409968	391848	361824	343536	313094	288563	304542	242052	232742	215560	165241	133406	89130	73335	54962	40947	31903
Ft0	203	5109577	68399	161517	275241	409838	451285	400925	381982	361358	347475	327541	278754	279772	293164	214603	208159	182549	143863	108132	71213	54722	37224	19916	12429
FiT1	203	3806065	36454	90372	167679	269766	307263	287684	282159	270475	258413	270540	209405	217512	245080	175958	168432	154449	119417	94218	60382	47137	32072	17726	9895
FiT2	203	3173270	59094	73875	141090	227053	264417	241791	237131	225927	216913	225425	170005	178185	201721	142470	138136	125135	97432	75201	47345	35980	22820	12131	6242
FiT3	203	3459666	28878	75973	151710	248430	290774	264496	262291	249715	239007	246345	186109	196975	220923	157894	153216	137673	106602	83952	52772	41130	28364	16245	9882
FiC1	203	3979675	41877	102393	191097	305346	343162	299708	311512	281726	282054	266048	221262	216014	253531	167422	176187	147297	114053	87525	61151	44015	30368	13126	8105
FiC2	203	4471477	59346	133891	235513	360945	393156	335536	357735	311574	312184	285401	241391	235790	276935	178859	191660	162000	124318	94801	64710	46671	31976	12980	6400
Ft0	203	5194020	66085	160449	280320	432756	451770	380559	414048	369651	344281	323261	292763	268903	293357	215480	222260	191264	152482	111571	78742	60944	36894	18288	8766
Ft0	203	5298277	66568	170723	291328	429437	474009	405701	414443	375725	373226	325062	285015	273110	321358	228145	206233	186140	142203	103720	71903	56213	40301	19775	15074
FiiT1	203	2772193	7393	24274	60472	125568	170854	184501	202811	209255	202718	228324	163807	190144	218627	137639	152451	131914	98972	82316	60076	43932	31827	20391	13380
FiiT2	203	3913854	31231	89536	174269	283327	316960	295033	291395	285081	263874	284263	200481	229231	252376	163779	179495	153857	114290	95845	68572	48945	35886	24027	16067
FiiT3	203	3242116	25171	70790	138676	225804	257350	246660	242301	237879	219985	239917	168086	192844	219736	135656	149096	128236	95871	80292	56993	41149	28925	18627	11379
FiiC1	203	3967035	7229	38060	99087	187248	298383	326903	301655	299621	290239	266605	284055	199488	229114	248876	161079	176739	151053	111319	95918	67697	49164	35167	17668
FiiC2	203	5336622	13706	66357	163588	289598	435244	474140	417292	413181	371317	366737	331212	307614	275447	294528	220059	219096	185775	149557	110205	77837	56382	45989	24438
Ft0	280	2529917	36570	84746	158313	251328	271216	233456	235027	214697	186331	169231	132973	114908	101824	75880	72952	57431	40856	30575	22269	18758	7893		
Ft0	280	2697078	43605	94268	173230	271730	292027	262974	255956	224949	201159	192800	138447	125214	111971	83810	67030	53596	33779	21394	13204	10603	6106		
FiT1	280	1807713	19382	49543	97722	156748	181819	180392	173973	157021	140813	141420	101294	96190	84127	59611	52932	40572	27887	18504	11482	9008	3300		
FiT2	280	1732496	19998	45881	93714	156681	176185	167296	159806	155331	141257	133315	93574	86587	79924	56882	49725	41412	27736	17108	12178	8205	4212		
FiT3	280	1548283	17135	41605	78853	134709	156173	148287	148447	136309	122008	123446	85723	77151	74539	54896	46072	37811	23986	15159	10896	7999	2460		
FiC1	280	2389829	30909	69292	134471	225584	254206	231034	227243	204523	184816	181894	131013	117574	102821	79822	65368	52762	34140	22237	13690	8994	4369		
FiC2	280	2948123	46279	107834	189335	287604	325457	277234	278260	257105	217531	204033	151271	141228	113580	90819	77319	59294	40604	24425	18456	12571	5038		
Ft0	280	2881068	42432	96186	177675	273571	312815	273301	274332	240347	232967	204394	146541	128644	121600	87799	80191	63490	39678	28481	19848	14914	7064		
Ft0	280	3187951	50648	110244	200048	320467	345460	312481	294916	276702	242890	230068	173585	146300	135375	95779	80235	61539	41302	25280	14397	8718	4814		
FiiT1	280	1890825	14307	44875	94547	167452	193098	183706	177247	172587	154169	150535	114081	94849	95102	63310	54520	42896	29962	18161	9336	5599	4204		
FiiT2	280	2122297	18378	55757	112237	194599	219994	203764	196389	189739	168795	164468	124769	104705	101963	69306	60136	47435	34538	21892	12941	9561	6396		
FiiT3	280	1759748	12449	42074	86426	151266	177040	170958	165568	162281	144633	138679	102807	87658	85547	60286	53075	42089	29182	17928	10526	9608	5865		
FiiC1	280	2297030	24856	61257	122855	216541	245807	227790	219374	210819	185973	177421	131162	109474	107703	72655	61392	46778	30838	17769	10141	7266	3868		
FiiC2	280	3053303	34778	98309	187163	311676	336179	304313	285757	267066	234409	218559	167802	140317	132219	93009	78847	60690	41369	25639	15207	8754	4153		
Ft0	280	3048138	44498	107934	187587	311476	331489	304616	280388	263303	232807	215522	164688	136286	127505	88447	78556	56799	41230	27054	14664	10114	7384		
Ft0	350	8362713	158717	346216	605174	879812	959295	820295	790286	705517	647979	564277	432980	367357	312168	206577	170066	118279	80314	52435	33675	23943	15998	7574	3962
Ft0	350	13090605	144293	331527	575937	850726	927075	807077	776631	707574	600537	580020	5391068	344197	322409	193578	153948	110202	74760	48867	36420	25916	17518	10833	5819
FiT1	350	5203866	34107	104392	224897	382170	473979	456710	454789	431702	375430	368893	250575	222064	225606	130880	108108	784174	52963	34078	23178	17833	11816	7731	5421

	Time days	Tot. ucm	C14-15	C15-16	C16-17	C17-18	C18-19	C19-20	C20-21	C21-22	C22-23	C23-24	C24-25	C25-26	C26-27	C27-28	C28-29	C29-30	C30-31	C31-32	C32-33	C33-34	C34-35	C35-36	C36-37
FiT3	350	3221894	33794	84358	163809	261584	328575	322709	321435	305461	267944	264726	177879	160389	171073	95371	81126	58854	39278	24823	16619	11288	7198	4710	3169
FiC1	350	5180132	47326	135636	287473	476880	582795	39500	524996	491621	421763	416725	273375	244283	224300	141199	114855	82003	55563	35680	24834	18313	11559	6415	4672
FiC2	350	8133008	117784	296626	551748	847620	958909	842020	806022	739365	632974	597187	409745	351094	309098	194375	149316	103434	68064	43090	29830	21221	12867	6860	3086
Ft0	350	7859258	145978	331612	560713	825768	918990	771457	779399	714326	563297	552715	377887	330259	290633	178912	145928	106084	67803	45973	32515	21133	17129	12105	5238
Ft0	350	8337001	150921	333183	591985	873226	947874	831295	771762	730982	638136	561568	427566	351336	311297	205475	164312	120585	82249	56392	39226	30556	23579	13878	11034
FiiT1	350	5073278	34556	107585	251273	434755	541619	511471	491735	482869	430827	385667	297288	248167	241831	157823	138450	97725	66506	44720	29934	23070	16469	9000	7014
FiiT2	350	8450323	68181	205997	437360	741484	911053	876747	810439	898790	697484	625421	488874	401367	369612	241858	198668	141229	95321	65642	45600	34958	25626	15350	12346
FiiT3	350	5240908	32155	109014	249079	410967	524821	539310	506325	505591	446109	408816	329285	271810	249839	169468	141743	104802	71048	48800	33207	26172	18151	10249	8524
FiiC1	350	6259815	46464	148369	324867	546907	680235	666034	614418	604806	527979	469791	368622	304624	263052	183212	148433	107567	72577	49631	34462	26292	19629	11413	9953
FiiC2	350	9244658	108614	309051	591339	947649	1057443	974132	882441	849045	728934	653034	503742	415582	354433	237836	186982	130266	87183	59126	41623	31831	23168	13157	10000
Ft0	350	9338061	181705	405136	681575	1003676	1088859	981316	880959	831483	706072	619381	477858	386537	330218	215557	168105	119133	78337	51788	33093	21667	10780	1735	
Ft0	407	10829817	223042	452468	779208	1133749	1230716	1066975	1013051	962303	808913	724944	580491	477804	434908	292166	216426	159982	95422	54039	26421	12647	5929	10945	
Ft0	407	12731677	254325	521880	879417	1280047	1400979	1214601	1358985	1082513	928142	815823	657788	535869	624992	324482	242346	183901	119615	79329	54698	42298	29626	23889	
FiT1	407	3750122	42951	95892	190821	280164	350796	349798	343880	340639	299501	276390	233178	196327	217207	131130	106736	86132	58640	41449	29079	24741	21256	19842	
FiT2	407	5437261	65771	143423	273972	419989	514137	513944	504685	496892	435166	400821	334657	284925	317192	184606	150824	120468	81210	55987	38463	32431	25207	21296	
FiT3	407	4775485	51166	110818	216080	337754	419515	427038	426671	429498	385172	366124	309457	258939	317854	175987	147991	123376	82654	56909	39291	32432	24764	20142	
FiC1	407	7706990	92464	203983	390512	605334	741947	732750	717118	711138	628759	577984	486016	433945	436901	259038	204367	156339	102228	68012	45387	35938	25973	21001	
FiC2	407	14206068	246931	535494	909813	1462584	1593394	1396866	1357114	1290858	1106493	981936	808649	659954	590045	376245	273186	198863	123612	78599	51757	40004	29662	23549	
Ft0	407	13709826	269449	576453	974079	1404282	1523242	1364164	1239552	1199918	1023434	923105	753010	601682	553465	368804	282696	214821	135857	90186	59120	40874	23200	11142	
Ft0	407	19744347	404073	876523	1486624	2088069	2329500	2043451	1814007	1771167	1558561	1357342	1022651	795340	762684	417342	306252	229504	137002	92699	56765	42585	24295	14627	
FiiT1	407	7124074	54239	154060	326972	553373	696605	702853	703544	677493	642169	574273	452705	387250	391108	239366	181329	145433	87079	58880	34563	26626	14638	8271	
FiiT2	407	3842208	32753	90657	188752	311730	389672	377320	375373	361440	326340	292897	244635	199642	203145	124164	100165	77802	49121	34627	21550	17205	9902	3406	
FiiT3	407	5855451	38862	114067	244083	431342	539056	560635	575513	559896	533720	476748	391102	330856	374406	200663	154570	125808	74890	49690	28989	21797	11501	7891	
FiiC1	407	8948888	70371	211119	438245	731908	888024	894781	896016	855120	804807	709507	579707	492732	472438	280379	210684	155439	92270	61888	36889	27962	15629	9135	
FiiC2	407	34287207	548104	1497442	2367609	3716128	4172059	3735285	3661003	3276218	2793355	2399290	1662174	1336993	1113716	593628	441245	295586	178812	125397	85431	71955	51250	40765	
Ft0	407	14826096	300020	632622	1092148	1563444	1711120	1564402	1347979	1369707	1127038	993380	785465	605002	603583	307398	241674	180050	117610	75230	48913	41183	22680	11141	
Ft0	473	6227270	138368	263872	426668	618291	660935	586839	619941	484838	447407	388358	299024	252035	274157	157711	128545	105888	81450	63911	55494	49393	44993	37051	31206
Ft0	473	5178756	122841	235021	402569	561470	610219	528878	469502	449105	375691	345350	247072	203237	234190	109164	84828	60803	37998	22527	16059	11457	7809	3461	2292
FiT1	473	1495674	19472	40384	74471	123971	149727	147663	135530	136740	115895	109687	80054	65745	148013	38485	33272	25603	16157	9596	6474	4929	3634	2134	2637
FiT2	473	1292243	18385	37825	75872	111016	140962	140087	128583	130166	111036	106100	78332	64982	130550	38302	33036	25046	15205	8438	4725	2878	1278	279	2575
FiT3	473	1128656	13260	27256	53402	85775	109076	110649	104536	102706	87044	83067	61026	49741	114874	29453	26073	21032	13743	8653	6479	5645	5914	4744	3408
FiC1	473	2449120	28422	64797	128413	207271	262254	267052	252405	245202	208965	198428	146318	137654	179494	69513	57554	40482	24688	13683	8446	5420	3588	2191	3690
FiC2	473	5934684	108039	231059	418537	633520	688209	619423	564507	511735	461659	401591	319975	241625	290125	136343	98171	68761	45227	28239	16919	11681	6390	4454	2436
Ft0	473	8374465	197934	394296	628639	913526	980719	819052	777868	729118	633351	551473	431208	313375	395120	172966	130812	90613	56345	34872	22759	17082	11517	7872	4276
Ft0	473	9848750	230125	449982	741968	1024648	1113978	973158	905676	859655	694761	656651	485304	396825	460632	228026	176264	128452	87460	54486	44958	30337	22745	11633	6777
FiiT1	473	2375882	27453	59256	109430	181175	225058	224773	211165	203315	190292	166428	135103	110494	208557	74399	59889	47097	34667	26995	22346	20515	15070	12507	2942
FiiT2	473	1934706	15822	44179	89482	150934	189948	186629	175478	167452	156776	171173	100842	95316	161611	63186	50342	37181	26187	18939	13419	9570	4320	2754	952
FiiT3	473	2330356	16283	46265	90233	161059	198551	212217	206765	199013	187138	271990	125114	110487	226405	75474	62239	49064	32982	22704	15441	10261	4547	2020	337
FiiC1	473	2153935	19277	52952	105157	181032	216008	220347	206478	195736	176779	165382	113509	107717	159373	66510	52578	36891	25272	18097	13323	9447	4276	3253	1256
FiiC2	473	8028993	123596	295301	540399	819469	919697	805818	760268	727177	611240	560838	418427	347024	431057	179130	157202	109294	72330	45831	34256	20850	14594	7282	4624
Ft0	473	9065566	182897	347951	585609	1020736	972935	958677	788214	726146	692626	520820	577341	428790	482394	279262	155215	106023	68920	44234	29727	19674	9421	6518	437

Alba microcosm analysis: ‘UCM segments’

	Time	g. sed extract	g. hexane recona.	Pr	Phy	C14_15	C15_16	C16_17	C17_18	C18_19	C19_20	C20_21	C21_22	C22_23	C23_24	C24_25	C25_26	C26_27	C27_28	C28_29	C29_30	C30_31	C31_32	C32_33	C33_34	C34_35	C35_36	C36_37
At0	0	10	6.39	16133	15042	211997	302567	342528	389321	398098	368709	367393	340372	336147	331373	316112	281292	277845	349147	232624	197494	167426	133970	103824	77823	61989	39113	25880
At0	0	10.02	6.22	21698	20635	371856	484525	502955	552533	542847	488171	470039	421267	403981	386123	354147	311790	301315	265716	245367	205861	173950	139030	107994	81466	65804	42418	27873
Ft0	0	11.82	6.37	40300	29596	55704	114280	176582	277183	291997	253145	250108	227440	218848	210588	206311	180041	186860	161916	149815	128099	109227	90584	72575	58335	47540	33043	25011
Ft0	0	13.59	6.2	39516	31288	73005	130481	189377	285972	292060	256144	239903	226879	211549	193958	184324	158527	164984	138366	128951	109993	96553	76097	61677	51221	40794	31114	21095
Ft0	28	10	6.477	35991	26791	45287	101031	163348	255265	263468	248527	220920	198753	189360	172628	144018	143284	137202	121860	110193	92640	80689	61832	49153	38426	27955	20623	15772
At0	28	10	6.3	18773	17728	258685	377190	433407	493222	485734	443427	434717	395976	389201	365814	357791	306031	279843	253576	229790	190956	163921	127466	97929	73801	56461	39652	28499
AiT1	28	10.03	6.36	16906	15479	260761	353466	389412	438574	424577	385127	371785	336820	329832	309336	298371	257357	234434	213849	193273	163075	140930	110549	87292	64693	49244	35392	24074
AiT2	28	10.05	6.3	17231	16606	257449	357950	402854	458953	447730	414165	401249	367760	362917	343500	333875	286802	269094	233334	207293	172427	146553	112881	87112	62371	46298	31960	20925
AiT3	28	10.03	6.36	17168	16025	257149	361375	403552	457496	445332	410463	397800	362259	354713	335327	320967	275076	243432	218904	192874	160490	135816	105153	80407	58787	44333	30835	20365
AiC1	28	10	6.38	17654	15093	200820	312714	371919	434473	424616	385585	373239	336359	326167	305060	301078	246565	221331	194729	171562	141858	119685	92328	69638	50565	37351	25145	15480
AiC2	28	10.04	6.2	18689	18121	268062	376940	417521	471246	450853	417027	403017	367221	358943	337495	337391	272589	234283	211974	185428	152699	129243	100201	75554	55676	40737	28146	17631
AiiT1	28	11.06	6.3	16414	15748	176573	285482	353702	416748	410296	380859	362765	354536	339163	320072	292971	255874	251719	215570	191691	153349	134594	105804	80288	60727	44806	33412	20488
AiiT2	28	12.12	6.33	18222	17604	215301	333137	402696	469733	461064	431204	412922	405790	387227	365333	329505	287118	270624	232629	203236	162396	140400	109742	84674	62430	46502	35019	20288
AiiT3	28	11.9	6.35	19162	19013	227531	359819	434750	504822	493883	465838	442377	433639	411956	389438	348729	304010	287625	245898	215550	173919	151965	121403	85258	71365	54794	42291	24516
AiiC1	28	10.23	6.26	17162	16523	167100	285174	362902	433203	425932	401504	381549	375047	357399	338194	305022	263893	248094	212525	186771	150545	131226	104562	81566	60571	45601	34393	19560
AiiC2	28	11.99	6.27	20155	19273	198554	335017	416223	489864	479459	449956	431325	423176	400716	376674	341504	284634	261885	220217	189324	150403	129086	101848	77691	57647	43798	32892	19515
Ft0	28	10	6.477	35437	26412	55961	107658	151220	248011	259564	229394	215904	211844	208864	182298	174656	150483	141410	114208	106227	79697	72881	57732	48213	36823	25582	23041	13574
Ft0	56	10.409	6.477	53398	40785	65939	162144	293136	452673	495685	442612	440100	429557	397358	374000	383089	319355	306465	256548	235581	212265	164615	139511	113068	90038	68210	50468	36171
At0	56	9.556	6.28	38361	34102	419066	651285	790760	880375	882170	828742	808089	784222	736432	699279	648180	581814	533178	458929	426586	370610	287048	236274	187074	139404	108337	77258	55884
AiT1	56	9.183	6.37	25329	21649	299293	455167	565937	637842	643775	603738	594024	582755	553896	532331	490549	444214	406703	355970	332810	291023	227555	188588	150107	113377	89354	64573	46350
AiT2	56	9.018	6.39	26862	26512	292746	453880	575844	664083	678703	645966	633180	621634	592817	568986	513105	474885	432929	377189	352631	306448	236652	194634	153039	114562	88814	62806	44072
AiT3	56	9.37	6.53	22807	23480	234900	400170	530562	622071	635417	603373	588547	575246	543637	518033	468405	424583	386543	333513	309526	268389	208333	172921	137682	104271	81612	58612	42011
AiC1	56	9.507	6.74	29152	25760	295724	474742	601797	682832	689193	645680	629945	612741	578313	550029	533037	453577	413150	357748	331922	286235	220648	180478	142087	106412	82558	57972	40530
AiC2	56	10	6.43	30827	27570	309276	493208	617599	693070	699970	661865	646145	629926	593818	564456	491252	467181	421110	369387	344452	300765	233074	192037	151252	113760	89116	63052	43454
Ft0	56	10.409	6.477	54936	49359	67573	162518	304032	472749	526076	472809	460139	444775	412489	378415	404433	303357	302481	240891	216605	187153	154743	128661	97763	79442	60318	46042	32427
Ft0	56	10.409	6.477	56351	46965	67286	163094	297419	461296	500579	455129	432646	428489	398266	367018	377467	299189	288038	231513	217006	188995	146532	127172	95125	79089	56348	44748	33441
AiiT1	56	8.88	6.21	26164	23838	292302	451164	568394	654030	657902	631663	590996	588192	560927	530302	479096	426160	410913	338069	320772	281274	216752	184257	136638	108316	79224	59362	42443
AiiT2	56	9.048	6.31	25397	26991	265807	423642	547398	638831	649396	626338	588539	587722	560449	529801	539682	429043	403551	338235	320495	280229	214683	182440	132852	105039	76124	56187	38352
AiiT3	56	9.104	6.19	27012	27864	378451	547187	658273	733640	732817	700528	712027	594500	619649	583240	587173	465897	437848	365328	344978	299784	228932	192853	140362	111112	81218	61079	43101
AiiC1	56	9.149	6.37	26271	23386	340074	490684	592930	659727	655549	621833	579924	586738	544142	510028	516440	406990	390704	318722	300347	261519	200130	169632	124890	99468	73830	56220	38853
AiiC2	56	9.442	6.35	31951	28889	401493	583996	685761	760063	746452	703219	657257	648168	612706	575662	561150	456133	431081	354857	333675	289868	221172	186175	136087	108132	79607	59311	40820
Ft0	56	10.409	6.477	51722	47612	69365	158480	297937	472911	514930	465938	457448	440882	409340	380148	389011	295852	280359	236734	207837	181004	141786	118493	91073	73213	56681	40554	30388
Ft0	56	10.409	6.477	57206	56243	76166	191468	265307	586137	607456	533685	525901	507283	481491	432529	454247	355749	329604	282715	243198	210471	164094	134475	104279	85373	61580	45116	35083
Ft0	98	9.889	6.72	58125	48970	92861	193948	294584	489565	543331	473801	481794	459096	431389	386600	368553	306432	338694	274037	247300	209484	181175	145686	112834	92439	69140	50417	35936
At0	98	10.038	6.29	46494	43310	624763	820865	849682	1031340	1035370	937539	921935	880117	836180	761734	767327	624424	684337	516900	470081	390782	330649	259988	194315	149344	11187	78352	54396
AiT1	98	10.001	6.45	25347	24170	381490	501088	533841	670376	688397	630118	619094	597843	576160	530445	616504	440277	451274	370788	344752	290915	250042	198727	150272	117997	89092	64092	44268
AiT2	98	8.985	6.47	24315	24266	395419	490048	509377	635161	656988	601758	591762	570537	553264	510199	604563	423166	434822	355467	329445	276765	236572	186832	140397	109681	83350	60589	42958
AiT3	98	9.196	6.56	23881	24076	386301	490860	516101	642173	654083	598549	581071	558913	537314	493821	560805	404706	412877	340798	316118	265360	229806	182809	139958	111224	85486	62079	42161
AiC1	98	8.837	6.51	26267	25885	427141	530070	553524	682319	689426	622578	609216	584433	558595	508858	524036	419723	429138	351308	323389	270077	231949	182546	138222	108488	82124	58639	39408
AiC2	98	8.94	6.3	28096	25223	429411	551802	571325	690192	691382	623342	608829	577240	548878	498988	480145	409140	420867	343635	317535	267726	231226	183922	140458	111499	85708	62731	43421
Ft0	98	9.889	6.72	61005	52874	87420	193207	341788	526862	569066	614674	510582	481422	452334	427504	373990	319359	341383	261524	246568	211011	168848	142637	105081	89089	62675	50681	35699
Ft0	98	9.889	6.72	60652	46657	80806	170549	317186	503775	540513	486578	468048	441469	423380	392057	329790	297213	319142	250077	234822	196393	159393	135547	106476	81727	63382	44836	33215
AiiT1	98	8.906	6.35	22755	22111	407191	484054	549608	627830	628132	600886	559214	530266	521389	498150	435489	403247	401368	328707	315692	267459	217193	183353	142591	103521	79997	56118	37819
AiiT2	98	9.009	5.84	35001	33156	424185	578904	805218	824938	828562	790798	741115	702652	687694	652851	598887	519946	509775	412685	390398	327967	263065	221531	170756	124951	98035	70594	49171
AiiT3	98	9.147	6.22	32046	30610	513774	612043	694188	724218	765941	725202	677385	639691	624274	591519	569001	471093	470500	372378	351386	293223	233972	194622	147230	105907	81372	57247	38529

