CRANFIELD UNIVERSITY

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NEW INSIGHTS INTO THE BIOTRANSFORMATION OF WEATHERED HYDROCARBONS IN SOIL

SCHOOL OF APPLIED SCIENCES

Ph.D. THESIS

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New insights into the biotransformation of weathered hydrocarbons in soil

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Abstract

Weathered petroleum hydrocarbons are a highly complex, important soil contaminant. After forty years of petroleum research, weathered hydrocarbons are still not sufficiently understood or appropriately accounted for in contaminated land risk assessments or the associated analytical methods that inform them. Improved insights into these contaminants potential for biotransformation and their residual toxicity are essential for improving risk assessments, bioremediation strategies and effective regeneration of previously contaminated land.

This thesis explores the biotransformation of weathered hydrocarbons in the context of risk assessment and management. The research includes a critical review and synthesis of six in-house historical pilot studies, implementation of a novel ultrasonic solvent extraction method for petroleum hydrocarbons and development of analytical tools, providing new insights for human and environmental risk assessments. The biotransformation potential and subsequent effect on the toxicity of two weathered hydrocarbon contaminated soils were investigated using soil microcosms. The use of a previously remediated soil provided novel insight into extended bioremediation potential for petroleum hydrocarbon residues to undergo further biotransformation.

The novel ultrasonic extraction method developed collaboratively is a preferred alternative to traditional Soxhlet methods with very high precision (RSD ≤ 10%) and extraction efficiencies. Key benefits of the technique include reduced costs, shorter extraction times (1 h. *vs*. 8 h.) lower solvent consumption (40 ml *vs*. 150 ml) and improved extraction efficiencies (recovery ≥ 95 %).

Ecotoxicological responses (using mustard seed germination and Microtox® assays) showed that a reduction in total petroleum hydrocarbon (TPH) load within soils could not necessarily be linked to a reduction in residual toxicity, thus reductions in TPH alone is not a suitable indicator of risk reduction. The residues in the previously remediated soil underwent further biotransformation with losses of up to 86 and 92 % in the aliphatic and aromatic fractions respectively. Grinding of this soil was shown to reduce the effectiveness of a nutrient treatment on the extent of biotransformation possible by up to 25% and 20% for the aliphatic and aromatic hydrocarbon fractions, respectively. Toxicity assays confirmed that biotransformation is not physically driven by surface area limitations, contrary to expectation, as responses of ground and un-ground soils were not significantly different ($P>0.05$). This may have implications for future studies using grinding as a pre-treatment, where biotransformation may be limited by grinding rather than other factors. Both the soils showed significant biotransformation ($P<0.05$) after 16 weeks of treatment. However, although the soil *not* previously treated had significantly less TPH losses, a loss of up to 92% shows that further degradation of this soil is possible even though previous investigations had suggested biotransformation had stopped. This has implications for bioremediation practitioners in that it questions whether bioremediation could be restarted and lower concentrations achieved, and warrants further investigation.

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Abbreviations/Acronyms

2-Fb	2-Fluorobiphenyl
ANOVA	Analysis of Variance
API	American Petroleum Institute
ASE	Accelerated Solvent Extraction
ASTM	American Society for Testing and Materials
ATSDR	Agency for Toxic Substances and Disease Registry
BTEX	Benzene, Toluene, Ethylbenzene and Xylenes
CCME	Canadian Council of Ministers of the Environment
CO_2	Carbon Dioxide
DERV	Diesel Engine Road Vehicle
DCM	Dichloromethane
DRO	Diesel range organics
EA	Environment Agency (United Kingdom)
E&P	Exploration and Production
EPH	Extractable Petroleum Hydrocarbon
EC_n	Equivalent Carbon number
EC_{50}	Effective concentration (required to induce a 50% effect)
EU	European Union
FT-IR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectroscopy
GC-FID	Gas Chromatography with Flame Ionisation Detection
GC-SIMDIS	Simulated Distillation Gas Chromatography

GC-TCD	Gas Chromatography with Thermal Conductivity Detection
GRO	Gasoline range organics
HMN	Heptamethylnonane
HPIC	High Performance Ion Chromatography
HPLC	High Performance Liquid Chromatography
IR	Infra-red spectroscopy
KD	Kuderna-Danish evaporation
KRO	Kerosene Range Organics
L^2TOFMS	Laser Desorption Laser Photoionisation Time-of-Flight Mass Spectroscopy
MaDEP	Massachusetts Department of Environmental Protection
MAE	Microwave Assisted Extraction
MRO	Mineral oil Range Organics
NAPL	Non-Aqueous Phase Liquid
OBM	Oil Based Muds
*o*TP	*o*-terphenyl
PAH	Polycyclic Aromatic Hydrocarbons
PERF	Petroleum Environmental Research Forum
PFE	Pressurised Fluid Extraction
PLE	Pressurised Liquid Extraction
PROMISE	Optimising the Biopiling of Weathered Hydrocarbons within a Risk Management Framework
Py-GC-MS	Curie point Pyrolysis GC-MS
RBCA	Risk-Based Corrective Action

RIVM	National Institute for Public Health and the Environment
RSD	Relative Standard Deviation
SD	Standard Deviation
SE	Standard Error
SFE	Supercritical Fluid Extraction
SPT	Solid Phase Test
Sq	Squalane
SWE	Subcritical Water Extraction
TBS	Tank Bottom Sludge
TLC	Thin-Layer Chromatography
TLC-FID	Thin-Layer Chromatography with Flame Ionisation Detection
TNRCC	Texas Natural Resource Conservation Commission
TPH	Total Petroleum Hydrocarbons
TPHCWG	Total Petroleum Hydrocarbon Criteria Working Group
UCM	Unresolved Complex Mixture
UK	United Kingdom
U.S. EPA	United States Environmental Protection Agency
VDLUFA	Verband Deutscher Landwirtschaqftlicher Forschungsanstalten
VPH	Volatile Petroleum Hydrocarbons
WHC	Water Holding Capacity

Chapter 1: Introduction and literature review

1.1 Introduction

Contamination of land due to anthropogenic activity, both present and historical, is a global problem. In the UK the Environment Agency estimates that more than 300,000 hectares of land are affected, amassing between 5,000 and 20,000 "problem sites" (Environment Agency, 2005). Contaminated land has become a subject of social, legal, environmental and economic concern within many of the world's industrialised countries (Whittaker et al., 1999; API, 2001; Environment Agency, 2005). Land may be contaminated because of past industrial activity, historic disposal practices, or due to an adverse event such as a chemical spill (Nathanail and Bardos, 2004). Although a large proportion of contaminated land may be attributable to historical practices, modern industrial processes also produce potential contaminants, Thus, contamination of land is an ongoing problem that requires active management.

Petroleum continues to be a widely utilised resource throughout the world. Its use has resulted in contamination through accidental spillage and leakage (Pollard et al., 1994). Certain components of petroleum contamination may pose risks to human health, property, watercourses, ecosystems, and other environmental receptors (Environment Agency, 2003b; Farrell-Jones, 2003). Petroleum, in its natural state, is a highly complex mixture of hydrocarbons with minor amounts of other heterogenic compounds such as nitrogen, oxygen and sulphur (Farrell-Jones, 2003). The composition of petroleum hydrocarbon products can vary substantially depending on the nature, composition, and degree of processing of the

source material (Pollard et al., 1999). Once released into the environment, petroleum products are subject to physical, chemical and biological processes that further change its composition, toxicity, availability and distribution (partitioning) within the environment (Figure 1.1). Such processes include adsorption, volatilisation, dissolution, biotransformation, photolysis, oxidation, and hydrolysis (Pollard et al., 1994; TPHCWG, 1998a; Jorgensen et al., 2000; Barakat et al., 2001; Environment Agency, 2003b). Oils that are weathered because the source term has aged significantly since release can be defined as weathered hydrocarbons. The extent of weathering experienced is particularly important when characterising petroleum contamination prior to remediation (Wang and Fingas, 2003), especially the heavy oils (the viscous (50-360 mPa s), high-boiling (ca. 300->600 °C) products such as No. 6. fuel oil with carbon ranges in excess of C_{20}). Whilst there is a large literature describing the composition and properties of petroleum products (TPHCWG, 1998a), due their highly complex nature there is a relative paucity of information on the toxicity, distribution, transport, and availability of weathered hydrocarbons in the environment (Pollard et al 1994; TPHCWG, 1998a).

As with all contaminants, their chemistry determines which environmental compartment they are found in and thus analysed and is also responsible for their environmental fate and transport characteristics. Analytical methods for determining concentrations of hydrocarbons in the soil need to be technically and economically feasible and capable of analysing the range of compounds key to the risk management protocols

applied (Environment Agency, 2003b). Various extraction and analytical methods are available for the characterisation of petroleum hydrocarbons, however, results from these methods can suffer from inter-method variation as illustrated by Buddhadasa et al. (2002). Additionally, as discussed by Whittaker et al. (1995), methods can suffer from both positive and negative analytical bias (Whittaker et al., 1995). Thus, even though a method may have high precision additional calibration is required to account for this bias (difference between the mean and the known concentration) within the results generated. Gas chromatography is a widely used technique for the analysis of petroleum hydrocarbons (Wang and Fingas, 1995; Wang et al., 1999). Biodegradation of more amenable components of the petroleum mixture leads to relative enrichment of the more recalcitrant species. Incomplete resolution of this more recalcitrant mixture leads to a characteristic “humped” appearance of the gas chromatograms output. The “hump” is the resulting signal produced by many hundreds of components such as cyclic and branched hydrocarbons and is widely referred to as the unresolved complex mixture (UCM). The shape and position of the UCM is not constant and depends on the nature of the original petroleum contamination and the extent of degradation that has taken place since release. These issues need to be addressed when implementing a national risk-based framework, as differences in analytical approach may inadvertently result in excessive or inadequate remediation being performed.

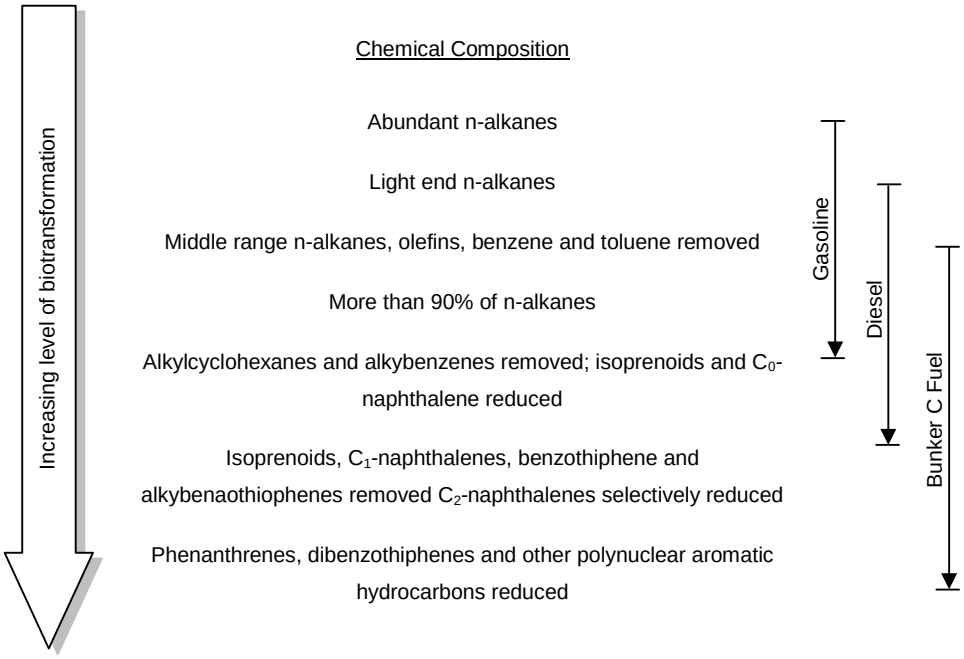


Figure 1.1: General petroleum hydrocarbon degradation pattern (modified after Kaplan et al., (1996)) (Note: right hand side illustrates the presence of the middle column in each of the compounds).

Risk assessment is now a well-established requirement for the management of contaminated land (ARCADIS, 2004) and support tool for environmental management decisions. It is widely used as a means of assessing and managing potential impacts to human- and ecosystem health (ARCADIS, 2004 and Vegter, 2001). Several risk-based frameworks for petroleum hydrocarbons in soil have been published under the auspices of the Total Petroleum Hydrocarbon Criteria Working Group (TPHCWG, 1998a), the American Society for Testing and Materials (ASTM, 1994), the Massachusetts Department of Environmental Protection (MaDEP, 2002), the Environment Agency of England and Wales (EA, 2003), the American

Petroleum Institute (API, 2001) and the Canadian Council of Ministers of the Environment (CCME, 2000), each reflecting national legislation and socio-economic issues (API, 2001; Vegter 2001 and Vegter et al., 2002). These frameworks, and the exposure assessment methods embedded within them, do not specifically address weathered hydrocarbons, although many acknowledge that petroleum products released to the environment will have undergone some degree of degradation (TPHCWG, 1998a; ATSDR, 1999; API, 2001; MaDEP, 2002; Environment Agency, 2003b; Environment Agency, 2005). Weathering of fresh petroleum product makes it very difficult to accurately predict the composition, toxicity and distribution of petroleum at a given site.

Historically the remediation of soil contaminated with petroleum hydrocarbons has been expressed in terms of reductions in total petroleum hydrocarbon (TPH) load rather than risk reduction. This practice still remains as standard in a number of countries; examples include Portugal and the UK (Ferguson, 1999; Environment Agency, 2003b). Recent stakeholder consultations in the UK, and subsequent publications from the Environment Agency, aim to adopt a risk-based framework where remediation is expressed in terms of risk, consistent with other countries (e.g. North America (TPHCWG, 1997), Canada (CCME, 2000) and the Netherlands (Barrs et al., 2001; Environment Agency, 2003b, 2004 and 2005).

There are a plethora of techniques available for, the remediation of contaminated land (Cookson, 1995; Wood, 1997; Eweis et al., 1998;

BIOWISE, 2000; Environment Agency, 2002; Hyman and Dupont, 2001; Wood, 2001), for which there are three main approaches; (i) containment (i.e. excavation & disposal, hydraulic, physical and chemical containment); (ii) separation (i.e. physical separation) and (iii) destruction (i.e. physical, chemical or biological destruction) (BIOWISE, 2000). Choice of approach depends on a number of environmental, economic and human health considerations (Kaufman, 1994). The UK adopts the 'suitable for use' approach as the most appropriate strategy for the sustainable development of contaminated sites (DETR, 2000; Holgate, 2000). Within the land remediation sector, the EU Landfill Directive (The Council of the European Union, 1999) is now encouraging the development and implementation of alternative remediation techniques and is expected to further increase the cost-effectiveness of bioremediation technologies (Sims and Sims, 1995; Pollard et al., 2005). This has resulted in increased interest and use of the technique for the remediation of hydrocarbon-contaminated soils.

A complete understanding of the contaminant in question is a key component when estimating potential risks to human health. To achieve this, adequate information regarding environmental fate, behaviour and distribution, toxicity, concentration, and potential exposure to a substance at a site is essential (Environment Agency, 2003) (Figure 1.2).

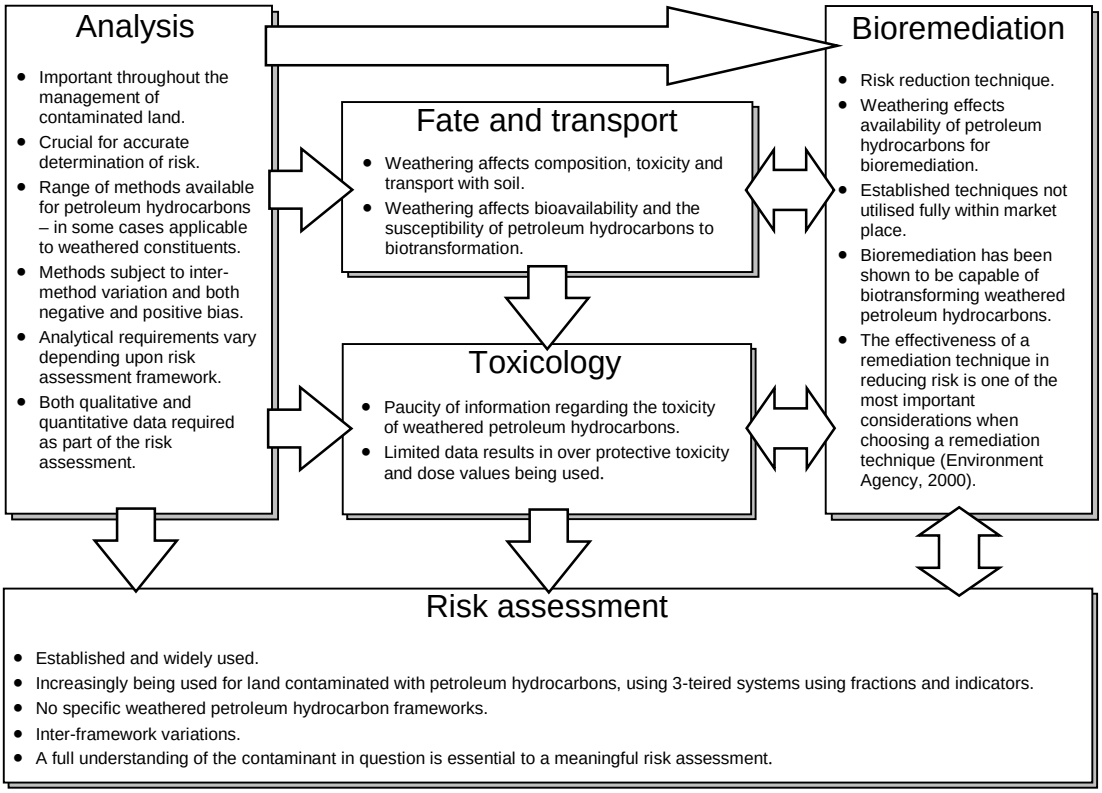


Figure 1.2: Illustration of the interactions of the key elements involved in remediation of weathered petroleum hydrocarbon contaminated land.

1.2 Characterisation of weathered hydrocarbons

Weathered hydrocarbons are those oils where the source term has aged significantly since release. This weathering of the source term further complicates these complex compounds, potentially giving rise to additional analytical issues.

There are several extraction and instrumental analysis techniques that can be used to characterise petroleum hydrocarbon contamination in soil. Method development is often driven by the objectives of published risk

assessment frameworks (Table 1.1) (ASTM, 1994; MaDEP, 1994; TPHCWG, 1998b; ATSDR, 1999; CCME, 2000; API, 2001). Many frameworks (e.g. TPHCWG, API, CCME, MaDEP) require the quantification of specific indicators and/or fractions; while others consider indicator compounds or chemicals of concern, such as the United States Environmental Protection Agency's (U.S. EPA) 16 priority polycyclic aromatic hydrocarbons (PAHs) (MaDEP, 1994; TPHCWG, 1998b; CCME, 2000; API, 2001). It is necessary to use analytical techniques capable of identifying and quantifying specified aromatic and aliphatic 'fractions' as well as the specific indicator compounds selected by the different protocols (summarised in Table 1.1). These compounds are known carcinogens including benzene, toluene, ethylbenzene and xylene (BTEX) and the 16 U.S. EPA PAHs (MaDEP, 1994; AEHS, 2000; CCME, 2000; Environment Agency, 2003b). Some frameworks stipulate quantification of a wide range of petroleum hydrocarbons *e.g.* UK approach suggests compounds from an equivalent carbon number of 5 to 70 be examined (Environment Agency, 2003b) (Table 1.1).

Table 1.1: Summary of the analysis methods developed for several risk assessment frameworks.

	Massachusetts Department of Environmental Protection (MaDEP, 1994)	Total Petroleum Hydrocarbon Criteria Working Group (TPHCWG 1997a & b, 1998a & b and 1999) .	Canadian Council of Ministers of the Environment (CCME, 2000).	New Zealand (Ministry for the Environment, 1999)	New South Wales (National Environment Protection Council, 1999)
Description	Use of two methods. Volatile petroleum hydrocarbon (VPH) method (MaDEP, 2004b) and extractable petroleum hydrocarbon (EPH) method (MaDEP, 2004a) developed by MaDEP. The EPH method refers to U.S. EPA methods for sample extraction (MaDEP, 2004a and 2004b)	Use of 'The Direct method' (AEHS) (AEHS, 2000) developed for the TPHCWG framework. Based upon U.S. EPA SW-864 test methods(U.S. EPA, 2005) and MaDEP EPH method (MaDEP, 2004a)	Recommends the use of benchmarked methods (CCME, 2000), however also allows the use of non-benchmarked methods providing that validation data demonstrate that the substitute method provides data comparable to the benchmark method.	Permits the use of a variety of methods, including those prepared by the Oil Industry Environmental Working Group (1999)(Oil Industry Environmental Working Group, 1999) which outlines methods for several different petroleum products often referring the reader to U.S. EPA documentation)(Oil Industry Environmental Working Group, 1999) .	Recommends the use of methods specified in the National Environmental Protection Councils (NEPC) Schedule B(3): Guideline on Laboratory Analysis of Potentially Contaminated Soils (1999) (National Environment Protection Council, 1999). Where no suitable analytical method is available it recommends the use of U.S. EPA, or equivalent methods. All chemical analysis should be carried out in laboratories currently accredited by the national association of testing authorities (NATA).
Reported Range	C_5 to C_{36}	C_6 to C_{35}	C_6 to C_{50}	C_6 to C_{36}	C_7 to C_{36}
Sample collection	EPH method uses amber glass wide mouth sample jars with Teflon lined screw caps. These are cooled immediately after collection and extracted within 14 days of receiving the sample. VPH method uses specially designed air tight collection vials with Teflon-lined septa screw caps stored at 4˚C and preserved with methanol before analysis within a maximum of 28 days.	Wide mouth glass jars with Teflon lined caps stored at 4˚C. Analysis must be performed within 14 days of sample collection.	Wide mouth glass jars with aluminium foil or Teflon-lined lids. Samples must completely fill the jars. Samples are not chemically preserved but are cooled to 4˚C. Laboratory sample handling procedure is also outlined.	100ml (volatiles) and 250ml (semi-volatiles) Borosilicate jars with Teflon-lined cap and completely filled. Stored at 4˚C in the dark.	Use of U.S. EPA or equivalent methods

(Continued on next page)

Table 1.1: Summary of the analysis methods developed for several risk assessment frameworks *(continued).*

	Massachusetts Department of Environmental Protection	Total Petroleum Hydrocarbon Criteria Working Group	Canadian Council of Ministers of the Environment	New Zealand	New South Wales
Extraction technique	VPH method uses Purge and trap with methanol. EPH method uses DCM for extraction and solvent exchanges into hexane. Using U.S. EPA methods 3540C (U.S. EPA, 1996b) (Soxhlet), 3545A (U.S. EPA 1996c) (pressurised fluid extraction (PFE)), 3541 (U.S. EPA, 1994) (Automated Soxhlet extraction), 3546 (U.S. EPA, 2000) (Microwave extraction) and 3570 (U.S. EPA, 2002) (microscale solvent extraction (MSE)).	Vortex or shaker method using n-pentane.	Purge and trap for C_6 to C_{10} range using methanol. Soxhlet is the benchmarked method for the C_{10} to C_{50} range.	For the C_{10} to C_{36} range any method that can be demonstrated to meet the performance criteria can be used. For the C_6 to C_9 range purge and trap is used.	U.S. EPA methods 3540B(U.S. EPA, 2005) or 3540C (U.S. EPA, 1996b) (Soxhlet extraction), 3550B (U.S. EPA, 1996d) (sonication extraction) or sequential bath sonication and agitation described by NEPC (National Environment Protection Council, 1999).
Evaporation	The EPH method uses those specified by the U.S. EPA. However, after fractionation the use of a gentle stream of air or nitrogen is recommended to bring the sample to the required volume. Evaporation is not applicable to the VPH method.	N/A	Uses an evaporation vessel after extraction for the C_{10} to C_{50} range. After silica gel cleanup rotary evaporator is the benchmarked method to reach the required sample volume.	Permits the use of any method that can be demonstrated to meet the performance criteria.	U.S. EPA methods specified for extraction using Kuderna-Danish (K-D) evaporation.
Clean up /fractionation	Silica gel clean up for EPH method. Not applicable to VPH method.	Extract fractionation using alumina or silica.	One of two specified clean up steps for C_{10} to C_{50} range, not fractionated.	Clean up steps and fractionation are optional as this may not be required for each sample/analytical approach.	Solvent exchange into hexane followed by K-D evaporation and treated with silica gel as described in U.S. EPA method 1664(U.S.EPA, 2005; (National Environment Protection Council, 1999)) .
Analysis Technique	EPH uses GC-FID[*]. VPH may use either GC/FID[*] or GC/PID[#].	GC-FID[*]	GC-FID[*]	For the C_{10} to C_{36} range GC-FID[*] is used and for the C_6 to C_9 range GC-MS[$] is used.	GC-MS[$], or GC-FID[*], however the use of GC/MS[$] to identify unusual mixtures is noted as being necessary when analysing by GC-FID[*].

[*]GC-FID refers to gas chromatography with flame ionisation detection, [#]GC-PID refers to gas chromatography with photoionisation detection, [$]GC-MS refers to gas chromatography with mass spectroscopy detection

1.2.1 Extraction of petroleum hydrocarbons from soil and class fractionation

Methods for the extraction of petroleum hydrocarbons from soil samples have been reviewed extensively in the open literature. They include purge and trap (volatiles), headspace (volatiles), manual shaking, Soxhlet, ultrasonic extraction, pressurised fluid extraction, microwave-assisted extraction and super-critical fluid extraction (TPHCWG, 1998a). For heavily weathered fuel oils, extraction of volatile hydrocarbons is rarely considered. Soxhlet extraction is commonly used in research, yet several risk assessment frameworks adopt manual shake methods, e.g. TPHCWG (AEHS, 2000; TPHCWG, 1999), the Agency for Toxic Substances and Disease Registry (ATSDR; 1999) and the Texas Natural Resource Conservation Commission (TNRCC; 2001). This method involves shaking or vortexing 10 g (typically) of soil with 10 ml of an appropriate solvent (typically *n*-pentane) for 1 hour, after which an aliquot is drawn for analysis (AEHS, 2000; MaDEP, 2004b). The popularity of manual shake/vortex methods is due to a combination of convenience and cost; it is quicker, easier, more accessible and cheaper than Soxhlet extraction, with no concentration step required prior to analysis (TPHCWG, 1998a; Farrell-Jones, 2003). Additionally legislative analysis requirements within some countries can be met using this method rather than a more exhaustive technique.

Soxhlet extraction (TPHCWG, 1998a; Farrell-Jones, 2003) is the benchmark method for the CCME C_{10}-C_{50} hydrocarbon range and a

component of the U.S. EPA methods for semi- and non-volatile organics in soil (U.S. EPA, 1996b; CCME, 2001b). Soxhlet extraction is a highly exhaustive extraction technique and can handle both air-dried and field-moist samples, the latter being facilitated through the addition of chemical drying agents, such as anhydrous sodium sulphate, prior to extraction. A wide range of solvent types can be employed making this technique versatile for different chromatographic end points. The Soxhlet method generates a relatively large volume of extract requiring concentration prior to chemical analysis. This may be seen as a disadvantage due to potential contamination and losses that may occur during concentration steps (TPHCWG, 1998a). However losses can be minimised through the use of concentration methods such as Kuderna-Danish.

The time taken to extract a sample using Soxhlet extraction and ultimately its cost has initiated investigations into alternative methods. Hawthorne et al. (2000), for example, reviewed methods available for the extraction of PAHs from historically-contaminated soils. Methods included Soxhlet extraction, pressurized liquid extraction (PLE), supercritical fluid extraction (SFE) and subcritical water extraction (at 300 and 250 ˚C) (SWE). Comparisons were made between hydrocarbon recovery, the effects on the sample matrix, the presence of co-extracted (non-target) matrix material and the relative selectivity for extracting different classes of target organics. The authors concluded that extraction methods that are relatively simple to perform yield the ‘dirtiest’ extracts; while those yielding cleaner, more specific extracts required methods that are relatively complex (Hawthorne et

al., 2000). Soxhlet and PLE yielded much darker and turbid extracts whereas subcritical water extracts were orange to dark orange in colour with moderate turbidity. SFE extracts were light yellow in colour and clear. Soxhlet and PLE yielded more artefact peaks in the gas chromatogram and, due to the extracts from these methods having a high soil matrix content, more frequent cleaning of GC injection ports was required in comparison with SFE extracts (Hawthorne et al., 2000). However, the development and enhancement of GC techniques negates this issue through enhanced sensitivity which allows the analysis of more dilute samples. In the study by Hawthorne et al. (2000) although there were minor differences in extraction efficiencies, the quantitative agreement between the methods investigated was reportedly good (Soxhlet extraction, pressurized liquid extraction (PLE), supercritical fluid extraction (SFE) and subcritical water extraction (at 300 and 250 ˚C) (SWE)), however it should be noted that methods such as SFE may be more expensive than their counterparts (Hawthorne et al., 2000). It has also been shown by Hollender et al., (2003) that ultrasonic extraction and accelerated solvent extraction can achieve higher extraction efficiencies when extracting PAHs than Soxhlet extraction. Saifuddin and Chua (2003) compared Soxhlet extraction to microwave-assisted extraction (MAE) (Saifuddin and Chua, 2003). Here, MAE was quicker (33 min vs. 24 hrs for Soxhlet extraction), used less solvent (4 ml of solvent compared to 20 ml for Soxhlet extraction) and capable of slightly higher extraction efficiencies (82 % rather than 77 % for Soxhlet extraction). However, samples needed to be free from metallic particles which clearly limits application of this technique

to contaminated soils (Dean, 1995; Farrell-Jones, 2003). Additionally, although MAE achieved higher extraction efficiencies, there was no significant difference between the data for MAE and Soxhlet extraction ($\alpha = 0.05$), thus the benefit of a slight increase in extraction efficiency is questionable (Saifuddin and Chua, 2003).

Soxhlet extraction is considered a harsh method that extracts a fraction closer to the full capacity of the soil for hydrocarbons, rather than extracting a more biologically relevant analogue of extractability (Reid at al., 2000). Soxhlet extracts all TPH within a soil, both contamination and naturally present some of which may naturally be highly bound to the soil and typically unavailable to microorganisms and transport within the soil. As such Soxhlet may extract TPH for which no source-pathway-receptor link is present. It has been suggested that methods that only extract environmentally relevant pollutant molecules should be used (those biologically available and for which a source-pathway-receptor link is present) (Hawthorne et al., 2000; Reid et al., 2000). Although any concentration determined by extraction is operationally defined, it may be more appropriate to employ a ‘weaker’ extraction that may determine a closer analogue of bioavailability and hence potential risk, depending on the use of the data.

Non-petroleum based hydrocarbons may result in spurious or elevated TPH concentrations especially when remediation methods employ the use of bulking materials such as woodchip. In order to limit interference, it is necessary to purify samples prior to analysis (Wang and Fingas, 2003). The

most commonly used methods of cleanup employ alumina or silica gel (U.S. EPA methods 3611B and 3630C respectively), used by the TPHCWG, ATSDR, TNRCC, CCME and MaDEP risk assessment frameworks (ATSDR, 1994; AEHS, 2000; CCME, 2001a; TNRCC, 2001; MaDEP, 2002b). This cleanup method also facilitates fractionation into aliphatic and aromatic fractions, which is required by MaDEP, TPHCWG, ATSDR and the EA (ATSDR, 1994; MaDEP, 1994; TPHCWG, 1999; Environment Agency, 2003). However it is likely that any moderately polar compounds will be retained in the silica matrix including any which increase in polarity as a result of biotransformation. This may be an issue when analysing weathered hydrocarbon wastes and those undergoing remediation. Attempts to automate the fractionation procedure have resulted in incomplete resolution of the aliphatic and aromatic fractions. Whilst some well-resolved components could be eliminated by subtraction, incomplete separation does not address any UCM present. The key fractions affected involve the mono- and di-aromatics.

Extracted samples often need to be concentrated prior to analysis, and before and/or after cleanup steps where an unacceptable level of dilution may be introduced, *e.g.* Soxhlet extraction (U.S. EPA, 1996b; TPHCWG, 1998a; Farrell-Jones, 2003) and class fractionation (U.S. EPA, 1996e; TPHCWG, 1998a; Farrell-Jones, 2003). There are several concentration methods that can be used: Kuderna-Danish concentration, nitrogen evaporation, and rotary evaporation. A concentration step is further source of error. For example, identification errors may occur if samples are

evaporated too exhaustively during sample preparation using methods such as rapid nitrogen evaporation, where volatile components are most likely to be lost (MaDEP, 1994). The use of a keeper solvent such as acetonitrile and methods such as Kuderna-Danish, as specified by the U.S. EPA Soxhlet extraction protocol, is considered to minimise such losses (TPHCWG, 1998a).

Due to the wide carbon range covered by hydrocarbon products and the tiered nature of some risk assessment frameworks, no single analysis technique is likely to be sufficient for analysing soils freshly and historically contaminated with petroleum hydrocarbons. It would seem sensible that if a tiered risk assessment (see section 1.3) is used then a systematic tiered analysis strategy be matched to it, as progression to higher tiers and thus higher levels of analytical complexity may not in all situations be necessary. The use of tiered analytical approaches are increasingly being applied in oil spill identification (Wang and Fingas, 1995 and Wang et al., 1997). For example, Wang et al*.* (1997) used a 5 tiered analytical approach, tier 1 determined oil residues, tier 2 determined *n*-alkanes and TPHs, tier 3 quantitatively identified target PAHs and biomarker components, tier 4 determined and compared diagnostic ratios of source-specific-marker compounds and tier 5 determined weathered percentages of residual oil (Wang et al., 1997). Overall these tiers enable the identification of oil type, degree of weathering and biodegradation (Wang et al., 1997).

Many of the risk assessment frameworks for petroleum hydrocarbons specify preferred extraction and analytical techniques; some having

published their own recommended methods (CCME, TNRCC, TPHCWG and MaDEP) (Table 1.1). The majority specify manual shake or vortexing methods with an appropriate solvent to extract the sample, followed by alumina or silica gel clean up and fractionation into aliphatic and aromatic compounds (MaDEP, 1994; AEHS, 2000; TNRCC, 2001). The MaDEP approach specifies volatile petroleum hydrocarbon (VPH) and extractable petroleum hydrocarbon (EPH) determinations. The VPH method uses a purge and trap approach, whereas the EPH method specifies extraction using dichloromethane (DCM), cross-referring to the U.S. EPA extraction method followed by silica cleanup and fractionation prior to analysis (MaDEP, 2004a and 2004b). The CCME method specifies purge and trap for the fraction range C_6-C_{10}, or Soxhlet extraction followed by silica gel clean up and fractionation for the C_{10}-C_{50} range (CCME, 2001b). However, it is stated that suitable alternative techniques can be used on the condition that validation data can demonstrate that the alternative method provides data comparable to the benchmark protocol (CCME, 2001b). The CCME method allows for use of U.S. EPA methods, adding further quality control measures (CCME, 2001b). Although in prescribing specific methods the CCME also allows laboratories to use in-house methods, the validation requirement of these methods should ensure the production of comparable data across laboratories with the presumption of comparable risk assessment and remediation outcomes. Neither the EA nor ASTM specify methods for the extraction of petroleum hydrocarbons in risk assessment, however the EA is to adopt performance criteria rather than prescribing

specific approaches (ASTM, 1994 and Environment Agency, 2003b). Here, as with the CCME, the emphasis is on quality and reliability of data rather than the use of specific ‘gold standard’ techniques.

1.2.2 Analytical methods used for petroleum hydrocarbons

The techniques used for the analysis of petroleum hydrocarbons can be grouped by their measurement outcome: (i) quantitation of the petroleum hydrocarbon load; (ii) the concentration of different groups of hydrocarbons; or (iii) the concentration of specific target compounds (TPHCWG, 1998a). There are also methods for the rapid on-site screening of contaminated soils. However, the majority of these are based on the measurement of vapours derived from the vadose zone by either *in situ* soil gas measurements or headspace analysis. In the case of weathered petroleum hydrocarbons, the relevance of such methods will depend upon time and alteration mechanisms. Further analysis would also be required to enable the analysis of components with low volatility present within weathered hydrocarbons (Wang and Fingas, 2003).

Methods that generate total petroleum hydrocarbon concentrations and group (fraction) concentrations are considered to be non-specific techniques (Wang and Fingas, 1995). These generate basic information that is a surrogate for contamination, *e.g.* a single TPH concentration. Such data are not suitable for risk assessment in isolation (TPHCWG, 1998a; Farrell-Jones, 2003). However, they are inexpensive, quick and easy and, as such,

can offer a useful screening tool (TPHCWG, 1998a; Farrell-Jones, 2003). The most commonly used specific methods include gas chromatography (GC), gas chromatography mass spectrometry (GC-MS), gas chromatography with flame ionization detection (GC-FID), fourier transform infrared spectroscopy (FT-IR), thin layer chromatography (TLC) and gravimetric analysis (TPHCWG, 1998a). Gas chromatographic methods are the most preferred TPH measurement techniques as they offer sensitivity, selectivity, and can be used to identify risk-critical compounds. As the compositions of crude oil and petroleum products are highly complex and display a high degree of between-oil variation, unique chemical 'fingerprints' for each oil can be isolated. These can be used to aid identification of the source of weathered oil contamination (Wang and Fingas, 2003). Techniques such as GC require additional skills/experience compared to other methods and require that samples are volatile at the operating temperature of the column (Dean, 1995). Issues also arise with co-elution of compounds as petroleum hydrocarbons comprise many isomers with similar boiling points and thus retention times. Weathered hydrocarbons typically exhibit low volatility, high boiling temperatures and require high column operating temperatures. This can vary depending upon the starting product and whether sorbed or mobile fractions are under analysis. GC techniques can be adapted to enable the analysis of specific hydrocarbon ranges, such as gasoline range organics (GRO) and diesel range organics (DRO) (Farrell-Jones, 2003) but are often unable to resolve a large proportion of unresolved complex mixtures (UCM), characteristic of weathered petroleum

hydrocarbons (Whittaker et al., 1999). This may become an issue as more toxicological data becomes available in the future.

Gas chromatography coupled with mass spectrometry detection (GC-MS) is routinely applied for the identification and measurement of individual petroleum hydrocarbons. These methods have a high level of selectivity, with the ability to confirm compound identity though the use of retention time and unique spectral patterns. GC-MS requires specialist operation and interpretation of the data and, as such, it can be more expensive than other GC methods depending on the market forces. GC-MS offers target analyte confirmation, non-target analyte identification and can be used to separate hydrocarbon classes (Hutcheson et al., 1996). Even with ready benchtop availability, some jurisdictions have felt unable to recommend GC-MS analysis of petroleum hydrocarbons to inform risk assessments (Hutcheson et al., 1996). The analysis requirements of current frameworks can be easily met, relatively cheaply by GC-FID. The MaDEP method adopts GC-FID methods along with the majority of risk assessment frameworks.

In response to the difficulties with traditional methods for the analysis of weathered petroleum hydrocarbons, alternative and specialised methods have been developed. Whittaker et al., (1995) in reviewing both conventional and novel analytical techniques for the characterisation of refractory wastes, highlighted several of these including simulated distillation gas chromatography (GC-SIMDIS), thin-layer chromatography with flame ionisation detection (TLC-FID), high-performance liquid

chromatography (HPLC) and laser desorption laser photoionisation time-of-flight mass spectrometry (L^2TOFMS) (Whittaker et al., 1995).

The coupling of curie point pyrolysis to GC-MS (Py-GC-MS) is an alternative method to conventional techniques for the analysis of non-volatile compounds such as rubbers, paints and synthetic plastics and has been applied to several sample matrices including soil (Buco et al., 2004). Curie point pyrolysis is an established technique during which maromoleucles are thermally dissociated into macro- and oligo-meric fragments, of which the macro-meric fragments are amenable to GC-MS analysis (van Loon et al., 1995). Buco et al., (2004) evaluated this technique for the analysis of the 16 PAHs included in the U.S. EPA priority pollutant list, and demonstrated repeatability within the range of classic techniques (RSD = 3.4%) with good accuracy for the measured PAHs (Buco et al., 2004). This technique is quick, involves no cleanup and does not require an extracting solvent. Particularly effective for low-molecular-mass PAHs, high molecular mass PAH quantification was complicated by reduced sensitivity. This may limit Py-GC-MS use for analysis of weathered petroleum hydrocarbons (Buco et al., 2004). Additionally, the small sample volume used makes the homogenization of samples critical for accurate analysis (Buco et al., 2004). The authors concluded that Py-GC-MS is suited to use as an alternative screening method for contaminated soil or sediment (Buco et al., 2004).

1.3. Risk management frameworks for hydrocarbons

Risk assessments should provide an “objective, scientific evaluation of the likelihood of unacceptable impacts to human health and the environment” (NICOLE, 1998). Where a ‘pollutant linkage’ between the source of a hazard and a receptor is present (Pollard et al., 1994; API, 2001; Vik et al., 2001; Vegter, 2002), estimates of exposure are often used to characterise risks to human health, comparing the potential intake of contaminants with acceptable or tolerable intakes inferred from toxicological or epidemiological studies. Many risk assessment frameworks adopt a three tiered approach with increasingly sophisticated levels of data collection and analysis (ASTM, 1994). As assessors move through the tiers, the generic and conservative approach of the earlier tiers is replaced with more detailed and site-specific assumptions (ASTM, 1994; API, 2001; Environment Agency, 2003b), although each tier aims to be protective of human health (ASTM, 1994; API, 2001; Environment Agency, 2003b; ARCADIS, 2004; Environment Agency 2005; MaDEP, 2005). The progression to higher tiers involves additional cost due to increased analytical and site investigation requirements. This expenditure enables a more complete characterisation of contaminants resulting in a more comprehensive risk assessment and more cost-effective corrective action (risk management) plans (ASTM, 1994). Site-specific assumptions resulting from use of the higher tiers may increase the cost-effectiveness of the remediation, and so assessors need to balance the increased cost and time against potential benefits before proceeding to the next level (ASTM, 1994). Cost-benefit analysis techniques are built into

some risk assessment frameworks to facilitate decision making for tier transmission (API, 2001).

Different countries and organisations consider aspects of risk assessment frameworks differently. For example, residential exposure scenarios have not been considered as relevant in the API framework (API, 2001). This is because the most realistic future use for exploration and production (E&P) sites are for ranch, agricultural or parkland land uses.

Hydrocarbon-contaminated soils contain many hundreds of different compounds. Although it may be feasible to identify each of the compounds present, this would be unnecessarily time consuming. Further, data describing the toxicity, partitioning, fate and transport characteristics of the different compounds are not currently available (API 2001, MaDEP, 2002b). Identification and assessment of all compounds would be burdensome which would not be practicable for stakeholders (Environment Agency, 2003b and 2005). Therefore, surrogate measures for carbon fractions of toxicological significance, such as boiling point and carbon number ranges, have been used to simplify the assessment process (ASTM, 1994). Furthermore, risk management frameworks have focused on a limited subset of key components, using broad observations regarding the characteristics of known petroleum hydrocarbons to group compounds into fractions and identify key toxic compounds for use as indicators (ASTM, 1994; API, 2001). Typically, petroleum fractions are used to consider threshold health effects while indicator compounds are used to evaluate non-threshold health effects (Environment Agency, 2005).

Approaches such as the ASTM (ASTM, 1994) risk-based corrective action (RBCA) framework use indicator compounds as a surrogate for risk. This approach was deemed by MaDEP (MaDEP, 1994) as insufficient for characterising risks posed at a petroleum hydrocarbon release site and fractions were introduced. The definitions of specific fractions are derived from either the carbon number (C_n) or equivalent carbon (EC_n) number. For example, MaDEP uses fractions to evaluate the threshold contaminants and indicator compounds (or 'target analytes') to evaluate non-threshold toxicity (ASTM, 1994; MaDEP, 1994; Environment Agency, 2003b). The MaDEP approach is one of the few approaches that use carbon numbers. Here TPH fractions are based upon "chemical structure, carbon number, and structure activity relationships" (MaDEP, 1994). The majority of frameworks use equivalent carbon numbers (EC_n), *e.g*. TPHCWG (TPHCWG, 1998a) because these are considered more closely related to the mobility of a compound in environmental media (Environment Agency, 2003b). As such, EC_n are based on "a range of physical-chemical properties and simple partitioning models" (TPHCWG, 1997a). In practice, the boiling point of the compound of interest on a non-polar GC column is used to derive EC_n, assuming the relationship between boiling point and EC is the same for both aromatics and aliphatics. In characterising the toxicity of a fraction, surrogate compounds or mixtures that are well characterised and characteristic of a particular fraction are often used (TPHCWG, 1998a; Environment Agency, 2003).

The validity of the equivalent carbon number may be challenged. For example, the TPHCWG derive EC_n using a simple empirical binomial model parameterised using data describing the boiling point (T_B, °C) and carbon number of 75 key hydrocarbons; where K_1 and K_2 are empirical constants, and C is the intercept (Equation 1).

$$EC_n = K_1[T_B]^2 + K_2[T_B] + C \qquad (1)$$

At best, this provides only a rough estimate of EC_n (e.g. a measured EC value of 31.3 for benzo[a]pyrene compared to the calculated value of 30.0 using Equation 1). Also, a T_B of 548 should relate to EC_{44}, however calculating this from Equation 1 provides a value of $EC_{34.6}$. Clearly there is a disparity between the TPHCWG model and the empirical data. Different parameterisations will have an effect on calculated EC_n. Figure 1.3 shows a series of fitted binomial models based on four different parameterisation data sets. As the boiling point increases, a clear disparity emerges between the *n*-alkanes and the PAHs. This can be seen most clearly in the "empirical" plot (Figure 1.3), between boiling point 450 and 550 °C, where PAHs have markedly lower EC numbers than the *n*-alkanes. Figure 1.3 suggests that the EC_n approach is unsuitable, particularly for substances $EC_{>20}$. Simple empirical models, such as equation 1, do not hold true; and the theory that T_B can be used to calculate EC_n representative of normalisation to the *n*-alkanes appears to be incorrect. However, the

implications for risk assessment are likely to be minimal, considering the heterogeneity of soils

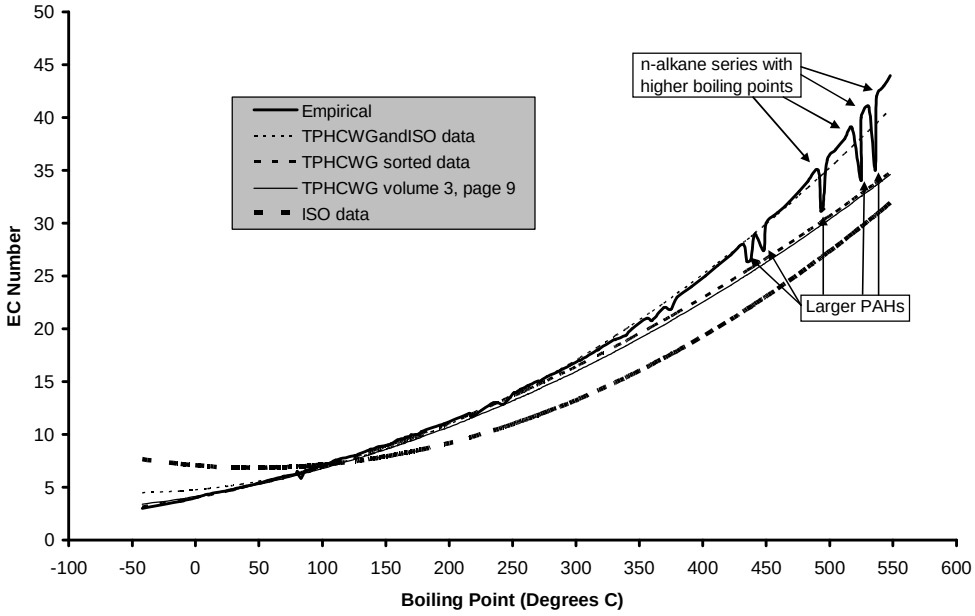


Figure 1.3: Estimated equivalent carbon number using Equation 1 parameterised with four different data sets. The measured data are also provided for comparison.

