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EFFECT-DIRECTED ANALYSIS OF TOXICANTS IN UNRESOLVED COMPLEX MIXTURES (UCMS) OF HYDROCARBONS FROM BIODEGRADED CRUDE OILS

by

ALAN GEORGE SCARLETT

A thesis submitted to the University of Plymouth

in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences
Faculty of Science
University of Plymouth

November 2007

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by

Alan George Scarlett

ABSTRACT

Contamination of the environment by petrogenic hydrocarbons continues to pose a threat to marine biota. Studies into the effects of hydrocarbon contamination have mainly been directed at a small number of polycyclic aromatic hydrocarbons (PAHs) that are known to be highly toxic to a wide range of biota. The majority of the hydrocarbons present in sediments and tissues are unresolved by conventional gas chromatography and have received little attention. Studies directed at these unresolved complex mixtures (UCMs) of hydrocarbons have previously identified the monoaromatic fraction as containing toxic UCM compounds.

The studies reported herein have explored the toxicity of UCM compounds to marine biota using an effect-directed analysis approach: (i) population-level effects on the amphipod *Corophium volutator* arising from chronic exposure to UCM hydrocarbon contaminated sediments; (ii) bioaccumulation and depuration of UCM hydrocarbons by the blue mussel, *Mytilus edulis* using comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-ToF-MS); and, (iii) the possible trophic transfer of UCM hydrocarbons from contaminated mussels to the predatory shore crab *Carcinus maenas*.

Chronic sediment exposure tests showed that oils dominated by UCM hydrocarbons reduced the growth rate and reproductive success of *C. volutator*. All fractions of the oils contributed towards the toxicity but the aromatic fraction produced effects at lower nominal sediment concentrations. The aromatic fraction was also responsible for the reduction of mussel filter-feeding clearance rates. Analyses of mussel tissue extracts by GC×GC-ToF-MS revealed that a range of aromatic compounds was rapidly accumulated, but most were readily depurated. Compounds that were more resistant to depuration, including branched alkylbenzenes (BABs), were also found in wild mussel populations previously reported to have poor health status. Tests using a commercially available complex mixture of $C_{12\text{-}14}$ BABs confirmed that these compounds were toxic to mussels and were not readily depurated. Crabs that consumed mussels contaminated with BABs were found to behave abnormally, but cellular and physiological effects were not significantly different to control organisms. Crab midgut gland tissues were found to contain low concentrations of BABs and fluorescence from urine suggested that the BABs were metabolised and/or excreted. The results did not support the hypothesis that BABs were likely to biomagnify within the marine food web.

The research reported herein supports the hypothesis that environmental UCMs are largely comprised of branched alkylated homologues of known petrogenic hydrocarbons. Of these, the BABs have been shown to bioaccumulate and cause adverse effects *via* a non-specific narcosis mode of action. Marine environment monitoring and regulatory bodies may benefit from taking into account the concentrations of UCM hydrocarbons, in particular the aromatic UCM, including the BABs.

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LIST OF COMMON ABBREVIATIONS

BAB	Branched alkylbenzene
BAT	Branched alkyltetralin
BIN	Branched alkylindan and alkylindene
BTEX	Benzene, toluene, ethylbenzene and xylene
DCM	Dichloromethane
EC_{50}	Effect Concentration 50 (concentration of a compound where 50% of its maximal effect is observed)
EDA	Effect-directed analysis
FID	Flame ionisation detector
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
GC×GC	Gas chromatography × gas chromatography (tandem GC)
GC×GC-ToF-MS	Gas chromatography × gas chromatography – time-of-flight – mass spectrometry
HPLC	High performance liquid chromatography
K_{ow}	Octanol-water partition coefficient
LAB	Linear alkylbenzene
LC_{50}	Lethal Concentration 50 (concentration of a compound which kills 50% of a sample population)
MS	Mass spectrometry
OC	Organic carbon
PAH	Polycyclic aromatic hydrocarbon
S*f*G	Scope for growth
SIM	Selected ion monitoring
TIE	Toxicity identification evaluation
ToF-MS	Time-of-flight – mass spectrometry
TPH	Total petroleum hydrocarbons
UCM	Unresolved complex mixture
UVF	Ultraviolet fluorescence
WAF	Water accommodated fraction
WSF	Water soluble fraction

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award. This study was financed by The Higher Education Innovation Fund (HEIF2) in collaboration with the Maritime and Coastguard Agency (project no. 562).

A programme of additional study was undertaken, which included a practical high performance liquid chromatography training course (2 – 3rd March 2006, LaserChrom Ltd. Rochester, Kent), a short course attended at the Setac Europe 15th Annual Meeting in Lille, France, 22-26th May 2005 entitled 'Statistical assessment of dose-response curves with free software' and gas safety courses administered by Gas Safe (Sandbach, Cheshire) undertaken at the University of Plymouth. Accredited teaching courses, General Teaching Associates (GTA and LTHE300), were also completed resulting in achievement of Certificate of Continued Professional Development - Learning, Teaching and Assessment: Theory and Practice. These teaching courses contributed towards the Postgraduate Certificate in Learning & Teaching in Higher Education and confirm associate membership of the Higher Education Academy.

Relevant scientific seminars and conferences were regularly attended at which research was presented.

Peer-reviewed publications arising from research described herein:

Scarlett, A., Rowland, S.J., Galloway, T.S., Lewis, A.C., Booth, A.M., (In press) Chronic sublethal effects associated with branched alkylbenzenes bioaccumulated by mussels. *Environmental Toxicology & Chemistry.*

Booth, A.M., Sutton, P.A., Lewis, C.A., Lewis, A.C., **Scarlett, A.**, Chau, W., Widdows, J. and Rowland, S.J., 2007. Unresolved Complex Mixtures of Aromatic Hydrocarbons: Thousands of Overlooked Persistent, Bioaccumulative, and Toxic Contaminants in Mussels, *Environmental Science and Tech*nology, 41: 457-464.

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Scarlett, A., Galloway, T.S., Rowland, S.J., (2007) Chronic toxicity of unresolved complex mixtures (UCM) of hydrocarbons in marine sediments. *Journal of Soils & Sediments*, 7:200-206.

Scarlett, A., Canty, M.N., Smith, E.L., Rowland, S.J. and Galloway, T.S., 2007. Can Amphipod Behavior Help to Predict Chronic Toxicity of Sediments? Human and Ecological Risk Assessment, 13 (3): 506 - 518.

Oral presentations and conferences attended:

Scarlett A, Booth A, Galloway TS, Rowland SJ (2007) Branched alkylbenzenes: a major toxic component of unresolved complex mixtures bioaccumulated in mussels? (poster presentation). In: Farrimond P (ed) The 23rd International Meeting on Organic Geochemistry, 9th -14th September 2007, Torquay, England. Book of Abstracts, Bideford UK.

Frenzel M, Booth AM, **Scarlett A**, Galloway T, Lewis AC, Burton SK, Lappin-Scott HM, Rowland SJ (2007) Bioremediation of 'the hump': assessment of the biodegradation of a toxic aromatic hydrocarbon Unresolved Complex Mixture (UCM) and measurement of remaining toxicity. In: Farrimond P (ed) The 23rd International Meeting on Organic Geochemistry, 9th -14th September 2007, Torquay, England.

Scarlett, A., Galloway, T.S. and Rowland, S.J., 2007. Does petroleum hydrocarbon monitoring of the marine environment require an upgrade? (poster corner presentation), SETAC Europe 17th Annual Meeting, 20-24 May 2007, Porto, Portugal.

Scarlett, A., Rowland, S.J. and Galloway, T.S., 2007. Effect-directed-analysis of unresolved complex mixtures of hydrocarbons (UCM). Identification of persistent, bioaccumulative and toxic components (poster spotlight presentation), SETAC Europe 17th Annual Meeting, 20-24 May 2007, Porto, Portugal.

Scarlett, A., Rowland, S.J. and Galloway, T.S., 2007d. The elephant in the room: unresolved complex mixtures of hydrocarbons (UCM) are widespread in marine sediments but are these ignored contaminants toxic? (poster presentation), University of Plymouth Vice Chancellor's Research and Innovation Conference, 3rd April 2007 Plymouth, United Kingdom.

Scarlett, A., Rowland, S.J. and Galloway, T.S., 2006. The elephant in the room: unresolved complex mixtures of hydrocarbons (UCM) are widespread in marine sediments but are these ignored contaminants toxic? (poster spotlight presentation), SETAC Europe 16th Annual Meeting, 7-11th May 2006 The Hague, The Netherlands.

Scarlett, A., Galloway, T.S., Canty, M., Smith, E.L. and Rowland, S.J., 2005. Let the organisms decide: an alternative approach as an adjunct to standard sediment toxicity tests (oral presentation). SETAC Europe 15th Annual Meeting, 22-26 May 2005, Lille, France.

Scarlett, A., Canty, M., Smith, E.L., Rowland, S.J. and Galloway, T.S., 2005. Seasonal variation in growth rates of laboratory-grown *Corophium volutator*: Implications for chronic toxicity tests (poster presentation), SETAC Europe 15th Annual Meeting, 22-26th May 2005, Lille, France.

Galloway, T.S., **Scarlett, A.**, Canty, M., Smith, E.L. and Rowland, S.J., 2005. Ecological impact of oil spills: life-cycle responses of the marine amphipod *Corophium volutator* to Alaskan North Slope crude-oil spiked sediments (poster presentation). SETAC Europe 15th Annual Meeting, 22-26th May 2005, Lille, France.

Oral presentations were made annually as part of the Biological Sciences, Ecotoxicology and Stress Biology Research Centre Seminar series within the University of Plymouth.

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Chapter 1

Introduction

This chapter presents a review of the current knowledge of unresolved complex mixtures (UCMs) of hydrocarbons in the marine environment, in particular the effect of components of the UCM on the health of marine organisms. Much of the research presented in this thesis has been published in peer-reviewed journals (Appendix A). The literature review presented within the Introduction has omitted reference to these papers other than when related to research performed by co-authors. The data presented within the published papers has been discussed in the context of the wider literature within **Chapter 7**.

1.1 General Introduction

The marine environment has long been subject to contamination by oil. This has occurred due to natural seepage and, more recently, due to human activities including drilling and transportation of crude and refined oils. Early studies of crude and refined oil toxicity focussed on the acute effects of high concentrations upon marine organisms (reviewed by NRC, 2003). From these studies, the highly volatile and water-soluble benzene, toluene, ethyl benzene and xylenes (BTEX) were identified as neurotoxic (Ritchie *et al.*, 2001) but were found to be rapidly removed from the marine environment. With the removal of BTEX compounds by weathering processes, the acute toxicity of hydrocarbons is reduced (Neff *et al.*, 2000) and the polycyclic aromatic hydrocarbons (PAHs) become more important contributors of toxicity. In recent years, most studies concerned with adverse biological effects arising from hydrocarbon contamination have focused on the PAHs, in particular the 16 priority PAHs identified by the US Environment Protection Agency as posing the greatest risk to biota (NRC, 2003). The PAHs often represent only a small percentage of the total hydrocarbons present within environmental samples and the majority of the compounds present, those which are unresolved by conventional gas chromatography (GC), tend to be overlooked by many researchers, including environmental monitoring and protection agencies, due to the analysis methods employed (Rowland *et al.*, 2001). The studies presented herein have focused on the potential for compounds within this 'unresolved complex mixture' (UCM) of hydrocarbons to cause adverse effects on marine biota.

Analysis of the hydrocarbon fraction of crude oils by GC reveals a complex mixture containing many thousands of individual components (Sutton *et al.*, 2005). Components that are resolved by GC have been extensively studied (e.g. Killops & Killops, 1993), and include *n*-alkanes, methylalkanes, acyclic isoprenoids, alkylated benzenes, naphthalenes

and phenanthrenes (Fig. 1.1). However, despite the application of many analytical techniques the remaining components have, until very recently, proved difficult to separate due to the large numbers of co-eluting compounds. Gas chromatograms of mature oils have prominent *n*-alkane peaks which distract attention from the underlying 'hump' of unresolved components. By way of illustration, the gas chromatography – mass spectrometry (GC-MS) total ion chromatogram (TIC), shown in Fig. 1.2a, contains large peaks corresponding to individual components such as *n*-alkanes. It appears that only a small UCM is present. However this is an artefact of the analytical technique and underlying UCM 'humps' are present even in fresh oils (Gough & Rowland, 1990). As the oil becomes more degraded and the *n*-alkanes removed, the UCM becomes more prominent (Fig. 1.2b) until only the unresolved components remains (Fig. 1.2c). Processes such as weathering and biodegradation result in a relative enrichment of the UCM component by removal of resolved components and the creation of new compounds (Peters *et al.*, 2005). It has been shown that both resolved and unresolved components of oils are subject to concurrent biodegradation (Gough & Rowland, 1990), i.e. it is not a sequential process, but due to the recalcitrant nature of some components, the rates of biodegradation of individual compounds greatly varies. Although often overlooked, except as a diagnostic tool for identifying petrogenic sources (Bouloubassi *et al.*, 2001; Hong *et al.*, 1995; Noboru Nishigima *et al.*, 2001; Steinhauer & Boehm, 1992; Zheng & Richardson, 1999), the UCM fraction often represents the major component of hydrocarbons within hydrocarbon-polluted sediments (Table 1.1) and biota (Table 1.2). A small number of studies has now demonstrated that aqueous exposure to components within the UCM can affect the health of marine organisms (Donkin *et al.*, 2003; Hokstad *et al.*, 2007; Rowland *et al.*, 2001) and high concentrations of environmental UCMs have been strongly implicated with impaired health in wild populations (Crowe *et al.*, 2004; Guerra-Garcia *et al.*, 2003).

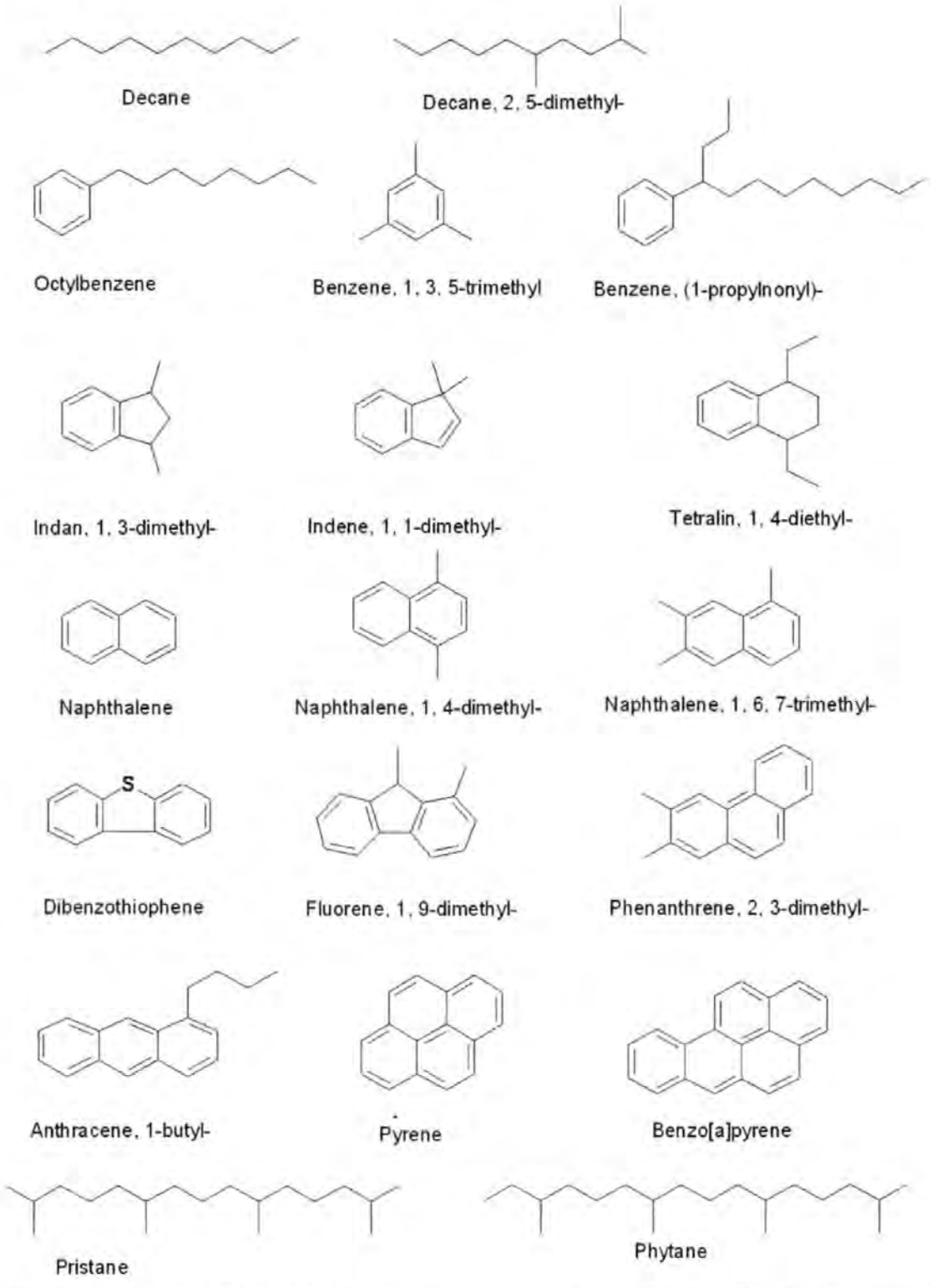


Figure 1.1 Examples of aliphatic and aromatic hydrocarbons, including some alkylated compounds, commonly found in the environment that can normally be resolved by gas chromatography.

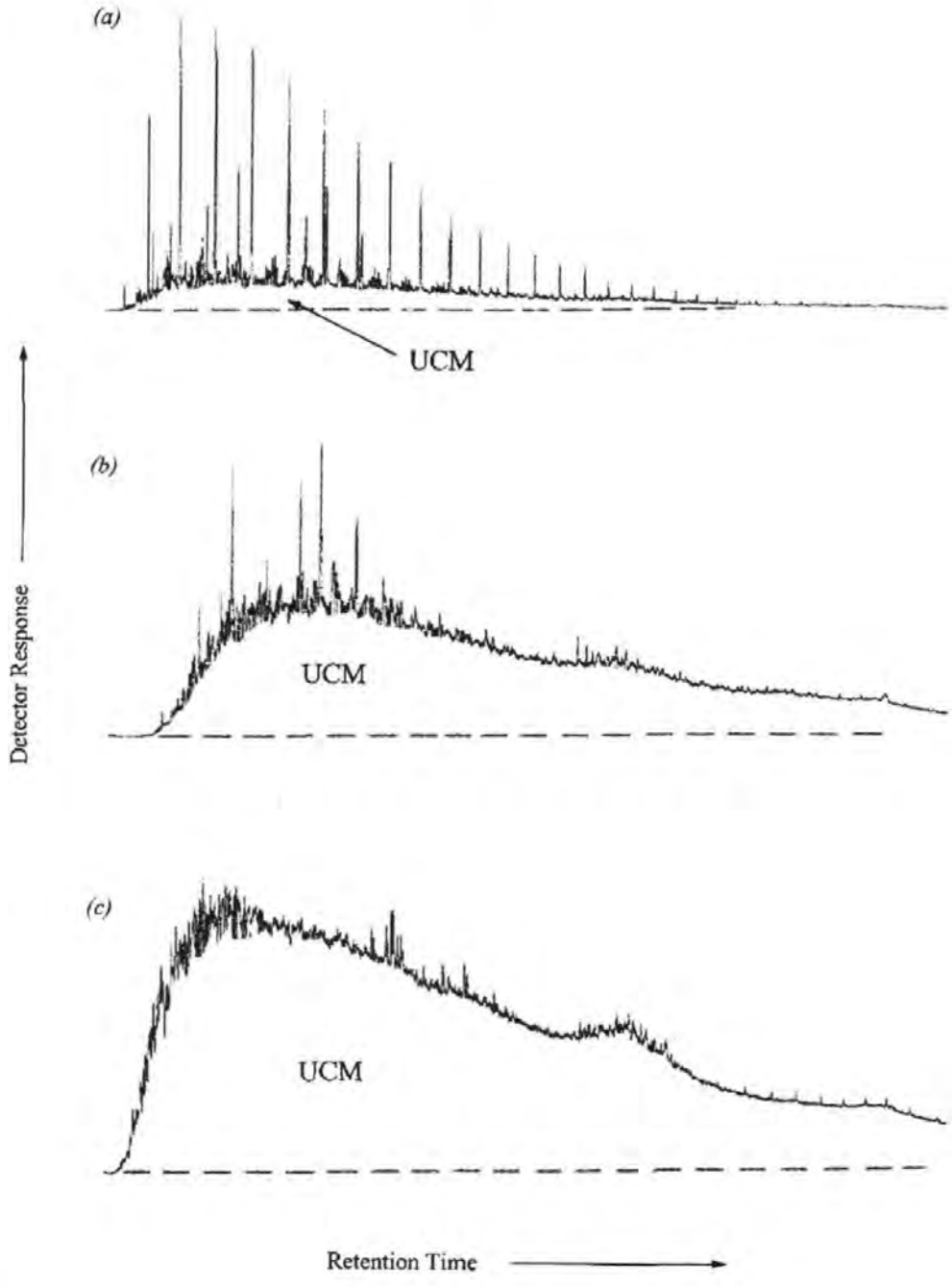


Figure 1.2 Gas chromatograms of three related Gulf Coast oils with differing levels of biodegradation: (a) nonbiodegraded; (b) moderately biodegraded; and, (c) very heavily degraded. Adapted from Peters & Moldowan (1993).