	Time	g. sed extract	g. hexane recona.	Pr	Phy	C14_15	C15_16	C16_17	C17_18	C18_19	C19_20	C20_21	C21_22	C22_23	C23_24	C24_25	C25_26	C26_27	C27_28	C28_29	C29_30	C30_31	C31_32	C32_33	C33_34	C34_35	C35_36	C36_37
AiiC1	98	8.902	6.28	29873	29853	364385	515395	637509	745149	746813	707505	690941	623927	607916	574154	531822	454828	451029	360150	339919	285778	229483	193694	159441	108939	85300	61142	43016
AiiC2	98	8.96	6.41	27189	27731	363043	508340	617431	691559	685183	647462	600632	562045	544894	513489	442679	464933	395546	320432	304044	255167	206192	174639	135674	100826	80308	58624	41072
Ft0	98	9.889	6.72	57124	48129	79974	182455	310878	490934	537106	468373	470007	447027	409311	374189	340132	307413	302381	247352	219392	183835	154735	122783	94844	75328	56493	42295	32177
Ft0	98	9.889	6.72	63123	57783	98121	206163	383552	588528	646285	575085	571564	570994	494319	483663	411308	359347	375042	301246	266743	234239	179535	151534	112559	92457	66941	51090	37329
Ft0	140	10.158	6.55	56368	29858	64391	122018	188150	371582	395810	348592	351361	334022	319908	313081	312132	264283	273447	244473	241823	222244	256912	194199	159516	150170	118469	93889	71558
At0	140	10.266	6.45	35304	38798	520945	574500	600236	803363	801123	741510	709261	682886	652760	654663	666426	572590	561869	549040	551661	505092	444298	398793	294218	245661	180835	130885	97472
AiT1	140	9.086	6.41	15030	15168	294205	297163	300775	413804	422541	393768	376256	361704	351101	356544	369930	316525	343828	313208	321009	303524	278619	262638	203134	178458	136800	102368	77128
AiT2	140	9.069	6.5	14956	14533	300655	292585	292280	392599	403919	377747	363309	349786	342196	350661	357466	314577	338078	311702	319693	305840	275294	256943	197946	172454	131495	97564	73512
AiT3	140	9.238	6.2	19429	19686	380459	375319	376693	514713	525589	491226	471471	453855	442330	450800	450643	402190	421335	395599	401836	371517	329099	296031	215869	179789	131627	94725	70508
AiC1	140	9.135	6.32	19142	16886	341882	335181	340772	467255	474352	443837	423850	408699	394253	395285	383249	341828	355480	322334	323002	296080	262892	238240	176591	149880	112082	82445	62036
AiC2	140	9.435	6.4	23503	22654	394437	405909	416283	545943	543280	503905	485384	458048	436042	435813	402263	381989	390545	358536	360525	327412	287968	256207	186325	154691	112976	81381	60523
Ft0	140	10.158	6.55	50923	39352	87547	156771	237748	440122	478947	429080	421269	411121	386937	375674	381592	316037	337583	297043	288562	262021	224922	198056	145722	121292	84567	58865	42065
Ft0	140	10.158	6.55	49960	36986	55823	140220	242101	400833	421655	377123	371867	354887	342836	315861	327290	375586	312668	239575	245967	228196	198582	175907	135285	113808	76311	53918	32799
AiiT1	140	8.908	6.32	14971	15777	225235	328326	363674	440282	444407	425090	411921	394219	396587	380014	391174	340111	399539	321410	341567	315756	273244	239693	176140	145529	95473	66940	42094
AiiT2	140	9.21	6.42	17929	18048	244364	352498	393470	479151	479728	45951	444715	418477	418300	398016	377311	352038	401282	330829	350217	322420	275834	240258	173794	143077	94136	66216	41319
AiiT3	140	9.411	6.44	20198	20367	289420	408723	460260	545697	532288	498075	479096	458140	453359	427890	446806	375055	416632	344527	359945	326182	275906	237031	169974	138735	90646	63626	40501
AiiC1	140	9.094	6.62	19023	17718	248854	356945	404147	485454	479339	453718	431415	412884	408924	384950	401837	335252	379100	305030	317480	286925	242802	209889	151949	125801	84672	62175	40234
AiiC2	140	9.101	6.37	22760	23645	292050	424664	483626	560021	545325	515114	499848	472621	466356	440371	410961	389576	456266	256353	369333	330724	275132	231180	162687	131008	85004	58984	36276
Ft0	140	10.158	6.55	54469	42647	71028	168318	273904	456520	484500	439967	432397	417744	398815	369718	378995	313294	358910	278972	280792	252555	206480	172604	123504	101031	62667	43622	27849
Ft0	140	10.158	6.55	55242	41220	66116	158299	273979	443449	474087	431413	428236	406032	384586	365080	373824	301673	336805	275968	269377	255854	194743	173623	128105	99121	67846	49080	33664
Ft0	203	9.922	6.45	62048	49844	70082	170251	304584	448348	485992	431980	409968	391848	361824	343536	313094	288563	304542	242052	232742	215560	165241	133406	89130	73335	54962	40947	31903
At0	203	9.942	6.24	36875	30225	527244	673101	798857	872996	861979	801783	761763	728462	664432	681176	600545	551142	541938	457017	435651	394064	294942	228593	140586	110857	75834	46804	25237
AiT1	203	9.106	6.33	13498	13270	261395	325743	382298	424366	432570	415248	388072	381781	353496	362658	326765	307699	327169	265068	259365	241678	186356	148898	94015	76963	54446	36104	21874
AiT2	203	9.301	6.37	12028	11189	206822	270084	320828	356941	366711	343748	329695	326064	302099	310217	280164	259377	279845	226089	221438	205711	157936	125522	78786	63642	44144	27926	17613
AiT3	203	9.424	6.38	13884	12287	309377	360240	404079	439279	441396	416422	398247	392892	364110	419185	340521	314869	335577	272680	265475	245432	188370	150453	95475	79425	58662	39865	23624
AiC1	203	9.171	6.43	17680	16145	318949	385172	447565	488658	482820	448393	429573	415755	381603	444794	352914	332098	346418	278299	268574	247541	190529	153326	98618	82970	61247	44200	29431
AiC2	203	9.493	6.77	22890	18221	366184	428224	481673	513737	500175	461582	438416	419524	381323	443194	348126	325866	328888	266761	255009	231391	174881	137515	85740	70076	50911	35251	21813
Ft0	203	9.922	6.45	57753	47806	68399	161517	275241	409838	451285	400925	381982	361358	347475	327541	278754	279772	293164	214603	208159	182549	143863	108132	71213	54722	37224	19916	12429
Ft0	203	9.922	6.45	60040	52356	66085	160449	280320	432756	451770	380559	414048	369651	344281	323261	292763	268903	293357	215480	222260	191264	152482	111571	78742	60944	36894	18288	8766
AiiT1	203	9.249	6.42	11674	10823	213696	289967	342614	402161	394566	362876	379579	356998	338522	334016	302545	279055	311804	234140	246698	216282	172577	130239	90986	72788	48724	27448	18651
AiiT2	203	9.193	6.55	11006	11250	201475	276940	326187	382168	374692	344241	358534	336848	319578	320429	285681	261721	292551	217811	227704	197614	156398	116419	80115	63174	40981	24127	14331
AiiT3	203	9.522	6.35	19490	19000	300224	416670	495343	575949	546250	498306	521621	482909	455568	483980	407245	371525	403596	302136	310470	265022	207550	153452	105682	83802	56386	33476	21641
AiiC1	203	9.376	6.48	17688	16599	253510	349676	419302	490993	466924	421166	440442	408996	385331	421473	345553	317947	345450	259526	269068	231073	183145	136560	94932	75887	51363	30551	20032
AiiC2	203	9.114	6.41	19407	16503	267365	350311	403680	458197	432430	388806	401590	369355	346481	340282	305493	280478	304564	225573	231529	197572	155642	115676	80364	64158	42982	24708	15126
Ft0	203	9.922	6.45	64744	50705	66568	170723	291328	429437	474009	405701	414443	375725	373226	325062	285015	273110	321358	228145	206233	186140	142203	103720	71903	56213	40301	19775	15074
Ft0	280	9.81	6.459	31087	30305	36570	84746	158313	251328	271216	233456	235027	214697	186331	169231	132973	114908	101824	75880	72952	57431	40856	30575	22269	18758	7893		
At0C3	280	9.39	6.203	22319	20099	369585	453936	504449	570039	536767	479063	464464	425702	364663	354785	265756	229014	196695	152685	140775	107923	75596	54553	37394	29077	21670		
AiT1	280	10.167	6.685	6125	6073	121137	167936	199899	237099	236057	220176	217613	206508	181133	181945	138274	122776	112722	89150	86890	70271	51480	39101	29200	23808	19036		
AiT2	280	9.932	6.369	5947	5561	130858	177183	209474	245839	242953	226249	223172	213379	189029	205501	147318	130629	122200	94898	92529	74886	54345	40554	28543	23099	15866		
AiC1	280	9.964	6.472	14753	13068	200901	268040	310890	359392	343101	309126	303123	282157	245498	262662	188019	17006	149061	114371	108013	84362	60131	43997	30829	24262	18435		
AiC2	280	10.103	6.374	8855	8845	155100	213026	253471	300012	292943	269638	267943	251671	219903	242981	166771	148720	132912	102278	97334	76275	54150	39870	28907	23603	17165		
Ft0	280	10.158	6.55	39726	35124	43605	94268	173230	271730	292027	262974	255956	224949	201159	192800	138447	125214	111971	83810	67030	53596	33779	21394	13204	10603	6106		
Ft0	280	10.158	6.55	42835	37082	42432	96186	177675	273571	312815	273301	274332	240347	232967	204394	146541	128644	121600	87799	80191	63490	39678	28481	19848	14914	7064		
AiiT1	280	9.78	6.306	6901	7095	113464	170956	216833	253394	271984	253367	251330	223534	218815	216695	143157	128574	127798	94167	88397	71135	44409	31765	21550	14147	5150		
AiiT2	280	9.729	6.328	5800	5984	126422	176599	214441	245079	263653	245764	242696	216755	213566	199782	139367	126384	123028	92930	87726	71304	45026	33718	22745	15480	12752		
AiiT3	280	9.987	6.416	11616	11975	201055	272647	327938	368939	385157	349286	344524	304014	296751	268330	190151	171587	165335	121738	111121	87220	54121	38977	25557	17767	13986		

	Time	g. sed extract	g. hexane recona.	Pr	Phy	C14_15	C15_16	C16_17	C17_18	C18_19	C19_20	C20_21	C21_22	C22_23	C23_24	C24_25	C25_26	C26_27	C27_28	C28_29	C29_30	C30_31	C31_32	C32_33	C33_34	C34_35	C35_36	C36_37
AiiC1	280	10.059	6.354	11278	11767	169400	238932	293073	333560	347630	313812	308834	274032	267053	266836	174446	158089	148361	111304	102380	80365	49452	35258	23156	16683	10332		
AiiC2	280	10.254	6.366	15591	13626	175429	254343	312877	353838	363924	326396	320104	278768	268236	243113	168995	152428	146969	105518	95565	73774	44974	31924	21925	14217	5266		
Ft0	280	10.158	6.55	42327	43397	50648	110244	200048	320467	345460	312481	294916	276702	242890	230068	173585	146300	135375	95779	80235	61539	41302	25280	14397	8718	4814		
Ft0	280	10.158	6.55	45344	39742	44498	107934	187587	311476	331489	304616	280388	263303	232807	215522	164688	136286	127505	88447	78556	56799	41230	27054	14664	10114	7384		
Ft0	350	10.465	6.376	124917	109558	158717	346216	605174	879812	959295	820295	790286	705517	647979	564277	432980	367357	312168	206577	170066	118279	80314	52435	33675	23943	15998	7574	3962
At0	350	10.285	4.984	81364	79826	1154000	1558570	1861960	2123860	2050540	1849810	1772240	1574390	1456150	1339440	979567	811939	644630	435733	349646	239987	163741	110249	70842	53756	37634	21835	11171
AiT1	350	11.117	5.026	21918	21644	352793	519350	668650	788536	834316	777833	755791	690708	651631	672533	455717	386296	343799	227120	193660	137849	94258	61518	37296	26392	16736	7897	3551
AiT2	350	9.858	5.265	12962	13034	261419	385411	500358	590420	638313	607537	593284	545264	517135	557103	364032	309159	282358	187168	164968	120190	84875	57726	36655	26866	17504	9061	4429
AiT3	350	9.836	5.23	14472	13595	290379	425128	549757	647282	802754	668044	652464	599452	570181	553447	400067	340176	304082	205424	179134	131416	91969	61945	38070	26981	16948	7618	2694
AiC1	350	9.568	5.475	16816	17252	392366	576065	729848	849216	878444	811117	783104	711282	666039	698033	463245	404294	351441	234792	202427	145977	102951	70055	44110	32082	20351	9988	3855
AiC2	350	10.278	5.42	45335	42002	502465	726194	904872	1036540	1036780	922475	880383	784222	723657	716550	493898	433094	346824	234137	193442	134904	82519	60886	37155	25916	15441	6362	1676
Ft0C3	350	10.158	6.55	121670	108475	144293	331527	575937	850726	927075	807077	776631	707574	600537	580020	391068	344197	322409	193578	153948	110202	74760	48867	36420	25916	17518	10833	5819
Ft0iC3	350	10.158	6.55	130457	107287	145978	331612	560713	825768	918990	771457	779399	714326	563297	552715	377887	330259	290633	178912	145928	106084	67803	45973	32515	21133	17129	12105	5238
AiiT1	350	10.113	5.352	14348	13258	232476	375194	485762	581680	644184	600223	607543	577858	468063	478420	329389	290845	272541	174571	151913	116382	75840	50072	34101	24067	19480	10226	4075
AiiT2	350	10.447	5.332	12451	12027	191819	338582	454290	550349	617892	580207	589252	559686	452709	462393	317932	281641	259169	171561	153128	115320	76329	51902	36586	25798	17438	8393	3553
AiiT3	350	10.06	4.979	11878	11955	444028	682363	835064	965103	1020480	939054	945851	885803	708630	715818	486791	424345	362037	243383	207963	148565	96691	65177	44181	30649	22675	11991	6177
AiiC1	350	10.245	4.964	31591	30551	485553	756201	942810	1107730	1182940	1066640	1074940	1019300	824339	841503	583085	526363	457349	308214	266926	197965	133296	93859	66763	48777	37235	21402	12883
AiiC2	350	10.535	5.118	42416	40248	440653	700255	873879	1016920	1045870	922875	926252	862505	688924	754765	487491	438134	379975	252654	219167	157346	105343	73802	52914	37974	29188	15994	8542
Ft0	350	10.158	6.55	132004	107625	150921	333183	591985	873226	947874	831295	771762	730982	638136	561568	427566	351336	311297	205475	164312	120585	82249	56392	39226	30556	23579	13878	11034
Ft0	350	10.158	6.55	146326	124017	181705	405136	681575	1003676	1088859	981316	880959	831483	706072	619381	477858	386537	330218	215557	168105	119133	78337	51788	33093	21667	10780	1735	
Ft0	407	10.047	6.312	167880	140001	223042	452468	779208	1133749	1230716	1066975	1013051	962303	808913	724944	580491	477804	434908	292166	216426	159982	95422	54039	26421	12647	5929	10945	
At0	407	10.293	6.246	74557	66876	1122410	1380740	1591630	1767410	1729130	1573610	1472760	1382560	1171270	1117650	861334	697311	608713	433682	324840	244298	150638	90482	47676	24864	9397	11929	
AiT1	407	11.175	4.968	13299	12368	347082	437491	534319	607992	648697	642948	619584	608820	536309	560473	419569	348685	343466	238612	192987	155363	97087	57663	27517	13707	7235	11102	
AiT2	407	10.917	5.5	13421	11768	360395	459199	567169	661154	716709	719571	703379	697202	619584	653140	486572	403663	415867	272795	219865	175603	109357	65368	32046	15962	7290	9664	
AiT3	407	10.629	5.204	13121	11140	322166	410864	507244	589148	636544	631549	608543	594470	521264	545748	400767	329064	335474	220034	175336	138115	83802	47704	20740	7797	2592	8841	
AiC1	407	10.557	5.287	15807	17736	545279	710398	882127	1028150	1077010	104055	984967	956823	829711	820377	620002	548908	470445	317065	241184	178847	107662	63863	31663	15576	8425	11734	
AiC2	407	10.918	5.391	39925	36361	586449	728011	850910	936029	925953	843344	785042	743423	633010	581537	462566	418251	353994	227438	168707	121265	71551	39940	17490	6684	2317	9418	
Ft0	407	10.047	6.312	185676	153728	254325	521880	879417	1280047	1400979	1214601	1358985	1082513	928142	815823	657788	535869	624992	324482	242346	183901	119615	79329	54698	42298	29626	23889	
Ft0	407	10.047	6.312	196401	170093	269449	576453	974079	1404282	1523242	1364164	1239552	1199918	1023434	923105	753010	601682	553465	368804	282696	214821	135857	90186	59120	40874	23200	11142	
AiiT1	407	10.677	5.411	18885	16462	452034	613520	768210	878722	935752	964162	876711	899315	787313	857477	637629	512720	506658	335913	274106	217092	136577	86614	53923	36100	19452	9005	
AiiT2	407	11.13	5.478	15951	13065	391803	527581	676304	788489	869508	906030	830731	850164	743768	710426	580644	457636	437204	286821	230020	177710	108610	68550	42917	29073	17343	8499	
AiiT3	407	11.157	5.473	18256	16015	691581	906758	1105720	1272470	1323830	1335860	1200960	1198770	1021670	1014860	763698	590421	528418	345766	263357	192915	117018	73704	47244	32296	18560	8038	
AiiC1	407	11.244	5.268	17114	18401	452312	622951	781663	896478	942752	928399	825452	825282	707069	662467	539430	441526	399311	274590	222281	169671	110589	74364	50308	35052	20397	8995	
AiiC2	407	11.139	5.407	101121	77762	1035050	1429490	1756160	1996150	1989770	1896120	1685920	1650330	1396340	1418710	1054980	831995	756986	467346	353774	255930	159728	103345	67690	45666	25839	10027	
Ft0	407	10.047	6.312	293698	260409	404073	876523	1486624	2088069	2329500	2043451	1814007	1771167	1558561	1357342	1022651	795340	762684	417342	306252	229504	137002	92699	56765	42585	24295	14627	
Ft0	407	10.047	6.312	204035	194377	300020	632622	1092148	1563444	1711120	1564402	1347979	1369707	1127038	993380	785465	605002	603583	307398	241674	180050	117610	75230	48913	41183	22680	11141	
Ft0	473	9.884	6.176	96563	78586	138368	263872	426668	618291	660935	586839	619941	484838	447407	388358	299024	252035	274157	157711	128545	105888	81450	63911	55494	49393	44993	37051	31206
At0	473	9.501	6.234	48700	44650	624212	812236	954132	1083980	1059390	983905	862918	804702	735080	682357	496534	405667	382450	239511	186685	143965	100465	69418	50264	38318	29422	20684	14702
AiT1	473	10.709	6.06	5260	3822	111847	149318	192625	232405	258107	264996	238766	233213	221365	203397	156575	132443	191734	89742	78183	66019	47028	32494	22776	16871	12655	8241	4853
AiT2	473	11.209	6.016	4011	2619	112863	147717	189364	229890	252076	263335	239336	233905	221143	239256	157593	132410	189408	87510	75133	62010	42763	28367	18579	12376	7970	4161	1243
AiT3	473	10.514	6.179	3308	2804	105364	138837	177453	214421	241774	249078	223399	217430	203560	184008	138194	113923	172625	72629	60633	48302	31777	19996	12365	7697	4467	1779	151
AiC1	473	10.587	6.262	6091	5554	199013	261715	323368	385633	410583	404337	356717	341312	317466	283707	213524	198169	216667	110862	90321	68931	46462	30196	19504	12652	7628	3585	930
AiC2	473	10.982	6.365	16904	15744	188367	261703	320536	369996	366216	341050	298369	280230	256425	262025	175224	166583	211877	86253	67555	51176	34760	23012	15565	10361	6501	2462	789
Ft0	473	9.884	6.176	85308	70661	122841	235021	402569	561470	610219	528878	469502	449105	375691	345350	247072	203237	234190	109164	84828	60803	37998	22527	16059	11457	7809	3461	2292
Ft0	473	9.884	6.176	129580	112302	197934	394296	628639	913526	980719	819052	777868	729118	633351	551473	431208	313375	395120	172966	130812	90613	56345	34872	22759	17082	11517	7872	4276
AiiT1	473	11.177	6.278	8355	6142	181010	236174	292746	335454	363570	352161	338253	330185	299866	331070	228169	172794	245917	114602	101952	78619	51987	33356	20139	13799	9052	5272	2886