Aromatic and aliphatic compounds differ in their toxicity, solubility and fate and transport characteristics (MaDEP, 2002a). Because of this, and the evidence shown in Figure 1.3, some frameworks employ fractions where aliphatic compounds are considered separately to aromatic, which are further fractionated by (equivalent) carbon number (Table 1.2). Each fraction may then be treated as if it were a separate compound in the environment (MaDEP, 1994; TPHCWG, 1998a; API, 2001). However, the 'New Zealand

Approach' (Ministry for the Environment, 1999) only considers aliphatic fractions while the aromatic faction is addressed separately by direct measurement of BTEX and PAH concentrations (Environment Agency, 2003b).

Toxicity values are assigned to the fractions and indicators used. This is achieved through the process of review and/or extrapolation of available toxicological data on hydrocarbon mixtures and specific hydrocarbon compounds (MaDEP, 1994). The number of fractions and their ranges vary between frameworks (Table 1.2), and in general build upon or adapt the fractions defined by TPHCWG and MaDEP. Various bodies have adapted these ranges. For example, The New Zealand approach uses three aliphatic fractions, while the TPHCWG approach employs 13 analytical fractions (6 aliphatic, 7 aromatic) covering the range from EC_5 – EC_{35} (TPHCWG, 1997a and 1997b; MaDEP, 2002a; Environment Agency, 2003b).

The API extended the fractions used by the TPHCWG so that there is a $>EC_{21}$-EC_{44} aromatic fraction and a $>EC_{16}$-EC_{44} fraction along with an additional EC_{44+} combined aliphatic and aromatic fraction (as it is not physically possible to separate hydrocarbons of this size into fractions) (API, 2001) (Table 1.2). This step was taken due to the TPHCWG fractions not encompassing hydrocarbons with carbon numbers greater 35 which can make up to 60 % $^{w}/_{w}$ of some crude oils (API, 2001) and is characteristic of weathered hydrocarbons. It was also considered that the TPHCWG fractions were appropriate for most refined products but not the crude oils present at the majority of E&P sites (API, 2001). Toxicological and fate and

transport data for these heavier hydrocarbons (>EC_{35}) are sparse (MaDEP, 2002a). As such, the API assigned the characteristics of the next closest aliphatic or aromatic carbon number fractions to the EC_{35}-EC_{44} aliphatic and aromatic ranges (MaDEP, 1994; TPHCWG, 1998a; ATSDR, 1999; API, 2001; Environment Agency, 2003) deriving oral and dermal reference doses of 0.03 mg kg^{-1} day^{-1} and 0.8 mg kg^{-1} day^{-1} respectively (as EC_{44} has extremely low volatility no inhalation reference dose was set by API (API, 2001)). The EA approach extends these carbon ranges further (Table 1.2), resulting in 16 fractions, giving an overall range from EC_5-EC_{70}. Further to the TPHCWG fractions, the EA added an aromatic EC_{35}-EC_{44} range, an aliphatic EC_{35}-EC_{44} range and a combined aromatic and aliphatic EC_{44}-EC_{70} range (Environment Agency, 2003b and 2005). The use of surrogate data from the next closest hydrocarbon fraction may be overly conservative and thus not cost-effective. In the case of the API approach, the next closest fraction usually encompasses petroleum hydrocarbons with lower molecular weights, and as such would be characterised with a greater degree of mobility within the environment (API, 2001). Further research into the characteristics of heavier compounds may provide a more complete understanding of their behaviour within the environment and potential risks to human health. It could also potentially result in a reduction in the analysis and remediation requirements enabling the risk assessment to become more streamlined.

Table 1.2: Summary of risk assessment used by several different jurisdictions (modified after Environment Agency, (2003b))

	American Society for testing and materials (ASTM, 1994)	Massachusetts Department of Environmental Protection (MaDEP, 1994)	Total Petroleum Hydrocarbon Criteria Working Group (TPHCWG 1997a & b, 1998a & b and 1999) .	Agency for Toxic Substances and Disease Registry (ATSDR, 1999)	Canadian Council of Ministers of the Environment (CCME, 2000)	American Petroleum Institute (API, 2001).	Environment Agency (Environment Agency 2005)	New Zealand (Ministry for the Environment, 1999)	New South Wales (New south Wales Environment Protection Agency, 2003)	National Institute for Public Health and the Environment (Barrs, 2001).
Indicator Compounds	Uses 'chemicals of concern' only.	Use most toxic and those most frequently tested for.	Uses most toxic compounds only.	Uses most toxic compounds only	Uses most toxic and those most frequently tested for	Uses most toxic compounds only	Most toxic and most prevalent in petroleum hydrocarbon-contaminated environment	Use of 'contaminants of concern' to address most toxic substances and aromatics	Individual compounds identified	Uses most toxic and those most frequently tested for
Fractions Number	None	6 Analytical fractions (3 aromatic, 3 aliphatic), using 4 toxicity values (3 aliphatic, 1 aromatic.	13 analytical fractions (6 aliphatic, 7 aromatic), using 7 toxicity values (3 aliphatic, 4 aromatic).	Similar to TPHCWG. Minor modification to aromatic groups to include BTEX compounds in same fraction	4 fractions, based on TPHCWG, separate evaluation of aliphatic and aromatic compounds not required	14 fractions based on TPHCWG (7 aromatic, 6 Aliphatic and 1 aliphatic and aromatic combined)	16 fractions based on TPHCWG and API (7 Aliphatic, 8 Aromatic and 1 aliphatic and aromatic combined)	3 aliphatic fractions	2 petroleum hydrocarbon fractions	7 fractions based on toxicity values (3 aliphatic and 4 aromatic)
Basis	N/A	Carbon number	Equivalent carbon number	Equivalent carbon number	Equivalent carbon number	Equivalent carbon number	Equivalent carbon number	Equivalent carbon number	Not defined	Equivalent carbon number
Application of approach	RBCA 3 tiered look-up tables for tier 1 and increasing use of site-specific information in tiers 2&3.	Not tiered as appropriate method is selected prior to assessment. 3 methods can be used – increasing specificity with methods 1 generic 3 site-specific.	RBCA 3 tiered look up tables for tier 1 and increasing use of site-specific into in tiers 2&3.	RBCA 3 tiered look up tables for tier 1 and increasing use of site-specific into in tiers 2&3.	RBCA 3 tiered look up tables for tier 1 and increasing use of site specific information in tiers 2 &3.	Modified TPHCWG approach.	Modified TPHCWG approach within UK context.	Use of a 3-tired approach, moving from generic guidelines to less conservative values using site-specific information.	None specified	Use of a tiered approach, moving from generic to less conservative values using site-specific information in tiers 2 and 3.

(Continued on next page)

Table 1.2: Summary of risk assessment used by several different jurisdictions (modified after Environment Agency, (2003b)) *(continued).*

	American Society for testing and materials	Massachusetts Department of Environmental Protection	Total Petroleum Hydrocarbon Criteria Working Group	Agency for Toxic Substances and Disease Registry	Canadian Council of Ministers of the Environment	American Petroleum Institute	Environment Agency	New Zealand	New South Wales	National Institute for Public Health and the Environment
Analysis	No recommended method of analysis	Use of two methods developed by MaDEP for volatile petroleum hydrocarbons (VPH) (MaDEP, 2004b)[55]and Extractable petroleum hydrocarbons (EPH) (MaDEP, 2004a)[54]	The 'Direct Method', developed by AEHS (AEHS, 2000)[1].	The 'Direct Method'(AEHS , 2000)[1].	Benchmarked methods for the C_6 to C_{10} and C_{10} to C_{50} ranges[19].	Modified 'Direct Method' for C_{44}+ range.	No specified methods, however are to adopt performance criteria – MCERTS (Environment Agency, 2005)[30]	Use of method prepared by the Oil Industry Environment Working Group(Oil Industry Environmental Working Group, 1999)[65].	Dependent on source of threshold concentration. Using NEPC methods (National Environment Protection Council, 1999)[62].	Single analytical method (NEN 5733) recommended.
Additivity effects	Not recommended	Precautionary based on addition of hazard quotients across fractions	Precautionary based on addition of hazard quotients across fractions	Precautionary. Developing index of concern based on addition of hazard quotients across fractions for compounds affecting same target organs of systems	Not advised due to different toxicological end points and exposure pathways of different fractions	Precautionary based on addition of hazard quotients across fractions	Assumes additivity of toxicological effects across all fractions, unless there are scientific data to the contrary.	Additivity of excess lifetime cancer risk for non- threshold substances. Precautionary approach, as for ATSDR	Not discussed in guidance document	Precautionary approach, based on addition of hazard quotients across fractions
Range		nC_5-nC_{36} Aliphatics, nC_9-nC_{22} Aromatics	EC_5-EC_{21} Aliphatics, EC_5-EC_{35} Aromatics	EC_5-EC_{21} Aliphatics, EC_5-EC_{35} Aromatics	EC_6-EC_{50}	EC_6 to EC_{44+}	EC_5 to EC_{70}	EC_7 to EC_{36}	EC_6 to EC_{40}	EC_5 to EC_{35}

1.4. Bioremediation

The bioremediation of contaminated soils has been extensively reviewed. Bioremediation methods utilise naturally occurring biological processes to transform, decrease or eliminate polluting substances (Eweis et al.,1998; BIOWISE, 2000; Environment Agency, 2002; Harries et al., 2004). Theoretically, optimal conditions are provided for bacteria or fungi to degrade or transform more complex compounds (*e.g.* contaminants) into relatively simple constituents that may pose a lesser potential risk to humans or ecosystems. An idealised bioremediation method would use harmless reagents, enable the process to be carried out quickly and efficiently (on-site), and result in an acceptable soil product that can be re-used with little/no further modification (Buddhadasa et al., 2002). Compared to popular remediation techniques such as excavation and disposal, bioremediation often has greater analytical and process control requirements, which are essential to ensure the conditions necessary for bioremediation are achieved and maintained during the treatment period. From an engineering perspective, the processes and logistics of bioremediation are relatively simple (Eweis et al., 1998). Any increased expense due to greater analytical and process requirements is usually offset by lower capital costs (Cookson, 1995; Environment Agency 2004). In 2000, an EA survey indicated that organic pollutants accounted for 83% of contaminants remediated at contaminated sites in England and Wales (Environment Agency 2000), demonstrating the applicability of bioremediation within the UK land remediation sector.

The disadvantages of bioremediation include the potential unpredictability of performance, difficulties in scaling up from laboratory to field and relatively long (weeks/months) remediation times. Bioremediation is not universally suitable for all contaminants (Hyman and Dupont, 2001) and have currently only been found to be capable of degrading organic contaminants susceptible to microbial attack including petroleum and aromatic hydrocarbons, PAHs, phenols, pesticides and oxygenated hydrocarbons. High concentrations of heavy metals and other highly toxic compounds can be prohibitive of microbial growth (Hyman and Dupont, 2001), or still leave the remediated soil unfit for purpose and classed as contaminated due to the residual presence of inorganic contaminants. Although bioremediation can breakdown potentially toxic contaminants, this process may result in the formation of metabolites that are toxic in their own right (Eweis et al. 1998). Contaminants need to provide an energy and carbon source to enable microbial growth, and so need to be biologically degradable or transformable (Environment Agency, 2000; Hyman and Dupont, 2001). Hence, biological remediation systems are more suited to organic contaminants, including weathered petroleum hydrocarbons (Hutcheson et al., 1996).

1.4.1 Bioremediation techniques

The choice of bioremediation technique can depend on a number of site specific factors, including the type, mobility, concentration and volume of a contaminant, the soil structure, surrounding geology, the proximity to

structures and potential receptors, and intended end use (Cookson, 1995; Eweis et al., 1998; BIOWISE 2000; Environment Agency, 2002). There is no single method for every situation and often combinations of techniques are implemented at sites with multiple contamination sources. Bioremediation processes can be divided into *in-situ* and *ex-situ. In-situ* methods include monitored natural attenuation (Margesin and Schinner, 1999; BIOWISE, 2000; Hyman and Dupont, 2001; Bhupathiraju et al., 2002; Environment Agency, 2002; Hejazi et al., 2003), biosparging (BIOWISE 2000; DTI, 2003) and bioventing (Flathman et al., 1994; Eweis et al., 1998; BIOWISE, 2000; Hyman and Dupont, 2001). They have the advantage of not requiring the excavation or removal of soil (Cookson, 1995; Eweis et al., 1998; BIOWISE, 2000). They are able to deal with deep contamination and enable remediation both under and around buildings (Cookson, 1995). These techniques minimise problems with dust, and hence worker exposure may be reduced (Cookson, 1995; Eweis et al., 1998). *In-situ* techniques can adapt, enhance and control bioremediation conditions. However, they are limited by the degree of process control that can be used. In comparison, *ex-situ* methods are contained and offer a higher degree of process control with greater control over time (Environment Agency, 2000). Techniques can be performed on or off site depending on the restrictions present at a particular site (Cookson, 1995). Overall, *ex-situ* methods are considered to be more efficient than *in-situ* techniques (BIOWISE, 2000) and can deal with higher concentrations of contaminants (Environment Agency, 2000). *Ex-situ* techniques include landfarming, composting, biopiling and bioreactor

treatments (Eweis et al., 1998). ‘Landfarming’ (also known as ‘land treatment’) is a simple technique used to treat large areas of land. Land farming has been used for the remediation of many waste types, but mainly for the remediation of hydrocarbon contaminated soils (BIOWISE, 2000; Cookson, 1995). Landfarming involves the excavation and spreading (to a 0.3-0.5 m thickness (Environment Agency, 2002)) of contaminated soil over a bunded area (incorporating a leachate collection system and impermeable liner material (BIOWISE, 2000; Environment Agency, 2002) which is tilled to aerate the soil at regular intervals (Cookson, 1995; Eweis et al., 1998; Environment Agency, 2002). Composting is an aerobic process using systems that involve the construction of piles, often using bulking agents to increase porosity and facilitate airflow (Eweis et al., 1998; BIOWISE, 2000). Anaerobic conditions can also be used to compost wastes; however, this can result in the synthesis of unpleasant odorous compounds such hydrogen sulphide (Cookson, 1995) and the generation of methane. Purpose built closed reactor composting systems can be used to compost wastes, and have been used as the basis of soil treatment centres in mainland Europe (Cookson, 1995; BIOWISE, 2000). Here, the soil is combined with water to form a slurry which is continuously mixed using mechanical agitators, giving rise to improved contact between the pollutants and the microorganisms (Eweis et al., 1998). Closed systems provide a high degree of process control over environmental conditions and allow for the control and treatment of volatile compounds. However they are more expensive than open systems such as windrows (Eweis et al., 1998).

Engineered biopiles are an intensive static pile version of composting that enable greater control over important environmental factors that effect biotransformation rates (i.e. oxygen, water and nutrient levels (BIOWISE, 2000) compared to other methods. This intensive method is especially useful when space is limited (BIOWISE, 2000). Details regarding biopile design and operation can be found elsewhere (Cookson, 1995; BATTELLE, 1996; BOIWISE, 2000; Guerin, 2000; Environment Agency, 2002).

As highlighted by some of the responses to the EAs survey (Environment Agency, 2000), the timescale in which pollutants can be remediated is an important consideration when selecting the most appropriate remedial treatment to use at a given site. Cost, guaranteed insurance, and risk reduction were also cited as reasons for not using bioremediation methods. Engineered biopiles offer a high degree of control, have a smaller footprint and are comparatively quick, yet they are not as expensive as closed bioreactor systems (*ca.* £10-40 per m^3 vs. *ca.* £30-150 per m^3) (BIOWISE, 2000). This makes biopiling attractive to contaminated land remediation specialists, especially as the high degree of control allows the processes to be optimised for biotransformation of specific pollutants of interest.

Bioremediation works well for remediating soils contaminated with petroleum hydrocarbons (Flathman et al., 1994; Hyman and Dupont, 2001). Most studies have reported biotransformation to be rapid in the initial stages of bioremediation, with rates seen to asymptote as the weathered proportion is biotransformed (Ellis, 1994; Fogel, 1994; Wood, 1997). Weathered

petroleum hydrocarbons have typically been present in the soil for a long period of time, they display relatively low bioavailability, and thus are more recalcitrant in the environment (Guerin, 2000). As a result, the optimisation of environmental conditions is imperative for the remediation of land contaminated with weathered petroleum hydrocarbons (Guerin, 2000). Giles et al. (2001) studied the bioremediation of weathered oil sludge (C_{20}-C_{38}) in composting piles. A biotransformation of 97 % $^{w}/_{w}$ TPH was achieved after 10 weeks. This study showed that indigenous bacterial populations were more suited to biotransforming the sludge (Giles et al., 2001). Unexpectedly, the bulking agent used had a greater effect on biodegradation than augmentation with a consortium of oil-degrading bacteria. The authors suggested that the bulking agent achieved higher degradation rates (complete compost) due to the presence of indigenous hydrocarbon-degrading microorganisms. However, this may have been due in part to the increased adsorption capacity of the amended soil matrix. It was suggested that this material was effective at modulating the temperature thus maintaining the bacteria within their optimal range (Giles et al., 2001).

1.4.2 Optimising bioremediation

The degradation process can be enhanced through biostimulation and bioaugmentation. The former refers to the enhancement of the bioremediation process by optimising specific environmental parameters such as temperature, pH, oxygen partial pressure, moisture and nutrient levels (Eweis et al., 1998), with regard to the current environmental

conditions, soil and contamination types present at a site. The latter describes the augmentation of bioremediation systems with commercially available microbial cultures which, in some cases, perform specific functions (BIOWISE, 2000; Guerin, 2000; Environment Agency, 2002; Nathanail and Bardos, 2004). Bioaugmentation may be required where native microbial populations are insufficient to achieve effective biotransformation. For example *Phanerochaete chrysosporium* (white rot fungus) can aid in the degradation of problematic recalcitrant compounds (Allard, A-S. and Neilson, A.H., 1997; Eggen, T. and Sveum, P., 1999; Zhang, X. X. et al., 2006). However, it should be noted that resulting increased costs are rarely justified by the benefits (Environment Agency, 2002). Additionally, it was shown by Trindade et al. (2005) and Giles et al. (2001) that indigenous microorganisms can be better adapted and more resistant to the contaminants present, potentially with greater remediation potential than exogeneous microorganisms (Giles et al., 2001; Environment Agency, 2002; Trindade et al., 2005). Typically, the addition of exogeneous organisms is not required when degrading hydrocarbons (BATTELLE, 1996; Giles et al, 2001; Trindade et al., 2005).

To grow, microorganisms require an electron donor (source of energy) and an electron acceptor as a means of extracting energy from the electron donor. Thus, electron acceptors play a key role in the biotransformation of a contaminant (the energy source – electron donor). Potential electron acceptors for microbial activity are (in order of energy yield, highest first): oxygen, nitrate, iron, manganease, sulphate, carbon dioxide and organic

carbon (Hyman and Dupont, 2001). Clearly as oxygen yields the highest amount of energy it is the preferred electron acceptor and is important to optimise its diffusion into- and concentration within the soil matrix (typically need to keep oxygen in the soil gas >2 %). Aeration within soils, were oxygen diffusion is not optimal for biodegradtion (such as clayey soils), can be enhanced by the use of bulking agents (to open up the soil structure) or manual aeration by turning the soil or using fixed aeration pipes.

Different microbial consortiums can require different temperature ranges to achieve optimum growth. For example, mesophilic microorganisms grow from about 15° to 45 °C (Hyman and Dupont, 2001) whereas thermophilic microorganisms grow best between 45 ° and 65 °C (Eweis et al., 1998; Hyman and Dupont, 2001). Typically during bioremediation mesophilic temperatures are common, with Giles et al., (2001) having found optimum growth for the bacteria present during the bioremediation of a weathered oil sludge to be less than 45 °C (Giles et al., 2001). As such the optimum temperature range for bioremediation is between 15 to 45 °C, thus remediation in cold climates can be restricted by low ambient temperatures. Conversely, in warm climates where temperatures remain within this optimum range remediation may still be difficult due to the rapid loss of moisture from the bioremediation system. Typically, during bioremediation (of contaminants that are not inhibitive of microbial growth) microbial numbers tend to increase during the early stages of remediation, with microbial numbers decreasing towards the end of a treatment as their energy source (the contaminant) is depleted (Nathanail and Bardos, 2004).

The pH of the soil can inhibit microbial activity and also affect the solubility of important nutrients such as phosphorus (Eweis et al., 1998; Hyman and Dupont, 2001). The typical optimum pH range for bioremediation is from pH 5.0 – 9.0, with a pH of 7.0 being preferable. Giles et al. (2001) reported a soil pH of 6.1 during the bioremediation of weathered oil sludge, suggesting that the 'typical' bioremediation pH range is likely to be suitable for weathered petroleum hydrocarbons.

Water is essential for microbial growth and maintenance and also serves as a transport medium through which organic compounds, contaminants and nutrients are transported into the cells and waste products from the cells (Eweis et al., 1998; Hyman and Dupont, 2001). Achieving a suitable water balance within the biopile can be critical as dry zones may result in decreased microbial activity (Eweis et al., 1998). Conversely, saturation inhibits gas exchange resulting in anaerobic conditions. The typical optimum water content range is within 55-80 % by weight of the water-holding capacity (BIOWISE, 2000; Hyman and Dupont, 2001). Bacteria also require nutrients (carbon, nitrogen, phosphorous, and in lesser quantities potassium, sodium, magnesium, calcium, iron, chloride and sulphur)(BIOWISE, 2000) for the assimilation and synthesis of new cell materials (Eweis et al., 1998; BIOWISE, 2000; Hyman and Dupont, 2001). The depletion of nutrients can affect the biotransformation of contaminants, in response bioremediation systems can be amended with fertilisers containing appropriate quantities of the rate-limiting nutrients (BATTELLE, 1996; Hyman and Dupont, 2001).

It is clear that successful bioremediation relies on the optimisation of several parameters. Thus, prior to the remediation of contaminated land it can be useful to assess the treatability of the soil and identify requirements for bioremediation.

1.5 Chapter Summary

The literature review has highlighted several knowledge gaps and methodological limitations within analysis and risk protocols. The key issues highlighted can be grouped into areas of analytical, toxicological, risk assessment and bioremediation limitations. Inter-laboratory and analytical method variations exist, with most analytical protocols not having been designed for the analysis extraction of hydrocarbons numbers up to $C_{70.}$ Knowledge on the toxicity of the hydrocarbon residues is still limited, and toxicological methods are not incorporated into the analytical protocols. Weathered petroleum hydrocarbons are acknowledged as having important qualitative and quantitative differences compared to fresh oil products, yet these criteria are not considered in the majority of risk assessment frameworks. In cases where weathered hydrocarbons are considered the lack of data on their toxicity, distribution, transport and availability results in over-conservative risk assessment and potentially unnecessarily overzealous remediation.

Although bioremediation technologies are sound, cost effective alternative treatment options, their use is still limited by negative stakeholder perceptions,

The literature highlights that without improvements in weathered hydrocarbon knowledge, the critical value of any risk assessment can be questioned.

Chapter 2: Research Objectives

2.1 Research gaps

The key knowledge gaps and methodological limitations within analysis and risk protocols highlighted by the literature review can be summarised as follows:

- Inter-laboratory and method variations exist giving rise to both positive and negative bias and the potential to give different results. Use of such unreliable results may potentially lead to inappropriate or inadequate remedial targets being set.
- Methods have not been designed to incorporate hydrocarbon numbers up to C_{70} required by recent risk assessment approaches (Environment Agency, 2005).
- Current extraction methods within risk assessment frameworks may not be suitable for all of the weathered hydrocarbon range, having typically been developed for fresher contamination of hydrocarbon numbers lower than 35.
- Toxicological methods are not incorporated into analytical protocols, even though reductions in TPH cannot currently be linked to reductions in toxicity.
- Lack of information on toxicity, distribution, transport and availability of weathered hydrocarbons, resulting in risk assessments being over-conservative.

- Majority of risk assessment frameworks do not consider all of the weathered hydrocarbon range, and thus may underestimate the risk at a site.
- Use of suitable bioremediation techniques is limited by negative stakeholder perceptions, associated with a lack of risk vocabulary within bioremediation.

2.2 Aims & Objectives

2.2.1 Aims

This work aims to assess the biotransformation potential of weathered petroleum hydrocarbons within two different types of soil. Both soils containing weathered petroleum hydrocarbons, but one has previously undergone treatment to a point where degradation had been assumed to have ceased. This will provide insight into weathered hydrocarbon degradation and toxicity within a soil/contamination type that has previously not been investigated, in addition it will also add to the general knowledge on weathered hydrocarbon degradation.

Overall, this work aims to increase the weathered hydrocarbon knowledge base, enhancing understanding of the degradation of weathered hydrocarbons, their toxicity and potential for risk. This work also informs the risk assessors and stakeholders, and helps increase confidence in bioremediation of weathered petroleum hydrocarbon contaminated soil.

2.2.2 Objectives

1. Review, summarise and critically analyse historical hydrocarbon pilot studies.

The literature review highlighted the need for the dissemination of bioremediation studies/trials both positive and negative, in order to reduce uncertainties associated with bioremediation of weathered hydrocarbons and increase the knowledge base. This would also help to improve contaminated land remediation decision making

Stakeholders hold unpublished internal reports containing potentially valuable information, which would be of benefit to the wider contaminated land community. A set of such reports were kindly provided by an oil industry facility. The facility provided a selection of In-house biotransformation and analytical reports, which detail the feasibility effectiveness, and optimisation of various bioremediation strategies. The general purpose of these reports was to establish and evaluate optimum remediation conditions for different petroleum hydrocarbon wastes and contaminated sites. The data within these reports is reviewed and analysed, highlighting key findings with them.

2. Develop and evaluate an analytical protocol that is practical and responsive to risk assessment.

As highlighted by the literature review Inter-laboratory and analytical method variations exist, with most analytical protocols not having been

designed for the analysis extraction of hydrocarbons numbers up to $C_{70.}$ Technology-knowledge transfer with support from TES-Bretby (Environmental Services Group, Burton upon Trent) contributed to the development and optimisation of a new exhaustive extraction method for contaminated soils with weathered petroleum hydrocarbons. The main objectives were to increase extraction efficiency and recovery, reduce time and cost and extend the identification of the compounds.

3. Assess the biotransformation potential of weathered petroleum hydrocarbons in sandy and clayey soils.

Using laboratory microcosms, the biotransformation potential of weathered petroleum hydrocarbons within two soils, kindly provided by oil industry facilities are assessed. Soil A is a hydrocarbon contaminated sandy soil that has previously undergone bioremediation to a point where it was believed that no further degradation was possible, and in theory no further degradation of the soil should be possible. Laboratory microcosms investigate the potential for further degradation of the oil contained in this soil, and also investigate if degradation is limited by the availability of the oil. Soil B is a clayey soil contaminated with weathered hydrocarbons and has not received any previous treatment. This soil has also been investigated by the PROMISE project, which found biostimulation (by nutrient addition) and combine biostimulation and bioaugmentation (by nutrient and inoculum amendment) treatments to have little effect on enhancing bioremediation. Investigation into any differences between the

microcosms here and those used by the PROMISE project gives insight into the factors limiting the bioremediation of this oil.

4. Investigate the relationship between chemistry and toxicity

Little knowledge is available on weathered hydrocarbons toxicity and associated risk. Improvement of our knowledge is urgently needed to aid in the development of guidelines for remediation strategies. Investigation of the soils used in the laboratory microcosms evaluates the relationship between chemistry and toxicity, and helps to improve our knowledge in this critical area.

Chapter 3: Critical analysis of hydrocarbon bioremediation pilot studies

3.1 Introduction

Petroleum hydrocarbons continue to be a widely utilised resource throughout the world. Their use has resulted in contamination through accidental spillage and leakage (Pollard et al., 1994). Certain components of petroleum contamination may pose risks to human health, property, watercourses, ecosystems, and other environmental receptors (Brassington et al., 2007). Throughout the United Kingdom (UK) there are thousands of sites that have been contaminated by previous industrial use, often associated with traditional processes which are now obsolete. These sites may present a hazard to the environment, for which there is a growing requirement for reclamation and redevelopment. Recent targets set by the Government demand that 60% of all new houses should be built on previously developed sites (brownfield) to relieve the pressure on Greenfield sites and preserve the countryside. A proportion of brownfield sites will be contaminated requiring a risk based approach in their redevelopment for residential use. As such any remedial technology used needs to be capable of being placed within such risk based approaches, and guaranteed reduction of risk. Although, local authorities, as part of the Environmental Protection Act 1990 development, have started to respond to the introduction of the proposed Section 143 registers, there is still no reliable estimate of the number of contaminated sites in the UK, or the overall scale of the consequent problem. The Environment Agency estimates that more than 300,000 hectares of land are affected, amassing between 5,000 and 20,000 "problem sites" (Environment Agency, 2005). Civil engineering

methods such as ‘dig and dump’ have commonly been used within the UK as a method of land remediation. Such methods are quick and reliable, reducing contaminant concentrations and risk quickly and effectively at a site. However, the recent implementation of the Landfill Directive, in particular the requirement for the pre-treatment of wastes before landfilling, and the separation of “hazardous“ and “non-hazardous“ waste streams, is expected to have a strong influence on current practice.

An Environment Agency survey conducted in 2000 showed that 94% of the remediation techniques used at contaminated sites were civil engineering methods of which 76% were excavation with off-site disposal. Concomitant use of biological treatments was not considered as a treatment option in most cases due to a lack of information on bioremediation technologies (Environment Agency, 2000). Moreover, the current negative stakeholder perception of these techniques is doing little to encourage their development and use, as they are often seen as being costly and time intensive without guaranteed risk reduction.

When selecting remedial techniques key issues of concern to stakeholders include cost, timescales, guaranteed insurance and risk reduction (Environment Agency, 2000). Although bioremediation techniques are clearly applicable to much of the contaminated land remediated within the UK, many stakeholders have difficulties aligning these key issues to bioremediation techniques. Several bioremediation techniques lend themselves to easily being manipulated to improve efficacy of difficult to desorb organic contaminants. Moreover bioremediation techniques can be

engineered to be effective for any combination of sites conditions and petroleum products.

To improve perception and uptake of bioremediation techniques within the contaminated land market, stakeholders need information on both successful and unsuccessful remediation trials, which will help guide decision and remedial management decisions for the clean-up of contaminated sites.

Data, generated during a series of historical bioremediation feasibility studies, has been kindly provided by an oil industry facility. The purpose of this study is to review and analyse this data, extracting key information of interest to establish optimum remediation conditions for different petroleum hydrocarbon contaminated sites. A total of six pilot studies performed over a period of several years were studied. Covering a range of different soils and oils, issues of optimisation, feasibility, contaminant composition differences, bioaugmentation and biostimulation methods. In analysing the data studies were, where possible, grouped together according to the purpose of the investigation.

3.2 Materials and Methods

3.2.1 Soil Characteristics

The oil facility routinely analysed a range of soil characteristics during each investigation these included moisture content, pH, WHC, particle size, organic carbon, nitrate, phosphate, and ammonium content. The methods

used were as follows. Moisture content was measured by reweighing duplicate samples of 20 g soil wet weight after drying at 105 °C. Soil pH was measured in a distilled water slurry (1 part soil: 2 parts water) after a 30 minute equilibration period. Maximum water holding capacity was determined in duplicate by flooding the wet weight equivalent of 100 g dry soil in a filter funnel and allowing it to drain overnight. Particle size analysis was performed by combination wet sieving (sand) and sedimentation (silt and clay) as described by Gee and Bauder (1986). Organic carbon was analysed by potassium dichromate oxidation as described by Schnitzer (1982). Soil (10 g) was extracted in 0.5 M potassium bicarbonate (adjusted to pH 8.5) and analysed by high performance ion chromatography (HPIC) for nitrate and phosphate and colourimetrically for ammonium.

3.2.2 Oil type and aging process (Experiments 1 & 2)

Biodegradability of nine different oils (Table 3.1) was assessed in two distinct microcosm experiments (Table 3.2). The soil used in experiment 1 was air dried and homogenised by screening through a 2-mm sieve. To improve drainage of the soil it was mixed in a 1:1 ratio with sharp building sand. Triplicate microcosms were established in sterile 50 ml serum bottles containing 10 g of soil and contaminated with 100 mg of the respective oil. Microcosms were established for each time point so that they could be destructively sampled. The water content of the soil was adjusted to 50 % using a fertiliser solution containing 25.7 g l^{-1} NH_4NO_3 and 21.4 g l^{-1} KH_2PO_4 dissolved in sterile deionised water, applied to achieve a C:N:P ratio of

100:10:1. Triplicate controls were also established using the same method as the microcosms but using a 2 % w/v mercuric chloride solution to re-hydrate the soil rather than fertiliser solution (to assess the abiotic losses). Previous investigations by the oil facility had showed mercuric chloride to be the most effective sterilant, and as such was the standard sterilant used for abiotic controls. All microcosms were incubated aerobically at 20 °C in darkness at high humidity, for a total of 30 weeks (at which point, through continued analysis, degradation had been deemed to have stopped) and sampled at 0, 2, 4, 6, 8, 12, 20 and 30 weeks.

Microcosms for experiment 2 were prepared in triplicate by placing 350 g of a soil historically contaminated with a mixture of crude oil and refined products (dry weight) into 500 ml glass jars. The water holding capacity (WHC) of the soil samples was then adjusted to 64 % using either (i) 5 % w/v mercuric chloride (to evaluate abiotic loss) (ii) fertiliser solution (to assess the effect of biostimulation) or (iii) sterile water (to asses intrinsic biodegradation). The fertiliser solution applied consisted of 23.8 g l^{-1} NH_4NO_3, 2.1 g l^{-1} KH_2PO_4 and 2.1 g l^{-1} K_2HPO_4 and was applied to achieve a C: N: P ratio of 100:1.7:0.17, adjusted to pH 6.8. Microcosms were incubated aerobically at 15 °C in darkness at high humidity, for a total of 37 weeks and sampled at 0, 2, 4, 8, 12, 16, 21, 30 and 37 weeks.

Table 3.1: Characteristics, degradation rates and percentage degradation of oils used in experiments 1 and 2 during the bioremediation pilot studies.

Oil Type			Degradation rates (mg kg^{-1} day^{-1})			Degradation (%)	
			2 weeks	6 weeks	20 weeks	20 weeks	End[#]
1. Crude oil mixture	Crude oil and refined products	light	1353	545	250	85	90 [37]
2. Diesel oil	Commercial grade summer DERV available from garage pumps	light	295	107	39	61	82 [30]
3. Brunei crude oil		light	107	51	30	54	77 [30]
4. Brent crude oil		light	277	120	45	72	74 [30]
5. Gas oil	A Burmah-Castrol/CONCAWE standard, light oil for biodegradation testing method development (CAS 64741-90-8)	light	304	176	66	74	74 [20]
6. XHVI gas oil	Comparatively biodegradable, light oil for biodegradation testing method development (lube 2613)	light	223	113	50	68	68 [20]
7 Lubricating oil	Automotive multigrade engine oil (Castrol GTX)	medium	188	79	31	56	57 [30]
8. 150 SN Dewaxed finished base oil	A Burmah-Castrol/CONCAWE standard, middle range lubricant base oil	Medium -heavy	<1	30	4	9	9 [20]
9. 1200 SN Brightstock	A Burmah-Castrol/CONCAWE standard heavy, de-asphalted distillation residue (CAS 64741-95-3)	heavy	68	110	32	42	42 [20]
10. Ondina 68	A marine oil of very low biodegradability (ST 85/128).	Heavy	<1	15	4	9	9 [20]

[#] Numbers in brackets denotes time in weeks

Table 3.2: Outline experimental set up for microcosm experiments 1 to 6

Investigation	Experiment Number	Experiment details	Duration (weeks)	Treatments					
				Controls		Fertiliser		Bulking agent	Augmentation
				Abiotic	*Biotic*	*Liquid*	*Pelleted*		
Oil type and aging	1	Uncontaminated soil spiked with one of nine different oils	30	✓*		✓		1:1 ratio with sharp building sand for all treatments	
	2	Historically contaminated soil	37	✓	✓	✓			
Bulking agent	3	Sandy loam soil spiked with untopped Brent crude oil	92	✓*	✓	✓		Fertiliser treatment bulked with either sterilised straw or pine bark	
Fertiliser type	4a	OBM & TBS	12	✓	✓	✓	✓	Dry wood chips in 1:1 ratio for all treatments	
	5	Two historically contaminated soils one sandy the other clayey	27	✓	✓	✓	✓		
Bioaugmentation	6	Three different soils two historically contaminated on freshly contaminated	64, 150, 52 for augmentation packages α, β and γ respectively	✓	✓	✓			In addition to other treatments each soil treated with one of three different commercial augmentation packages (α, β and γ)
Leaching	4b	OBM & TBS	12	✓	✓	✓	✓	Dry wood chips in 1:1 ratio for all treatments	

* This control was unsuccessful.

3.2.3 Bulking agents (Experiment 3)

The sandy loam soil used in this experiment was air-dried, homogenised and mixed with sand as previously described in section 3.2.2. Duplicate microcosms were established in shallow trays using 2.5 kg of soil, into which "untopped Brent Crude oil" was mixed to achieve a final concentration of 9409 mg kg^{-1}. Four microcosm conditions were established as follows (i) soil mixture + mercuric chloride (5 % w/v) (to assess abiotic losses), (ii) un-amended soil mixture (to assess intrinsic biodegradation), (iii) soil mixture + sterilised wet chopped straw (250 g) and fertiliser powder (1 g) (to assess the effect of the bulking agent), and (iv) soil mixture + sterilised wet pine bark (250 g) and fertiliser powder (1 g) (to assess the effect of the bulking agent). The fertiliser powder used consisted of NH_4NO_3 and NaH_2PO_4 in a 4:1 ratio and was applied to achieve a C: N: P ratio of 100:10:1. All the trays were adjusted to 50 % WHC using sterile deionised water and incubated aerobically in darkness at 25 °C, for a total 21 months with sampling at 0, 1, 2, 3, 6, 12, and 21 months.

3.2.4 Biostimulation treatments (Experiments 4a & 5)

The efficacy of liquid and pelleted fertiliser formulations in improving the biodegradation was assessed on different types of oily wastes using duplicate microcosm experiments (Table 3.2).

Two oily-waste samples, oil based mud (OBM) and tank bottom sludge (TBS) were collected from an old oil storage tank (Experiment 4a). Before use, both oily wastes were mixed with dry wood chips (1:1 ratio) to aid oxygen transfer and drainage. The microcosms were then established in sterile shallow glass trays using 1 kg of the OBM or TSB mixture. Four experimental treatments were set up as follows: (i) oiled mixture + 5 % w/v mercuric chloride (killed abiotic control), (ii) oiled mixture + sterile distilled water (natural biodegradation), (iii) oiled mixture + liquid fertiliser, (iv) oiled mixture + pelleted slow-release fertiliser and sterile deionised water. The liquid fertiliser solution used contained 1.5 g l^{-1} NH_4NO_3, 0.25 g l^{-1} KH_2PO_4 and, 0.25 g l^{-1} K_2HPO_4 which was applied at a C:N:P ratio of 100:1:0.1 and adjusted to a pH of ~7. Pelleted slow-release fertiliser was applied to achieve a C: N: P ratio of 100:2:0.2. This ratio was the closest to the standard ratio that could be achieved due to the size of the pellets and scale of the experiment. The microcosms were incubated aerobically at 25 °C in darkness for a total of 12 weeks and sampled at 0, 2, 4, 8 and 12 weeks.

The soils used in experiment 5 were collected from two different locations. One was sand from a cable ducting area and the other a clayey soil from a condensate manifold area. Separate microcosms for each soil were established using 250 g soil (dry weight) in 500 ml glass jars. Microcosms were brought to 65 % of their water holding capacity by the addition of either (i) 5 % w/v mercuric chloride solution (ii) sterile deionised water (no fertiliser) (iii) liquid fertiliser or (iv) pelleted slow-release fertiliser and sterile deionised water. The

liquid fertiliser consisted of 14.5 g l^{-1} NH_4NO_3, 0.8 g l^{-1} equimolar KH_2PO_4 and K_2HPO_4, applied at a ratio of 100:1:0.1. The pelleted fertiliser was applied at a C: N ratio of 100:10 as a ratio of 100:1 could not be achieved accurately due to the size of the pellets. Microcosms were incubated aerobically in the dark at 15 °C, adjusted to a pH of ~7, and incubated for a total of 17 weeks with sampling at 0, 3, 13 and 27 weeks.

3.2.5 Bioaugmentation trials (Experiment 6)

The efficacy of three commercial bioaugmentation packages (α, β and γ) was assessed in a microcosm experiment. The bioaugmentation package characteristics were as follows: (α) microbial inoculum and mixture of biodegradable surfactants (nonylphenol ethoxylates and alkoxylated linear alcohols); (β) microbial inoculum, mineral nutrient solution and biocatalyst solution and (γ) microbial inoculum, mineral nutrient solution and natural surfactant of plant origin. Each of the augmentation packages were used according to the manufacturers instruction. Duplicate microcosms were established in 500 ml sterile glass jars containing 350 g (dry weight) of either a sandy soil historically contaminated with crude oil (packages α and β) or a sandy soil freshly contaminated with Diesel (package γ). The WHC of the soil was adjusted to 65%. The following duplicate controls using the same conditions were also established: (i) abiotic control (soil and mercuric chloride at 5 % w/v, to determine whether any abiotic degradation was occurring), (ii)

soil and deionised sterile water (to compare with natural attenuation) and (iii) soil and fertiliser solution (to compare with biostimulation approach). The liquid fertiliser consisted of 23.8 g l^{-1} NH_4NO_3, 2.3 g l^{-1} KH_2PO_4 and 2.1 g l^{-1} K_2HPO_4, applied at a C:N:P ratio 100:1:0.1. Microcosms were incubated aerobically at 15 °C in the dark for 64, 150 and 52 weeks for packages α, β and γ respectively. Microcosms were sampled at 0, 3, 6, 10, 27, 42, 52 and 64 weeks for package α; 0, 3, 6, 12, 18, 29, 32, 40, 50, 56, 62, 79, 90, 100, 112, 131 and 150 weeks for package β; and 0, 3, 5, 10, 14, 23, 34 and 52 for package γ. The microcosm for package β was established as described with the exception of the use of 2 kg soil in a shallow glass tray and incubated at 30 °C.

3.2.6 Leaching tests (Experiment 4b)

Using the same microcosms and soil as used in experiment 4a (section 3.2.4) leaching tests were performed. Leaching tests were only performed in experiment 4b and were used to evaluate the amount of benzene, toluene, ethyl benzene and xylene (BTEX) that may leach from the oily wastes prior to bioremediation and after 12 weeks of bioremediation. Tests were performed in duplicate by mixing and shaking the oily wastes with ultra pure water containing mercuric chloride (5 % w/v) in completely filled (no headspace) 50 ml Teflon centrifuge tubes for 72 hours followed by centrifugation at ~2500 x*g* (3000 rpm) for 15 minutes. This leachate was then analysed for BTEX by purge and trap gas chromatography and by IR spectroscopy (section 3.2.7).

3.2.7 Chemical analyses

These experiments were performed over a long time-scale, as a result several different extraction and analysis techniques were used (Table 3.3). Generally samples were extracted either by Soxhlet (acetone: hexane, 1:1) or ultrasonic extraction (Freon-113). Depending on the experiment, extracts were then used to gravimetrically determine the content of total petroleum hydrocarbons (TPH), aliphatic, aromatics and the 'oil and grease' fractions. TPH content was analysed using either Infra-red spectroscopy (IR) and/or gas chromatography with flame ionisation detection (GC-FID). Typically GC-FID was used to characterise the contamination at the start and end of the experiment and IR used throughout to monitor degradation.

Soxhlet extracted samples were extracted in standard Soxhlet apparatus using 300 ml acetone: hexane in a 1:1 ratio, for 16 hours.

Ultrasonically extracted samples were chemically dried with anhydrous sodium sulphate (10 g, thermally treated overnight at 120 °C and stored in a desiccator prior to use) and ultrasonically extracted using Freon-113 (20 ml), for 30 minutes in a Decon FS200 sonic bath. Samples were thoroughly mixed before being allowed to settle overnight in the dark. The extracts were then shaken for 1 hour and left to settle before the solvent was decanted. This process was then repeated a further two to four times depending upon the sample.

Polar compounds were removed using Florisil (30-60 mesh, previously heat treated at 500 °C stored in a desiccator and activated prior to use by the addition of 6% w/v distilled water).

Aliphatic and aromatic portions were separated using column chromatography after the drying and re-dissolving of the Freon extract in hexane.

Depending upon the analysis to be performed samples were blown down under a gentle stream of nitrogen prior to gravimetric analysis or re-dissolving into an appropriate solvent for further analysis.

TPH content was analysed in all samples by fourier transform infrared (FT-IR) spectroscopy on Freon extracts using a PE 881 IR scanning spectrophotometer. Samples were scanned at wavelengths between 3200 and 2700 cm^{-1} against a solvent blank. The peak heights at wavelengths 2960, 2930 and 2860 were summed and the concentration of the TPH extract determined by reference to a standard curve prepared with oil extracted from the original contaminated soil covering a range of 0 - 500 $\mu g\ ml^{-1}$, using diesel as a standard

Table 3.3: Analysis techniques employed during bioremediation pilot studies

Experiment	Sample preparation				Analysis		
	Extraction		*Cleaning*	*Class Fractionation*			
	Soxhlet	Ultrasonic	Florisil	Column chromatography	Gravimetric	GC-FID*	IR*
1		✓	✓	✓		✓	✓
2	✓	✓	✓	✓	✓	✓	✓
3	✓	✓	✓	✓	✓	✓	✓
4a +b		✓	✓	✓	✓	✓	✓
5		✓	✓	✓		✓	✓
6		✓	✓				✓

* With the exception of experiment E, where used IR analysis was performed at each time point to determine TPH content and GC-FID analysis was used to characterise the TPH and/or aliphatic and aromatic fractions of the contaminant at the start and end of the experimental period.

TPH and prepared aliphatic and aromatic extracts were analysed by gas chromatography using a HP 5890A gas chromatograph fitted with a HP 7673 autosampler and a flame ionisation detector (GC-FID). The column used was a Chrompack fused silica capillary column (30 m x 0.32 mm x 0.12 µm), onto which 1 µl of sample was injected in split ratio mode (17:1) at 250 °C. Different temperature programs were used for the aliphatic and aromatic fractions. The initial oven temperature for the aliphatic program was 80 °C held for 5 minutes before being raised to 300 °C at a rate of 5 °C per minute then held for 15 minutes. The initial oven temperature for the aromatic program was 90 °C held for 5 minutes before being raised at a rate of 5 °C per minute to 160 °C, then raised to 300 °C at a rate of 10 °C per minute, and held for 17 minutes. Mixed *n*-alkanes and polycyclic aromatic hydrocarbons (PAHs) were analysed concomitantly for calibration purposes for aliphatic and aromatic fractions, respectively.

3.2.8 Monitoring

During the experimental period of all the microcosms the soil moisture levels were maintained though regular weighing. Depending upon the experiment the water holding capacity (WHC) was adjusted as required by the addition of sterile deionised water, fertiliser solution or sterilant solution.

The pH, ammonium, nitrate, phosphate and moisture content was determined as described in section 3.2.1. Soil respiration in experiments 2 and 5 microcosms was monitored at 25 °C by differential manometry on a Gilson respirometer.

3.3 Results

3.3.1 Influence of the oil type and aging on biodegradation

The type and the degree of oil weathering may affect an oils susceptibility to degradation (Kaplan et al., 1996). Lighter, fresher oil contamination is typically expected to degrade quicker than the heavier and weathered oils. Weathered oils may be bound tighter to the soil matrix and contain a greater proportion of recalcitrant compounds, reducing their degradability. Here various oils of different composition were investigated. At the end of the experimental period a total degradation of between 42 – 90 % had occurred in all but the Ondina 68 and 150SN dewaxed finished base oil (Table 3.1), both of which only degraded by 9 %. When grouped together (light, medium and heavy) a general pattern can be drawn; lighter oils degrade more extensively than the heavy oils. This general pattern displays a link between oil type and the extent of degradation achievable (Table 3.1). The lighter oils degraded up to ten times more than both the medium and heavy oils with an overall degradation rate of up to 62 times greater than that of the heavier oils investigated. Whilst there is a clear difference in the degradation achieved between the lighter oils and the heavy/medium oils there does not appear to be a clear difference between the medium and heavy oils in this investigation. The crude oil mixture (no. 1 table 3.1) was historical contamination that was expected to have weathered and thus display a recalcitrant nature, however up to 90 % of this oil was observed to degrade in this investigation (Table 3.1). Subsequent characterisation of this contaminant (data not made available) showed the oil to have a carbon

range form C_{12} to C_{26} which has undergone partial weathering as shown in the loss of hydrocarbons in the range $<C_{12}$. Thus this oil is actually a relatively light contaminant which would be expected to degrade reasonably well, as was seen here.