Table 1.1 Comparison of sediment hydrocarbon and UCM data

Location	*UCM* ($\mu g\ g^{-1}$ *dw*)	*Total n-hydrocarbons* ($\mu g\ g^{-1}$ *dw*)	*n-Alkane* ($\mu g\ g^{-1}$ *dw*)	*CPI*	*Pr:Ph*	*Reference*
Argentina – Chubut river, Rawson Port	284	ns	741	ns	0.9	(Commendatore & Esteves, 2004)
Argentina – Rio de la Plata Estuary	0 – 1445[•]	0 – 1532[•]	ns	ns	ns	(Colombo *et al.*, 2005)
	0 - 205.7[#]	0 - 207				
Antarctica	-	< 0.5	-	ns	ns	(Lenihan *et al.*, 1990)
Australia – Great Barrier Reef	-	0.5 - 2	-	ns	ns	(Volkman *et al.*, 1992)
Azerbaijan – S. Caspian Sea	1 - 310		1 - 5	1 – 9.1	ns	(Tolosa *et al.*, 2004)
Canada – Halifax harbour Nova Scotia	153 - 546	ns	19 - 46	ns	ns	(Hellou *et al.*, In press)
China – Xiamen Harbour	2.7 – 30[•]	3.1 – 33[a]	0.4 – 3.4	ns	ns	(Hong *et al.*, 1995)
China – Yangtze River Estuary	1.8 – 9[•]	2.2 – 11.8	0.2 – 1.9	1.1 – 4.9	ns	(Bouloubassi *et al.*, 2001)
Crete – Eastern Mediterranean	0.3 – 4.8[•]	0.5 – 5.7[a]	0.1 – 0.9	ns	ns	(Gogou *et al.*, 2000)
Egypt – Alexandria, Eastern Harbour	54 – 1214[•]	-	7 - 143	ns	ns	(Aboulkassim & Simoneit, 1995)
France - Rhone river (Mediterranean)	18 – 146[•]	25 - 170	-2 - 12	1.1 – 11.2	ns	(Bouloubassi & Saliot, 1993)
France – Rhone prodelta	76 - 275	ns	10 - 27	2.6 – 4.3	ns	(Tolosa *et al.*, 1996)
France – Gulf of Lions	7 – 17	ns	0.8 – 2.3	1.6 – 5.5	2.7 – 5.5	(Tolosa *et al.*, 1996)
France- Gulf of Foz, Refinery outlet, (Mediterranean)	++	9400 - 32980	ns	ns	ns	(LeDreau *et al.*, 1997)
France- Gulf of Foz, Canal, (Mediterranean)	++	1280 - 47580	ns	ns	ns	(LeDreau *et al.*, 1997)
France- Gulf of Foz, Deep Creek	~50 - 4500	50 - 4500	ns	ns	ns	(LeDreau *et al.*, 1997)

Table 1.1 continued

Location	*UCM* ($\mu g\ g^{-1}$ *dw*)	*Total hydrocarbons* ($\mu g\ g^{-1}$ *dw*)	*n-Alkane* ($\mu g\ g^{-1}$ *dw*)	*CPI*	*Pr:Ph*	*Reference*
France- Gulf of Foz, Open Sea, (Mediterranean)	+	40 - 3370	ns	ns	ns	(LeDreau *et al.*, 1997)
Hong Kong- Coastline	5.6 – 1760	5.9 – 1996	0.2 - 296	ns	ns	(Zheng & Richardson, 1999)
Hong Kong – Victoria Harbour	56 – 626[•]	60 – 646[a]	3.1 - 20	ns	ns	(Hong *et al.*, 1995)
Ireland - Liffey estuary	++	ns	210-8129	~1	ns	(Choiseul *et al.*, 1998)
Ireland - E. coast	++	ns	0 - 137	1.2	ns	(Choiseul *et al.*, 1998)
Kazakhstan – N. Caspian Sea	1 - 12		0 - 2	2 - 9	ns	(Tolosa *et al.*, 2004)
Kuwait, Gulf	28 – 5300[•] 5.9 – 80[#]	40 - 240	0.3 – 2.2	ns	0.7 – 2.9	(Readman *et al.*, 1986)
Mediterranean Sea – W. & E. Deep Basin	7 - 13	ns	0.7 – 1.7	3 – 4	1.7 – 3.1	(Tolosa *et al.*, 1996)
Russia – N. Caspian Sea	<0.5 - 4	ns	<0.3	1 – 4.5	ns	(Tolosa *et al.*, 2004)
Russia – S. Caspian Sea	<0.5 - 3.5	ns	<0.1 – 0.1	1.3 – 2.7	ns	(Tolosa *et al.*, 2004)
Russia – Sochi, Black Sea	2.9 – 140[•] 2.5 – 16[#]	7.6 - 170	0.7 – 3.4	ns	1 – 5.2	(Readman *et al.*, 2002)
Saudi Arabia, Gulf	6.4 – 5300[•] 1- 1400[#]	11 - 6900	0.2 - 28	ns	0.3 – 1.3	(Readman *et al.*, 1996)
Saudi Arabia, Ras Al Ghar	420[•] 160[#]	671	ns	ns	2.1	(Fowler *et al.*, 1993)

Table 1.1 continued

Location	*UCM* ($\mu g\ g^{-1}$ *dw*)	*Total n-hydrocarbons* ($\mu g\ g^{-1}$ *dw*)	*n-Alkane* ($\mu g\ g^{-1}$ *dw*)	*CPI*	*Pr:Ph*	*Reference*
Spain - Barcelona	488	ns	7.7	1.5	1.4	(Tolosa *et al.*, 1996)
Spain – Catalan coast	17 - 23	ns	1.1	2.4 – 4	1 – 1.4	(Tolosa *et al.*, 1996)
Spain – Ceuta harbour (N. African coast)	~400-4500	496-6972	ns	0.9-1.2	0.8-3.1	(Guerra-Garcia *et al.*, 2003)
Spain – Ebro shelf & slope	0 - 15	ns	0.3 – 1.1	3.5 – 5	1.9 – 2.9	(Tolosa *et al.*, 1996)
Spain – Ebro prodelta	8 - 17	ns	1.1 – 1.8	4.4 – 7.6	2.2 – 5.1	(Tolosa *et al.*, 1996)
Turkey – Bosphorus, Black Sea	4 – 38*	12 - 76	1.3 – 2.6	ns	2.1 – 3.9	(Readman *et al.*, 2002)
	3.6 – 30#					
UK - Severn estuary	++	ns	105	~1	ns	(Thompson & Eglinton, 1978)
Ukraine – Danube coastline, Black Sea	33 – 160*	49 -220	1.2 - 2.1	ns	0.7 - 1	(Readman *et al.*, 2002)
	13 – 15#					
Ukraine –Coastline, Black Sea	1 – 3.1*	2.1 - 6.6	0.1 – 0.6	ns	1 - > 45	(Readman *et al.*, 2002)
	0.5 – 2#					
Ukraine –Odessa, Black Sea	78 – 232*	110 - 310	1.4 – 1.6	ns	0.1 – 0.9	(Readman *et al.*, 2002)
	28 – 63#					
USA – Baja California	0 – 221	1 – 71	ns	~1	ns	(MaciasZamora, 1996)
USA – New York Bight	++	35 - 2900	ns	ns	ns	(Farrington & Tripp, 1977)
USA – Mississippi-Alabama, Continental Shelf	1 – 131*	-	0.1 - 3.2	ns	ns	(Kennicutt *et al.*, 1995)
USA – West Falmouth, Massachusetts	~8000	~8000	ns	ns	ns	(Reddy *et al.*, 2002)

+ UCM present ++ UCM concentration not given but dominant, ns = not stated, CPI = Carbon Preference Index, Pr:Ph = pristane:phytane ratio* aliphatic UCM, # aromatic UCM

Table 1.2 Comparison of biota hydrocarbon and UCM data

Location	*Biota*	*UCM* ($\mu g\ g^{-1}\ dw$)	*Total hydrocarbons* ($\mu g\ g^{-1}\ dw$)	*n-Alkane* ($\mu g\ g^{-1}\ dw$)	*CPI*	*Reference*
Argentina – Rio de la Plata Estuary	Macrophytes	0 – 2262[•]	12 – 2405[•]	ns	ns	(Colombo *et al.*, 2005)
		0 – 457[#]	463.9[#]			
Argentina – Rio de la Plata Estuary	Clams	418 – 1943[•]	1981.7[•]	ns	ns	(Colombo *et al.*, 2005)
		46 – 515[#]	529.4[#]			
Bahrain	Bivalves	26 – 113[•]	40 – 124[•]	ns	ns	(Fowler *et al.*, 1993)
		12 – 62[#]	16 – 67[#]			
Australia (west) – Rowley shelf	Oysters	+	ns	1 - 5	~1	(Pendoley, 1992)
Baltic	Mussels	+	ns	14 - 49	ns	(Law & Andrulewicz, 1983)
Baltic (north) clean	Bivalves	-	ns	10	ns	(Broman & Ganning, 1985)
Baltic (north) polluted	Bivalves	+	ns	60 - 1461	~1	(Broman & Ganning, 1985)
Ireland – E. coast (rural)	Mussels	-	ns	0 - 352	0 – 2.3	(Choiseul *et al.*, 1998)
Ireland – E. coast (urban)	Mussels	+	ns	146 - 1361	0.5 – 1.9	(Choiseul *et al.*, 1998)
Mexico - Tabasco	Oysters	5 - 57	ns	ns	ns	(GoldBouchot *et al.*, 1995)
Oman	Bivalves	21 – 80[•]	27 – 88[•]	ns	ns	(Fowler *et al.*, 1993)
		54 – 69[#]	58 – 78[#]			
Saudi Arabia, Gulf	Bivalves	21 – 420[•]	143 – 475[•]	ns	ns	(Fowler *et al.*, 1993)
		12 - 210#	27 – 240[#]			
Saudi Arabia, Gulf	Fish	8 – 1700[•]	10 – 2290[•]	ns	ns	(Fowler *et al.*, 1993)
		3 – 210#	7 - 339			

Table 1.2 continued

Location	*Biota*	*UCM* ($\mu g\ g^{-1}\ dw$)	*Total hydrocarbons* ($\mu g\ g^{-1}\ dw$)	*n-Alkane* ($\mu g\ g^{-1}\ dw$)	*CPI*	*Reference*
UK - Cleethorpes	Mussels	170 - 401*	ns	ns	ns	(Rowland *et al.*, 2001)
		102 - 136#				
UK - Teesmouth	Mussels	188 – 275*	ns	ns	ns	(Rowland *et al.*, 2001)
		83 – 94#				
UK - Whitby	Mussels	3280 – 3610*	ns	ns	ns	(Rowland *et al.*, 2001)
		365 – 496#				
UK – Whitsand	Mussels	5 – 9*				(Rowland *et al.*, 2001)
UK – New Brighton	Mussels	708 – 764*	ns	ns	ns	(Donkin *et al.*, 2003)
		235 – 265#				
USA - California	Bivalves	+	ns	18 - 270	ns	(Risebrough *et al.*, 1983)
Venezuela (Central Coast)	Clams (*Tivela mactroidea)*	0 – 56*	ns	1.5 - 14	~1	(Jaffe *et al.*, 1995)

+ UCM present ++ UCM concentration not given but dominant, ns = not stated,

CPI = Carbon Preference Index, Pr:Ph = pristane:phytane ratio

* aliphatic UCM, # aromatic UCM

1.2 Characterisation of UCMs

Petroleum hydrocarbons may enter the marine environment from numerous sources. By far the largest input comes from atmospheric emissions with municipal and industrial wastes, urban run-off, bilge and fuel oil, tanker operations and tanker accidents making smaller contributions (Clark, 2001). Once in the marine environment these petroleum products are subject to weathering and biodegradation and ultimately can be detected as UCMs within sediments and marine biota (Tables 1.1 and 1.2). Clearly, with such a diverse source of inputs there will be great variation in components between UCMs and local sources may strongly influence the composition. A number of processes are involved in the weathering of petroleum and shaping the UCM: evaporation removes the most volatile compounds, dissolution removes the more polar and water-soluble compounds and biodegradation mainly attacks the linear alkanes, branched alkanes, and then the cycloalkanes and aromatics (Volkman *et al.*, 1992). Biodegradation of these compound groups occurs simultaneously but degradation rates are faster for the simple alkanes (Larter *et al.*, 2003; NRC, 2003).

Environmental UCMs result from highly degraded petroleum hydrocarbons and once formed they can stay largely unchanged in sediments for many years. For example, in 1969 a diesel oil spill contaminated saltmarsh sediment within Wild Harbor River, US; by 1973 only a baseline hump was observed, which remained largely unchanged within the anaerobic sediment for 30 years (Reddy *et al.*, 2002). In a study of the potential for UCM-dominated oil to be further degraded, McGovern (1999) concluded that even using bacteria specifically adapted for complex UCM hydrocarbons in conjunction with nutrient enrichment, biodegradation rates would still be relatively slow. Bacterial degradation of hydrocarbons is complex and will depend on environmental conditions (e.g. aerobic or anaerobic, temperature, nutrient availability, available species of bacteria). Sediment

UCMs can therefore persist for decades possibly posing an ongoing threat to benthic organisms.

Numerous analytical techniques have been developed to investigate the chemical composition of UCMs. These include: open-column silica gel chromatography, silver-impregnated silica gel chromatography and thin layer chromatography (TLC) (Killops & Al-Juboori, 1990). Chemical modification prior to analysis using chromic acid or ruthenium tetroxide has also been applied (Gough & Rowland, 1990; Killops & Al-Juboori, 1990; Warton *et al.*, 1999). Spectroscopic methods for UCM analysis include infrared (IR) (Killops & Al-Juboori, 1990), fluorescence (Mason, 1987), nuclear magnetic resonance (NMR) (Killops & Al-Juboori, 1990) and mass-spectrometry (Rodgers *et al.*, 1999). Analyses of UCMs have also been performed using GC (ASTM, 1990) and HPLC (Killops & Readman, 1985b) or combined methods such as GC-MS (Boehm *et al.*, 1997; Wang & Fingas, 1995; Wang *et al.*, 1999).

A recent analytical tool for the separation of complex mixtures is comprehensive two-dimensional GC (GC×GC). This powerful technique, introduced by Liu and Phillips (1991) combines two GC columns with different separation mechanisms: typically a primary column that separates compounds based on volatility coupled to a second short column that separates by polarity. The two columns are connected by a modulator, a device that traps, focuses and re-injects the peaks that elute from the first column into the second column (Fig. 1.3). Each peak eluting from the first column (which may be a number of co-eluting peaks) is further separated on the second column. The second separation is rapid, allowing the introduction of subsequent fractions from the first column without mutual interference. Dallüge *et al.* (2003) reviewed the principles, advantages and main characteristics of this technique. One of the main advantages is the very high

separation power, making the technique ideal for unravelling the composition of complex mixtures (Dallüge *et al.*, 2003). Another important feature of GC×GC is that chemically related compounds show up as ordered structures within the chromatograms, i.e. isomers appear as distinct groups in the chromatogram as a result of their similar interaction with the second dimension column phase (Phillips & Beens, 1999). The use of GC×GC for the characterization of complex petrochemical mixtures has been described previously (Beens *et al.*, 2000; Blomberg *et al.*, 1997; Schoenmakers *et al.*, 2000; Vendeuvre *et al.*, 2004; Vendeuvre *et al.*, 2005a; Vendeuvre *et al.*, 2005b). Applications of GC×GC coupled to Flame Ionisation Detection (FID) in the field of petrochemical environmental pollution include oil spill source identification (Gaines *et al.*, 1999), crude oil biomarker analysis (Frysinger & Gaines, 2001) soil remediation (Van De Weghe *et al.*, 2006) and characterisation of UCMs in petroleum-contaminated sediments (Frysinger *et al.*, 2003). Most research into petrochemical hydrocarbons using GC×GC has utilised FID and although attempts were made to use GC×GC with quadrupole mass spectral analysis (Frysinger & Gaines, 1999; Reddy *et al.*, 2002), the peaks eluting from the second dimension column were very narrow (typically 100–200 ms) and only a time-of-flight MS (ToF-MS) can deliver the high acquisition rates necessary for quantitative description of the peaks (van Deursen *et al.*, 2000). The use of GC×GC-ToF-MS raises the opportunity for direct analysis of tissue extracts from UCM-contaminated organisms without the need for multiple fractionations. The improved resolution of the GC×GC, together with the ToF-MS analysis, may enable identification of compound groups and hence allow toxicity testing of these compounds in order to determine which components of the UCM effect the health of marine organisms.

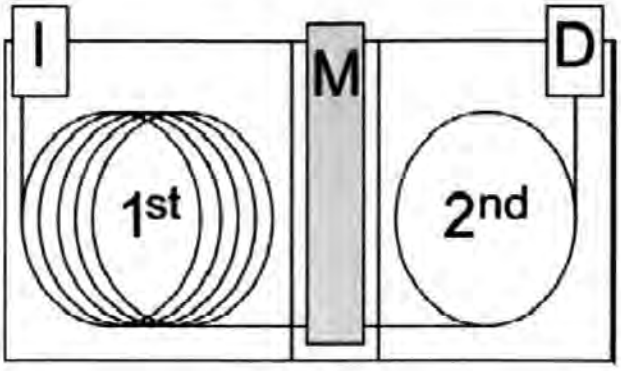
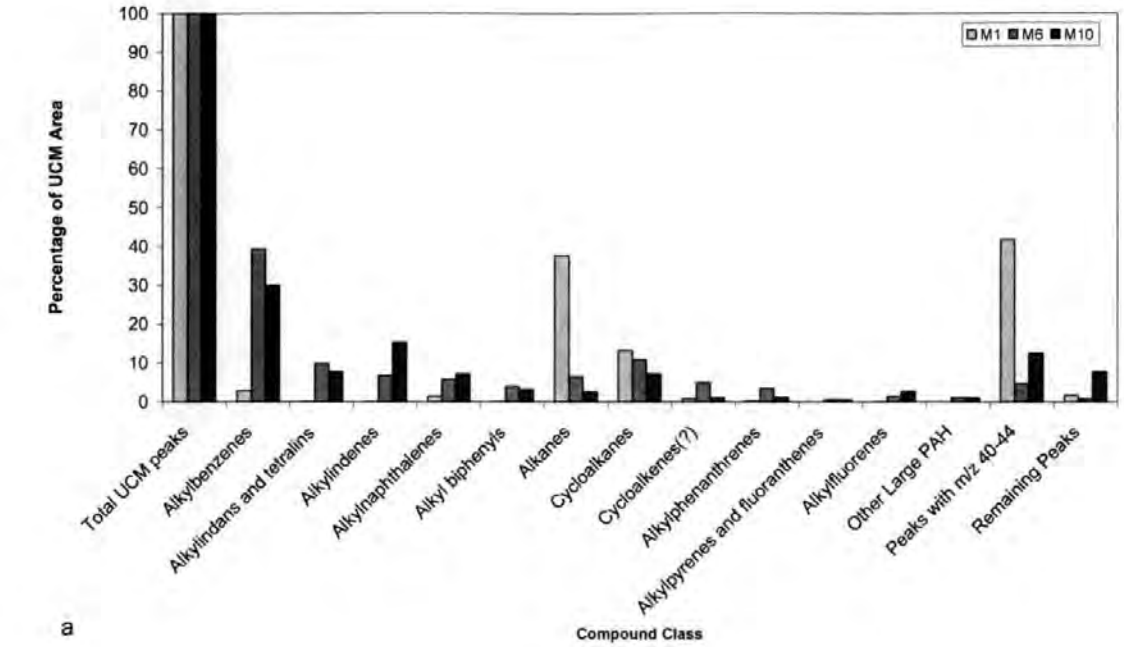


Figure 1.3 Schematic of a GC×GC system. I, injector; M, modulator; D, detector; 1st, GC oven with first-dimension column; 2nd, separate, GC oven with second-dimension column. Reprinted from Dallüge *et al.* (2003).

Using GC×GC, Frysinger *et al.* (2003), were able to fully resolve monoaromatic, diaromatic and multi-ring PAH compounds from UCMs and reported that UCMs may contain an array of alkanes, branched alkanes, cycloalkanes, monoaromatics, multi-ring aromatics, heteroatomic aromatics, steranes, and cyclic triterpenoids. It was also reported that the Wild Harbor River sediment UCM was comprised mainly of saturates including branched alkanes, alkylcycloalkanes and alkylbenzenes (~85 % by mass) and that it was possible to resolve many individual branched alkanes and cycloalkanes from the saturates fraction of the UCM. These branched isomers have very similar properties to each other and would normally elute close together using conventional GC thus appearing as part of the characteristic UCM 'hump'. Recent analysis by GC×GC-ToF-MS of UCMs extracted from the tissues of the blue mussel *Mytilus edulis* L, has shown that they contain a vast array of both known and unknown compounds (Booth, 2004; Booth *et al.*, 2006; Booth *et al.*, 2007). The comparative analysis of UCMs extracted from mussels known to possess high, moderate and low Scope for Growth (S*f*G, a measure of the capacity for growth and reproduction (**Chapter 2**: section 2.5)) revealed that alkylbenzenes represented the largest structural class within the UCM of mussels with low S*f*G; also, alkyltetralins, alkylindans

and alkylindenes were prominent in the stressed mussels (Fig. 1.4) and were absent only in mussels with high S*f*G.



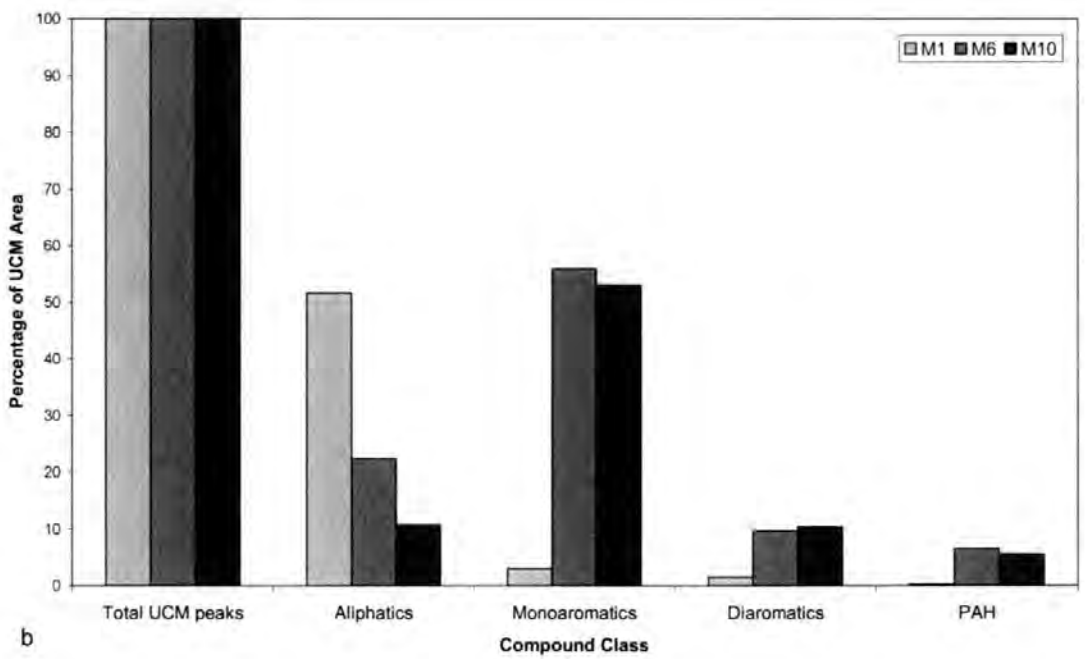


Figure 1.4 Structural classes of hydrocarbons (a) and more general classes (b) of compounds derived by tandem gas chromatography analysis of three UCMs that elicit high (M1), medium (M6) and low (M10) Scope for Growth (a measure of the capacity for growth and reproduction) in *Mytilus edulis*. Graphs and data republished from Booth *et al.* (2006).

1.3 Environmental concentrations of UCMs

Sediment aliphatic UCM concentrations in excess of 5000 μg g^{-1} have been reported in sediments from the gulf of Kuwait (Readman *et al.*, 1986) and Saudi Arabia (Readman *et al.*, 1996) but estuarine sediment concentrations rarely exceed 200 μg g^{-1} with coastal sediments mostly < 30 μg g^{-1} (Table 1.1). Aromatic UCM fractions within sediments are less frequently reported but generally do not exceed 50 μg g^{-1} other than in the gulf of Kuwait and Saudi Arabia (Readman *et al.*, 1996; Readman *et al.*, 1986) although concentrations in excess of 200 μg g^{-1} were reported by Colombo *et al.* (2005) in the Rio de la Plata Estuary, Argentina (Table 1.1). Total UCM sediment concentrations of about 8000 μg g^{-1} were measured in the vicinity of an oil spill site in West Falmouth, Massachusetts USA (Reddy *et al.*, 2002). The UCM of hydrocarbons within sediments may not be readily bioavailable to organisms but the UCM tissue burden has been reported in a number of species (Table 1.2) suggesting that many UCM compounds are available for uptake by marine organisms. Experiments conducted in the vicinity of the oil platform site L-4-a in the North Sea showed that translocated mussels (55 days exposure) bioaccumulated up to 190 μg g^{-1} UCM (ash-free dry weight) during drilling operations that released 'low-tox' oil-based muds. Mussels placed up to 5 km away still possessed detectable UCM concentrations within their tissues (Bedborough *et al.*, 1987; van het Groenewoud *et al.*, 1988). A further experiment the following year (1987) after termination of drilling revealed that only mussels at the platform site had significantly elevated concentrations of UCM. Most of biota tissue concentrations reported (examples are provided in Table 1.2) are for bivalve molluscs, although burdens of around 2500 μg g^{-1} within macrophytes were reported in the Rio de la Plata Estuary, Argentina (Colombo *et al.*, 2005) and up to 210 μg g^{-1} aromatic UCM reported in fish from the Arabian Gulf (Fowler *et al.*, 1993). Aliphatic UCM tissue concentrations in excess of 3000 μg g^{-1} have been reported in mussels from Whitby, UK (Rowland *et al.*, 2001) and aromatic UCMs of

around 500 μg g^{-1} have been found in mussels, clams and macrophytes (Colombo *et al.*, 2005; Rowland *et al.*, 2001). Although relatively few studies give detailed concentrations of UCM hydrocarbons within sediment or biota, others provide total hydrocarbon and/or aliphatic concentrations and many comment that a UCM was present or dominated the sample (Tables 1.1 and 1.2).