	Time	g. sed extract	g. hexane recona.	Pr	Phy	C14_15	C15_16	C16_17	C17_18	C18_19	C19_20	C20_21	C21_22	C22_23	C23_24	C24_25	C25_26	C26_27	C27_28	C28_29	C29_30	C30_31	C31_32	C32_33	C33_34	C34_35	C35_36	C36_37
AiiT2	473	11.524	6.24	6755	5045	152233	197812	249890	292791	342418	323501	312950	307419	280961	265625	211379	159436	188978	108691	99828	79615	53466	34325	20177	13723	8555	4640	1158
AiiT3	473	10.982	6.658	9571	7232	307044	386768	459580	520378	567380	532833	506558	488629	440283	417589	323942	244931	295046	159536	139523	105937	71918	47603	29740	20498	10421	4540	438
AiiC1	473	11.638	6.288	11501	9965	349679	449969	537635	617670	656970	611917	576956	555719	499029	465925	366852	293856	351680	177606	153888	109706	72509	46653	28513	19940	12767	6784	1883
AiiC2	473	11.416	6.404	27686	25453	379356	503143	589836	651007	660120	577363	534040	501379	439952	401946	313089	240934	297938	145139	124765	92422	64434	44194	29417	22377	15811	9560	4866
Ft0	473	9.884	6.176	147007	123257	230125	449982	741968	1024648	1113978	973158	905676	859655	694761	656651	485304	396825	460632	228026	176264	128452	87460	54486	44958	30337	22745	11633	6777
Ft0	473	9.884	6.176	147625	120616	182897	347951	585609	1020736	972935	958677	788214	726146	692626	520820	577341	428790	482394	279262	155215	106023	68920	44234	29727	19674	9421	6518	437

D.4 GCMS TIC total ion abundance data for analysis of microcosm samples (see chapter 6.5.1)

Alba microcosms - GCMS TIC scan data (ion abundances)

	Time Days	55amu	57amu	69amu	71amu	82amu	83amu	85amu	91amu	92amu	95amu	97amu	99amu	105amu	77amu
Alba	0	207000	180000	147000	102000	47900	106000	58200	35400	11200	101000	114000	22300	46200	20000
Ait0	0	263000	249000	185000	132000	54400	146000	84400	44400	10255	151000	172000	35100	64000	18600
Ait0	0	239000	226000	168000	120000	49700	134000	78100	41200	12600	140000	160000	32700	59800	17000
Alba	0	160000	145000	111000	78800	36400	94600	54100	30400	9220	103000	113000		44100	13500
Aiit0	0	229000	212000	159000	112000	49300	131000	75900	42000	12500	137000	150000		59400	17600
Aiit0	0	221000	204000	154000	108000	48600	130000	75200	42300	12400	137000	155000		59700	17300
Alba	0	124000	109000	91500	63300	31000	80700	45800	26700	7930	88800	96900		38200	11800
Alba	56	217000	189000	203000	128000	50800	148000	81800	43900	10500	154000	160000	27300	53900	22800
AiT1	56	168000	132000	156000	95300	37500	113000	61300	32900	7620	118000	121000	20900	41500	16700
Alba	56	223000	184000	201000	126000	51200	143000	79800	42200	10300	147000	153000	26900	53800	22600
AiT2	56	184000	155000	171000	106000	40700	125000	67200	35800	8230	129000	131000	22600	45400	18400
Alba	56	221000	190000	209000	134000	53700	152000	83900	43600	10600	150000	156000	27500	54900	23800
AiT3	56	160000	130000	148000	93500	35400	110000	58200	30600	7040	108000	111000	19100	39100	16300
Alba	56	202000	167000	182000	120000	49400	138000	75400	38600	9480	130000	135000	23800	48100	21800
AiC1	56	149000	127000	136000	90900	34900	104000	55600	27800	6780	95600	99200	17500	35600	15200
Alba	56	173000	137000	159000	106000	43000	123000	66100	33400	8140	111000	115000	20100	41200	19500
AiC2	56	129000	98000	121000	81400	31200	92600	50200	24100	5930	81600	85500	15200	30700	13600
Alba	56	160000	128000	149000	100000	40700	114000	61100	30600	7510	99700	103000	18000	37200	18100
Alba	56	148000	122000	135000	91420	29700	98300	51900	26800	5720	85000	90400	14800	31900	16400
AiiT1	56	117000	93100	108000	71000	23200	76100	39600	20500	4390	67600	69700	11700	25000	12000
Alba	56	118000	91500	110000	73500	26200	75900	40500	21200	4910	66700	69400	11800	25100	13200
AiiT2	56	105000	83300	98300	64800	21200	66100	34100	18200	4050	60000	61100	10300	22100	10500
Alba	56	117000	87300	112000	73400	25300	73500	39200	21000	4860	67300	69100	11700	24900	12600
AiiT3	56	112000	88900	106000	69900	22800	68700	35900	19300	4380	63500	64500	11000	23200	11100
Alba	56	55400	44600	40500	24600	6700	22500	11900	7860	1300	20000	19800	2980	7300	4820
AiiC1	56	107000	86200	95900	65500	19700	64200	33500	17400	3700	54300	56700	94700	20600	10600
Alba	56	122000	96000	112000	76900	25400	75500	40900	21100	4700	64500	67300	11500	24400	13500
AiiC2	56	123000	102000	112000	77600	24100	76400	40700	20300	4560	64500	67400	11500	24300	42300
Alba	56	126000	100000	118000	79800	25900	77200	42000	21700	4860	67300	69900	12000	25200	13600
Alba	56	78900	56100	46800	29500	8860	28700	15100	10500	1790	23200	23300	3380	8130	7040
At0	56	56600	36900	33400	20600	4720	20200	10300	6940	1040	15700	15700	2170	5560	4390
Alba	56	62600	37900	35900	21700	5590	21400	11000	7820	1130	16700	16500	2240	5780	5300
Alba	203	14800	14100	11600	8560	4300	9900	5310	2900	9060	8540	9300	1590	3010	1560
AiT1	203	6460	5240	5120	3070	1660	4090	1920	1340	379	4090	3790	682	1350	677
AiT1	203	6200	4990	4820	2900	1550	3860	1800	1280	342	3870	3600	532	1270	642
Alba	203	13700	13500	10300	7710	3620	8640	4780	2550	853	7300	8530	1480	2550	1260
AiT2	203	5720	4420	4220	2410	1340	3310	1480	1150	310	3380	3040	471	1110	585
AiT2	203	4680	3600	3420	1920	1050	2630	1170	926	229	2700	2410	365	887	452
Alba	203	13400	13200	10300	7520	3500	8580	4670	2630	863	7320	8400	1440	2860	1310
AiT3	203	8210	6530	6060	3550	1890	4770	2170	1620	441	4890	4400	696	1610	778
AiT3	203	6380	4990	4690	2730	1500	3740	1670	1290	359	3780	3440	526	1260	650
Alba	203	8440	7850	6290	4530	2110	5280	2850	1600	515	4530	5160	867	1730	765
AiC1	203	7890	7070	5740	3940	1840	4810	2450	1540	434	4380	4620	748	1660	674
AiC1	203	8040	7080	5960	3940	1960	4860	2410	1550	440	4520	4560	753	1590	747

	Time Days	55amu	57amu	69amu	71amu	82amu	83amu	85amu	91amu	92amu	95amu	97amu	99amu	105amu	77amu
Alba	203	9560	9210	7180	5190	2400	5870	3210	1850	597	5000	5700	1010	1980	939
AiC2	203	8310	7930	6140	4440	212	5060	2720	1540	479	4380	4760	840	1650	790
AiC2	203	7900	7560	5930	4340	2060	4950	2650	1460	453	4220	4590	809	1560	752
Alba	203	10200	9540	7820	5630	2740	6580	3540	1990	634	5420	6360	1050	2120	1010
Alba	203	11700	10300	50600	32300	12800	32300	18900	13500	3780	43800	48300	9440	17200	5900
AiiT1	203	56100	43500	31900	15300	54000	13500	6370	5420	1370	17400	16000	2820	7570	3080
AiiT1	203	40800	32200	25800	12400	44600	11100	5150	4330	1110	14000	12900	2320	4140	2480
Alba	203	68700	37200	28400	17200	5360	12500	6830	4310	1210	13000	14600	2940	4380	2660
AiiT2	203	20500	15900	13400	6590	2410	5950	2760	2340	594	7720	7210	1280	3400	1300
Alba	203	14900	14600	11400	7210	2440	6080	3450	2050	622	6990	8360	1720	2690	1030
AiiT3	203	12800	11100	9510	5360	2040	5170	2620	1980	551	6740	7060	1330	3270	889
AiiT3	203	8480	7250	6410	3560	1420	3570	1800	1390	394	4740	4910	919	2280	611
Alba	203	10500	9970	9440	5840	2180	5220	2920	1940	560	6430	7370	1490	2700	898
AiiC1	203	8090	7020	6530	3680	1480	3780	1920	1460	408	5120	5350	996	2500	621
AiiC1	203	7250	6240	6050	3410	1400	3550	1810	1380	394	4740	4910	938	2260	592
AiiC2	203	7570	7290	6850	4240	1670	4100	2320	1510	456	5140	5830	1190	2610	640
AiiC2	203	6320	6020	5560	3410	1380	3390	1900	1250	374	4270	4800	972	2170	528
Alba	203	9770	9330	9445	5650	2410	5620	3030	2120	635	6520	7610	1480	3460	967
Alba	203	7840	7740	9540	6060	2250	5320	2960	1920	575	6200	6980	1380	2500	924
At0	203	8480	8580	9900	6240	2310	5620	3110	1980	610	6440	7200	1430	3180	939
At0	203	8350	8320	9910	6280	2330	5640	3060	1980	611	6330	7070	1400	3140	952
Alba	203	6500	6970	8820	5990	2080	5050	2890	1650	483	5540	6500	1300	1880	806
Alba	473	107000	107000	129000	90600	33400	78500	40800	22200	6420	70100	71800	13500	22700	13400
AiT1	473	33500	17700	39300	16600	8660	21200	7000	8300	1860	27900	18200	2480	8050	4920
AiT1	473	29300	14500	34800	14530	7660	18600	6070	7440	1690	25100	16100	2210	7240	4350
Alba	473	109000	108000	132000	94800	36600	84700	43900	22400	6560	70500	74100	14300	25200	13500
AiT2	473	29200	16500	34400	14600	7800	19200	5590	7340	1630	24800	15300	1990	7330	4340
AiT2	473	23900	8080	29100	11900	6560	16200	4610	6220	1410	21200	13100	1680	6290	3580
Alba	473	86300	8530	106000	75500	28900	67400	34900	18600	5520	59500	62100	11800	20600	10600
AiT3	473	31300	14200	37700	14700	8370	20300	6240	8420	1930	28300	17800	2320	8330	4880
AiT3	473	25800	12200	30600	11600	6700	16300	4910	6980	1590	23700	14600	1870	6820	3800
Alba	473	85000	85600	106000	75500	28500	66000	35000	19200	5610	60900	63200	12200	20200	11400
Alba	473	83200	83000	101000	71100	26800	62600	33200	18900	5670	59700	62000	11900	20000	10200
AiC1	473	42300	27700	50200	25000	11600	29000	11000	9930	2570	35500	27800	4090	11200	4800
AiC1	473	45100	27600	54400	27300	12600	31700	11900	10900	2770	38100	30300	4450	12200	5690
Alba	473	84900	81900	102000	72900	27900	65500	34900	19600	5910	63000	66700	1270	21900	10300
Alba	473	32400	29000	38900	28800	9500	24000	12500	6860	2040	23200	24500	4580	8710	3670
AiC2	473	27200	23700	32300	24100	7990	20000	10600	5830	1740	19600	20600	3880	7330	2880
Alba	473	60900	60500	75800	54400	20500	47700	25800	14800	4360	47100	48400	9250	15900	8100
Alba	473	184000	165000	206000	146000	42800	110000	59300	31200	7980	94100	95600	19500	32000	21800
AiiT1	473	55600	24900	61000	29200	11500	29800	10800	10900	2340	33800	23300	3580	10200	7690
AiiT1	473	51500	24500	55700	26900	10500	27100	9690	9650	21000	31000	20500	3240	8900	6160
Alba	473	134000	117000	140000	101000	32800	76600	41700	21900	6300	65100	66800	13700	21100	14800
AiiT2	473	31900	7730	33200	13700	6470	15600	5570	6380	1450	19700	12200	1920	5790	3790
AiiT2	473	30300	7590	31100	12700	5890	14200	5080	5880	1330	18000	11300	1780	5330	3560
Alba	473	101000	89800	102000	71700	22900	53700	29200	15300	4510	45700	46900	9740	15400	9650
Alba	473	89400	76000	88900	59800	19300	45600	24500	13100	3870	39400	40800	7610	13100	8130
AiiT3	473	52500	29100	52200	24200	9990	24700	9730	9040	2210	28000	21700	3420	9100	6170
AiiT3	473	40700	24100	39700	17800	7500	18700	7180	6540	1590	21600	15700	2540	6600	3430

	Time Days	**55amu**	**57amu**	**69amu**	**71amu**	**82amu**	**83amu**	**85amu**	**91amu**	**92amu**	**95amu**	**97amu**	**99amu**	**105amu**	**77amu**
Alba	473	120000	108000	117000	80800	26500	62100	34100	18400	5380	53900	55800	11500	19600	11400
AiiC1	473	53000	30500	52200	26000	10100	25700	10800	8350	2110	27500	22700	3810	9070	4600
AiiC1	473	41300	21100	40900	19700	7910	19800	8260	6750	1680	21700	17900	2950	7300	3870
Alba	473	122000	107000	117000	80000	26700	62700	34600	19100	5510	55500	57500	11900	19100	12000
AiiC2	473	48400	38800	46300	31100	9680	23900	13400	7040	2040	21300	22700	4870	8580	3900
AiiC2	473	50200	43800	45900	30500	9500	23500	13100	6970	2010	21000	22200	4770	8410	3960
Alba	473	109000	97400	98500	64700	21800	50900	28200	16200	4650	46300	47100	9990	15500	9610
Alba	473	126000	111000	106000	68400	23800	55400	30400	17300	5030	50400	53200	11200	18300	10300
Alba	473	227000	176000	198000	134000	60300	149000	82800	46600	13800	141000	141000	25400	38100	228000
At0	473	188000	172000	173000	118000	50400	126000	69200	37300	11200	118000	124000	22700	40800	16600
At0	473	195000	184000	184000	123000	52400	133000	72600	40600	12100	128000	133000	24000	43900	19200
Alba	473	166000	147000	158000	106000	47400	115000	63500	35600	10600	112000	114000	20800	34100	16100
Alba	473	156000	133000	150000	101000	46200	111000	61400	34800	10500	107000	110000	20200	32500	15100

Ion abundance ratios normalised to Alba reference oil for Alba microcosms

	Time Days	57/55	71/69	85/83	83/97	83/91	91/105	97/105	83/95	97/99	99/95	85/97	85/95	97/105	83/69
AiT1	56	0.927	0.972	0.977	1.004	1.016	0.992	1.003	0.991	0.997	0.984	0.981	0.968	1.003	1.006
AiT1	203	0.834	0.808	0.860	1.035	0.892	1.020	0.880	0.853	0.954	0.787	0.890	0.734	0.880	0.945
AiT1	473	0.515	0.591	0.633	1.037	0.691	1.106	0.736	0.647	0.718	0.448	0.656	0.409	0.736	0.859
AiiT1	56	0.991	0.977	0.981	1.002	1.023	0.974	0.994	0.980	1.008	0.987	0.983	0.962	0.994	0.991
AiiT1	203	1.443	0.793	0.857	0.994	0.870	0.846	0.740	0.815	0.883	0.724	0.852	0.698	0.740	0.969
AiiT1	473	0.520	0.673	0.665	1.131	0.788	1.076	0.750	0.749	0.762	0.505	0.752	0.498	0.750	0.904
AiT2	56	1.000	0.978	0.969	0.999	1.016	0.999	1.015	0.976	0.980	0.956	0.968	0.945	1.015	1.016
AiT2	203	0.783	0.767	0.813	1.072	0.861	1.086	0.872	0.829	0.889	0.688	0.871	0.674	0.872	0.930
AiT2	473	0.776	0.583	0.556	1.116	0.704	1.112	0.701	0.658	0.674	0.397	0.621	0.366	0.701	0.872
AiiT2	56	1.043	0.996	0.967	1.003	1.026	0.976	0.998	0.988	0.994	0.979	0.970	0.956	0.998	0.999
AiiT2	203	1.432	0.812	0.849	0.964	0.877	0.699	0.636	0.802	0.882	0.733	0.818	0.680	0.636	1.009
AiiT2	473	0.280	0.575	0.657	1.107	0.694	1.082	0.678	0.672	0.764	0.464	0.727	0.441	0.678	0.861
AiT3	56	0.963	0.973	0.963	0.994	1.019	0.981	1.005	0.983	0.976	0.965	0.958	0.947	1.005	1.002
AiT3	203	0.819	0.804	0.832	1.062	0.893	1.100	0.925	0.839	0.916	0.724	0.883	0.698	0.925	0.948
AiT3	473	0.841	0.541	0.581	1.061	0.673	1.097	0.696	0.635	0.675	0.404	0.617	0.369	0.696	0.852
AiiT3	56	1.037	1.026	0.982	0.986	1.070	0.928	1.007	0.984	1.033	1.030	0.968	0.966	1.007	1.030
AiiT3	203	0.891	0.895	0.897	1.016	0.916	0.820	0.739	0.904	0.921	0.819	0.912	0.811	0.739	1.012
AiiT3	473	0.650	0.669	0.716	1.041	0.815	1.030	0.806	0.758	0.806	0.587	0.745	0.543	0.806	0.903
AiC1	56	1.051	1.008	0.986	1.004	1.032	0.969	0.995	1.005	1.005	1.005	0.990	0.991	0.995	0.999
AiC1	203	0.937	0.933	0.925	1.026	0.968	1.022	0.965	0.929	0.946	0.856	0.949	0.859	0.965	0.998
AiC1	473	0.645	0.705	0.710	1.050	0.876	0.969	0.808	0.790	1.436	1.081	0.745	0.561	0.808	0.920
AiiC1	56	1.016	1.026	0.969	1.006	1.090	0.925	1.002	1.019	10.047	10.177	0.975	0.987	1.002	1.042
AiiC1	203	0.908	0.926	0.927	0.987	0.967	0.905	0.886	0.888	0.951	0.855	0.915	0.823	0.886	1.015
AiiC1	473	0.616	0.714	0.761	1.017	0.905	0.952	0.847	0.811	0.806	0.642	0.774	0.617	0.847	0.916
AiC2	56	0.955	1.006	1.010	0.996	1.038	0.962	1.002	1.009	1.017	1.030	1.006	1.019	1.002	0.995
AiC2	203	1.007	1.008	0.989	1.037	1.029	0.998	0.990	0.974	1.032	0.970	1.026	0.964	0.990	0.999
AiC2	473	0.905	1.038	0.979	0.992	1.054	0.869	0.923	1.000	2.052	2.069	0.971	0.979	0.923	0.971
AiiC2	56	1.049	1.017	0.981	1.018	1.055	0.968	1.003	1.022	0.996	1.000	0.999	1.003	1.003	1.027
AiiC2	203	1.039	1.055	1.061	0.966	1.030	1.071	1.142	0.988	1.047	1.071	1.025	1.048	1.142	0.970
AiiC2	473	0.947	0.995	1.011	0.972	1.051	0.808	0.874	1.004	1.026	1.060	0.983	1.016	0.874	0.975

	Time Days	**57/55**	**71/69**	**85/83**	**83/97**	**83/91**	**91/105**	**97/105**	**83/95**	**97/99**	**99/95**	**85/97**	**85/95**	**97/105**	**83/69**
At0	0	1.068	1.019	1.037	0.954	1.073	0.949	1.067	0.979	1.039	1.066	0.989	1.014	1.067	1.020
At0	56	0.981	0.996	0.979	1.022	1.064	0.948	0.987	1.025	0.979	0.981	1.000	1.003	0.987	0.998
At0	203	0.979	0.963	0.971	1.026	0.979	0.769	0.734	0.998	0.998	0.971	0.996	0.970	0.734	1.006
At0	473	1.131	1.001	0.988	0.973	1.035	0.808	0.859	1.009	1.003	1.040	0.962	0.997	0.859	0.978
At0	0	1.034	1.002	1.016	1.025	1.008	1.020	1.004	1.042			1.041	1.059	1.004	0.963

Forties microcosms - GCMS TIC scan data (ion abundances)