Additional investigations (Figure 3.1) performed with the contaminated soil in experiment 2 show that fertiliser addition increased the overall amount of degradation by approximately ten times with degradation of aliphatic and aromatic compounds being increased eleven and seven times, respectively. The addition of fertiliser more than doubled the degradation rate of the aliphatic fraction, whilst for the aromatic fraction the degradation rate increased to sixteen times greater than the rate in the untreated soil. In both untreated and fertilised microcosm the aliphatic fraction was degraded to a greater extent than the aromatic fraction. However, the addition of fertiliser has the greatest effect on increasing degradation of the aromatic fraction (Figure 3.1). As indicated in Figure 3.1 statistical analysis showed that there is no significant difference between the two controls ($P > 0.05$), suggesting biostimulation of the soil is essential for degradation to occur in this soil. Microbial respiration analysis (data not made available) confirmed that the abiotic control was effective and thus this initial high rate of degradation can be attributed to abiotic loss (e.g. volatilisation) of the lighter compounds within this contamination, which accounted for 36 % of the overall degradation achieved (Figure 3.1). The increase in degradation rate and overall percent degraded between the fertilised and non-fertilised microcosms showed that degradation within this soil was severely limited by

a lack of available nutrients within the soil. Biodegradation within this soil slowed towards the end of the experimental period. However, analyses showed that some resolvable compounds remained at the end and bioremediation was not limited by nutrient availability or adverse pH (data not made available). As conditions remained favourable for bioremediation it may be possible that degradation was still occurring but at a much slower rate than was previously observed.

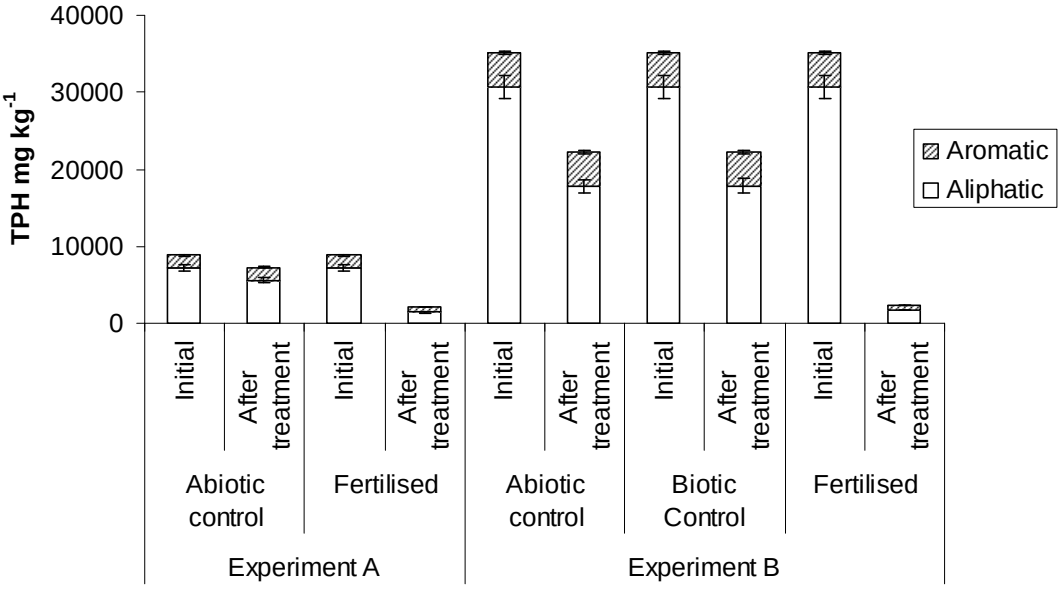


Figure 3.1: The effect of biostimulation on aliphatic and aromatic ratios (Experiments 1 and 2) (mean of duplicate samples is displayed, bars indicate standard error).

3.3.2 Influence of bulking agent type on bioremediation efficiency

Bulking agents have frequently been used during bioremediation for several purposes (BIOWISE, 2000; Eweis et al., 1998; Giles et al., 2001).

Bulking agents can improve soil structure, break up and mix the soil, lower the bulk density and increase porosity to facilitate better transfer of oxygen, water and nutrients into the soil and the removal of waste products (Eweis et al., 1998; Hyman and Dupont, 2001; Rojas-Avelizapa et al., 2006; Wellman et al., 2001). Bulking agents also increase the amount of contaminated soil exposed to oxygen and microbial attack by increasing the surface to volume ratio (BATTELLE, 1996). Organic matter addition can improve water holding capacity, pH and ion exchange capacity, which enhances microbial activity and as such bioremediation (Rojas-Avelizapa et al, 2006).

Although abiotic controls were employed in this experiment analyses during the experiment showed that they had been unsuccessful and were abandoned and data not used. Within this investigation the use of bulking agents was expected to enhance bioremediation and increase degradation rates, as others have demonstrated (Giles et al., 2001; Rhykerd et al. 1999). However, an overall TPH reduction of 78% (aliphatic and aromatic fractions were degraded by 82% and 66%, respectively) was achieved in the un-amended microcosm (Figure 3.2 and Table 3.4), with the bulking agents showing no additional benefit to TPH reduction (Table 3.4). This is unexpected as the soil used had a high clay content. As such, the addition of a bulking agent should improve biodegradation by opening up the structure of this soil and facilitating better transfer of oxygen, water and nutrients within the soil. This unexpected result may have been due to some of the oil components becoming less available through binding to the

bulking agents, hence resulting in more extensive degradation within the non amended microcosm. Pine bark appears to be the least effective at enhancing degradation of the aromatic fraction (Figure 3.2), as both the unamended and straw amended microcosms showed a greater degree of aromatic fraction removal than the pine bark treatment. Here, the straw and unamended microcosms degraded the aromatic fraction by 15 % and 24 % more than the pine bark amended microcosm (Table 3.4).

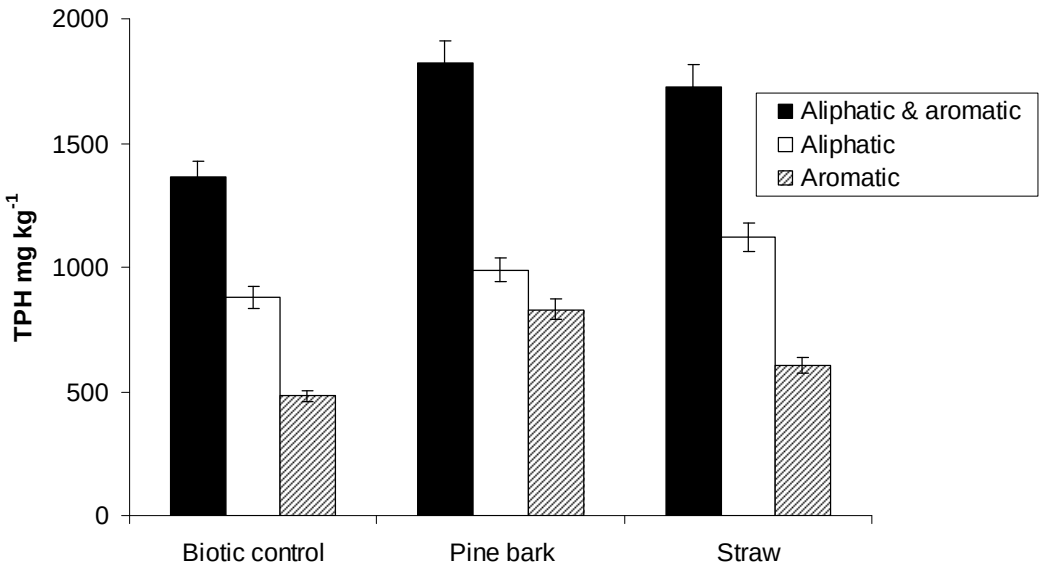


Figure 3.2: The effect of pine bark and straw bulking agents on aliphatic and aromatic fraction degradation after 84 weeks (Note: the abiotic control is not shown as it was unsuccessful and subsequently abandoned.)

Table 3.4: Aliphatic and aromatic fractions concentration and distribution at the start and end of the bulking agent experiment (experiment 3), and the percentage lost.

Treatment	Start					End					
	Total	Aliphatic		Aromatic		Total		Aliphatic		Aromatic	
	mg kg^{-1}	*mg kg^{-1}*	*%*	*mg kg^{-1}*	*%*	*mg kg^{-1}*	*% loss*	*mg kg^{-1}*	*% loss*	*mg kg^{-1}*	*% loss*
Biotic Control	9490	7250	77	2160	23	1360	78	878	82	482	66
Pine Bark						1820	71	990	79	829	42
Straw						1730	72	1120	76	606	57

3.3.3 Biostimulation treatments: comparative analysis of different fertilisation approaches

Carbon, nitrogen and phosphorous and other minor nutrients are essential for microbial growth, a low level of which within a contaminated soil may limit bioremediation (Cookson, 1995; Eweis et al., 1998; BATELLE, 1996). Hydrocarbon-contaminated soils naturally contain a high level of carbon as an energy source for microbial growth, but often require the addition of N and P containing fertilisers to bring up levels to that conducive of bioremediation. Several forms of fertiliser are available for this purpose. Slow-release pelleted forms such as Osmocote™ may have advantages over traditional liquid forms as they reduce leaching and loss of fertiliser nutrients (which could potentially contaminate protected watercourses with nitrate) and lower maintenance costs (Röling et al., 2004; Xu et al., 2004; Xu and Obbard 2004). Here pelleted fertiliser (Osmocote™) is compared against a traditional liquid fertiliser application.

As seen in other investigations, the addition of fertiliser increased degradation within the soil compared to natural attenuation. The addition of fertiliser increased degradation by up to 42 %, and initial and final degradation rates by up to 2.3 and 2.4 times greater respectively than without fertiliser (Table 3.5). No significant difference in the overall degradation achieved was observed between the two different fertilisers, suggesting that pelleted fertilisers are a good alternative to liquid fertiliser for the degradation of oil in contaminated soils. Although achieving a comparable amount of degradation, the rate of degradation in the pelletized fertiliser treatments was initially slower in all but the clay soil than that in the liquid fertiliser treatments which achieved initial degradation rates of up to 1.3 times faster.

Here the type of contaminant being treated appears to have had the greatest effect on the bioremediation achieved. Degradation in the Oil based muds (OBM) microcosm is significantly less than that seen in the Tank bottom sludge (TBS), sand and clay microcosms and may be due to recalcitrance of the compounds present (Figure 3.3). The OBM and TBS were bulked up using exactly the same soil, thus structure is not a limiting factor in this case. Soil analyses (data not made available) confirm that biodegradation within the OBM microcosm was not nutrient, oxygen or pH limited as these were stated to have remained within ranges suitable for bioremediation to occur.

Table 3.5: Initial and final TPH concentration, degradation rates and overall degradation achieved during the biostimulation experiments (experiments 4a and 5).

Experiment		Treatment	TPH (mg kg^{-1})		Degradation		
			Initial	*Final*	Overall (%)	Rate (mg kg^{-1} day^{-1}) *Initial* (2 weeks)	*Final*
4a	TBS	Abiotic Control	28000	21900	22	616	73
		Biotic Control		19400	31	750	103
		Liquid fertiliser		8800	69	1170	229
		Pelleted fertiliser		7500	73	1020	244
	OBM	Abiotic Control	87500	68000	22	1960	232
		Biotic Control		67500	23	2140	238
		Liquid fertiliser		51300	41	2860	432
		Pelleted fertiliser		45000	49	2140	506
5	Sand	Abiotic Control	7410	4060	45	73	18
		Biotic Control		1550	79	61	31
		Liquid fertiliser		1270	83	139	32
		Pelleted fertiliser		1410	81	121	32
	Clay	Abiotic Control	7880	7190	9	33	4
		Biotic Control		4000	49	72	20
		Liquid fertiliser		3000	62	72	25
		Pelleted fertiliser		2890	63	72	25

In the case of the soils used in experiment 5, fertiliser only had an additional benefit in the degradation of the oil within the clayey soil (Figure 3.3), here degradation within the sandy soil was shown to not be limited by nutrients as comparable degradation was achieved in the unammended microcosm (Figure 3.3).

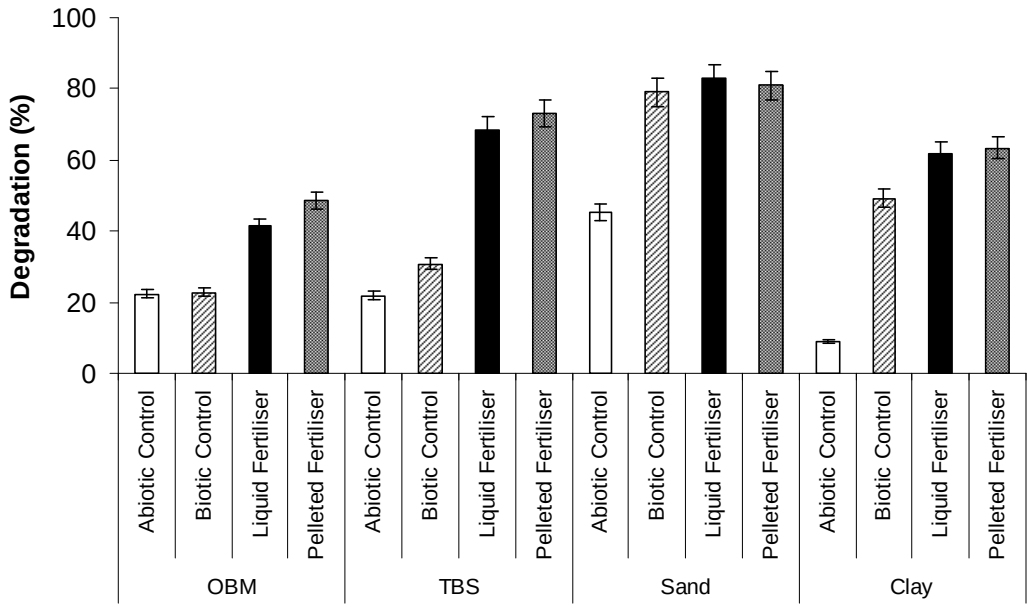


Figure 3.3: Influence of fertiliser type on degradation over a 27 week period

Biodegradation within the sandy and clayey soils (Experiment 5) is seen to occur without the addition of any form of fertiliser. A final reduction of 49 and 79% for the clayey and the sandy soils respectively was achieved by the end of the 27 week period (Table 3.5), without fertiliser amendment. Addition of fertilisers increased this by up to 13 and 4% in the clayey and sandy soils, respectively (Table 3.5). This observation shows a clear difference between the sandy and clayey soils, and indicates that bioremediation was nutrient limited within the clayey soil. There is a negligible difference between the two fertiliser types used for both soil types, showing that they could be used interchangeably. Degradation within the sandy soil occurred nearly twice as fast as in the clayey soil achieving 18 to 21 % more biodegradation than the clayey soil (Table 3.5). Results from this

suggest that bioremediation is occurring *in-situ* through natural attenuation at the site from which the sandy soil was taken and could potentially be left to remediate itself through natural attenuation. However, the action of removing the soil and setting up of the experiment will have aerated the soil which may also be an influencing factor. As the soil was incubated and water levels maintained this may have affected bioremediation in the unamended soil. In all cases bioremediation slowed towards the end of the experimental period yet showed no sign of coming to a halt, suggesting further bioremediation was possible but at a much slower rate, this is confirmed by the presence of resolvable peaks in the gas chromatograms for the residues (data not made available).

3.3.4 Bioaugmentation trials

Bioaugmentation packages stating that the product can improve the rate and extent of remediation are commercially available. In addition to these microbial consortia have often been used by researchers over the years in an attempt to improve bioremediation (Giles et al., 2001). In some situations an indigenous microbial population may not be sufficient for bioremediation of a soil and as such it may be necessary to add cultured hydrocarbon degrader organisms (Cookson, 1995; Eweis et al., 1998) but in other cases the indigenous microbial population is better adapted to the soil conditions and may perform better than a exogenous consortium (Trindade et al. 2005). Bioaugmentation packages can include within them nutrient

solutions, a microbial consortium and a surfactant, and as such aim to provide a complete package rather than a microbial consortium alone.

In the trials performed here all the augmentation packages increased the amount of degradation achieved by between 1.1 to 1.4 times compared to natural attenuation (Table 3.6). It might be expected that a nutrient solution alone without any additional microbial consortia (as is included in the augmentation packages) would increase degradation to a lesser extent than augmentation packages (for package details see section 3.2.5). This was not the case here as the addition of just a fertiliser alone increased degradation by between 1.3 to 1.6 times performing comparatively well if not better than the augmentation packages tested (Table 3.6). Additionally, the fertiliser treatment achieved lower final concentrations of TPH than both packages α and β (Table 3.6). However, it can be seen that these packages achieved a faster initial rate of degradation than the fertilised treatments, yet the overall degradation rate for these packages was slower than that achieved by the fertiliser alone treatment (Table 3.6). Bioaugmentation package γ appeared to perform the best out of the three packages tested, yet the standard fertiliser solution alone treatment performed just as well, as such any benefits of this augmentation package is debateable (Figure 3.5). The augmentation packages tested did improve bioremediation compared to natural attenuation but gave no additional benefit compared to a fertiliser solution alone that would make the packages a worthwhile alternative to the fertiliser solution used here (Figure 3.5).

Table 3.6: Initial and final TPH concentration, degradation rates and overall degradation achieved during the bioaugmentation experiment (experiment 6).

Augmentation Package	Treatment	TPH (mg kg^{-1})		Degradation		
				Overall (%)	Rate (mg kg^{-1} day^{-1})	
		Initial	*Final*		*Initial* *(2 weeks)*	*Final*
α	Abiotic control	43000	37500	13	171	12
	Biotic control		17500	59	100	57
	Fertiliser		1970	95	29	92
	Augmentation Package		7803	82	100	78
β	Abiotic control	65000	44000	35	194	23
	Biotic control		30000	56	197	36
	Fertiliser		20000	71	389	46
	Augmentation Package		28000	59	794	38
γ	Abiotic control	4600	4250	8	20	1
	Biotic control		1000	78	34	9
	Fertiliser		100	98	106	12
	Augmentation Package		100	98	<1	12

All of the augmentation packages used in this study contained a mineral nutrient addition (for details see section 3.2.5). However, no adjustment to the concentration and rate of mineral nutrients applied could be made, thus could not be optimised in line with the initial soil conditions. However, soil monitoring analysis confirmed that bioremediation within the augmentation package treated soils was not limited by pH or nutrients. Indeed tests of package α treated soil showed nutrient levels higher than that in the fertiliser treated soil yet still below a concentration toxic to remediation. Further investigation (data not made available) highlighted the surfactant within package α as being potentially limiting to bioremediation as when it was not

applied bioremediation achieved levels much closer to that achieved in the fertilised microcosm.

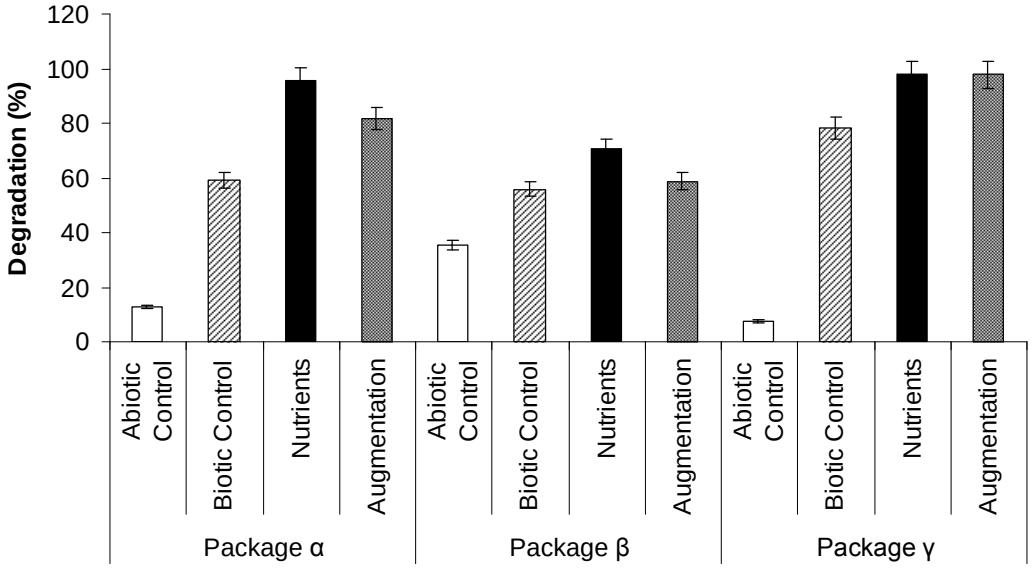


Figure 3.4: Influence of bioaugmentation on the extent of TPH degradation at the end of 64, 150 and 52 week trails for packages α, β and γ respectively.

3.3.5 Influence of bioremediation on leaching

Once a contaminant is within the ground, the water-soluble aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes (BTEX) have the potential for dissolution and leaching into the vadose zone and groundwater environments (Salanitro et al., 1997). BTEX have been recognised as the predominant leachable components within petroleum hydrocarbon contamination (Salanitro et al., 1997). Such compounds have the potential to contaminate protected watercourses. Bioremediation and

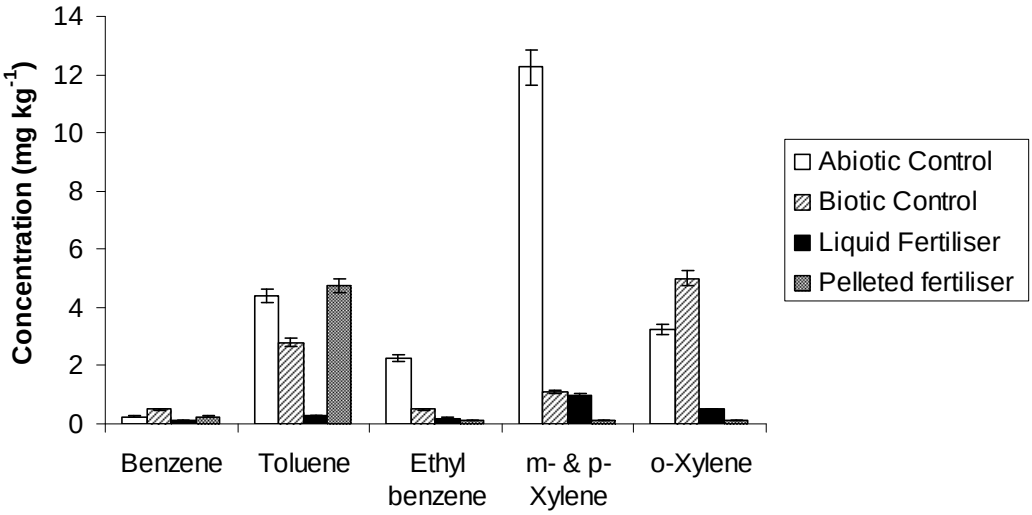
civil engineering techniques can be employed to reduce and/or stop the migration of contaminants through the soil as well as dilute and degrade them. However, bioremediation techniques themselves may increase leaching of a contaminant. Thus, it is important to consider the degree of potential leaching so that appropriate collection/decontamination systems may be employed where necessary.

It might be expected that the addition of an organic bulking agent may reduce leaching due to absorption. However, in this study this was not the case (data not made available). The addition of woodchips to tank bottom sludge (TBS) and oil based mud (OBM) had a negligible effect on hydrocarbon leachability (data not made available), and as such leachate collection is essential for the remediation of these particular wastes. Here, therefore, the main purpose of the bulking agent was to enhance oxygen, water and nutrient transfer thought the oily wastes and facilitate microbial growth. After 12 weeks (of the bioremediation treatment) leaching of BTEX in all the treatments was reduced by at least 85 % (Table 3.7). Leaching of the BTEX compounds from both of the oily wastes was recued by between 95 to >99 and 85 to >99 % in the pelleted and liquid fertiliser treatments respectively by the end of the experiment (Table 3.7). More leaching of BTEX was seen from the TBS than OBM, however leaching of xylenes for both TBS and OBM were reasonably comparable (Figure 3.5).

Table 3.7: Initial and final BTEX compounds concentration and overall reduction in BTEX leaching in experiment 4b.

Experiment		Treatment	Concentration leached *(mg kg^{-1})*		Reduction in leaching (%)
			Initial	***Final***	
OBM	Benzene	Abotic Control	25	0.25	92
		Biotic control		0.5	98
		Liquid fertiliser		0.125	>99
		Pelleted Fertiliser		0.25	95
	Toluene	Abotic Control	94	4.4	95
		Biotic control		6.25	97
		Liquid fertiliser		5	>99
		Pelleed Fertiliser		0.1	>99
	Ethylbenzene	Abotic Control	22	2.25	90
		Biotic control		0.25	98
		Liquid fertiliser		0.1	99
		Pelleted Fertiliser		0.1	>99
	m + p-xylene	Abotic Control	75	12.3	94
		Biotic control		1.1	99
		Liquid fertiliser		1	99
		Pelleted Fertiliser		0.1	>99
	o-Xylene	Abotic Control	81	3.25	96
		Biotic control		5	94
		Liquid fertiliser		0.5	99
		Pelleted Fertiliser		0.1	>99
	Total	Abotic Control	297	22.4	92
		Biotic control		9.9	97
		Liquid fertiliser		2.13	99
		Pelletd Fertiliser		5.3	98
TBS	Benzene	Abotic Control	113	9.38	92
		Biotic control		0.6	99
		Liquid fertiliser		0.2	>99
		Pelleted Fertiliser		0.1	>99
	Toluene	Abotic Control	163	22.5	86
		Biotic control		6.25	96
		Liquid fertiliser		5	96
		Pelleted Fertiliser		0.1	>99
	Ethylbenzene	Abotic Control	84	2.54	97
		Biotic control		2.5	97
		Liquid fertiliser		0.1	97
		Pelleted Fertiliser		0.1	>99
	m + p-xylene	Abotic Control	88	5.5	94
		Biotic control		13.4	85
		Liquid fertiliser		0.1	85
		Pelleted Fertiliser		0.5	>99
	o-Xylene	Abotic Control	100	5.63	94
		Biotic control		5	95
		Liquid fertiliser		0.3	95
		Pelleted Fertiliser		0.5	>99
	Total	Abotic Control	547	45.5	92
		Biotic control		27.8	95
		Liquid fertiliser		5.7	95
		Pelleted Fertiliser		1.3	>99

(a)



(b)

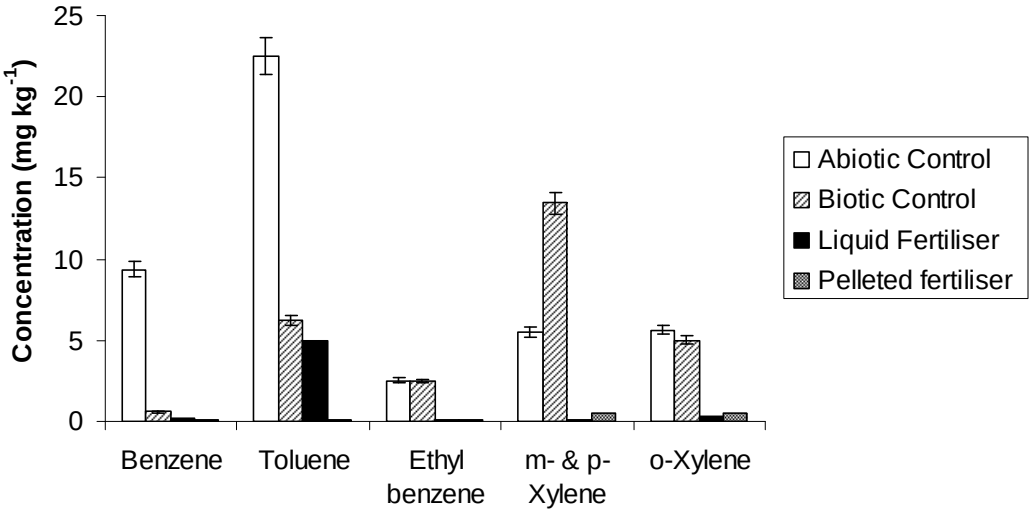


Figure 3.5: Effect of bioremediation treatment on BTEX leaching from oil based mud samples (a) and tank bottom sludge samples (b), respectively.

3.4 Chapter Summary

The data within the bioremediation feasibility studies, provided by an oil industry facility, were grouped together according to the purpose of the investigation. Overall the studies showed that the extent of degradation achievable can be affected by the oil type, bulking agent used, fertilisation and bioaugmentation. The main findings from each of the study groups are briefly summarised below:

- The extent of degradation seen in the oils investigated varied greatly from 9 - 90%, with lighter oils degrading more extensively than heavier oils. Demonstrating a link between oil type and degradation achievable.
- Nether pine bark or straw were shown to be effective at improving degradation when used as a bulking agent. This may have been due to some compounds binding to the bulking agent being used. Pine bark amendment was also observed to reduce degradation of the aromatic fraction to a greater extent than straw, suggesting a greater affinity of these compounds to pine bark than straw.
- The use of a fertiliser during bioremediation was shown to increase degradation by up to 42% increasing initial and final degradation rates by up to 2.3 and 2.4 times compared to no treatment. Liquid and pelleted fertiliser performed comparably well. Here the greatest effect on degradation was seen in the soil type as the OBM showed significantly less degradation than the TBS, sand and clay soils.

- The augmentation packages tested, although achieving degradation of up to 1.1 to 1.4 times greater than natural attenuation had no beneficial additional effect over that achieved by the addition of a fertiliser alone.
- Bioremediation using fertilisation as a treatment dramatically reduced the leaching of all BTEX compounds by between 85 to >99 %.

Overall, the studies suggest on the basis of physicochemical parameters that the success of bioremediation considering both biostimulation and bioaugmentation approaches is largely dependent on the oil contaminant and soil structure characteristics. For the majority of the contaminated soils investigated mineral nutrients played an essential role without which in some cases bioremediation could not occur. Slow-release fertilisers were shown to be an important alternative to liquid fertilisers, in mitigating issues arising from the addition of liquid fertilisers. Combining bioaugmentation strategies with biostimulation may improve the rate and extent of degradation while the potential benefit of bioaugmentation still needs further investigation. Bioremediation may also be used to reduce the risk caused by the potential leaching of BTEX from the contaminated soils. Ex-situ bioremediation for treatment will allow greater control over soil temperature, water holding capacity and leaching. The design of an efficient bioremediation system always requires a careful site assessment. Consideration of the physical, chemical and biological properties of the contaminated sites is essential in establishing appropriate response and recovery methods. Despite the ability of indigenous microorganisms to

degrade petroleum hydrocarbons, there are still situations where use of a microbial inoculum might enhance petroleum hydrocarbon biodegradation.

Chapter 4: Chemical analysis development

4.1 Introduction

The literature review (Chapter 1, section 1.2) highlighted that robust analytical techniques are required to enable a complete as possible characterisation of weathered hydrocarbon wastes. However, there are several important limitations to the methods used for the analysis of weathered petroleum hydrocarbons. Briefly, inter-laboratory and analytical method variations exist, toxicological methods are not included and the methods used have not been designed for and are not suitable for the extraction and analysis of hydrocarbons up to C_{70} that recent risk frameworks incorporate (Environment Agency, 2005).

Method development and choice is driven by several factors including cost (capital and operating), solvent consumption, legislative requirements, availability and the degree of technical skill required (Banjoo and Nelson 2005; Brassington et al., 2007). Within commercial and public sector environments cost and legislative requirements are typically the main drivers in method choice.

Risk assessment is a well-established and important tool for environmental management decisions, which is increasingly being used in petroleum hydrocarbon contaminated land management (for review see Brassington et al., 2007). However, these frameworks are not always supported by suitable and robust analytical protocols, especially in the case of matrices contaminated with weathered hydrocarbons (TPHCWG, 1998, Brassington et al., 2007). Many hydrocarbon-contaminated sites (former refineries, coal carbonisation plants, and integrated steelworks) contain (i)

oils that are weathered because the source term has aged since release (Westlake, 1974; Pollard et al., 1994); (ii) heavy fuel oil residues (Uhler et al., 2002); and/or (iii) viscous tars and solid bituminous process residues that are difficult to treat biologically (Gray et al., 2000).

Comparison of reference analytical methods used for petroleum risk assessment protocols (Brassington et al., 2007) highlights the need for practical and simple extraction procedures that allow a better characterization of both aliphatic and aromatic hydrocarbon fractions within oil-contaminated samples, including soil and sediment samples with high moisture levels (allowing analysis of as received samples and negating volatile losses associated with sample drying) (Environment Agency, 2005; Brassington et al., 2007). Within a UK context, the development of novel methods should also allow the identification of risk-indicator compounds within each hydrocarbon fraction, and the monitoring of recalcitrant biomarkers to enable verification of treatment success as described by the UK Environment Agency (2005). It is evident that clear guidance on the analytical techniques and compounds to be analyzed, which can be consistently applied to generate comparable risk assessments (Environment Agency, 2005) and eliminate inter- laboratory and method variations is needed. Additionally methods must be sufficiently adaptable to allow for future changes in the fractions and indicators required by risk assessments.

Currently petroleum hydrocarbon risk assessment frameworks typically adopt one of three different approaches to the analysis of petroleum hydrocarbon in soils. Frameworks either: (i) specify the method of analysis;

or (ii) have a benchmarked method but allow use of other methods under certain criteria or; (iii) use performance criteria (Brassington et al., 2007). The approach used can often be a reflection of the data requirements of a particular framework (see sections 1.2.1 & 1.3, tables 1.1 & 1.2). Those frameworks that do provide guidance on analytical protocols, typically involve the use of two methods for the extraction of the samples, one for volatile petroleum hydrocarbons (VPH) and one for extractable petroleum hydrocarbons (EPH).

Protocols within the published risk assessment frameworks typically involve the extraction of the soil using a manual shake/vortex method, as used by TPHCWG (AEHS, 2000), ATSDR (ATSDR, 1999) and CCME (CCME, 2001) (Chapter 1, section 1.2.1, table 1.1). However such methods are not suitable for the heavier weathered hydrocarbons, as they have been designed for the lower hydrocarbon range contamination (C_5-C_{36}) that are relatively fresh and un-weathered (Chapter 1, section 1.2.1, table 1.1). Methods are also typically not exhaustive enough to extract weathered hydrocarbons which are typically more strongly bound to the soil matrix due to the weathering/ageing process.

Most commercial environmental analytical laboratories possess the expertise to perform the more exhaustive analysis that is needed for these weathered petroleum hydrocarbons. However, clear guidance is needed as inter-laboratory variations can potentially arise due to extraction and sample clean-up inconsistencies (Environment Agency, 2004). Currently no single method can adequately characterise all forms of petroleum hydrocarbons in

soils (Environment agency, 2004), as different methods are needed for the volatile petroleum hydrocarbons and the weathered hydrocarbons. Concerns exist over the performance of the current reference methods used, specifically in terms of poor extraction efficiencies and analytical losses imparted by sample handling. The alteration of chemical composition with time may also affect the accuracy of final measurements and lead to misrepresentations of human health risk. In this respect, the impact of calibration on final measurement needs to be evaluated for a range of weathered products. Varying degrees of detail can be achieved using the different methods available to the analyst. Mills et al (1999) showed that the method and consequently the level of detail chosen for analysis can affect the evaluation of a sample. They showed that the outcome of a bioremediation study is highly dependent upon the analytical methods used to interpret the results. By using different analytical methods, completely opposite conclusions could be reached.

To date, while the UK approach sets out guidelines for evaluating human health risks from petroleum hydrocarbon contaminated soils, as yet there has been no specification or adoption of recommended analytical procedures for these contaminants. Furthermore, the framework itself notes that currently adopted methods for petroleum hydrocarbon analysis may not be suitable for the heavier compounds and questions whether it is practical or relevant for analysing weathered hydrocarbons (Environment Agency, 2005). These observations further highlight the need to develop a suitable and robust analytical procedure to inform risk assessment.

4.2 Soxhlet extraction

Soxhlet is an exhaustive technique that can handle both air-dried and field-moist samples, which has routinely been used by many assessing these types of weathered hydrocarbon wastes. A wide range of solvent types can be employed making this technique versatile for different chromatographic end points.

Soxhlet was the method initially chosen and used for extraction of weathered petroleum hydrocarbons within this piece of work. Soxhlet extraction was followed by Kuderna-Danish evaporation and column chromatography. Technology-knowledge transfer with support from TES-Bretby (Environmental Services Group, Burton upon Trent) allowed for the set up of this method and subsequent use for the initial analysis of project PROMISE samples as part of the work here. A brief outline of the Soxhlet methodology initially used is given in the following sections.

The PROMISE project (Optimising the Biopiling of Weathered Hydrocarbons within a Risk Management Framework) is a DTi funded collaborative project. Work of PROMSIE is closely linked to and in some cases has been performed as part of the work described here. As project PROMISE also concerns weathered hydrocarbons both projects have worked along side each other sharing information, work and knowledge to the benefit of both projects.

4.2.1 Standards, solvents and reagents

All solvents used were HPLC grade and purchased from Patterson Scientific (Cambridge, UK). Silica gel grade 923, *o*-terphenyl (CAS 92-94-4), Squalane (CAS 111-01-3), 2,2,4,4,6,8,8-Heptamethylnonane (CAS 4390-04-9) and 2-Fluorobiphenyl (CAS 321-60-8) were purchased from Sigma-Aldrich (Dorset, UK). Anhydrous sodium sulphate was purchased from Fisher Scientific (Loughborough, UK). Silica gel and anhydrous sodium sulphate were baked at 110˚C for 12 h and at 400˚C for 4 h, respectively before use. The removal of any total petroleum hydrocarbons (TPH) was confirmed by the analysis of the blank control method. Diesel fuel and Mineral Oil Standard (neat motor oil, 15w-50) used as quality control standards were purchased commercially from car product stores.

4.2.2 Extraction protocol

Field moist samples (10 ± 0.05 g) were manually blended and chemically dried with ~10 g anhydrous sodium sulphate (Na_2SO_4) using a clean stainless steel rod. Dried samples were transferred to thimbles (glass thimbles with a porosity 1 glass frit), stoppered with glass wool (low-lead, rinsed in dichloromathane (DCM), sonicated for 15 min, air-dried in a fume hood and baked overnight at 600 °C). In addition to the samples, a solvent blank, a spiked blank (spiked with 5000 µg ml^{-1} each of diesel fuel and mineral oil) and an uncontaminated soil (as a reference material) were also extracted. In order to evaluate the recovery percentage for the extraction

method, samples were spiked with 1 ml of a surrogate solution containing *o*-terphenyl (oTP), Squalane (Sq), Heptamethylnonane (HMN) and 2-Fluororbiphenyl (2-Fb) at a concentration of 100 µg ml^{-1} each in methanol. Samples were then extracted for 6-8 hours at 4 – 6 cycles per hour with 150 ml 90:10 DCM : Acetone.

4.2.3 Kuderna-Danish (KD)

Solvent extracts were carefully rinsed into a 250 ml KD evaporation flask attached to a 10 ml receiver tube, placed in a hot water bath (~60-70 °C) and evaporated down to 1 ml where possible (weathered hydrocarbon extracts are typically dark and can become quite thick on concentration making 1 ml a difficult volume to achieve, in such cases samples were taken down to a larger volume), first using a large 2 ball snyder column followed by a small 2 ball snyder column.

4.2.4 Solvent exchange

Samples that were cleaned and/or fractionated by column chromatography had to be exchanged into cyclohexane prior to fractionation. Here, during the KD step, extracts were reduced down to 5 ml rather than 1 ml at which point 5 ml of cyclohexane was added and the evaporation continued until a volume of 1 ml was reached.

4.2.5 Silica gel column chromatography: clean-up and class fractionation

Column chromatography was used to reduce interferences from polar materials and clean and/or fractionate. Here columns were prepared in Thistle columns using ~10 g of silica gel slurry with ~40 ml of DCM. Columns were conditioned with 40 ml of pentane after which the sample was loaded onto the column, and eluted with 25 ml of pentane then 25 ml of DCM: pentane (50:50) for the aliphatic and aromatic fractions respectively. Aliphatic and aromatic extracts were concentrated down to a volume of 1 ml using KD as previously described and placed in 2 ml autosampler amber glass vials.

4.2.6 Instrumental analysis

Total hydrocarbon petroleum (TPH), aliphatic and aromatic fractions were identified and quantified by gas chromatography-mass spectrometry (GC-MS) using a Perkin Elmer AutoSystem XL gas chromatograph coupled to a Turbomass Gold mass spectrometer operated at 70 eV in positive ion mode. The column used was a Restek fused silica capillary column (30 x 0.25 mm internal diameter) coated with RTX®-5MS (0.25 µm film thickness). Using diesel and PAH standards of concentrations ranging from 0.5 to 2500 µg ml^{-1} and from 1 to 5 µg ml^{-1}respectively, the temperature program was developed using the literature and advice from Perkin Elmer as a guide, resulting in the MS and GC methods described as follows.

Splitless injection with a sample volume of 1 µl was applied The oven temperature was increased from 60 °C to 220 °C at 20 °C min^{-1} then to 310 °C at 6 °C min^{-1} and held at this temperature for 15 min. The mass spectrometer was operated using the full scan mode (range *m/z* 50-500) for quantitative analysis of target alkanes and PAHs. For each compound, quantification was performed by integrating the peak at specific *m/z*. External multilevel calibrations were carried out for both oil fractions and surrogates, quantification ranging from 0.5 to 2500 µg ml^{-1} and from 1 to 5 µg ml^{-1}, respectively. Surrogate standards were used to determine the extraction efficiency of the method. For quality control, a 500 µg ml^{-1} diesel standard and mineral oil were checked every 10 samples. In addition, duplicate blank control and reference material were systematically used. The blank control was treated in exactly the same manner as the samples but contained no soil. The reference material was an uncontaminated soil of known characteristics, and was spiked with a diesel and mineral oil standard at a concentration equivalent to 16,000 mg kg^{-1}.

4.3 Sequential ultrasonic solvent extraction

The largest piece of collaborative work performed between this work and the PROMISE project was the analytical development described here. Initial Soxhlet extracts of PROMISE samples (extracted as part of this work and passed to TES-Bretby for instrumental analysis, due to GC-MS facilities being unavailable at Cranfield at that time), showed extractions efficiencies that were unacceptable by TES-Bretby standards. This led to the

collaborative development of a sonication method. Whilst the work was performed collaboratively the required laboratory work was performed collaboratively within TES-Bretby laboratories.

The sonication method described here has several benefits over the Soxhlet extraction method. The method can take ~1 h whereas Soxhlet took a minimum of 8 h. It uses only 40 ml of solvent rather than 150 ml. The method also produces cleaner samples which don't require concentration or cleaning prior to analysis. Technology-knowledge transfer with the support from TES-Bretby (Environmental Services Group, Burton upon Trent) contributed to the development and optimisation of this new exhaustive extraction method for contaminated soils with weathered petroleum hydrocarbons. The main objectives were to increase extraction efficiency and recovery, reduce time and cost and extend the identification of the compounds. This new method and validation performed is detailed in the following sections. The method described is investigated and evaluated using four different soil matrices i) silty soil, ii) clay soil, iii) sandy soil and iv) a granular matrix comprising ash, brick and concrete fragments selected to represent the surface soils found at many industrial sites ('made' ground). Each soil matrix was spiked with a mixture of diesel and lubricating oil at levels corresponding to 20% (10,000 mg kg^{-1}) and 80% (40,000 mg kg^{-1}) of the concentration range typical of environmental soil samples (API, 2001).

4.3.1 Standards, solvents and reagents

Standards solvents and reagents were as detailed in section 4.2.1 in addition to which the following compounds were used. Florida Total Recoverable Petroleum Hydrocarbon standard (C_8-C_{40} Florida TRPH) and Semi Volatile Calibration Mix #5 (16 priority EPA PAH mix – EPA 8310) were supplied by Thames Restek Ltd (Saunderton, UK). Soil certified reference material was purchased from RTC Corporation, Catalogue Reference Number CRMPR9583 containing TPH at 9510 mg kg^{-1}.

Polished Deionised water

Deionised water was polished by shaking 1 l of deionised water with 50 ml of hexane in a separating funnel for two minutes. The water was then decanted into a clean 1 l bottle and the hexane layer discarded. After cleaning the separating funnel with hexane the process repeated on the cleaned water using a fresh aliquot of hexane. Polished water was kept in a clean 2.5 l bottle and used within 48 hrs.

4.3.2 Extraction protocol

Soil samples (5 ± 0.05 g) were chemically dried with 5 g of anhydrous sodium sulphate (Na_2SO_4). In order to evaluate the recovery from the extraction method, dried samples were spiked with 1 ml of a surrogate solution containing *o*-terphenyl (oTP), squalane (Sq), heptamethylnonane

(HMN) and 2-fluororbiphenyl (2-Fb) at a concentration of 200 µg ml^{-1}each in acetone.

Spiked soil samples were extracted with 4 ml of acetone and sonicated for 2 min at 20 °C. Hexane and acetone were added to the samples in a 1:1 ratio. Samples were sonicated for a further 10 min, followed by manual shaking to break up and mix the sample matrix. This step was repeated once more and followed by centrifugation for 5 min at 750 rpm. After passing the supernatant through a filter column fitted with glass receiver tube, a sequential step series, including resuspension of the samples in 10 ml of acetone: hexane (1:1), sonication for 15 min at 20 °C, centrifugation for 5 min at 750 rpm and then decantation into a filter column, was repeated twice. The final extract volume was adjusted to 40 ml with a mixture of acetone: hexane (1:1) and homogenized by manual shaking before analysis.

4.3.3 Micro-scale silica gel column chromatography: clean-up and class fractioning.

The extract from the ultrasonic process can be directly used to determine TPH content without further need for concentration or dilution. A silica gel column chromatography procedure was used to split the extracted hydrocarbons into aliphatic and aromatic class fractions. Approximately 80 ml of polished water and a spatula of sodium chloride (baked at 400 °C for 4 h) were added to the sample extracts, partitioning out any acetone into the water and ensuring the removal of the non-polar content. TPH silica cleanup

was then carried out by passing 1 ml of the upper phase from the partitioned samples through a silica gel column, eluting with 3 ml of dichloromethane (DCM). The split of the aliphatic/aromatic fractions can be realized by eluting with 3 ml of hexane followed by 3 ml of DCM, respectively.

4.3.4 Instrumental analysis

Analysis was performed as described previously in section 4.2.6. In addition, Texas banding compounds (Texas1 to 5), diesel range organic (DRO), kerosene range organic (KRO) and mineral oil range organic (MRO) compounds in soils (Table 4.1) were identified and quantified using was GC and by comparison of these peaks with the response of a known concentration of diesel and mineral oil standards.

Table 4.1: Total petroleum hydrocarbons and sub-ranges of hydrocarbons used to characterize aliphatic and aromatic fractions, and method reporting limits for each. These fractions are based on approximate boiling point/carbon number ranges with respect to *n*-alkanes

Analyte	**Method Reporting Limit (mg kg^{-1})**
TPH (>nC8->nC40)	10
DRO (<nC10->nC24)	10
KRO (>nC8->nC14)	10
MRO (>nC22->nC34)	10
TEXAS1 (>nC8->nC10)	2.00
TEXAS2 (>nC10->nC12)	2.00
TEXAS3 (>nC12->nC16)	2.00
TEXAS4 (>nC16->nC21)	2.00
TEXAS5 (>nC21->nC35)	4.38
ALI/ARO TEXAS1 (>nC8->nC10)	4.00
ALI/ARO TEXAS2 (>nC10->nC12)	4.00
ALI/ARO TEXAS3 (>nC12->nC16)	4.00
ALI/ARO TEXAS4 (>nC16->nC21)	4.00
ALI/ARO TEXAS5 (>nC21->nC35)	8.75

4.3.5 Validation procedure

Validation provides confidence that the established performance characteristics are based on robust experimental determinations and are statistically sound. Each spiked soil matrix was allowed to stand for 24 h at room temperature before commencing extraction, to allow the spike to interact with the soil matrix. Performance characteristics were determined with a minimum of ten degrees of freedom by analyzing each certified reference material or spiked samples in duplicate in different analytical batches. Eleven batches of duplicates were analyzed for each matrix at each spiking level thus providing ten degrees of freedom in each validation experiment.