The source of hydrocarbons in environmental samples can sometimes be diagnosed from assessment of the types of hydrocarbons present. Although the presence of a UCM in hydrocarbon chromatograms is considered to be linked to weathered petroleum contamination (LeDreau *et al.*, 1997; Readman *et al.*, 2002), smaller contributions (low μg g^{-1} dry weight) may relate to bacterial reworking of sedimentary organic matter from weathering of ancient rocks (Readman *et al.*, 2002; Volkman *et al.*, 1992). The ratio of unresolved to resolved compounds has been used as a diagnostic criterion of anthropogenic inputs (Bouloubassi *et al.*, 2001; Readman *et al.*, 2002), and significant contamination by petroleum products assumed to have ocurred when the ratio is ≥ 2 according to Simoneit (1986) or ≥ 4 according to Lipiatou and Saliot (1991). The Carbon Preference Index (CPI) is another useful indicator of biogenic sources of hydrocarbons i.e. a CPI ratio > 4 together with a dominance of *n*-alkanes with more than 24 carbon atoms indicates a source of *n*-alkanes from terrestrial plants whereas a CPI ratio closer to 1 together with a predominance of *n*-alkanes with less than 24 carbon atoms often provides evidence of contamination from petrogenic sources (Hong *et al.*, 1995; Zheng & Richardson, 1999). A further indicator of the origin of environmental hydrocarbons is the ratio of isoprenoids pristane (2, 6, 10, 14 – tetramethylpentadecane) and phytane (2, 6, 10, 14 –tetramethylhexadecane, Fig. 1.1). In sediments uncontaminated by petrogenic hydrocarbons the pristane:phytane ratio (pr:ph) is higher than 1 and typically between 3 and 5 (Steinhauer & Boehm, 1992). From the examples of sediment hydrocarbon data presented in Table 1.1, it is apparent that sites with

high UCM concentrations typically have CPI and Pr:Ph values consistent with petrogenic sources; one exception to this is the data from the Rhone prodelta, France, where UCM concentrations of 76 – 275 $\mu g\ g^{-1}$ were reported with CPI and Pr:Ph values of 1.6 – 5.5 and 2.7 – 5.5 respectively (Tolosa *et al.*, 1996); this suggests that a non-petrogenic input may, at least in part, be the source of the UCM.

Contamination of the marine environment by UCM hydrocarbons would appear to be common, with localised high concentrations within both sediments and tissues. The reporting of the UCM hydrocarbon concentrations has tended to be used to provide evidence for petrogenic sources of the contamination. Toxicity studies associated with these reports have mainly been concerned with the effects of PAHs which are known to be toxic to a range of marine species. It has sometimes been reported that the observed adverse effects cannot be explained by the measured concentrations of known contaminants and some authors (e.g. Martins *et al.*, 2005) have suggested that the high UCM hydrocarbon concentrations may contribute to the observed effects.

1.4 Toxic effects & modes of action of petroleum hydrocarbons

Numerous studies have investigated the effects of hydrocarbon contamination of the marine environment, including effects on bacteria, micro and macro algae, angiosperms, invertebrates and vertebrates (reviewed by NRC, 2003). All phyla have been shown to suffer adverse effects resulting from exposure to hydrocarbons. The acute and chronic toxicity of petroleum hydrocarbons to marine biota has been shown to be dependent upon:

- The persistence and bioavailability of specific hydrocarbons.
- The concentration and length of exposure.
- The ability for organisms to accumulate and metabolise hydrocarbons and the fate of the metabolised products.
- The interference of specific hydrocarbons, or metabolites, with normal metabolic processes.
- The specific narcotic effects of hydrocarbons on neurotransmission (NRC, 2003).

Early investigations of crude and refined oil toxicity focussed on the acute effects of high oil concentrations upon marine organisms. From these early studies it was found that the acute toxicities of individual hydrocarbons were mainly related to their water solubility and that the toxicity of a specific oil type was the result of additive toxicity of individual compounds, especially aromatic compounds (Peterson, 1994). An important component of acute toxicity is the narcotic effects of individual compounds and this is strongly related to low molecular weight volatile compounds (Donkin *et al.*, 1989). BTEX are highly volatile and are therefore rapidly removed from the marine environment but they are also relatively soluble in seawater and may come into contact with biota long enough to cause acute toxicity. BTEX are generally neurotoxic as they are rapidly absorbed and distributed within organisms' lipid-rich and vascular tissues where they induce physical and chemical

changes to nervous system membranes (Ritchie *et al.*, 2001). Benzene has also been found to be carcinogenic to mammals, including humans, *via* the action of active metabolites (reviewed by Schnatter *et al.*, 2005). With the removal of BTEX compounds from the water column by weathering processes, the acute toxicity of the water-accommodated fraction (WAF) of hydrocarbons is reduced (Neff *et al.*, 2000). Thus PAHs become more important contributors of toxicity of weathered oils.

Anthropogenic inputs of PAHs into the marine environment may occur from petrogenic sources or from the incomplete combustion of fossil fuels. Contamination from petrogenic sources tends to result in a relatively large fraction of two and three ring PAHs compared to pyrolytic sources that produce a greater fraction of the higher molecular weight compounds (Neff, 1979). The USEPA has designated 16 toxic compounds as priority pollutant PAHs. These range from the highly water-soluble two-ring naphthalene, (molecular weight 128, water solubility 31 mg L^{-1} (EPISuite™, 2000)) to the relatively insoluble six-ring, benzo(ghi)perylene (molecular weight 276, water solubility 0.00026 mg L^{-1}(EPISuite™, 2000)). The higher solubility of the lower molecular weight compounds makes them more bioavailable to marine organisms as these compounds are more likely to exist in true solution rather than adsorbed to particles. Consequently, naphthalene has been shown to cause acute sublethal narcotic toxicity (Donkin *et al.*, 1991). Additional factors that may alter the toxicity of petroleum hydrocarbons are photodegradation and photoactivation (Boese *et al.*, 1999; Garrett *et al.*, 1998; Little *et al.*, 2000; Mallakin *et al.*, 1999). Sublethal effects following acute or chronic exposure to petroleum hydrocarbons include: disruption to energetic processes; interference with biosynthetic processes and structural development; and direct toxic effects on developmental and reproductive stages (Capuzzo *et al.*, 1988).

The toxic effect of petroleum hydrocarbons can be observed at four levels of biological organisation (NRC, 2003):

1. Biochemical and cellular.
2. Organismal, including the integration of physiological, biochemical and behavioural responses.
3. Population, including alterations in population dynamics.
4. Community, resulting in alternations to community structure and dynamics.

In a review of the biological effects of petroleum hydrocarbons, Capuzzo (1987) reported that data gathered following oil spills during the 1970s and 1980s showed that the medium and higher molecular weight aromatic compounds, such as the alkylated phenanthrenes and alkylated dibenzothiophenes, were among the most persistent compounds in both animal tissues and sediments although the degree to which these body burdens interfere with normal metabolic processes has been the focus of much debate and research (NRC, 2003). Sublethal effects of hydrocarbons can occur at much lower concentrations than those which induce acute responses (Donkin *et al.*, 1991; Lotufo, 1997) and can result in impairment of feeding mechanisms, growth rates, energetics, reproduction, recruitment rates and increased susceptibility to disease and other histopathological disorders (Capuzzo, 1987). Organisms may be particularly vulnerable to exposure to hydrocarbons during the early developmental stages (Heintz *et al.*, 1999) and chronically contaminated sediments may result in recruitment failure - although life-history traits of benthic organisms may contribute to slow recovery of populations following a spill (Dauvin, 2000; Dauvin & Gentil, 1990; Sanders *et al.*, 1980).

In a study of the toxicity of three environmentally-weathered middle distillate oils differing in aromatic hydrocarbon content, it was demonstrated that the oil with the lowest aromatic

hydrocarbon content had the greatest toxicity. Also, the toxicity of the three weathered oils was consistent with the reported toxicity of unweathered oils of similar type under similar conditions and was more similar to one another when reported as total petroleum hydrocarbons than as total PAHs or total naphthalenes. As a consequence, Barron et al. (1999) concluded that heterocyclic compounds and other soluble compounds within the WAF may contribute to the observed toxicity. This is consistent with the theory of a narcosis mechanism of toxicity (non-specific toxicity) in which all soluble components of oil bioaccumulated in aquatic organisms contribute to toxicity (Barron *et al.*, 1997; Peterson, 1994).

Most of the toxicity studies concerned with petroleum hydrocarbons have been focussed on components accommodated within the water column as these are more readily bioavailable to aquatic organisms (Barron *et al.*, 1997; Peterson, 1994). Non-polar organic chemicals including many petroleum hydrocarbons have a low aqueous solubility and high lipid solubility. In aqueous solution, hydrocarbons diffuse down a fugacity gradient from the water phase into lipid-rich tissues of marine biota (Mackay, 1979). According to equilibrium partitioning theory (Bierman, 1990), non-polar organic chemicals dissolved in water will partition across permeable membranes into the tissues of aquatic organisms until equilibrium, approximated by the octanol/water partition coefficient (K_{ow}), is reached. When a critical concentration is achieved, toxic responses within the organism occur (McCarty & Mackay, 1993). Although the log K_{ow} of PAHs increase with molecular weight (Neff & Burns, 1996) the relationship between PAH log K_{ow} and bioavailability, measured as log bioconcentration factor (BCF), is not linear (Baussant *et al.*, 2001b). For sediment-associated hydrocarbons the sediment organic carbon-water coefficient (K_{oc}) is useful for predicting uptake into organisms (Di Toro *et al.*, 2000). Organic carbon content may influence uptake of organic compounds by biota due to the strong sorption affinity of

contaminants to the sediment organic carbon matrix (Gunnarsson *et al.*, 1999). High molecular weight PAHs may be less bioavailable than predicted by equilibrium theory due to limitations on uptake rates by organisms, their lower solubility in tissue lipids and rapid metabolism by some species (NRC, 2003). However, a study by Spehar *et al.* (1999), cited by Di Toro & McGrath (2000), of the toxicity of 13 PAHs with log K_{ow} values > 5.3 showed that toxicity can occur by additive narcosis at tissue concentrations predicted by a target lipid model even with PAHs above the log K_{ow} range of the model (Di Toro & McGrath, 2000).

Most laboratory studies concerned with hydrocarbon toxicity have focused on acute effects arising from aqueous exposure however wild organisms may be subject to longer-term chronic exposures. Chronic toxicity may be defined as either the effects of long-term and continuous exposure to a toxicant or the long-term sublethal effects of acute exposure (Connel & Miller, 1984). The chronic toxicity of petroleum hydrocarbons may arise from continuous exposures such as from ongoing seepage from oil platforms or from persistent fractions following an oil spill. Alterations in bioenergetics and growth of bivalve molluscs following exposure to petroleum hydrocarbons appear to be related to tissue burdens of specific aromatic compounds (Donkin & Widdows, 1990; Donkin *et al.*, 1989; Gilfillan *et al.*, 1977; Widdows *et al.*, 1982; Widdows *et al.*, 1987). Widdows *et al.* (1982) reported a negative correlation between cellular and physiological stress indices (lysosomal properties and S*f*G) and mussel tissue concentrations following long-term exposure to low concentrations of North Sea crude oil. Depuration of aromatic hydrocarbons from tissue resulted in recovery of mussels (Widdows *et al.*, 1987). Donkin *et al.* (1989) suggested that depression in mussel S*f*G was related to the accumulation of two- and three-ring aromatic hydrocarbons as these compounds induced a narcotising effect on the ciliary feeding mechanism. The toxicity of subtidal sediment following a spill of No. 2 fuel oil

from the barge *North Cape* was investigated by Ho *et al.* (1999): toxicity to the amphipods *Ampelisca abdita* declined with decreasing concentrations of PAHs over a six month period. In urban areas where there are numerous sources of hydrocarbon inputs, PAHs and other hydrophobic hydrocarbons may accumulate and persist within sediments.

The processes controlling the uptake and persistence of PAHs in marine biota, with particular regard to chronic exposure and highlighting differential mechanisms of uptake, tissue distribution and elimination, was reviewed by Meador *et al.* (1995). The transfer of contaminants to marine organisms and toxicological effects on the ecosystem are dependent on the availability and persistence of the contaminants within benthic environments. Bioaccumulation of lipophilic compounds is influenced by chemical factors such as solubility and particle adsorption-desorption kinetics of specific compounds and biological factors such as the transfer of compounds through food webs, the quantity of lipid in exposed organisms and metabolic processes. The possession of tumours and other histopathological disorders in benthic fish from hydrocarbon-contaminated areas suggest a link between the concentrations of lipophilic contaminants (including PAHs) and the increased incidence of histopathological disorders (Khan, 2003; Lyons *et al.*, 2004; Neff & Boehm, 1985; Reynolds *et al.*, 2003). Additional sublethal effects resulting from chronic hydrocarbon exposure include impairment of physiological processes that may alter the energy available for growth and reproduction, measurable by S/G analysis (e.g. Widdows *et al.*, 1990; Widdows *et al.*, 1995) and other effects on reproduction, developmental processes including direct genetic damage and suppression of immune response (Capuzzo, 1987; Capuzzo *et al.*, 1988; Galloway & Depledge, 2001). Alterations to reproductive and developmental potential of organisms can lead to population or ecosystem level effects but demonstrating causality between contamination and perturbations within marine communities is challenging for researchers. It was argued by Cairns (1983) that the

detection of toxic effects at higher levels of biological organisation is limited by the lack of reliable predictive tests at population, community and ecosystems levels. Kooijman & Metz (1984) suggested that the sublethal effects of contaminant exposure should be interpreted in the light of the survival probabilities and reproductive success of populations, thus bridging the gap between individual and population responses.

Many of the adverse effects associated with exposure to higher molecular weight PAHs are due to biotransformation of the parent compounds by metabolic processes, resulting in toxic products. Vertebrates have a high capacity for metabolising aromatic hydrocarbons, including PAHs, through cytochrome P450 1A mediated oxidation (Stegeman *et al.*, 1992) but although there is a large literature that links elevated P450 1A levels in fish, many additional compounds can also induce these enzymes. Metabolism of hydrocarbons may result in excretion of some compounds but also activation to form toxic metabolites which can then form DNA adducts through reaction with DNA (Wirgin *et al.*, 1994). Metabolites can also react with cells active within the immune system leading to immunotoxic responses (Davila *et al.*, 1996). There are three principal pathways currently proposed for metabolic activation of PAHs: (i) *via* bay region dihydrodiol epoxide by cytochrome P450 enzymes (CYPs), (ii) *via* radical cation by one-electron oxidation, and (iii) the *ortho*-quinone pathway dihydrodiol dehydrogenase (reviewed by Xue & Warshawsky, 2005). Invertebrate species lack the arylhydrocarbon receptor (AhR), a ligand-dependent transcription factor that is believed to mediate many of the toxicological effects of PAHs in vertebrates, but recent studies have suggested the presence of AhR homologues in a wide range of invertebrates, including nematodes, insects and gastropods and hence hydrocarbons may affect the immune response of a wide diversity of species (Coteur *et al.*, 2001; Galloway & Depledge, 2001; Weisner *et al.*, 2003). Another hydrocarbon toxicity model based on reports of high early life-stage toxicity in fish embryos exposed to

alkylphenanthrenes substituted with two to four carbons (Brinkworth *et al.*, 2003) has gained further support from an evaluation of toxicity models of chronic embryonic exposure to complex PAH mixtures (Barron *et al.*, 2004). Sublethal effects caused by PAH embryonic exposure include edema of the yolk sac and pericardium, haemorrhaging, disruption of cardiac function, mutations and heritable changes in progeny, spinal deformities, neuronal cell death, anaemia, reduced growth, and impaired swimming (Barron *et al.*, 2004; Barron *et al.*, 2003; Brinkworth *et al.*, 2003; Incardona *et al.*, 2004; White, 2002). Such effects are inconsistent with the narcosis mechanism of action which is characterised by central nervous system depression and rapidly reversible effects. Alkylation of phenanthrene has been reported to increase the toxicity compared to the parent compound by over 100 times (Hawkins *et al.*, 2002) and tissue concentrations as low as 0.15 $\mu g\ g^{-1}$ have been found to cause early life-stage toxicity in 100 % of trout exposed as embryos (Brinkworth *et al.*, 2003). The mechanism of alkylphenanthrene toxicity is unknown but may occur from oxidative stress and effects on cardiovascular morphogenesis (Brinkworth *et al.*, 2003; Incardona *et al.*, 2004).

From the many studies concerned with the effects of exposure to petrogenic hydrocarbons, a great wealth of knowledge has been accumulated with regard to toxicities of individual compounds to individual species which has allowed the effects of oil contamination to be modelled (e.g. French-McCay, 2002). Such models rely heavily on studies utilising acute toxicity tests; fewer studies have investigated the long term effects of exposure. The majority of studies have related effects to aqueous concentrations rather than to tissue concentrations and studies involving depuration are in the minority. *In-situ* studies provide a much greater environmental realism (reviewed by Chappie & Burton, 2000) but are difficult to maintain under strictly controlled conditions. Community studies of wild habitats (e.g. Guerra-Garcia *et al.*, 2003) can highlight differences in community structure

between contaminated and clean sites but it is often difficult to establish causality with a high degree of confidence. Chronic tests under controlled laboratory conditions provide a valuable link between effects observable on individuals and those at the population or community level. Bioavailability of PAHs from sediments and food is less than that from solution (Pruell *et al.*, 1986) so investigations into the long term effects associated with sediment exposure are particularly useful.

The potential for hydrocarbons to transfer from one trophic level to another has received very little attention. Trophic transfer has not been well studied generally, although there have been a few investigations into the trophic transfer of metals (Seebaugh *et al.*, 2005; Wallace & Lopez, 1996; Wallace & Luoma, 2003). The limited evidence available indicates that trophic transfer of PAHs is not important. For example, Albers' (2006) study into PAHs in birds concluded that trophic transfer was unlikely. A recent study by Wan *et al.* (2007) in which 18 PAHs in phytoplankton, zooplankton, five invertebrate species, five fish species, and one seabird species were analysed from Bohai Bay, China, demonstrated trophic transfer of the PAHs in the food web. The results indicated that PAHs undergo trophic dilution in the marine food web; the authors suggested that this was likely due to low assimilation efficiencies and efficient metabolic transformation at higher trophic levels. In contrast, the results of a pilot study by Rice *et al.* (2000) suggest that uptake of hydrocarbons *via* the food web may be important. In this study, polychaete worms were exposed for 28 days to clean sediments spiked with the PAH benzo(a)pyrene (BaP) and field sediments collected from two sites in Puget Sound, Washington, contaminated predominantly with PAHs and chlorinated compounds. Exposed worms were then fed live to predatory juvenile flatfish, English sole (*Pleuronectes vetulus)* for 10 or 12 days. Growth was reduced in all but one of eight groups of fish fed contaminant-exposed worms. Juvenile fish from all contaminant-exposed groups showed increased expression of

CYP1A, and fish exposed to BaP-exposed worms showed clear evidence of hepatic PAH-DNA adducts. Interestingly, the results suggested that sediments deemed to be nontoxic by invertebrate bioassays have the potential to cause adverse effects at higher trophic levels. The paucity of data concerning trophic transfer does not permit confident prediction of how consumption of hydrocarbon contaminated organisms will affect predatory species and deserves further study.

1.5 Potential toxicity of UCM hydrocarbons

Although numerous studies have investigated the effects arising from exposure to compounds resolved by GC, very few studies have examined the potential for toxic effects arising from exposure to the unresolved components present within weathered oils. The UCM often represents the majority of compounds within sediments and tissues contaminated by hydrocarbons but insufficient data exists to evaluate the risk to marine biota. In order for monitoring and regulatory agencies to predict effects and set maximum exposure guidelines, it is necessary to establish:

- If UCM components can elicit adverse biological effects and, if so, which components are most important
- Whether UCM components within the environment are sufficiently bioavailable to be readily taken up be organisms and, if so, whether they are depurated or continue to accumulate.
- If effects on individuals can impact populations and communities.

The potential for UCMs in the environment to be toxic to marine biota was first highlighted by Rowland *et al.* (2001) in an investigation into the effect of the monoaromatic hydrocarbon fraction from a weathered Gullfaks (North Sea) crude oil upon the feeding rate of the blue mussel, *M. edulis*. Feeding rate (aka clearance rate) is a major component within S*f*G and is therefore a meaningful measure of an organism's capacity for growth and reproduction (see **Chapter 2**, Section 2.5). Rowland *et al.* (2001) reported that feeding rate was significantly reduced in mussels in all exposure concentrations i.e. ≥ 50 $\mu g\ L^{-1}$ corresponding to approximately ≥ 15 $\mu g\ g^{-1}$ wet tissue weight (~ 60 $\mu g\ g^{-1}$ dry weight) and the highest nominal exposure concentration of 200 $\mu g\ L^{-1}$ (90 $\mu g\ g^{-1}$ wet weight; ~ 350 $\mu g\ g^{-1}$ dry weight) resulted in a feeding rate reduction of over 40 %. The body burden of UCM hydrocarbons within wild mussels from the east coast and Cornish

coast of the UK was also measured by Rowland *et al.* (2001): concentrations were in the range 271 to 3975 $\mu g\ g^{-1}$ dry weight for east coast mussels compared to a maximum of 9 $\mu g\ g^{-1}$ for mussels from an unpolluted Cornish site (Table 1.2). Mussels previously collected from the east coast sites were found to have reduced S/G (Widdows *et al.*, 1995); this had been attributed to PAHs but further analysis by Rowland *et al.* (2001) suggested that the impaired health was likely, in part, due to the high tissue burden of aromatic UCM.

Rowland *et al.* (2001) focused on the aromatic fraction of the UCM as Thomas *et al.* (1995) had found that an aliphatic UCM isolated from a base stock of Silkolene-150 lubricating oil did not elicit an acute toxic effect upon *M. edulis* following 24 h aqueous exposure, unless the UCM was oxidised using chromium (V1) oxide-acetic acid (i.e. oxidation increased the solubility and bioavailability of components within the oil). The lack of acute toxicity of the aliphatic UCM fraction may have been due to its low solubility (Thomas *et al.*, 1995) but this does not exclude the possibility of chronic toxic effects due to long term exposure to bioavailable components. In a study of macrobenthic assemblages within sediments contaminated by aliphatic hydrocarbons with a dominant UCM component (Guerra-Garcia *et al.*, 2003), a relationship was found between the distribution of marine organisms and the presence of high concentrations of asphaltenes. It is possible however, that the altered macrobenthic assemblage in the presence of high concentrations of asphaltenes could be related to physical rather than chemical effect since these compounds have a thick and viscous character (Guerra-Garcia *et al.*, 2003).

Compelling evidence for the ability of UCMs to elicit a non-specific narcotic toxic response was provided by Donkin *et al.* (2003) in a study to determine whether UCMs accumulated by mussels in the field were toxic. The study involved the transplantation of

mussels from clean to contaminated locations and used an effect-directed fractionation approach. Evidence for UCM toxicity provided by Donkin *et al.* (2003) included:

1. Mussels from oil-contaminated sites showed reduced clearance rates but this improved following a period of depuration.
2. The water in which the oil-contaminated mussels had depurated was found to contain a UCM.
3. Steam distillation extracts of mussel tissues that contained UCMs were shown to be toxic to juvenile mussels as determined by reduction in clearance rate (distillates from clean site mussels did not elicit a response).
4. A monoaromatic fraction derived by HPLC fractionation of tissue extracts reduced mussel clearance rates by 70 % (additional aromatic fractions also produced smaller depressions but these were only significant at the 90 % confidence level).
5. The resolved component in the toxic extracts was very low relative to that of the total UCM compounds.