	Time Days	55amu	57amu	69amu	71amu	82amu	83amu	85amu	91amu	92amu	95amu	97amu	99amu	105amu	77amu
Alba	0	124000	109000	91500	63300	31000	80700	45800	26700	7930	88800	96900		38200	11800
Fit0	0	118000	198000	84000	148000	30700	79200	118000	16900	6980	47100	88600		25700	5780
Fit0	0	124000	209000	89500	158000	33400	85300	126000	17800	7480	49200	95400		27000	6070
Alba	0	120000	108000	93300	67000	31800	82500	47900	28000	8380	91400	102000		38500	12000
Fiit0	0	132000	220000	98200	168000	36100	92900	134000	19900	8410	54400	103000		29200	6700
Fiit0	0	150000	251000	111000	187000	40600	103000	148000	21600	9270	59200	112000		31200	7310
Alba	0	158000	144000	123000	88200	41800	108000	63300	35400	10600	115000	126000		46700	15000
Alba	56	167000	135000	144000	97300	34500	116000	60900	30800	6730	98400	105000	17400	36500	18000
FiT1	56	114000	193000	97600	161000	28000	81700	102000	14900	5020	44900	74300	28900	21200	7150
Alba	56	161000	127000	147000	99500	36500	113000	60000	30700	6950	99300	105000	17600	37200	18200
FiT2	56	108000	190000	98600	159000	26800	78900	97500	15000	4970	45500	74200	28400	21100	7130
Alba	56	145000	114000	141000	94100	33800	104000	55200	28700	6520	93600	98500	16500	34700	16900
FiT3	56	166000	290000	150000	246000	41600	120000	151000	21900	7600	66600	111000	43700	31500	10300
Alba	56	146000	114000	145000	95800	34800	103000	54700	28700	6680	93200	97100	16400	34800	17100
FiC1	56	101000	185000	91700	157000	25800	70500	94300	13000	4700	38700	64800	26700	18500	6190
Alba	56	118000	90800	114000	76100	28400	80100	42400	22400	5630	71800	73900	12600	27000	13700
FiC2	56	115000	217000	101000	187000	28400	77200	112000	13800	5390	40100	69600	31200	19600	6500
Alba	56	127000	101000	122000	81100	30200	83400	44200	23700	5730	75400	77800	13400	28600	14700
Alba	56	762000	687000	636000	453000	146000	503000	276000	137000	28500	420000	443000	75600	160000	83900
FiiT1	56	98600	188000	83500	100000	18700	57900	79700	10500	3570	30700	51400	21800	14100	4880
Alba	56	698000	652000	631000	444000	136000	445000	247000	124000	26300	391000	412000	71200	150000	74700
FiiT2	56	62700	110000	44600	75900	9830	29600	41000	5560	1740	16000	26200	10900	6550	2400
Alba	56	583000	552000	516000	354000	104000	332000	188000	98000	20500	317000	329000	55000	115000	55600
FiiT3	56	53800	81500	32400	48500	6740	22100	26700	4270	1060	11600	17800	6310	4520	2000
Alba	56	97600	69300	61200	38900	12300	38200	20500	12600	2360	32700	31600	14900	10700	7770
FiiC1	56	30900	45700	17300	28000	3200	11700	15300	2260	541	5830	8860	3360	2260	1090
Alba	56	82300	56800	49600	31300	9520	30800	16200	10500	1900	25600	24900	3740	8520	6770
FiiC2	56	28300	39800	15200	24800	2560	10300	13500	1970	458	4400	7500	2840	1890	961
Alba	56	72300	47200	42700	26600	7860	26000	13600	9230	1570	21200	20700	3030	7180	6020
Alba	203	24900	23600	29100	13700	6490	15100	8540	4540	1370	13600	15100	2750	3870	2210
FiT1	203	11200	17200	8270	10600	2850	7000	6660	1600	556	4960	6800	2010	2050	638
FiT1	203	7140	9930	5270	6150	1710	4170	3700	1010	293	2950	3830	1040	1160	428
Alba	203	11200	10500	8190	6330	3170	7350	3960	2190	651	6640	7360	1210	1780	1120
Alba	203	12500	12000	9750	7170	3370	8340	4540	2410	763	7350	8520	1440	2610	1240
FiT2	203	3840	5760	2840	3520	989	2440	2280	587	167	1790	2450	656	744	214
FiT2	203	3930	5920	2920	3630	1030	2510	2360	627	214	1840	2490	710	782	254
Alba	203	11600	10800	9090	6340	2980	7220	3850	2260	679	6540	7280	1240	2400	1170
FiT3	203	4030	5770	2960	3450	988	2420	2180	640	212	1860	2440	662	785	261
FiT3	203	3420	4870	2470	2890	839	2030	1820	549	178	1540	2020	556	662	227
Alba	203	11400	11200	8690	6440	2900	7120	3930	2100	712	6280	7280	1240	2190	1000
FiC1	203	6930	11800	4930	7070	1760	4160	4470	947	327	2640	4070	1310	1200	361
FiC1	203	7170	12100	5100	7230	1820	4290	4570	991	343	2720	4190	1330	1250	382
Alba	203	13700	13000	10500	7480	3560	8290	4500	2580	822	7570	8260	1410	2760	1370
FiC2	203	9390	21200	6470	13200	2300	5370	8170	1100	475	2810	5070	2370	1410	422
FiC2	203	8120	18300	5590	11400	2000	4640	7080	953	410	2450	4370	2050	1230	363
Alba	203	12500	11100	9650	6570	3320	7380	3920	733	691	7190	7190	1230	2510	1340
Alba	203	7250	7140	7170	4490	1800	4310	2460	1610	477	5300	6080	1220	1960	711
FiiT1	203	3670	5470	3050	3320	783	2000	1890	570	179	2020	2820	937	1110	183
FiiT1	203	2900	4210	2450	2590	618	1570	1460	462	148	1560	2130	704	850	159
Alba	203	8850	8630	9380	5760	2320	5480	3010	2000	588	1530	7440	1490	2590	915
FiiT2	203	3120	5020	2630	3160	704	1720	1770	487	153	1550	2320	821	891	171

	Time Days	55amu	57amu	69amu	71amu	82amu	83amu	85amu	91amu	92amu	95amu	97amu	99amu	105amu	77amu
FiiT2	203	3210	5140	2660	3080	690	1720	1760	469	159	1580	2390	849	934	166
Alba	203	7760	7430	8060	4860	2010	4730	2580	1740	517	5800	6400	1260	2150	805
FiiT3	203	3870	5840	3500	3800	895	2230	2090	672	232	4930	3040	1010	1230	253
FiiT3	203	3410	5230	2970	3170	754	1880	1770	571	170	1900	2600	851	1070	201
Alba	203	7850	7560	8850	5450	2110	5120	2850	1840	557	1430	6850	1340	2460	849
Alba	203	7640	7700	7800	4790	1890	4610	2570	1640	467	5630	6430	1260	2180	764
FiiC1	203	5220	8970	4870	5860	1280	3080	3200	815	292	2720	4290	1560	1670	291
FiiC1	203	5290	9260	5080	6300	1300	3180	3390	828	307	2750	4340	1670	1680	287
Alba	203	7590	7480	8870	5570	2110	5060	2800	1820	548	5980	6650	1320	2360	846
FiiC2	203	6060	13500	5960	10500	1590	3670	5340	893	403	2570	4780	2440	1760	328
FiiC2	203	6440	14500	6500	11600	1720	3940	5790	940	420	2760	5020	2640	1330	348
Alba	203	7840	7740	9540	6060	2250	5320	2960	1920	575	6200	6980	1380	2500	924
Alba	473	60900	60500	75800	54400	20500	47700	25800	1480	4360	47100	48400	9250	15900	8100
FiT1	473	9830	9740	11000	10900	2570	6610	4840	2110	599	7250	6220	1710	2480	720
FiT1	473	8330	8370	9160	8910	2120	5500	4020	1820	504	6220	5210	1410	2100	558
Alba	473	70000	67300	82500	57700	21900	52600	28100	16500	4920	53100	55400	10400	18000	8240
Alba	473	64000	61000	75600	52400	19900	47700	25800	15400	4640	49100	50800	9420	16700	7920
FiT2	473	13000	12000	14000	13500	3350	8750	6190	2780	816	9480	8320	2200	3110	942
FiT2	473	15900	14600	16800	15800	4040	10500	7340	3480	982	11300	10100	2610	3760	1160
Alba	473	67100	63600	76500	53300	20400	48900	480	16300	4900	51600	53300	9930	16100	7920
FiT3	473	9430	7020	9300	8670	2360	6130	4380	2300	621	6940	5750	1490	2170	796
FiT3	473	7960	5660	7770	7020	1960	5130	3670	1910	510	5960	4730	1220	1770	484
Alba	473	74500	70400	80100	54600	22000	53800	29700	17700	5350	56400	59000	10800	18000	7940
FiC1	473	18000	19200	18100	14800	4420	11900	7430	3920	1090	12700	12100	2540	4150	1520
FiC1	473	14900	14000	15000	12300	3680	9960	10700	3270	912	10700	10000	2120	3450	1200
Alba	473	68300	65000	76500	53100	20500	49500	27400	16400	4950	52200	54500	10200	17600	7640
FiC2	473	68900	66800	77600	53100	20500	49800	27400	16700	5010	53500	51600	10200	16600	7630
FiC2	473	47200	102000	49200	91800	14800	34900	51300	7360	3270	21100	37400	18300	9940	2540
FiC2	473	47800	104000	49300	93000	15000	35500	52800	7560	3380	21200	37900	18700	5380	2630
Alba	473	64800	61400	71900	48700	19500	47200	26000	15700	4740	49900	52400	9650	16500	7530
Alba	473	126000	111000	106000	68400	23800	55400	30400	17300	5030	50400	53200	11200	18300	10300
FiiT1	473	17200	17000	13600	11900	2680	6540	5310	2190	584	6610	5930	1950	2440	1060
FiiT1	473	21000	23000	15800	13600	3080	7500	6030	2550	688	7450	6700	2240	2790	1270
Alba	473	114000	102000	91300	58000	19600	45900	25500	14700	4120	42000	43000	9390	14500	8810
FiiT2	473	23500	21300	17500	17000	3420	8700	6480	2730	749	8400	7960	2450	3220	1190
FiiT2	473	24600	23500	17700	14500	3480	8820	6500	2790	788	8600	8060	2450	3300	1340
Alba	473	115000	100000	88100	56500	19400	45800	25300	14700	4240	42500	43900	9540	14000	8640
FiiT3	473	14500	4770	9860	8220	1940	5070	3480	1760	430	5220	4030	1330	1860	844
FiiT3	473	14100	11600	8710	7680	1740	4540	3160	1610	396	4760	3660	1220	1650	722
Alba	473	107000	90900	74500	47500	16800	39500	21800	12800	3680	37300	39400	8390	12900	7310
Alba	473	107000	91100	72800	46800	16700	39100	21900	12800	3690	37800	39400	8450	12700	7340
FiiC1	473	27900	20100	18400	14800	3680	9730	6580	9600	817	9360	9260	2580	3570	1350
FiiC1	473	28800	9760	19400	15200	3890	10300	6900	3120	856	9840	9980	2760	3810	1460
Alba	473	111000	89600	76100	48400	17300	40700	22700	13300	3860	39500	40700	8890	13100	7480
FiiC2	473	73200	129000	47600	83700	11400	26900	41300	5420	2310	14200	26400	15800	7560	2250
FiiC2	473	67300	135000	41700	77900	9820	23400	38600	4650	2050	12100	23000	14700	6660	1660
Alba	473	121000	100000	84700	54700	19900	46200	25800	15400	4440	44800	46300	10100	15800	8760
Alba	473	166000	147000	158000	106000	47400	115000	63500	35600	10600	112000	114000	20800	34100	16100
Ft0i	473	165000	300000	148000	236000	50000	117000	158000	24200	10500	65500	114000	53100	29900	9410
Ft0i	473	159000	289000	140000	226000	49500	114000	156000	23800	10300	62500	115000	51600	29000	9310
Alba	473	156000	133000	150000	101000	46200	111000	61400	34800	10500	107000	110000	20200	32500	15100

Ion abundance ratios normalised to Alba reference oil for Forties microcosms

	Time days	57/55	71/69	85/83	83/97	83/91	91/105	97/105	83/95	97/99	99/95	85/97	85/95	97/105	83/69
FiT1	56	2.12	2.44	2.36	1.01	1.47	0.84	1.23	1.57	2.33	3.64	2.38	3.71	1.23	1.06
FiT1	203	1.57	2.30	1.67	1.05	1.28	0.68	0.83	1.27	1.63	1.97	1.75	2.12	0.83	1.37
FiT1	473	1.02	1.39	1.36	1.10	0.55	1.62	0.82	0.90	1.44	1.18	1.49	1.22	0.82	0.95
FiiT1	56	2.08	1.69	2.50	1.02	1.52	0.88	1.32	1.61	2.47	3.92	2.53	4.03	1.32	0.93
FiiT1	203	1.50	1.73	1.68	1.00	1.28	0.66	0.85	0.70	1.65	1.16	1.67	1.17	0.85	1.10
FiiT1	473	1.18	1.35	1.46	1.06	0.94	0.93	0.82	0.91	1.55	1.34	1.55	1.33	0.82	0.93
FiT2	56	2.23	2.40	2.33	1.00	1.44	0.86	1.24	1.54	2.28	3.53	2.32	3.59	1.24	1.06
FiT2	203	1.59	1.73	1.74	1.02	1.22	0.85	1.03	1.22	1.63	1.95	1.77	2.12	1.03	1.04
FiT2	473	0.97	1.37	2.58	1.13	1.01	0.94	0.84	0.97	1.40	1.20	2.91	2.49	0.84	0.98
FiiT2	56	1.87	2.45	2.47	1.08	1.52	1.01	1.43	1.69	2.44	3.82	2.67	4.17	1.43	0.98
FiiT2	203	1.66	1.94	1.87	0.99	1.32	0.66	0.88	0.79	1.78	1.42	1.86	1.48	0.88	1.11
FiiT2	473	1.06	1.40	1.34	1.04	1.02	0.82	0.81	0.95	1.40	1.29	1.39	1.27	0.81	0.97
FiT3	56	2.23	2.47	2.37	1.02	1.52	0.84	1.25	1.63	2.34	3.73	2.42	3.85	1.25	1.11
FiT3	203	1.49	1.62	1.66	1.01	1.14	0.87	0.97	1.17	1.60	1.85	1.68	1.94	0.97	1.02
FiT3	473	0.77	1.33	2.43	1.17	0.89	1.07	0.81	0.92	1.40	1.09	2.86	2.23	0.81	1.01
FiiT3	56	1.66	2.20	2.15	1.21	1.55	1.07	1.37	1.80	1.83	2.72	2.59	3.86	1.37	1.06
FiiT3	203	1.58	1.77	1.70	0.98	1.20	0.70	0.85	0.44	1.68	0.76	1.67	0.75	0.85	1.09
FiiT3	473	0.67	1.34	1.25	1.22	0.92	0.94	0.71	0.90	1.54	1.14	1.53	1.13	0.71	0.99
FiC1	56	2.36	2.58	2.52	1.02	1.51	0.85	1.27	1.64	2.43	3.93	2.56	4.14	1.27	1.09
FiC1	203	1.76	1.97	1.96	1.03	1.32	0.84	1.07	1.42	1.87	2.57	2.02	2.77	1.07	1.05
FiC1	473	1.06	1.19	1.50	1.09	1.00	0.99	0.91	0.98	1.14	1.03	1.63	1.47	0.91	1.00
FiiC1	56	2.11	2.55	2.46	1.08	1.73	0.83	1.33	1.70	1.15	1.80	2.66	4.17	1.33	1.09
FiiC1	203	1.74	1.97	1.90	0.98	1.36	0.64	0.89	1.37	1.90	2.66	1.86	2.60	0.89	1.08
FiiC1	473	0.64	1.24	1.20	1.04	0.52	1.70	0.84	1.01	1.28	1.24	1.26	1.22	0.84	0.99
FiC2	56	2.41	2.78	2.74	1.03	1.58	0.85	1.30	1.73	2.62	4.40	2.82	4.75	1.30	1.10
FiC2	203	2.45	2.93	2.84	1.05	1.61	0.79	1.22	1.79	2.74	4.70	2.96	5.08	1.22	1.07
FiC2	473	2.26	2.76	2.69	1.00	1.58	0.99	1.56	1.77	2.57	4.56	2.69	4.77	1.56	1.10
FiiC2	56	2.09	2.60	2.50	1.10	1.82	0.83	1.37	1.93	2.55	4.46	2.75	4.82	1.37	1.10
FiiC2	203	2.27	2.81	2.64	1.02	1.50	0.77	1.13	1.68	2.62	4.30	2.69	4.42	1.13	1.08
FiiC2	473	2.30	2.82	2.85	1.02	1.65	0.71	1.15	1.86	2.83	5.15	2.90	5.28	1.15	1.04
Ft0	0	1.89	2.50	2.58	1.09	1.59	0.92	1.35	1.89	-	-	2.81	4.87	1.35	1.07
Ft0	473	2.09	2.39	2.46	1.00	1.50	0.77	1.16	1.75	2.50	4.37	2.46	4.30	1.16	1.09
Ft0	0	1.84	2.37	2.47	1.09	1.57	0.92	1.33	1.87	-	-	2.69	4.61	1.33	1.06

An assessment of variability of measurement based on measurements of At0 samples over the course of the trials

	57/55	**71/69**	**85/83**	**83/97**	**83/91**	**91/105**	**97/105**	**83/95**	**97/99**	**99/95**	**85/97**	**85/95**	**97/105**	**83/69**
Mean	1.045	0.997	0.999	0.984	1.060	0.895	0.969	1.002	1.037	1.058	0.983	1.000	0.969	0.999
Standard Deviation	0.059	0.019	0.025	0.049	0.078	0.095	0.153	0.031	0.076	0.106	0.045	0.036	0.153	0.024
Relative Standard Deviation	5.6%	1.9%	2.5%	5.0%	7.3%	10.6%	15.7%	3.1%	7.3%	10.0%	4.6%	3.6%	15.7%	2.4%
95% Confidence Interval	0.047	0.015	0.020	0.039	0.062	0.076	0.122	0.025	0.067	0.093	0.036	0.028	0.122	0.019
95%CI as % of mean	4.5%	1.5%	2.0%	4.0%	5.8%	8.5%	12.6%	2.5%	6.4%	8.8%	3.7%	2.8%	12.6%	1.9%
n	6	6	6	6	6	6	6	6	5	5	6	6	6	6

D.5 GCMS SIR data for analysis of microcosm samples (see chapter 6.5.2, 7.4 and7.6)

GC-MS SIR data for Alba microcosms (substituted phenanthrene and dibenzothiophene peak areas –appendix B for key)

	Time days	C1P-1	C1P-2	C1P-3	C1P-4	C2P-1	C2P-2	C2P-3	C2P-4	C3P-1	C3P-2	C3P-3	C3P-4	C1D-1	C1D-2	C1D-3	C2D-1	C2D-2	C2D-3	C2D-4	C3D-1	C3D-2	C3D-3	C3D-4	C3D-5	C3D-6
Alba	0	1450352	1600355	3032540	1617958	3874384	1733680	866132	1256566	876826	1502304	2024150	1644905	2018630	1387685	949457	1400829	2819179	2410840	1155802	627060	1251844	858519	1168775	1168775	544385
Ait0	0	1232997	1351894	2510133	1363514	3290530	147068	744716	1069055	786513	1256132	1733480	1411968	1643070	1086938	750877	1126495	2368432	2087931	997847	540080	1076161	842810	799358	1043501	485637
Ait0	0	1303304	1284508	2588234	1436440	3358425	1485743	673107	1015102	765344	128190	1833873	1437867	1643256	1102587	768081	1178005	2370000	2096910	1029521	553497	1083762	727862	805560	1014360	468000
Alba	0	1152551	1192708	2292071	1184501	2973461	1326765	639980	903972	662320	1057588	1437150	1148830	1465510	971593	682526	997365	1983761	1684045	788732	450977	877874	607707	655592	836978	389587
Aiit0	0	1381413	1407665	2667510	1482035	3543394	1594730	730625	1040668	755839	1307666	1797217	1440456	1749802	1184609	788992	1170735	2381318	2059374	1001464	537712	1087263	711455	784752	1014017	471765
Aiit0	0	1286190	1261727	2442900	1359668	3231541	1440064	653083	911037	649266	1085571	1555784	1202842	1632979	1087267	738012	1047439	2104368	1832886	893615	475412	933492	642281	678403	905189	411729
Alba	0	827812	886853	1674348	949559	2109640	866699	497578	626360	456690	738258	1073483	853818	1105103	722298	517891	710602	1432972	1207613	575977	324527	635513	439838	480135	596528	274648
Alba	203	195185	277852	452236	282852	512913	237833	146458	193737	103142	136933	185209	129692	30642	204249	148174	167948	331345	296506	135270	81886	154254	92318	118557	141060	55997
AiT1	203	6424	11303	27145	20774	95781	61246	40894	49968	45498	62549	89177	55430	27808	22348	14459	61394	93119	112369	41432	38326	72583	46966	66545	71550	28124
AiT1	203	3784	5720	14245	10675	49666	33881	24654	27013	24006	31724	48566	27097	16026	12320	7691	32098	47585	59253	21192	19102	36455	21745	35255	36147	13032
Alba	203	72568	95965	168463	114137	180115	91737	62712	68207	36025	41868	62121	65191	119002	68270	56821	58437	115293	99282	41627	25317	44036	24239	36504	40804	13421
AiT2	203	1203	2181	5685	5434	20033	14428	10675	10494	11683	12995	20630	9689	5584	4803	3247	9087	18221	23124	7922	6472	11459	7846	15127	15218	3917
AiT2	203	441	2078	3399	3094	10584	8858	6079	7957	5706	6302	10366	4599	3213	2787	1957	3853	9531	11847	4022	3135	5803	3902	7034	7128	1841
Alba	203	48703	73773	109792	92544	132621	69356	41560	54395	25618	27338	41988	24087	81195	47827	37840	37643	80235	71078	28505	15946	28723	15853	25783	27054	7736
AiT3	203	432	1819	2805	2898	13800	10391	7109	7174	7909	9402	15269	6686	3180	3244	2246	6258	13358	18113	5919	4591	8366	5797	9686	9954	2585
AiT3	203	561	2499	3289	3271	15610	12365	9282	9760	9290	10649	16736	8986	4107	3715	2264	7690	15520	20347	6289	5125	9591	6708	11062	11732	3040
Alba	203	49097	68466	111134	84236	104906	55154	35674	35156	20023	21883	35194	19701	79865	45057	35915	34414	72639	60182	24005	12894	22583	12520	20041	23198	7344
Alba	203	41251	58954	92367	69786	87666	47875	30040	31306	16416	18281	30066	16615	64916	36080	29002	28199	58296	46886	18720	9916	17476	10716	17039	18869	4976
AiC1	203	4851	14937	26402	21315	50921	32088	19659	25373	9459	12008	19544	10357	21301	11298	11578	15001	30248	29454	11695	7111	11935	6309	10663	10035	2672
AiC1	203	4709	14180	26164	19365	45642	27134	15928	20872	9061	11981	19466	9756	23006	10742	11407	15575	29900	28795	10977	6047	10234	6160	9681	9655	2525
Alba	203	41958	67334	110202	74797	103496	53831	31996	40010	18857	22905	36608	19141	74896	43579	35499	33897	67538	55704	21520	11683	18877	11739	11369	19791	5116
AiC2	203	21903	41521	58112	60733	58094	33051	18143	20976	10647	12450	19077	11545	38693	21837	19382	16811	35260	29165	11513	5506	9541	6088	9817	10436	2763
AiC2	203	33921	56291	79477	79583	82997	45574	24157	28421	16189	15840	26512	16031	55723	30360	25909	24951	51201	41480	17192	8700	14576	8687	14167	15600	4377
Alba	203	43087	63765	102932	82939	84353	47354	32076	32286	16141	16336	28247	16557	70911	40330	32476	31990	64883	51524	20043	11415	18604	9868	16525	19050	4926
Alba	203	139019	242510	341371	307009	456564	251590	169127	17331	90398	105521	172452	91843		160989	123236	133502	261786	228074	91184	56010	100863	56296	87113	95882	27917
AiiT1	203	3477	7425	16697	15387	73772	45682	30638	39922	30216	41551	68318	36239		15205	9846	39295	56576	70581	23453	21995	43943	25157	43810	43929	13886
AiiT1	203	3117	6973	15695	12041	65424	49720	27588	35868	28276	42110	68834	32236		14183	9589	35740	53363	66700	22583	22096	39857	24399	41968	42179	13765
Alba	203	86966	120675	211970	150395	272475	137801	84294	107238	48276	65177	93840	52596		88715	69037	77652	153322	135840	58429	34185	62810	33520	51755	57008	20867
AiiT2	203	1188	4489	7129	6615	38952	24909	15236	20423	18544	23021	36458	16455		7214	6222	17101	30636	40326	12693	12568	22876	14304	26275	24564	7132
AiiT2	203	1238	4539	7372	6169	33962	22331	14220	19498	15001	23073	35136	17194		6522	5182	15774	26949	37729	11710	10084	19620	11836	22152	21919	5801
Alba	203	77288	111980	182281	136326	225559	111817	66969	87930	38914	46718	68667	47977		78424	61799	66800	130552	115805	48187	27786	49615	26915	44887	45837	15444
Alba	203	73934	120861	173977	128520	222812	115629	71528	90442	38908	51360	80197	45505		74985	60259	67508	127076	112537	46262	27174	48563	25580	43344	45147	13259
AiiT3	203	4398	12891	26107	19308	79272	52220	37490	36894	26308	32596	51678	28389		17464	17237	37777	62089	68338	25657	16705	30430	18241	31898	30830	8295