4.4 Results

Soxhlet is a widely used exhaustive technique that is easily standardised for the extraction of petroleum hydrocarbons, and as such was initially selected for use within this and project PROMSIE work. Analysis of early samples extracted and analysed as part of the PROMISE gave unsatisfactory recovery (~70%) which lead to the development of the ultrasonic method described here. Analysis of early Soxhet extracted samples were analysed by TES-Bretby and as such data is unavailable for inclusion here.

Ultrasonic extraction has been investigated elsewhere and has a high potential for wider use in this area of analysis (Banjoo and Nelson, 2005; Sanz-Landaluze et al., 2006). Ultrasonication is a quick, easy and cost-effective method that is now widely used in environmental analysis. However, analytical procedures using ultrasonication vary not only in the method used (e.g. type and volume of solvents, cycle duration etc.), but also in the type of ultrasonic apparatus used (sonic probe or ultrasonic bath). Some of the more detailed investigations have shown that ultrasonic methods have the potential to produce equivalent or better efficiencies than currently used methods such as Soxhlet (Heemken et al., 1997; Sun et al., 1998; Banjoo and Nelson 2005; Sporring et al., 2005). Conversely, other investigations have shown the opposite to this with worse efficiencies compared to alternative methods (Song et al., 2002; Hollender et al., 2003). Therefore, if sonication is to be used in place of traditional methods, it needs to be clearly defined and optimized. Whilst sonication has been investigated

and developed by other researchers none have used the sequential step sequence described in section 4.3.2 or sonicated the sample with acetone prior to the addition of hexane.

The novel ultrasonic solvent extraction method presented here has been evaluated using several different soils and two different concentration levels in addition to the use of a certified reference material. The mean TPH concentration achieved for each tested soil, concentration levels, standard deviation (precision) and difference between the mean and the known concentration of the spiked soil (bias) of the samples are shown in Table 4.2. Here, accuracy of the test method is assessed by comparing results to the known actual value (thus inaccuracy is evaluated in terms of bias) (Miller and Miller, 1993).The results showed that the method had good extraction efficiency and recovery independently of soil type with relative standard deviation (RSD) values for all the soils of below 10% for all of the spiked soils. Here an acceptable bias for the method of -2.6 to 5 % is recorded. The highest degree of variability was obtained in the clay soil (Table 4.2), a trend also reported by Shin and Kwon (2000), which they suggested was due to the stronger binding of compounds to the clay matrix, reducing extractability and increasing variability.

Table 4.2: Mean concentration extracted, precision and bias (inaccuracy) for different spiked soil matrices.

Matrix	Spike concentration mg kg^{-1}	Mean concentration extracted mg kg^{-1}	Precision mg kg^{-1}	Precision % RSD	Bias mg kg^{-1}	Bias %	Total Error %
Silty soil	0	65	6.8	10.5			
	10000	9988	301	3.0	-77	-0.8	6.1
	30000	29280	800	2.7	-785	-2.6	7.74
Clay soil	0	81	22.6	27.9			
	10000	10142	680	6.7	61	0.6	14.2
	30000	30104	2171	7.2	23	0.1	14.6
Sandy soil	0	9.4	5.8	61.7			
	10000	9727	377	3.9	-282.4	2.8	10.4
	30000	28759	611	2.1	-1250.4	4.2	8.2
Madeground	0	286	63.5	22.2			
	10000	10802	320	3.0	516	5.0	11.6
	30000	31166	1497	4.8	880	2.9	12.9

It is important to validate extraction efficiencies using certified reference materials as the recoveries obtained with spiked compounds may not be representative of those obtained with native compounds. This is because spiked analytes are usually lightly coated on the surface of the matrix, whereas native compounds can be strongly absorbed to the soil matrix. Although important, very few certified commercial data sets are available within the open literature. Extraction of certified reference matrix RTC CRMPR 9583 gave good precision. Here a RSD of 3.4% (Table 4.3) was achieved. Here the relatively high bias of 17 % (inaccuracy) obtained may be due to the lack of an evaporation step within the method (Table 4.3).

Table 4.3: Mean concentration extracted, precision and bias (inaccuracy) for reference soil Matrix RTC DRMPR 9583

Matrix RTC CRMPR 9583	
Certified Value	9510 ± 666 mg kg^{-1}
Mean	11124 mg kg^{-1}
Precision	374 mg kg^{-1}
Precision	3.4% RSD
Bias	1614 mg kg^{-1}
Bias	17.0%
Total Error	24.8%

Ensuring reliability and validity of the results is an important consideration. Quality control is built into this method to allow for continued evaluation and validation of the method, through the analysis of a reagent blank and a spiked reference material with every ≤ 20 samples. Additionally, sample recovery is monitored using surrogate spikes. Analysis of KRO, DRO and MRO hydrocarbon ranges shows a variability ranging from an

RSD of 1.7 to 13.0, with the highest overall degree of variability occurring when analysing the DRO range (Table 4.4). With the exception of the made ground soil, a higher degree of precision was typically observed when extracting higher concentrations of hydrocarbons.

Texas risk carbon banding fractions RSD values ranged between 1.7% and 14.5% (Table 4.5). It is only when analyzing the aromatic fraction (Table 4.6) where RSD values rise above 20%. The greatest degree of variability was observed when extracting low concentrations of the C_8-C_{10} range for all of the soils tested, possibly due to volatile losses or thermal decomposition of compounds. Here, the diminution curve showed that precision was lower due to the detection limits of the GC used (data not available). This is consistent with the precision obtained for ultrasonic methods elsewhere. Shin and Kwon (2000) and Sanz-Landaluze et al. (2006) demonstrated RSD values of <10% and 14.4% respectively when optimising sonication methods. Within the silty and sandy soils a greater degree of precision was achievable at higher concentrations, whereas within the clay and made ground soils precision is higher when extracting lower concentrations. The results showed that ultrasonic solvent extraction method has a good degree of accuracy, achieving an extraction efficiency of ≥ 95%.

Table 4.4: DRO, KRO and MRO hydrocarbon banding mean concentration extracted and precision for soils spiked with 20 and 80% of typical hydrocarbon concentration found in environmental samples

		Silty soil			**Clay soil**		
Carbon Band	Spike level*	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision %RSD	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision %RSD
DRO C_{10}-C_{24}	20%	8401	1095	13.0	6574	545	8.3
	80%	18957	642	3.4	19801	1528	7.7
KRO C_8-C_{14}	20%	1663	65	3.9	1641	148	9.0
	80%	4963	180	3.6	4986	510	10.2
MRO C_{22}-C_{34}	20%	3933	159	4.0	3708	328	8.8
	80%	11415	331	2.9	10862	776	7.1
		Sandy soil			**Madeground**		
Carbon Band	Spike level*	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision %RSD	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision %RSD
DRO C_{10}-C_{24}	20%	6468	318	4.9	7052	228	3.2
	80%	19135	317	1.7	20677	1006	4.9
KRO C_8-C_{14}	20%	1639	75	4.5	1724	66	3.8
	80%	5005	171	3.4	5201	375	7.2
MRO C_{22}-C_{34}	20%	3527	138	3.9	4123	120	2.9
	80%	10451	545	5.2	11513	425	3.7

* Percentage of expected concentration range encountered in environmental samples.

Table 4.5: Texas Risk bandings mean concentration extracted and precision for soils spiked with 20 and 80% of typical hydrocarbon concentration found in environmental samples.

		Silty Soil			**Clay soil**		
Carbon Band	Spike level*	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision % RSD	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision % RSD
TPH C_8-C_{10}	20%	224	12	5.4	234	30	12.9
	80%	669	23	3.4	673	50	7.4
TPH C_{10}-C_{12}	20%	531	23	4.3	550	33	5.9
	80%	1593	51	3.2	1697	108	6.4
TPH C_{12}-C_{16}	20%	2317	111	4.8	2383	163	6.9
	80%	7173	282	3.9	7615	529	7.0
TPH C_{16}-C_{21}	20%	2363	61	2.6	2523	178	7.1
	80%	6783	254	3.7	7352	534	7.3
TPH C_{21}-C_{35}	20%	4304	204	4.7	4173	337	8.1
	80%	12143	354	2.9	12100	799	6.6
		Sandy soil			**Madeground**		
Carbon Band	Spike level*	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision % RSD	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision % RSD
TPH C_8-C_{10}	20%	231	28	12.2	228	23	10.2
	80%	655	31	4.7	642	36	5.6
TPH C_{10}-C_{12}	20%	536	25	4.6	556	21	3.9
	80%	1633	41	2.5	1690	109	6.4
TPH C_{12}-C_{16}	20%	2328	100	4.3	2502	114	4.6
	80%	7340	161	2.2	7832	517	6.6
TPH C_{16}-C_{21}	20%	2445	76	3.1	2653	83	3.1
	80%	6919	173	2.5	7531	318	4.2
TPH C_{21}-C_{35}	20%	3880	170	4.4	4471	126	2.8
	80%	11461	454	4.0	12678	412	3.2

* Percentage of expected concentration range encountered in environmental samples.

Table 4.6: Aliphatic and aromatic fractions, mean concentration extracted, precision and bias for TPH DRO, KRO, MRO and texas risk bandings

		Aliphatic				
Carbon band	Spike value mg kg^{-1}	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision %RSD	Bias mg kg^{-1}	Bias %
TPH	160	160.1	23.7	14.8	100.1	0.1
DRO		82.8	12.6	15.2		
KRO		30.6	4.5	14.8		
MRO		76.6	13.4	17.5		
>C_8->C_{10}*		10.1	1.6	15.6		
>C_{10}->C_{12}		10.1	1.5	14.9		
>C_{12}->C_{16}		21.0	3.4	16.2		
>C_{16}->C_{21}		21.9	3.9	178.8		
>C_{21}-C_{35}		77.1	13.7	17.7		
		Aromatic				
	Spike value mg kg^{-1}	Mean mg kg^{-1}	Precision mg kg^{-1}	Precision %RSD	Bias mg kg^{-1}	Bias %
TPH	160	166.1	26.6	16.0	103.8	3.8
DRO		84.2	13.5	16.0		
KRO		12.3	1.8	14.4		
MRO		88.9	15.1	17.0		
>C_{10}->C_{12}		7.7	1.7	22.3		
>C_{12}->C_{16}		28.7	5.6	19.6		
>C_{16}->C_{21}		32.7	5.4	16.4		
>C_{21}-C_{35}		94.0	15.7	16.7		

NOTE: Certified reference materials for aliphatic and aromatic fractions are not available consequently matrix spike data has been used to determine the bias (inaccuracy) and precision of the method.
*PAH mixed used to spike the soil contained no aromatic PAH >C8-C10 fraction

4.5 Chapter Summary

It is clear that robust analytical methods are required to enable appropriate risk assessments and adequate remediation to be performed. However appropriate extraction methods for weathered hydrocarbons are limited. The traditionally used Soxhlet extraction method, although a benchmarked exhaustive method has its disadvantages, and as such alternative methods are sought.

The ultrasonic method described here is an improvement on traditionally used methods as it saves time (1 h compared to ~8 h required for traditional Soxhlet) and costs, reduces solvent consumption (40 ml compared to 150 ml for Soxhlet, avoids evaporation steps and is easy and simple to use. Overall the results show that the method is capable of achieving extraction efficiencies of ≥ 95 %. The results show that the method has good precision as an RSD value of 3.4 % for the certified reference material used was recorded, with RSD values of below 10% for the all the spiked soils investigated being recorded.

The method is also more environmentally friendly due to reduced solvent usage, with reduced health and safety risks as unlike Soxhlet extraction chlorinated solvents (i.e. DCM) are not used. The method has shown a good potential for implementation as a standard method, potentially capable of providing (through use) further insight and knowledge to the contaminated land sector.

Chapter 5: Biotransformation of weathered hydrocarbons: the relationship between chemistry, toxicity and risk

5.1 Introduction

As was previously discussed in Chapter 1 there is a paucity of information concerning weathered hydrocarbons, their degradation and toxicity. As such there is sparse information available on the relationship of chemical change, toxicity and risk during the remediation of weathered hydrocarbon, with no known investigation where this has been analysed specifically for weathered hydrocarbons.

To assess the potential for biotransformation of weathered oils in soil, microcosms were designed. These microcosms used two soils both historically contaminated with high levels of weathered hydrocarbons, which were made available by oil industry facilities. Soil A was taken from a windrow where bioremediation had been completed and soil B was taken from a site prior to remediation where oil drums had leaked contaminating the soil.

Soil A is a weathered hydrocarbon contaminated sandy soil that has previously undergone bioremediation to a point where it was believed that no further degradation was possible, and in theory no further degradation of the soil should be possible. Laboratory microcosms investigated the potential for further degradation of the oil contained within this soil, and also investigated if degradation is limited by the availability of the oil. Investigation into the bioremediation potential of soils contaminated with weathered hydrocarbons, that have been previously remediated have not previously been performed. Additionally although many researchers grind soil as part of their soil homogenisation process, no study could be found

where the effect of this grinding upon bioremediation was investigated. Soil B was clayey soil contaminated with weathered hydrocarbons and had not received any previous treatment. This soil has also been investigated by the PROMISE project, which found nutrient and inoculum additions to have little effect on enhancing bioremediation. Thus any differences seen in the investigation here give insight into factors affecting the biodegradation of this soil.

5.2 Materials and methods

5.2.1 Physicochemical and biological soil characterisation

Soil samples collected from two contaminated sites (A and B), with high levels of weathered petroleum hydrocarbons were kindly provided by an oil facility. The soils were stored at 4 °C until use. Each soil was homogenised using a 2 mm sieve prior to establishing the microcosms, and stored at 15 °C for acclimatisation to the planned experimental temperature.

Soil properties were previously determined as part of the project PROMISE (Tables 5.1 and 5.2) using standard methods as described by Allan (1989). The grain-size analysis showed that the A and B soils were of granular soil structure and clay soil structure, respectively. The pH values in both soils were near neutral and corresponded to the preferable pH for a maximum rate of growth for microorganisms in soil (Eweis et al., 1998 and Hyman & Dupont, 2001). The typical optimum water content range for microbial activity is within 55-80 % by weight of the water holding capacity

(BIOWISE, 2000 and Hyman & Dupont, 2001). Soils A and B have a water content below this range, 15 % and 21 % respectively, which is 39 and 48 % respectively of their maximum WHC showing that degradation may have been reduced due to low water content. The bulk density of both soils are indicative of un-compacted soil suggesting that there is sufficient pore space for adequate aeration of the soils, thus bulking agents are unnecessary for the bioremediation of these soils. Low concentrations of available nitrogen and phosphate sources show that both of these soils have become nutrient-limited. Biostimulation of these soils may increase the biotransformation of the oil within the soils. Whilst some metals such as iron are required for microbial growth in many instances, some metals such as mercury can be toxic if made available. One of the most toxic metals in soil is mercury, however bioremediation has been seen to occur with concentrations up to 100 mg kg^{-1} of mercury (Riis et al, 1999) present within the soil. This was attributed to heavy metals such as mercury being highly bound to the soil matrix. Researchers have also shown that heavy metals become available at toxic concentrations when soil pH becomes acidic (Riis et al, 1999). As such soil pH needs to be monitored and adjusted to prevent the soil becoming acidic where heavy metals will become available at toxic concentrations.

Comparison of the concentration of the metals in Table 5.2 to guideline values from the New Dutch list and from U.S. DoE (Environment Agency, 2004a), highlights that the levels of Iron (U.S. DoE microbial level 200 mg kg^{-1}), cadmium (Dutch target level 0.8 mg kg^{-1} intervention level 12 mg kg^{-1};

U.S. DoE microbial benchmark 20 mg kg^{-1}), silver (soil A only (U.S. DoE 50 mg kg^{-1})) and Antimony (Dutch target level 3 mg kg^{-1}, intervention level 25 mg kg^{-1}; U.S. DoE microbial bench mark 100 mg kg^{-1}) are above intervention or benchmark levels and would need action to be taken to reduce the levels of these metals within soils (Environment Agency, 2004a). As such these metals may if available prove inhibitive of microbial activity and degradation of the TPH contamination within these soils. However, as already mentioned maintaining a neutral soil pH will help to reduce these heavy metals from becoming available and toxic to microbial activity.

Table 5.1: Characteristics of soils A and B.

	Soil A		Soil B	
	Mean	**Stdev**	**Mean**	**Stdev**
TPH[1] in mg kg^{-1}	22700		31500	
Bulk Density g ml^{-1}	0.973		0.823	
Moisture content in %	15	0.79	21	0.77
Moisture content in % at WHC[2]	38	3	44	1
pH in Water	6.8	0.4	7.5	0.1
pH in 0.01 M $CaCl_2$	6.5	0.0	6.6	0.0
LOI[3] in %	12	1	15	7
% organic Carbon	7	0	9	4
DOC[4] in µg g^{-1}	75	21	152	60
TOC[5] in µg g^{-1}	168	13	280	116
% Carbon	9	1	8	1
% Nitrogen	5	1	2	1
Ammonium	No significant levels detected in the samples			
Nitrate	No significant levels detected in the samples			
Phosphate in %	0.0016	0.0029	0.0000	0.0000
Respiration ml/h*kg	0.53	0.01	0.20	0.20
CFU[6] per g in TSA[7]	5.89E+05	7.56E+04	7.07E+07	1.75E+07
CFU in BH[8] with 0.1% diesel	Not practical for present environmental samples			
MPN[9] per g in TSB[10]	2.53E+03	6.48E+02	1.27E+11	1.27E+11
MPN per g in BH with 0.1% diesel	8.28E+02	1.40E+02	3.44E+02	3.44E+02

[1]TPH refers to Total Petroleum hydrocarbon, [2]WHC refers to Water holding capacity, [3]LOI refers to Loss on ignition, [4]DOC refers to dissolved organic carbon, [5]TOC refers to total organic carbon, [6]CFU refers to Colony forming Unit, [7]TSA Tryptic Soy Agar, [8]BH refers to Bushnell Haas medium, [9]MPN refers to Most probable number, [10]TSB Tryptic Soy Broth

Table 5.2: Metal content of soils A and B in mg kg^{-1}

Metal	Soil A	Soil B
Iron	32500 ± 1630	23600 ± 1180
Zinc	448 ± 22.4	248 ± 12.4
Manganese	274 ± 13.7	134 ± 6.7
Cobalt	nd	nd
Copper	nd	nd
Selenium	nd	nd
Cadmium	136 ± 6.8	24 ± 1.2
Lead	111 ± 5.55	61 ± 3.05
Mercury	8.4 ± 0.42	7 ± 0.35
Nickel	nd	nd
Silver	123 ± 6.15	12 ± 0.6
Antimony	820 ± 41	297 ± 14.9
Rubidium	68 ± 3.4	53 ± 2.65
Strontium	nd	122 ± 6.1
Tin	610 ± 30.5	348 ± 17.4

5.2.2 Experimental design

Triplicate microcosms were established in sterile 1 l amber glass wide mouth jars containing 700 g and 256 g of soils A and B (dry weight), respectively. These soils were already contaminated with oil at a total petroleum hydrocarbon concentration of 22700 and 31500 mg kg^{-1} soil, respectively. The concentrations of the aliphatic and aromatic fractions within different carbon bands from C_8 to C_{35} are shown in table 5.3. Each soil was homogenised through a 2 mm sieve and stones and vegetation debris were removed prior to establishing the microcosms. Four different microcosms conditions were established, as follows: (i) no amendments, (ii) addition of nitrogen and phosphate (C: N: P ratio 100:10:1), (iii) addition of nitrogen and phosphate (C: N: P ratio 100:10:1) and inoculum (10^6 cells per g soil dry weight) and (iv) for soil A only the soil was ground first and treated

as per condition (ii). Mercuric chloride (52 mg l^{-1}) as a sterilant for the abiotic controls using the treatment conditions of (i) for both soils and (iv) for soil A only.

Table 5.3: Initial TPH concentration within soils A and B.

	Soil A		**Soil B**	
Total TPH (mg kg^{-1})	22700		31500	
Carbon Fraction	**Aliphatic (mg kg^{-1})**	**Aromatic (mg kg^{-1})**	**Aliphatic (mg kg^{-1})**	**Aromatic (mg kg^{-1})**
C_8-C_{10}	<13.8	<13.8	<13.8	<13.8
C_{10}-C_{12}	32.5	<13.8	10.7	156
C_{12}-C_{16}	1560	399	1130	2580
C_{16}-C_{21}	3980	1730	1840	5990
C_{21}-C_{35}	7480	3450	2710	5570
Total	13052	5592	5787	14296

Nitrogen and phosphorus were added to the microcosms in the form of ammonium nitrate (NH_4NO_3) and potassium phosphate (pH 7), respectively. The Phosphate buffer was prepared 38.5 ml of 1 M monopotassium phosphate (KH_2PO_4) and 61.5 ml of 1 M Potassium phosphate, dibasic (K_2HPO_4) to achieve a pH 7 buffer, confirmed using a pH meter (Jenway 3540, pH and conductivity meter). These were prepared using sterile de-ionised water and glassware which had been autoclaved prior to use, sterilising solutions as appropriate using an autoclave. The fertilizer was added to give a C: N: P ratio of 100:10:1. Summary of nutrients and bioaugmentation within the soil microcosms is presented in Table 5.4.

Table 5.4: Microcosm design.

Soil	Microcosm	Treatments*						Sampling+		Total number of Microcosms
		Soil pre-treatment		Mercuric chloride solution	Sterile deionised water	Fertiliser solution	Inoculum	Destructive	Non-destructive	
		Sieved	Ground							
A	Abiotic control	✓		✓				✓		18
	Abiotic ground control	✓	✓	✓				✓		18
	Biotic control	✓			✓				✓	3
	Biostimulated	✓				✓			✓	3
	Biostimulated & ground	✓	✓			✓			✓	3
	Biostimulated & bioaugmented	✓				✓	✓		✓	3
B	Abiotic control	✓		✓				✓		18
	Biotic control	✓			✓				✓	3
	Biostimulated	✓				✓			✓	3
	Biostimulated & bioaugmented	✓				✓	✓		✓	3

* All treatments were incubated aerobically in the dark at 15 °C in high humidity
+ Sampling for all microcosms was performed at 0, 7, 14, 28, 56 and 112 days.

The inoculum was a mixture of hydrocarbon degraders (kindly provided by Aberdeen University), which had been formulated onto fine woodchips. For the amendment a few woodchips were added to 10 ml of Bushnell-Haas broth supplemented with 1 g l^{-1} salicilic acid and 1 % ethanol, adjusted to pH 7. This was placed in an orbital shaker at 150 rpm in the dark at 20 $^{\circ}$C and left overnight, after which 1% was added to 100 ml of fresh medium and grown on to stationary phase (about 24 hr, checked by optical density readings at 600 nm). The cell number at stationary phase was 10^8 cells ml^{-1} and 10^6 cells g^{-1} (dry weight) were added to the soils.

Once all amendments (nutrients and inoculum) were added the moisture content of the microcosms was adjusted to 80 % of the soil's water holding capacity using sterilised deionised water.

5.2.3 Incubation conditions and sampling

The microcosms were incubated in amber glass bottles aerobically in the dark at 15 °C. High humidity was maintained using damp cotton wool and moisture checked periodically to ensure it was maintained at 80 % of the soils water holding capacity. To ensure that the microcosms did not become oxygen-limited they were mixed weekly and capped loosely to allow oxygen transfer, except the abiotic controls.

Triplicate microcosms were then used for analysis after 0, 7, 14, 28, 56, and 112 days, sampling non-destructively for the biotic microcosms and destructively for the abiotic microcosms.

Analyses of the microcosms consisted of (i) respiration to assess microbial activity, (ii) nitrogen (N) and phosphate (P) sources to monitor the nutrients consumption, (iii) total petroleum hydrocarbons (TPH) concentrations to assess oil biodegradation and to identify recalcitrant compounds, (iv) seed germination and Microtox® bioassay to assess residual toxicity, (v) pH and moisture monitoring to ensure conditions were not limiting bioremediation and (v) statistical analyses to compare results.

5.2.4 Sample analyses

Carbon dioxide analysis

Carbon dioxide production was monitored during the experiment. This was performed by sealing 1 g (dry weight) of the respective microcosm soil in a headspace vial, which was incubated under the same conditions as the microcosms for 24 h. After mixing the headspace within the vial, 1 ml of gas was withdrawn with a gastight syringe. This was injected directly into a Gas Chromatograph with Thermal Conductivity Detection (GC-TCD) (Cambridge Scientific Instruments). TCD detection is a well established method based upon the changes in thermal conductivity of a gas stream associated with the presence of target analyte molecules (Skoog et al. 1998). The resultant CO_2 peak was measured and percentage CO_2 determined. Calibration was performed using a 1 % CO_2 standard in N_2, which was checked at every injection by injecting 1 ml of the same standard. Calibration was performed using standards of CO_2 at 0.2 %, 0.4 %, 0.6 %, 0.8 % and 1 %. Ambient air

samples were also analysed to determine the CO_2 level prior to incubation. The GC-TCD was a Cambridge Scientific Instruments 200 Series Gas Chromatograph which used helium as a carrier gas at 20 psi with injector and column temperatures of 125 and 110 °C, respectively. The column was a CTR1 concentric packed column (Alltech, USA). The outer column was 6 ft x ¼ inch packed with activated molecular sieve, and the inner column was 6 ft x $^1/_8$ inch porous polymer mixture. Data was quantified using DataApex software program Clarity version 2.4.4.105. Injection was performed with a 10 ml min^{-1} split.

pH

The pH of the soil was determined using deionised water and a solution of 0.01 M $CaCl_2$. This was measured by adding 50 ml of solution to 4 g soil (wet weight) in a flask and shaken for 30 minutes. Samples were then left to settle for 30 minutes before a reading was taken. The calcium chloride method was used in addition to the water method as results can vary due to seasonal variations. The calcium chloride method also better approximates field conditions.

Moisture

The moisture content of the microcosms was maintained at 80 % of the soil water holding capacity (WHC), by weighing the microcosms and adding sterile de-ionised water if required.

Nutrients analysis

Nitrate and ammonium were extracted using the method defined by the cell test. Briefly, 5 g of soil was suspended in 10 ml of 25 mM $CaCl_2$ solution with a spatula of charcoal activated for soil tests in a closed bottle. The mixture was then stirred for 1 h, left to settle and filtered through a fluted filter prior to analysis following the manufacturer's instructions (MERCK, 2006a and MERCK, 2006b).

Phosphate was extracted using the method defined by the cell test. Briefly an extraction solution was prepared by dissolving 120 g of calcium lactate in 800 ml of boiling water to which 40 ml of hydrochloric acid was added and made up to the mark. This stock was diluted 1:20 with water. The soil was extracted by adding 5 g of dry soil to 250 ml of diluted extractant, shaking for 90 minutes after which the sample was filtered through a fluted filter prior to analysis following the manufacturer's instructions (MERCK, 2006c).

Hydrocarbons analysis

Extraction and subsequent analysis of the soils for total petroleum hydrocarbons (TPH) was performed using the methods described previously in section 4.2. Additionally, the compounds defined in Table 5.5 were also determined. Using the GC-MS software (TurboMass ver. 4.4.0, Perkin Elmer) *n*-alkanes (right hand side of Table 5.5) with carbon numbers from 10 to 40 were identified and quantified at m/z 57 using the diesel standards

as described in section 4.2.6 and one of two internal standards (Nonadecane-d_{40} and Triacontane-d_{62} purchased from Sigma Aldrich, Dorset, UK). All other compounds listed in Table 5.5 were quantified on the ion listed using one of four internal standards (Napthalene d_8, Phenanthracene-d_{10}, Chrysene-d_{12} and Perylene d_{12} purchased from Sigma Aldrich, Dorset, UK). The conserved biomarker 17α(H)21β(H)-hopane was chosen for use in assessing degradation within the soil, however delays beyond our control in acquiring a standard prevented its use. Alternatively the isoprenoids pristane and phytane were determined to evaluate bioremediation with the soils.

Table 5.5: Compounds determined.

Compounds	Carbon number	Q_{ion} *(m/z)*	Compounds	Carbon number	Q_{ion} *(m/z)*
Naphthalene*	10	128	Decane*	10	57
C_1-Naphthalenes*	11	142	Undecane*	11	57
C_2-Naphthalenes*	12	156	Dodecane*	12	57
C_3-Naphthalenes*	13	170	Tridecane*	13	57
C_4-Naphthalenes*	14	184	Tetradecane*	14	57
Acenaphthylene*	12	152	Pentadecane*	15	57
Acenaphthene*	12	154	Hexadecane*	16	57
Fluorene*	13	166	Heptadecane*	17	57
C_1-Fluorenes$^+$	14	180	Pristane*	19	57
C_2-Fluorenes$^+$	15	194	Octadecane*	18	57
C_3-Fluorenes	16	208	Phytane*	20	57
Phenanthrene*	14	178	Nonadecane*	19	57
Anthracene*	14	178	Eicosane*	20	57
C_1-Phenanthrenes/anthracenes$^+$	15	192	Heneicosane*	21	57
C_2-Phenanthrenes/anthracenes*	16	206	Docosane*	22	57
C_3-Phenanthrenes/anthracenes*	17	220	Tricosane*	23	57
Fluoranthene*	16	202	Tetracosane*	24	57
Pyrene*	16	202	Pentacosane*	25	57
C_1-Fluoranthenes/pyrenes*	17	216	Hexacosane*	26	57
C_2-Fluoranthenes/pyrenes*	18	230	Heptacosane*	27	57
C_3-Fluoranthenes/pyrenes	19	244	Octacosane*	28	57
Benz(a)anthracene*	18	228	Nonacosane*	29	57
Chrysene*	18	228	Triacontane*	30	57
C_1- Chrysenes/benz(a)anthracenes$^+$	19	242	Hentriacontane*	31	57
C_2- Chrysenes/benz(a)anthracenes	20	256	Dotriacontane*	32	57
C_3- Chrysenes/benz(a)anthracenes	21	270	Tritriacontane*	33	57
C_4- Chrysenes/benz(a)anthracenes	22	284	Tetratriacontane*	34	57
Benzo(b)fluoranthene*	20	252	Pentatriacontane*	35	57
Benzo(k)fluoranthene*	20	252	Hexatriacontane*	36	57
Benzo(e)pyrene*	20	252	Heptatriacontane*	37	57
Perylene$^+$	20	252	Octatriacontane*	38	57
Benzo(a)pyrene*	20	252	Nonatriacontane*	39	57
Indeno(1,2,3-c,d)pyrene$^+$	22	276	Tetracontane*	40	57
Dibenz(a,h)anthracene$^+$	20	278			
Benzo(g,h,i)perylene$^+$	22	276			
Dibenzo(a,l)pyrene$^+$	24	302			
2-Methylnaphthalene*	11	142			
1-Methylnaphthalene	11	142			
Dibenzofuran$^+$	12	168			
Dibenzothiophene	14	184			
C_1-Dibenzothiophenes$^+$	13	198			
C_2-Dibenzothiophenes$^+$	14	212			
C_3-Dibenzothiophenes	15	226			
C_4-Dibenzothiophenes	16	240			

* = identified within both soil A and B, $^+$ = identified in soil B only.

Note: Alkyl homologs of compounds are grouped by the number of carbon groups attached and denoted by C_n where n equals the number of carbon groups attached.

Toxicity bioassays

Seed germination

Seed germination tests were performed at the start and end of the microcosm experiment in triplicate by adding 10 mustard seeds to 20 g of test soil (wet weight) in 120 ml bottles. The seeds were left to germinate for 4 days at 25 $^{\circ}$C in darkness.

Microtox® Solid Phase Test (SPT)

Microtox® analysis was performed at the start and end of the experiment in triplicate. The toxicity of oiled soils was evaluated by the response of the luminescent bacteria *Vibrio fischeri* using the Microbics Microtox® solid phase test (Microtox® SPT). Briefly, 0.4 g of dry soil was suspended into 10 ml glass bottle filled with 4 ml of Microtox® SPT diluent and stirred for 20 min with a vortex about half the height of the vial. Following 15 min of settling time, a one-ml aliquot of the aqueous phase was transferred into appropriate cuvettes to make a twelve-dilution series (1:2). Each sample was analyzed using the solid phase test protocol as described by the manufacturer. A standard 100 g l^{-1} phenol solution was used to check the performance of both operator and analytical system and the 95% confidence range was maintained below 15% variation throughout the study. Tests were done in triplicate. The toxicity decreases when the EC_{50} value increases.

Statistical analysis

Statistical analysis of the results was performed using Excel and SPSS (Statistical Product and Service Solutions) version 15. Mean, standard deviation (SD), standard error (SE), Analysis of variance (ANOVA) and One-Way ANOVA's were performed on the data to determine any significant differences between data points when appropriate.

5.3 Results

5.3.1 Changes in carbon dioxide concentration

Carbon dioxide production was monitored within the soil microcosms throughout the microcosm experiment. The CO_2 within the ambient air was 0.074, 0.065, 0.072, 0.049, 0.073 and 0.073 mg CO_2 per ml when samples from 0, 7, 14, 28, 56 and 112 days respectively were analysed. After incubation all soils demonstrated a CO_2 evolution greater than the ambient air concentration (Figure 5.1), suggesting that all microcosms contained a viable microbial population.

Whilst CO_2 production in soil A barely changed during the experiment, a decline of CO_2 production was observed in soil B (Figure 5.1).

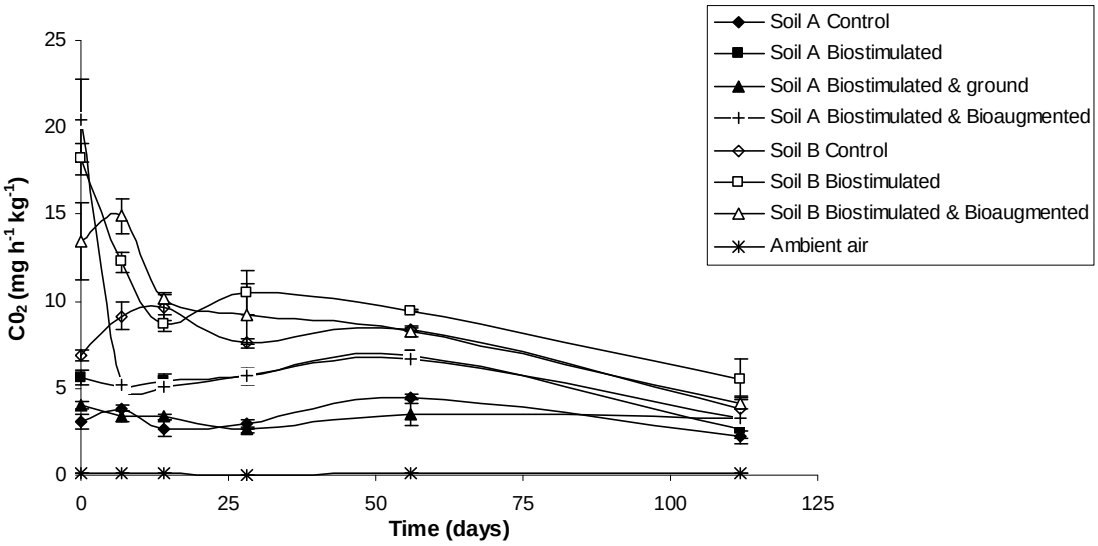


Figure 5.1: Mean (±SE) CO_2 concentration during the microcosm experiment (112 days) for all soils and treatments.

The addition of an inoculum at the beginning of the microcosm experiment resulted in significantly increased CO_2 production within the combined biostimulated and bioaugmented soil A microcosm (P= <0.001, <0.001 and <0.001 compared to the control, biostimulated and ground biostimulated treatment, respectively) (Figure 5.2). This increase was short-lived as, by the 7-day sampling, CO_2 evolution had reduced to levels comparable to that in the biostimulated treatment. With the exception of the sampling at 0 days, this suggests that the addition of an inoculum did not increase CO_2 production, also suggesting that the soil may have been inhibitive to the consortium added and that the CO_2 produced was generated by the existing microbial population.

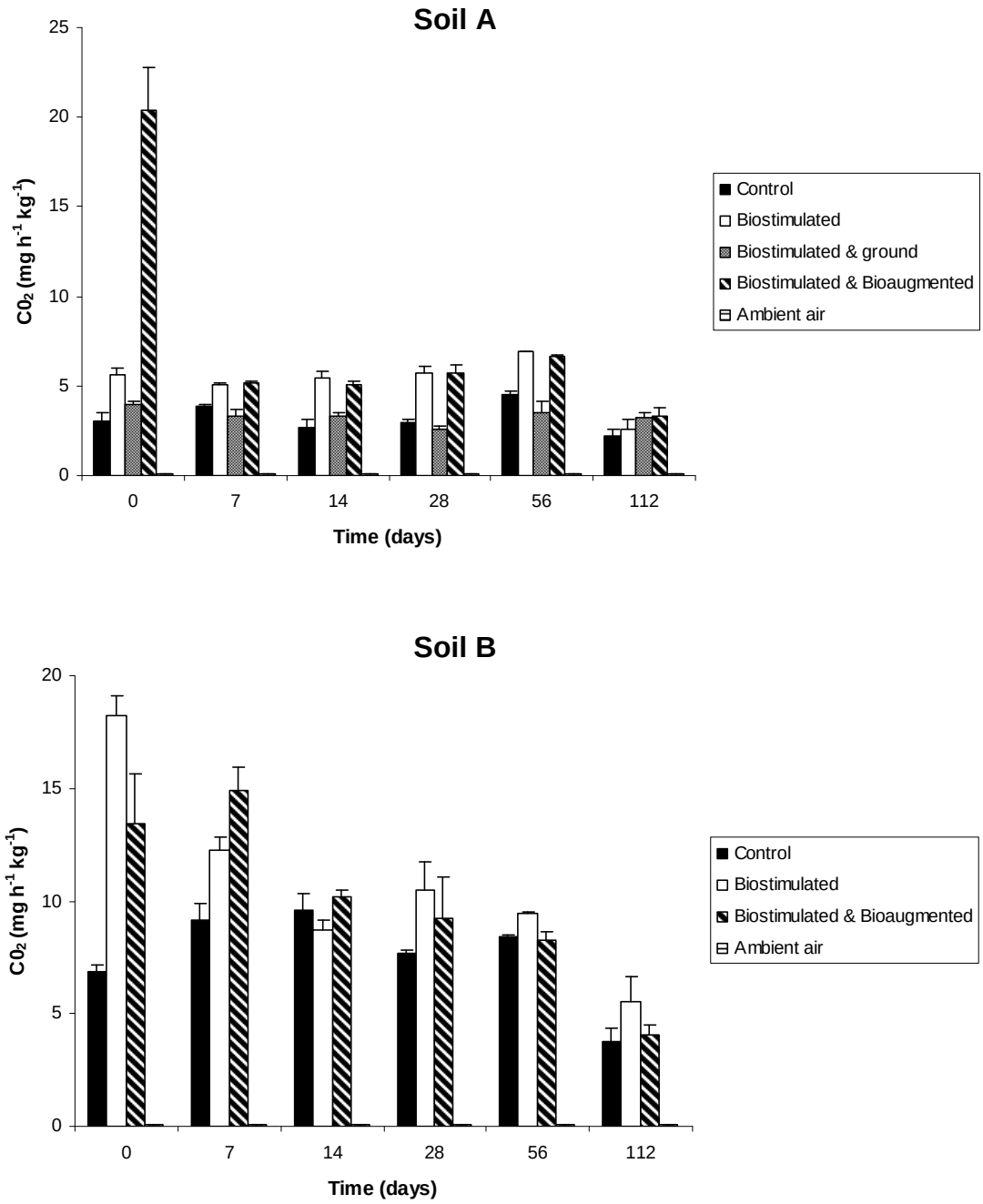


Figure 5.2: Mean (+SE) carbon dioxide concentration from triplicate samples over the experimental period (112 days) from soil A and B microcosms.

The treatments observed to have the most beneficial effect on CO_2 evolution within soil A were the biostimulated and the biostimulated & bioaugmented (Figure 5.2). These treatments maintained significantly higher CO_2 concentrations for the duration of the experiment compared to the control (P-values were: 0.005 & 0.003 at 7 days, 0.003 & 0.007 at 14 days, 0.002 & 0.002 at 28 days and 0.004 & 0.008 at 56 days for the biostimulated and biostimulated & bioaugmented treatments respectively when compared to the control). No difference in CO_2 evolution was seen at the end of the treatment period between the different treatments. The ground and biostimulated treatment showed no additional CO_2 evolution compared to the control, whereas the biostimulated treatment did, suggesting that the ground structure of the soil has had a limiting effect upon CO_2 evolution within this soil.

Within the soil B microcosms the initial influx of nutrients and inoculum significantly increased CO_2 production over the first 7 days ($p=0.003$ and 0.036 for the biostimulated and biostimulated & bioaugmented treatments respectively), after which evolution was observed to gradually decrease over time to levels comparable to the concentration produced within the control and other treatments (Figure 5.2). With the exception of the sampling at 0 days, this suggests that the addition of an inoculum did not increase CO_2 production, also suggesting that the soil may have been inhibitive to the consortium added and that the CO_2 produced was generated by the existing microbial population. Here, within soil B, no

treatment significantly increased CO_2 evolution over the duration of the experiment.

5.3.2 pH

The soil pH was determined throughout the microcosm experiment and although differences were observed between different soils the pH within the treatments remained within the pH 5 – 9 limits determined to be viable for microbial activity (see chapter 1). As such, no adjustments were required.

5.3.3 Change in petroleum hydrocarbons composition

Initial analysis of the two soils showed that soil B contained higher concentrations of total petroleum hydrocarbons than soil A. In addition, hydrocarbon composition analysis showed that soil B had a higher concentration of aromatic compounds while soil A had the higher concentration of aliphatic compounds (Table 5.3). Neither soil contained all of the aromatic compounds that were initially selected for analysis (Table 5.5), and soil A had considerably less of those compounds than soil B (Table 5.5). The bulk of the contamination within both soils ranges between C_{16}-C_{35} (Table 5.3). The limited presence of hydrocarbons below C_{16} gives an indication of the extent of weathering that has already occurred.

Aliphatic hydrocarbons

n-Alkanes ranging from C_{10} to C_{40} were determined for both soils, the distribution of which at the start and end of the experiment is shown in Figure 5.3(a). Here a clear difference between the soils is observed. As previously shown (Table 5.3), soil A has much higher concentrations of these compounds, with a distinctively concentrated group in the C_{32}-C_{36} range (Figure 5.3(b)). With the exception of this range the profile is comparatively flat compared to soil B (Figure 5.3 (b)). Overall soil B is less concentrated with a distinctively different profile to soil A (Figure 5.3 (c)). The bulk of the *n*-alkanes present within soil B are spread over a much broader range from C_{16}-C_{40} (Figure 5.3 (c)). Suggesting that less degradation/weathering prior to treatment has occurred within this soil. Although widely spread, two main groups can be seen around C_{17} and C_{30}-C_{38} (Figure 5.3(c)).

At the end of the experiment the concentration of the *n*-alkanes analysed has clearly reduced within both soils, whilst maintaining some of their original profile (Figure 5.3). As expected the control for both soils shows distinctively less reductions in the concentrations of the *n*-alkanes analysed. Biostimulation treatments have reduced concentrations greatest within both soils with no beneficial effects of augmentation (both soils) or grinding (soil A only) being shown (Figures 5.3 (b) and (c)). Within both soils the lighter *n*-alkanes have been preferentially degraded, resulting in profiles displaying greater proportions of the $\geq C_{32}$ compounds at the end of the experiment.

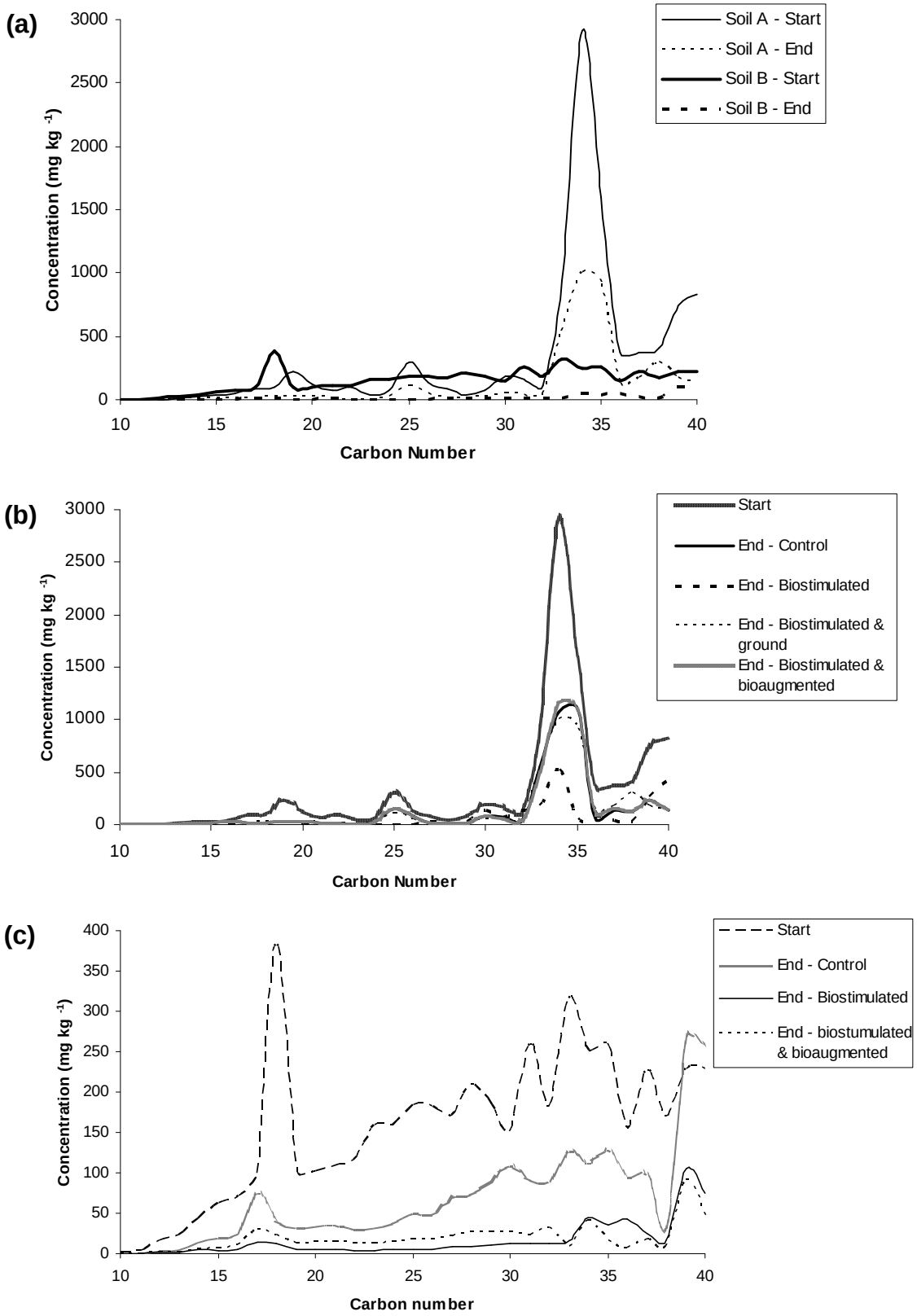


Figure 5.3: *n*-Alkane C_{10} –C_{40} distributions at the start and end of the microcosm experiment in (a) both soils, (b) soil A and (c) soil B.

C_{17}: Pristane and C_{18}: Phytane ratios

Initial C_{17}: Pristane and C_{18}: Phytane ratios of 0.03 and 0.01 respectively for soil A, and 0.10 and 0.16 respectively for soil B were observed. Within soil B these ratios were both significantly higher than those in soil A (P-values for both ratios were < 0.001), reflecting a clear difference between the hydrocarbon contamination within these soils. Here the low ratio values for both soils indicate that the hydrocarbon contamination present has undergone biodegradation prior to this investigation. Degradation is shown to be occurring within both soils as both ratios decline throughout the experimental period, with a faster decline being observed in soil B (Figure 5.4). Here the C_{18}: phytane ratio is shown to have decreased by the greatest amount. The final ratio values for all treatments within both soils show significant decreases (P-values all <0.001), however these isoprenoids are themselves known to degrade (Riser-Roberts,1998) (Figure 5.4). Thus this should only be viewed as an indication of degradation occurring, as it may not be an accurate representation of the extent of degradation that has occurred. Use of such ratios could underestimate the extent of degradation and thus it is necessary to analyse the degradation in more detail to establish the full extent of degradation that has occurred.