Taken as a whole, the study provides compelling evidence for UCM toxicity. Although the study does not provide concentrations of specific resolved fractions which may have contributed towards the observed toxicity, the fraction that caused the greatest toxic response comprised mainly aromatic UCM hydrocarbons with about four to six double bond equivalents (Donkin *et al.*, 2003) and therefore any contribution by the resolved fractions would appear to be minimal. Smith *et al.* (2001) suggested that among the possible compounds in hydrocarbon UCMs were disubstituted alkyltetralins; these were shown to have oxidation products (Thomas *et al.*, 1995), aqueous solubilities, sublethal narcotic toxicities and a resistance to biodegradation, consistent with some aromatic hydrocarbon UCM fractions (Smith *et al.*, 2001). Disubstituted cyclic alkyltetralins (Fig.

1.5) have six double bond equivalents and elute in the same HPLC retention time window as the most toxic fraction found by Donkin *et al.* (2003) but these cyclic alkyltetralins have not been shown to be present within environmental UCMs to date (Booth, 2004).

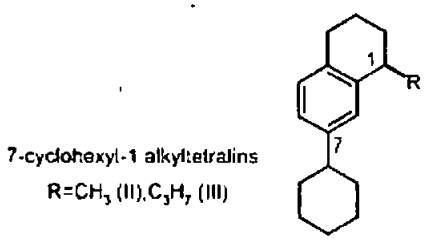


Figure 1.5 Example of disubstituted cyclic alkyltetralin. Reproduced from Smith *et al.* (2001).

Scope for Growth (S*f*G) has been used as an integrated measure of sublethal stresses on mussels (Smaal & Widdows, 1994) and should translate into population and community level effects (Bayne, 1985) but this has rarely been directly tested. Hence the reduced S*f*G attributed to UCMs (Donkin *et al.*, 2003) may not necessarily cause impacts at higher levels of organisation. This question was however addressed by Crowe *et al.* (2004) in a study linking physiological measurements (S*f*G of mussels), the diversity of macrofaunal communities associated with mussels and the UCM body burdens. Possible confounding factors were also investigated. The study compared three sites at which mussels exhibited low S*f*G with three clean sites with mussels with high S*f*G. Biodiversity was reduced within mussel beds with low S*f*G and high UCM concentrations. Although there was generally a good relationship between S*f*G and UCM concentration, mussels from one site with low S*f*G had a low aliphatic UCM and the aromatic fraction was not detected; the authors suggested that the low S*f*G in the mussels may have been due to a local sewage input rather than to UCM hydrocarbon pollutants. Although contaminants other than the UCM may have been present, this study does suggest that UCMs are, at least in part, responsible for impaired health of wild mussels and have effects at the community level for associated macrofauna.

Culbertson *et al.* (2007) conducted an interesting study into the behaviour and feeding rate of fiddler crabs *Uca pugnax* (S. I. Smith, 1870) resident in marsh sediments contaminated with oil from the Florida barge spill in 1969. Although the authors related biological effects to sediment concentrations in terms of total petroleum hydrocarbons (TPH), the vast majority of this was UCM hydrocarbons when analysed by conventional GC (Reddy *et al.*, 2002). It was found that crabs from oiled sites avoided burrowing into oiled layers, had delayed escape responses and reduced feeding rates. This altered behaviour, which make the crabs more vulnerable to predation, together with the lowered physiological condition, may have been responsible for the lower densities of crabs found within the UCM hydrocarbon contaminated site (Culbertson *et al.*, 2007).

Of the very limited number of studies that have reported UCM hydrocarbon concentrations within animal tissues (Table 1.2), most were reports of bioaccumulation within bivalves. Partition theory (Bierman, 1990) suggests that some UCM hydrocarbons would accumulate from the water to the lipid-rich tissues of bivalves but whether the consumption of contaminated bivalves would provide an alternative route of uptake with consequential potential for toxic effects is unknown. For PAHs, bioaccumulation *via* trophic transfer is thought to be of limited importance and biomagnification does not appear to occur due to the ability of higher organisms to metabolise and excrete the compounds (Bierman, 1990; NRC, 2003). Highly lipophilic compounds (e.g. organochlorines) with log $K_{ow} > 6.3$ were found by Russell *et al.* (1999) to strongly biomagnify but compounds with $K_{ow} < 5.5$ did not show any such tendency. The highly alkylated structures of compounds found within the UCM of some mussels (Booth *et al.*, 2007) may be less readily metabolised and excreted than the PAHs and hence may transfer to higher trophic levels. Due to the paucity of literature concerning trophic transfer of

hydrocarbons and the limited reports of UCMs within biota, the capacity for UCM hydrocarbons to transfer to higher trophic levels, and possibly biomagnify through the food web cannot be assessed without experimentation.

Thomas *et al.* (2002) in a study of potentially genotoxic compounds from a number a UK estuaries found that extracts from sediments with dominant UCMs elicited a positive response using the Mutatox™ bioassay. Mutatox is a commercially available mutagenicity assay using a dark variant strain of marine bioluminescent bacteria. When exposed to mutagenic compounds the bacteria revert to the normal genotype and emit light. The presence of a UCM has also been reported in mutagenic fractions obtained from marine sediments (Grifoll *et al.*, 1988; Ho & Quinn, 1993) although, as with the UK estuary sediments (Thomas *et al.*, 2002), the contribution towards the mutagenic activity of the UCM was unknown. Fractionation of sediment extracts from Hebburn (Tyne, UK) showing mutagenic activity revealed the presence of alkyl substituted two-ring PAHs with an underlying UCM (Thomas *et al.*, 2002). Alkylated polycyclic aromatic hydrocarbons have also been found within UCM fractions (Frysinger *et al.*, 2003; Thomas, 1995) but little information exists concerning their mutagenic activity. Individual alkylated PAHs have been observed to have potentially mutagenic, tumour-promoting, or carcinogenic activity (Baird *et al.*, 2007) but, with the exception of 1- and 2-methylnaphthalene, insufficient toxicity data are available to quantify the mutagenic risk from exposure to individual alkylated PAHs or mixtures of alkylated PAHs.

Reineke *et al.* (2006) studied the sublethal toxicity (clearance rates of *M. edulis*) of the monoaromatic and total aromatic hydrocarbon fractions of two pairs of undegraded and moderately biodegraded crude oils from the Santa Maria basin (California) and the Vienna basin (Austria), all of which were dominated by UCM hydrocarbons. The total aromatic

and monoaromatic hydrocarbon fractions from sulphur-rich (~3.1 %) Monterey Formation crude oils (California) were found to be slightly more toxic than the fractions isolated from sulphur-lean Vienna basin oils. The total aromatic hydrocarbon fractions of the two Monterey oils reduced mussel clearance rates by *ca.* 50 % compared to control mussels. The majority of mussels accumulated tissue concentrations of up to approximately 50 $\mu g\ g^{-1}$ wet weight (~300 $\mu g\ g^{-1}$ dry weight) oil within 24 h exposure. Some mussel groups exposed to the aromatic fraction of undegraded Monterey Formation oil accumulated up to 177 $\mu g\ g^{-1}$ wet weight (~1000 $\mu g\ g^{-1}$ dry weight), but this did not lead to a further significant reduction of clearance rate. Reineke *et al.* (2006) extracted whole tissues but Wraige (1997) showed that gill tissue concentrations correlate better with the reduction of clearance rate than total body burdens, especially at high total body burden values. This is consistent with the assumption that the gills (the presumed site of toxic action) have a certain capacity for lipophilic compounds which cannot be exceeded. The mussel clearance rate tests did not show any significant differences in toxicity of aromatic compounds from undegraded or in-reservoir biodegraded crude oils from the same oilfield. The authors suggested that organic sulphur compounds were the cause of the slightly higher toxicity of the aromatic hydrocarbon fractions from the Monterey oils.

Recent research into the effects of the unresolved complex mixture (UCM) of petrogenic oils in the marine water column has identified a number of effects arising from acute exposure to the water-soluble-fraction (WSF) of UCM-rich oil from the Troll oilfield in the North Sea (Hokstad *et al.*, 2007). The research used a battery of tests to explore the toxicity of water-soluble components of the UCM. Having created WSFs of UCM-rich and UCM-poor oils by slow vortex mixing with water, the WSFs were solvent extracted, concentrated, then separated into 14 fractions, based on polarity, using HPLC. The battery of toxicity test endpoints, arising from acute exposure to a primary culture of rainbow trout

(*Oncorhynchus mykiss*) hepatocytes, included: metabolic inhibition and cytotoxicity, DNA-damage and sub-lethal biomarker responses for planar dioxin-like chemicals (7-ethoxyresorufin *O*-deethylase activity (EROD)) and environmental estrogens (vitellogenin induction). In addition, Acetylcholine Esterase inhibition (AchE) was determined in purified AchE from electric eel (*Electrophorus electricus*), and toxicity towards a number of algae species was determined using fluorescence measurements. Hokstad *et al.* (2007) reported that a polar fraction (F11), containing mostly UCM hydrocarbons, was associated with the highest effect levels in most bioassays, including EROD activity, estrogenecity, cytotoxicity, and DNA damage. The only other fraction (F1) to show similar metabolic inhibition contained non-polar compounds. Chemical analysis of the fractions showed that decalins, phenols, naphthalenes and PAHs were associated with non-polar and medium-polar fractions (fractions 1- 7). In contrast, the only compounds detected in fraction 11 of significance were reported to be C_1-C_3 dibenzothiophenes and C_2-fluorenes. Fraction 11 contained the greatest mass of material i.e. >70 % by gravimetric analysis. In order to test if known compounds could be responsible for the observed toxicity, synthetic mixtures of decalins, naphthalenes, PAHs and phenols, representing the concentrations of individual compounds found in the non-polar, medium-polar and polar HPLC-fraction (fractions 1, 6 and 11, respectively) were prepared and analysed in the trout hepatocyte bioassays. Metabolic inhibition and estrogenecity were found to be associated with fraction 6 (rich in alkylated phenols). EROD activity was not detected in any of the fractions. From this the authors concluded that the predominant toxicity of fraction 11 was associated with the unresolved compounds and not with 'well-known' WSF-associated compounds. Analysis of fraction 11 by GC×GC-ToF-MS identified sulfoxides as a notable group of compounds (Booth, personal communication). Little is known of toxicities of sulfoxides, although Seymour *et al.* (1997) reported that dibenzothiophene sulfoxide inhibited bacterial bioluminescence (Microtox® assay). Although the toxicity of the sulfoxide was found to

be considerably less than that of the parent compound (dibenzothiophene),the sulphoxide was more soluble and hence would be more bioavailable. The implications for marine biota of toxic compounds from within the polar fraction are difficult to quantify. Clearly, these compounds are bioavailable, but unless present in the water column at sufficient concentrations to cause acute effects, they may not bioaccumulate, unlike lipophilic compounds and therefore may not cause longer-term effects. As well as the WSFs of oils, Hokstad *et al.* (2007) also tested a number of hydrocarbon groups for cytotoxicity in the trout hepatocyte bioassay; these included alkylated branched compounds of tetralins, benzenes, pentanoic acid and naphthenic acids. It was found that cytotoxicity occurred at low concentrations for all analysed compounds, except the branched alkylbenzenes. Although the study by Hokstad et al. (2007) demonstrated the potential for a UCM water-soluble components to elicit a range of effects, the implications for marine biota are less clear as concentrations may not have been environmentally realistic.

The foregoing rather small number of studies into UCM toxicity (viz. Donkin *et al.*, 2003; Hokstad *et al.*, 2007; Rowland *et al.*, 2001; Thomas *et al.*, 1995) together with studies of community effects linked to tissue and sediment UCM concentrations (Crowe *et al.*, 2004; Guerra-Garcia *et al.*, 2003) strongly suggest that UCMs have the potential for causing effects at various levels of biological organisation. In particular, the monoaromatic UCM fraction has been implicated with adverse effects. However, to date, laboratory studies have all been concerned with acute aqueous exposures to a single species of filter-feeding bivalve mollusc. What is not clear from the literature is:

1. Which components within the UCM elicit adverse biological effects.
2. Whether organisms can accumulate sufficient UCMs from environmental contaminant sinks such as marine sediments to impair health.

3. Whether the effects are observable at the population level.
4. Whether effects are exhibited after chronic exposures at environmentally realistic concentrations.
5. Whether components of the UCM can transfer from one trophic level to another and biomagnifications can occur.

1.6 Present study

There are two main approaches to determining which compounds from within a complex mixture are contributing towards observed toxic effects. One approach is to extract and analyse the chemicals present within polluted organisms. If the effects are greater than that expected from identified compounds with known toxicities, then additional compounds from within the mixture are assumed to be contributing to the observed effects. If possible, samples of these pure compounds can then be obtained or synthesised and their toxicities measured. Due to the extreme complexity of the UCM, this approach has, in the past, proved difficult as compounds could not be resolved by conventional GC. An alternative approach is coupling of biotesting and fractionation, often termed 'effect-directed analysis' (EDA; Fig. 1.6). The present study utilises both approaches.

To investigate aqueous toxicity of UCM hydrocarbons, whole organism tests using *M. edulis* were employed to provide comparative data with previous laboratory studies and comparison with tissue extract analyses of wild mussels. The *V. fischeri* bioluminescence inhibition assay was used in parallel with the *M. edulis* clearance rate tests to provide rapid screening of toxic solutions. The estuarine sediment-dwelling amphipod *Corophium volutator* Pallas was chosen to explore the potential for population-level effects resulting from chronic exposure to UCM hydrocarbon contaminated sediment. The advantages and possible weaknesses of the species and their associated tests are discussed in **Chapter 2**, Section 2.5.

Following a review of the literature relevant to the potential toxicity of UCM hydrocarbons (**Chapter 1**) a number of gaps in our current knowledge were identified. **Chapter 2** presents the experimental details relevant to **Chapters 3 – 6**. In **Chapters 3 and 4**, research based on the EDA approach is presented which explores the toxic components

within three UCM-dominated oils. **Chapter 3** outlines the development of a chronic sediment exposure using the amphipod *C. volutator* and establishes baseline toxicities for the UCM-rich oils. **Chapter 4** investigates the toxicity of primary fractions derived from the UCM-rich oils. The recent availability of GC×GC-ToF-MS has potentially enabled greater resolution of the UCM and thus allowed the possibility of identifying, and determining the toxicity of, components within the UCM of wild biota. Research presented within **Chapters 5 and 6** concentrate on one group of UCM compounds: the branched alkylbenzenes (BABs). This group of compounds was found previously to represent a substantial component of the UCM detected in the tissues of North Sea mussels which had low health status as reflected in S*f*G (Booth *et al.*, 2007) but no toxicological information has been available up until now for these highly branched monoaromatic compounds. Hence, **Chapter 5** investigates the acute and chronic sublethal toxicity of BABs, towards the mussel *M. edulis*. **Chapter 6** explores the possibility of trophic transfer and biomagnifications of the BABs, and investigates whether toxic effects can be elicited *via* this route of uptake. **Chapter 7** provides a general discussion related to the main findings of the research presented in **Chapters 3 – 6** and publications resulted from these studies, together with a summary of the main conclusions and suggests areas for future research. Peer-reviewed papers arising from these studies are reproduced within **Appendix A**. Previous papers published within peer-reviewed journals are reproduced within **Appendix B**.

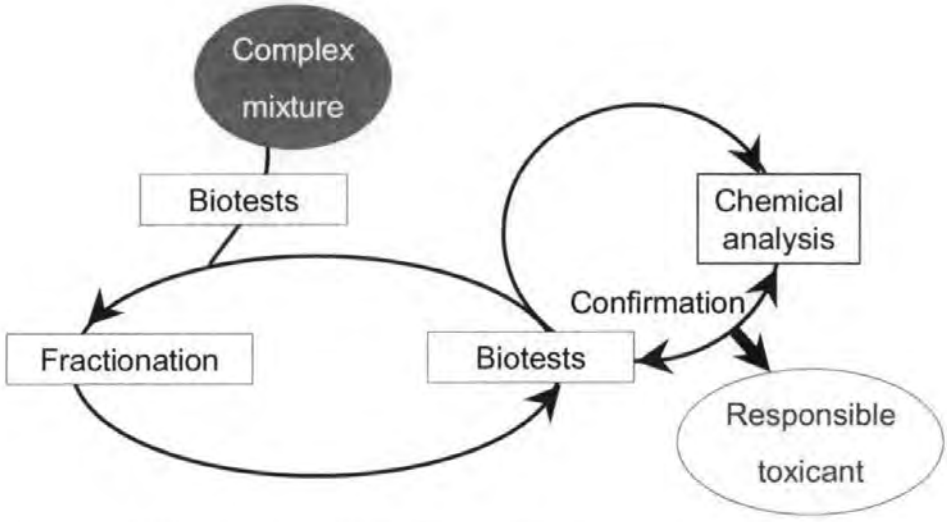


Figure 1.6 Scheme of effect-directed analysis of a complex mixture. Adapted from Brack (2003).

Chapter 2

Experimental procedures

This chapter describes the experimental procedures, the species and biotests performed, and details of analyses performed in the course of this study.

2.1 General procedures

2.1.1 Cleaning of glassware

Glassware was cleaned by soaking in a 2 – 5 % solution of 'Decon-90' (Decon Laboratories Ltd, Hove, UK) for between 2 – 24 h then thoroughly rinsed as per manufacturer's instructions. The glassware was dried (110 °C) then rinsed with DCM to remove any residual organics prior to use.

2.1.2 Solvents and chemicals

Solvents were HPLC grade or higher and obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Solvents were routinely analysed by GC-MS concurrently with experimental samples to ensure adequate purity.

Open column chromatography utilised aluminium oxide (Al_2O_3, grade 1, neutral, 150 mesh, BDH Ltd, Poole, UK) and silica gel (SiO_2, grade 645, 60-100 mesh, Sigma Aldrich Chemical Ltd, Gillingham, UK). The aluminium oxide was activated at 450 °C and stored at 110 °C until required then deactivated with Milli-Q grade water (mechanical shaker, 500 rpm, >45 min) as necessary for separation of fractions and stored short-term in a desiccator. The silica gel was activated at 130 °C then stored at 110 °C until use.

Anhydrous sodium sulphate and cotton wool were pre-extracted (DCM, 24 h), dried (110 °C) and stored in a desiccator prior to use.

Specific chemicals and reagents are described within experimental procedures.

2.2 General instrument details

2.2.1 Gas chromatography - mass spectrometry (GC-MS)

Instrument 1: Hewlett Packard GC-MSD. This comprised a HP5890 Series II gas chromatograph fitted with a HP7673 auto-sampler and a HP5970 quadrupole mass selective detector.

Column: HP1-MS fused silica capillary column, 30 m x 0.25 mm id × 0.25 μm film thickness.

Injector: Autosplitless injection, 1.0 μL, 250 °C.

Carrier gas: Helium (40 kPa head pressure).

Mass spectrometer conditions:

Ion source temperature: 280 °C.

Ionisation energy: 70 eV.

Full Scan mode: 50-550 Daltons (constant flow of 1.0 mL min^{-1}).

Selected Ion Monitoring mode: *m/z* 91, 105, 119, 246, 260.

Ionisation mode: Electron impact (EI).

The oven temperature was programmed from 40-300 °C at 10 °C min^{-1} and held for 10 min. Data and chromatograms were monitored and recorded using ChemStation (version B.02.05) software.

All samples were analysed in Full Scan mode. Due to the low abundances of target compounds within crab tissue extracts, these were also analysed in Selected Ion Monitoring (SIM) mode.

For confirmation of compound identification, extracts of crab tissues were analysed using a second, more sensitive GC-MSD.

Instrument 2: Agilent 5975C Series GC-MSD. This comprised an Agilent gas chromatograph fitted with an Agilent auto-sampler 7683B Series and an Agilent 7890A quadrupole mass selective detector.

Column: Agilent HP5-MS column, 30 m x 0.25 mm id × 0.25 μm film thickness.

Injector: Autosplitless injection, 1.0 μL, 250 °C.

Carrier gas: Helium (constant flow: 1.75 mL min^{-1}).

Mass spectrometer conditions:

Ion source temperature: 230 °C.

Ionisation energy: 70 eV.

Multiplier: 1200 EM volts

SIM mode: *m/z* 91, 105, 119, 246.

Ionisation mode: Electron impact (EI).

The oven temperature was programmed from 40-300 °C at 10 °C min^{-1} and held for 10 min. Data and chromatograms were monitored and recorded using Enhanced ChemStation software.

2.2.2 Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-ToF-MS)

Instrument: Pegasus 4D (Leco Corporation, USA) GC×GC-ToF-MS system, based on a Agilent 6890 Gas Chromatograph (Agilent Technologies, Wilmington, DE, USA) interfaced to a Pegasus III time-of-flight mass spectrometer (LECO, St Joseph, MI, USA) with an Agilent Technologies 7863 Series Autosampler.

1st dimension column: 5 % Phenyl- 95 % methyl-polysiloxane 28.9 m × 320 μm × 0.25 μm DB-5 (J&W Scientific, Wilmington, DE, USA).

2nd dimension column: 50 % Phenyl Polysilphenylene-siloxane 2.0 m × 100 μm × 0.1 μm DPX-50 (SGE, Melbourne, Australia).

Injection temperature: 300 °C.

Transfer line: 280 °C.

Carrier gas: Helium 99.9999 %.

Injector: Autosplitless injection, 1.0 μL.

The following conditions were those used for the analysis of BABs and BABs-exposed mussels' tissues. The conditions were modified for the analysis of oil fractions and associated tissue extracts; details of these conditions, where different from that of the BABs analysis, are provided in parenthesis.

The first-dimension oven temperature was held at 70 °C for 0.2 min, then raised from 70-240 °C at 5 °C min^{-1} (120 °C at 10 °C min^{-1}) then further raised to 270 °C at 20 °C min^{-1} (4 °C min^{-1}) and held at this temperature for 5 min. The second-dimension oven was held at 85 °C for 0.2 min, then raised from 85-245 °C at 5 °C min^{-1} (135 °C at 10 °C min^{-1}) then further raised to 285 °C at 20 °C min^{-1} (4 °C min^{-1}) and held at this temperature for 5 min. A second dimension modulation period of 4 s was employed. The modulator hot temperature was offset 30 °C above secondary oven temperature with a hot pulse time of 1.0 s, and cool time between stages of 1.0 s; the cold temperature during trapping was estimated at -140 °C; electronic pressure control was used in constant flow mode at 1.5 mL min^{-1}. A time-of-flight mass spectrometer (ToF-MS) was used as the detector, and operated at a spectrum storage rate of 100 Hz (100 spectra s^{-1}) based on 5 kHz transients.

The system used the following parameters: ion source 250 °C, EM 1750 V. The mass range monitored was from 40-500 Daltons. The automated data processing was achieved using LECO® ChromaToF™ software (version 2.01, Leco Inc., USA). The software was used to complete a peak finding routine, the deconvolution of mass spectra from partially coeluting compounds and a preliminary NIST library search.