	Time days	C1P-1	C1P-2	C1P-3	C1P-4	C2P-1	C2P-2	C2P-3	C2P-4	C3P-1	C3P-2	C3P-3	C3P-4	C1D-1	C1D-2	C1D-3	C2D-1	C2D-2	C2D-3	C2D-4	C3D-1	C3D-2	C3D-3	C3D-4	C3D-5	C3D-6
AiiT3	203	4607	12040	23545	17238	72246	53924	26903	36099	22993	28668	48415	27362		15572	15973	33069	57409	61820	23447	15438	28965	17048	28023	28405	7841
Alba	203	65390	110554	159439	131142	204884	104316	67845	73540	36626	47588	74833	41977		69332	54411	62463	120921	108350	44182	26000	46477	24343	41646	43604	13000
AiiC1	203	4285	14352	24542	19747	76356	57791	30395	42701	23816	28265	46310	25157		15589	17825	34855	59738	65204	24644	16402	30325	16753	29468	28738	8005
AiiC1	203	4190	14140	26037	19187	81757	60923	30432	39763	27077	32119	53647	28798		18150	19786	38778	68228	73863	28122	19002	34503	20239	33132	33606	9819
Alba	203	69068	117712	170473	150895	223952	116309	82856	89373	39204	53107	80197	43181		76787	60943	68474	132096	117071	47612	28723	50716	28848	44815	47610	14603
AiiC2	203	43655	86115	108409	104858	146417	92472	44291	61254	28769	34356	53490	28430		47340	40270	45045	87865	78479	30829	18288	32408	19321	31041	32350	8727
AiiC2	203	43583	86869	111638	104740	161125	87454	61080	64140	30266	37900	58644	30811		48420	41663	47958	95460	84126	34783	20385	35652	20470	32824	34881	9339
Alba	203	87143	141548	211539	158847	273536	135322	82573	105890	49641	60305	87629	49925		87868	69294	77067	154835	138822	56623	33593	62058	33195	54332	56138	19101
Alba	203	71419	113322	164804	131610	201243	102492	64933	86902	33601	46371	67936	36945		70717	56440	57914	114852	101882	42341	22551	40155	21149	36812	38532	12501
At0	203	109260	146747	250174	195682	309676	155133	106073	117669	54260	67985	102669	61174		111799	87783	89014	184177	167633	72073	36250	66227	35956	54816	61873	21541
At0	203	105175	145935	244303	177612	291759	147716	89450	114440	52058	66082	90970	57893		110180	88192	83915	175515	158356	67792	34755	63196	34699	55877	59820	20288
Alba	473	157943	185974	312538	179305	460360	201199	121324	159338	107473	151298	214104	158828	256627	179195	126762	139333	290100	241764	117614	67472	132905	82789	100788	129156	55079
AiT1	473	558	643	868	782	5453	3057	1615	2049	2954	5115	8319	5285	1862	1108	729	2768	4532	4391	1881	2373	4715	3190	4190	4970	2076
AiT1	473	848	723	1031	681	6455	3396	1976	2378	3714	5751	9831	5846	1935	1489	918	3101	5476	5195	2222	2764	5645	3853	5078	5877	2415
Alba	473	161665	188931	309423	192499	471831	227343	142343	170000	132598	193276	267862	219238	361954	244945	176433	179577	328720	308454	148850	88219	177752	115775	135977	174927	76660
AiT2	473	339	443	707	441	4156	1812	902	1303	1961	4273	6790	4176	1226	892	533	1785	2769	2838	1301	1752	3485	2460	3301	3641	1662
AiT2	473	404	442	569	631	3632	1599	765	1199	2192	3682	6435	3939	1140	947	404	1535	2631	2644	1160	1617	3287	2306	3248	3570	1582
Alba	473	159186	199583	326706	191616	495287	227353	159184	176239	134678	194345	263140	226612	351706	239788	187141	186698	353763	326011	160016	90631	177557	120085	136041	172291	80115
AiT3	473	866	830	1113	811	7397	3061	1901	2442	3859	6702	11523	4779	2659	1629	1171	3005	4989	5159	2338	3075	6189	4427	6231	6614	2402
AiT3	473	604	697	1294	721	5768	2686	1403	2169	2740	5618	9071	4623	2128	1611	895	2323	3868	4225	1861	2360	4741	3606	4726	5057	1974
Alba	473	233204	27361	438001	248731	644421	296911	189185	228993	155380	235402	337401	284383	517079	339146	255063	315924	449572	419947	211344	121090	235858	153139	171267	226656	101519
Alba	473	240546	287101	473701	291224	675465	309514	205910	238847	163153	242274	345104	210644	500837	344385	247733	252796	482636	427689	218106	125151	238483	157387	178013	229268	102265
AiC1	473	1632	2259	3469	2316	27822	17009	8304	13458	45042	69520	91125	39023	6201	5798	3047	13398	21278	60185	18616	29396	60732	50802	75962	81898	41366
AiC1	473	1580	1821	3922	2948	30534	17684	9627	13946	50979	74410	98265	42354	7198	7518	4393	14627	22401	65936	18876	34786	67002	59475	86413	92212	38206
Alba	473	271746	347002	437408	332227	745040	370329	208839	277737	189169	268770	369028	287965	545781	369758	266630	276797	565137	465868	224720	133440	264796	169678	196462	258853	113470
AiC2	473	104323	134599	219673	123150	320078	146328	95757	109735	76541	108072	148245	123096	191047	130529	93641	132590	187513	176000	82741	48308	96674	62666	73274	93059	39944
AiC2	473	102554	125479	207535	119712	288528	132703	85890	99344	67809	93712	135718	105161	171340	116929	85164	95550	171908	164376	74733	43512	86737	56543	64327	84698	36064
Alba	473	334563	382105	622621	388199	897414	404211	237330	298116	216241	296112	413289	313858	562981	382033	271233	383129	549358	500202	235737	145843	279139	175219	206941	269552	115424
Alba	473	522936	654735	1076761	624727	1283702	677532	327734	403921	287221	389807	527503	411138	676613	470492	323222	383552	748921	677556	323877	214259	419872	256649	310237	390103	167570
AiiT1	473	3152	4299	7294	4552	28023	13186	9394	10502	11761	17618	29950	18700	7055	4778	2757	10970	18913	18127	8393	10247	21431	14362	18788	20915	8618
AiiT1	473	1794	2713	4045	2678	14706	6956	4562	5468	5895	9007	15277	9306	4034	2753	1883	6522	10816	10409	4871	5721	12079	7966	10018	11889	4717
Alba	473	220487	263140	446960	243180	543956	257597	143495	173093	100039	136103	190604	152084	290435	200959	141633	169936	331603	301257	141774	88865	173722	109494	127869	163491	72344
AiiT2	473	1871	2669	3935	2360	12677	6178	3714	3916	4377	6634	11104	7112	3352	1952	1526	4990	8344	8340	3790	4367	9174	5628	7318	8560	3592
AiiT2	473	1512	2312	2930	2064	11170	5466	3403	3298	3807	5956	9543	5577	2924	1950	1127	4437	8035	7768	3545	3872	8099	5339	6654	7999	3236
Alba	473	153927	204913	302623	197449	385331	180369	108685	116628	75679	103274	150677	110074	216980	149494	104904	131495	262737	234692	111201	70613	141474	87450	100443	133209	57939
Alba	473	117409	155224	226494	152559	300765	143858	88081	92259	56602	73106	108655	83747	168662	113147	81985	102313	198891	183344	84659	56091	111547	70325	78587	103792	44754
AiiT3	473	1484	1597	2577	1750	13115	5844	3345	4453	5426	8490	12099	7002	2823	2226	1204	5220	9180	9706	4024	5664	13019	8604	12290	13248	4855
AiiT3	473	1046	2011	2646	1309	12307	5586	3299	4261	5756	8318	12712	7103	2810	2065	1612	5388	9473	10100	4135	5647	13122	8652	12895	13836	5226

	Time days	C1P-1	C1P-2	C1P-3	C1P-4	C2P-1	C2P-2	C2P-3	C2P-4	C3P-1	C3P-2	C3P-3	C3P-4	C1D-1	C1D-2	C1D-3	C2D-1	C2D-2	C2D-3	C2D-4	C3D-1	C3D-2	C3D-3	C3D-4	C3D-5	C3D-6
Alba	473	97676	109632	177708	116541	276476	127889	80164	72520	54744	72384	104556	80090	144308	95341	70379	97183	188396	172508	76896	56894	113606	70427	80535	104448	45627
AiiC1	473	1353	1311	1982	1875	11436	7232	4192	4749	13973	18753	27117	18328	2526	1853	1504	6323	10994	25811	7274	16462	31484	25157	68741	38969	15485
AiiC1	473	920	1236	1804	1221	11288	7199	4199	5581	14815	19477	28520	18459	2732	1799	1505	6740	11541	27047	7910	17426	33190	25919	41320	41984	16779
Alba	473	103308	127409	185861	135485	308920	151480	98068	102419	67142	88906	129907	96433	161408	105102	75961	116912	231187	210451	94796	70313	141236	84713	99328	126050	55152
AiiC2	473	31105	39868	61426	39111	97176	43435	23732	28198	17007	20954	30091	22040	46293	30230	22354	33477	65821	59441	26369	18413	36492	22396	27241	33056	14334
AiiC2	473	32519	44494	70333	44876	107003	50462	27539	32489	19357	25798	35377	28154	51961	33139	23818	49659	76357	67869	31069	21978	42692	26298	31431	37425	17204
Alba	473	100703	121270	193550	139707	307634	147919	94298	91995	66299	82864	131672	96373	159743	106092	76738	117196	234444	212813	92688	70222	145826	87918	102456	129535	56588
Alba	473	85061	105558	167899	100864	269413	149951	70466	82145	60116	74921	114353	82890	140840	83276	66501	115579	227194	200944	90516	71644	136580	82400	98069	122691	51933
At0	473	85881	97043	159480	111550	275039	135812	75323	78840	60954	79557	114317	76255	143835	91854	68798	117127	223829	206977	94632	71809	136300	81535	93436	122289	53671
At0	473	95417	130081	189738	129014	347803	174726	97746	106444	84631	102474	138411	101607	180226	114725	82579	147202	291955	254621	114737	87271	169241	103620	123046	153017	65284
Alba	473	98571	135914	193451	134460	353199	175479	97321	104778	80294	100959	150908	103840	177438	116605	84548	142564	285641	252172	115171	86330	161273	97013	116314	145392	63654

GC-MS SIR data for Alba microcosms (sterane, triaromatic sterane, pentacyclic triterpane & βsterane peak areas –appendix B for key)

	Time days	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	TARS1	TARS2	TARS3	TARS4	βster1	βster2	βster3	βster4
Alba	0	349683	225205	339913	138077	109872	431979	584314	154924	204861	139556	225456	218166	171212	287908	455454	322170	167162	1157903	87334	1532729	137843	744950	489991	156712	544002	430911	467627	294323	472651	358357	1637654	873968	983688	981398	855803	814604	991033	169473
Ait0	0	316559	210841	264634	208048	104662	401664	530948	152854	184365	126247	184668	221158	151337	281720	444528	262061	195140	1067714	87524	1461522	126468	726068	478610	155470	550680	371554	422786	279268	451295	338556	1610735	863112	1085130	959114	816696	1025534	1035930	199781
Ait0	0	326459	224883	316618	208002	89964	432612	564861	158520	173046	130901	231487	231400	169129	307913	499648	323994	189878	1138639	121819	1544819	117141	733913	504593	163501	538205	410499	450126	287270	454540	349477	1622328	961129	1055034	1064458	901050	1061159	1114542	213969
Alba	0	255187	172300	237030	173161	80683	334928	463910	130489	158941	93342	178635	194677	147774	218815	371696	237263	168790	875904	72556	1161074	115236	560078	388670	132699	424119	325896	337036	222897	355242	246783	1210034	660353	806260	788031	708376	846379	834639	160003
Aiit0	0	335027	211784	242011	234797	107448	452024	568712	170400	178587	125601	223822	228056	179637	268794	458825	300892	166391	1140784	90921	1472140	138417	626509	430402	137875	553345	366718	400557	261379	382779	278192	1502137	806314	975858	937819	888556	1042903	1029065	202289
Aiit0	0	276944	185607	259878	189961	88862	382867	507954	157820	189207	119554	202393	202560	159961	245648	415607	287787	143556	1006068	78333	1315258	107005	630057	409866	132759	492128	328726	354449	223948	338733	265369	1343827	740475	844587	808424	795837	951278	937018	171759
Alba	0	190437	130236	178386	123428	55497	257901	326475	101761	124935	76896	140956	140013	91889	148090	279806	183946	112949	636582	62281	852350	74471	381358	241640	80748	284636	188570	220662	132940	207687	157374	854910	463758	428360	556759	854910	463758	428360	556759
Alba	203	25806	17379	21243	13029	6187	29229	31602	7850	13006	5814	11540	13981	9006	15793	56266	11789	14635	98044	8229	118251	11523	49911	38912	13854	28553	21738	27845	15278	23768	14689	118079	50808	53754	56851	63885	62076	65101	14186
AiT1	203	18621	10445	15363	11720	6640	21820	21154	5673	10258	4506	9618	12345	7256	12573	41174	28923	11249	70692	5354	84693	8624	37796	25862	8465	17428	13931	21178	9570	17969	11836	74170	27168	29075	29111	38074	42429	42341	9262
AiT1	203	9612	5989	9683	7384	3845	15324	12617	2539	5025	1925	5192	6214	5685	7063	27126	20066	6267	45488	2815	53677	5431	21500	20137	5190	13697	9509	10841	6916	10771	6065	45534	15226	15096	19471	28566	27218	26970	5831
Alba	203	8910	6017	7703	4495	2564	11580	10923	1987	5105	1550	3980	5437	6001	5081	21761	14504	5431	35899	2149	44841	4279	16948	16846	3651	10013	7771	10075	5794	8650	4377	37213	11626	13186	17237	23531	23247	23954	4774
AiT2	203	7152	4389	5772	4321	1517	8047	6217	1100	2883	877	2452	3133	3260	3901	15900	10619	3749	25372	1045	29472	3193	10353	10034	2265	6404	4424	5191	3835	5034	2886	21011	6248	6627	5600	15318	16507	15486	2967
AiT2	203	4415	2300	3192	2039	874	4768	4513	903	2097	399	1288	1383	1438	2085	11546	7586	1916	18291	1546	25003	1949	9102	8300	1931	4826	3696	4268	2482	3379	2547	13708	4740	4248	4608	10083	12275	9885	1839
Alba	203	7563	4412	5768	4411	1715	7964	8530	1549	4426	745	3414	4427	5495	4323	17808	13446	4321	32932	2743	39943	4384	14395	14447	3641	9042	6345	8661	5420	8024	3846	30114	9102	10810	12293	16724	17483	19438	4054
AiT3	203	6037	3134	4259	3003	1475	6875	6445	1155	2874	845	2450	3684	3106	3556	15463	10333	3705	26979	1170	34940	2505	13061	13872	2992	7418	5303	6471	3600	6755	4157	23067	6792	8148	9011	14263	14864	15879	2893
AiT3	203	6837	3817	5542	3206	2545	8128	7356	1326	3054	937	2965	3752	2979	4128	16699	13870	3923	33759	1878	41412	4006	15725	16378	4194	9942	6329	7871	5954	7700	4844	27185	8118	8786	11639	15460	16723	16557	3460
Alba	203	6588	3622	4271	3851	1044	6435	8051	1201	2746	1026	2601	3895	3160	4271	18958	15149	6127	34286	2081	45664	4556	18370	19842	4211	10476	7188	10056	5943	10147	7034	30946	9361	11529	15783	15393	16181	19265	4415
Alba	203	4076	2072	3749	2853	663	4413	5142	1114	894	470	1635	2436	2780	2414	12680	8181	2661	18709	787	25797	1998	7934	8719	1479	3523	2721	3545	2705	3379	1619	15356	5067	4533	6113	10943	10733	10537	1869
AiC1	*203*	5660	2766	3797	2042	1129	6365	4997	494	1651	422	1560	2031	2883	2250	12218	8722	2096	20773	1141	27133	2325	8696	8277	2000	4467	3184	3252	1929	2624	1088	16279	4942	5809	4476	12082	11153	11826	2360
AiC1	203	5400	3008	3918	2342	1317	5717	4518	562	1757	230	2322	2643	2804	2356	11908	8913	2898	20687	1342	*26297*	*1954*	7793	7741	*1582*	4075	3436	4021	2987	3299	2375	16966	4852	5282	4535	12085	10323	11270	2161
Alba	203	5460	3191	3483	2721	1002	4732	4883	662	1533	400	1819	3205	2626	2537	13260	8264	2380	20419	1278	26817	1417	7993	7492	1982	4028	2848	4472	2787	4101	2721	19878	4429	6132	7392	11388	10930	12092	2154
AiC2	203	3980	2107	3136	1993	509	3484	3765	380	1098	312	1303	1604	1206	1866	10694	6311	1736	16586	1000	23597	1744	6919	6395	1329	3550	2464	3256	1992	2741	3216	12339	3744	3061	4037	8880	8720	9093	1764
AiC2	203	7174	3573	4438	3084	969	6644	7388	968	2859	719	2435	3775	3671	3516	15842	10944	4917	28577	2118	37160	3684	12645	11645	2920	7470	5123	6416	3265	6110	4613	25851	7254	7129	9157	16408	15594	16622	3160
Alba	203	4544	2362	3265	2212	883	4621	5249	784	1857	490	1795	2271	2317	2154	13508	9581	2921	22131	1746	28664	2635	9868	10973	2075	5421	3775	5682	3267	5701	2347	19207	5332	5804	7458	10691	11581	12167	2110
Alba	203	43784	27452	33755	18352	12259	49313	47986	7209	23672	6330	19685	24539	32659	25840	85146	55558	19477	142945	11667	175221	18100	58807	73131	15238	36505	33092	32308	20903	33012	12652	95033	28965	35114	43225	96419	101139	108765	23562
AiiT1	203	22250	13282	19268	11598	9025	27509	25399	4445	11625	3131	11262	13973	16640	14846	46409	31847	12255	84690	7960	105955	9534	36602	42413	7896	18339	13130	19052	12953	13377	8450	54201	19542	22425	17032	55901	56711	63909	11770
AiiT1	203	23597	14850	18063	9714	8055	30466	25875	4718	12468	3313	10343	13110	13156	14060	44175	32142	12919	81061	5335	101193	8145	33478	38701	7276	18849	12681	20296	16277	13063	7219	54262	18307	24115	17185	54844	57787	61727	11439
Alba	203	23472	13983	17062	8974	6337	23878	19776	3477	10848	2556	7621	10587	14179	10554	34573	22018	8097	59326	3879	69389	5821	25730	25913	5133	11700	9149	11120	7308	9820	5930	46137	15382	16036	10062	47108	43180	45912	9451
AiiT2	203	15126	7284	11585	6820	5314	17919	15291	2758	8458	1538	6289	8030	12026	8444	29404	21604	6442	54894	5155	64685	5464	19351	21260	5037	10652	7324	9527	6211	7573	6101	29685	10245	11041	9744	30622	34557	37007	7277
AiiT2	203	12981	7629	9725	5369	4352	17768	11889	2238	7113	1159	5028	8142	8186	6895	27923	18514	6950	49361	4131	63918	5714	18910	23682	4139	8892	7531	8586	6319	7370	3813	23538	9121	8845	6437	37204	30472	32039	5468
Alba	203	18480	10810	13190	7139	4969	17724	16337	2249	7342	1771	6583	8595	10715	7526	31056	20542	6859	48240	3868	60692	5075	18654	22786	4680	10267	6720	11032	7614	7910	3947	33801	10811	12320	11479	36466	34801	38782	7575
Alba	203	18787	9395	12816	7147	4362	18340	15056	2152	7310	1519	5568	7660	11163	6590	29606	19307	6266	46334	2703	56990	3582	16039	21732	2507	8136	6071	8333	4879	5915	4945	26352	8496	10147	7473	36057	33443	33848	6655
AiiT3	203	15529	9398	12080	6166	4191	16408	15097	2218	7077	1190	6525	10293	9301	7601	31411	20497	7924	56821	4173	70009	6057	20779	26508	4310	9736	6359	10241	6408	6700	6133	28986	11107	10086	7715	32126	35494	37583	7168
AiiT3	203	14691	8391	11190	6434	4503	17425	13941	2560	6737	1125	5937	7966	11895	6971	28729	19614	6073	51008	3306	63458	5379	20010	21237	3813	9298	7029	8412	5997	8371	5034	24495	8391	9861	6482	31617	32219	34729	6088
Alba	203	16703	8393	11309	7067	4425	17978	14836	2190	7475	1542	5544	8436	8808	6759	29797	19663	6266	47631	3154	60051	5209	19388	20194	4801	9285	8696	9630	4542	7289	5297	28523	10199	11591	8764	33437	32709	35512	6032