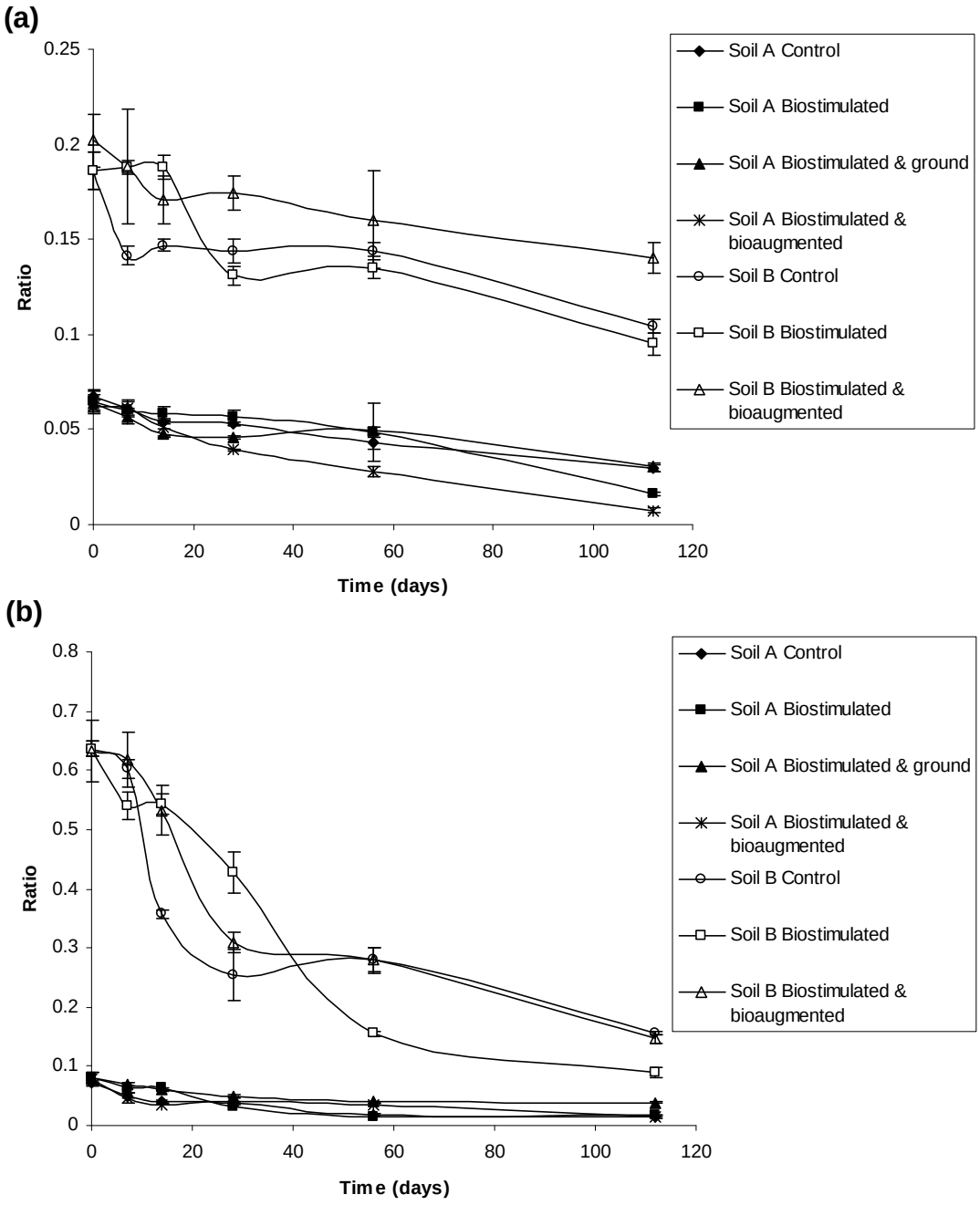


Figure 5.4: Change in the Mean (±SE) ratios for (a) C_{17}: Pristane and (b) C_{18}: Phytane during the soil microcosm experiment.

Comparison of the treatment used for both soils shows that the treatments have had different effects on the C_{17}: pristane ratio over time

(Figure 5.5). The decline in the C_{17}: pristane ratio for the contamination in soil A shows no sign of stopping, suggesting that degradation was still occurring at the end of the experiment. Here, the combined biostimulation & bioaugmentation treatment has had the greatest effect on this ratio and is significantly lower than the ratios for all the other treatments by the end of the experiment (P-values of <0.001, <0.001 and 0.001, when compared against the control, biostimulation and ground biostimulation treatments respectively were recorded). Although not to the same extent, the biostimulation treatment has also had a significant effect on the reduction of this ratio (P-values of <0.001 and <0.001 when compared to the control and ground biostimulation treatment respectively). This suggests that the addition of an inoculum has an additional beneficial effect, over and above that arising from the biostimulation treatment alone.

The pattern of degradation shown by the of C_{17}: pristine ratio for soil A is different to that shown in soil B. Here, the addition of an inoculum has not had a beneficial effect on degradation, and is significantly less reduced than both the control and the biostimulation treatment (P-values of 0.001 and < 0.001 compared to the control and the biostimulation treatments respectively) (Figure 5.5). There is no significant difference in the C_{17}: pristine ratio between the control and the biostimulated treatment ($p> 0.05$), this could suggest that adjustment of soil water, incubation and aeration has been the major influence in enhancing degradation here.

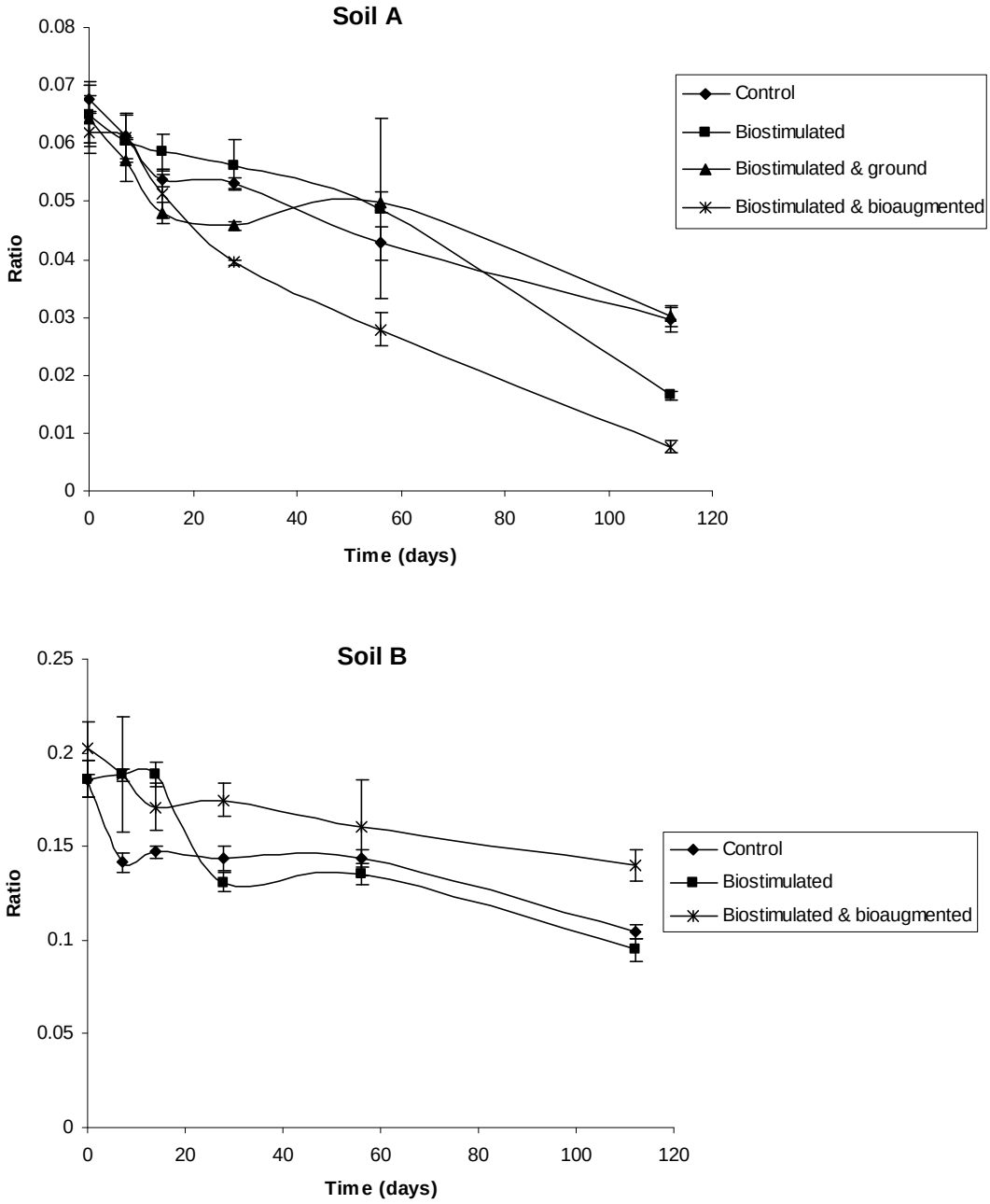


Figure 5.5: Change in mean (±SE) C_{17}: Pristane ratio over 112 days treatment.

Evaluation of the final ratio for C_{18}: phytane within soil A shows that none of the treatments have significantly reduced the ratio compared to the

control ($P > 0.05$), and the ground biostimulated treatment has negatively affected the ratio as it is significantly more than the control ($P < 0.001$) (Figure 5.6). This would suggest that treatment has had no effect on enhancing degradation but the pattern of ratio change in the combined biostimulated and bioaugmented treatment is different to that observed within the other treatments. Here the ratio appears to still be decreasing suggesting that a longer incubation period would see the ratio for C_{18}: phytane decreasing further (Figure 5.6).

At the end of the experimental period biostimulation treatment for soil B has reduced the C_{18}: Phytane ratio of the contamination by the greatest amount and is significantly different to both the control and the combined biostimulation and bioaugmentation treatment ($P < 0.001$ and < 0.001 compared to the control and biostimulated treatment respectively). However the shape of the graph for the control and the combined biostimulation and bioaugmentation treatment suggest that degradation is still occurring and as with soil A further reduction in this ratio may be achieved through a longer incubation period. However, the different shape of the biostimulated & bioaugmented treatment may also be a result of a slower rate of degradation and thus may with additional incubation only reach the same value as the biostimulated treatment.

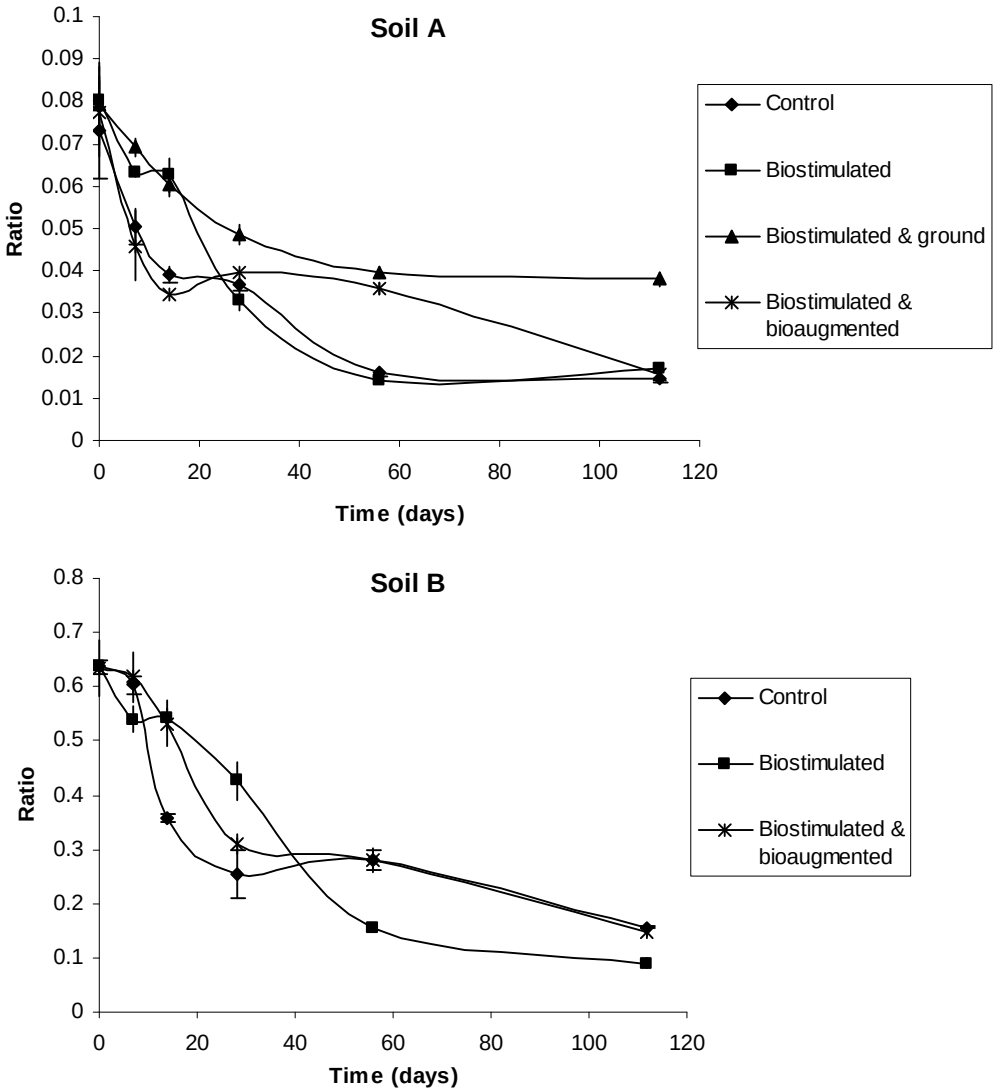


Figure 5.6: Change in mean (±SE) C_{18}:Phytane ratio over 112 days of treatment.

Detailed aliphatic fractions analysis

It has been shown by the C_{17}: pristane and C_{18}: phytane ratios that the hydrocarbon contamination within both of these soils has seen some degradation/weathering prior to investigation, and that treatment has further reduced this contamination. However as pristane and phytane themselves

are susceptible to degradation it is necessary to look at the change in composition in more detail to assess the extent of degradation achieved.

Additionally, to get a better understanding of the pattern of degradation, and in order to inform risk analysis, it is necessary to evaluate the contamination using class fractioning and banding of the hydrocarbons present (Chapter 1, section 1.3).

Analysis of the hydrocarbon fractions suggested by the Environment Agency (2005) shows that the most prominent fractions present are the C_{16}-C_{35} and C_{35}-C_{40} fractions. Although lighter fractions are present, they are significantly less concentrated ($P < 0.001$) (Figure 5.7). The relative absence of these lighter fractions is typical of weathered hydrocarbons where the lighter compounds have been preferentially degraded leaving higher proportions of the heavier compounds behind. Additionally, it should be noted that hydrocarbons above C_{40} where not detected within these soils (Figure 5.7)

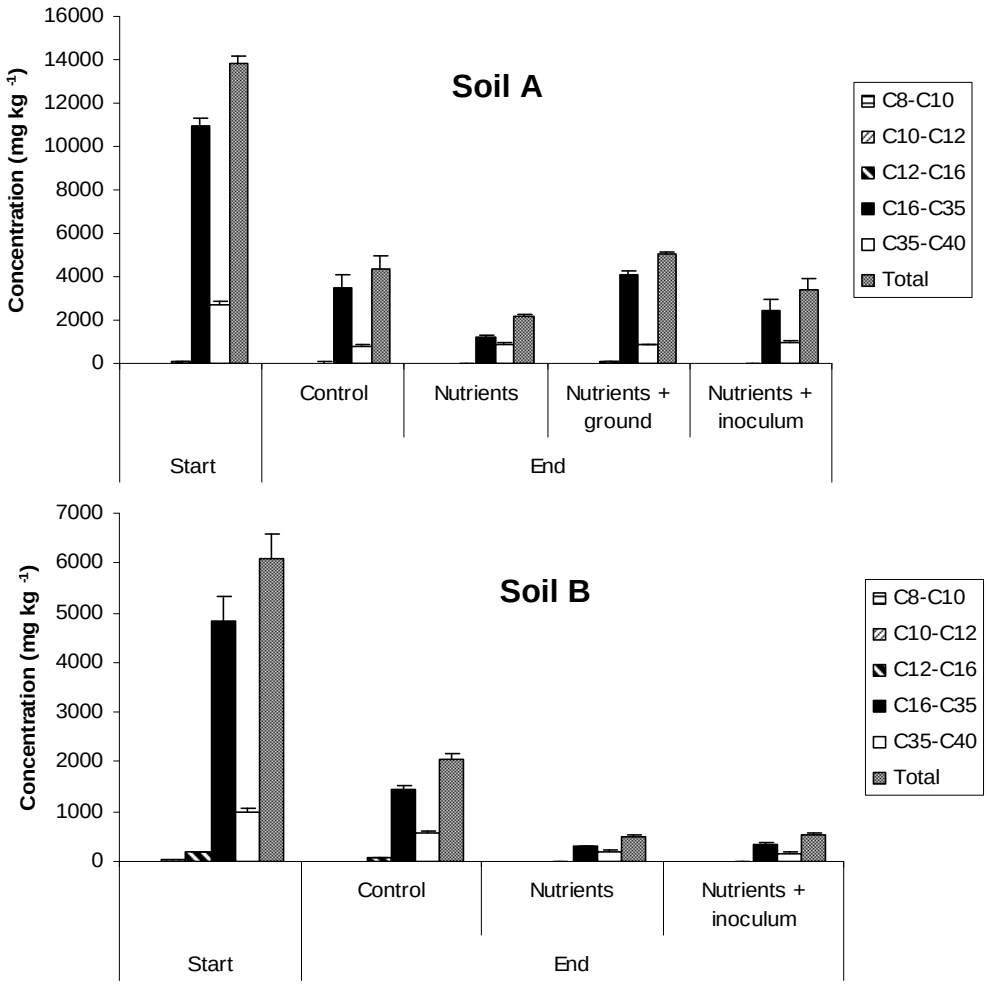


Figure 5.7: Mean (+SE) concentrations of the aliphatic C_8-C_{10}, C_{10}-C_{12}, C_{12}-C_{16}, C_{16}-C_{35} and C_{35}-C_{40} fractions for both soils at the start and end of the microcosm experiment.

The degradation pattern of the aliphatic compounds is shown in Figures 5.8-5.11 and Figures 5.12-5.14 for each of the treatments for soils A and B respectively. Here, there is a clear difference in the characteristics of contamination within the two soils. The profile of the *n*-alkanes determined is far flatter for soil B compared to A which varies between carbon numbers

to a greater extent (Figure 5.8 and 5.12). Overall, the *n*-alkanes determined within both soils show degradation over time within both soils. However there is a clear difference between the controls and the biostimulated and biostimulated & bioaugmented treatments for both soils (Figures 5.8, 5.9, 5.11 and 5.12-5.14) especially in concentration at 112 days. Additionally soil B shows a broader distribution of the *n*-alkanes determined. The biostimulated and biostimulated & bioaugmented treatments are both shown to reduce the concentrations of the *n*-alkanes within soil A faster and to a greater extent than shown in the control. Only slight differences between the two treatments are shown, where the biostimulated treatment shows a greater reduction of the C_{10}-C_{25} range (Figures 5.10 and 5.11).

The degradation profile for soil A ground biostimulated treatment is similar to the control with only slight differences in the rate of degradation shown in the C_{20}-C_{30} range, additionally within the C_{26}-C_{30} range it appears that grinding has reduced the initial concentration of some of these compounds.

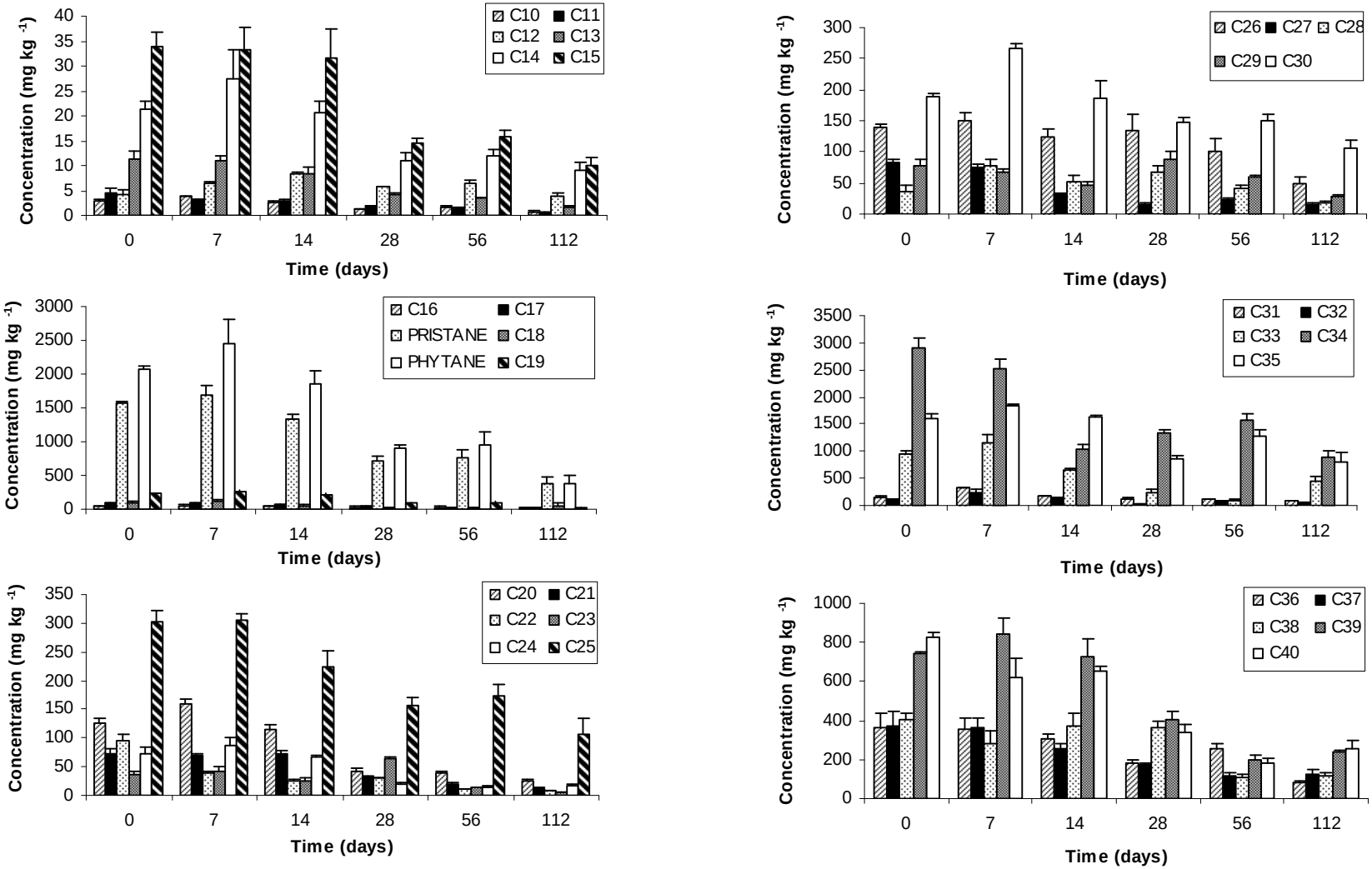


Figure 5.8: Change in the mean (+SE) concentration of *n*-alkanes C_{10}-C_{40} within soil A control.

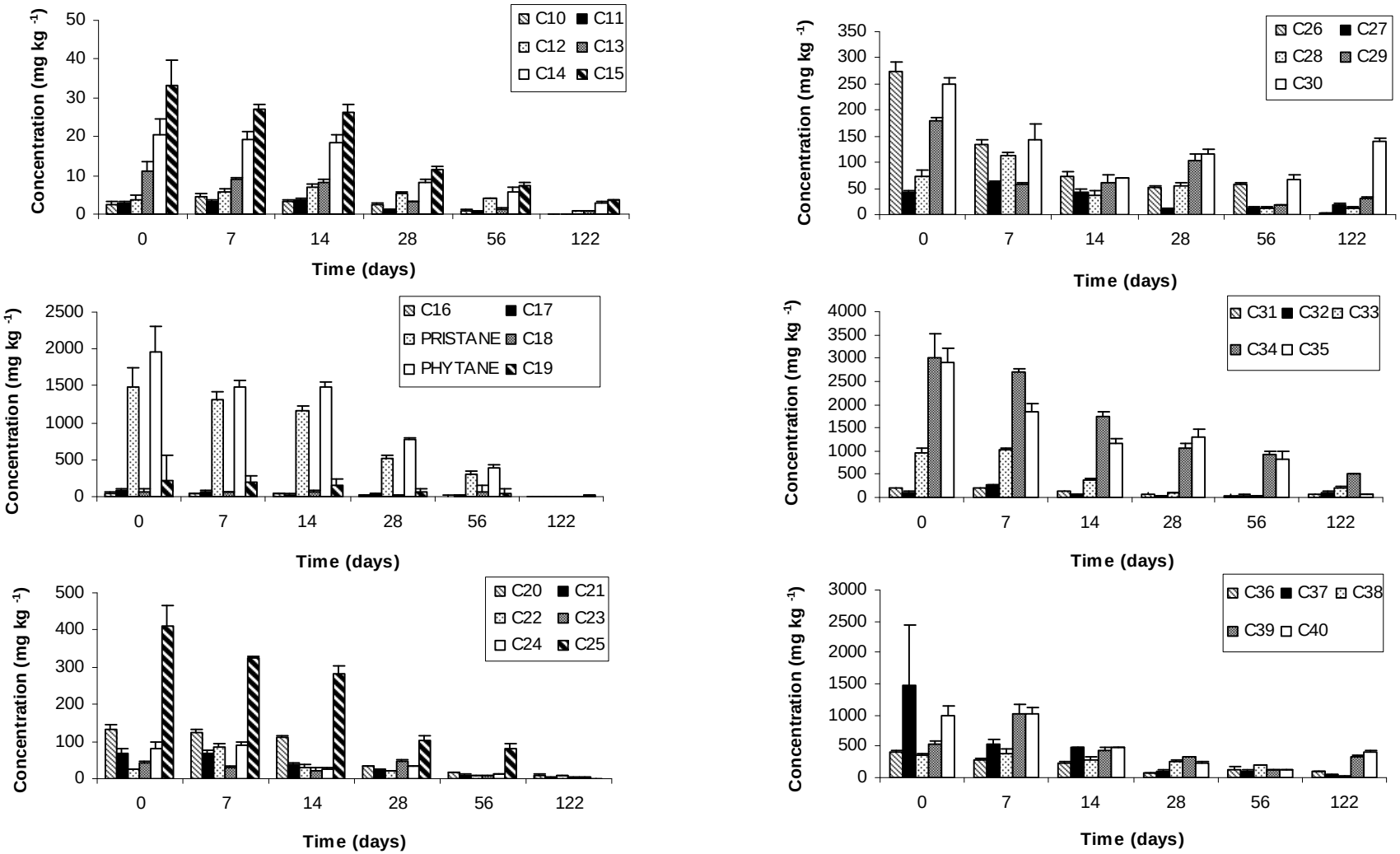


Figure 5.9: Change in the mean (+SE) concentration of *n*-alkanes C_{10}-C_{40} within soil A biostimulated treatment.

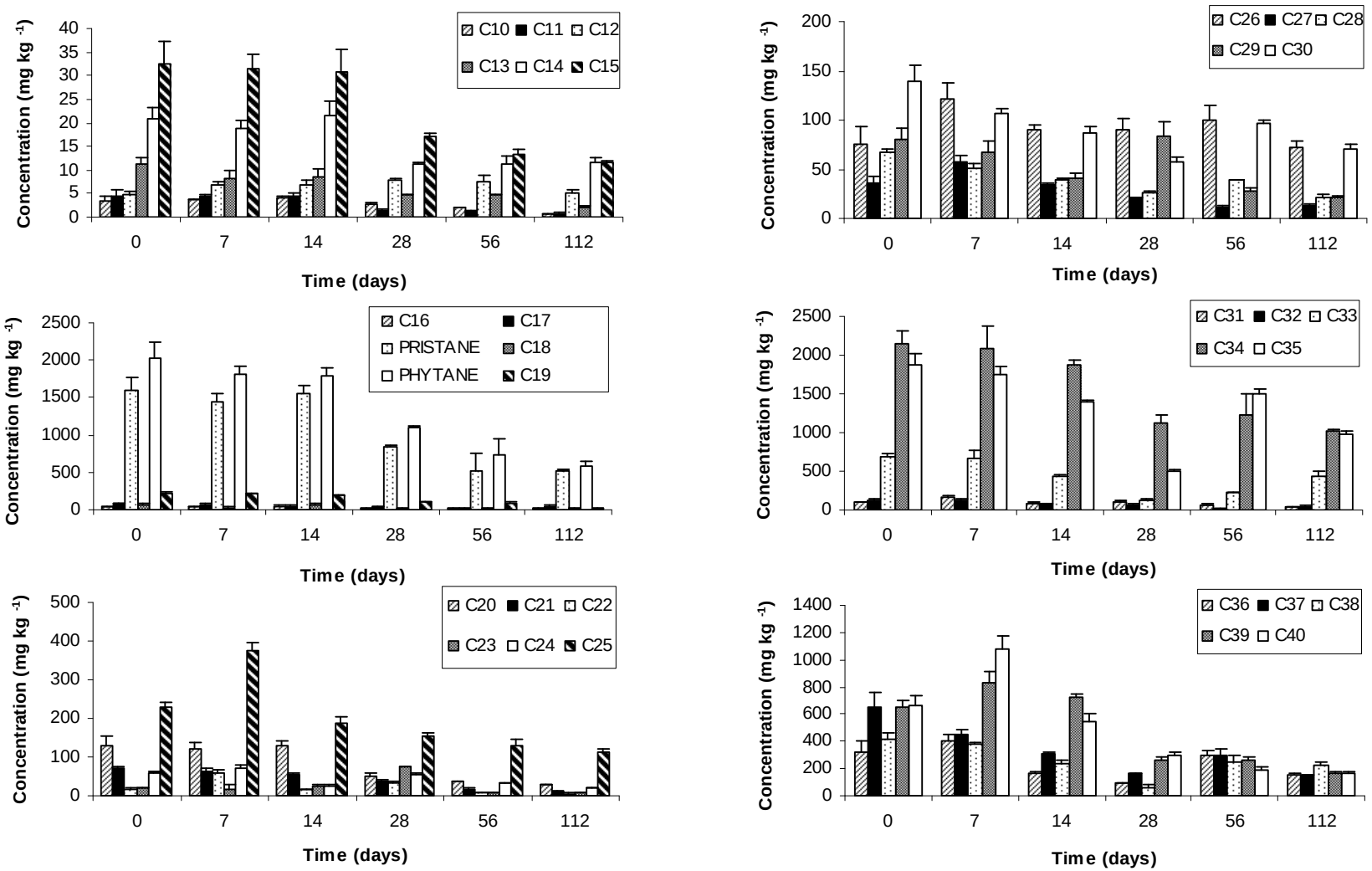


Figure 5.10: Change in mean (+SE) concentration of *n*-alkanes C_{10}-C_{40} in soil A ground biostimulated treatment.

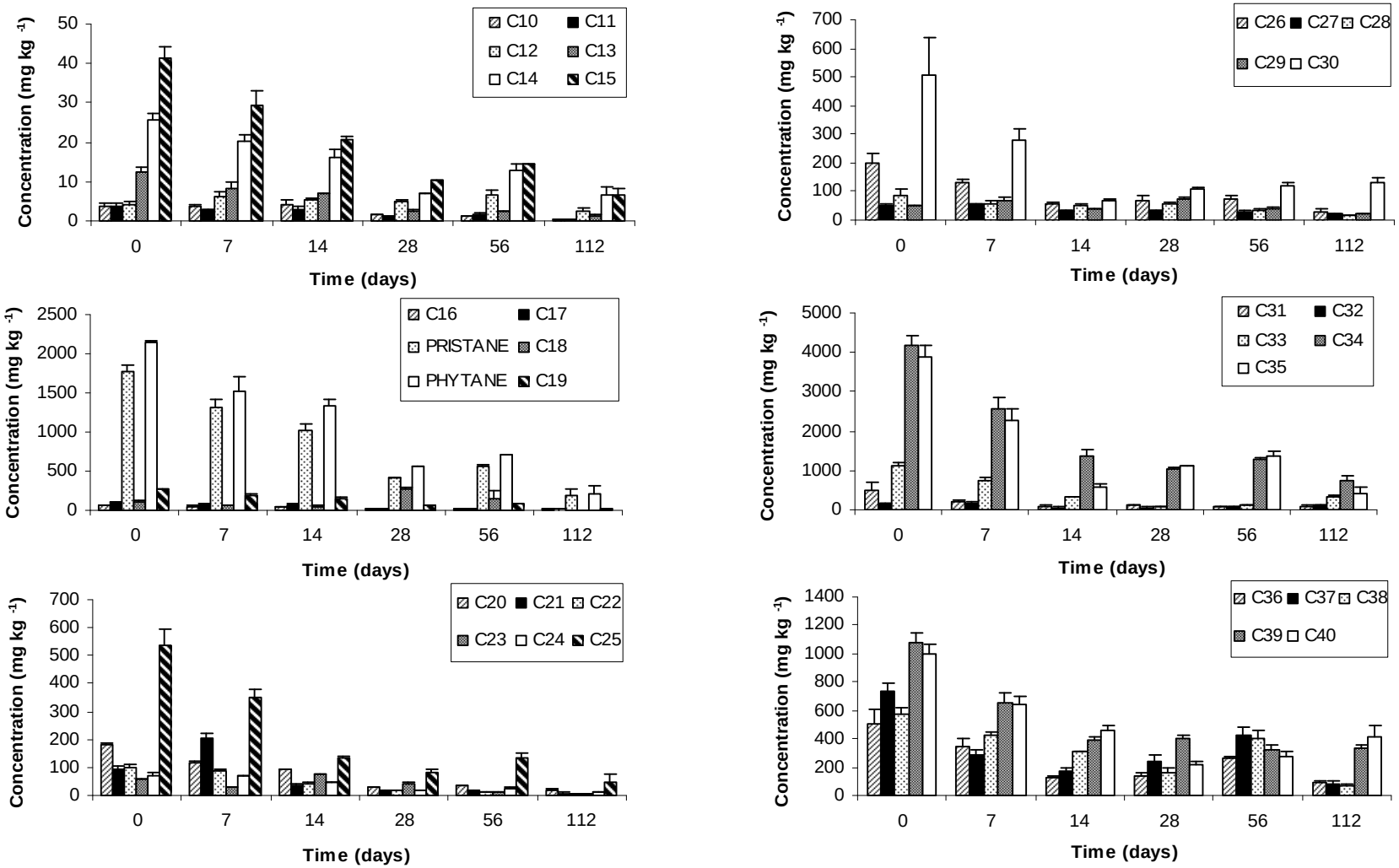


Figure 5.11: Change in mean (+SE) concentration of *n*-alkanes C_{10}-C_{40} in soil A biostimulation & bioaugmentation treatment.

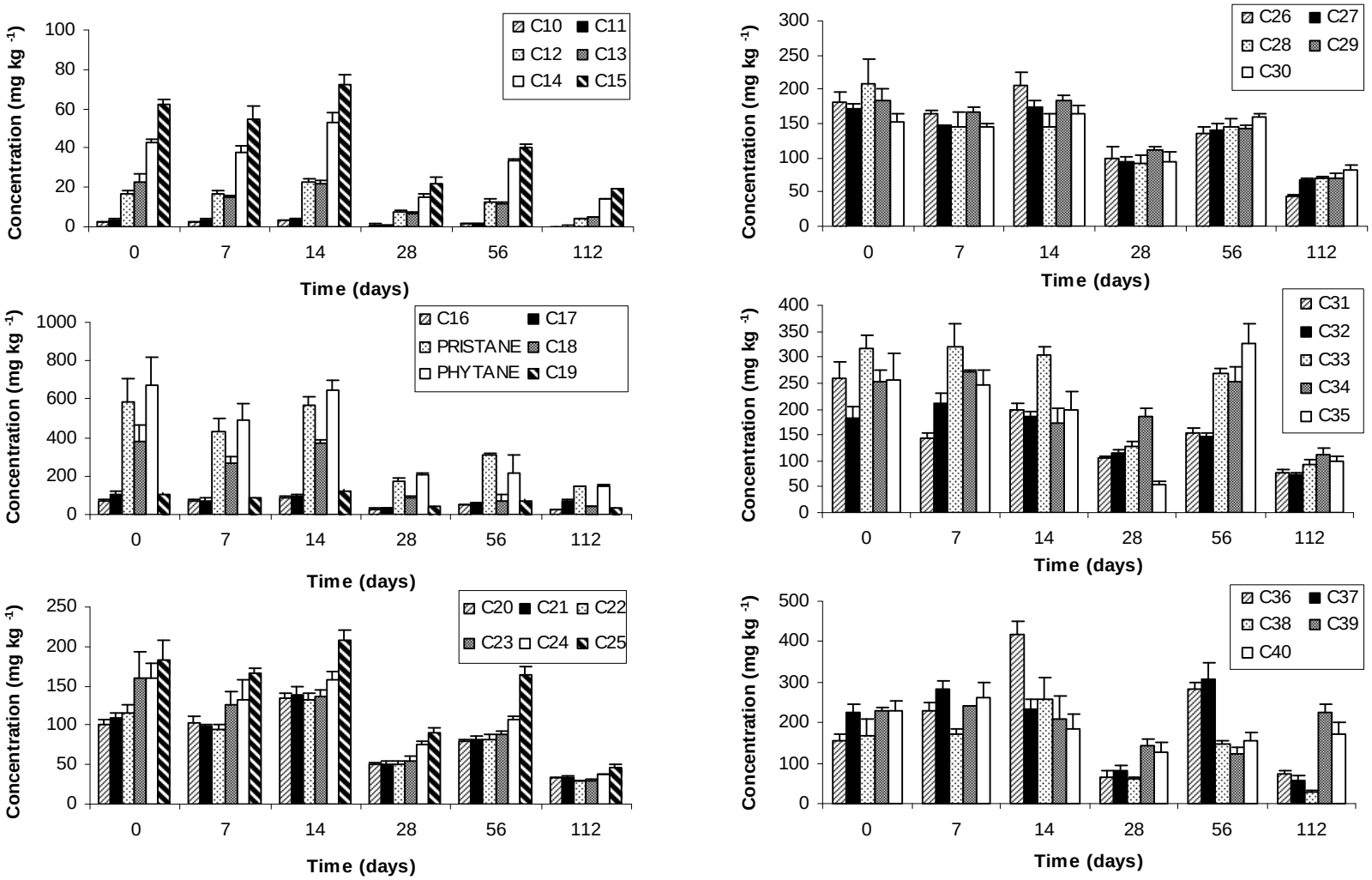


Figure 5.12: Change in mean (+SE) concentration of *n*-alkanes C_{10}-C_{40} in soil B control.

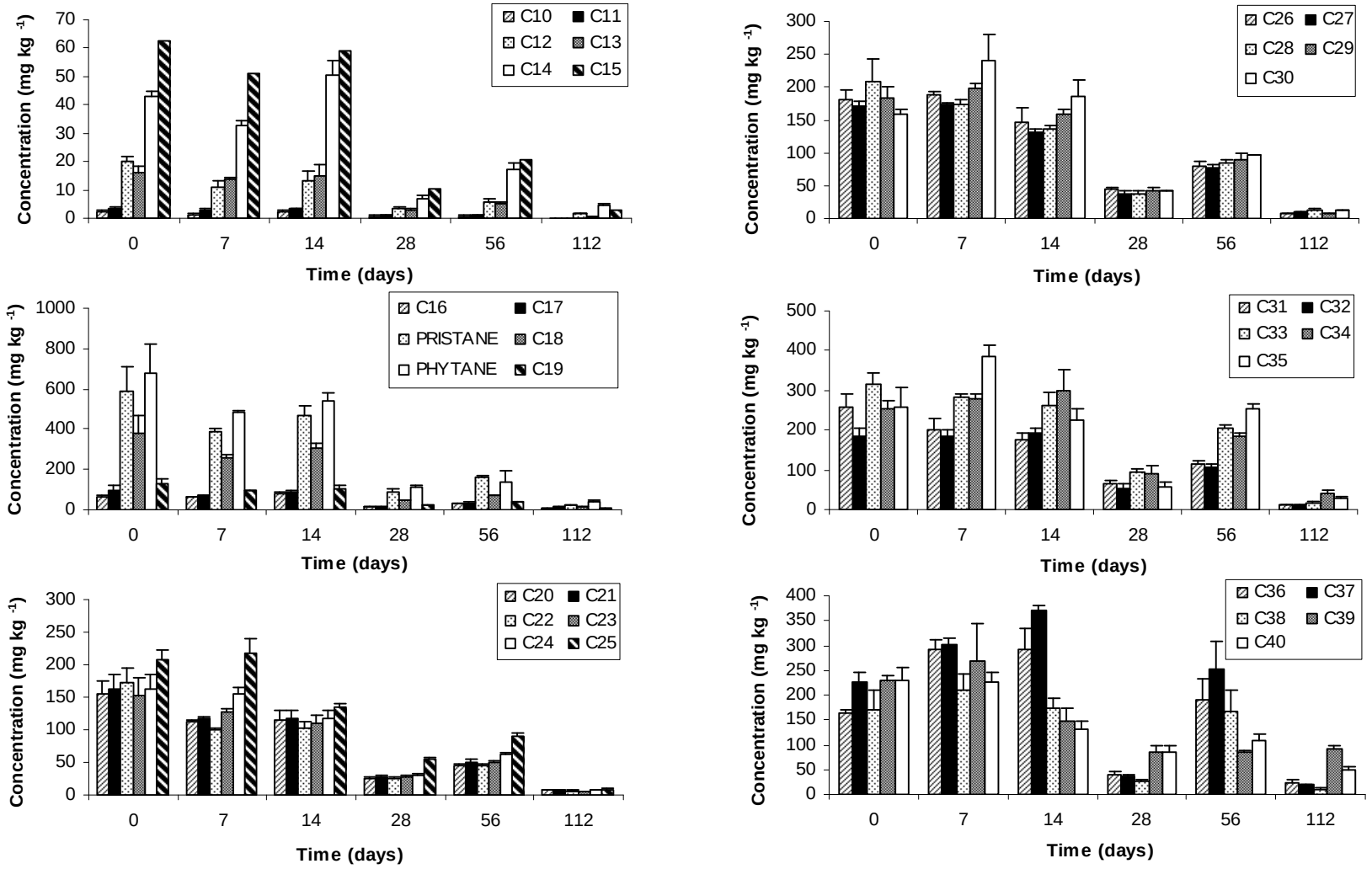


Figure 5.13: Change in mean (+SE) concentration of *n*-alkanes C_{10}-C_{40} in soil B biostimulation treatment.

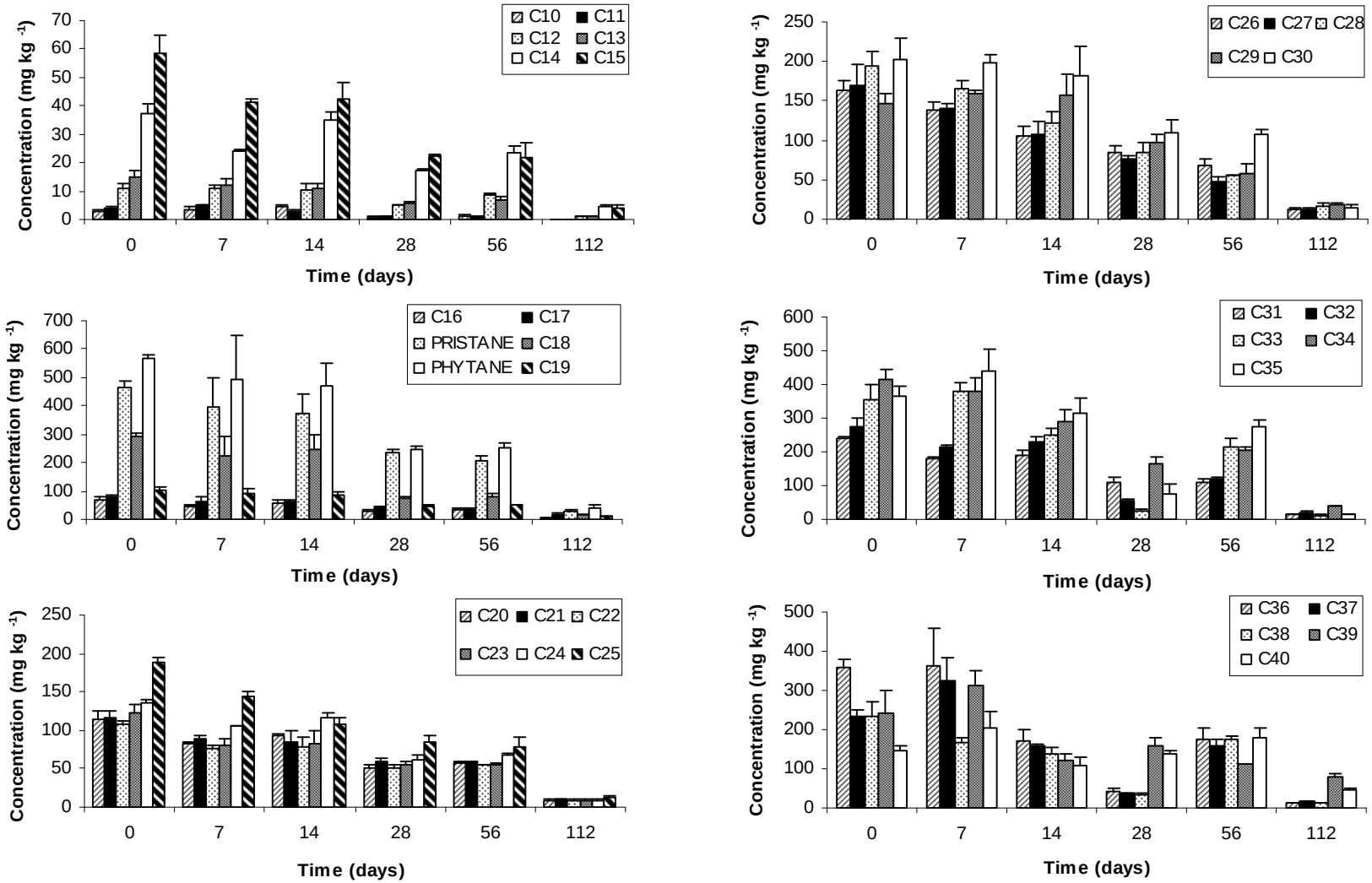


Figure 5.14: Change in mean (+SE) concentration of *n*-alkanes C_{10}-C_{40} in soil B biostimulation & bioaugmentation treatment.

Whilst degradation within the control for soil B is shown, degradation is faster and reaches a lower concentration for all of the *n*-alkanes in both the treatments (Figures 5.12 – 5.14). A difference is observed between the degradation resulting from the two treatments (Figure 5.13 and 5.14). The soil receiving an inoculum shows a gradual reduction in *n*-alkane concentration whereas the concentration of the *n*-alkanes in the biostimulated treatment suddenly sees a reduction at 28 days followed by an increase and then further reductions. Both treatments for soil B reach comparable *n*-alkane concentrations by 112 days.

Overall the total degradation of the *n*-alkanes within soil A was shown to be 68, 86, 61 and 83 % in the control, biostimulated, ground biostimulated and biostimulated & bioaugmented treatments respectively (Table 5.6). In soil B an overall degradation of 66, 92 and 92 % was shown for the control, biostimulated and biostimulated & bioaugmented treatments respectively (Table 5.7). Whilst no difference is shown between the soils in the overall extent of degradation within the control, the biostimulated and biostimulated & bioaugmented treatments do show a significant difference ($P < 0.001$ and $=0.009$ for the biostimulated and biostimulated & bioaugmented treatments respectively). This difference between the soils demonstrates characteristic differences in the oil contamination, as the contamination within soil A is likely to be more highly bound to the soil matrix and contain a higher proportion of recalcitrant compounds, which would affect the overall extent of degradation as shown. Within both soils the degradation resulting from the biostimulated and biostimulated & bioaugmented treatments is

significantly different to the controls (Soil A $P < 0.001$ and $=0.005$, Soil B $P < 0.001$ and < 0.001 compared to the control for the biostimulated and biostimulated & bioaugmented treatments, respectively).