2.3 General procedures for chemical analyses of water, sediment and tissues

2.3.1 Preliminary tests

Procedural details of preliminary tests involving water-accommodated-fractions (WAFs) and chemically dispersed WAFs (DWAFs) of lightly weathered (20 %) ANS, henceforth termed ANS^{i}, are described in Scarlett *et al.* (2007c) and Smith *et al.* (2006). Water, sediment and tissue from preliminary exposure tests with lightly weathered ANS^{i} used extraction and analysis procedures as detailed by Kelly *et al.* (2000). This procedure utilised *n*-pentane as the carrier solvent for sediment and tissue extracts and quantitation was by comparing ultra violet fluorescence values to a standard curve derived from weathered ANS^{i} oil dissolved in DCM (WAFs and DWAF) or *n*-pentane (sediment and tissue). Fluorescence was measured using a Hitachi Fluorescence Spectrometer F-4500 (Finchampstead, Berkshire, UK) with excitation wavelengths (λ) of 254 nm (DCM) 310 nm (*n*-pentane) and emission λ 360 nm.

2.3.2 Main tests

Due to concerns following a re-assessment of risk with regard to storage of samples in *n*-pentane, subsequent extraction procedures utilised *n*-hexane. As highly volatile compounds were not present within the more weathered and biodegraded oils, *n*-hexane was deemed adequate for purpose.

Aliquots of water samples (100 mL) were extracted into dichloromethane (DCM, 3 × 25 mL + 25 mL rinse of separating funnel) for quantification of hydrocarbon concentrations or BABs by GC-MS. Sediment and tissue samples were extracted using an alkaline saponification method adapted from Kelly *et al.* (2000). In brief: frozen sediment and tissue samples were allowed to defrost at room temperature then mixed with a stainless

steel spatula. The dry weight percentage of each sample was determined by weighing subsamples (×2) in pre-weighed foil dishes then re-weighing after drying at 105 °C for 16 h. Approximately 50 g of wet sediment and 15 g of wet tissue samples were digested with potassium hydroxide pellets (15 g and 5 g for tissue and sediment respectively) and methanol (100mL), under reflux for 2 hours. When cool, the digests were filtered through solvent-rinsed filter papers (Whatman 113v) into 250 mL separating funnels. The digests were extracted with *n*-hexane (2 × 50 mL) and the combined extract dried with anhydrous sodium sulphate. Extracts requiring quantitation using UVF were made up to 100 mL prior to analysis (detailed above). Extracts were reduced in volume to a few mL by rotary evaporation then transferred to preweighed 7 mL vials and gently reduced under nitrogen until dry. The vials were reweighed and the extracts made up to 1 mL. Aliquots (100 µL by µL syringe) were transferred to 2 mL GC-MS vials and the volume adjusted to 1 mL by µL syringe with DCM. The procedures described above were altered as necessary for extraction and analyses of small samples (e.g. mussel tissues from fractionated oil toxicity tests and crab tissue extracts were reduced in volume to 100 µL). Deviations from these procedures are detailed within relevant sections.

2.4 Description of oils and quantitation of hydrocarbons

2.4.1 Description of oils rich in UCMs

Two oils were selected as they were rich UCM hydrocarbons (viz. dominated by unresolved hydrocarbons when analysed by conventional GC): An in-reservoir biodegraded crude oil Tia Juana Pesado (TJP) that was spilled previously in the Mersey UK (Davies & Wolff, 1990) and a feed stock of the lube oil Silkolene-150 (SLK). For comparative purposes the crude oil Alaskan North Slope was evaporatively weathered to produce an oil with a greater proportion of resolved hydrocarbons than the other oils but lacking the acutely toxic volatile BTEX compounds. A comparative description of the oils is given below (Table 2.1)

Table 2.1 Properties of Alaskan North Slope (ANS) and Tia Juana Pesado (TJP)

	API (Group)	Pour point (°C)	Sulphur content (%)	Reference
ANS (fresh)	27.5 (III)	-18	1.1	*i*
TJP	12.1 (IV)	-1	2.7	*ii & iii*

i Environment Canada, Emergencies Science Technologies Division (http://www.etc-cte.ec.gc.ca/databases/spills/pdf/WEB_Alaska_North_Slope_(SOCSEX).pdf 17.09.2007).

ii McQuilling Services, LLC IMO 13H MARPOL Regulation - Carriage of Heavy Crude Oil, Garden City, NY. (http://www.meglobaloil.com/MARPOL.pdf 17.09.2007).

iii The International Tanker Owners Pollution Federation, ITOPF Handbook 2007/2008 (http://www.itopf.com/itopfhandbook2007.pdf 17.09.2007).

2.4.2 Artificial weathering of oil

The fresh ANS crude oil was initially weathered in order to simulate evaporative losses of *ca* 20 % that typically occur during the first 2 – 3 h at sea following a spill; this represents the earliest optimal time that dispersants may be deployed at sea (K. Colcomb, Maritime & Coastguard Agency (MCA), personal communication). Full procedures and results

obtained are detailed by Smith *et al.* (2006). This lightly weathered oil was used for preliminary chronic sediment exposures with *C. volutator*. Analysis had revealed that volatile compounds remained within the lightly weathered (*ca.* 20 %) ANS[i] crude oil (Smith *et al.*, 2006), so this was further weathered until no further evaporative losses were apparent and a stable weight was achieved (72 h). The additionally weathered oil was then rotary evaporated at 40 °C for 30 min to ensure loss of volatile components (no further loss was observed). Once weathered the oil was stored in completely filled amber glass bottles at 4°C until use. Henceforth, any reference to ANS will refer to the additionally weathered ANS unless otherwise stated.

2.4.3 Quantitation of hydrocarbons by GC-MS and GC×GC-ToF-MS

Calculation of UCM component within oils using GC-MS

Gas chromatograms of the three oils (Fig. 2.1) showed the dominance of the UCM within all three oils but a number of resolved compounds were visible in the chromatogram of the weathered ANS oil. The relative proportion of resolved and unresolved compounds within the whole oils, aliphatic and aromatic fractions of ANS and TJP oils were calculated by subtracting the areas of resolved fractions (MS Data analysis – 'integrate' function) from the total area (MS Data analysis – 'areasum' function). From this, the percentages of UCM of the total hydrocarbons and ratio of resolved to UCM compounds were derived (Table 2.2).

Quantitation of BABs using GC-MS and GC×GC-ToF-MS

Quantitation of BABs using GC-MS was by measurement of the major resolved component *via* integration of total ion current (TIC) and *m/z* 246 ($M^{+\cdot}$) responses for which a linear calibration of GC-MS response was obtained ($r^2 = 0.999$; 0-0.06 mg mL^{-1} injected). Quantitation of BABs by GC×GC-ToF-MS was achieved by summation of all

the resolved peak areas with fragment ions consistent with alkylbenzenes (*m/z* 91, 92, 105, 119 and 133). A linear calibration of the GC×GC-ToF-MS response was obtained (r^2 = 0.994; 0-0.01 mg mL^{-1} injected) for the C_{12-14} BABs mixture.

Table 2.2 Resolved (*r*) and unresolved compounds (UCM) determined by GC-MS analyses of whole and primary fractionated oils

	Fraction							
	Whole oil		F1		F2		F3	
Oil	UCM (%)	*r*/UCM (%)	UCM (%)	*r*/UCM (%)	UCM (%)	*r*/UCM (%)	UCM (%)	*r*/UCM (%)
SLK	95.6	4.6	94.9	5.4	97.3	2.8	98.6	1.4
TJP	97.8	2.3	98.4	1.7	97.7	2.3	96.3	3.9
ANS	87.2	14.7	81.3	23.0	92.4	8.2	94.7	5.6

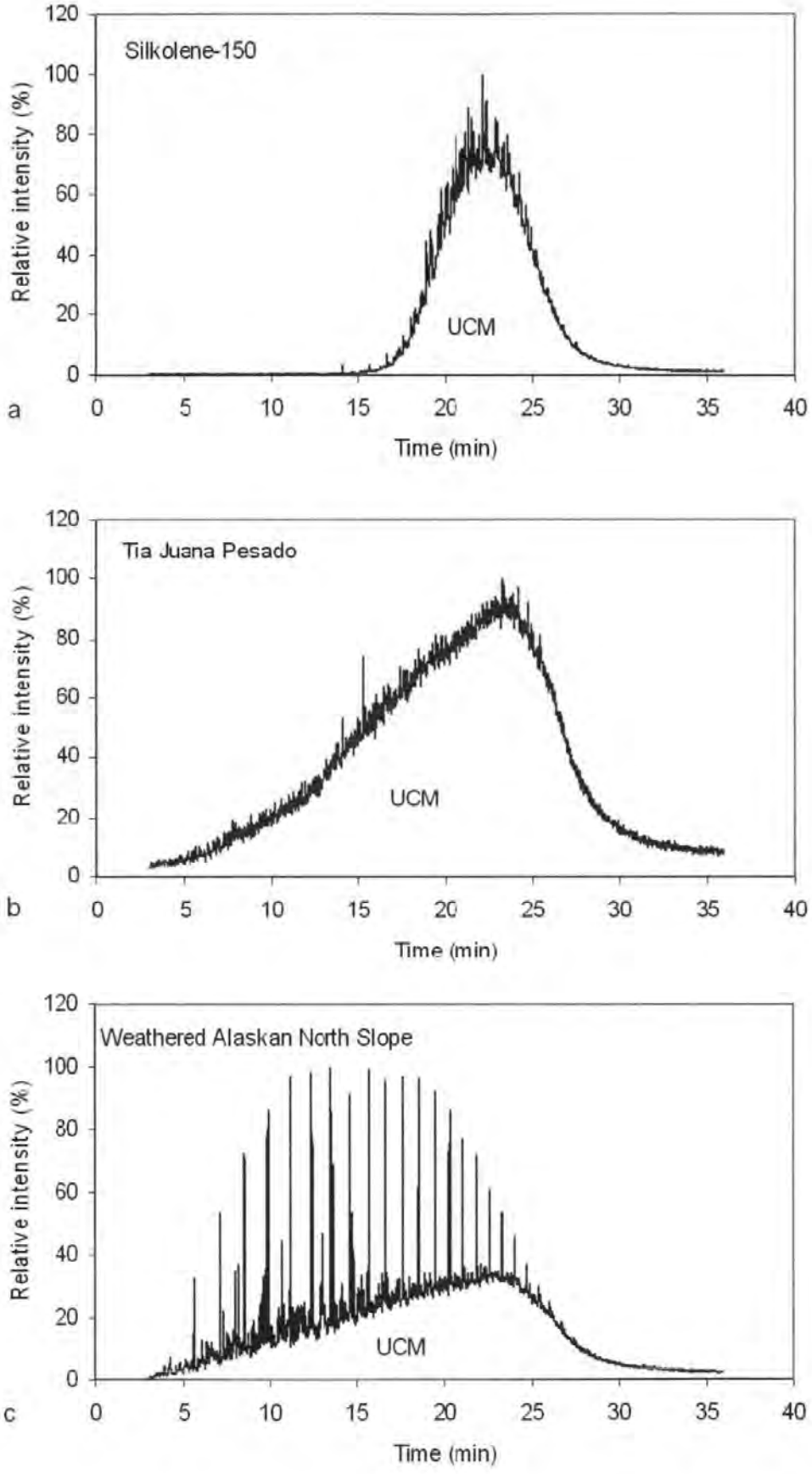


Figure 2.1 Total ion chromatograms of whole oils rich in UCM hydrocarbons.

2.4.4 Primary fractionation of oils

The oils were fractionated by open column chromatography (Fig. 2.2) adapted from methods described by Wraige (1997), Sutton *et al.* (2005) and Brack *et al.* (2005). Aliquots of *ca*1.5g of oil were adsorbed onto *ca*10 g of deactivated alumina (4.5 % MilliQ water w/w) by rotary evaporation of the oil and alumina with hexane (20 mL) at 40 °C until near dry. The column was packed with alumina (4.5 % MilliQ water w/w) over activated silica (ratio 1:1 w/w) and eluted with increasingly polar solvents: 100 % hexane (F1 - aliphatic), 90:10 (v/v) hexane:DCM (F2 - aromatic 1), 100 % DCM (F3 - aromatic 2) and 100 % methanol (F4 – polar). Elution volumes (× column volumes) were 1.5× for F1 and 2× for F2, F3 & F4. Analysis by GC-MS of column extracts spiked with a mixture of known hydrocarbons showed that the F1 fraction contained only aliphatic compounds and that the F2 fraction contained aromatic hydrocarbons such as alkylbenzenes to fluoranthene. The column chromatography process was repeated three times to produce sufficient quantities of fractions for aqueous biotests using the bioluminescence assay, feeding rates of *M. edulis*, and sediment exposures using *C. volutator*. Subsamples of fractions F1, F2 and F3 of all oils from each column run were analysed by GC-MS. The F4 fractions were retained but were not analysed by GC-MS. Gravimetric analysis of the fractions confirmed that SLK was dominated by aliphatic (F1) hydrocarbons but also that it contained a small aromatic (F2) fraction representing about 16 % of the whole oil (Fig. 2.3). There was little variation between repeated gravimetric analyses of fractions (n = 3) with CVs ranging from 0.1 % to 6.1 % for F1, F2 and F3 fractions. Gas chromatograms of replicated fractions appeared identical and were all dominated by UCM hydrocarbons with very little apparent resolved petrogenic hydrocarbons. Total ion chromatograms of the aliphatic and aromatic fractions of SLK (Fig. 2.4), TJP (Fig. 2.5) and weathered ANS (Fig. 2.6) are shown below.

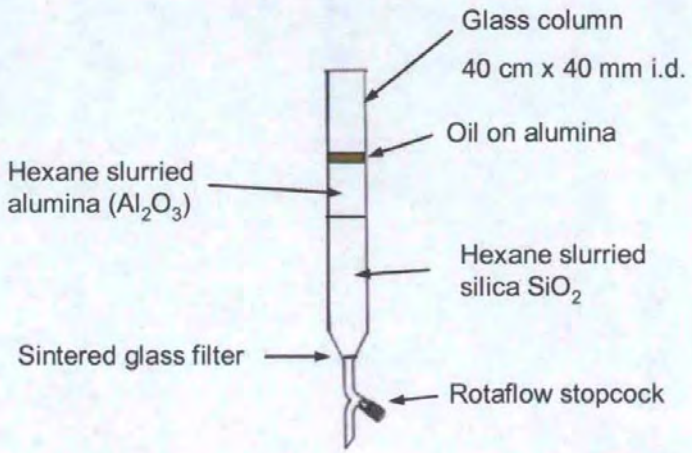


Figure 2.2 Apparatus used for large scale open column chromatography fractionation of oils.

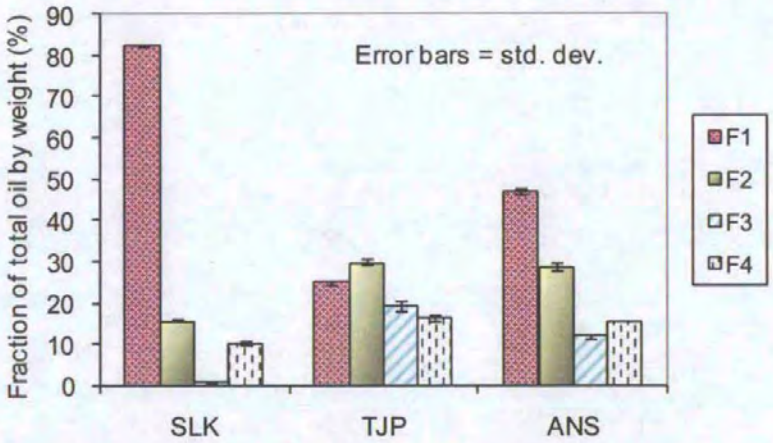


Figure 2.3 Gravimetric analysis of fractions of oils created by open column chromatography (n = 3). F1 = aliphatic fraction eluted by 100 % hexane; F2 = aromatic 1 fraction eluted by 90:10 (v/v) hexane:DCM; F3 = aromatic 2 eluted by 100 % DCM; and, F4 = polar fraction eluted by 100 % methanol.

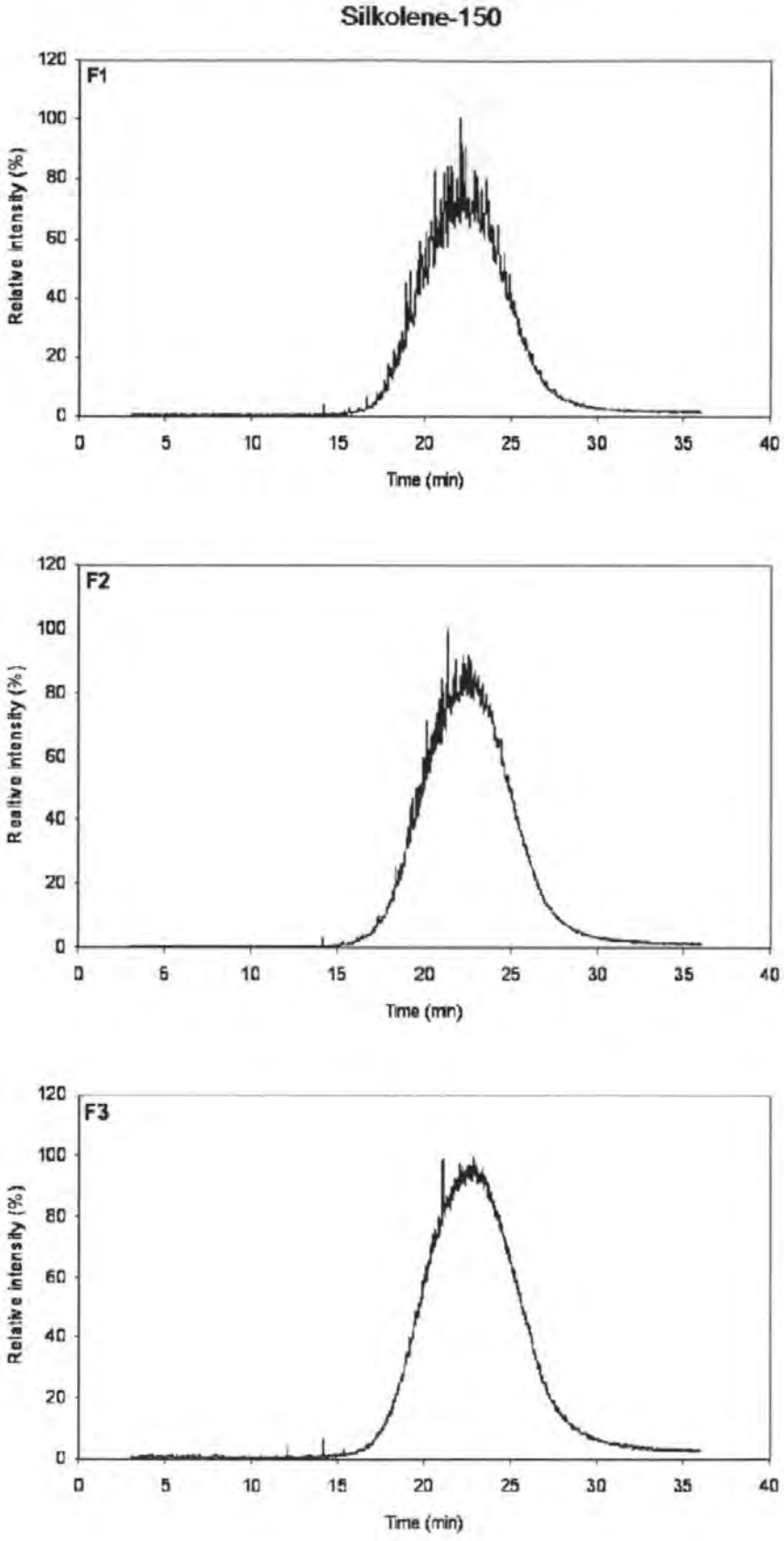


Figure 2.4 Gas chromatograms of fractions of Silkolene-150: aliphatic (top), aromatic 1 (middle) and polar aromatic 2 (bottom).

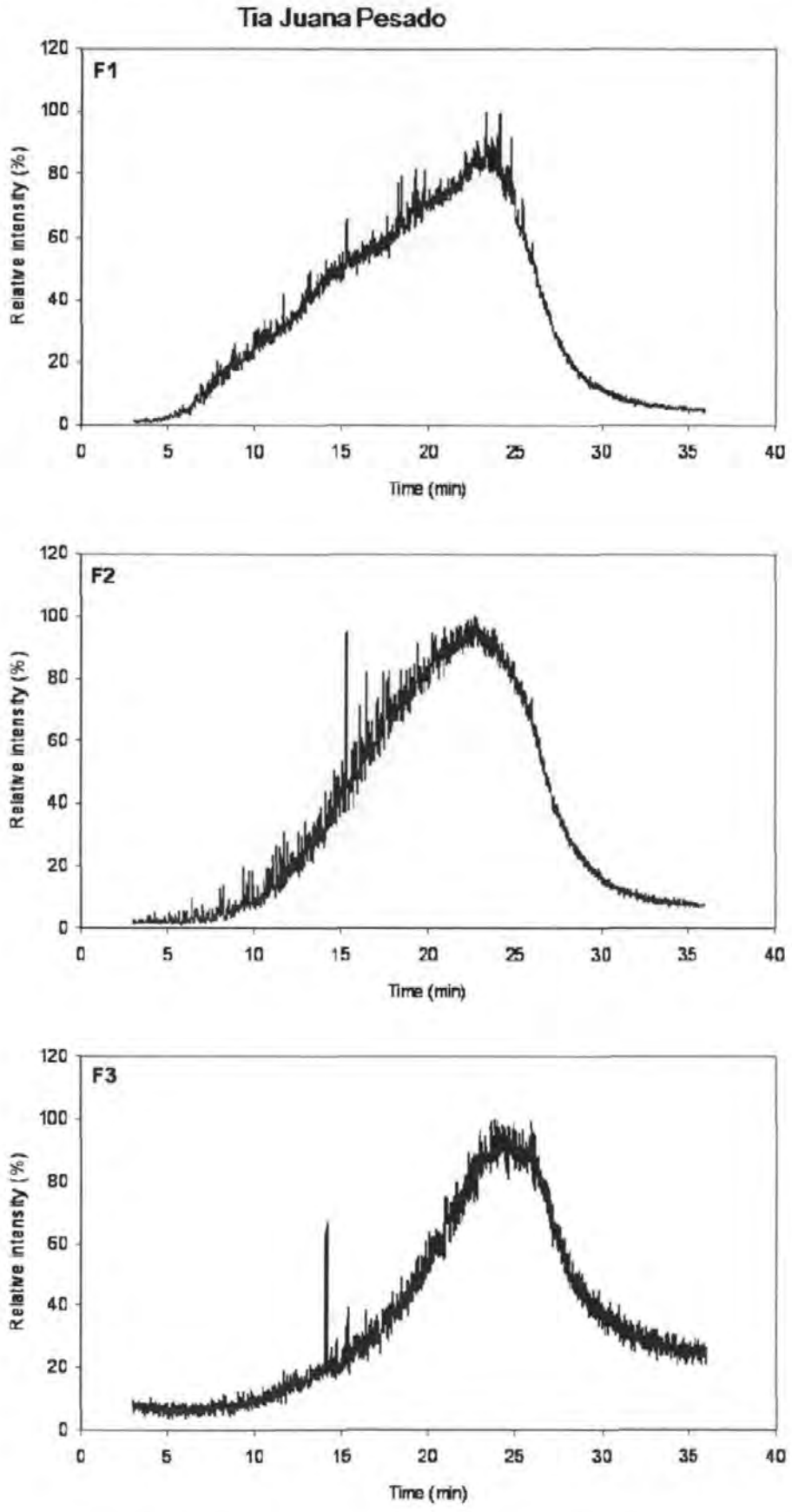


Figure 2.5 Gas chromatograms of fractions of Tia Juana Pesado: aliphatic (top), aromatic 1 (middle) and polar aromatic 2 (bottom).