	Time days	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	TARS1	TARS2	TARS3	TARS4	βster1	βster2	βster3	βster4
AiiC1	203	15025	8694	10798	5838	4083	16491	13423	1858	7145	1109	5881	7313	10767	7435	30749	18977	8553	50460	3621	62508	5210	19535	21041	3986	8988	6016	8885	5276	6380	4542	24859	9286	9867	6005	31024	31561	33227	5754
AiiC1	203	18403	10553	12882	6892	5603	20411	15886	2301	8099	1381	5929	8778	13025	7143	31461	21167	6934	55096	3436	65795	4887	19794	51510	4186	9664	8811	9296	6907	8689	5409	29241	9420	11429	6171	37546	35232	37830	6666
Alba	203	19809	11139	13747	8238	6812	21753	17264	3807	8668	1915	7634	12226	11890	8686	33745	23196	7771	55144	4567	68856	5332	23287	24105	4283	10141	7533	10692	5452	8750	5652	35640	11532	13729	9795	40864	38978	41686	7612
AiiC2	203	16127	9369	11139	6341	4338	17770	14227	2465	7628	1175	5016	6814	10025	6328	28568	19993	5817	46845	4027	60832	4306	17065	17641	3925	8398	6716	7096	4770	5581	4957	22808	7444	9208	6442	32732	32533	33155	5574
AiiC2	203	18553	10331	12990	7834	5153	20546	16395	2387	8016	1904	6798	10446	12008	8408	33500	21009	6971	55582	4094	70845	4438	22214	25470	4078	10959	9138	10185	6101	7896	5205	27807	10759	10399	8385	37670	36489	39945	5114
Alba	203	23793	13818	15693	8400	6212	25687	19338	3344	8650	2229	6961	10449	11045	8350	33756	22547	6017	51132	4403	58910	5328	20372	24809	4068	9833	8134	9303	5219	7624	4245	35345	11722	12525	10436	43902	41548	42398	8044
Alba	203	12591	8135	11439	6653	4793	17087	14333	2407	7764	1755	6407	9299	7716	7332	20200	11452	6147	39170	2995	51126	5370	20701	18744	3921	9651	7927	9126	5931	7756	5063	44124	15345	21363	11596	32267	30834	32359	6652
At0	203	19599	12035	13835	8627	7414	22461	18967	4702	8216	3059	9644	14153	11961	12036	32512	15992	8548	61167	3869	70827	6926	29436	24003	6613	15512	10035	15619	9168	12979	9074	76951	28501	37864	22776	42909	40957	43878	9448
At0	203	17072	10159	13282	7635	5900	20075	16193	2753	7312	2619	7524	9162	10488	8803	27938	13602	7929	46989	3268	59417	5882	21597	20722	4985	11596	8649	11633	6412	10170	5947	56751	18985	30864	15380	35343	35885	36397	7203
Alba	473	28383	21509	28512	18370	8447	31406	36747	12445	17140	8845	19829	22726	14702	19456	25205	18005	14632	80261	3847	90452	7254	40232	28740	10623	26424	19819	20947	13266	18201	14167	171381	81749	84185	100564	65213	66032	83035	14169
AiT1	473	5845	3479	11073	10783	1911	18246	20202	6160	11475	2653	9964	16027	12335	9313	10472	2423	3166	14808	1245	13599	1653	7151	4430	4318	5890	4797	5518	2807	6994	8252	115389	69700	67290	80038	21558	54568	49576	11136
AiT1	473	6610	2633	17406	12599	4002	20922	23599	6376	10035	2933	10109	19311	12809	11389	11911	3211	3776	18103	1250	14978	3147	7701	5548	3089	4545	3876	5924	2928	6395	5262	123554	77945	77724	84207	26637	53437	62791	12455
Alba	473	39154	21605	24831	16828	8836	35735	39883	15522	20767	8963	25443	25211	13773	22450	21384	19543	10074	52438	5791	76805	8711	39799	24004	9035	27475	21465	21886	15093	18599	15164	246676	130782	116312	138412	71788	71154	77434	14443
AiT2	473	5166	5075	17472	11033	2872	19734	23002	6895	11968	2755	12215	15206	12562	9683	11734	2185	2649	13015	1516	13269	1585	5777	3858	3710	5528	3771	5546	4321	7193	6866	129720	80121	73140	83661	26290	52125	54072	12171
AiT2	473	7654	5596	16385	9460	1997	17634	19623	6641	12072	1849	12533	14906	12790	9647	11046	2656	1429	16699	1196	12055	2058	5956	4048	2329	4047	5422	8104	5561	16549	9368	112879	76410	73206	83634	22844	50707	52863	9439
Alba	473	32936	21067	30327	18560	8087	35030	39129	13577	16589	9348	24191	23979	13206	21498	22634	16846	9414	55294	2754	72966	7292	34997	23471	5943	27586	19400	19260	13422	19430	10168	224033	108461	111027	128875	64356	75688	71010	13631
AiT3	473	13949	6242	26460	18577	3088	27807	29158	6602	13818	1924	11988	19590	13054	12369	19288	4608	3287	21488	3181	17426	2848	7985	4904	4547	6644	4655	8448	3081	10737	8149	164918	110077	108176	122953	43567	81584	75495	18077
AiT3	473	9586	7191	17370	13451	1810	22033	24553	7528	15874	2302	11301	17292	11714	9882	13939	5180	3153	20277	2353	14516	2459	6651	3823	4372	7234	3551	9005	3337	11121	9050	135102	88757	90254	100947	33535	65072	61947	15934
Alba	473	43024	22133	29572	22044	8333	39681	46531	18726	22465	10320	30358	28784	18004	22769	33359	18854	15361	80470	6427	97931	10097	48329	32382	11995	33953	30382	30505	20382	31297	25426	266421	127878	132527	154024	83833	85866	85926	15293
Alba	473	44920	23694	38405	22995	11371	41460	51740	19717	23660	12022	27608	31433	19605	26783	32297	22357	12801	84755	5245	110033	12270	51362	37145	10005	39695	28028	33570	21758	38894	23636	277438	144984	139818	171212	89815	93889	94265	18173
AiC1	473	33783	17637	31044	17368	10006	38898	39441	17025	24088	6953	24472	27482	17050	21132	41366	19426	13072	71880	2480	99312	10431	49095	29348	11757	32323	23369	32601	19909	28982	17249	238738	127182	135055	148905	68756	89384	87597	16056
AiC1	473	34520	17789	28681	18472	7167	39166	47736	17395	22659	6749	25321	29747	20927	22567	34009	22571	14147	79434	3003	99567	9673	49883	30279	11742	32438	23358	29864	23241	31635	15742	213876	125748	123247	141673	71268	90397	95510	19862
Alba	473	56616	29381	43685	24072	12320	53126	59778	24316	28744	13213	39043	28913	22263	34712	46381	33124	42314	101668	7022	139751	11261	66441	45303	15790	50951	37501	46117	29761	47493	28875	280596	150344	161601	173504	110684	112987	115531	20064
AiC2	473	20667	13525	19671	12953	5058	23074	29835	11544	14525	6314	18250	17000	11715	16287	19084	13614	7199	50294	3468	67140	7275	32354	22123	7700	23177	16509	17595	15267	19459	12974	144444	79109	75263	92547	51175	53887	55426	11974
AiC2	473	23487	13841	21034	13307	4276	23063	30241	10681	13467	5944	15342	18102	11208	15744	22253	12764	6950	46658	3335	61776	5719	28849	20279	6940	21723	17547	17110	11441	16839	9711	130636	67715	64936	82675	49242	57463	55740	12809
Alba	473	65306	29042	48735	30584	10915	55700	68408	22115	26602	14497	34356	37178	21981	29648	55996	62215	21349	106716	6192	139183	13688	66195	44780	18760	46730	38211	37796	25099	41012	31251	287715	152805	138078	146646	103802	133236	116306	21429
Alba	473	159050	97514	117886	82955	56406	163053	180595	48954	84276	40745	82914	101605	63453	85041	188613	170590	73450	416858	25751	522814	54429	238726	151398	50573	172498	132132	165471	103797	159325	110907	494042	250638	269393	301828	613392	373704	325751	70256
AiiT1	473	37756	26878	65693	44369	5940	70670	86009	23663	35765	8778	38044	63895	38034	34429	85221	45609	21495	133730	2456	111518	18853	45532	33424	21568	34077	25631	45624	20554	44965	34784	217649	145180	135152	135490	105201	197759	189230	35918
AiiT1	473	19284	14144	3111	19565	2584	34448	45780	12987	21089	4362	19545	29400	19612	18502	51106	17256	11758	80337	4100	69660	6338	30050	28806	11541	20939	15532	24740	10570	31833	28619	120751	73525	77375	89113	52196	98231	108716	24982
Alba	473	54926	29104	41350	24874	18800	60764	66395	21055	30393	14031	32751	38546	28183	33775	70089	57050	25792	157898	10812	201229	20419	94251	68273	23679	72139	54214	67672	44799	73623	52682	201805	108375	115389	139128	108232	116668	126569	29556
AiiT2	473	8750	4522	29649	27555	2595	32192	42281	9901	17386	3855	12477	27280	18716	17104	34405	10126	7178	54925	3818	54009	7283	21766	21077	9351	14145	12572	17233	10442	20746	16852	112271	80729	74953	82200	29222	95962	95748	23031
AiiT2	473	9002	4264	29081	23656	1698	28910	34740	8362	19190	3055	14951	26818	19849	15287	29839	10211	5442	50966	1680	46656	5982	19199	14249	7800	12310	10630	15607	9364	18770	16242	110250	73434	69623	90657	29069	85791	85899	19193
Alba	473	42009	24290	29034	17815	6995	36634	49601	14089	22579	10106	24228	27708	15217	26813	40984	32559	16340	92950	8099	130669	14631	58520	42578	14931	39509	31637	39958	27900	46173	33030	166992	89890	89108	116337	80042	89894	87232	20347
Alba	473	30979	18609	19058	12876	8811	29981	34111	9876	14557	6358	16047	19311	9671	16648	30273	18805	10602	60768	4846	85991	8886	34243	26198	8801	24673	19975	27049	14930	27415	18221	130375	64130	63395	76462	56485	60788	61268	14645
AiiT3	473	18489	9987	17516	11209	2089	19658	23572	7515	11077	1815	13120	15823	9213	12807	26373	19874	6273	46231	2808	52997	5156	26630	16837	6518	18916	12159	20077	11876	19595	12259	95364	54585	53034	66913	38084	49418	50772	13112
AiiT3	473	16108	9080	14307	8830	4168	18993	20205	6491	9499	1774	10778	14060	9151	9632	20899	16653	6565	38634	3266	41851	4652	21042	18940	5381	15059	8815	18409	8723	17203	12135	83021	47080	41227	49856	30755	42125	46374	10083
Alba	473	26367	15291	18966	11651	5870	26061	28394	9222	14421	6524	14441	15987	9012	14467	20650	19010	6701	48221	2743	62147	6098	27867	20870	6334	19931	14717	20314	12569	20112	14605	119545	64491	63501	75510	52752	58126	62836	15939

	Time days	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	TARS1	TARS2	TARS3	TARS4	βster1	βster2	βster3	βster4
AiiC1	473	22928	12193	18665	11329	5329	23799	24341	9463	13568	3453	13590	13534	7765	10344	22547	16810	9517	44242	3156	52443	4728	24434	16399	5718	17842	13673	18224	11541	18758	13122	103591	52380	55034	62066	47931	52117	53886	11743
AiiC1	473	23691	12072	16687	10594	4008	19981	22673	7567	11468	2670	12649	16311	6691	11908	19061	12666	8482	43667	3184	52646	6345	22790	16324	5904	16938	11880	16501	11289	16543	12972	104840	52715	54915	64088	44362	50147	48453	11715
Alba	473	29306	17068	19279	12993	6728	25782	30608	8529	13308	5973	14938	15572	7480	12610	18874	15000	5310	38507	2661	45106	5504	18958	12200	3606	14692	12510	15316	8103	15002	10486	110720	59650	56048	60435	53662	54248	47378	9978
AiiC2	473	9703	4363	5985	4113	2572	8872	9145	2946	4258	1752	4323	5053	2738	4521	8232	5307	2910	18799	858	23325	2562	10036	7108	2256	7241	5629	7870	4875	6394	3323	35018	18828	19156	21891	15403	17866	18829	4707
AiiC2	473	11119	5911	7531	4751	2766	9600	9508	3429	4065	2040	5353	5022	3519	4861	7726	6000	2237	16359	1647	22045	1546	8441	6392	1648	6855	4456	6662	4575	7151	3646	40910	19256	17986	23358	19058	18161	19217	4394
Alba	473	28577	15511	19726	11064	7095	26841	27279	8632	10454	4735	13398	15094	7762	14687	20183	16508	6657	32920	2873	45289	4270	19274	15664	4108	14352	9875	15145	9401	13480	10631	119320	62010	62572	72911	48948	53504	50324	13973
Alba	473	23283	13646	16119	10683	3193	18755	23664	6747	9522	4277	11191	11684	6806	9186	11617	8840	3374	17668	959	25935	1104	11896	9097	2970	7475	6061	7933	5824	7771	5635	110435	55811	55010	63762	43223	40287	41799	11898
At0	473	24860	14215	15813	9723	3908	19081	24617	7073	10635	5080	12734	13234	6474	9432	10852	9601	3477	20106	1000	25422	2815	11259	8643	2022	7797	4804	7125	6762	7890	5297	110466	54122	52558	60252	40550	44922	41964	9426
At0	473	31259	15273	18455	11722	5333	22397	29796	8398	14985	5367	12926	14355	8543	11935	12052	9001	3651	20290	1103	28466	1953	11962	11195	2624	9061	5476	8118	5149	8067	5987	127609	63294	58468	74481	49554	52474	53444	11177
Alba	473	31047	16481	19710	12875	6018	26662	32454	10222	13002	4616	15787	14363	9557	12836	13068	11308	3198	21364	886	29918	2536	13658	12076	3187	9690	6521	9349	5039	9348	5462	129188	64132	62122	73339	52142	55044	56886	12496

GC-MS SIR data for Forties microcosms (substituted phenanthrene and dibenzothiophene peak areas –appendix B for key)

	Time days	C1P-1	C1P-2	C1P-3	C1P-4	C2P-1	C2P-2	C2P-3	C2P-4	*C3P-1*	C3P-2	C3P-3	C3P-4	C1D-1	C1D-2	C1D-3	C2D-1	C2D-2	C2D-3	C2D-4	C3D-1	C3D-2	C3D-3	C3D-4	C3D-5	C3D-6
Alba	0	827812	886853	1674348	949559	210964	866699	497578	626360	456690	738258	1073483	853818	1105103	722298	517891	710602	1432972	1207613	575977	324527	635513	439838	480135	596528	274648
Fit0	0	731930	806053	1444904	987828	1396085	671708	564321	492398	310879	486949	842254	555041	886439	502508	364672	466873	1044882	789854	300246	187368	382412	281971	212467	293839	137322
Fit0	0	642293	709508	1222234	874303	1240370	612482	497634	433741	285993	428995	733305	507595	762837	439598	321744	419328	916743	696772	271372	169508	340718	246565	193389	265779	124865
Alba	0	703302	741198	1380076	807042	1698195	695835	422113	502002	376262	647739	865896	735076	906766	604090	427546	601397	1211751	1032075	487087	279550	545486	674697	417481	518412	238029
Fiit0	0	659970	648363	1211839	867451	1191977	552436	476317	424678	284678	428504	740597	484367	797424	442583	333609	416560	925688	705776	269210	168662	348180	261888	193389	268020	127494
Fiit0	0	664465	649921	1200031	864084	1183519	546562	491473	430220	299994	441934	768062	512041	777957	435531	320289	410378	928891	706048	267519	172647	343670	257746	197005	272932	129728
Alba	0	616884	662565	1224000	693391	1526540	664461	340244	473435	348335	583843	809678	680542	806017	535665	381949	534462	1081793	908781	427568	253902	493012	339256	369879	481437	216938
Alba	203	43087	63765	102932	82939	84353	47354	32076	32286	16141	16336	28247	16557	70911	40330	32476	31990	64883	51524	20043	11415	18604	9868	16525	19050	4926
FiT1	203	166	1246	1100	2605	3650	3905	4924	5891	2501	2218	4318	2183	757	455	262	1464	3586	4761	1443	904	1476	779	2073	1375	862
FiT1	203	189	1133	1079	2448	4062	4662	6102	7807	2406	2138	4544	2629	962	477	287	1580	4255	5737	1142	1128	1668	881	2273	1521	722
Alba	203	49248	69355	114690	99585	114260	59355	35129	44157	21747	24170	39719	23908	84398	48287	40565	38172	77271	68477	26637	14728	24614	13990	22690	27409	7190
Alba	203	46557	84747	112868	105091	101502	55073	30917	40161	19816	19563	37448	19169	79919	46153	37449	36084	71452	60712	22587	12749	21384	12202	20451	22125	5975
FiT2	203	79	603	580	1187	2188	2198	2580	2529	1028	972	2321	1182	434	115	174	913	2280	2899	635	638	899	586	1215	817	371
FiT2	203	72	540	374	1085	2024	2035	2083	2439	1069	1009	2382	894	449	220	116	869	2151	2706	508	475	847	569	1150	760	430
Alba	203	44772	72336	102634	83253	97987	49273	26661	34309	15868	18018	26871	15759	72913	39540	33841	30075	57781	52084	19468	10276	17893	10225	16208	16528	5050
FiT3	203	91	561	515	1633	2627	2742	2817	3233	1066	1247	2410	1292	379	280	123	1162	2728	3141	506	612	972	525	1176	640	364
FiT3	203	85	686	692	1670	3764	2795	4350	3472	1474	1414	3003	1316	612	308	189	1314	3447	4111	1009	552	1168	552	1315	797	501
Alba	203	47203	75056	109659	102258	120036	63161	37875	50176	18337	19598	33686	18890	74229	42705	35254	33133	68077	57127	23121	11806	20999	11437	19340	20773	5657
FiC1	203	100	669	570	1531	3395	4487	6898	4962	1920	1553	4205	2176	539	468	197	2088	4677	6065	1595	819	1595	806	2245	1247	647
FiC1	203	22	395	432	1069	2894	3829	6222	4625	1626	1450	3920	1818	588	336	224	1868	4298	4834	1184	750	1329	682	1930	1147	439
Alba	203	48456	66910	106987	79828	110422	58040	34513	39920	17838	17990	33183	19013	67890	40765	32668	31874	63798	54822	22382	11391	19352	10994	18206	20349	6068
FiC2	203	12468	63901	37141	60650	28948	25137	23190	18863	4948	4017	10248	6036	15038	8035	3529	4935	17413	28893	2733	2280	3967	1200	10287	2311	1277
FiC2	203	13783	73592	39419	65822	33104	29271	27644	23174	5279	4709	12916	6842	15090	8339	3061	5645	19560	33108	3715	2808	4371	1575	11949	3131	1164
Alba	203	139019	242510	341371	307009	456564	251590	169127	17331	90398	105521	172452	91843		160989	123236	133502	261786	228074	91184	56010	100863	56296	87113	95882	27917
Alba	203	67244	96689	150598	109740	248170	120740	86391	88343	70940	93489	132298	89594		83366	60919	96067	187614	164069	69146	58455	111396	64522	81940	98853	42257
FiiT1	203	1275	2874	4574	5043	41021	21791	27476	23060	25204	28697	48275	24896		3941	3535	20098	44536	44055	13581	15897	30217	16459	20914	26831	10791
FiiT1	203	1209	3069	4889	5786	36338	24180	21621	22516	21751	23443	42321	21289		3778	3498	18951	42980	41250	12830	15434	28643	14803	20278	24407	9402
Alba	203	79981	111370	170534	124116	256767	127563	88699	88666	65266	79463	115793	70679		104835	80936	110556	219460	194275	85314	61338	112836	78491	88352	10444	42085
FiiT2	203	3792	9358	14764	14645	54942	37866	37479	32762	25503	26330	51913	20357		8863	8040	26574	56634	53338	13596	14095	21757	11475	19852	20192	5591
FiiT2	203	1931	8712	10597	13791	42749	25175	30806	29387	17822	17593	36131	13669		5187	5326	14007	35078	32804	6834	7897	10738	6178	12025	11324	3180
Alba	203	66976	100307	147393	111988	20402	103804	71388	74755	40765	52845	76648	44214		85217	68536	72093	143877	131920	56177	30931	55484	31577	47412	54085	16892
FiiT3	203	581	2888	1522	4788	9834	7703	10105	10432	6439	5114	12816	4857		1047	376	3657	8609	11195	1310	2071	3497	2141	5231	4251	1150
FiiT3	203	374	2079	1545	4792	9584	10170	11170	10991	6253	6274	12589	5863		1177	546	3691	8986	12727	1282	2462	3482	2636	4946	4375	1464
Alba	203	67073	102467	156210	129632	201720	102545	68125	70282	37449	46984	70831	41690		83424	66631	67949	137121	122886	51232	25349	45718	26429	40099	45453	16047