Whilst considerable degradation is shown within both soils, the extent of degradation starts to become less at carbon numbers over C_{27} and C_{34} within soils A and B, respectively, for the biostimulation and biostimulation & bioaugmentation treatments (Tables 5.6 and 5.7), again suggesting that a greater proportion of the heavier compounds within soil A contamination have become more highly bound to the soil matrix and/or more recalcitrant to degradation. An increase in surface area should allow for greater microbial attack, yet the ground soil A demonstrates that this has not occurred. Thus degradation here may be limited due to the recalcitrance of the compounds rather than their availability.

Table 5.6: Mean *n*-alkane concentrations and percentage of degradation of triplicate samples from soil A microcosms.

Compound		Initial Concentration	Final concentration (112 days)							
			Control		Biostimulated		Ground & biostimulated		Biostimulated & bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss
Decane	10	2.78	0.78	71.8	0.17	93.6	0.76	78.6	0.42	89.2
Undecane	11	4.41	0.66	85.1	0.15	94.6	0.85	80.4	0.41	89.2
Dodecane	12	4.15	3.76	9.44	0.75	80.2	5.30	0	2.41	41.8
Tridecane	13	11.2	1.54	86.3	0.74	93.2	2.20	80.7	1.22	90.0
Tetradecane	14	21.2	8.90	58.0	2.95	85.8	11.7	44.4	6.78	73.4
Pentadecane	15	34.0	9.95	70.8	3.70	88.9	11.6	64.4	6.76	83.7
Hexadecane	16	48.2	17.5	63.6	6.48	876	19.1	50.6	11.9	81.6
Heptadecane	17	92.7	13.7	85.2	3.19	96.3	43.1	47.2	8.13	91.4
Pristane	19	1570	376	76.0	7.94	99.5	528	66.7	189	89.3
Octadecane	18	94.4	48.1	49.0	8.28	89.0	20.4	72.2	9.11	91.0
Phytane	20	2060	379	81.6	3.58	99.8	578	71.4	213	90.0
Nonadecane	19	229	24.5	89.3	26.5	88.0	19.3	90.7	25.9	90.2
Eicosane	20	126	25.1	80.0	10.3	92.1	29.4	77.3	18.2	89.8
Docosane	22	95.5	7.48	92.2	7.33	70.2	5.81	68.3	6.52	93.4
Tricosane	23	35.6	5.41	84.8	2.78	93.6	7.20	66.3	4.64	91.8
Tetracosane	24	71.6	16.8	76.6	2.32	97.2	20.5	63.7	8.95	87.7
Pentacosane	25	301	108	64.2	1.52	99.6	112.4	50.5	49.4	90.8

(Continued on next page)

Table 5.6: Mean *n*-alkane concentrations and percentage of degradation of triplicate samples from soil A microcosms *(continued.)*

Compound		Initial concentration	Final concentration (112 days)							
			Control		Biostimulated		Biostimulated & ground		Biostimulated& Bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss
Hexacosane	26	138.8	48.4	65.1	3.70	98.7	72.6	3.02	29.2	85.4
Heptacosane	27	82.1	15.6	81.0	18.3	56.4	13.1	64.1	23.3	56.0
Octacosane	28	36.1	17.5	51.6	13.3	81.6	21.6	67.8	15.9	81.7
Nonacosane	29	77.5	27.7	64.3	31.1	82.8	21.2	73.4	21.5	58.2
Triacontane	30	189	105	44.3	141	43.2	70.1	49.6	133	73.8
Hentriacontane	31	160	85.0	47.0	69.0	64.6	47.2	51.6	92.7	81.6
Dotriacontane	32	117	58.8	49.8	111	5.13	49.4	62.8	107	29.6
Tritriacontane	33	938	445	52.5	208	78.1	432	38.0	312	71.6
Tetratriacontane	34	2920	896	69.3	506	83.1	1010	53.0	73	82.6
Pentatriacontane	35	1590	794	50.2	54.7	98.1	987	47.67	398	89.7
Hexatriacontane	36	368	84.2	77.1	106	73.2	155	52.2	92.6	81.5
Heptatriacontane	37	376	126	66.6	39.3	97.3	152	77.0	84.7	88.5
Octatriacontane	38	409	114	72.0	31.8	91.1	223	46.2	71.3	87.5
Nonatriacontane	39	740	236	68.1	326	38.7	168	74.2	328	69.6
Tetracontane	40	827	257	68.9	401	59.2	1645	75.4	415	58.6
Total		13800	4370	68.4	2150	86.1	5020	60.5	3430	83.0

Table 5.7: Mean *n*-alkane concentrations and percentage of degradation of triplicate samples from soil B microcosms.

Compound		Initial concentration	Final concentration (112 days)					
			Control		Biostimulated		Biostimulated & Bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	Mg kg^{-1}	% Loss	Mg kg^{-1}	% Loss
Decane	10	2.34	0.23	90.2	0.14	94.0	0.11	96.4
Undecane	11	3.61	0.39	89.2	0.12	96.8	0.09	97.6
Dodecane	12	16.9	4.01	76.2	1.64	91.9	1.30	88.3
Tridecane	13	22.5	5.22	76.8	0.81	94.9	0.89	94.2
Tetradecane	14	43.1	14.0	67.5	4.55	89.5	4.53	87.9
Pentadecane	15	62.5	19.0	69.6	3.13	95.0	4.21	92.8
Hexadecane	16	69.6	24.4	64.9	5.56	91.1	6.52	90.8
Heptadecane	17	99.6	71.2	28.5	15.0	84.9	19.6	75.3
Pristane	19	587	144	75.4	22.9	96.1	27.7	94.0
Octadecane	18	383	39.0	89.8	14.0	96.4	16.5	94.3
Phytane	20	675	151	77.7	42.2	93.6	42.8	92.5
Nonadecane	19	103	30.7	70.0	6.69	94.7	8.90	91.5
Eicosane	20	102	32.8	67.7	6.32	95.9	8.86	92.2
Heneicosane	21	108	33.8	68.8	7.29	95.5	9.69	91.7
Docosane	22	117	28.8	75.2	5.78	96.7	7.97	92.6
Tricosane	23	159	30.3	80.9	4.87	96.8	7.94	93.5
Tetracosane	24	159	36.9	76.8	6.62	95.9	9.51	92.9
Pentacosane	25	183	46.9	74.4	8.20	96.0	11.7	93.9

(Continued on next page)

Table 5.7: Mean *n*-alkane concentrations and percentage of degradation of triplicate samples from soil B microcosms *(continued).*

Compound		Initial concentration	Final concentration (112 days)					
			Control		Biostimulated		Biostimulated & Bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss
Hexacosane	26	182	44.2	75.7	7.19	96.0	11.6	92.9
Heptacosane	27	172	68.0	60.4	9.40	94.5	12.6	92.5
Octacosane	28	209	69.5	66.7	12.3	94.1	16.8	91.3
Nonacosane	29	184	71.0	61.4	6.70	96.4	17.8	87.8
Triacontane	30	153	82.8	45.7	12.2	92.3	15.4	92.4
Hentriacontane	31	259	78.1	69.8	12.6	95.1	13.6	94.3
Dotriacontane	32	182	72.7	60.2	11.4	93.7	21.8	92.1
Tritriacontane	33	317	92.4	70.8	16.6	94.8	11.7	96.7
Tetratriacontane	34	252	113	55.2	41.9	83.4	37.6	91.0
Pentatriacontane	35	258	98.5	61.8	29.3	88.6	16.1	95.6
Hexatriacontane	36	155	75.5	51.2	23.6	85.7	11.7	96.8
Heptatriacontane	37	226	58.9	74.0	18.9	91.7	15.7	93.3
Octatriacontane	38	169	28.2	83.3	10.7	93.7	11.5	95.0
Nonatriacontane	39	229	225	1.49	90.4	60.5	79.5	67.0
Tetracontane	40	229	172	24.9	48.3	78.9	45.1	68.6
Total		6070	2060	66.0	508	91.9	527	91.5

Aliphatic fractions

Grouping the *n*-akanes into the fractions suggested by the Environment Agency (2005) gives further insight into the effectiveness of the treatments used and the pattern of degradation of the oil contamination within the two soils (Figures 5.15 and 5.16).

A difference between the two soils is shown in the distribution of these fractions. Whilst both contain high proportions of the C_{16}-C_{35} and C_{35}-C_{40} fractions soil B has a larger proportion of the C_{12}-C_{16} fraction, as would be expected from a soil that has undergone less degradation prior to remediation thus having a higher proportion of this fraction still remaining. However this fraction remains comparatively unchanged within all soil B treatments for the duration of the experiment, where it would typically have been expected to decrease as it is preferentially degraded over the heavier and more recalcitrant compounds (Figure 5.16).

At the start of the experimental comparing soil A to soil B shows that the proportions of the C_8-C_{10}, C_{10}-C_{12}, C_{12}-C_{16} and the C_{35}-C_{40} fractions are significantly different from each other ($p < 0.05$). At the end of the experiment this has changed and the fractions whose proportions are significantly different between two soils are the C_8-C_{10}, C_{10}-C_{12}, C_{12}-C_{16} fractions. This further illustrates how different the contamination in these two soils are as the proportion of the C_8-C_{10}, C_{10}-C_{12}, C_{12}-C_{16} fractions are consistently significantly higher ($p < 0.05$) in soil B than soil A at both the start and end of the experiment. Comparing the C_{35}-C_{40} fractions shows a different pattern, here soil A has a significantly higher proportion of this

fraction at the start compared to soil B by the end of the experiment however whilst the proportion of this fraction has increased within both soils there is no longer any significant difference between the two soils for this fraction ($p > 0.05$).

At the end of the experimental period all of the fractions determined have seen a significant reduction within all of the treatments for soil B and the biostimulated and combined biostimulated and bioaugmented treatments for soil A ($p < 0.001$). Within soil A the only treatments not showing a significant difference for all the fractions between the start and end of the experimental period is the control and the biostimulated and ground treatment, as here there is no significant difference in the C_{10}-C_{12} fraction between the start and end ($p > 0.05$).

Comparing treatments and soils, the results show that the biostimulated and the combined biostimulated and bioaugmented treatments have had the greatest effect on changing the distribution of these fractions (Figures 5.15 and 5.16). Both of these treatments have significantly reduced the concentration all of the fractions for soil B compared to the control ($p < 0.001$). However, within soil A a different pattern is observed, here no difference is seen between any treatments in the concentration of the C_{35}-C_{40} fraction, whilst for the remainder of the fractions it is only the biostimulated treatment that has significantly reduced the fractions to the control by the end of the experiment. Whilst the ground and biostimulated treatment has significantly reduced most of the fractions by the end of the experiment this treatment appears have had no effect on the distribution of

the aliphatic fractions, as no significant change is shown in the proportions of these fractions between the first sampling point (0 days) and the last (112 days) (Figure 5.15) ($p > 0.05$), the concentration of these fractions are also not significantly different to the control at the end of the experiment ($p > 0.05$).

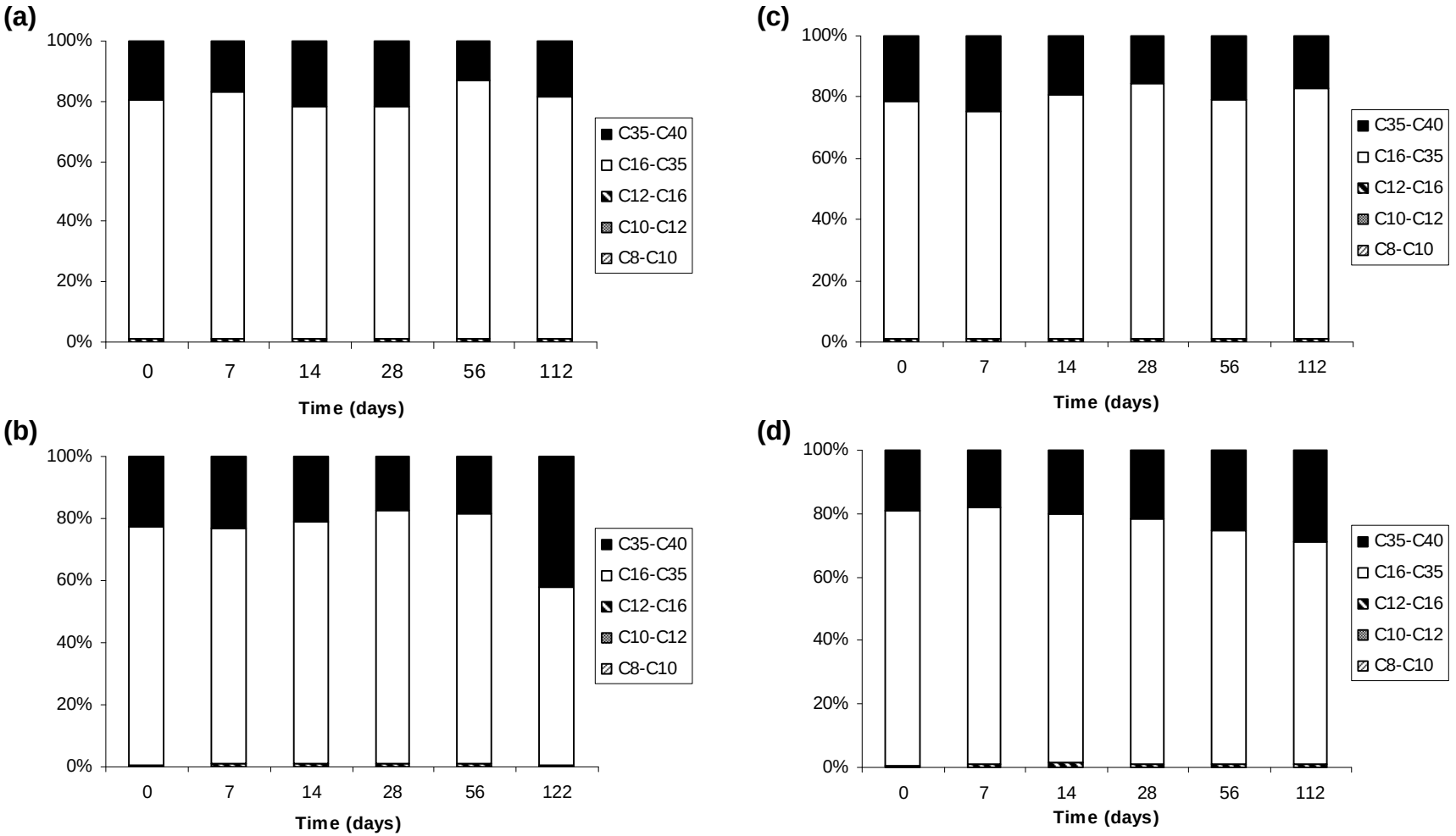


Figure 5.15: Change in mean aliphatic fraction proportions for soil A (a) control, (b) biostimulation, (c) ground biostimulation and (d) biostimulation and bioaugmentation treatments.

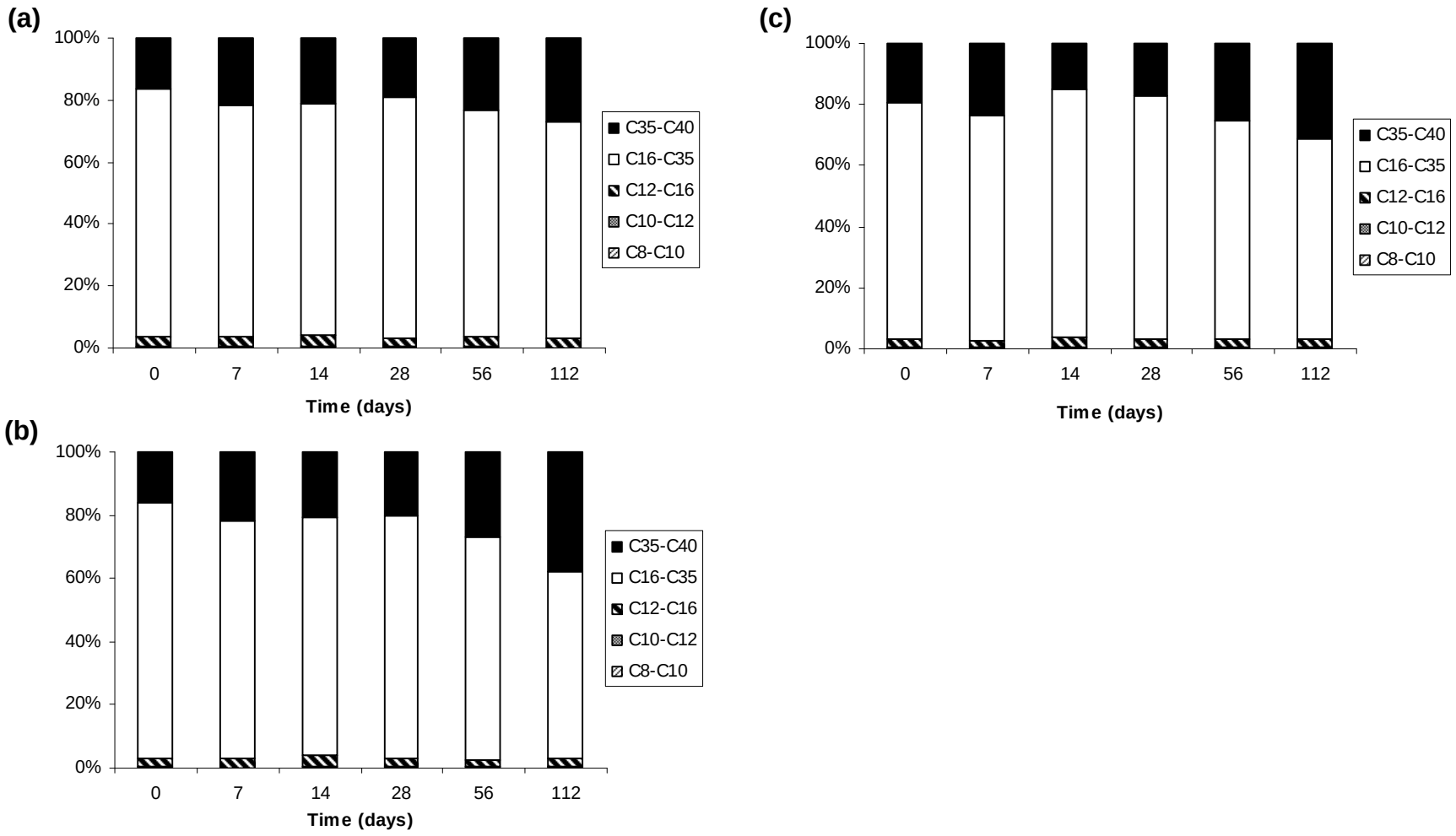


Figure 5.16: Change in mean aliphatic fraction proportions for soil B (a) control, (b) biostimulation and (c) biostimulation and bioaugmentation treatments.

Aromatic hydrocarbons

Whilst both aliphatic and aromatic compounds are important in assessing the risk at petroleum hydrocarbon contaminated sites, aromatic compounds (and thus PAHs) are typically more hazardous to human health and as such have lower threshold concentrations (Environment Agency, 2005). Therefore it was important to include these in the analysis of the soil microcosms.

The PAH compounds that were selected for analysis are listed in Table 5.5, of which not all have been found within the soils (as indicated in Table 5.5). Here it should be noted that alkyl homologs of compounds are grouped together by the number of carbon groups attached to the compound, as such C_1-Naphthane refers to a group of alkyl naphthalene compounds that have one carbon group (i.e. one methyl group) attached denoted by C_1. Hence, C_2-Naphtalene has two carbon groups attached.

The degradation pattern of these PAHs is shown in figures 5.17-5.20 and figures 5.21-5.28 for each of the treatments for soils A and B respectively. Comparison of the PAHs present within the two soils shows a clear difference, soil A contained 22 of the selected PAHs whereas soil B contained 34, showing soil B to have a much wider distribution of PAHs (Figures 5.17, 5.21 and 5.22). Overall a gradual degradation of the PAHs detected is shown within both soils.

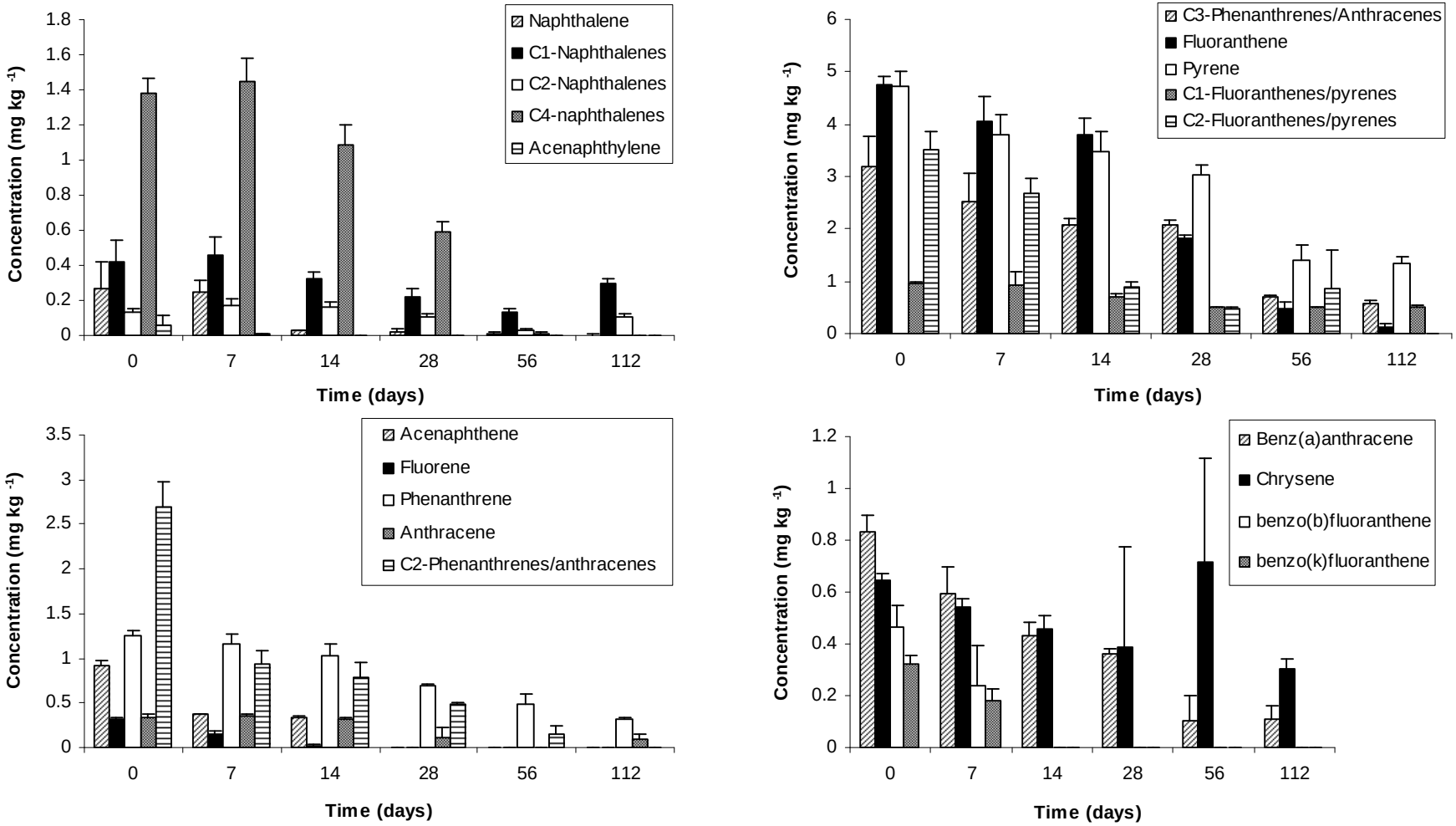


Figure 5.17: Change in mean (+SE) concentration of selected PAHs within soil A control.

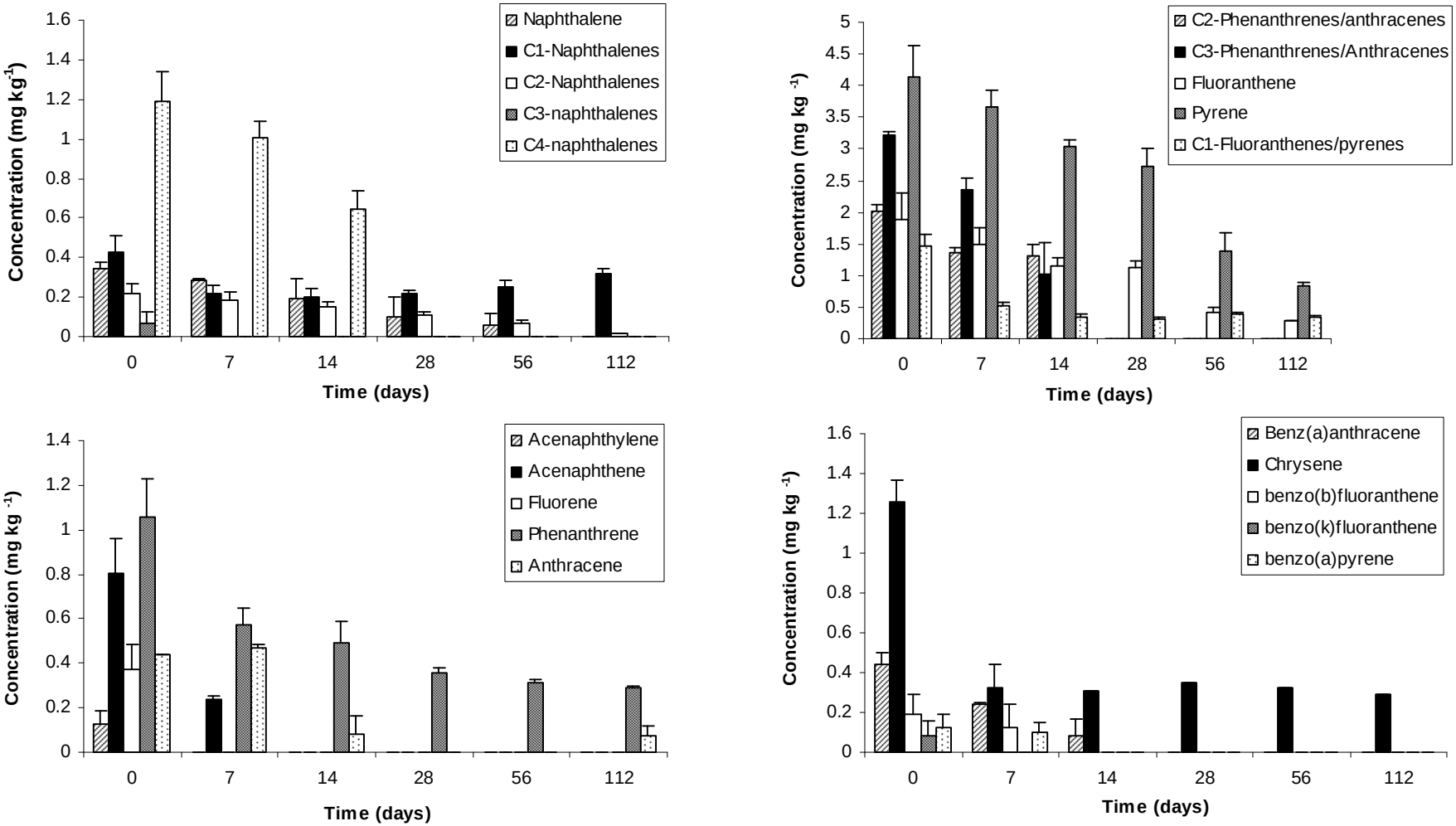


Figure 5.18: Change in mean (+SE) concentration of selected PAHs within soil A biostimulation treatment

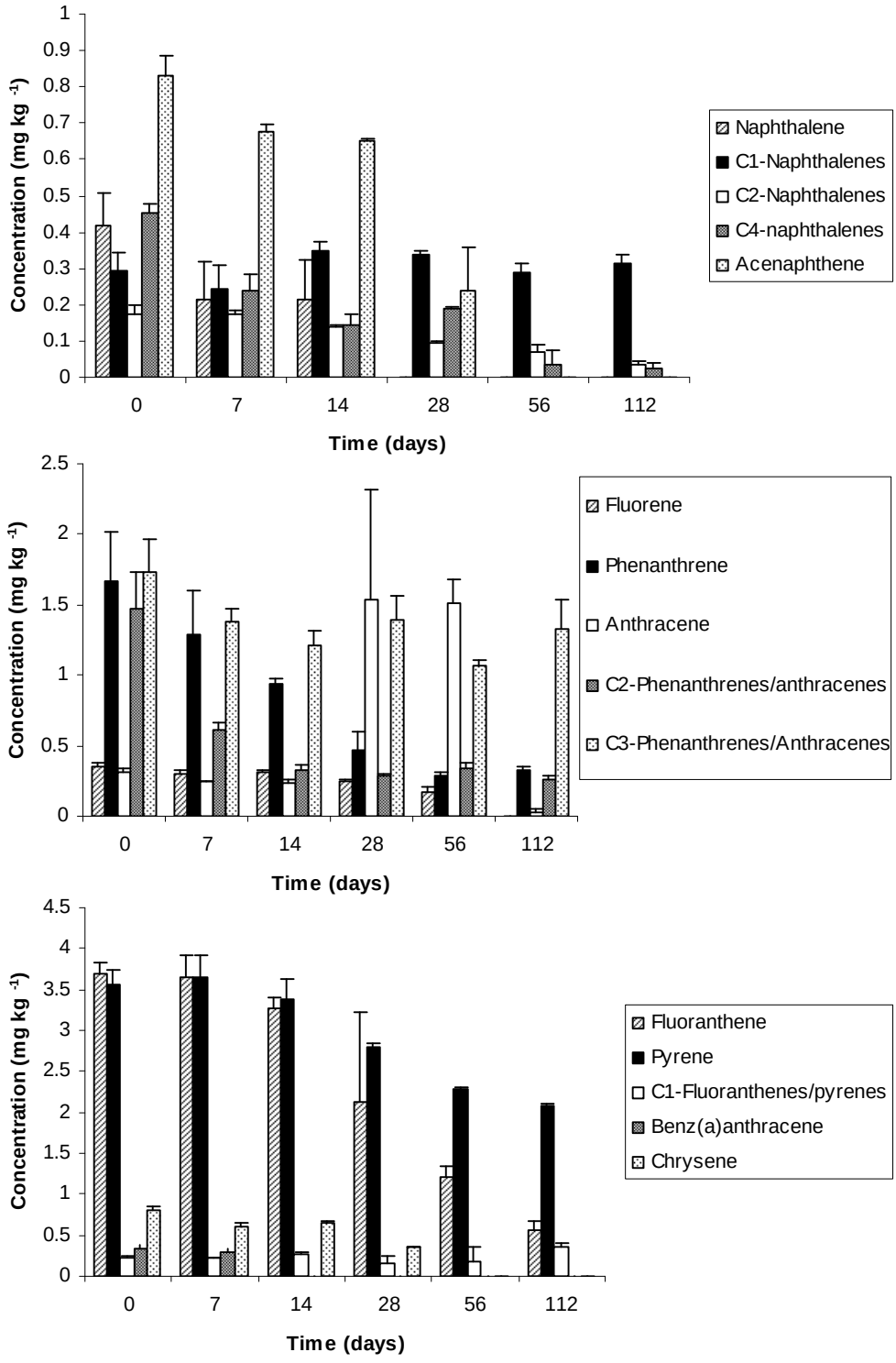


Figure 5.19: Change in mean (+SE) concentration of selected PAHs within soil A ground biostimulated treatment.

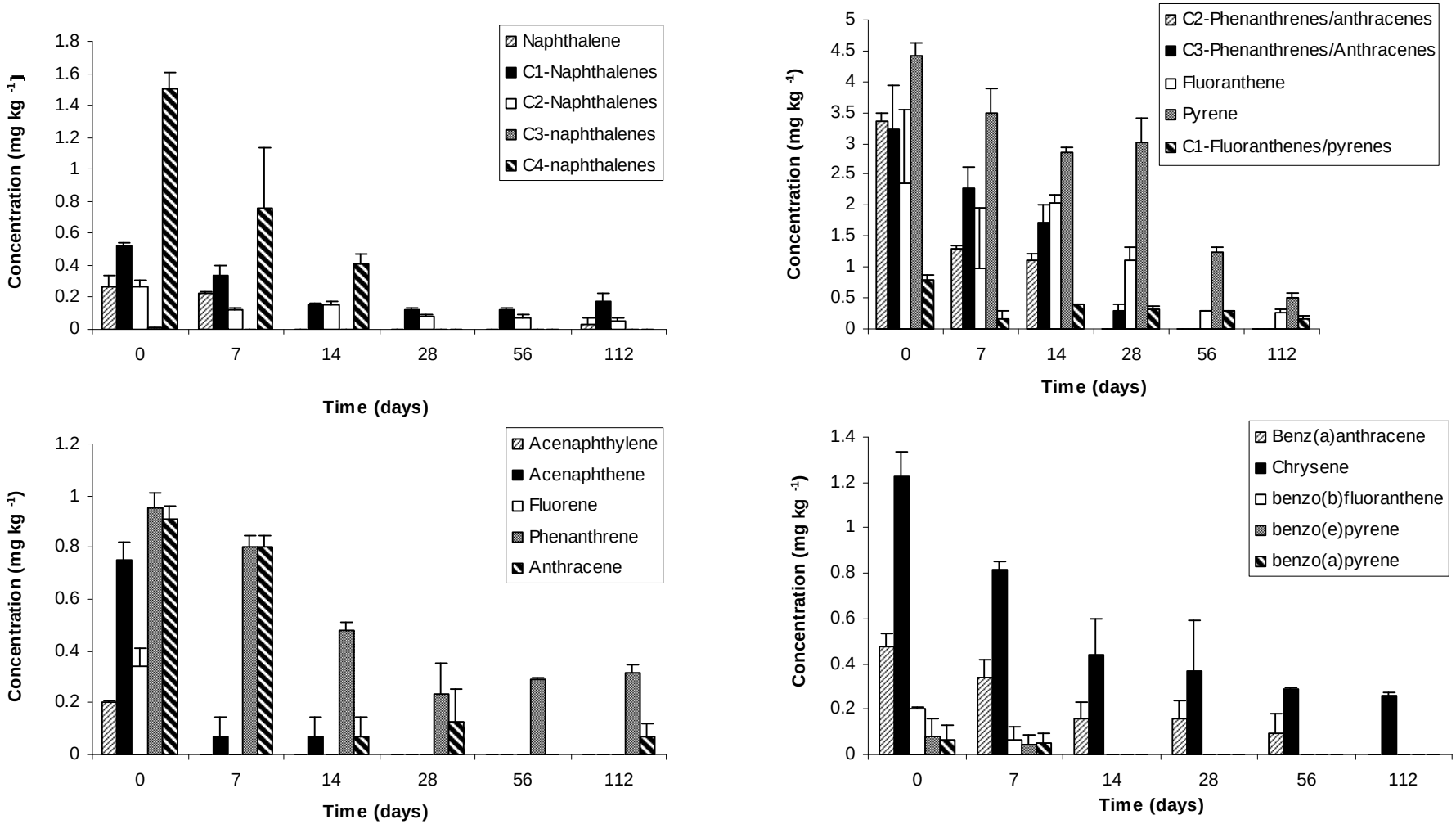


Figure 5.20: Change in mean (+SE) concentration of selected PAHs in soil A biostimulation and bioaugmentation treatment.

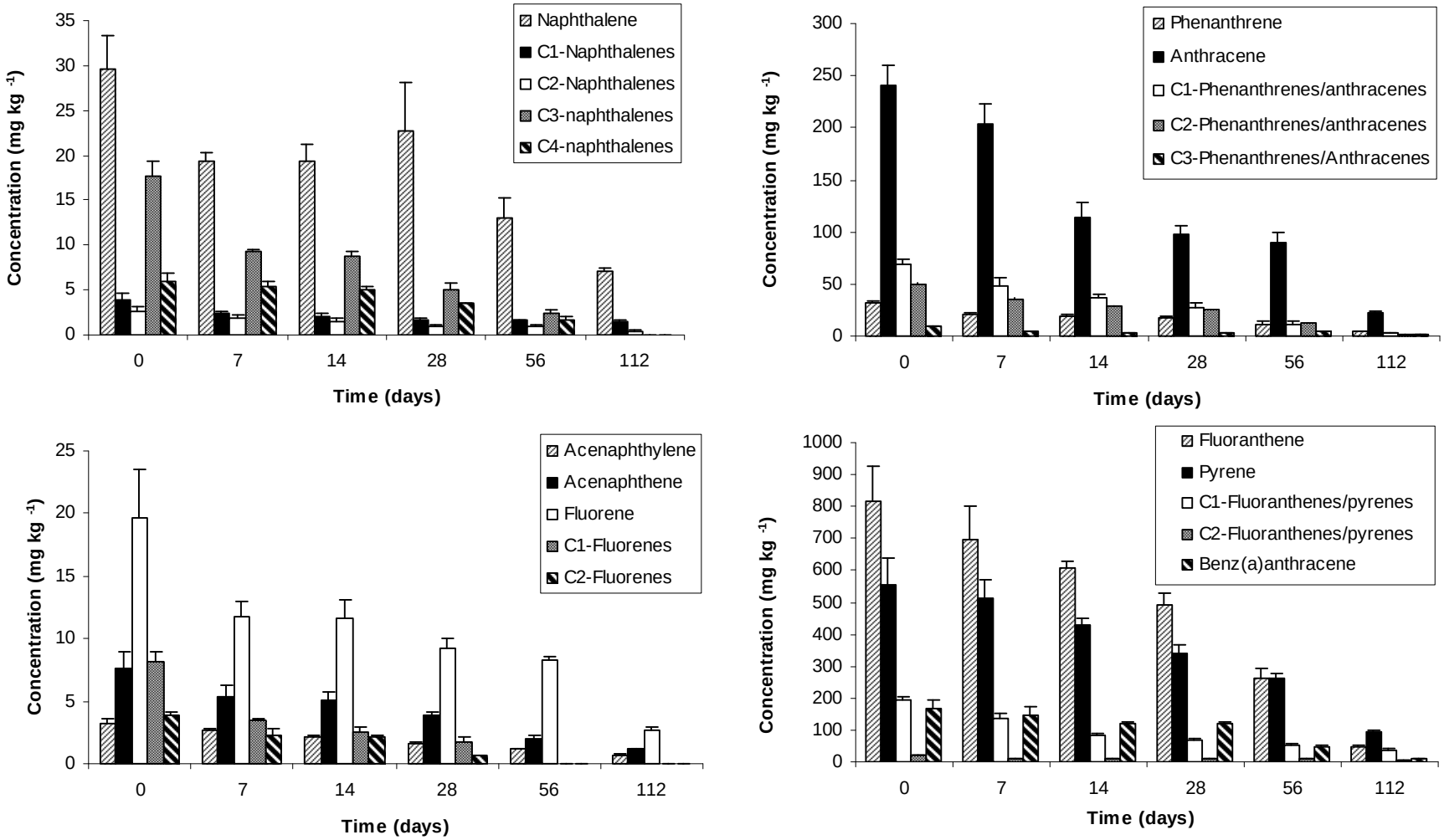


Figure 5.21: Change in mean (+SE) concentration of selected PAHs in soil B control.

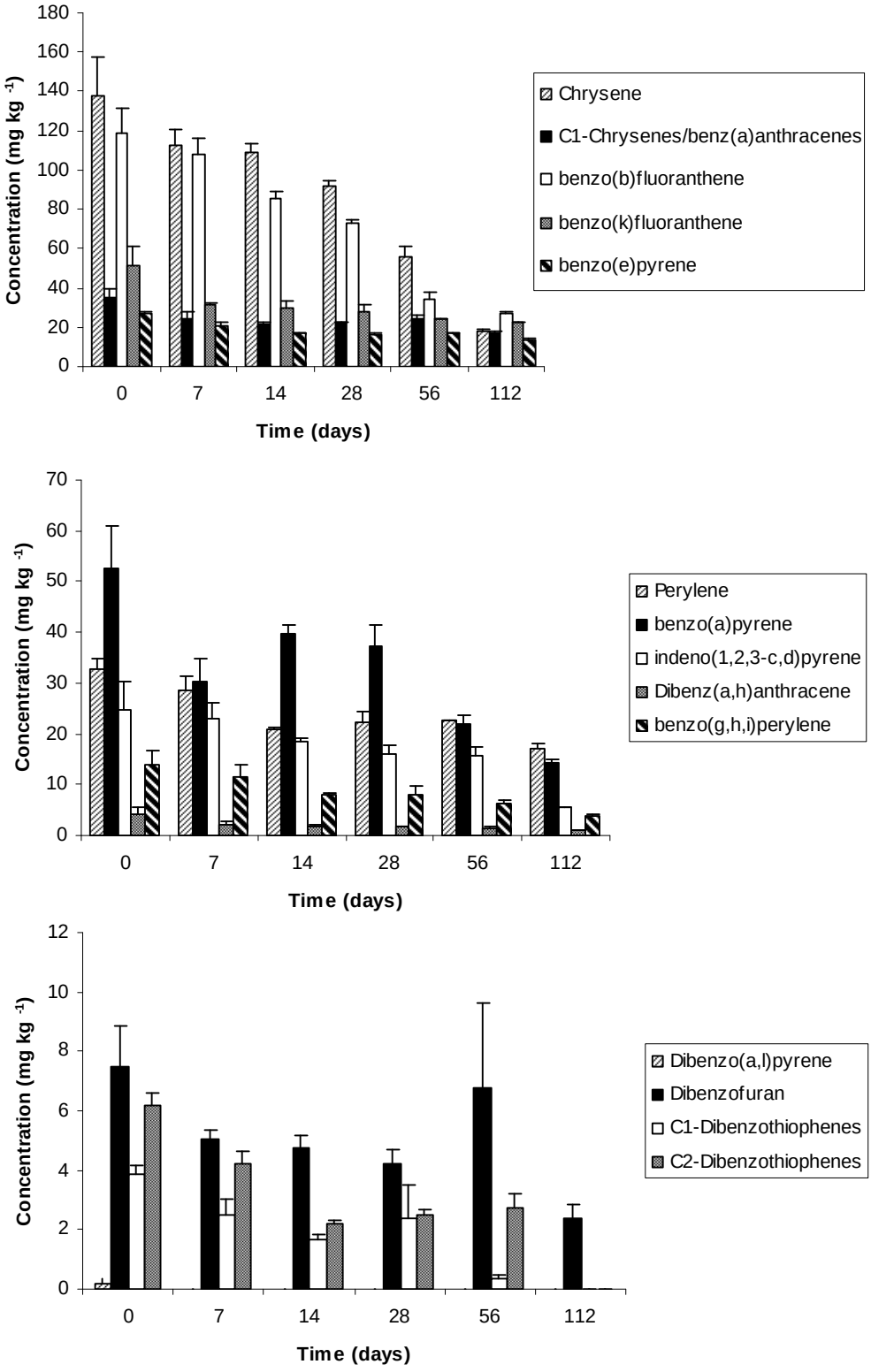


Figure 5.22: Change in mean (+SE) concentration of selected PAHs within soil B control.

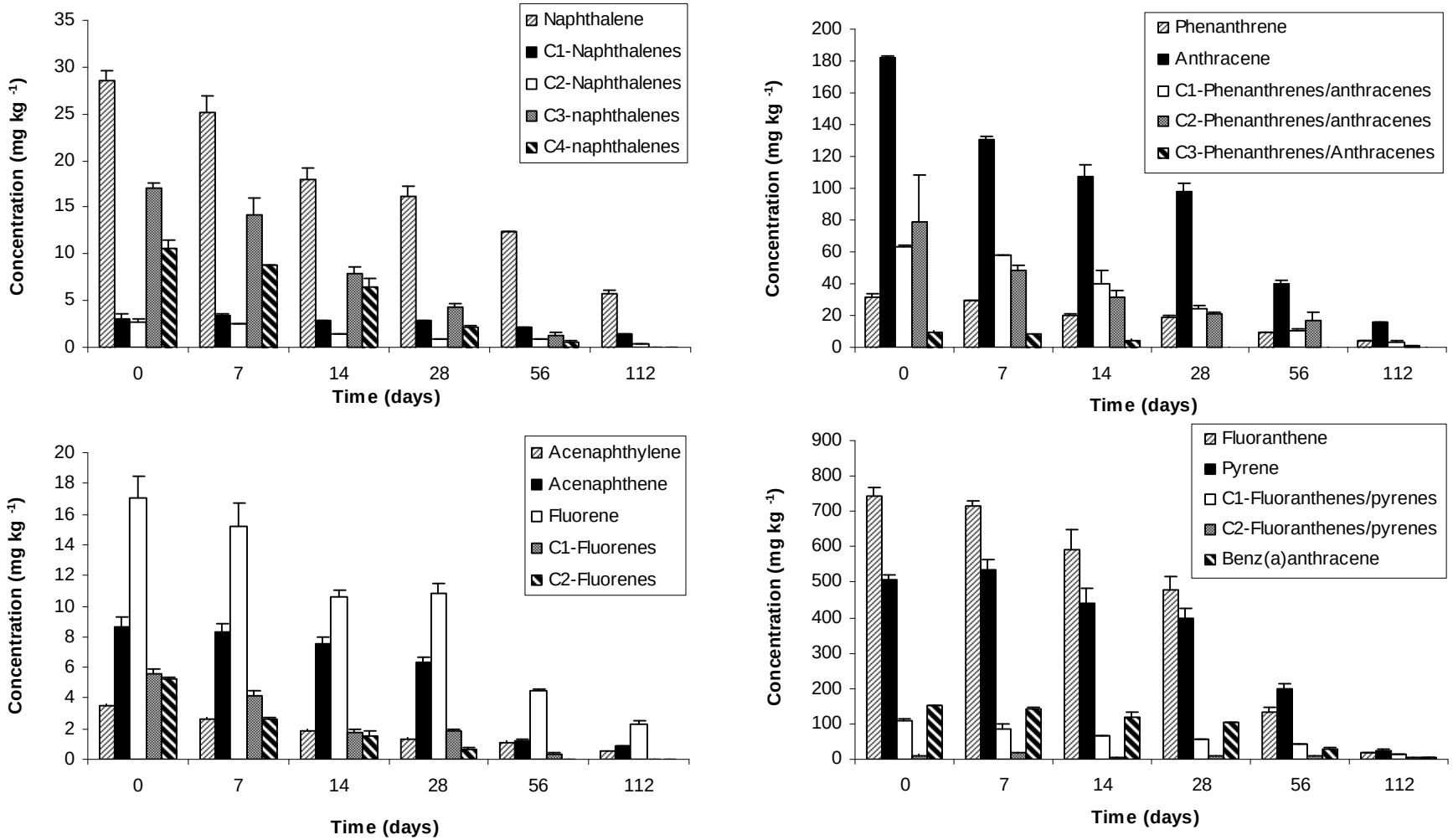


Figure 5.23: Change in mean (+SE) concentration of selected PAHs in soil B biostimulation treatment.