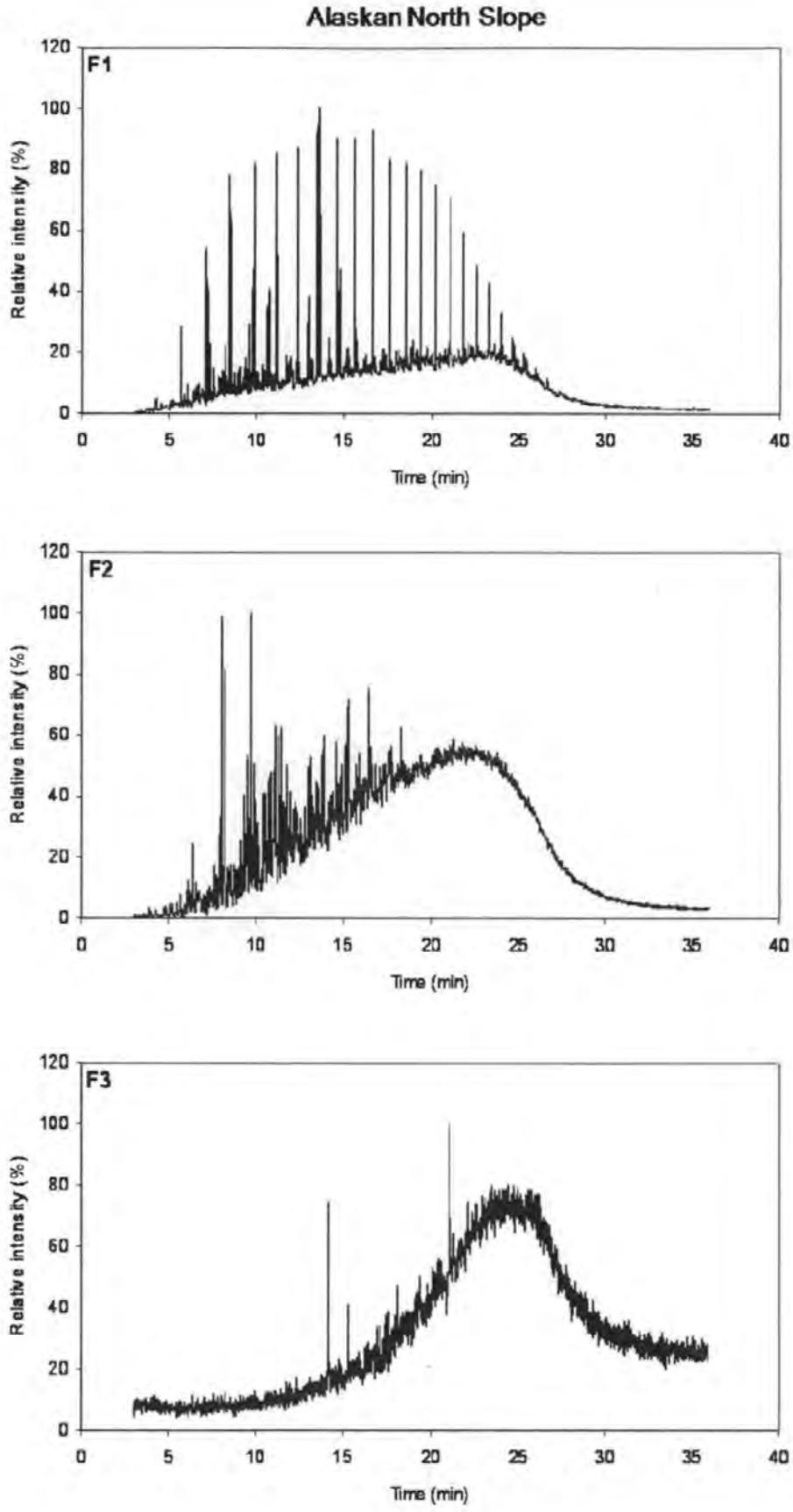


Figure 2.6 Gas chromatograms of fractions of weathered Alaskan North Slope: aliphatic (top), aromatic 1 (middle) and polar aromatic 2 (bottom).

2.5 Selection of organisms used for toxicity tests

2.5.1 *Corophium volutator*

Amphipods are reported to be sensitive to hydrocarbon exposure (Dauvin, 1998; Dauvin, 2000; Dauvin & Gentil, 1990; Gesteira & Dauvin, 2000; Gesteira *et al.*, 2003; Lee & Page, 1997; Nikitik & Robinson, 2003; Poggiale & Dauvin, 2001; SEEEC, 1998). The reason for this relative sensitivity compared to some polychaete worm species, is not clear. It is possible that the amphipods' position in the top layer of sediment rather than within deep burrows, coupled to their high respiration rate while ventilating water through their burrows, may be a factor. Boehm *et al.* (1982) suggested that exposure to the fine-floc (contaminated by hydrocarbons) at the sediment-water interface was responsible for their sensitivity. Amphipods have been used for both acute and chronic standard sediment exposure tests in the US (USEPA, 1994; USEPA, 2001). *Corophium volutator* is an estuarine and coastal sediment-dwelling amphipod of the family Corophiidae (Wilson & Parker, 1996) with a widespread distribution within Europe and the eastern coast of the US and Canada (Connor *et al.*, 2004), and sub species in Japan (Omori & Tanaka, 1998). Adults (6-10 mm) can live for about a year and can produce up to three generations of offspring (~1mm) that are born from eggs held within the females' pouches until independent. The species is a standard European sediment acute toxicity test species (PARCOM, 1993; Roddie & Thain, 2001) and has been extensively used for toxicity studies both in Europe and the US (Bat & Raffaelli, 1998; Ciarelli *et al.*, 1997; Conradi & Depledge, 1998; Conradi & Depledge, 1999; Grant & Briggs, 2002; Hellou *et al.*, 2005; Hyne & Everett, 1998; Kirkpatrick *et al.*, 2006; Kravitz *et al.*, 1999; Roddie *et al.*, 1994; Roddie & Thain, 2001; Stronkhorst *et al.*, 2003a; Stronkhorst *et al.*, 2003b; van den Heuvel-Greve *et al.*, 2007).

Life-cycle toxicity tests have been performed using *C. volutator* (Brown *et al.*, 1999; Conradi & Depledge, 1998; Conradi & Depledge, 1999) but prior to commencement of the present study no chronic sediment exposure tests had been reported for this species. The importance of conducting chronic exposure tests and the suitability of *C. volutator* for such tests, has increasingly been recognised, resulting in a recent description of a chronic test method by van den Heuvel-Greve *et al.* (2007) which is very similar to the method arising from the research reported herein (Scarlett *et al.*, 2007c).

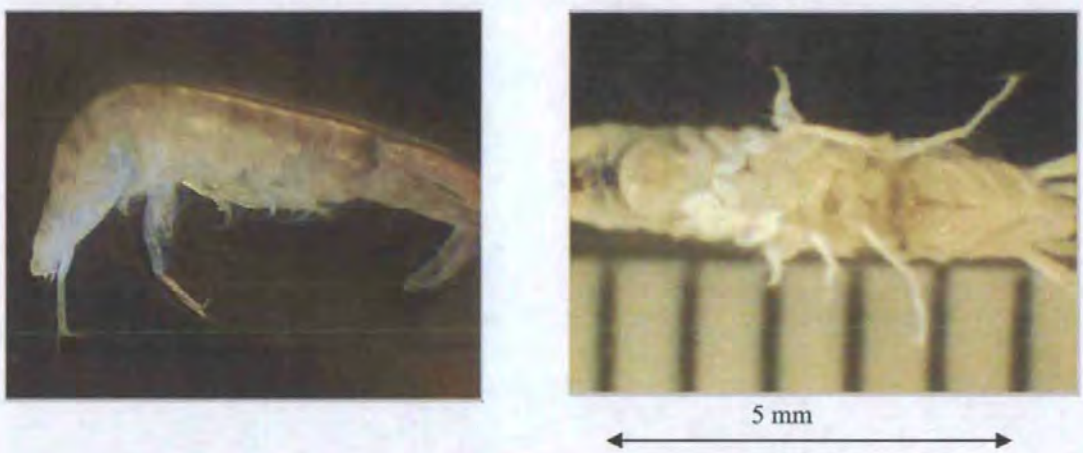


Figure 2.7 Photograph of gravid adult female *C. volutator* (left) with detail of eggs held within the female's pouch (right).

2.5.2 *Mytilus edulis*

The blue mussel *M. edulis* is a sessile filter-feeding bivalve mollusc species commonly found in coastal and estuarine rocky shores and has a widespread geographic range. The species has been extensively used as a 'bioindicator' of contamination in marine environments and has been used in numerous toxicity studies including the worldwide 'Mussel Watch' program (Farrington *et al.*, 1983; NAS, 1980; Risebrough *et al.*, 1983; Wade *et al.*, 1998). The sensitivities of *M. edulis* to a wide range of hydrocarbons have been reported (e.g. Baussant *et al.*, 2001b; Donkin & Widdows, 1986; Donkin *et al.*, 1991; Donkin *et al.*, 1989; Widdows & Donkin, 1988; Widdows & Donkin, 1991; Widdows *et*

al., 1995; Widdows *et al.*, 1987) and they have been commonly used to assess environmental stress at a cellular level (e.g. Brown *et al.*, 2004; Galloway *et al.*, 2004b; Galloway & Depledge, 2001; Galloway *et al.*, 2002; Moore *et al.*, 2006; Moore *et al.*, 2004; Smith *et al.*, 2000). In addition, *M. edulis* has been used to assess the effects of hypothesised UCM components (e.g. Booth, 2004; Crowe *et al.*, 2004; Donkin *et al.*, 2003; Reineke *et al.*, 2006; Rowland *et al.*, 2001; Smith *et al.*, 2001; Thomas *et al.*, 1995; Wraige, 1997).

A principal assay used to assess the health of *M. edulis* is S*f*G (Smaal & Widdows, 1994). This is an integrated physiological parameter that quantifies the energetic balance between the processes of energy acquisition (feeding and absorption) and energy expenditure (metabolism and excretion). Quantitative and predictable reduction in S*f*G has been reported in response to accumulation a wide range of contaminants including petroleum hydrocarbons (see references above). Riisgard (2001) suggested that a large proportion of published studies reporting S*f*G have used methods that are flawed and that only those studies that show maximum filtration rates measured under optimal laboratory conditions are valid. However, this was strongly rebutted by Widdows (2001). The main determinant of S*f*G is the clearance rate of the mussels. Toxicants may also reduce absorption efficiency and/or increase respiration (Widdows *et al.*, 1987) and therefore S*f*G is likely to be a more sensitive assay than comparison of clearance rates alone. The *M. edulis* clearance rate assay has however proved to be a robust and reproducible measure of hydrocarbon sublethal toxicity (Donkin *et al.*, 2003; Donkin *et al.*, 1991; Donkin *et al.*, 1989; Scarlett *et al.*, 2005).

2.5.3 *Carcinus maenas*

The decapod crustacea *Carcinus maenas* Linnaeus, 1758 , commonly known as the shore crab or European green crab, has been widely used to detect effects of a range of environmental contaminants (e.g. Bjerregaard & Depledge, 1994; Brown *et al.*, 2004; Camus *et al.*, 2004; Dissanayake & Galloway, 2004; Dissanayake *et al.*, 2006; Dissanayake *et al.*, 2007; Elumalai *et al.*, 2007; Fillmann *et al.*, 2002; Galloway *et al.*, 2004a; Galloway *et al.*, 2004b; Gamble *et al.*, 1995; Gowland, 2002; Hebel *et al.*, 1997; Lewis *et al.*, 1999; Lundebye & Depledge, 1998; Martin-Diaz *et al.*, 2007; Spaargaren, 1990; Watson *et al.*, 2004a; Watson *et al.*, 2004b). Whereas the bivalve mollusc *M. edulis* has limited ability to metabolise hydrocarbons (Stegeman *et al.*, 1992) *C. maenas* are known to biotransform PAHs into more hydrophilic compounds prior to excretion (Sole & Livingstone, 2005). Analysis by UVF of crab urine has been used to detect PAHs (Dissanayake & Galloway, 2004; Galloway *et al.*, 2004b; Watson *et al.*, 2004a; Watson *et al.*, 2004b) and therefore adaptation of this analytical method may provide a useful means for detecting excretion of other hydrocarbons.

Carcinus maenas is a predatory species that commonly feeds upon bivalve molluscs (Vernberg & Vernberg, 1983); they are therefore more likely to be indirectly exposed to toxicants *via* contaminated prey as well as direct exposure to dissolved contaminants. In their natural environment, shore crabs perform diurnal and seasonal migration cycles (Naylor, 1962; Vernberg & Vernberg, 1983) and this may allow individuals not continually exposed to UCM hydrocarbons to accumulate compounds by trophic transfer *via* consumption of prey resident in contaminated water. A major drawback of the use of *C. maenas* to detect hydrocarbon toxicity is that the organism appears to be relatively insensitive to oil contamination. For example, Galloway *et al.* (2004b) reported that cellular viability was not significantly affected in *C. maenas* from sites in the Solent (UK) heavily contaminated by petroleum hydrocarbons but a population of the filter-feeding

bivalve mollusc *C. edule* (cockle) did have significantly different cellular viability from other sites in Southampton water.

2.5.4 *Vibrio fischeri*

The bioluminescent bacterium *V. fischeri*, formally known as *Photobacterium phosphoreum*, has been extensively used within the Microtox system for the testing of both aqueous and sediment samples; (see Cronin & Schultz, 1998; Hermens *et al.*, 1985a; Kaiser & Palabrica, 1991; Leftley, 2000; van Beelen, 2003 and references therein). The rationale for using microbial assays has been summarised by Qureshi *et al.* (1984):

- They possess the majority of the same biochemical pathways present within higher organisms;
- They exhibit a significant degree of organisation in membrane structure;
- They generally elicit toxic responses to many chemicals through mechanisms similar to those in higher organisms;
- They represent the lowest common denominator in the marine food chain.

It has been generally accepted that the bioluminescent system can be viewed as a branch of the electron transport system in which electrons are shunted to oxygen at the level of flavin, (reviewed by Leftley, 2000). However, the sites of cellular action responsible for the decrease in luminescence are not known and the diverse nature of active compounds would suggest that more than one site might be involved, e.g. membranes, cell permeability, electron transport, ribosomes and protein synthesis (Hastings *et al.*, 1985). The measured response of over a thousand pure chemicals has been documented (Kaiser & Devillers, 1994; Kaiser & Esterby, 1991). Both qualitative and quantitative relationships between Microtox test data and acute and subchronic toxicity data for other species, including invertebrates and fish, have been reported (Kaiser & McKinnon, 1992).

Although there is generally a good correlation between bioluminescence inhibition EC_{50} values and toxic endpoint of other species, there can be discrepancies. A comparison between bioluminescence inhibition EC_{50} values for some hydrocarbons, reported within Kaiser & Palabrica (1991), with sublethal toxicity EC_{50} values for *M. edulis* (Donkin *et al.*, 1991; Donkin *et al.*, 1989), suggested that the bioluminescence inhibition test may be a factor of ×10 to ×20 less sensitive. Johnson & Long (1998) reported EC_{50} values 60, 400 and 1000 mg L^{-1} for Fuel Oil no. 2, crude oil and recycled motor oil respectively.

Vibrio fischeri bioluminescence inhibition is known to detect log K_{ow}-dependent non-specific effects including narcosis (Hermens *et al.*, 1985a), uncoupling (Schultz & Cronin, 1997) and some electrophilicity-based effects (Cronin & Schultz, 1998). The *V. fischeri* is not suitable for detecting PAH toxicity arising from metabolic activity due to the lack of AhR and may not be sensitive to compounds that produce long-term effects (Backhaus *et al.*, 1997; Kaiser, 1988). Another problem with *V. fischeri* is that it is sensitive to elemental sulphur (Pardos *et al.*, 1999) which occurs naturally in anaerobic sediments (Gagne *et al.*, 1999). Therefore if tests are applied to sediment extracts, sulphur must be removed (e.g. by reaction with activated copper) prior to testing with *V. fischeri* although this may affect thiol-, nitrogen-, and oxygen-bearing components (Brack *et al.*, 1999). The carrier solvent has been shown to affect the toxicity of chemical. For example, Johnson & Long (1998) reported that dimethyl sulfoxide (DMSO) as a carrier solvent produced no significant changes in the toxicity of phenol whereas DCM increased toxicity and both ethanol and acetone decreased toxicity. However, the bioluminescence inhibition test can particularly useful for the rapid screening of small volumes of chemicals with known mode of action.

Port Quin, Cornwall

Avon Estuary, Devon

Figure 2.8 Location of organism and sediment collection sites in the southwest of UK (top): satelite images of Port Quin, Cornwall (botton left), and the Avon estuary near Aveton Gifford, Devon (bottom right). Images from Google™ Earth (2007).

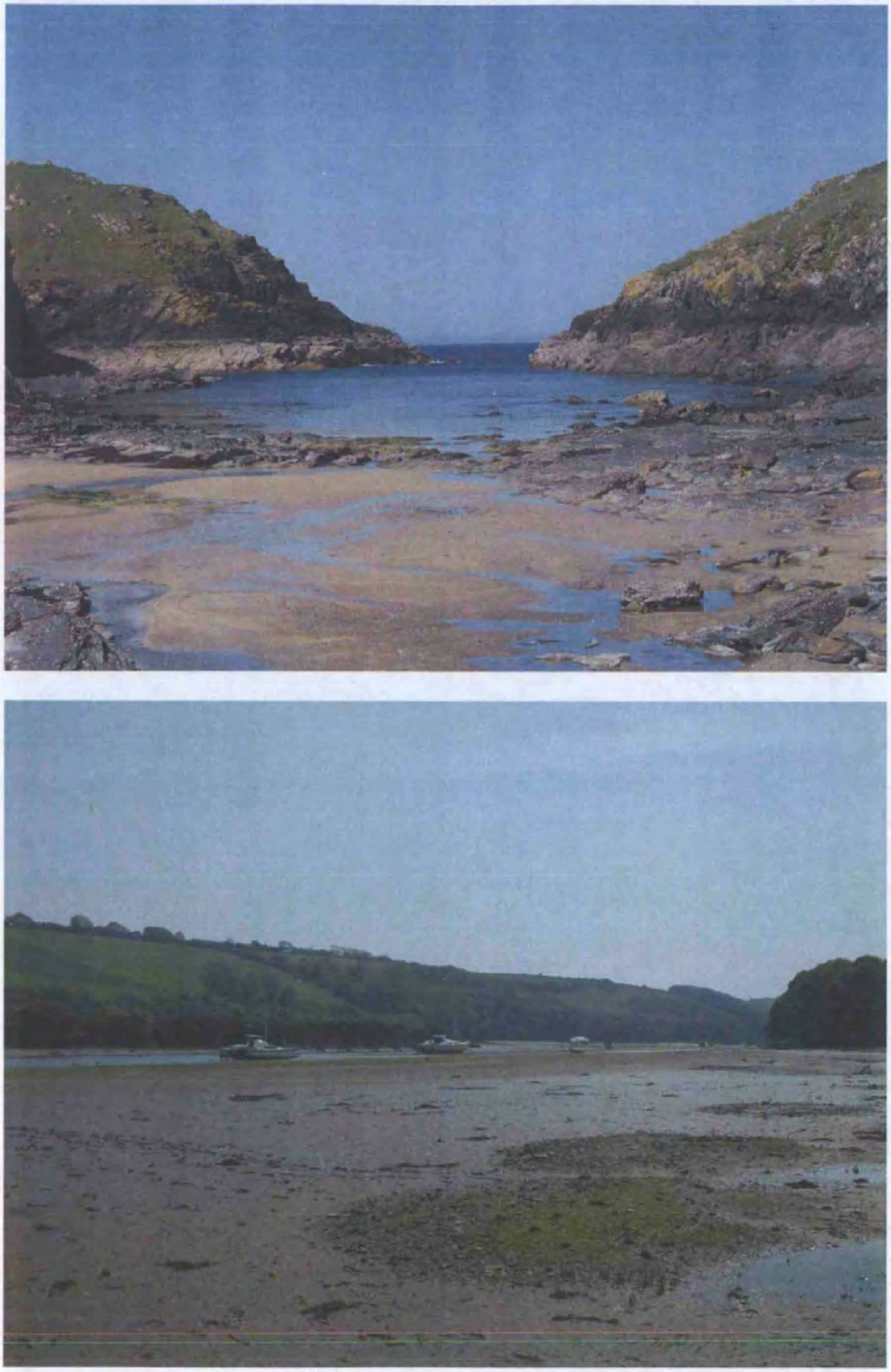


Figure 2.9 Photographs of collection sites for *Mytilus edulis* at Port Quin, Cornwall (top), and *Corophium volutator* and sediment on the Avon estuary, Devon (bottom).

2.6 Supply and maintenance of organisms

2.6.1 Collection of sediment and *Corophium volutator*

Sediment was collected from an intertidal area of the Avon estuary near Aveton Gifford, south Devon UK (N 50° 18.388' W 003° 50.920'; Figs 2.8 and 2.9). This site contains sediment characterised by sandy mud with a large population of *C. volutator*. The habitat is classified by the Joint Nature Conservation Committee (JNCC) as LS.LMu.UEst.Hed.Cvol (Connor *et al.*, 2004). Sediment from the top 10 cm was transferred into a white polypropylene bucket and transported to the laboratory within one hour. The amphipods were sieved (1 mm mesh) from the top 5 cm of sediment and placed in white polypropylene buckets and transported to the laboratory within one hour. The sediment was then sieved (300 μm) to remove macrofauna, stones and large organic debris. The grain size of the sediment was typically 33 % sand, 67 % silt/clay. Organic carbon content was estimated by loss on ignition at 400 °C (Schumacher, 2002) to be 3.8 % of dry weight. Sediment was oxygenated by aeration *via* Pasteur pipette and overlying water quality (dissolved oxygen (DO_2), salinity, pH, temperature and ammonia content measured prior addition of amphipods and commencement of sediment exposure tests.

For preliminary and fractionated oil sediment tests, adult and juvenile *C. volutator* were collected from an intertidal area of the Avon estuary near Aveton Gifford, south Devon UK, as specified above. Due to an unexplained collapse in the *C. volutator* population at the Aveton Gifford collection site, amphipods were later supplied by Guernsey Sea Farms (Vale, Guernsey, UK) for use within baseline sediment tests. Amphipods were maintained in 5 L culture tanks lined with field-collected sieved (<300 μm) sediment. The tanks were filled with filtered seawater (25 ± 1 psu)which was aerated and maintained at 15 ± 1 °C with a 12:12 h light/dark cycle. The animals were fed weekly with 2 drops of aquarium invertebrate food (Waterlife Invert Food, Waterlife Research Industries, Longford, UK;

Liquifry Marine, Interpret Ltd., Dorking, UK; Roti-Rich, Florida Aqua Farms, Dade City, FL, USA; and dried algae) per litre of overlying water and the water replaced 24 h after feeding. Amphipods were maintained under the above conditions for 7-10 days after removal from the field to acclimate them to the experimental conditions. Water quality measurements: (DO_2, salinity, pH, temperature and ammonia content were measured prior to water changes. Test conditions and acceptability requirements are given in Table 2.3.

2.6.2 Collection and maintenance of *Mytilus edulis*

Mussels were collected from Port Quin, a narrow rocky inlet on the North Cornwall coast, UK (N50° 35.363', W004°52.043'; Figs 2.8 and 2.9), taking care not to rip the byssal threads with which the animals attach themselves to rock. Mussels from this site have been reported to contain negligible or no UCM or aromatic hydrocarbons (Donkin *et al.*, 2003; Widdows *et al.*, 1995). Mussels were transported to the laboratory within two hours and maintained in filtered seawater at 15 °C (± 1°C), 35 psu (± 2 psu), with a 12:12-h light:dark cycle within a climate controlled room. The mussels were not fed for the first couple of days to allow them time to acclimate to laboratory conditions and then water exchanged followed by feeding with the brown flagellate microalga *Isochrysis galbana* in accordance with supplier's recommendations i.e. *ca.* 6 mL (0.5 g dry weight) *Isochrysis* per 100 g mussel wet tissue weight (Reed Mariculture Inc., Campbell, CA). The animals were maintained under laboratory conditions for a minimum of one week prior to exposure tests.