	Time days	C1P-1	C1P-2	C1P-3	C1P-4	C2P-1	C2P-2	C2P-3	C2P-4	*C3P-1*	C3P-2	C3P-3	C3P-4	C1D-1	C1D-2	C1D-3	C2D-1	C2D-2	C2D-3	C2D-4	C3D-1	C3D-2	C3D-3	C3D-4	C3D-5	C3D-6
Alba	203	60779	91762	134471	103247	17281	87273	51881	71896	29792	37755	52879	32194		64765	52781	52779	103043	91519	36716	19070	33471	18341	30440	33549	9759
FiiC1	203	200	761	1511	3613	10465	16326	27975	22584	7784	6587	17222	7551		1594	654	8878	18065	16817	1245	4016	4902	2071	4479	3835	1678
FiiC1	203	113	1148	1633	3983	14057	16503	33736	26440	9874	6544	22343	9544		1563	697	10436	19761	20328	1401	4644	5657	2856	4025	5101	2232
Alba	203	80078	122257	175562	125576	209851	102077	58763	73112	35017	45998	68803	40061		79379	61197	61359	123029	110048	45664	23280	42141	23522	38386	43321	13895
FiiC2	203	34306	93809	10613	144347	80755	62140	59992	54938	15714	13673	33916	13157		26342	24972	16737	51640	35756	4604	6217	8482	4543	6588	6499	2477
FiiC2	203	24113	75508	78562	125351	57149	47930	47995	43536	10962	9136	21685	9337		16662	13276	11084	35833	25505	2414	4733	6353	3325	5703	3297	2053
Alba	203	72352	127769	169669	132295	201243	102492	64933	86902	33601	46371	67936	36945		70717	56440	57914	114852	101882	42341	22551	40155	21149	36812	38532	12501
Alba	473	334563	382105	622621	388199	897414	404211	237330	298116	216241	296112	413289	313858	562981	382033	271233	383129	549358	500202	235737	145843	279139	175219	206941	269552	115424
FiT1	473	1788	2257	3581	2585	12830	6856	5820	4522	7153	8929	16638	9790	3498	1780	1668	4660	8908	8079	2847	3786	7570	4447	5286	6671	2835
FiT1	473	1893	2165	3180	2645	12725	6943	5831	4798	6651	7957	15425	10145	3542	1855	1735	4819	9238	8268	2998	3873	7633	4525	5131	6728	2705
Alba	473	324215	388162	640703	373577	85510	372815	243161	284519	200580	275327	397273	325363	561158	374282	271719	281224	591872	499820	236784	139803	270116	171970	201037	258251	111980
Alba	473	294782	329243	571273	329577	771717	343894	211901	267878	184106	267963	376034	278103	517113	352271	249290	262959	544985	460379	218991	134215	255148	160828	185272	242382	102512
FiT2	473	866	1005	1792	1669	5873	3495	2836	2564	3987	5387	9476	5117	1829	918	893	2620	4561	4490	1581	2205	4283	2616	3274	3934	1580
FiT2	473	1551	1915	2835	2581	10977	5970	4874	4544	7006	10435	18255	9688	2971	1579	1719	4772	8559	7838	2813	4109	8091	4853	5853	7235	2989
Alba	473	289797	341426	557279	346414	763428	324209	199763	244956	179552	250457	349130	280166	475175	329808	230815	253124	508086	426690	198486	122983	236909	148069	176697	227492	105360
FiT3	473	1820	2223	3114	2719	11064	5859	5984	4078	5886	7394	14010	7509	2881	2089	1389	4166	7924	6621	2464	3231	6531	3823	4424	5618	2147
FiT3	473	1969	2531	3490	2640	11684	6341	5858	4083	6077	7980	15512	8392	3015	1595	1546	4420	8485	7078	2532	3415	7090	4142	4829	5773	2343
Alba	473	381669	440110	760651	432907	983696	430228	270718	321994	240246	320311	46517	369131	608485	419089	296898	320820	661028	552170	263019	161284	313366	192504	229437	295527	128400
FiC1	473	1831	1979	2240	2026	11880	8158	7920	6206	23141	23645	47070	27488	3509	1671	2770	6522	12045	16751	4495	11458	24253	18733	29932	29645	10575
FiC1	*473*	*1487*	2220	2074	2771	13492	8939	8059	6352	24640	25984	49488	29283	3435	1733	2660	6889	12114	17219	4908	12142	25174	19225	31165	31333	11307
Alba	473	352226	422391	738958	393002	934634	404508	249824	298510	211651	306188	425867	336528	586432	392857	273432	290979	608029	504961	239176	146415	285381	171176	205853	267553	113015
Alba	473	420220	483824	849656	480168	1036277	462152	280467	322158	246145	353876	498242	371741	670696	455618	320452	331678	689294	582843	276054	17404	335335	200180	231272	311847	135208
FiC2	473	291062	343534	551779	468504	694571	340125	289282	246324	200748	219407	394233	235466	391985	243581	157352	199977	425552	316034	132336	91527	182519	104150	105590	147510	67178
FiC2	473	276709	324555	523990	440232	667785	326403	267152	229934	184070	211053	373278	219341	374349	232211	150388	184378	419078	298821	124985	82940	169249	96265	96473	135858	58237
Alba	473	367625	416367	695668	395103	904471	401160	241614	276369	202905	281809	399672	214185	545945	376044	262726	266331	565524	478058	225454	137771	274503	166326	195164	249253	108440
Alba	473	74047	108834	152964	118892	232017	116985	67790	66347	44546	53836	83378	56855	124288	81308	59202	87254	178506	153983	71265	51006	99423	61507	68772	90028	38856
FiiT1	473	490	663	740	856	3636	2040	1916	1242	1638	1953	4067	2215	685	404	277	1800	3568	2900	817	1501	3400	1855	2214	2789	1102
FiiT1	473	366	565	620	745	2658	1525	1333	849	1196	1376	2945	1359	563	318	249	1684	2767	2381	862	1291	2588	1476	1823	2144	852
Alba	473	84738	114915	166543	122373	256070	124696	73398	70583	44078	61313	93602	65244	137549	90711	66816	101956	201254	174241	79943	58430	113980	67179	78898	102681	45013
FiiT2	473	585	636	866	763	3852	2076	1936	1507	2121	2978	5765	2576	953	450	465	1882	4234	3686	1209	2231	4594	2610	3430	3977	1450
FiiT2	473	515	618	668	824	3131	1669	1666	1246	1562	1995	3931	1663	627	378	198	1542	3259	2747	800	1744	3385	1900	2475	2735	1088
Alba	473	110605	152508	216290	149380	329313	159250	89701	100140	6672	77801	120446	8477	185328	122889	86026	123625	250923	219502	98214	74789	145297	87439	106896	129052	56742
FiiT3	473	512	732	792	842	2939	1651	1598	1022	1294	1572	3108	1538	755	336	306	1440	2822	2454	798	1468	2869	1579	2055	2362	980
FiiT3	473	316	460	467	539	2346	1335	994	780	914	1036	2231	1028	533	253	233	1004	2211	1770	674	1008	2110	1150	1415	1702	659
Alba	473	73129	99222	142491	93901	253165	118476	68006	78144	54026	66341	97599	68731	125212	82318	58546	99319	201009	176033	79563	64189	124580	76398	88300	111102	49395
Alba	473	61684	80266	121393	77249	205109	94444	53314	62335	43860	55929	79173	57892	111477	70942	50413	81708	169662	146156	64011	51972	103485	62130	73603	92994	39184
FiiC1	473	996	995	1141	1319	2878	2436	1992	1403	2201	2353	5384	2479	840	459	319	1967	3617	3113	1004	2324	4982	2884	4275	4639	1700

	Time days	**C1P-1**	**C1P-2**	**C1P-3**	**C1P-4**	**C2P-1**	**C2P-2**	**C2P-3**	**C2P-4**	*C3P-1*	**C3P-2**	**C3P-3**	**C3P-4**	**C1D-1**	**C1D-2**	**C1D-3**	**C2D-1**	**C2D-2**	**C2D-3**	**C2D-4**	**C3D-1**	**C3D-2**	**C3D-3**	**C3D-4**	**C3D-5**	**C3D-6**
FiiC1	473	722	1148	820	1342	3116	2344	1931	1242	1827	1722	4385	1968	859	464	351	1889	3502	2860	1057	2079	4524	2521	3736	4057	1481
Alba	473	67713	81420	122315	94258	215172	105515	66740	67599	44766	57949	88550	68639	116834	74023	53088	88189	176243	155676	71589	55012	108433	65016	77229	96628	42832
FiiC2	473	60828	76834	110109	109112	169450	88142	76962	56341	41078	41942	79537	47735	94166	54128	33015	64578	149367	102917	40448	37207	71855	38032	41275	54262	22765
FiiC2	473	55019	72131	97967	95512	152440	82634	72887	53554	35470	37002	71822	41090	81974	47137	29526	57450	131460	93699	36527	32523	65819	34516	37781	48613	21011
Alba	473	85061	105558	167899	100864	269413	149951	70466	82145	60116	74921	114353	82890	14084	83276	66501	115579	227194	200944	90516	71644	136580	82400	98069	122691	51933
Alba	473	98571	135914	193451	134460	353199	175479	97321	104778	80294	100959	150908	103840	177438	116605	84548	142564	285641	252172	115171	86330	161273	97013	116314	145392	63654
Ft0	473	106135	141308	208739	177223	268036	155553	134923	97246	74597	75005	140982	80326	177095	102352	60764	107657	248478	174782	67494	58547	111003	59465	66301	83952	34197
Ft0	473	104326	140754	200914	172460	283794	141884	114542	83172	70443	78180	132861	72793	168552	100848	61859	103940	237518	169447	68036	54735	104588	53958	60533	79576	33203
Alba	473	126559	191919	261571	190110	476537	247968	148220	131126	113649	147355	216522	159804	249552	162172	115398	189011	382482	331063	139404	109451	216671	130625	146948	180236	83436

GC-MS SIR data for Forties microcosms (sterane, triaromatic sterane, pentacyclic triterpane & βsterane peak areas –appendix B for key)

	Time days	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	TARS 1	TARS 2	TARS 3	TARS 4	βster1	βster2	βster3	βster4
Alba	0	190437	130236	178386	123428	55497	257901	326475	101761	124935	76896	140956	140013	91889	148090	279806	183946	112949	636582	62281	852350	74471	381358	241640	80748	284636	188570	220662	132940	207687	157374	854910	463758	428360	556759	854910	463758	428360	556759
Fit0	0	108167	95837	104968	74173	25405	139914	177175	55325	59728	40702	125998	61154	38114	66023	152503	69287		276482	19699	402369	86450	188991	132526	56081	139767	95902	105345	64429	69888	55331	480578	209121	225887	241778	180578	209121	225887	241778
Fit0	0	88943	71064	76032	55346	21812	105684	128324	41931	42095	28895	100747	47934	30174	56338	139560	57437		212665	19986	317651	75328	149162	98630	45925	99551	73652	74469	47496	50411	41852	387697	164667	206759	185722	178102	200202	188046	30076
Alba	0	150395	95815	145129	100914	43171	202694	240128	73967	94294	59057	109177	94887	73398	118320	248030	163901	91826	534201	42420	689112	52216	277773	218680	56436	227283	166958	161900	103360	152912	113796	722222	416252	442471	461434	398472	458609	431201	77491
Fiit0	0	94547	82036	82584	57956	20478	113015	147074	45919	50404	35009	125424	49374	32111	55974	182266	58175		235139	15888	358415	104358	161123	104921	65705	114234	86466	78602	49532	50755	43562	438069	173740	218183	215511	191684	214196	204318	32704
Fiit0	0	95497	88854	85835	58394	22694	111822	141119	41255	43186	32461	121485	45121	31039	59138	154437	55941		221972	13415	319986	98616	151825	112852	56371	106666	76931	81891	48338	54818	48200	415755	181245	207311	204296	184941	211161	192134	36571
Alba	0	134220	83490	110261	83583	41008	170888	208978	63673	68463	48875	79770	79313	60685	99641	189479	121480	60801	434078	29210	542771	42416	242538	149282	52505	171343	118200	141344	87021	130843	94395	630397	350020	393258	402731	331858	380268	365633	66011
Alba	203	4544	2362	3265	2212	883	4621	5249	784	1857	490	1795	2271	2317	2154	13508	9581	2921	22131	1746	28664	2635	9868	10973	2075	5421	3775	5682	3267	5701	2347	19207	5332	5804	7458	10691	11581	12167	2110
FiT1	203	1519	730	1253	863	484	1437	777	385	595	62	348	268	1134	539	2422	2076	1040	5786	1045	10502	714	3408	4520	337	1715	1539	1921	625	1133	977	2925	1259	3042	1115	2505	3052	3193	535
FiT1	203	2163	855	1322	845	850	1793	1315	742	636	93	268	380	2202	682	2437	2337	1064	7426	1772	14268	858	4829	5902	954	2083	1508	2540	1147	811	1575	3797	1022	1588	1759	3173	3653	4226	736
Alba	203	5711	3178	3733	2980	1158	5158	5608	972	2184	331	1662	1842	1939	2091	16329	11214	3638	24015	2979	33599	2254	10073	11888	2461	6239	4423	6267	4910	4294	3363	16809	4456	5951	5088	11361	12097	10178	2482
Alba	203	4854	2766	3517	2661	659	4677	4251	711	1453	470	1773	2194	3252	2857	15670	11210	3287	29156	1183	31693	3129	10964	11044	2659	6664	4950	6101	3780	5210	3775	18283	5524	5253	6733	10897	11509	11516	2376
FiT2	203	1256	458	961	610	478	1157	675	466	642	118	214	232	880	434	1707	2057	494	6378	1411	11492	806	4014	4864	458	2729	2100	2499	1052	1517	1101	1763	520	1153	973	2060	2515	2764	473
FiT2	203	914	433	753	558	467	1123	706	478	657	81	200	211	903	407	2131	1935	499	5448	1244	11029	978	3677	3951	571	1760	1182	1712	1011	1002	1100	1393	541	716	1075	1981	2182	2476	509
Alba	203	4207	2304	3054	2203	882	3904	4421	758	1514	421	1756	1694	3056	3021	17417	11560	5601	30028	2934	41407	3103	12776	13967	2386	7323	5496	7712	5356	7616	5413	15600	4933	5613	4779	9534	10563	10946	2394
FiT3	203	910	515	963	695	436	1190	837	428	554	135	262	333	839	472	2550	2124	324	6208	902	11439	656	3643	4371	590	2107	2408	2871	1867	475	737	1355	614	756	864	1917	2605	2700	503
FiT3	203	1348	616	1159	745	685	1831	927	657	1018	79	280	416	1407	581	2170	2678	515	7311	1076	11928	746	3945	5675	493	2807	2173	2781	1332	1626	1011	1562	723	848	1283	2670	3982	4083	751
Alba	203	5973	3395	4072	2892	1304	6820	5727	618	2553	255	2125	3343	4990	2820	17545	12948	2789	31018	2681	45188	2831	13752	14355	2275	7393	5678	8219	6061	7726	4795	18246	5729	5308	6708	14302	14115	14698	2989
FiC1	203	2123	1109	1995	1371	1714	2798	1919	2163	1368	138	596	636	2732	1146	3052	2771	1294	9064	3213	17369	1211	6754	7198	625	3549	2859	4152	3242	2722	1700	2282	1530	1689	1865	5251	6002	6441	1268
FiC1	203	2105	1077	1574	933	1814	2554	1539	1427	1637	278	466	658	2615	944	2466	2387		8542	2933	16693	1506	6152	7134	677	3908	2036	3460	2287	2205	2483	1977	1036	1791	1841	4229	4792	6212	1151
Alba	203	6800	2778	4700	2548	1615	8169	7113	1333	3317	656	2457	4008	4905	3750	19455	13404	3891	33280	3394	46930	4352	16687	18768	2829	8311	6504	7426	5249	8195	5331	22028	7121	6786	7748	15206	15886	17060	3921
FiC2	203	3106	1802	2461	1394	3887	5196	2923	3319	2605	265	1154	1366	6072	1542	4284	3539		13481	2569	21839	1833	9049	10038	1074	5687	2994	5974	3236	4526	3377	3074	2441	2538	2347	7924	7275	10278	1645
FiC2	203	3287	1716	2626	1688	4201	5359	2570	3061	2470	228	928	937	5342	1041	4472	2568		13090	2381	24859	1533	9638	10048	1134	4934	2768	5405	2858	2882	2683	2688	1920	2405	2286	7846	8457	9892	1824
Alba	203	43784	27452	33755	18352	12259	49313	47986	7209	23672	6330	19685	24539	32659	25840	85146	55558	19477	142945	11667	175221	18100	58807	73131	15238	36505	33092	32308	20903	33012	12652	95033	28965	35114	43225	96419	101139	108765	23562
Alba	203	19555	12850	16592	9278	8590	23652	19742	7955	9180	5185	11964	13282	6787	10909	20364	16907	5866	47781	3961	56258	6173	24892	19625	7156	17191	13467	17531	10131	14965	4427	86595	44951	46053	54314	40555	39528	42886	10425
FiiT1	203	8308	5556	7217	4787	3258	9565	9281	3188	3145	1614	3379	3832	3540	4449	4198	3946		12953	1265	18856	1649	8586	7220	1488	5403	3454	4878	2632	3160	1748	20308	11520	12409	13909	13562	12488	16289	3692
FiiT1	203	6110	4053	5640	3958	2828	8123	7021	2555	2535	1408	2478	3054	3258	3967	4422	4122		13449	1503	19360	2130	8619	6771	1293	5378	3751	5142	2964	3973	1980	20540	8995	9392	12298	10515	11783	1330	3159
Alba	203	12494	7674	10121	5257	5162	15797	17486	4060	6776	2459	6618	8702	6728	8104	18877	10202	6067	37097	3529	44733	4327	19013	16739	4960	11806	8728	11264	6651	9426	6378	60336	23399	25297	30216	27867	25832	29379	7247
FiiT2	203	7872	4646	6426	4305	3084	8414	7326	2647	2753	1012	1899	2948	2773	3939	6229	5857		16841	2624	23956	2837	11054	9106	1500	5963	3733	5921	3237	3557	2686	20896	7317	7620	9939	12643	13748	13444	3115
FiiT2	203	6038	3599	5102	3057	2594	6889	5874	2567	2491	653	1889	2525	3135	3089	5484	4142		15701	1938	23023	1941	9918	8791	1244	5089	3544	4838	2604	3077	1384	14803	4811	5779	5047	11628	12423	11803	2430
Alba	203	8634	5004	6785	4181	2555	10093	9462	1943	3792	1297	4489	6568	5795	4668	15177	8568	4258	29454	1612	36420	3894	14428	12882	3071	7812	5866	7400	4480	6289	3994	38665	11641	14225	13777	18713	17386	20052	4220
FiiT3	203	3818	2327	3132	1767	1038	4168	3119	942	1807	304	1229	1806	2565	1410	3743	2880		10077	538	17324	1031	6531	6097	487	3249	2341	2712	1581	1458	697	5064	2518	2684	2874	6694	7456	7167	1356
FiiT3	203	5215	3093	4092	2381	1757	5836	4251	1623	2512	404	1525	1991	3789	1954	4899	4891		14157	837	21178	2261	9384	9221	945	4935	2836	4304	1909	2636	1534	7777	3415	4421	3551	8942	9997	10011	2068
Alba	203	10641	5578	8694	3868	3202	11914	9948	2034	5430	1348	4479	6559	5780	4752	17717	11494	4687	31339	1935	36256	3734	13320	13486	3428	7256	4003	6781	4085	5050	2729	39754	11858	12066	15965	21548	20160	20960	3857