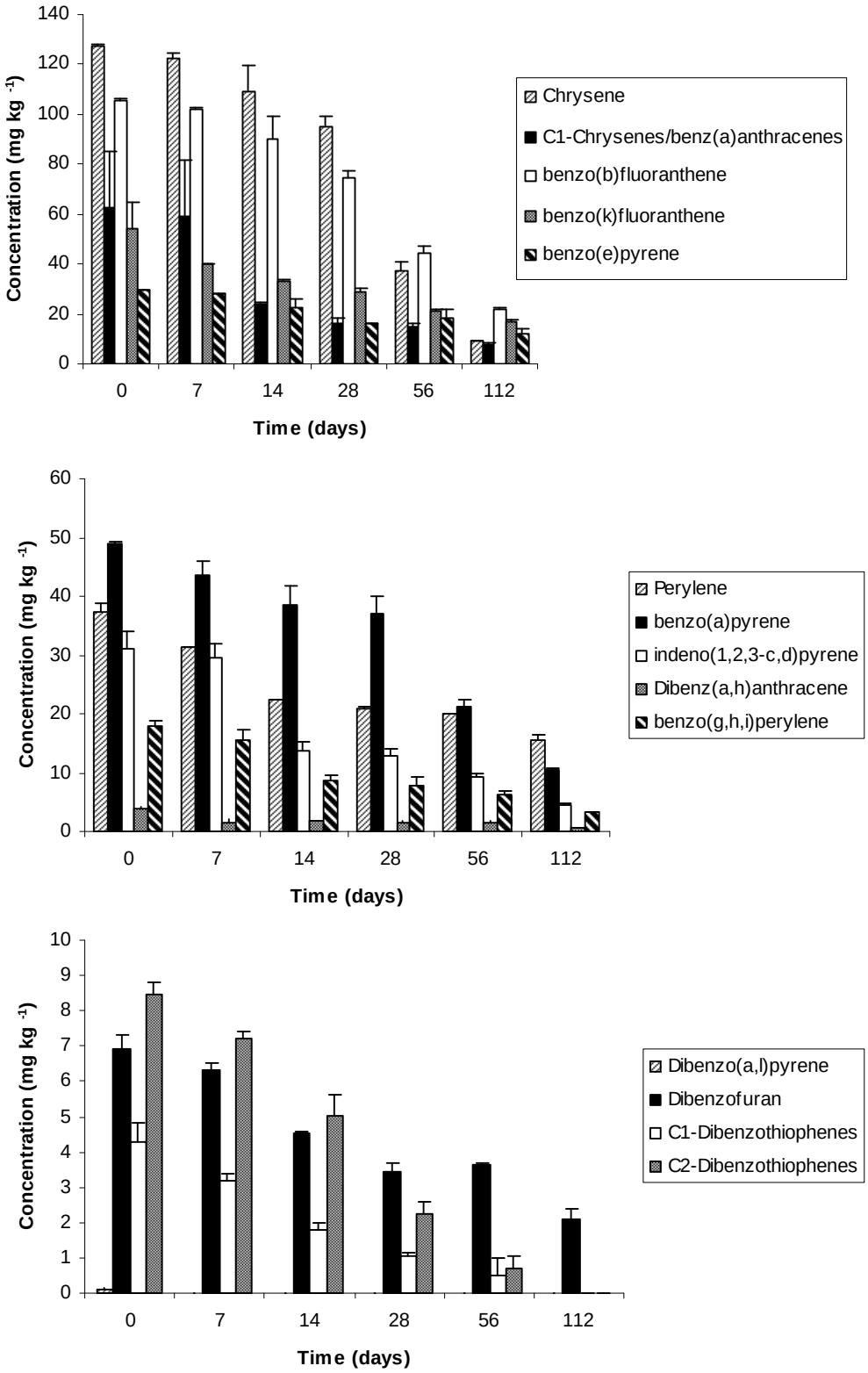


Figure 5.24: Change in mean (+SE) concentration of selected PAHs within soil B biostimulation treatment.

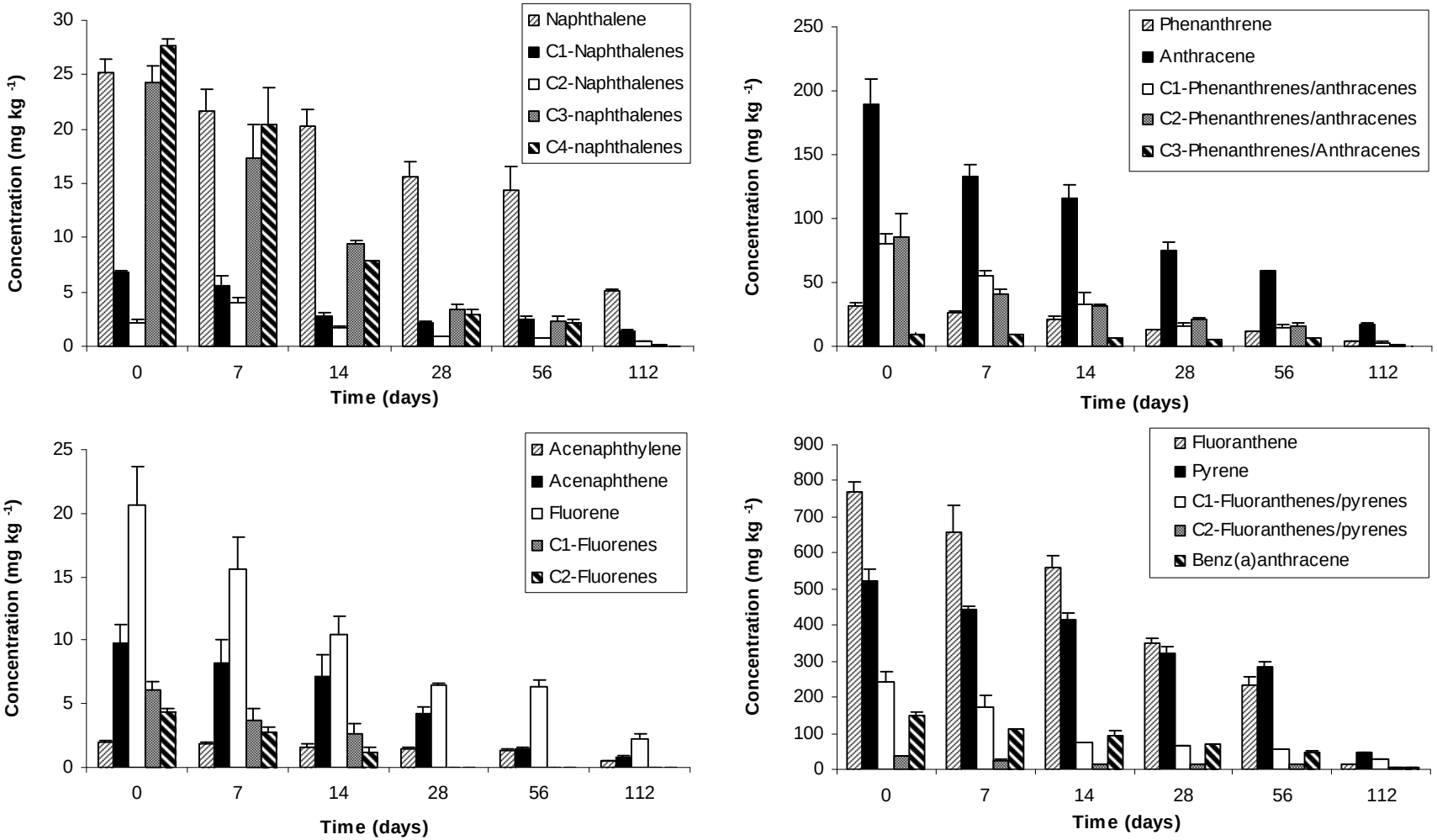


Figure 5.25: Change in mean (+SE) concentration of selected PAHs in soil B biostimulation and bioaugmentation treatment.

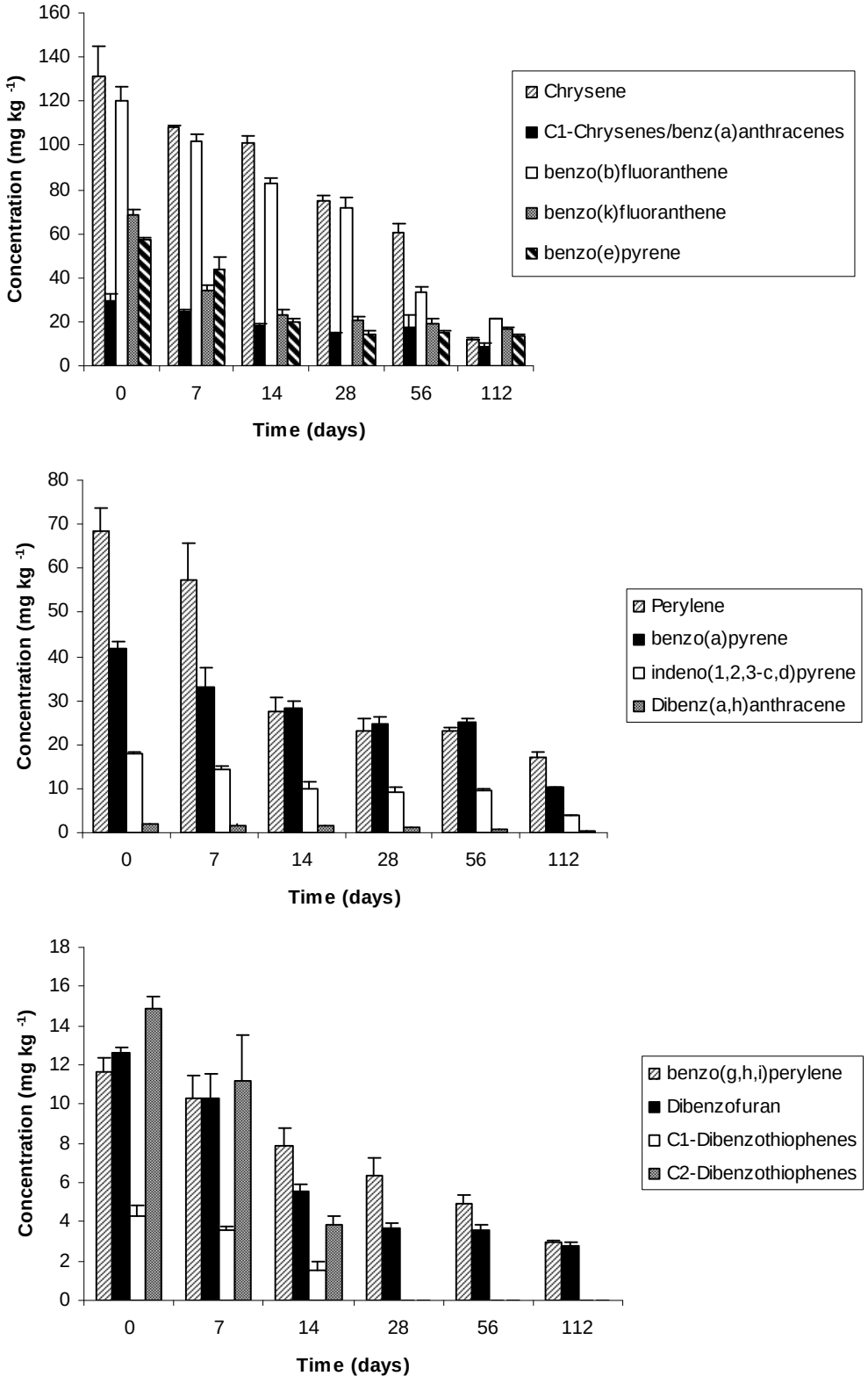


Figure 5.26: Change in mean (+SE) concentration of selected PAHs within soil B biostimulation and bioaugmentation treatment.

There is a clear difference between the degradation within the control and the biostimulated and biostimulated & bioaugmented treatments for soil A. Both the biostimulated treatments show a far more rapid reduction in the PAHs compared to the control, with the C_1-Naphthalenes, C_2-Naphthalenes, phenanthrene, anthracene, fluoranthene, pyrene, and C_1-fluoranthenes/pyrenes remaining throughout the microcosms at the end (Figures 5.17-5.20). The ground treatment shows less degradation of the PAHs than that seen in the control, again suggesting that grinding may have inhibited degradation within soil A (Table 5.8).

A gradual reduction in all the PAHs concentration within all of the soil B microcosms is shown, with all treatments showing a similar pattern of degradation. Noticeable differences in degradation patterns shown, were an initially slower rate of reduction of the C_3-naphthalens and C_4-naphthalenes within the biostimulated & bioaugmented treatment (Figures 5.21-5.28).

Overall the total degradation of the PAHs selected for analysis within soil A was shown to be 86, 88, 68 and 92 % in the control, biostimulated, ground biostimulated and biostimulated & bioaugmented treatments respectively (Table 5.8). In soil B an overall degradation of 86, 92 and 91 % was shown for the control, biostimulated and biostimulated & bioaugmented treatments respectively (Table 5.9). As was shown by the initial analysis of the soils, soil B has far more PAHs than soil A with a much greater variety of PAH present and in higher concentrations (Tables 5.8 & 5.9).

Comparing treatments between the two soils shows a similar overall extent of degradation. Only the biostimulated treatments show a significant

difference between the two soils ($p < 0.001$), as significantly more degradation of these PAHs was observed in the biostimulation treatment for soil B rather than soil A (Tables 5.8 and 5.9). The combined biostimulated and bioaugmented treatment for soil A shows the greatest overall degradation of the selected PAHs and was significantly different to all the other treatments ($P=0.007$, 0.009 and < 0.001 compared with the control, biostimulated and the ground and biostimulated treatments respectively). Grinding had no positive effect on PAH degradation in soil A, indeed a negative effect was observed as the overall degradation achieved was significantly less than that observed within the control for soil A ($p < 0.001$). Although the degradation of the PAHs in the biostimulated treatment was shown to be quite high, it was not significantly different to the control, thus showing no beneficial effect of the treatment. This result was unexpected and it is unclear why it this was seen and requires further investigation beyond the scope of this study. However, as grinding increases surface area this will have also increased the availability (and thus increased the concentration available) of toxic compounds to microorganisms which may have had an adverse effect. Results for soil A show that the addition of an inoculum was required to enhance degradation of the selected PAHs over that which may occur naturally. A difference between soils is shown when observing the effects of treatment upon total PAH reductions. Unlike soil A, in soil B, the biostimulation did have a significant effect in degrading the PAHs as both the biostimulation and biostimulation & bioaugmentation treatments showed significantly more total reductions in the PAH compared

to the control (P-values for both compared to the control were < 0.001). No difference was shown between the treatments suggesting that, unlike soil A, an inoculum is not required to aid degradation of the selected PAHs.

Within soil A the PAHs remaining and thus showing the most resistance to degradation were: C_1-Naphthalenes, C_2-Naphthalenes, phenanthrene, anthracene, fluoranthene, pyrene, and C_1-fluoranthenes/pyrenes (Table 5.8). Of these remaining C_1-naphthalenes reduced the least.

Soil B contained far more PAHs at the start of the investigation and as such more remain at the end however the compounds showing the least degradation were: naphthalene, C_1-naphthalene, acenaphthylene, C_1-chrysenes/benz(*a*)anthracenes, Benzo(*k*)fluoranthene, benzo(*e*)pyrene, perylene, indeno(*1,2,3-cd*)pyrene, dibenz(*a,h*)anthracene, benzo(*g,h,i*)perylene and dibenzofuran (Table 5.9). Showing that different PAHs within the two soils have been preferentially degraded, with the only common PAHs remaining being the C_1-naphthalene. During biodegradation lighter compounds are typically degraded faster resulting in a comparative increase in the higher molecular weight hydrocarbons. However, the PAHs determined here included alkyl homologs (i.e. C_1-napthalene, C_2-Napthalene etc.), with carbon groups that are typically easy to degrade and as such, degradation of these carbon groups could result in the relative enrichment of alkyl homologs with less carbon groups attached (i.e C2-Napthalene degrading to C1-napthalene). This could account for the pattern seen in Tables 5.8 and 5.9, where as the number of carbon groups attached increases so does the percent degraded.

Table 5.8: Mean PAH concentrations and percentage of degradation of triplicate samples from soil A microcosms.

Compound		Initial concentration	Final concentration (112 days)							
			Control		Biostimulation		Ground & Biostimulated		Biostimulated & Bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss
Naphthalene	10	0.27	0.00	100	0.00	100	0.00	100	0.03	87.3
C_1-Naphthalenes	11	0.42	0.30	29.4	0.32	25.7	0.32	25.7	0.17	66.8
C_2-Naphthalenes	12	0.14	0.11	22.2	0.02	92.1	0.03	80.5	0.05	82.3
C_3-Naphthalenes	13	0.06	0.00	100	0.00	100	0.00	100	0.00	100
C_4-Naphthalenes	14	1.38	0.00	100	0.00	100	0.03	94.4	0.00	100
Acenaphthylene	12	0.06	0.00	100	0.00	100	0.00	100	0.00	100
Acenaphthene	12	0.92	0.00	100	0.00	100	0.00	100	0.00	100
Fluorene	13	0.33	0.00	100	0.00	100	0.00	100	0.00	100
Phenanthrene	14	1.25	0.32	74.0	0.29	72.6	0.32	80.8	0.32	66.7
Anthracene	14	0.34	0.10	70.6	0.07	83.3	0.03	91.2	0.07	92.2
C_2-Phenanthrenes/anthracenes	16	2.69	0.00	100	0.00	100	0.26	82.7	0.00	100
C_3-Phenanthrenes/anthracenes	17	3.20	0.57	82.3	0.00	100	1.33	23.1	0.00	100

(Continued on next page)

Table 5.8: Mean PAH concentrations and percentage of degradation of triplicate samples from soil A microcosms *(continued).*

Compound		Initial concentration	Final concentration (112 days)							
			Control		Biostimulation		Ground & Biostimulated		Biostimulated & Bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss
Fluoranthene	16	4.76	0.14	97.0	0.29	84.6	0.57	84.7	0.26	88.8
Pyrene	16	4.73	1.34	71.7	0.84	79.7	2.08	41.7	0.50	88.7
C_1-Fluoranthenes/pyrenes	17	0.97	0.52	46.8	0.35	75.9	0.35	100	0.16	79.9
C_2-Fluoranthenes/pyrenes	18	3.52	0.00	100	0.00	100	0.00	100	0.00	100
Benz(*a*)anthracene	18	0.83	0.11	86.9	0.00	100	0.00	100	0.00	100
Chrysene	18	0.64	0.30	53.1	0.29	77.0	0.00	100	0.26	79.0
Benzo(*b*)fluoranthene	20	0.47	0.00	100	0.00	100	0.00	100	0.00	100
Benzo(*k*)fluoranthene	20	0.32	0.00	100	0.00	100	0.00	100	0.00	100
Benzo(*e*)pyrene	20	0.08	0.00	100	0.00	100	0.00	100	0.00	100
Benzo(*a*)pyrene	20	0.13	0.00	100	0.00	100	0.00	100	0.00	100
Total		27.51	3.81	86.0	2.48	87.5	5.31	67.5	1.82	91.7

Table 5.9: Mean PAH concentrations and percentage of degradation of triplicate samples from soil B microcosms.

Compound		Initial concentration	Final concentration (112 days)					
			Control		Biostimulated		Biostimulated & Bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss
Naphthalene	10	29.6	7.02	76.3	5.66	80.1	5.07	79.9
C_1-Naphthalenes	11	3.98	1.49	62.5	1.42	54.4	1.43	78.7
C_2-Naphthalenes	12	2.70	0.46	82.8	0.38	86.0	0.41	81.3
C_3-Naphthalenes	13	17.6	0.01	100	0.05	99.7	0.08	99.7
C_4-Naphthalenes	14	5.90	0.02	99.7	0.00	100	0.03	99.9
Acenaphthylene	12	3.15	0.71	77.4	0.57	84.0	0.50	75.4
Acenaphthene	12	7.58	1.16	84.7	0.88	89.9	0.86	91.2
Fluorene	13	19.7	2.66	86.5	2.29	86.6	2.28	88.0
C_1-Fluorenes	14	8.15	0.00	100	0.02	99.7	0.00	100
C_2-Fluorenes	15	3.94	0.00	100	0.00	100	0.00	100
Phenanthrene	14	31.8	4.90	84.6	4.09	87.0	4.33	86.3
Anthracene	14	240	22.4	90.7	15.4	91.6	17.8	90.6
C_1-Phenanthrenes/anthracenes	15	68.3	2.78	95.9	3.58	94.2	2.96	96.3
C_2-Phenanthrenes/anthracenes	16	50.1	2.13	95.7	1.08	98.6	0.95	98.9
C_3-Phenanthrenes/anthracenes	17	9.24	1.16	87.4	0.00	100	0.41	95.5
Fluoranthene	16	815	45.8	94.4	19.0	97.5	14.6	98.1
Pyrene	16	556	96.2	82.7	24.8	95.1	44.5	91.5

(Continued on next page)

Table 5.9: Mean PAH concentrations and percentage of degradation of triplicate samples from soil B microcosms *(continued).*

Compound		Initial concentration	Final concentration (112 days)					
			Control		Biostimulated		Biostimulated & Bioaugmented	
		mg kg^{-1}	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss	mg kg^{-1}	% Loss
C_1-Fluoranthenes/pyrenes	17	12	38.1	80.1	15.3	86.1	26.7	889.0
C_2-Fluoranthenes/pyrenes	18	18.4	6.42	65.1	3.99	64.2	5.84	84.2
Benz(*a*)anthracene	18	166	9.10	94.5	5.78	96.2	4.92	96.7
Chrysene	18	137	17.6	87.2	8.82	93.0	12.0	90.9
C_1- Chrysenes/benz(*a*)anthracenes	19	35.3	16.9	52.2	7.92	87.4	8.70	70.5
Benzo(*b*)fluoranthene	20	118	26.9	77.3	21.8	79.3	21.4	82.2
Benzo(*k*)fluoranthene	20	51.1	22.2	56.6	16.7	69.4	17.0	75.1
Benzo(*e*)pyrene	20	27.0	13.7	49.4	12.0	59.2	13.7	76.1
Perylene	20	32.8	16.9	48.4	15.7	57.9	17.2	74.9
Benzo(*a*)pyrene	20	52.7	14.3	72.9	10.4	78.8	10.3	75.3
Indeno(*1,2,3-cd*)pyrene	22	24.7	5.42	78.0	4.57	85.2	4.04	77.7
Dibenz(*a,h*)anthracene	24	4.27	0.91	78.7	0.60	84.5	0.53	72.9
Benzo(*g,h,i*)perylene	22	13.9	3.96	71.5	3.28	81.7	2.96	74.7
Dibenzo(*a,l*)pyrene	24	0.19	0.00	100	0.00	100	0.00	100
Dibenzofuran	12	7.47	2.38	68.2	2.11	69.6	2.75	78.2
C_1-Dibenzothiophenes	13	3.87	0.00	100	0.00	100	0.00	100
C_2-Dibenzothiophenes	14	6.20	0.00	100	0.00	100	0.00	100
Total		2760	384	86.1	208	91.8	244	91.4

Aromatic fractions

The distribution of the Environment Agency's suggested aromatic fractions, for all the treatments in both soils is shown in figures 5.27 and 5.28. There is a clear difference between the fractions within the two soils as soil B contains higher proportions of the C_{12}-C_{16} and C_{16}-C_{21} fractions, and soil A contains a higher proportion of the C_{10}-C_{12} fraction.

A clear difference is shown between the ground and biostimulated treatment and all other treatments for soil A. At the start and throughout the experiment this treatment has a consistently higher proportion of the C_{12}-C_{16} fraction, which may be a result of the grinding process as it is evident from the start. The distribution of the fractions within soil A varies greatly over the experimental period with no real clear trend, however at the end the C_8-C_{10} fraction in all but the biostimulated & bioaugmented treatment has been greatly reduced. It should be noted that the initial concentrations of these compounds were significantly lower than shown in soil B (Tables 5.3 and 5.8), and will have already undergone significant degradation prior to this experiment. Thus, the compounds remaining are more likely to be highly bound to the soil and more recalcitrant in nature, which could account for the varied pattern of degradation.

Within soil B a clear trend in the change in fraction distribution is shown in all treatments. Here the C_{12}-C_{16} fraction is reduced and the C_{21}-C_{35}, C_{10}-C_{12} and C_8-C_{10} fractions are increased within both the treatments, and to a lesser extent within the control as well. Potentially indicating some PAHs

that may be in part resistant to degradation present within the later two fractions (C_{10}-C_{12} and C_8-C_{10}).

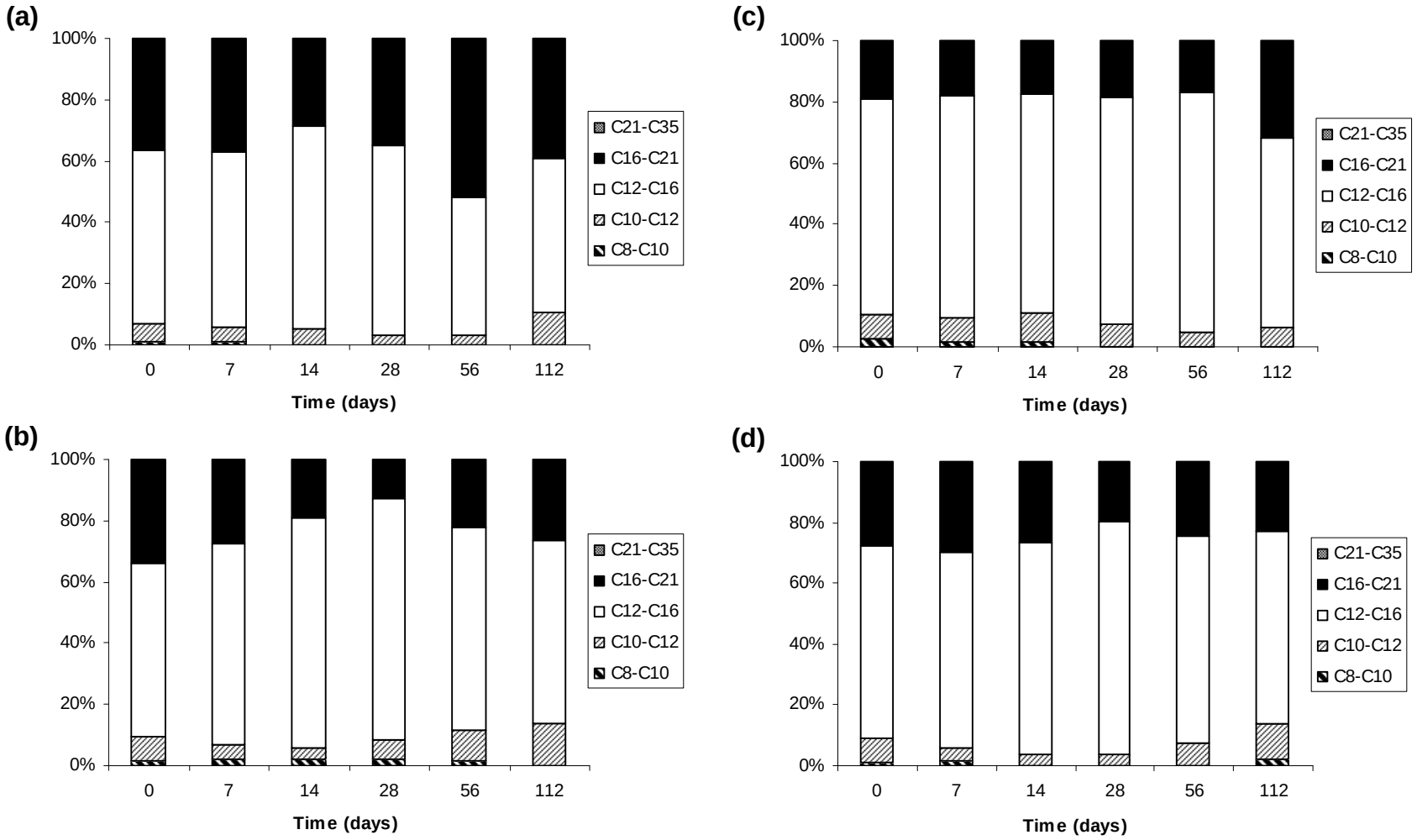


Figure 5.27: Change in mean aromatic fraction proportions for soil A (a) control, (b) biostimulation, (c) ground biostimulation and (d) biostimulation and bioaugmentation treatments.

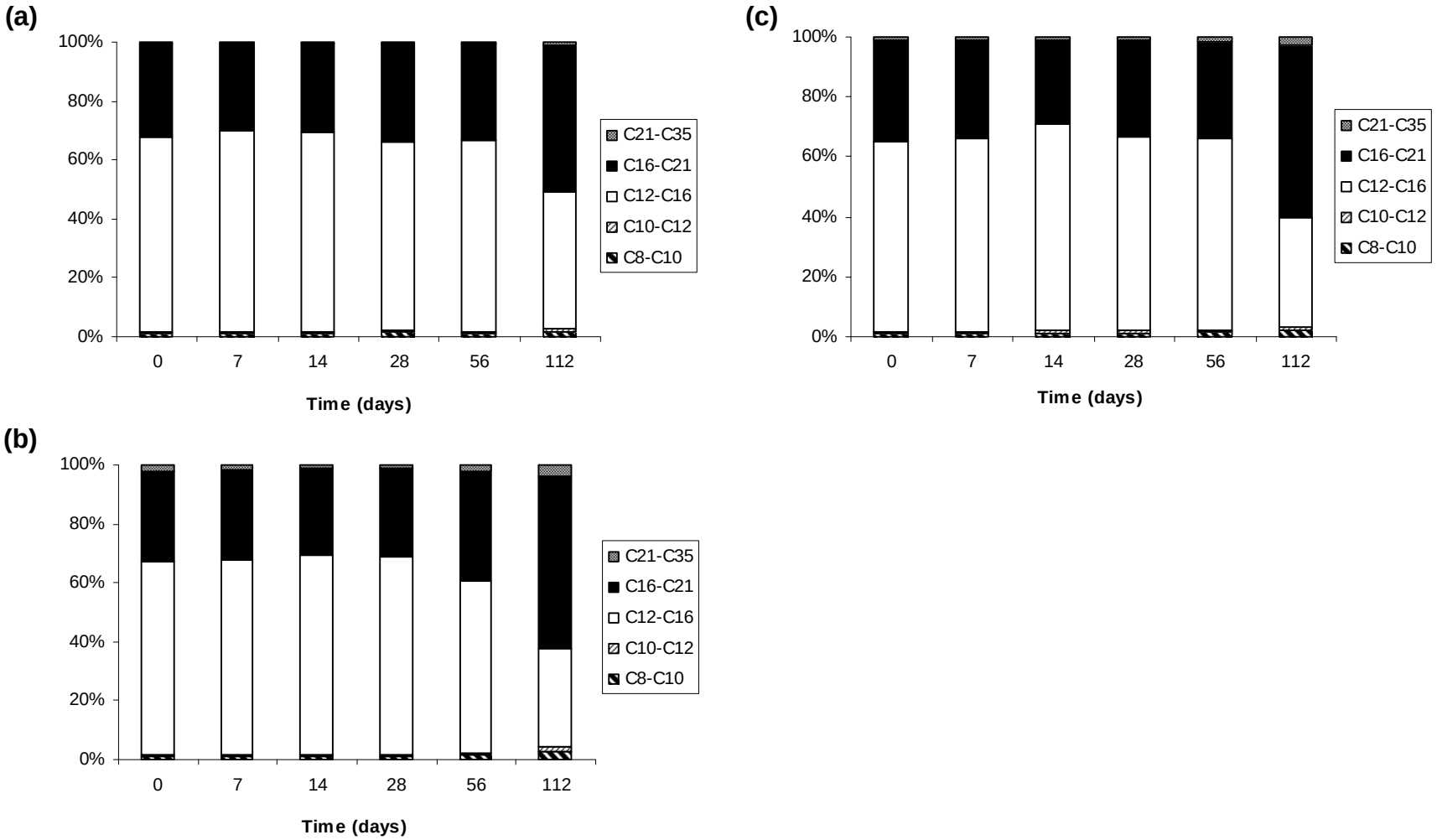


Figure 5.28: Change in mean aromatic fraction proportions for soil B (a) control, (b) biostimulation and (c) biostimulation and bioaugmentation treatments.

5.3.4 Ecotoxicological response

Seed germination

The results for the seed germination experiments were normalised to a clean uncontaminated soil, to take into account the germination rate of the seeds used. Visual observations showed that within the uncontaminated soil seeds germinated quickly with 90% germinating over the experimental period. Whilst seed germination was observed within both of the contaminated soils and each of the treatments used, it should be noted that visual observations over the experimental period showed that the rate of germination and subsequent seedling growth was visually reduced, compared to the un-contaminated soil. Thus even though the control treatment for soil B achieved 100% germination at the end of the microcosms experiment, the rate and degree of growth was not equivalent to that seen in uncontaminated soils (Figure 5.29).

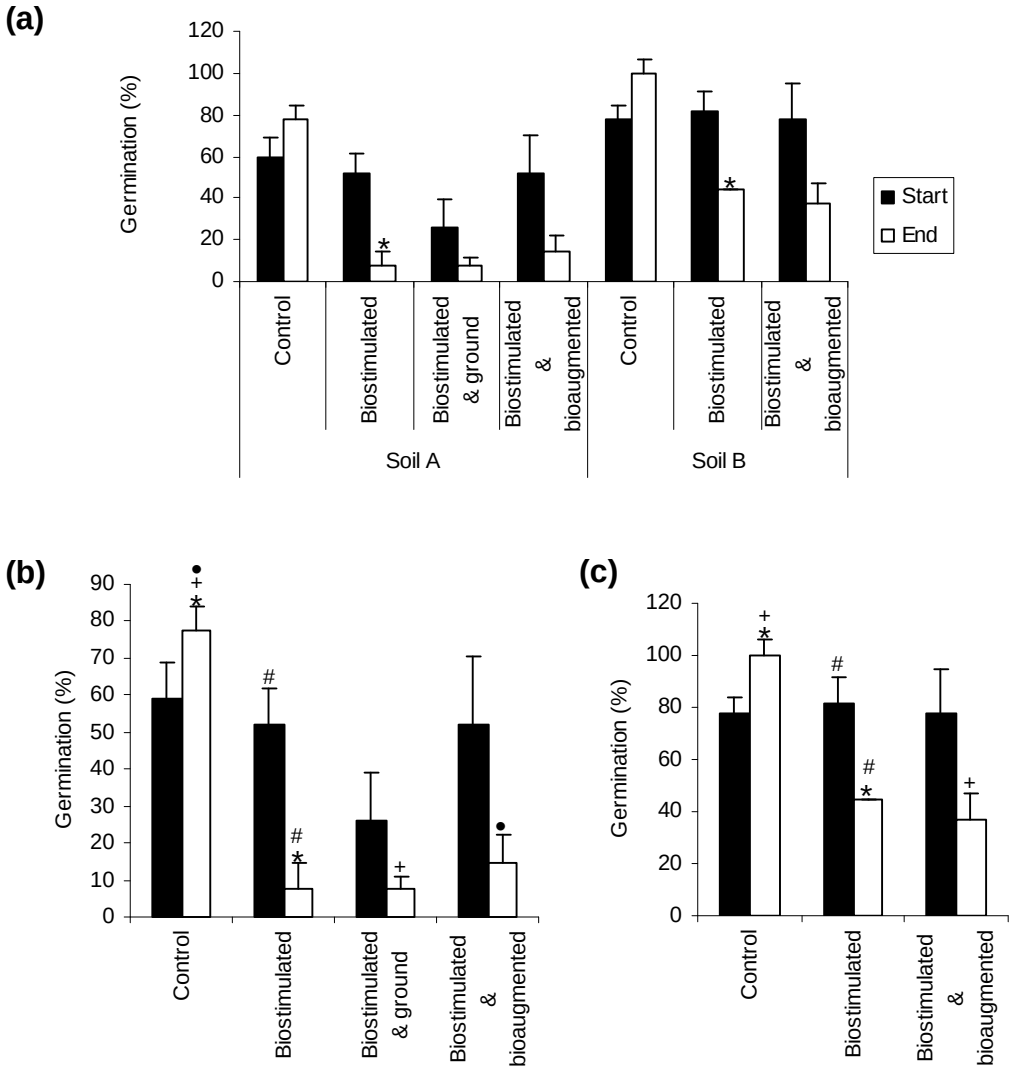


Figure 5.29: Mean (+SE) seed germination within (a) soils A and B, (b) soil A and (c) soil B. Significant differences (p<0.05) between points is indicated by matching symbols above points.

Overall the results from the seed germination assay show a reduction in germination within the treated soils, whereas the control microcosm soil for both soils shows an increase in germination (Figure 5.1 (a)). Between the soils, comparing treatments there is only a significant difference between

the biostimulated microcosms at the end of the microcosm experimental period (P=0.007) (Figure 5.29 (a)).

Evaluation of soil A seed germination results shows that the only treatment showing a significant difference in germination between the start and end of the microcosm experimental period was the biostimulation treatment where a significant reduction in germination is observed (p=0.022) (Figure 5.29 (b)). The seed germination results for soil A at the end of the microcosms experiment show that there has been a significant effect of the treatments compared to the control (p= <0.001, <0.001 and 0.001 for biostimulated, biostimulated & ground and biostimulated & bioaugmented respectively). The treatments used have reduced germination, however there is no significant difference in that reduction between the treatments (Figure 5.29 (b)).

Seed germination in soil B shows a similar pattern to that of soil A showing a significant effect of the treatments compared to the control (Figure 5.29 (c)) (P= 0.003 and 0.001 for biostimulated and biostimulated & bioaugmented treatments compared to the control respectively). Although the treatments for soil B appear show a greater germination rate to soil A, statistically there was only a significant difference between the soils within the biostimulated treatments.

Microtox® Solid Phase Test (SPT)

Overall the Microtox® results show a significant difference in the toxicity between the two soils ($P < 0.05$) (Figure 5.30 (a)). Soil A had larger EC_{50} concentrations than soil B for each of the comparable microcosms at both the start and end of the microcosm experimental period (Figure 5.30 (a)) (P-values were <0.001, <0.001, and 0.041 at the start for the control, biostimulated and biostimulated & bioaugmented treatments respectively at the start and <0.001, <0.001 and <0.001 at the end).

At the start of the microcosm experiment the biostimulated & ground treatment for soil A is the only treatment for which a significant difference to the control was observed (p= 0.001), which is also significantly different to the EC_{50} concentration of the other treatments (p= 0.001 and 0.000 for the biostimulated and biostimulated & bioaugmented treatments) (Figure 5.30 (b)). After the treatment period a significant difference is observed between the control and both the biostimulated & ground microcosms (p= 0.010) and the biostimulated & bioaugmented microcosm (p =0.036). Significant differences are observed for all the treatments when comparing EC_{50} values at the start to the end of the experiment. Here the biostimulated and biostimulated & bioaugmented treatments reduce toxicity within the soil whereas the biostimulated & ground treatment increase toxicity. The control for soil A also changes significantly, out performing the treatments at reducing toxicity.

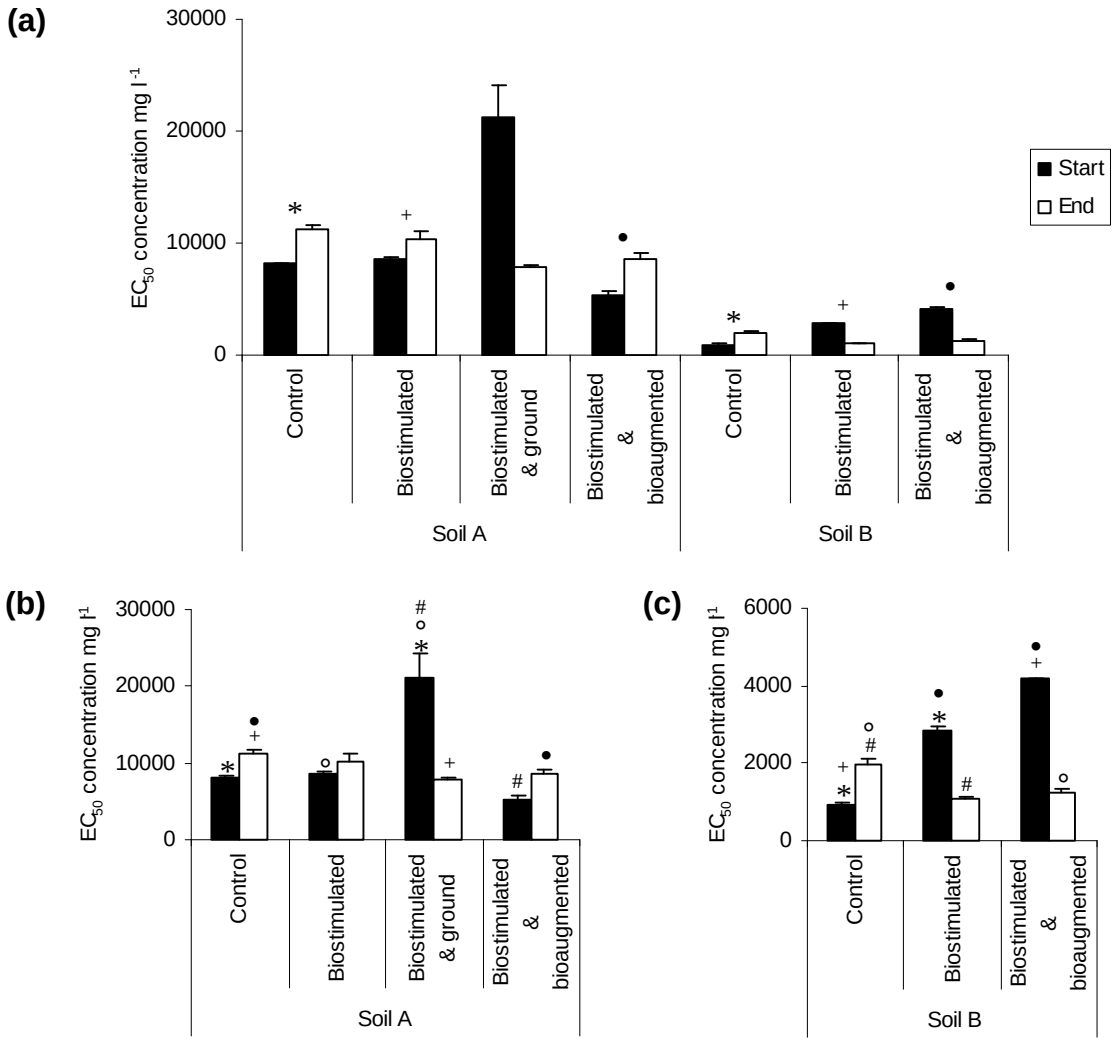


Figure 5.30: Mean (+SE) EC_{50} concentration recorded for (a) both soils, (b) soil A and (c) soil B. Significant differences ($p<0.05$) between points is indicated by matching symbols above points.

The change in EC_{50} concentration within soil B displays a similar pattern to that observed with soil A (Figure 5.30 (c)). There are significant differences for all the treatments when comparing EC_{50} values at the start against the end of the experiment for soil B. Additionally at the start of the experimental period the toxicity of the treatments and the control are all

significantly different from one another with the treatments showing higher EC_{50} concentrations. After treatment there is no difference between the toxicity of the treatments however they are different to the control which is significantly less toxic than the treatments.

5.4 Chapter Summary

Overall, both of the soils investigated showed significant biotransformation ($P < 0.05$) after 16 weeks of treatment. The microcosm experiments have shown that there is potential for extended bioremediation of petroleum hydrocarbon residues. The residues in the previously remediated soil underwent further biotransformation with losses of up to 86 and 92 % in the aliphatic and aromatic fractions respectively (Tables 5.6 & 5.8). Degradation of the oil contamination within soil B was also observed, displaying a degradation profile typical to that seen during hydrocarbon degradation (Figures 5.12 – 5.14 and 5.21 – 5.26). Here, within soil B a reduction of 92% for both the aliphatic and aromatic fractions occurred within biostimulated soil (Tables 5.7 & .9). Grinding of soil A was shown to reduce the effectiveness of a biostimulation treatment on the extent of biotransformation possible by up to 25% and 20% for the aliphatic and aromatic hydrocarbon fractions, respectively (Tables5.6 & 5.8).

The results show clear differences between the two soils. Soil A has a higher concentration of the aliphatic fraction and soil B has a higher concentration of the aromatic fraction. The most prominent aliphatic and

aromatic fractions found within the contamination in both soils were the C_{16}-C_{35} and C_{35}-C_{40} and the C_{16}-C_{21} and C_{12}-C_{16} hydrocarbon ranges respectively (Figures 5.7, 5.15, 5.16, 5.27 & 5.28). Contamination within soil B was far more widely distributed over the hydrocarbon range analysed (C_{10}-C_{40}) than the contamination within soil A which had far lower concentrations of the lighter hydrocarbons (Figure 5.3). At the end of the treatment the most prominent hydrocarbons remaining were above a hydrocarbon number of 32 (Figure 5.3).

C_{17}:pristine and C_{18}:phytane ratios of 0.03 and 0.01 for soil A and 0.10 and 0.16 for soil B respectively show that both soils have been degraded prior to this investigation. Significant differences in these ratios between soil A and B ($p < 0.001$) show that soil A has seen a greater extent of degradation than soil B as would be expected due to the nature of these soils. Within both soils the C_{17}:pristine ratio profile indicates that degradation is still occurring at the end of the experiment, with only the combined biostimulation and bioaugmentation treatment in soil A resulting in a significant difference in this ratio by the end of the experiment ($p < 0.001$) (Figure 5.5). At the end of the experiment none of the treatments achieved a significantly lower C_{18}: phytane ratio than the control, however degradation appeared to still be occurring within the combined biostimulated and bioaugmented treatment (Figure 5.6). Within soil B the biostimulated and bioaugmented treatment significantly reduced the C_{18}: phytane ratio compared to the other treatments (control and biostimulated) (Figure 5.6).

Whilst biostimulation had an important effect on increasing degradation rates bioaugmentation significantly increased degradation of the aromatic hydrocarbons beyond that seen in the biostimulated treatment for soil A. This trend was not replicated in the fresher contamination in soil B, where biostimulation achieved similar results to biostimulation and bioaugmentation combined. This suggests that soils that have undergone treatment or are at an apparent end in degradation, may benefit from the addition of an inoculum at this stage, and could then see further degradation of the contaminants.

Carbon dioxide analysis confirmed microbial activity within all treatments for both of the soils treated (Figure 5.1). Within soil A biostimulation and biostimulation & bioaugmentation treatments were shown to have the greatest effect on respiration. However, within soil B treatment did not increase respiration within the soil (Figure 5.2).

Ecotoxicological responses (using mustard seed germination and Microtox® assays) showed that a reduction in total petroleum hydrocarbon (TPH) load within soils could not necessarily be linked to a reduction in residual toxicity (Figures 5.29 and 5.30). Toxicity assays confirmed that biotransformation is not physically driven by surface area limitations, contrary to expectation, as responses of ground and un-ground soils were not significantly different ($P > 0.05$). The ecotoxicological response of both soils showed changes as a result of treatment, however the results from the assays seem unreliable with opposing results. Here seed germination indicated that all the treatments for both of the soils increased the toxicity of

the soils, whereas Microtox® results indicated that the biostimulation and the biostimulation and bioaugmentation treatments reduced the toxicity of soil A. Thus, showing a clear disparity between these ecotoxicological tests.

Chapter 6: Discussion

6.1 Literature insights

The literature review identified the key elements involved in remediation of weathered petroleum hydrocarbon contaminated land, and the interactions between them (Chapter 1, Figure 1.2) (Brassington et al., 2007). These elements were chemical analysis, bioremediation, fate and transport, toxicology and risk assessment. These are all key components for the successful remediation and remedial technology efficiency demonstration for land contaminated with fresh and weathered hydrocarbons. Also identified were several important knowledge gaps and methodological limitations within analysis and risk protocols in relation to the weathered portion of hydrocarbon contamination in soil (Chapter 1, Tables 1.1 &1.2).

Many hydrocarbon-contaminated sites posing potential risks to human health harbour weathered, ‘mid-distillate’ or heavy oils. These sites present considerable challenges to remediation over and above those posed by fresh or more refined petroleum distillates. Critically, there are important scientific components that drive risk management for these wastes, and specifically the partitioning of risk-critical compounds within the oil/soil matrix.

Risks posed by weathered petroleum hydrocarbons can be actively managed through optimising treatment process parameters during bioremediation. This said, the ‘in-field’ verification of *ex-situ* technologies such as biopiling, continues to be expressed in many countries in terms of reductions in total petroleum hydrocarbon (TPH) load, or ‘losses’ from the

soil being treated, rather than by reference to reductions in risk. An observation from the UK is that the absence of risk from the vocabulary of many remediation operators and remediation projects reduces stakeholder (regulatory, investor, landowner and public) confidence in technology performance, and in doing so, limits the market potential of these technologies. The work presented here can be used to start to address some of these short comings, through the use of chemical and toxicological data in addition to the novel extraction protocol developed (Chapter 4).

The weathering of petroleum hydrocarbons further complicates this complex source term, affecting bioavailability and the performance of remedial technologies. Additionally, this complicates risk management decisions concerning these wastes. Weathered petroleum hydrocarbons are acknowledged as having important qualitative and quantitative differences compared to the fresh product, yet they are not considered in the majority of risk assessment frameworks. In cases where weathered hydrocarbons are considered, the lack of data on their toxicity, distribution, transport and availability results in over-conservative risk assessment and potentially unnecessarily overzealous remediation (Chapter 1, section 1.3). For heavy oils (the viscous (50-360 mPa s), high-boiling (ca. 300 - >600 °C) products such as No. 6. fuel oil with carbon ranges in excess of C_{20}), their inherent complexity is further compounded as they weather in the environment on account of biotic and abiotic losses that shift their chemical composition towards recalcitrant, asphaltenic products of increased hydrophobicity.