2.6.3 Collection and maintenance of *Carcinus maenas*

Common shore crabs *C. maenas* were collected from Bantham on the river Avon estuary, S. Devon (N50°16.696' W003°52.176'), i.e. downstream of the *Corophium* collection site (Fig. 2.8). Traps were baited with catfood and bacon, and deployed 2 h before high water.

The crabs were removed from the traps at highwater and sorted by sex, size and colour. Male green crabs with carapace width of 60 – 70 mm were retained and transported back the laboratory within one hour. Male crabs were selected as it has been shown that handling times differ between the sexes (Spooner *et al.*, 2007). Crabs (mean carapace width = 65.7 mm (standard error (SE) = 0.87, CV = 5.6 % ; mean weight = 73.4g, SE = 2.7 g, CV = 15.8 %) were placed individually in glass vessels containing 10 L of filtered seawater (15° C) within a temperature-controlled (15° C) room. The vessels were aerated *via* glass Pasteur pipettes. The crabs were held for three days without feeding prior to commencement of the trophic transfer test.

2.6.4 Storage of *Vibrio fischeri*

The bacteria were supplied frozen and maintained at -20 °C until required.

2.7 Toxicity test procedures

2.7.1 Toxicity tests with *Corophium volutator*

Reference toxicity tests

In order to test that the Guernsey supplied *C. volutator* population was consistent, in terms of sensitivity to a standard toxicant, with the local *C. volutator* population from Aveton Gifford used previously and the general *C. volutator* population as a whole, the test organisms were exposed to $CdCl_2$ using a method described by Ciarelli *et al.* (1997) and their sensitivity compared with published data (Ciarelli *et al.*, 1997) and those obtained during previous reference toxicity testing. In brief, static aqueous tests of 72 h exposure were performed in the absence of sediment. Twenty adult amphipods were placed in a nominal $CdCl_2$ concentration ranging from 0 to 14.0 mg L^{-1}, two replicates per treatment, with a salinity of 31 psu and gentle aeration *via* a glass Pasteur pipette. The organisms were monitored daily throughout the experiment and the number surviving and deceased recorded after 72 h. A 72 h LC_{50} value was derived using the trimmed Spearman-Kärber method and compared with literature values. Further tests were performed to check for any changes to sensitivity or differences between populations by exposing *C. volutator* to 7.0 mg L^{-1} (i.e. close to the LC_{50} value recorded previously), two replicates per treatment, conditions as above. The mortality was compared with the 72 h LC_{50} value recorded previously.

Preliminary tests

Procedural details of preliminary tests involving water-accommodated-fractions (WAFs) and chemically dispersed WAFs (DWAFs) of lightly weathered (20 %) ANS[i] are described in Scarlett *et al.* (2007c) and Smith *et al.* (2006).

Table 2.3 Test conditions and acceptability limits for chronic sediment toxicity tests with *Corophium volutator*. Water quality measurements and performance criteria achieved, mean and coefficient of variance (CV), given in parenthesis

Test type:	Whole sediment toxicity test, static-renewal
Test sediment:	Mud, sandy mud or muddy sand. Sieved through 300 µm
Overlying water:	Filtered seawater. Salinity: daily limits: 25 psu ± 3 psu. (Mean = 25.2, CV = 0.4 %)
Temperature:	Daily limits: 15 °C (±2 °C). (Mean = 15.1 °C, CV = 1.4 %)
Photoperiod:	12 h light: 12 h dark
Test vessel:	2-L glass Pyrex squat-form beaker
Sediment volume:	160 mL (15 mm depth)
Overlying water volume:	Fill to 1200 mL mark in test vessel (ca.1000 mL)
Water renewal:	Once per week: siphon off and replace 800 mL
Life stage and size of amphipod:	Life-cycle test: neonates, retained between 300 µm and 500 µm mesh screens Partial life-cycle: Juveniles, < 4 mm mean length with CV < 10 %
Number test organisms/vessel:	Life-cycle test: 30 Partial life-cycle: 20
Number of replicate vessels:	Minimum of 5 + dummy vessels for chemical analyses
Feeding:	Standard aquarium invertebrate diet: 2 drops per vessel, once per week, 24 h prior to water exchange
Aeration:	Constant 1-2 bubbles s^{-1} *via* glass Pasteur pipette
Dissolved oxygen (DO):	Daily limits: >3.6 mg L^{-1} (Mean = 6.73 mg L^{-1}, CV = 1.4 %)
pH:	Within 7.0 – 9.0 pH units (Mean = 8.22, CV = 0.9 %)
Test duration:	When reproduction occurs in all control replicates
Endpoints:	Survival, growth rate and reproduction upon test termination Survival and growth rate (µg $individual^{-1}$ day^{-1}) at 28 days if used.
Performance criteria:	Control survival at test termination ≥ 70 %. (Mean = 95 %, CV = 9.0 %)

Baseline and fractionated oils sediment tests

Spiking of sediments

Sediments were spiked with ANS, TJP and SLK oils to give nominal concentrations of 1000 μg g^{-1} (dry wt.) for acute tests and 500 μg g^{-1} (dry wt.) for baseline chronic tests. Nominal concentrations for tests with fractionated oil were whole oil equivalents based on gravimetric analysis of oils (e.g. a fraction representing 20 % of the whole oil would be spiked at a concentration of 100 μg g^{-1} dry wt. (Table 4.1)). The spiking method was based on that of Roddie and Thain (2001). Oils were dissolved in dichloromethane (DCM) as they were not readily soluble in acetone or methanol and spiked (5 mL) onto 20 g aliquots of dry sediments. The spiked sediment was left overnight for the solvent to completely evaporate and then mixed with 320 mL aliquots of wet sediment and 100 mL of 25 psu seawater in wide neck 500 mL glass bottles (Schott). The combined spiked sediments were shaken vigorously by hand, then by orbital shaker at 200 rpm for 3.5 h. The bottles were again vigorously shaken by hand and the slurry from each divided equally between two 2 L Pyrex beakers. Solvent controls were created using 5 mL of DCM as above. Additional replicates were created for chemical analysis and behavioural tests.

Acute sediment exposure toxicity test

Acute sediment tests were based on standard 10-day sediment toxicity tests (ASTM, 2000; Roddie & Thain, 2001; USEPA, 1994) but with the slight alteration to the standard protocol to give greater consistency with the preliminary and chronic tests i.e. a 12:12 h light:dark regime was imposed in preference to continuous light. Juvenile *C. volutator* were exposed to a nominal concentration of 1000 μg g^{-1} (dry wt. oil). Details of lengths and weights of amphipods are provided within methods sections of relevant chapters. Test conditions were as described for preliminary tests. Five replicate vessels were used for each of the five treatments; a total of 100 amphipods per exposure treatment. Water

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

Chronic sediment exposure toxicity tests

Chronic tests were based on the acute 10-day sediment test (Roddie & Thain, 2001) and the USEPA (2001) amphipod chronic sediment tests. The test was essentially as described for the preliminary chronic study except that the test was shortened by initiating the test with juvenile amphipods. Juvenile *C. volutator* (details of lengths and weights of amphipods together with exposure periods are provided within methods sections of relevant chapters) were exposed to nominal oil concentrations as detailed above. Amphipods (n = 20) were transferred to 25 mL beakers *via* plastic Pasteur pipettes and then randomly allocated to exposure vessels (2 L squat-form Pyrex beakers). Care was taken to ensure that the amphipods were not trapped by the surface tension of the water. Five replicates for each of five treatments were used to assess the chronic toxicity of the oils; a total of 100 amphipods per treatment. The animals were fed weekly with 2 drops of standard aquarium invertebrate food as described above and the overlying water 80 % replaced 24 h after feeding. Water quality measurements were measured before addition of amphipods and prior to water exchanges during the test. Test conditions and water quality acceptability limits are given in Table 2.3.

The tests were terminated when reproduction was apparent in all replicates of the control treatment. Survivorship, wet weights and lengths of organisms were recorded. The surviving amphipods were collectively weighed to 0.1 mg (amphipods were carefully blotted on absorbent paper to remove excess water) to obtain wet weights then separated

into groups of mature adult (≥5.0 mm excluding antennae), sub-adult (<5.0 mm), gravid females and males with the aid of a dissecting microscope. Following chronic tests involving fractionated oils, all adult and subadult amphipods were measured. The amphipods were rinsed with deionised water then dried at 60 °C for 24 h to obtain dry weights. The numbers of neonate *C. volutator* were also counted. Although the majority of neonates could be detected from their movement and separated from the debris for enumeration, the separation from the debris of the remaining organisms was facilitated by the addition of 70 % isopropanol plus a few drops of Rose Bengal solution (*ca.* 1g L^{-1}). All neonates were preserved in the 70 % isopropanol /Rose Bengal solution for recounting for quality assurance (QA) purposes.

Behavioural tests using *Corophium volutator*

Details of the behavioural tests using *C. volutator* that were performed alongside acute and chronic preliminary and baseline tests are provided within Scarlett *et al.*(2007a). The same methodology was applied to sediment spiked with oil fractions prior to chronic exposure tests (**Chapter 4**).

2.7.2 Aqueous exposure tests with *Mytilus edulis*

Preparation of test solutions and exposure conditions

All aqueous exposure tests were semi-static with complete water exchanges every 24 h. Lengths of exposure varied depending on the physical and chemical properties of the contaminant under investigation, and the information being sought; details of exposure are given within the relevant chapter sections. Tests were similar to those described by Donkin *et al.* (1991) except that groups of nine mussels were exposed in 9 L of test compound instead of 16 mussels in 18 L as previously reported. Unless otherwise stated, test solutions were prepared by injecting 0.5 mL of an acetone solution of the test

compound into 10 L of filtered seawater held at 15 °C in a glass aspirator (i.e. acetone conc. 0.005 % v/v). The test solution was vortex mixed (magnetic system with Teflon-coated follower) for a minimum of 2 h prior to use. The test solutions were added to the mussel exposure vessels and replaced every 24 h. Mussels were fed continuously with *Isochrysis galbana* (Reed Mariculture Inc., Campbell, CA, 0.11-0.15 mg dry weight mL^{-1}) delivered *via* glass Pasteur pipettes by means of a peristaltic pump at a rate of ~20 mL h^{-1}. Aeration was supplied *via* glass Pasteur pipettes which also aided dispersion of the *Isochrysis* suspension. Water quality measurements of dissolved oxygen, pH, salinity, and temperature were recorded daily prior to water exchange.

Measurement of clearance rates

The feeding rate assay was adapted from Donkin *et al.* (1991; 1989) and as reported by Scarlett *et al.* (2005). In brief: mussels were placed individually in 400-mL glass beakers containing 350 mL of clean filtered (2 μm) seawater at 15 °C. After an acclimation period with slow vortex mixing, 500 μL of *Isochrysis* algal suspension was added to give ~25 × 10^3 cells mL^{-1}. A 20 mL water sample was removed immediately from all the beakers upon the addition of the algae and retained in vials for algae enumeration. Further samples were taken after 15 and/or 30 min. Algal particles (3 to 10 μm) were enumerated using a Beckman Z2 Coulter particle count and size analyzer (Beckman Coulter, Wycombe, UK). From the loss of algal particles during the 30-min period, the feeding rates of the mussels were determined (Equation 2.1). Mussels were stored at -80 °C prior to extraction and quantitation of hydrocarbons.

$$\text{Clearance rate (L hr}^{-1}) = (v \times 60 / t)\ (\text{Ln } t_0 - \text{Ln } t_2)$$

Equation 2.1 Calculation of mussel feeding rates (L hr^{-1}) derived from algae cell counts. Where: v = volume of water in feeding rate beaker (L), t = duration of assay (minutes), t_0 = initial cell count and t_2 = final cell count after t minutes.

Assessment of cellular viability (Neutral Red Retention)

The cellular viability of mussels exposed for 14 days to BABs was compared to that of the control organisms. The procedures were based on Pipe *et al.* (1999), modified by Rickwood & Galloway (2004).

Platereader: Optimax tunable microplate reader spectrophotometer, Global Medical Instrumentations Inc, Ramsey, Minnesota.

Microplates: Sero-Wel 120 microwell plate, Sterilin, Barloworld Scientific Ltd. (Stone, Staffs., UK).

Chemicals and reagents: Sigma-Aldrich (Poole, UK), unless otherwise stated.

Marine Bivalve Physiological Solution: Hepes 4.77 g, sodium chloride, 25.48 g, magnesium sulphate, 13.06 g, potassium chloride, 0.75 g, calcium chloride, 1.47 g. Made up to 1 L with distilled water.

Neutral Red solution: Neutral Red, 0.004 g (Sigma N-7005). Made up to 100 mL with Marine Bivalve Physiological Solution.

Acidified ethanol: Glacial acetic acid 1 mL (Sigma A-6283), ethanol 50 mL (28719 4N, BDH Chemicals Ltd., Poole, UK). Made up to 100 mL with distilled water.

Additional: Poly-L-lysine (Sigma P-8920), syringes and needles (Sigma Z-23,072-3 and Z-19,240-6), multiwell plates (Sterilin 612F96), Siliconised eppendorfs (Sigma T-3406), microplate sealers (DIS-961-050K Fisher), BioRad protein assay (500-0006, BioRad Laboratories, München, Germany).

Microwell plates were coated (100 µL per well) in dilute Poly-L-lysine (1:10 ratio Poly-L-lysine: distilled water) 24 h prior to assay, and the wells drained. Haemolymph was extracted by syringe from the posterior abductor muscle of BABs-exposed and solvent control mussels (8 mussels per treatment) and stored on ice within Eppendorf tubes prior to pipetting 50 µL (in triplicate) to microplate wells. The microplate was agitated at 400 rpm for 60 s then incubated for 50 min at 15 °C. Following incubation the excess liquid was removed from the wells (*via* the grooves in the well sides to ensure that adhered cells were not disturbed) and 200 µL of Neutral Red solution (4 mg Neutral Red in 100 mL marine bivalve physiological saline solution) added then incubated in darkness at 15 °C for 3 h. The supernatant was then removed and washed with marine bivalve physiological saline solution to remove residual Neutral Red. Acidified ethanol (200 µL) was placed in the wells, the microplate covered and incubated for 10 min. The plate was agitated for 30 s and then the absorbance at 550 nm read using a platereader. The supernatant was then removed from the wells.

The protein within wells was then quantified by reference to a standard curve derived from protein standards. Standard solutions were prepared from 2 mg mL^{-1} protein standard (Sigma PO834) in distilled water to give concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1}. Bio-Rad reagent (500-0006) was diluted with distilled water to give a 25 % solution. The protein standards were pipetted (10 µL) into to empty wells and the dilute Bio-Rad reagent added to all wells including those containing the mussel haemolymph. The microplate was covered and incubated in darkness for 15 min then the absorbance read at 595 nm. The Neutral Red retention was then calculated per unit of protein.

2.7.3 Bioluminescence inhibition tests using *Vibrio fischeri*

Quality control and reference tests

The bioluminescence inhibition assays was performed using the Microtox model 500 analyser. Pipetting accuracy and precision are important determinants for ensuring repeatable results when using the Microtox system. For quality assurance purposes, basic tests were performed on standard reference toxicants phenol and zinc sulphate and the results compared with those provided by the bacteria suppliers (SDI Europe, Hook, UK). Tests performed using reference materials showed a good agreement with published data with high precision. Repeated Microtox tests using reference toxicants $ZnSO_4$ and phenol produced EC_{50} values within confidence limits provided by suppliers (SDI Europe Ltd) and coefficients of variation (CV) <1 0 % comparable to the lowest CV of >10 % (mean 28 %) reported for a recent interlaboratory comparison exercise (Riva & Bibó, 2005).

Microtox test protocols

The rationale for the use of the bioluminescence test is provided within Section 2.5 The test protocols were either the standard 'basic test' protocol (i) or the standard 'comparison test for marine and estuarine samples' protocol (ii) (MicrotoxOmni™, 2005). For both tests, the frozen Microtox acute toxicants test reagent was reconstituted in 1000 µL of Microtox reconstitution solution, placed in the reagent well (15 °C ± 0.5 °C) and used within two hours. In brief: (i) the bacteria *V. fischeri* were exposed to serial dilutions of test solutions of oils dissolved in solvent and spiked into Microtox diluent (SDI Europe, Hook, UK) for 5 and 15 minute periods and their response compared to that of the reference solution (solvent control in diluent at equivalent solvent concentration). The maximum exposure concentration for the basic test was 49 %. Where possible, the concentration at which bioluminescence was inhibited by 50 % (IC_{50}) was calculated; (ii) the bacteria *V. fischeri* were exposed to five replicates of test solutions for 5 and 15 minute

periods and their response compared to that of the reference solution (solvent control in seawater at equivalent solvent concentration). Reference sample solutions (1500 µL) were placed in 6 cuvettes and test sample solutions (1500 µL) placed in 5 cuvettes; these were left in the analyser incubator (15 °C ± 1 °C) for 5 min whilst the test reagent was reconstituted. The reconstituted reagent (150 µL) was pipetted into one of the cuvettes containing the reference sample solution and mixed by aspiration. Aliquots of the mixed reference solution (100 µL) were pipetted into 10 empty cuvettes and left for 15 min; the light emission from each was read. Immediately, 900 µL of reference sample and test sample solutions were transferred. The light emission was re-measured after 5 and 15 min. The maximum exposure concentration for the comparison test was 90 %.

2.7.4 Trophic transfer tests with *Mytilus edulis* and *Carcinus maenas*

Exposure procedures

Common shore crabs, *C. maenas* were fed with mussels contaminated by BABs to test for transfer of the contaminants from one trophic level to another. Following a seven day laboratory exposure, the health of the crabs was assessed in terms of cellular biomarkers, physiology, behaviour and tissue burden. Crab urine was also analysed for the presence of BABs or their metabolites.

Mussels (collected from Port Quin and maintained as previously described, Section 2.6) were semi-statically exposed to 40 µg L^{-1} BABs for 72 h with water exchanges every 24 h. Water spiking and mussel exposure conditions were as described previously (Section 2.7.2) except that 10 mussels were exposed in 10 L of test water. Mean length was 40.6 mm (SE = 0.13mm, CV = 5.0 %, n = 240). The mussels were exposed sequentially to provide two contaminated mussels per crab per day i.e. one mussel from each of two exposure vessels. The remaining mussel from each exposure vessel was retained and

stored frozen at -80 °C. Based on previous experiments (see **Chapter 5**), the crabs were exposed to ~15 µg BABs per day, i.e. a total maximum exposure of about 100 µg from their diet of contaminated mussels.

Previous 72 h mussel exposure tests of BABs had shown no significant differences in terms of effects upon clearance rates or chemistry of extracted tissues), between solvent (0.005 % acetone) and seawater controls; for logistical reasons, the trophic transfer test was performed with solvent control mussels only. The mussels were cut with a scalpel and opened slightly to facilitate ease of feeding by the crabs and to avoid confounding factors. Mussels contaminated by BABs may have less ability to withstand attack from the crabs which may cause differences in energetic expenditure. Crabs (n = 9 per treatment, mean carapace width = 65.7 mm (SE = 0.87 mm, CV = 5.6 %; mean weight = 73.4 g, SE = 2.7 g, CV = 15.8 %)) were each fed two exposed mussels per day for seven days with seawater exchanged every 48 h. Mussels that were found to be unopened after 4 h within the crab exposure vessels were manually opened fully. Unconsumed mussels were removed during water exchanges. The behaviour of the crabs, in terms of their ability to feed and general reactions during feeding and water exchanges, was monitored throughout the exposure. Following exposure to the mussels, the crabs were not fed for two days prior to behaviour and physiological tests. After testing the crabs were weighed and measured then stored frozen at -80 °C prior to chemical extraction and analysis of tissues.

Behavioural response

Effects on behaviour were assessed by measurement of the time taken to achieve specified actions associated with feeding. Time measurements and behaviour response recognition were aided by digital video filming. Glass aquaria (×2) with sides and lid impenetrable to light visible to *C. maenas,* except the front panel, were filled with 15 L of seawater (15

°C). Opaque partitions were fitted at one end of the aquaria and a frozen cockle in shell, *ca.* 30 mm shell length (Gamma Foods, Tropical Marine Centre Ltd., Chorleywood, Hertfordshire), was placed at a designated position. A crab with heart rate transmitter/detector attached (see below) was placed at a designated position at the opposite end to the cockle and allowed to acclimate for 10 min after which the partition was removed. Time points recorded were:

1. Time taken to engage and break into the cockle
2. Time taken to eat the cockle

The amount of food consumed before abandonment of the cockle was also noted. A 15 min cut-off time was allocated. If a crab failed to engage the cockle within 15 min, or failed to break into the cockle following 15 min from initial engagement, the test was terminated. Crabs were returned to their exposure vessels with 10 L of clean seawater. Uneaten cockles were placed within the exposure vessels of their respective crabs. The behaviour test aquaria were cleaned prior to subsequent tests and alternated between BABs-exposed and solvent-exposed test organisms. Video recordings of the tests were later inspected to aid quantification of behaviour.

Physiological response

The physiological condition of the crabs was assessed by measurement of the heart rate during the behavioural response test (above). The heart rates of the crabs were measured using the Computer-Aided Physiological Monitoring System (CAPMON). The CAPMON technique was originally described in detail by Depledge and Anderson (1990). In brief, an infrared transmitter/detector unit was positioned dorsally over the heart and attached to the carapace using three small spots of cyanocrylate glue (Loctite, Hemel Hempstead, Hertfordshire, UK). The CAPMON system emits infrared light through the carapace onto the heart surface. Conformational changes of the heart alter the intensity of the reflected

light received by the detector. Each complete cardiac cycle was recorded and cumulative data stored at one minute intervals.

Cellular biomarkers of exposure and stress

Following the behaviour and physiological tests, the crabs were placed in clean seawater overnight and their behaviour monitored. The following day, 3 days after the crabs were last fed contaminated mussels, urine and haemolymph were removed from the organisms as described by Watson *et al.* (2004b). In brief, crabs were drained of residual seawater and restrained with the ventral surface uppermost on a plastic board using rubber bands. The third maxillipeds were moved aside and kept apart by inserting absorbent paper between the base of the appendage and top of the sternum. The operculum of each antennal gland bladder was lifted using a hooked seeker, causing urine to flow from the bladder, through the opercula, where it was collected using a 200 µL pipette. Samples (20–400 µL per crab) were then transferred to siliconised microcentrifuge tubes and frozen at −80 °C until analysis. With the crab still restrained, the absorbent paper holding the maxillipeds was removed and a haemolymph sample was taken from a suitable arthrodial membrane at the base of the fourth pereiopod using a disposable syringe. Haemolymph samples and crabs were stored at −80 °C until analysis. The crabs were subsequently thawed and dissected to remove the whole of the midgut gland (hepatopancreas). The tissues were weighed and stored frozen at −80 °C until further analysis.

Cellular viability was assessed using the Neutral Red Retention assay as described in section 2.7.2 The only difference being additional washing steps (×3) of the plate wells with marine bivalve physiological saline solution.

Impairment to the crabs' immune response was assessed using the Phagocytosis assay as originally described by Pipe et al. (1999), refer to Rickwood & Galloway (2004).

Details of the platereader, together with most of the chemicals and reagents used are as described for the Neutral Red Retention assay (section 2.7.2). Additional chemicals and reagents are described below.

Bakers Formal Calcium (B.F.C): 2 % sodium chloride (Sigma S-7653), 1 % calcium acetate (Sigma C-4705), 4 % formaldehyde (Sigma F-1635). Made up with distilled water.