	Time days	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	TARS 1	TARS 2	TARS 3	TARS 4	βster1	βster2	βster3	βster4
Alba	203	11930	6715	8597	4526	3764	13288	11310	2355	5419	1414	4557	6900	7529	5270	19166	10190	4936	34653	2602	43684	4322	16918	16397	3787	8433	6397	8631	4897	6342	4489	36557	11921	13866	11055	24515	23063	25443	5562
FiiC1	203	5993	3344	4310	3387	2129	6254	5066	1793	2985	439	1264	2107	3767	2607	4637	3801		14757	1600	23584	1366	9595	10004	1157	4851	2682	4190	2277	2818	2457	5641	3502	3557	2500	11197	11749	12050	2100
FiiC1	203	6885	4524	5011	3302	2369	8079	5291	1611	3526	442	1455	1866	3593	2545	5489	3681		15893	3087	25443	1828	8813	9624	994	4097	3076	4167	1933	1969	1690	5857	3040	3463	2690	12171	12521	12231	2338
Alba	203	16091	8898	10822	5588	4389	16077	12702	2592	6951	1377	6141	7895	9991	7936	23166	11325	6460	40541	2546	47032	4160	18802	15338	5197	8774	5686	8330	4024	7931	5141	45307	15387	13908	17040	31593	29750	31104	6796
FiiC2	203	8517	4642	6142	4046	3476	10408	7710	2828	3947	344	2222	2781	5575	3472	6794	6133		19104	1566	28317	3051	12279	12474	1619	5727	2835	4887	2991	2818	1916	7553	4497	4990	3650	16261	17314	16087	3227
FiiC2	203	5716	3448	4900	2952	3250	8666	5263	2527	3172	380	1226	2090	4240	2703	6526	6726		15384	1381	22765	1599	8246	8282	1467	3765	2445	4496	1918	3458	1455	4980	2774	3403	2203	12906	12941	12960	2449
Alba	203	12591	8135	11439	6653	4793	17087	14333	2407	7764	1755	6407	9299	7716	7332	20200	11452	6147	39170	2995	49956	5370	20701	18744	3921	9651	7927	9126	5931	7756	5063	38682	12446	12672	11775	32267	30834	32359	6652
Alba	473	65306	29042	48735	30584	10915	55700	68408	22115	26602	14497	34356	37178	21981	29648	55996	62215	21349	106716	6192	139183	13688	66195	44780	18760	46730	38211	37796	25099	41012	31251	287715	152805	138078	146646	103802	133236	116306	21429
FiT1	473	7136	3989	17313	12267	1128	16680	19709	6970	6551	1573	7202	9768	8562	7124	8546	4750		18950	1257	23486	2929	13804	8382	2121	9024	6177	8931	5065	5882	6038	69579	44766	41261	49230	17434	38927	39814	8270
FiT1	473	7012	4699	16235	11895	1404	14361	19628	6212	6350	1260	6025	9583	5406	6370	8308	4079		19542	944	22149	3835	13044	7618	2424	7008	5468	5597	6434	5388	3740	65296	39680	37144	38805	17756	32535	35360	8390
Alba	473	49985	30749	45813	24049	12607	52728	64747	18512	29945	13123	28116	38930	20623	27115	53387	33413	17188	107546	6684	147122	14315	67404	57875	18464	46110	36489	43300	27157	46029	31533	283338	150688	148188	172019	113364	121257	114465	22257
Alba	473	59593	33739	43241	25163	11054	52632	68104	19643	30192	12797	33090	36372	24256	34136	51248	35178	21086	105091	5522	151891	15154	71958	48194	16574	50270	37898	44003	30647	44740	35759	303086	162451	148804	185099	114556	126986	130753	21970
FiT2	473	4111	2579	10050	5856	654	8956	11505	4333	4514	902	4524	6119	4324	4321	7090	4793		17122	2024	24949	4548	13651	12075	3182	10392	8132	6033	4038	5948	6191	36341	23940	22186	24928	12184	21985	20643	3903
FiT2	473	10642	6415	18802	13255	1841	17743	23498	9295	10160	2153	8333	11675	77002	7827	10957	8373		29872	2207	47225	4324	24359	16315	4429	15226	9050	11864	7234	9033	6205	70926	41992	42455	46474	42860	42471	47701	9087
Alba	473	55745	30266	40066	26809	10193	50452	62019	21829	28581	11282	28782	31438	21570	30476	51484	34370	18059	109028	8450	152435	12553	70594	45711	16355	47635	35490	49807	31473	48666	32764	269138	138559	139989	163999	109096	118691	114919	19108
FiT3	473	5854	2470	17726	12145	965	16152	20502	6653	8169	2251	6328	9782	6296	6717	9028	6403		24511	2336	36327	4681	18059	11238	3420	11786	8645	10605	6354	7588	6309	65220	41792	37346	43451	12866	37171	37540	8571
FiT3	473	5685	2738	18188	14541	782	18457	23365	7730	8645	2808	7389	10340	8439	8479	10385	6024		26047	1813	40529	4524	19275	12847	3962	11637	7232	9445	4987	8244	4313	65793	39145	35521	43824	17506	44864	43639	8969
Alba	473	82157	42699	56285	37685	15828	70558	84835	26853	37174	17144	40627	39304	30612	39135	72744	44253	32074	145865	7467	209290	17453	87310	60450	21293	69177	48760	65315	41604	60646	42281	343092	173270	176357	195724	152633	157023	156400	30030
FiC1	473	42231	25041	35872	24364	5756	39727	40944	14293	19364	2721	15355	18550	12798	12066	19915	14930		59509	3463	96471	7871	44002	30261	5137	28340	20511	24050	15965	16101	13183	112530	61374	61132	72845	70933	76703	71927	14100
FiC1	473	43683	24914	35622	24314	5704	39705	42838	15689	21267	3243	15543	20275	13126	17899	18278	16817		67417	5240	104726	9568	49978	33513	5067	30761	22941	27936	16320	20300	15937	123738	69086	64922	74031	72949	79923	80133	13774
Alba	473	81076	43692	56959	39498	14384	69494	89644	25273	41240	18771	42084	45706	27423	41305	77584	58400	33882	174936	13121	232100	22091	102903	73248	27191	78903	62214	75678	46664	84317	49455	333093	168734	171675	206955	154672	165660	157667	31138
Alba	473	79165	52149	64034	43612	21131	81232	94238	33688	48651	24498	49272	57296	32879	46784	91751	51866	37668	200179	11184	250337	25678	124375	90681	27735	87024	71012	87826	52973	84417	59462	367655	205739	205497	230759	179849	192614	188190	35176
FiC2	473	55674	33857	43063	27916	9223	48360	58698	19246	21301	10442	21085	25377	14059	22682	39887	19782		76412	5038	113489	13990	55384	37336	6037	34312	24180	30311	20350	22085	17559	133304	73325	70290	82328	92483	94196	83962	16246
FiC2	473	52203	27853	40166	26349	7349	45058	59154	18727	22173	10423	19015	22160	14173	21301	38113	21009		75189	3837	121484	17101	59246	38516	6743	39869	30838	35446	24725	27939	20581	132396	77941	78150	85988	81820	92126	82298	18397
Alba	473	81279	47522	58063	37310	27292	82945	92625	27566	41156	16343	40317	48367	27349	39649	85359	89184	31860	186700	10772	229856	22579	112742	86225	29729	80343	63632	74342	50645	79781	53306	312881	170437	168892	203744	163839	177049	165876	31972
Alba	473	19970	12388	13267	8441	3931	17331	21139	5889	8743	3441	9352	10490	4970	8141	13698	11086	4575	25605	1535	30400	4104	14817	9318	4011	10001	6526	10511	7110	10444	7747	85043	42091	39958	52019	35708	36258	32264	8082
FiiT1	473	3214	1391	6290	4190	365	5602	6773	2844	3089	493	2486	3062	2015	2309	3203	2478		7118	352	9123	925	4310	2809	808	2905	1533	2407	1960	2907	1569	17802	11107	9799	11081	5253	11122	11767	3157
FiiT1	473	1996	912	4177	3506	216	4398	5422	1647	1930	387	1646	2594	1792	1698	2692	1917		5532	1226	8929	1226	3995	3151	778	3092	2231	2971	1509	2081	778	15821	8628	7948	10003	4066	9650	9997	2099
Alba	473	21043	10136	11793	6768	2710	15206	20296	6566	7875	2930	8680	10625	3647	9560	10841	10415	3127	24094	1700	30549	2639	13613	9130	2537	10468	7425	8998	5806	9725	6828	88075	42074	39168	49372	28909	31991	31015	7111
FiiT2	473	6882	4364	6873	4838	967	6711	6355	2456	3444	983	2226	3100	1657	3291	3468	3235		10895	1396	17704	1159	8099	5722	589	5402	3962	4797	3245	3461	2219	20910	12116	11295	13256	9092	11289	12024	3106
FiiT2	473	4959	2960	4754	3307	501	5283	4957	2348	2822	1127	2075	2452	1488	2431	2822	2889		9795	655	15552	1457	6765	5174	771	4614	3181	3750	2614	2513	1610	17780	9327	8774	10492	7657	9560	9448	2633
Alba	473	25972	14602	15591	7981	4110	20086	25213	7773	10648	4320	11562	11530	5912	9775	14256	10501	6688	28155	2084	34819	3118	14636	11227	3154	10272	8466	10954	6947	9892	6383	102701	51814	48761	54152	41460	39370	38217	7733
FiiT3	473	1650	418	6193	4567		6257	5744	1623	2309	313	1286	2297	1713	2057	2045	1096		3545	406	6640	455	3027	2529	510	2118	1718	1930	947	1942	1109	22484	13716	12624	14341	4731	10042	10147	3175
FiiT3	473	1200	622	3999	3333	227	3634	3834	1042	1350	206	726	1897	972	1462	1342	696		2966	284	4699	376	2116	1596		1191	815	1504	1026	796	367	14378	9002	6786	8865	2599	7164	7527	1677
Alba	473	19860	11683	13031	7357	3216	15324	18385	5358	7733	2803	9533	10020	4817	8704	8949	7261	3141	15724	1078	22988	2234	10370	7203	2242	6152	4712	7000	4615	6583	4435	89534	43382	46536	51784	30844	32245	35151	8378
Alba	473	16206	9193	10575	7390	2868	14607	15119	5746	6593	2519	8580	8547	4372	7349	8933	8242	2524	14480	1519	21441	2237	9026	6864	1489	6040	4429	7053	5462	6418	3951	82827	39167	38169	44350	28014	27927	24701	6951
FiiC1	473	6379	3935	3967	3216	1309	5668	4199	1703	1943	385	2434	2618	1334	1560	2622	2532		6263	346	10071	1216	4496	3216	266	2757	1930	2563	2151	1809	1262	19803	10324	9488	12539	7573	8919	7860	2105

	Time days	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	TARS 1	TARS 2	TARS 3	TARS 4	βster1	βster2	βster3	βster4
Alba	203	11930	6715	8597	4526	3764	13288	11310	2355	5419	1414	4557	6900	7529	5270	19166	10190	4936	34653	2602	43684	4322	16918	16397	3787	8433	6397	8631	4897	6342	4489	36557	11921	13866	11055	24515	23063	25443	5562
FiiC1	203	5993	3344	4310	3387	2129	6254	5066	1793	2985	439	1264	2107	3767	2607	4637	3801		14757	1600	23584	1366	9595	10004	1157	4851	2682	4190	2277	2818	2457	5641	3502	3557	2500	11197	11749	12050	2100
FiiC1	203	6885	4524	5011	3302	2369	8079	5291	1611	3526	442	1455	1866	3593	2545	5489	3681		15893	3087	25443	1828	8813	9624	994	4097	3076	4167	1933	1969	1690	5857	3040	3463	2690	12171	12521	12231	2338
Alba	203	16091	8898	10822	5588	4389	16077	12702	2592	6951	1377	6141	7895	9991	7936	23166	11325	6460	40541	2546	47032	4160	18802	15338	5197	8774	5686	8330	4024	7931	5141	45307	15387	13908	17040	31593	29750	31104	6796
FiiC2	203	8517	4642	6142	4046	3476	10408	7710	2828	3947	344	2222	2781	5575	3472	6794	6133		19104	1566	28317	3051	12279	12474	1619	5727	2835	4887	2991	2818	1916	7553	4497	4990	3650	16261	17314	16087	3227
FiiC2	203	5716	3448	4900	2952	3250	8666	5263	2527	3172	380	1226	2090	4240	2703	6526	6726		15384	1381	22765	1599	8246	8282	1467	3765	2445	4496	1918	3458	1455	4980	2774	3403	2203	12906	12941	12960	2449
Alba	203	12591	8135	11439	6653	4793	17087	14333	2407	7764	1755	6407	9299	7716	7332	20200	11452	6147	39170	2995	49956	5370	20701	18744	3921	9651	7927	9126	5931	7756	5063	38682	12446	12672	11775	32267	30834	32359	6652
Alba	473	65306	29042	48735	30584	10915	55700	68408	22115	26602	14497	34356	37178	21981	29648	55996	62215	21349	106716	6192	139183	13688	66195	44780	18760	46730	38211	37796	25099	41012	31251	287715	152805	138078	146646	103802	133236	116306	21429
FiT1	473	7136	3989	17313	12267	1128	16680	19709	6970	6551	1573	7202	9768	8562	7124	8546	4750		18950	1257	23486	2929	13804	8382	2121	9024	6177	8931	5065	5882	6038	69579	44766	41261	49230	17434	38927	39814	8270
FiT1	473	7012	4699	16235	11895	1404	14361	19628	6212	6350	1260	6025	9583	5406	6370	8308	4079		19542	944	22149	3835	13044	7618	2424	7008	5468	5597	6434	5388	3740	65296	39680	37144	38805	17756	32535	35360	8390
Alba	473	49985	30749	45813	24049	12607	52728	64747	18512	29945	13123	28116	38930	20623	27115	53387	33413	17188	107546	6684	147122	14315	67404	57875	18464	46110	36489	43300	27157	46029	31533	283338	150688	148188	172019	113364	121257	114465	22257
Alba	473	59593	33739	43241	25163	11054	52632	68104	19643	30192	12797	33090	36372	24256	34136	51248	35178	21086	105091	5522	151891	15154	71958	48194	16574	50270	37898	44003	30647	44740	35759	303086	162451	148804	185099	114556	126986	130753	21970
FiT2	473	4111	2579	10050	5856	654	8956	11505	4333	4514	902	4524	6119	4324	4321	7090	4793		17122	2024	24949	4548	13651	12075	3182	10392	8132	6033	4038	5948	6191	36341	23940	22186	24928	12184	21985	20643	3903
FiT2	473	10642	6415	18802	13255	1841	17743	23498	9295	10160	2153	8333	11675	77002	7827	10957	8373		29872	2207	47225	4324	24359	16315	4429	15226	9050	11864	7234	9033	6205	70926	41992	42455	46474	42860	42471	47701	9087
Alba	473	55745	30266	40066	26809	10193	50452	62019	21829	28581	11282	28782	31438	21570	30476	51484	34370	18059	109028	8450	152435	12553	70594	45711	16355	47635	35490	49807	31473	48666	32764	269138	138559	139989	163999	109096	118691	114919	19108
FiT3	473	5854	2470	17726	12145	965	16152	20502	6653	8169	2251	6328	9782	6296	6717	9028	6403		24511	2336	36327	4681	18059	11238	3420	11786	8645	10605	6354	7588	6309	65220	41792	37346	43451	12866	37171	37540	8571
FiT3	473	5685	2738	18188	14541	782	18457	23365	7730	8645	2808	7389	10340	8439	8479	10385	6024		26047	1813	40529	4524	19275	12847	3962	11637	7232	9445	4987	8244	4313	65793	39145	35521	43824	17506	44864	43639	8969
Alba	473	82157	42699	56285	37685	15828	70558	84835	26853	37174	17144	40627	39304	30612	39135	72744	44253	32074	145865	7467	209290	17453	87310	60450	21293	69177	48760	65315	41604	60646	42281	343092	173270	176357	195724	152633	157023	156400	30030
FiC1	473	42231	25041	35872	24364	5756	39727	40944	14293	19364	2721	15355	18550	12798	12066	19915	14930		59509	3463	96471	7871	44002	30261	5137	28340	20511	24050	15965	16101	13183	112530	61374	61132	72845	70933	76703	71927	14100
FiC1	473	43683	24914	35622	24314	5704	39705	42838	15689	21267	3243	15543	20275	13126	17899	18278	16817		67417	5240	104726	9568	49978	33513	5067	30761	22941	27936	16320	20300	15937	123738	69086	64922	74031	72949	79923	80133	13774
Alba	473	81076	43692	56959	39498	14384	69494	89644	25273	41240	18771	42084	45706	27423	41305	77584	58400	33882	174936	13121	232100	22091	102903	73248	27191	78903	62214	75678	46664	84317	49455	333093	168734	171675	206955	154672	165660	157667	31138
Alba	473	79165	52149	64034	43612	21131	81232	94238	33688	48651	24498	49272	57296	32879	46784	91751	51866	37668	200179	11184	250337	25678	124375	90681	27735	87024	71012	87826	52973	84417	59462	367655	205739	205497	230759	179849	192614	188190	35176
FiC2	473	55674	33857	43063	27916	9223	48360	58698	19246	21301	10442	21085	25377	14059	22682	39887	19782		76412	5038	113489	13990	55384	37336	6037	34312	24180	30311	20350	22085	17559	133304	73325	70290	82328	92483	94196	83962	16246
FiC2	473	52203	27853	40166	26349	7349	45058	59154	18727	22173	10423	19015	22160	14173	21301	38113	21009		75189	3837	121484	17101	59246	38516	6743	39869	30838	35446	24725	27939	20581	132396	77941	78150	85988	81820	92126	82298	18397
Alba	473	81279	47522	58063	37310	27292	82945	92625	27566	41156	16343	40317	48367	27349	39649	85359	89184	31860	186700	10772	229856	22579	112742	86225	29729	80343	63632	74342	50645	79781	53306	312881	170437	168892	203744	163839	177049	165876	31972
Alba	473	19970	12388	13267	8441	3931	17331	21139	5889	8743	3441	9352	10490	4970	8141	13698	11086	4575	25605	1535	30400	4104	14817	9318	4011	10001	6526	10511	7110	10444	7747	85043	42091	39958	52019	35708	36258	32264	8082
FiiT1	473	3214	1391	6290	4190	365	5602	6773	2844	3089	493	2486	3062	2015	2309	3203	2478		7118	352	9123	925	4310	2809	808	2905	1533	2407	1960	2907	1569	17802	11107	9799	11081	5253	11122	11767	3157
FiiT1	473	1996	912	4177	3506	216	4398	5422	1647	1930	387	1646	2594	1792	1698	2692	1917		5532	1226	8929	1226	3995	3151	778	3092	2231	2971	1509	2081	778	15821	8628	7948	10003	4066	9650	9997	2099
Alba	473	21043	10136	11793	6768	2710	15206	20296	6566	7875	2930	8680	10625	3647	9560	10841	10415	3127	24094	1700	30549	2639	13613	9130	2537	10468	7425	8998	5806	9725	6828	88075	42074	39168	49372	28909	31991	31015	7111
FiiT2	473	6882	4364	6873	4838	967	6711	6355	2456	3444	983	2226	3100	1657	3291	3468	3235		10895	1396	17704	1159	8099	5722	589	5402	3962	4797	3245	3461	2219	20910	12116	11295	13256	9092	11289	12024	3106
FiiT2	473	4959	2960	4754	3307	501	5283	4957	2348	2822	1127	2075	2452	1488	2431	2822	2889		9795	655	15552	1457	6765	5174	771	4614	3181	3750	2614	2513	1610	17780	9327	8774	10492	7657	9560	9448	2633
Alba	473	25972	14602	15591	7981	4110	20086	25213	7773	10648	4320	11562	11530	5912	9775	14256	10501	6688	28155	2084	34819	3118	14636	11227	3154	10272	8466	10954	6947	9892	6383	102701	51814	48761	54152	41460	39370	38217	7733
FiiT3	473	1650	418	6193	4567		6257	5744	1623	2309	313	1286	2297	1713	2057	2045	1096		3545	406	6640	455	3027	2529	510	2118	1718	1930	947	1942	1109	22484	13716	12624	14341	4731	10042	10147	3175
FiiT3	473	1200	622	3999	3333	227	3634	3834	1042	1350	206	726	1897	972	1462	1342	696		2966	284	4699	376	2116	1596		1191	815	1504	1026	796	367	14378	9002	6786	8865	2599	7164	7527	1677
Alba	473	19860	11683	13031	7357	3216	15324	18385	5358	7733	2803	9533	10020	4817	8704	8949	7261	3141	15724	1078	22988	2234	10370	7203	2242	6152	4712	7000	4615	6583	4435	89534	43382	46536	51784	30844	32245	35151	8378
Alba	473	16206	9193	10575	7390	2868	14607	15119	5746	6593	2519	8580	8547	4372	7349	8933	8242	2524	14480	1519	21441	2237	9026	6864	1489	6040	4429	7053	5462	6418	3951	82827	39167	38169	44350	28014	27927	24701	6951
FiiC1	473	6379	3935	3967	3216	1309	5668	4199	1703	1943	385	2434	2618	1334	1560	2622	2532		6263	346	10071	1216	4496	3216	266	2757	1930	2563	2151	1809	1262	19803	10324	9488	12539	7573	8919	7860	2105

	Time days	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	TARS 1	TARS 2	TARS 3	TARS 4	βster1	βster2	βster3	βster4
FiiC1	473	7037	3453	4809	3105	1155	5701	4831	2039	1801	261	2196	2544	1726	1647	3262	2093		5764		11501	1166	4803	3588	811	3353	2442	3615	2287	2743	1632	18584	9302	9087	11928	8250	8626	9334	1875
Alba	473	18545	9455	12885	7059	4903	15615	19928	5671	7418	2947	8142	10584	3490	8178	9354	8022	2999	17456	1695	23296	2113	10430	7335	2818	8171	5533	7159	4626	7003	4817	91970	43738	39680	47427	28534	31466	29525	6842
FiiC2	473	11627	5499	8216	5198	1469	8617	9935	1950	3217	1736	2579	5653	2289	3888	2930	2360		6565	299	12382	1065	5446	4414	658	4123	3345	3672	2319	2044	1819	34219	18481	17407	19683	15814	14814	15464	2293
FiiC2	473	12133	5127	6457	5165	1054	7952	10573	2970	3160	1841	2891	4943	1607	3049	2351	1755		7012	465	11528	1268	5256	3873	1131	3432	2510	3096	2283	2369	1983	32301	15938	15226	17756	13691	13002	12876	2158
Alba	473	23283	13646	16119	10683	3193	18755	23664	6747	9522	4277	11191	11684	6806	9186	11617	8840	3374	17668	959	24591	1104	11896	9097	2970	7475	6061	7933	5824	7771	5635	110435	55811	55010	63762	43223	40287	41799	11898
Alba	473	31047	16481	19710	12875	6018	26662	32454	10222	13002	4616	15787	14363	9557	12836	13068	11308	3198	21364	886	23602	2536	13658	12076	3187	9690	6521	9349	5039	9348	5462	129188	64132	62122	73339	52142	55044	56886	12496
Ft0	473	21162	10019	12953	8856	2435	13942	18609	5908	5326	2646	5577	10149	2931	7342	3742	3638		10914	662	11978	785	7993	5419	593	5362	2659	4762	3227	3622	1718	56995	29386	27246	30887	27947	27237	24193	4754
Ft0	473	23946	10903	13452	9868	2670	14790	20410	6063	7190	2841	6729	11344	4276	8229	4087	4237		12660	656	11360		9652	7469	931	7027	4743	5744	4251	4309	2540	62073	31912	31222	37139	31534	31736	30197	5491
Alba	473	53977	27944	33832	22769	8278	45567	49166	14350	22290	8688	22362	23210	13123	19580	21421	18839	7027	37547	2196	24594	4516	21500	16249	5287	15604	10471	14212	9917	16894	5707	187239	96463	92209	106818	93737	101380	93914	20958