These changes raise an important feature of hydrocarbon contaminated land that is often overlooked – that the source term, the oil matrix, is itself a strong partition medium for risk critical compounds and weathering imparts further hydrophobicity to the oil matrix. Compositional changes dramatically affect the partitioning behaviour of these source terms prior to, during and following biological treatment. Effecting the soil compartment to which they preferentially partition into/found in. Risk critical components (*e.g.* the higher ring polynuclear aromatic hydrocarbons (PAH)) in weathered oils are less bioavailable because they are effectively partitioned within the source term in accordance with Raoult's Law (Kotz et al., 2005). Sun and Boyd (1991) first suggested the concentration of residual oil within a oil-soil matrix required for it to act as a discrete partition medium (*ca.* 1000 mg kg^{-1}) and suggested that this residual oil, as the original source of priority contaminants, could typically be ten times more effective as a partition medium than soil organic matter for hydrophobic organics. This is rarely represented within the fate and transport models that support the environmental exposure assessment of hydrocarbons with the possibility that regulatory exposure assessment models may dramatically over estimate the availability of risk-critical compounds through exposure routes. Zemanek et al. (1997) showed that between 71-96%$^{w}/_{w}$ of PAH in weathered diesel-contaminated loam soils were partitioned to residual oil (at 2-6%$^{w}/_{w}$ of the total soil composition) in petroleum and weathered creosote-contaminated soils, with 84% $^{w}/_{w}$ of benzo[*a*]pyrene partitioned to the residual oil phase. Woolgar and Jones (1999) estimated oil - water partition

coefficients (termed log K_{mw}) for a series of PAH to be between 4.5 - 6.5, dependent on the source term. Under these conditions, highly partitioned constituents in weathered hydrocarbon waste matrices may be biologically inaccessible to microbial communities and resistant to biotransformation. However, their very inaccessibility may, but not necessarily, also restrict the dose available to receptors. Clearly, attempts to improve the bioavailability of these components to microorganisms during bioremediation may also result in increased human exposure were a source pathway receptor linkage ins present. In estimating the fate of pollutants in complex environmental matrices, the application of fugacity models (Mackay, 1979) for predicting the relative phase distributions and concentrations of contaminants and their metabolites during treatment (Sims, 2003; Sims and Sims, 1995) is now proving valuable for informing exposure assessments and the optimisation of *in-situ* remediation. Recently Pollard et al. (2008) using fugacity modelling highlighted that the oil source term may play an important role in organic partitioning, and subsequent bioavailability. Here the authors using a typical biopile design used level I and II approaches to model the environmental distribution of selected risk critical contaminants (Pollard et al., (2008). They demonstrated that non-aqueous phase liquid (NAPL) and soil phases were the dominant partition medium, illustrating the importance of inclusion of these phases during risk assessment of oil-contaminated sites.

The relationships between chemical presence, toxic response, bioavailability and risk for weathered hydrocarbons have yet to be fully

elucidated and coupled into a meaningful risk management framework, though work is progressing (Tien, 1999; Environment Agency, 2003a, 2003b, 2004 and 2005;). One of the obvious research needs is to authenticate human exposures to oil/soil matrices in the context of contaminated land and, in particular, to explore the bioavailability of risk-critical compounds (benzene, benzo[*a*]pyrene) in light of these newly revealed partition relationships.

The regulation of site remediation now requires adoption of a risk-based approach and this extends to technology verification (Environment Agency, 2002). Whereas the effectiveness of an environmental technology in treating pollution has historically been expressed as a percentage reduction in the pollutant concentration released to, or found in, a media of concern, regulators are increasingly concerned with mass, toxicity and risk reductions within the multimedia, multiphase environment. For petroleum hydrocarbons in soil, international regulatory guidance on the management of risks from contaminated sites is now emerging. The literature review showed that much of this guidance promotes the use of risk management frameworks to guide decision-making, the application of reference analytical methodologies and the derivation and use of acute, sub-chronic, and chronic toxicological criteria for these wastes. The review showed that these frameworks adopt a variety of approaches to the evaluation of risk-critical components within the hydrocarbon waste-soil matrix (Chapter 1, section 1.3, table 1.2). Not only do they vary in their approach but in the analytical methods used to evaluate the contamination within a soil (Chapter 1,

section 1.2.1, table 1.1). Giving rise to the potential for varied remediation targets to be set.

In the U.S., a substantive research effort has focused on integrating hydrocarbon fate and transport, petroleum microbiology and environmental diagnostics to inform regulatory processes for site management under the Superfund Program. ThermoRetec (2000), reporting for the Petroleum Environmental Research Forum (PERF), provide an authoritative account of the central importance of partitioning within soil-bound hydrocarbons in developing environmentally acceptable endpoints (remedial objectives). Drawing on a detailed understanding of NAPL and residual oil fate and behaviour, this work is now influencing the development of remediation criteria for petroleum hydrocarbon in soils in the US for human health, groundwater and ecological receptors, and a reappraisal of the level of residual petroleum hydrocarbons that can be left at remediated sites without posing an unacceptable risk. In contrast, weathered, mid-distillate and heavier oil sources are generally given a narrow treatment by these reviews and frameworks. The Environment Agency (2003b) have recognised this in their recent consultation on principles for evaluating the human health risks from petroleum hydrocarbons in soils, and have called for views. One of the few environmental exposure assessments explicitly to address heavy oils has been discussed in a recent article relating to worker and visitor exposure following the wrecking of the oil tanker ‘Erika’ in 65 km south of the Brittany coast (Baars, 2002). Here, inhalation, dermal and oral PAH exposures from beached No. 6 fuel oil were estimated and found to be

negligible for beach cleaners and tourists (occasional visitors) coming into to contact with heavy oil, demonstrating the feasibility of this level of risk analysis for these problematical wastes.

The move towards risk-based corrective action (RBCA) has been slow in the UK and, whilst some progress has been made in integrating the aspects of analysis, exposure assessment and technology verification (Environment Agency, 2003a), there are gaps in the current knowledge base. Specifically: (i) analytical strategies in the UK are not generally targeted at the bioavailability of risk-critical components; (ii) risk assessments do not regularly account for highly weathered residues encountered at many sites; and (iii) treatment 'success' is still supported by reductions in hydrocarbon load in isolation of combined reductions in toxicity, chemical mass and risk. As such many studies follow a pattern of reporting reductions in TPH load as a presumed surrogate for risk reduction (Al Awadhi et al., 1996; Milne et al., 1998; Tien et al., 1999; Guerin, 2000; Bourgouin, 2003).

A contributing factor to the over-reliance on TPH as an indicator of treatment performance in isolation of other parameters, has been the cost of implementing more sophisticated diagnostic techniques and their low uptake within the sector. This has been, in part, as result of the absence of a regulatory framework. Nevertheless, researchers have been concerned with improved diagnostics methods (the analysis of specific carbon number ranges); the fingerprinting of hydrocarbon wastes for source identification (for liability disputes) and in tracking biotransformation; and with biological

techniques as indicators of the impact of hydrocarbon contamination on soil function. Initiatives have included the development of reference methods for the analysis of petroleum hydrocarbons from nC_6-C_{50} (CCME, 2001; TNRCC, 2001), the application of biomarker analysis (*n*-alkane: substituted *n*-hopane indices) to bioremediation verification (Hough, et al., 2006; Moldowan et al., 1995) and the validation of microbial bioassays for petroleum. Hough et al., (2006), building on that of Prince *et al.* (1994) demonstrated that the ratio of total alkanes ($\sum n$-alkanes) to 17α(H)21β(H)-hopane to be the most sensitive of a series of biomarker ratios in reflecting oily waste depletion in a 256-day soil microcosm study. The biomarker 17α(H)21β(H)-hopane was not used within this study for the reason mentioned in section 5.2.4.

Many analytical protocols are available for weathered petroleum hydrocarbons. Inter-laboratory and analytical variations exits, with most analytical protocols not having been designed for the extraction of hydrocarbons numbers up to C_{70} (Chapter 1, section 1.2.1, table 1.1). Additionally many do not incorporate a 'clean-up' step to ensure only petrogenic TPH is determined or the class fractionation now required by several of the frameworks. Accurate analysis of weathered hydrocarbons is essential. It was shown by Mills et al (1999), how whilst different analytical strategies give varying degrees of detail about the contamination in a sample (which can be useful), the level of detail chosen for analysis can affect the evaluation of a sample. They showed that by using different analytical methods, completely opposite conclusions could be formed. This

is unacceptable, especially when analytical data is used within human health risk assessment and helps to set remediation goals, in such situations poor analysis of the soil could have detrimental human health impacts. Clearly these observations demonstrate the need for the development of validated robust analytical protocols for such wastes, where inter-laboratory variances can not cause a significant effect on results.

The insights and knowledge gaps found can be briefly summarised as: (i) limited analytical reliability due to important analytical differences between laboratories, methods and risk assessment protocols, further compounded by limited heavy hydrocarbon analysis capabilities (> C_{40}) (ii) paucity of chemical, toxicological and environmental distribution data regarding weathered hydrocarbons and no assessment of toxicity within risk assessment protocols, limiting knowledge generation and resulting in potentially over-conservative remediation goals and (iii) lack of appropriate incorporation of weathered hydrocarbons within risk assessment.

Although highly suited to these wastes, bioremediation techniques are limited by complicated legislation and negative stakeholder perceptions of these technologies, which can be linked back to the lack of knowledge concerning these wastes. The literature highlights that without improvements in weathered hydrocarbon knowledge, the value of any risk assessment where these wastes are concerned are in doubt. This consequently limits the use of bioremediation for these wastes. Without a full understanding of the contaminant in question, a risk assessment is meaningless.

6.2 Critical analysis of hydrocarbon bioremediation pilot studies

6.2.1 Influence of the oil type and ageing on biodegradation

A review of hydrocarbon bioremediation pilot studies (Chapter 3) confirmed that oil type has an effect on the extent of its susceptibility to degradation, a trend demonstrated elsewhere (Kaplan et al., 1996) (Chapter 3, section 3.3.1). A clear difference in the degradation achieved was seen between the lighter oils (up to 90% loss) and the heavy/medium oils (up to 57% loss), although no clear distinction was observed between the degradation achieved between the heavy and medium oils used within this study (Chapter 3, table 3.1). Differences in the extent of degradation achievable has been reported elsewhere with a range of 49-90% being shown by Raymond et al., (1976) when investigating 6 different oils. Yet it is clear that some of the oils in the current investigation were far more resistant to degradation as shown by the lack of degradation seen in the Ondina 68 and 150SN dewaxed finished base oil, both of which only degraded by 9% (Chapter 3, section 3.3.1, table 3.1). Additionally studies by Huesemann, (1995) and Sugiura et al., (1997), have indicated that the extent of oil and total petroleum hydrocarbon (TPH) biodegradation is affected most by the oil type rather than the treatment used (Huesemann, 1995). The effect of the composition of the oil was studied in greater detail and closely linked to the type of oil and its molecular composition

(Huesemann, 1995) with alkanes being shown to be more susceptible to biodegradation (Sugiura et al., 1997).

It is widely acknowledged that as oils are weathered, they tend to become more tightly bound to the soil leaving behind a greater proportion of recalcitrant compounds (Pollard et al., 1994; Whittaker et al., 1995) and thus bioremediation is often expected to be difficult. However, evaluation of this historical data showed that this is not always the case. A soil historically contaminated with crude oil and refined products that had aged in the field achieved comparatively high levels of degradation (90% TPH loss) (Chapter 3, table 3.1). However, subsequent characterisation of the original soil contaminated oil showed the oil to have a hydrocarbon range C_{12} to C_{26} which had undergone partial weathering as shown by the loss of hydrocarbons $<C_{12}$, here the type and degree of weathering has had an effect on the oils susceptibility to degradation. As previously discussed (Chapter 1) petroleum hydrocarbons are a highly complex soil contaminant, which due to their wide use are of global concern. As such any additional information on the degradation of both fresh and weathered petroleum hydrocarbons is useful to the contaminated land sector. However, due to the sparse knowledge available concerning weathered hydrocarbons and the large number of sites contaminated globally (as discussed in Chapter), new data concerning these contaminants is of even greater importance on a global scale.

6.2.2 Influence of bulking agent type on bioremediation efficiency

The benefit of bulking agents in enhancing bioremediation has been widely reported in the literature (Eweis et al., 1998; Hyman and Dupont, 2001; Wellman et al., 2001; Rojas-Avelizapa et al., 2006). As such the expected outcome of this investigation was an increase in the rate and extent of TPH loss within the test soil in line with that demonstrated elsewhere (Rhykerd et al. 1999; Giles, et al., 2001). Although a loss of TPH was seen within pine bark and straw amended microcosms, no additional benefit of the bulking agents was seen beyond the benefit of fertilisers alone (Chapter 3, section 3.3.2, figure 3.2). This was unexpected as the soil had a high clay content, as such the addition of bulking agents should have improved degradation by opening up the structure of the soil to bioremediation. This may have been due to the oil binding to the bulking agents making them less available to microbial attack. As discussed by Namkoong et al (2002) in some cases bulking agents can represent a preferential carbon source and can be degraded in preference to target compounds, which may also account for the reduced degradation seen here.

Interestingly, less of the aromatic fraction degraded within the pine bark amended treatment than the straw amended treatment (Chapter 3, section 3.3.2, figure 3.2). The pine bark used presumably had a high lignin content, which being an aromatic polymer of random phenylpropane subunits may have absorbed aromatic compounds strongly thus making them unavailable for bioremediation. Thus, illustrating variations in the affinity of different

compounds for binding to different bulking agents, resultantly removing compounds from the bioremediation system. Chaíneau et al. (2003) demonstrated a significant increase in biodegradation due to the addition of a bulking agent and nutrients to a clayey soil. A 45% increase in degradation was observed when straw and nutrients were added together and a further 11% increase when organic matter was added. This was a trend that was expect to occur here however, as discussed by Namkoong et al. (2002) in some cases bulking agents can represent a preferential carbon source and can be degraded in preference to target compounds, which may account for the reduced degradation shown here. It is clear from the investigation here and within the literature that use of bulking agents can have varying biotransformation results for weathered hydrocarbons (Eweis et al., 1998; Rhykerd et al. 1999; Giles et al., 2001; Hyman and Dupont, 2001; Wellman et al., 2001; Namkoong et al., 2002; Chaíneau et al., 2003; Rojas-Avelizapa et al., 2006). This further complicates bioremediation choices for stakeholders and suggests that it may be advantageous to trial potential bulking agents rather than relying on published literature. Whilst time consuming and costly this type of analysis can only reduce the paucity of knowledge on these weathered hydrocarbon contaminants.

6.2.3 Biostimulation treatments: comparative analysis of different fertilisation approaches.

It is widely known that low levels of nutrients essential to microbial growth (carbon, nitrogen, phosphorous and other minor nutrients) can inhibit bioremediation of hydrocarbon contaminated soil (Cookson, 1995; Eweis et al., 1998; BATELLE, 1996). In the bioremediation pilot trials studied, the effect of pelleted forms of fertiliser was investigated. These fertilisers can have advantages over liquid forms by reducing leaching and loss of fertiliser nutrients, the loss of which could not only reduce bioremediation efficiency but also contaminate protected watercourses with nitrate (potentially resulting in 'blue-baby' syndrome). In the investigations performed the addition of fertiliser enhanced TPH loss by up to 50% compared to un-fertilised treatments (Chapter 3, section 3.3.3, figure 3.3). However, both fertiliser types performed equally well. The initial slower rate of degradation that was seen within the pelleted fertiliser treatment was not unexpected due to the nature of such fertilisers.

Pelleted fertilisers may be preferred in many cases as re-application is often not required, and will not leach out of soil as easily as liquid fertilisers, reducing the risk of groundwater contamination by nitrate. Application of all the fertiliser a soil needs in one treatment (as with liquid fertiliser) has the potential to cause toxic effects to the bioremediation system, and leaching to watercourses. However, there has been no evidence of this in this experiment. Likewise, pelleted fertilisers seem to be a good alternative to liquid fertilisers. The use of pelleted slow-release fertilisers may be

advantageous in difficult and remote locations where in situ remediation is being performed. Xu et al. (2004) have previously shown that slow-release fertiliser has the ability to supply sufficiently high levels of nutrients to sea sediments to increase microbial activity and increase bioremediation, when compared to an un-ammended contaminated sediment. Röling et al., (2004) also experienced significant increases in oil degradation when using fertiliser, without any significant difference in oil chemistry between liquid and slow-release amended soils. As shown here, even though significant differences were observed initially in the respiration and microbial population between the two fertiliser treatments, this has no effect on the degradation of the oil (Röling et al., 2004). In an investigation by Xu and Obbard (2004) the slow-release fertiliser Osmocote™ out performed soluble nutrients and was able to stimulate the biodegradation rates of PAHs with a ring number higher than 2 where the soluble nutrients could not. In oil contaminated beach sediments Xu and Obbard (2003) demonstrated the potential advantages of slow-release fertiliser when comparing several different types of fertiliser, here treatments receiving a soluble inorganic nutrient and a slow-release fertiliser (Inipol) showed nutrient levels similar to unamended treatments after only 15 days due to their susceptibility to leaching loss from irrigated sediments. Whereas treatments receiving the slow-release fertiliser Osmocote™ maintained nutrient levels beneficial for bioremediation, showing a difference between these types of slow-release fertiliser and indicating that appropriate selection of a slow-release fertiliser effects efficacy can be beneficial using the correct formulation.

6.2.4 Bioaugmentation trials: benefits and limitations

Within the literature, bioaugmentation, through the use of commercial bioaugmentation packages or cultured hydrocarbon degraders has shown varying degrees of efficacy (Giles et al., 2001). The bioaugmentation trials performed within the bioremediation pilot studies employed the use of three different bioaugmentation packages, each of which incorporated nutrient solutions and surfactants in addition to the microbial consortium. It was shown that although the treatment package increased the amount of degradation achieved by 1.4 times compared to natural attenuation, overall they had no additional benefit over the use of a standard fertiliser solution (Chapter 3, section 3.3.5, figure 3.5). Here the indigenous microbial population was sufficient for bioremediation to proceed on the addition of a fertiliser, with the addition of fertiliser increasing degradation by up to 1.5 times at a rate that was up to 1.2 times faster than the bioaugmentation package (Chapter 3, section 3.3.5).

This trend was also observed by Cunningham et al. (2004) who found that a commercial product did not perform as well as a biostimulated system, with the commercial product also being out-performed by the control. Cunningham et al. (2004) showed however that a system augmented with an enrichment culture in addition to fertiliser could perform better than a system using fertiliser alone. This suggests that the microbial consortium within commercial packages may not be targeted enough to see an improvement in hydrocarbon degradation at such sites. Microbial consortium addition has also been shown to have little effect on increasing

degradation elsewhere as shown by Cho et al. (1997) where not only was the addition of a microbial culture shown to give no improvement in degradation but also impeded bioremediation. Additionally in investigations performed by Trindade et al. (2005) and Giles et al. (2001) the addition of a foreign microbial consortium was shown to perform no better than the indigenous population. Indeed, a view that is now being expressed by many as a general rule is that microbial cultures are ineffective at enhancing biodegradation (Van Hamme et al., 2003), which is confirmed by the trend seen in the current investigation.

The augmentation packages were each tested on different contaminated soils, which may have been an influencing factor on the efficacy of the augmentation package. Packages α and β were used on historically contaminated soil whereas package γ was tested on freshly contaminated soil. Package γ treatment performed better than both package α and β treatment, which will have contained a greater proportion of lighter hydrocarbons than the other two soils, allowing for a greater initial degree of degradation. Here further investigations into the efficacy of package γ on historically contaminated soils could be advantageous.

6.2.5 Influence of bioremediation on leaching

It is important during bioremediation design to consider a contaminant's leaching potential, to enable appropriate collection/decontamination systems to be employed. The influence of bioremediation on the leaching of

benzene, toluene, ethylbenzene and xylenes (BTEX) was investigated in the bioremediation pilot studies. During these woodchips were added as bulking agents and were expected to slightly reduce leaching through absorption, yet this was not seen (Chapter 3, section 3.3.4). Addition of these bulking agents to tank bottom sludge's (TBS) and oil based mud's (OBM) had negligible effect on hydrocarbon leachability (Chapter 3, section 3.3.4). After a 12 week period of bioremediation the amount of BTEX and TPH that could be leached from the OBM and TBS composts was reduced by 97 and 99% respectively, showing a considerable effect of bioremediation on leaching (Chapter 3, section 3.3.4). As has also been demonstrated elsewhere (Salanitro et al, 1997), bioremediation in the form of biopiling dramatically reduced the concentrations of BTEX and TPH that could be leached from the piles by 97->99 % over 12 weeks. BTEX concentrations declined by between 10-234 μg L^{-1} before remediation to < 01 μg L^{-1} after. Chaîneau et al., (2003) demonstrated that water soluble hydrocarbon leached during the first stages of bioremediation and decreased when degradation ceased. When investigating the leaching of PAH from soil Haeseler et al. (1999) demonstrated a reduction in PAH leaching after biological treatment. Also highlighting that in this case the accessibility of the PAHs was the limiting factor for bioremediation, when after biological treatment the PAHs seemed to lose the capacity for leaching. Both Haeseler et al. (1999) and Salanitro et al. (1997) works concluded that although hydrocarbon residues are still present within soil after bioremediation they are no longer available for leaching and biodegradation.

6.3 Chemical analysis development

Methods for the extraction and subsequent analysis of petroleum hydrocarbons used within risk assessment frameworks have been shown to be inappropriate for the analysis of both heavy and weathered hydrocarbons (Chapter 1, section 1.2). Thus the selection of an appropriate technique for the analysis of the hydrocarbon content of the soil microcosms was essential, for which Soxhlet was initially selected (Chapter 4). Whilst Soxhlet extraction, as with many of the other methods available has several disadvantages, using large volumes of solvent with long extraction times, the literature indicated that it was one of the most reliable exhaustive techniques available for weathered hydrocarbon extraction (Chapter 1). Soxhlet extraction is a widely used, benchmarked, exhaustive and easily standardised technique for the extraction of petroleum hydrocarbons contaminated soils (Shu et al., 2003).

Collaboration in this research with TES-Bretby resulted in the development of a novel ultrasonication method (Risden et al., '*submitted*'). This subsequently replaced Soxhlet extraction for the extraction of soil samples from the soil microcosm experiments. As a result solvent use, extraction times and sample throughput were all greatly improved. The ultrasonication method presented is a quicker, easier to use, uses less solvent than traditional Soxhlet.

Ultrasonic extraction has previously been investigated, with the potential for wider use in this area of analysis (Banjoo and Nelson, 2005; Sanz-Landaluze et al., 2006). However, as with traditionally used methods used

within the risk assessments (Chapter 1, section 1.2), current ultrasonication methods can be limited by inter-method variations. The novel solvent utlrasonic method presented here (Chapter 4, section 4.3) has good extraction efficiency and recovery independently of soil type with relative standard deviation (RSD) values for all the soil types tested of below 10% for all of the spiked soils (Chapter 4, section 4.4, table 4.2). The method is low cost, fast and scalable with low capital outlay using non-clorinated solvent. The relatively high bias (inaccuracy) obtained may be due to the lack of an evaporation step within the method (Chapter 4, section 4.4, table 4.2). The highest degree of variability was obtained in the clay soil (Chapter 4, table 7), a trend also reported by Shin and Kwon (2000), which they suggested was due to the stronger binding of compounds to the clay matrix, reducing extractability and increasing variability.

Validation of the method using reference matrix RTC CRMPR 9583 gave good precision, achieving an RSD of 3.4% (Chapter 4, section 4.4, table 4.3). This is a better degree of precision than that achieved by Sanz-Landaluze et al., (2006) of 14.4% when validating their method using a reference material. With the exception of the made ground soil, a higher degree of precision was typically observed when extracting higher concentrations of hydrocarbons, higher variability at lower concentrations may be attributed to the detection limits of the GC used. This is consistent with the precision obtained for ultrasonic methods elsewhere.

An extraction efficiency of ≥ 95% was attained which is in good agreement with those of Banjoo and Nelsons (2005), where extraction

efficiency greater than 90% for optimized sonication method was obtained. Differences between the method used in here and those of Banjoo and Nelson (2005) occur in extraction duration, solvent volume and addition sequence. Additionally, samples were not agitated in the Banjoo and Nelson method. In the present study, agitation was used to ensure full contact between sample and solvent increasing extraction efficiency. Banjoo and Nelson (2005) used evaporative techniques in their post extraction sample preparation. Here, no such step is required, which may account for some of the differences in extraction efficiencies of these two methods, as losses may have occurred through during extract concentration steps.

As with any analytical method it is important to ensure reliability and validity of the results. Quality control is built into the method through the analysis of reagent blanks and spiked reference materials, and sample recovery is monitored using surrogate spikes (Chapter 4).

Sanz-Landaluze et al., (2006) optimised sonication parameters when using an ultrasonic probe to give good extraction efficiencies with satisfactory accuracy confirmed using certified reference materials, when extracting the 16 U.S. EPA priority PAHs. The method described by the authors does not require a clean-up step; however concentration through evaporation is employed which could lead to volatile losses. Ultrasonic probes are more expensive to purchase than ultrasonic baths, yet in real terms, this cost is in no way prohibitive. However, the additional health and safety requirements of ultrasonic probe use could be an issue. Sonic probes as shown by Sanz-Landaluze et al., (2006) also require an understanding of

and optimisation of amplitude and ultrasonic power/energy. Use of ultrasonic baths as used within the method developed here (chapter 4) negates these issues and as a commonly used piece of laboratory equipment is easily and quickly available.

Previous investigations have shown that the water within soil samples can significantly influence extraction efficiency of organic compounds (Shu et al., 2003). A mixture of two solvents such as 1:1 acetone/hexane or 1:1 hexane/dichloromethane (DCM) is commonly used for organics extraction (Shu et al., 2003; Coulon et al., 2004). Extraction of field moist samples can suffer interference from water when hydrophobic solvents (hexane and DCM) are used, reducing extraction efficiencies. Within the method described in this work (chapter 4) acetone, a hydrophilic solvent, allows penetration and extraction of contaminants from field moist samples, simultaneously disrupting the soil matrix and enhancing extraction rates. This, followed by the addition of hexane, enables the extraction of non-polar compounds. This initial use of a polar solvent negates the need for oven- or freeze-drying which has been shown to reduce extraction efficiencies (Schwab et al., 1999; Bergknut et al., 2004; Banjoo and Nelson 2005), particularly for the lower molecular weight equivalent carbon (EC) fractions. The effectiveness of this step in overcoming this interference from water has also been reported elsewhere (Schwab, et al 1999; Banjoo and Nelson 2005).

The effectiveness of polar solvents such as acetone was also shown by Schwab, et al (1999), when investigating the effect of solvent, soil type,

extraction cycles, soil quantity and aging on the efficiency of mechanical shaking. The authors found that soil moisture played a key role and of the solvents studied acetone was the least affected by soil moisture and type.

Banjoo and Nelson (2005) optimized an ultrasonic extraction procedure for the determination of PAHs and aliphatic hydrocarbons ranging from C_{12} to $C_{24,}$ in sediments. This was compared against a reflux with methanolic KOH method. The investigation showed that ultrasonic extraction of dried sediment with acetone: hexane mix (1:1) gave comparable concentration of the PAHs studied to the reflux method, with lower variation in the reproducibility. The advantage of using acetone prior to the addition of other solvents was highlighted. The authors found that extraction efficiency increased when samples were initially sonicated with acetone only prior to addition of hexane.

The effect of solvent choice has also been demonstrated by Shin and Kwon (2000) and Sanz-Landaluze et al., (2006), where acetone:DCM (1:1, v/v) and hexane respectively were shown to be the solvents of choice during sonication.

Bergknut et al., (2004) compared pressurized liquid extraction (PLE) to Soxhlet extraction and also assessed the effects of other parameters of the PLE process. The effects of different organic solvents, pre-treatment and extraction time on the availability of polycyclic aromatic hydrocarbons (PAHs) extracted by PLE was assessed by sequentially extracting soil in water, methanol, *n*-butanol, acetone, *n*-hexane and toluene by PLE. Here the sample was extracted using the solvents one after the other. PLE

extraction using methanol demonstrated equivalent extraction efficiency in 14 minutes as a 2 h toluene Soxhlet extraction. The studies highlighted the different solvents affinity for different molecular weight PAHs. Toluene and acetone extracted roughly even concentrations of all PAHs tested in the study comprising 2-, 3-, 4- and 5-fused-rings benzene, whereas *n*-butanol extracted higher concentrations of high molecular weight PAHs (> 4- and 5-fused-rings benzene).

Many of the methods currently in use for the analysis of weathered petroleum hydrocarbon contaminated soils incorporate, where necessary, a sample clean up method (Chapter 1, table 1.1)(AEHS, 2000; CCME, 2001; New South Wales Environment Protection Agency, 2003; MaDEP, 2004). In some cases methods will, in the same step, fractionate a sample into aliphatic and aromatic fractions (Chapter 1, table 1.1) (Ministry of the Environment, 1999; AEHS, 2000). Risk assessments are increasingly evaluating aliphatic and aromatic compounds separately; therefore it is important that this is incorporated into methods not only to satisfy the risk assessment requirements but to ensure the results are not skewed by the presence of biogenic TPH. The method described here (Chapter 4) achieved this though the use of a micro-scale silica gel column chromatography method to fractionate extracts into aromatic and aliphatic fractions, and where required remove interfering polar compounds (Chapter 4).

Comparison against other available methods is helpful in evaluating the applicability of a method to the extraction of weathered hydrocarbons in soil

on a broader analytical scale, enabling its position within the group of weathered hydrocarbon soil extraction methods to be defined. Alternative methods have been demonstrated to give a range of efficiencies from 75% and 77% for supercritical fluid extraction (SFE) (Heemken et al., 1997) and Soxhlet (Saifuddin and Chua, 2003), respectively up to ≥ 97% and 99% for accelerated solvent extraction (ASE) (Heemken et al., 1997) and microwave assisted extraction (MAE) (Saifuddin and Chua, 2003), respectively. In this study, a minimum recovery of ≥ 95% has been demonstrated with maximum recoveries in the range > 99%, easily positioning this methods within the best of the alternatives to Soxhlet methods.

6.4 Microcosm study - Biotransformation of weathered hydrocarbons

As described in Chapter 5, the two soils used in the microcosm study were from two different sites. Soil A had been historically contaminated with oil, which had then undergone bioremediation in windrows. Bioremediation of this soil had reduced it's concentration from *ca.* 50,000 mg kg^{-1} down to *ca.*15, 000 mg kg^{-1}, and to a point at which it had been deemed that no further degradation could be achieved.

Soil B had been taken from a site at which waste oil drums had leaked, prior to any remedial treatment at an unknown concentration and composition. Subsequent initial TPH analysis of the soils showed concentrations of 22,700 mg kg^{-1} and 31,500 mg kg^{-1} TPH for soils A and B

respectively. The previous analysis of soil A was performed by a different unknown laboratory. The discrepancy that can be seen between the two reported concentrations for soil A further demonstrates the need for the use of a robust analytical method, such as that described in Chapter 4 (section 4.3), when analysing these types of wastes.

The treatments investigated within the soil microcosms were typically used treatments that have shown success with other hydrocarbon contaminated soils. However, the potential for further bioremediation of a soil that has previously undergone treatment has not been previously investigated. The evaluation of the potential for biotransformation within this soil is important as the ability to reinitiate and extend biotransformation has not been investigated before. As such this provides valuable knowledge concerning, chemical and toxicological change on a soil type not previously investigated. Additionally any significant reductions in TPH may have important implications for bioremediation strategies.

It was also noted during the literature survey that although soils are often ground during the course of homogenisation prior to bioremediation, the use of grinding as a treatment in itself had not been investigated. Thus both of these aspects were investigated during the microcosm experiment.

6.4.1 Changes in carbon dioxide production

Changes in carbon dioxide evolution provide insights into the growth of the microbial population present within a soil. Within the investigation

described here CO_2 monitoring showed the presence of a microbial population with in all treatments (Chapter 5, section 5.3.1, figure 5.1). Within both soils the addition of an inoculum had no effect on increasing CO_2 evolution above that observed in the biostimulated treatment, which may have been due soils being toxic to the exogenous organisms. Changes in CO_2 evolution can be indicative of TPH reduction, here it suggested that little degradation is occurring in soil A and microorganisms are maintaining at a steady state (Chapter 5, section 5.3.1, figure 5.2). Whereas higher CO_2 levels in soil B and subsequent reductions suggest more degradation initially that is subsequently decreasing as microbial food sources are consumed over time. However, hydrocarbon analysis shows that whilst this holds true for soil B it does not represent the degradation seen in soil A. (Chapter 5, section 5.3.3).

6.4.2 Changes in petroleum hydrocarbon composition

Analysis of residues within the two soils showed that they are quite different, soil A has significantly higher concentrations of aliphatic compounds whereas soil B has significantly higher concentrations of aromatic compounds ($P<0.001$) (Chapter 5, section 5.3.3). Additional differences in the oils characteristics were shown in their *n*-alkane profiles Soil A had a reasonably flat profile with a distinct peak at C_{32}-C_{36}, whilst in soil B the *n*-alkanes were far more evenly distributed about the C_{16}-C_{40} range. Demonstrating that soil A although containing higher concentrations

of the *n*-alkanes has been more significantly weathered and degraded during prior treatment. These profiles are also observed when looking at the individual *n*-alkanes that make up this profile (Chapter 5, section 5.3.3, figures 5.7-5.14).

C_{17}: pristane and C_{18}:phytane ratios can be used as indictors of bioremediation and weathering. Initial C_{17}: pristane and C_{18}: phytane ratios of 0.03 and 0.01 respectively for soil A, and 0.10 and 0.16 respectively for soil B, showed that the soil had undergone a substantial degree of weathering prior to this investigation. Monitoring of these ratios showed further reductions over time, illustrating that degradation was occurring. However these ratios should not be used as indicators of the extent of degradation as both pristane and phytane themselves have been shown to degrade (Riser-Roberts, 1998; Riccardi et al., 2008). However the shape of curve for C17: pristane ratio for both soils suggest that degradation may still be occurring at the end of the experiment. The curve for the C18:phytane ratio showed a similar pattern but only in the augmented soil A and the control and augmented treatment for soil B. Suggesting that further incubation may result in additional degradation, however as these ratios cannot be relied upon it is necessary to investigate this degradation in more detail (Riser-Roberts, 1998; Riccardi et al., 2008).

Chemical analysis of the soils further confirmed that they where both reasonably weathered, with ~ 89% and ~80% of the oil having a carbon number greater than 16 (Chapter 5, table 5.3).

Both soils displayed a typical degradation curve, with degradation slowing down towards the end of the experimental period as would be expected (Chapter 5, section 5.3.3, figures 5.7-5.14 and 5.17-5.26). This reduction in TPH is of greater interest with regard to soil A, as it demonstrates that further degradation of the contamination was indeed possible even though remediation had been initially assumed to come to a halt. This shows that under the right conditions further reductions may be possible, within soils where degradation appears to have come to a halt. This additional degradation could suggest that optimum conditions were not established for the remediation of this soil previously or that a change in treatment strategy is required during bioremediation of weathered hydrocarbons to achieve the optimum level of degradation possible within the soil. This has implications for both present and past remediation schemes, and depending upon the end use of a remediated soil could warrant additional investigation into remediated soils. Soils that may have been placed in landfill sites when bioremediation techniques failed to achieve required concentrations, should pose no harm to human health. However those soils that have reached targets suitable for other uses, could pose risks as these soils may be capable of further biotransformation which could leach toxic metabolites out of the soil and into important receptors such as watercourses.

Appropriate investigations were performed by the oil facility that provided the treated soil, to enable the determination of any limiting factors, yet none were evident. However, as soil analysis at the beginning of this

investigation showed, this soil was nutrient limited (N=5%), suggesting that nutrients have been lost during storage.

Whilst a significant reduction in the aromatic fraction within soil B has been seen, further degradation of the aromatic compounds within soil B appears to be capable as although degradation of these compounds slowed towards the end it had yet to reach a plateau (Chapter 5). All microcosms for soil A saw an overall reduction in TPH however only the nutrient treated microcosm was seen to be significantly different to the control showing that grinding had no beneficial effect on remediation and as such TPH loss was not driven by surface area in this case. Both treatment used on soil B resulted in TPH reduction that were statistically different to the control, however no difference was shown between the treatments themselves. Suggesting that the addition of the inoculum had little or no effect of reducing TPH a trend that has been mirrored in the literature.

Soil B has also been used in microcosms as part of project PROMISE, however unlike here no additional benefit of these treatments compared to the control has been seen (Chapter 5). Experimental microcosms used in this investigation were essentially identical to those used by PROMISE for soil B, the only difference being the size of the microcosms and the aeration regime. In the microcosm experiment described here soil was mixed weekly, providing better aeration of the soil than the PROMISE microcosms which were not mixed. This could suggest that degradation within the PROMISE microcosms may have been partially oxygen limited. This also further illustrates the importance in optimising methods.

Evaluation of the aliphatic fractions suggested by the Environment Agency (2005) showed that the most noticeable effect of the microcosm treatments for both soils was in the C_{16}-C_{35} and C_{35}-C_{40} hydrocarbon ranges (Chapter 5). Although a statistically significant change was seen in the change in the concentration of all the fractions evaluated, it was the C_{16}-C_{35} and C_{35}-C_{40} ranges that showed the most noticeable change, as would be expected form the initial analysis of the soils at the start of the investigation (Chapter 5). Due to the low concentrations in the C_8–C_{16} hydrocarbon range present within both soils, changes in the fractions within this range were negligible, with the most significant change occurring in the hydrocarbon ranges above C_{16}. Within soil A the nutrient treatment effected the greatest amount change in the proportions of these fractions with a reduction of 19% in the C_{16}-C_{35} and subsequent increase by the approximately the same amount in the C_{35}-C_{40} range. Within soil B the nutrient treatment again performed the best with a change of 21% for these fractions.

The PAHs that were selected for analysis were not all present within either of the soils, however as earlier analysis indicated soil B had a far greater concentration and wider distribution of these PAHs (Chapter 5, tables 5.8 and 5.9, figures 5.17-5.26). Within both soil losses in these PAHs was seen and whilst several remained at the end of the incubation period, the only PAH to remain in both was C_1-naphthalene. Although degrading these PAHs significantly greater than the control, no significant difference between the treatments for soil B was highlighted. However within soil A the addition of an inoculum was necessary to improve degradation over that

seen in the control (by 6%), suggesting that inoculation plays an important part in reinitiating the degradation in PAHs within previously treated soils.

6.4.3 Ecotoxicological response

It is important to evaluate the change in toxicological response resulting from bioremediation treatments as it has been shown that reduction in TPH does not always infer a reduction in toxicity (Baud-Grasset et al., 1993). It has also become clear that reduction in toxicity may not be measured by the disappearance of the parent compound alone (Baud-Grassest el al., 1993).

The toxicity of the two soils were evaluated prior to and after treatment within microcosms using seed germination and Microtox® Solid Phase Test (SPT) (Chapter 5, section 5.2.2). Additionally carbon dioxide production was monitored throughout the experiment, which can also give an indication of toxicity to the microbial population.

Seed germination showed similar patterns between soil A and B, where all of the treatments used on both soil resulted in an inhibition of germination at the end of the bioremediation period, compared to the control microcosms for both soils (Chapter 5, section 5.3.4 figure 5.29). A ~50% reduction in germination was seen over the three treatments for soil A, and a ~40% reduction over the two soil B treatments. This would indicate an increase in the toxicity of the soil even though a reduction in TPH was seen within the microcosms, a trend also shown by Baud-Grasset et al (1993). Within soil B no significant difference between the control and the

treatments at the start indicate that the treatments themselves were not toxic at this stage to germination, and thus the reduction in germination over the duration of the microcosm experiment was a result of the effect of the treatments upon the soil/oil chemistry. It was expected that the grinding of soil A would have an effect on seed germination, however this was not confirmed by the experiment (Chapter 5, section 5.3.4 figure 5.29).

When toxicity was evaluated using Microtox® a significant difference was shown between the two soils, with soil A having higher EC_{50} concentrations and therefore less toxicity (Chapter 5, section 5.3.4, figure 5.30(a)). Although the toxicity of nutrient and the nutrient & inoculum treatments were seen to improve over the experimental period neither performed better than the control microcosm at reducing toxicity (Chapter 5,section 5.3.4, figure 5.30(a)). As with soil A the soil B control microcosm out performed the treatments used. However where toxicity decreased in soil A, it increased within soil B over the experimental period, suggesting that intermediate compounds generated during the degradation of the soil had a greater toxic effect than the source term, even though TPH concentration reduced. Here, although both soils demonstrated a reduction in TPH this cannot be directly related to a reduction in toxicity. Further analysis using an additional range of toxicity indicators may enable a more complete picture of the change in toxicity to be drawn. However, toxicity bioassays are known for being unreliable (Baud-Grasset et al., 1993) as such further research into robust toxicity assays is also required.

Comparison of the seed germination and Microtox® results shows that an increase in toxicity in soil B was seen using both methods, whereas for soil A opposing results were achieved, this variability in results from bioassay methods is replicated elsewhere (Baud-Grassest el al., 1993). With only two ecotoxicolgical method being used it is not possible to determine which method could be giving a false positive or negative, further investigation into toxicological assays is required to established which of these two methods is more reliable.

6.5 Chapter Summary

In summary the literature review highlighted important research gaps concerning weathered hydrocarbon biotransformation, toxicity and analysis. Important steps towards addressing these issues have been made by the work presented here.

A robust, repeatable extraction protocol suitable for weathered hydrocarbon extraction from soil, which fulfils current risk assessment requirements has been developed.

Two soils containing weathered hydrocarbons have been biotransformed, providing additional compositional and toxicological data. Importantly it has been shown that further degradation of previously remediated soils is possible, which has implications for bioremediation strategies and risk assessment protocols.

Grinding of remediated soils containing weathered hydrocarbons limited biotransformation, raising issues for treatments where grinding has been used during sample homogenisation, as in such cases this may have reduced the potential for bioremediation in such soils, also illustrating that remediation was not driven by surface area.

The toxicological data generated illustrates the need for toxicity to be assessed at remediation sites in addition to TPH reductions, as reductions in TPH did not infer a reduction in toxicity and additionally risk.

Chapter 7: Conclusions and recommendations for future work

7.1 Conclusions

Based upon the work presented here several conclusions can be drawn:

1. The novel ultrasonic method presented is a viable alternative to traditional extraction methods. This is demonstrated by good accuracy (% bias -2.6 to 5), very high precision (RSD ≤ 10%) and extraction efficiencies (≥ 95%), across a range of soils and oil concentrations (Chapter 4, section 4.4., tables 4.2-4.6).
2. The novel method presented is capable of several different layers of analysis making it compatible with the current risk assessment frameworks, enabling carbon banding and class fractionation analysis, as evidenced by the precision and reproducibility of a variety of hydrocarbon ranges (Chapter 4, section 4.4, tables 4.2-4.6). Whilst also enabling the provision of data important for bioremediation studies, additionally, minor modifications to instrumental analysis protocols will allow this method to adapt to meet changes in risk assessment requirements.
3. The ultrasonic method presented is an improvement over conventional methods. As the method reduces costs, shortens extraction times (1h. vs. 8 h.), lowers solvent use (40 ml vs. 150 ml), has a smaller laboratory footprint (24 samples per every one Soxhlet extracted in terms of space requirements), is easier and reduces potential sources of error (such as reduced transfer and evaporative losses) (Chapter 4, section 4.4).

4. The ultrasonic method has shown a good potential for implementation as a standard method, potentially capable of providing (through use) further insight and knowledge to the contaminated land sector.
5. Previously remediated soils containing weathered hydrocarbon residues have the potential for further biotransformation. This is evident from significant losses of 86 % ($P<0.001$) and 92 % ($P=0.007$) of the aliphatic and aromatic fractions respectively within the treated soil investigated in this work (Chapter 5, section 5.3.3, tables 5.6 and 5.8).
6. Further biotransformation of a previously remediated soil is not limited by the availability of the residues to microbial attack, and is thus not driven by surface area. This is demonstrated by no significant reductions being shown in any of hydrocarbon fractions within the ground and biostimulated treatment for the previously remediated soil (Chapter 5, section 5.3.3, tables 5.6 and 5.8).
7. Careful, amendment with hydrocarbon degraders can significantly enhance the extended biotransformation of PAHs within treated residues by up to 6 % ($P=0.007$). Within the work here significant differences in PAH degradation were only seen on the addition of an inoculum, thus biostimulation alone cannot be relied upon to remove/reduce these compounds within treated soils (Chapter 5, section 5.3.3).
8. Grinding of a previously treated soil has no beneficial long term effect on contaminant toxicity. Although the EC_{50} concentration was initially significantly higher, the work here demonstrated that after 112 days no improvement in toxicity had occurred (Chapter 5, section 5.3.4, figure

5.30). This is an observation that is further confirmed by seed germination assays (Chapter 5, section 5.3.4, figure 5.29).

9. Toxicological bioassays demonstrated that the toxicity of both the treated and un-treated soils could not be linked to a reduction in TPH (Chapter 5, sections 5.3.3 and 5.3.4). Whist toxicological assays are omitted from inclusion in risk assessment protocols, this casts doubt upon the actual risk reduction achieved at remedial sites which follow such protocols. Although, risk critical compounds were reduced this did not infer a reduction in toxicity (Chapter 5).
10. Increased dissemination of weathered hydrocarbon remediation is achieved not only through the analysis of the soils in this work but by review of the historical pilot studies. These demonstrated several things including (i) remediation success was largely dependent upon oil contaminant and soil structure characteristics (ii) for the majority of the contaminated soils investigated mineral nutrients played an essential role, (iii) the potential benefit of bioaugmentation still needs further line of investigation.

7.2 Recommendations for future work

The additional biotransformation of residues within a previously treated soil, have important implications for both bioremediation strategies and risk assessments. However, it was only possible to investigate one soil within this work, as such further investigation into extended biotransformation in a

range of soil types is required. This would enable the evaluation of the extent to which these implications could occur.

Other potential treatments that were not investigated here could be analysed for their potential to extend bioremediation of treated soils (i.e. bulking agents and surfactants etc.).

Analysis of potential limiting factors for bioremediation were undertaken by the oil facility that provided the remediated soil for this project, yet none were identified. Thus, it was assumed that no further biotransformation could occur, yet in the investigation here it did. This poses an important question – how could biotransformation have occurred in this investigation if nutrient, moisture, pH, microbial population issues were not found to be limiting factors previously? What other factors could have influenced this and is a period of soil resting required prior to additional biotransformation? Investigation into this may bring other influencing factors to light, which may have not been considered and could help extend the biotransformation.

An important analytical method for the determination of weathered hydrocarbons has been developed within this research, yet it is not supported by an equally robust toxicological protocol. Whilst such bioassays are known to be highly variable in their response, it is still important for these assays to be used to ensure risk reductions are protective of human health. As such continued research into these protocols and any toxicological links to TPH is still required.

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Chapter 9: Appendix

9.1 Publications

Published refereed papers:

Brassington, K.J., Hough, R.L., Paton, G.I., Semple, K.T., Risdon, G., Crossley, J., Hay, I., Askari, K. and Pollard, S.J.T. (2007) Weathered hydrocarbon wastes: A risk management primer. CRC Reviews in Environmental Science and Technology. 37: 199-232

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Papers in preparation:

Brassington K.J., Coulon F., Bazin R., Linnet P.E., Thomas K.A., Mitchell T.R., Lethbridge G., Pollard S.J.T. (In prep). A critical analysis of hydrocarbon bioremediation pilot studies. *Environment International*.

Risdon G., Pollard S.J.T., Brassington K.J., Paton G., Semple K. and Coulon F. (Submitted). Development of a novel robust analytical procedure for weathered hydrocarbons contaminated soils within a UK risk-based framework. *Analytical Chemistry*.

Kirsty J. Brassington, Graeme Paton, Kirk Semple, Gordon Lethbridge, Simon J.T. Pollard, Frédéric Coulon. (In prep) Biotransformation of weathered hydrocarbons: the relationship between chemistry, toxicity and risk.

Conference Presentations:

Brassington, K. J., Hough, R.L. & Pollard, S.J.T. (2005). Risk assessment frameworks for weathered petroleum hydrocarbons: current status and limitations. SEESOIL Winter Meeting – Soil Protection & Policy, University of Reading, UK, 7 December 2005.

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Pollard, S.J.T., Hough, R.L. and Brassington, K.J. (2005) Optimising biopile processes for weathered hydrocarbons within a risk management framework. *In proceedings of the CL:AIRE and FIRSTFARADAY joint conference on contaminated land*. International Convention Centre Birmingham, UK 27-28th April, 2005.