Zymosan particles (Sigma Z-4250)

The phagocytosis activity of haemocytes was determined by measuring the uptake of zymosan particles (derived from *Saccharomyces cerevisiae*) dyed with Neutral Red dye. In brief: Microplate wells were pre-coated in poly-L-lysine solution. BFC (100 µL) was added to negative control wells and incubated at 10 °C for 10 min. Zymosan particle/Neutral Red solution (50 µL) was then added and the microplate incubated for a further 30 min. BFC (100 µL) was added to all wells and the microplate span at 200 rpm for 5 min. The supernatant was removed and the wells washed (×3) with Marine Bivalve Physiological Saline solution. Prior to the final spin, zymosan particles standards ranging from 0.3125×10^7 to 10^8 mL^{-1} were pipetted (100 µL) into free microplate wells. The Neutral Red dye was resolubilised by the addition of acidified ethanol (100 µL). After 10 min the microplate was gently shaken at 200 rpm for 1 min. Particle uptake by adhered cells was estimated by the degree of absorbance at 540 nm against a standard curve of zymosan particles. The BioRad protein assay was used to determine the protein concentration of each haemolymph sample.

Crab urine analysis

Fluorescence was measured using a Hitachi Fluorescence Spectrometer F-4500 (Finchampstead, Berkshire, UK). The C_{12-14} BABs mixture was dissolved in ethanol to give a concentration series of 0 – 5 mg mL^{-1}. Excitation scans of the BABs standard were performed using an emissions λ of 295 - 310 nm, based on reported emission λ of benzene (Ali, 1994). From this an excitation λ of 273 nm was derived which produced a broad emission λ of 290 – 305 nm, presumably due to the complex nature of the large number of isomers present. A linear relationship between fluorescence area and concentration was established ($r^2 = 0.976$, $P = 0002$). Crab urine was diluted in ethanol (1:20) and BABs quantified by interpolation from the standard curve. Sufficient urine for analysis was available for only six of the BABs exposed crabs. Triplicate analysis of crab urine showed a high degree of precision (CV = 2.3 %). The maximum fluorescence peak was shifted from 292 nm in the BABs standards to 296 nm in the crab urine samples.

Chemical extraction and analysis of midgut tissue

The combined tissues from eight crabs from each treatment were split to give two replicates per treatment and placed in preweighed amber glass jars and reweighed to establish tissue wet weight (~14 g per replicate). An internal standard (phenanthrene d_{10}) was added to the tissues which were then extracted by alkaline saponification (see Section 2.3). Following clean up the extracts were reduced in volume to 100 μL and analysed by GC-MS in Full Scan mode and SIM mode with selected fragment ions of *m/z* 91, 105 and 119, and molecular ions *m/z* 246 and 260. Alkylbenzenes were identified by their retention times and mass spectra with reference to the standard BABs mixture. Due to the low abundance of identified BABs, the extracts were further analysed using a more sensitive GC-MS (Agilent 5975C Series GC-MSD) to confirm the identity of the compounds. The tissue concentrations were quantified by reference to two relatively resolved peaks.

2.8 Statistical Analyses

Statistical analyses of results were performed using Statgraphics Plus 5.1. Proportional data were arcsine transformed prior to analysis. Following checks for variance using Cochran's C test, data with >2 groups were analysed by Analysis of Variance (ANOVA). Where there was a significant difference ($P \leq 0.05$) of means, the data were further analysed by the Student Newman Keuls test to determine significant differences ($P \leq 0.05$) between treatments. Unpaired two-sample data were first tested for normality by standardised skewness and standardised kurtosis tests. If within acceptable limits, the data were analysed by *t*-tests, otherwise by Mann-Whitney U-tests.

Chapter 3

Effect-directed analysis: preliminary and baseline toxicity tests

Very few TIE/EDA studies involving hydrocarbons have been directed at sediment-bound contamination and chronic exposure tests are extremely rare. A method for conducting chronic whole sediment tests was therefore developed to determine if such tests were sensitive to subtle changes in growth and reproduction. The preliminary amphipod life-cycle test was carried out using lightly weathered ANS[1] crude oil to allow additionally weathered and biodegraded oils to be compared. To ascertain if environmentally realistic concentrations of UCM hydrocarbons within sediments were toxic to the *C. volutator*, baseline EDA tests were performed on weathered and UCM-dominated oils and the results compared with preliminary tests. To test if amphipod behavioural responses to oil-contaminated sediment could be used as a predictor of chronic effects, a behaviour test was developed using burrowing activity of *C. volutator*. Aqueous exposure tests were performed using *M. edulis* and *V. fischeri* to establish baseline toxicity. Some of the results from these studies have been published (Scarlett *et al.*, 2007a; Scarlett *et al.*, 2007b; Scarlett *et al.*, 2007c).

3.1 Introduction

3.1.1 General introduction

One approach for the determination of components responsible for adverse effects from within a complex mixture is effect-directed analysis (EDA). The terms EDA and 'bioassay-directed fractionation' (BDF) are frequently used interchangeably and with 'toxicity identification evaluation' (TIE). All of these terms describe toxicity assessment of complex samples using biotests coupled with chemical analysis to identify the components responsible for the observed toxicity (Fig. 1.6). Prior knowledge of key contaminants is not necessary, thus avoiding expensive chemical analysis, and interactive toxicity among the components is reflected in the results arising from TIE/BDF/EDA studies. TIE investigations may best be viewed as a specific standardised protocol of EDA with a focus on inorganic and organic toxicants in aqueous samples, while EDA often pre-selects organic toxicants by the extraction procedure and focuses on the total amount of these toxicants rather than the bioavailable fraction (Brack, 2003). More recently, whole sediment TIEs have been under development (e.g. Burgess *et al.*, 2003; Burgess *et al.*, 2004; Ho *et al.*, 2004; Stronkhorst *et al.*, 2003b).

3.1.2 Selective review of effect-directed analysis

For the purposes of this review BDF and EDA will be treated together and referred to as EDA. Both EDA and TIE have the aim of identification of chemical causes of measurable effects i.e. the establishment of cause and effect relationships (Brack, 2003). Marine habitats are often reported to be contaminated with complex mixtures of both known and unknown chemicals. Adverse effects may be observed within such contaminated habitats but determination of which compounds are responsible for the observed effects is difficult to establish. Similarly, UCMs contain many thousands of compounds (Sutton *et al.*, 2005) so determination of which compounds may be responsible for any observed effects is very challenging. With EDA/TIE studies, complex mixtures are tested for biological effects and

subjected to one or more physiochemical fractionation procedures (Fig. 1.6). Following each fractionation further biotesting is performed and active samples further fractionated until the level of complexity is reduced to a few compounds that can be identified and quantified. In order to fractionate environmental samples it is necessary to extract the potential toxicants from the relevant matrix. Extraction procedures are selective and therefore certain components remain in the sample and are not analysed. For example, an efficient extraction method for the collection of non-polar organics may not also be efficient for the collection of metals. The method chosen for extraction is therefore dependent upon what is assumed to be the causative agent of toxicity. If the initial TIE Phase I manipulations have been performed this will help provide direction as to what chemical extraction method and fractionation procedure will be most relevant but otherwise the type of toxicant may be suspected based on additional information. A selective review on the use of EDA to investigate organic contaminants in the marine environment (Table 3.1) found that a variety of toxicity tests and endpoints were utilised. Tests included both acute and chronic Microtox and Mutatox tests, *Salmonella* microsome mutagenicity assay, unicellular algal tests, recombinant yeast estrogen screen (YES assay) and clearance rates of juvenile mussels (see references cited in Table 3.1). No particular assay proved to be of greater sensitivity and Brack *et al.* (2003) reported that the major toxicants were quite different for the different test systems and therefore the results were not consistent with a unique pathway of toxicity but suggested organism-dependent modes of action. From this it is clear that EDA should not rely on a single test species and should involve dissimilar phyla with potentially different modes of toxicant uptake. The main toxicants identified were typically PAHs including alkylated and sulphur-heterocyclic compounds, although many studies concluded that the toxicants remained unidentified (Table 3.1). In a study of creosote-contaminated groundwater, Hartnik *et al.*, (2007) reported that PAHs only accounted for 13 % of the observed toxicity (bioluminescence inhibition) despite representing up to 85 % of pure creosote. Methylated benzenes, phenolics and heterocyclics

accounted for 80 % of the toxicity with alkylated quinolines the most toxic single fraction, accounting for 26 % of the total measured toxicity.

Table 3.1 Effect-directed analysis (EDA) studies pertaining to organic compounds in the marine environment

Sample type / Location	*Biotest*	*Toxicants identified/ suspected*	*Additional notes*	*Reference*
Coastal sediment / Barcelona, Spain	*Salmonella* microsome mutagenicity assay	140 aromatic compounds, 57 of them classified as mutagenic or belonging to mutagenic chemical classes included:1- nitropyrene, 6-nitrochrysene, and 6-nitrobenzo[a]pyrene	Sediments from main polluted sources exhibited lower mutagenic activity than distant locations	(Fernandez *et al.*, 1992)
Dissolved and Particulate Water Phases / Barcelona , Spain	*Salmonella* microsome mutagenicity assay	Candidates for fraction mutagenicity included: o-tolidine, nitroquinoline, nitroaniline, dichlorobenzidine and several aromatic quinones	Study found sediment chronically polluted by frameshift and base-pair substitution mutagens and promutagens	(Grifoll *et al.*, 1992)
Estuarine sediment / Black Rock Harbor, Connecticut	Mutatox™	Known mutagenic polycyclic aromatic hydrocarbons (PAHs)	Operational blank also gave active result	(Ho & Quinn, 1993)
Sediment / Sydney Harbour, Nova Scotia, Canada	*Salmonella typhimurium* microbiological assays	High molecular mass PAHs including benzo[a]pyrene, benzo [ghi] perylene, indeno[1,2,3-cd] pyrene, and compounds of molecular weight 302 Daltons were found to be responsible for the majority of the mutagenic activity	Sediment known to be coal tar contaminated	(Marvin *et al.*, 1995)

Table 3.1 continued

Sample type / Location	*Biotest*	*Toxicants identified/ suspected*	*Additional notes*	*Reference*
Sediment / Sydney Harbour, Nova Scotia, Canada	*Salmonella typhimurium* strain YG1025	Compounds eluting in the most active subfractions included naphtho[2,1- a]pyrene and naphtho[2,3-a]pyrene	PAHs with molecular mass of 302 biologically active in complex environmental mixtures.	(Marvin *et al.*, 1999)
Benthic and suspended sediment / Hamilton Harbour, Western Lake Ontario, Canada	*Salmonella* microsome mutagenicity assay *Salmonella typhimurium* strains YG1025	5- to 7-membered ring PAHs with molecular masses between 252 and 302 Daltons, including the known mutagens and carcinogens benzo[a]pyrene, indeno[1,2,3-cd]pyrene and dibenz[a,h]anthracene	Required oxidative metabolism to activate	(Marvin *et al.*, 2000a)
Sediment / Tyne, Tees, Mersey, and Thames estuaries, Southampton Water, UK	Mutatox	Numerous PAHs, alkyl substituted PAHs, nitro-polycyclic aromatic compounds (nitro-PACs), polycyclic aromatic ketones, oxygenated-PACs, and other known mutagens contributing to the genotoxicity measured in the samples.	Only solvent extract showed mutagenic activity Some potentially genotoxic compounds remain unidentified	(Thomas *et al.*, 2002)

Table 3.1 continued

Sample type / Location	*Biotest*	*Toxicants identified/ suspected*	*Additional notes*	*Reference*
Biota /-New Brighton, Port Quin, Plymouth, UK	Juvenile *M. edulis* clearance rate	Aromatic Unresolved Complex Mixture with 4-6 double bond equivalents major component of toxic fraction.	Toxic fraction eluted with non-toxic naturally occurring squalene	(Donkin *et al.*, 2003)
Photomodified anthracene N/A	Microtox, reproduction of the green algae *Scenedesmus vacuolatus*, and genotoxicity in the *umuC* test	1-hydroxyanthracene-9,10-dione and 1,4-dihydroxyanthracene-9,10-dione were identified and confirmed as genotoxicants. Anthracene-1,4-dione, a trace photometabolite, was identified as a very potent toxicant dominating the toxicity of photomodified anthracene to *V. fischeri*.	Major toxicants were quite different for the different test systems. Hence not a unique pathway of toxicity such as oxidative stress but suggests organism-dependent modes of action	(Brack *et al.*, 2003)
Sediment / Tyne, Tees, Mersey, and Thames estuaries, Southampton Water, UK	Recombinant yeast estrogen screen (YES assay)	Nonylphenol, cirmarizine, and cholesta-4,6-dien-3-one	Only solvent extract showed activity Important estrogen-receptor (ER) agonist substances that contaminate marine sediments remain unidentified	(Thomas *et al.*, 2004)

Table 3.1 continued

Sample type / Location	*Biotest*	*Toxicants identified/ suspected*	*Additional notes*	*Reference*
Sediments from a PCB polluted bay	EROD activities and malformations investigated in rainbow trout (*Oncorhynchus mykiss*) larvae	Fraction mainly composed of dicyclic aromatic compounds (DACs), including PCBs, less teratogenic than the fraction mainly composed of polycyclic aromatic compounds (PACs) -subfraction mainly composed of three- and four-ring compounds (including alkylated and sulphur-heterocyclic compounds).	Results imply that non-additive effects get more pronounced the more complex the exposure	(Sundberg *et al.*, 2005)
Sediment sample from the west coast of Sweden	Chronic algal test *Scenedesmus vacuolatus* and acute, chronic bioassays using *Vibrio fischeri*	Compounds identified as potentially relevant toxicants by chemical analysis of toxic fractions: anthracene, fluoranthene, pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[a]pyrene, benzo[k]fluoranthene, and indeno[1,2,3-cd]pyrene	Chronic algal toxicity was a powerful tool for discriminating between toxic and nontoxic fractions. Acute and chronic bacterial toxicity failed to identify toxic fractions	(Grote *et al.*, 2005a)
Harbour sediment from Zierikzee in Zeeland, The Netherlands	ER- and DR-CALUX	Dioxin-like activity could be explained by the presence of various PAHs. Natural estrogenic hormone 17-beta-estradiol and its metabolite estrone were identified as the main contributors to the estrogenic activity.	Some estrogenic activity of a relatively non-polar nature remained unidentified	(Houtman *et al.*, 2006)

Table 3.1 continued

Sample type / Location	*Biotest*	*Toxicants identified/ suspected*	*Additional notes*	*Reference*
River, sediment, suspended matter and waste water samples from sewage treatment plants on the Upper Danube	Neutral Red assay, comet assay, *Arthrobacter globiformis* dehydrogenase assay, YES assay, fish egg assay with the zebrafish (*Danio rerio*) and Ames test with TA98		Pilot study: authors concluded that it was not possible to elucidate that chemically induced alterations were responsible for the an observed fish decline.	(Keiter *et al.*, 2006)
Creosote-contaminated groundwater, Hommelvik, Norway	Microtox	PAHs, methylated benzenes, phenolics and n-heterocyclics	Methylated benzenes, phenolics and n-heterocyclics accounted for 80 % of toxicity. PAHs only accounted for 13 % toxicity despite representing up to 85 % of pure creosote. Alkylated quinolines were the most toxic single fraction, accounting for 26 % of the total measured toxicity.	(Hartnik *et al.*, 2007)

3.1.3 Effect-directed analysis of sediment-associated UCM hydrocarbons

Non-polar organic contaminants readily associate with sediment particles and colloidal phases within interstitial and overlying water and, to a much lesser degree, the truly dissolved phase which is considered the primary bioavailable phase to aquatic organisms (Burgess & McKinney, 1999). Although sediment-bound UCM hydrocarbons have generally been considered non-bioavailable, sediment-dwelling organisms may accumulate components of the UCM leading to adverse effects. Based on this possibility, Le Blanc *et al.* (1999), cited by Smith (2002), carried out studies into the bioavailability of UCM compounds by incubating New York harbour sediment with macroporous anion-exchange and adsorbent (XAD) resins (used to estimate the desorbable fraction) for sixty days. It was found that a 5-30 % desorption of the sediment-bound UCM occurred and this predominantly contained lower molecular weight compounds but no details were provided of the composition of the UCM. Further study of the potential for sediment-bound UCM hydrocarbons to elicit effects on sediment-dwelling organisms is therefore required. Indeed, the National Research Council's (2003) study *Oil in the Sea III* stated that more information was needed about the chronic biological effects resulting from petroleum hydrocarbons in sediment and highlighted this as a priority area of research.

Previous TIE and EDA studies have concentrated on acute toxicity tests which are very useful for identifying highly toxic chemicals but acute tests may not test key life stage events such as moulting and reproduction during which sensitivity to toxicants may be increased. Also, marine and estuarine sediments are more likely to contain moderately toxic contaminants that fail to cause significant acute mortality. In response to these issues, the USEPA have developed a 28 day life-cycle test using the estuarine amphipod *Leptocheirus plumulosus* (USEPA, 2001) and this was used by McGee *et al.* (2004) to compare chronic with acute toxicity of sediment from Chesapeake Bay, USA. It was

reported that sublethal toxicity arising from chronic toxicity was only found in sediments tested at one laboratory and was not replicated at another testing laboratory. The authors suggested the differences in amphipod diet may have been responsible for the discrepancy but this could have been due to temperature regime differences. One laboratory conducted the tests at 2 °C below the USEPA (2001) guidelines. The *L. plumulosus* test should be conducted at 25 °C and the organism's life-cycle completed within the 28 day exposure period. A similar but slower growing amphipod test species in northern European waters is *C. volutator*, which resides in muddy intertidal sediment with summer seawater temperatures typically around 15 °C. *Corophium volutator* is now a standard European test organism for acute sediment toxicity testing (PARCOM, 1993; Roddie & Thain, 2001) and has been used in many acute studies (Bat & Raffaelli, 1998; Briggs *et al.*, 2003; Ciarelli *et al.*, 1997). *Corophium volutator* has also been used in long-term life-cycle tests (Brown *et al.*, 1999; Conradi & Depledge, 1998; Conradi & Depledge, 1999) but the amphipods were only exposed to aqueous toxicants and not contaminants associated with whole sediments. Peters & Ahlf (2005) have demonstrated that *C. volutator* can be successfully cultured in the laboratory and the authors recommended the use of this species for chronic exposure toxicity testing. A chronic sediment test using the amphipod *Gammarus locusta* L has recently been described (Costa *et al.*, 2005). However, the test has to be conducted at a temperature above normal environmental conditions in order to complete the life-cycle within 28 days (Neuparth *et al.*, 2002) and *Gammarus* spp. are known to display cannibalistic behaviour (Dick, 1995) which suggests the use of *G. locusta* for chronic studies is less than ideal. Because sediments contaminated by UCM hydrocarbons are unlikely to exhibit acute effects upon sediment-dwelling organisms due to the limited bioavailability of sediment-bound compounds it was beneficial to complement the standard acute tests with chronic testing using *C. volutator* to evaluate the

toxicity of whole sediments such that the effects of UCM hydrocarbons upon the survival, growth and reproduction can be evaluated.

Chronic exposures are advantageous but represent a significant commitment in time and resources. One possibility for screening potentially chronically toxic samples is the use of an organism's behaviour. An investigation into the use of amphipod behaviour as a surrogate for chronic toxic effects was carried out in conjunction with chronic sediment toxicity tests and a preliminary study has been published. This paper (Scarlett *et al.*, 2007a) details all the methodology, results and discussion pertaining to the behavioural tests associated with the preliminary whole sediment test with ANS^{i} and the baseline toxicity tests with UCM-dominated oils, and therefore these will not be replicated herein, but the publication is included within the Appendices.

3.1.4 Effect-directed analysis of aqueous-phase UCM hydrocarbons

The creation of aqueous-phase test media for oils is problematic as oil contains many thousands of compounds with highly differing degrees of solubility. In addition, the solubility of individual hydrocarbons is dependent upon the other hydrocarbons present, in accordance with Raoult's law (Page *et al.*, 2000). The most toxic components of oil are regarded to be present within the WSF (Tsvetnenko & Evans, 2002) but reproducible WSFs are difficult to create and have been criticised as environmentally unrealistic (Baussant *et al.*, 2001b). The relatively heavy UCM-dominated oils are particularly difficult to solubilise within a time period acceptable for toxicity testing. An alternative to the creation of WSFs is oil in water dispersions (Baussant *et al.*, 2001a; Baussant *et al.*, 2001b; Sanni *et al.*, 1998). However, these require sophisticated devices in order that reproducible oil dispersions are formed. A pragmatic approach is to enhance the solubility of the hydrocarbons using a water-miscible solvent and to relate any observed toxic effects

with compounds accumulated into the tissues of test organisms rather than in the aqueous phase. The profile of the bioaccumulated compounds can be compared with that from tissue extracts of wild organisms. Such an approach would be unacceptable if using conventional gas chromatography due to the lack of resolution of UCM compounds but the improved resolution permitted by GC×GC-ToF-MS enables useful information, including the possibility of identifying specific groups of compounds, to be gleaned from aqueous tests using *M. edulis* as a test species.

As an adjunct to biotesting with *M. edulis*, the Microtox bioluminescence inhibition assay may provide a rapid screening tool for the identification of toxic solutions. From the literature (e.g. Leftley, 2000; van Beelen, 2003), it would appear that the bioluminescence inhibition assay may be less sensitive (*ca.* 10-20×) to hydrocarbon toxicity than sublethal tests with *M. edulis*. In addition, *V. fischeri* is reported to be sensitive to solvents and their presence also affect the toxicities of other compounds (Johnson & Long, 1998). The bioluminescence inhibition test does however have the advantages of rapidity and the requirement of very small toxicant test volumes. If correlations between adverse effects on *M. edulis* and bioluminescence inhibition can be established, further EDA studies can use the bioluminescence inhibition test as a proxy for effects on *M. edulis*.

3.1.5 Aims of the present study

The research described in this chapter was concerned with establishing baseline acute and chronic, aqueous and sediment, toxicity of the three selected oils: SLK, TJP and ANS. Prior to proceeding with this it was necessary to develop suitable methods for the chronic testing of sediments to determine if long-term sediment exposures were sufficiently sensitive to detect differences between treatment groups given the logistical constraints. By using the less weathered ANS^{i} oil for the method development, information concerning

the relative toxicities of resolved components of the oil could be derived by comparison with results derived from tests using the more highly weathered ANS. Due to the long exposure time necessary to establish population-level effects using *C. volutator*, a behavioural test was developed to determine if behaviour could serve as a proxy for chronic test endpoints. The predominantly aliphatic SLK had been found previously not to cause adverse effects to *M. edulis* (Thomas, 1995) so it was hypothesised that SLK would be less toxic than the oils with a greater aromatic content to all species tested. Aims were:

1. To develop a method for testing chronic exposure to sediment-bound hydrocarbons using the amphipod *C. volutator*, and quantify effects.
2. To develop a method for quantifying amphipod behaviour and test for association with chronic test endpoints.
3. To establish baseline toxicity for the three oils when associated with sediment and to test for differences that could be related to oil composition.
4. To establish baseline toxicity for the three oils when in the aqueous phase (i.e. dissolved or accommodated within seawater) and to test for differences that could be related to oil composition.
5. To compare the results of 1 and 3 to derive the relative toxicities of the resolved and unresolved components of sediment-bound hydrocarbons.
6. To compare the results of 3 and 4 to derive information concerning routes of uptake of UCM hydrocarbons.

3.2 Methodology

3.2.1 Preliminary sediment tests using lightly weathered ANS[i] crude oil

Preliminary acute and chronic sediment exposure tests were performed using sediments spiked with lightly weathered (*ca.* 20 % evaporative loss by mass) ANS. Sediments were spiked with oil to produce concentrations consistent with subtidal petrogenic hydrocarbon concentrations reported following real and artificial oil spills (NRC, 1989). In addition, sediments were also spiked with WAF and dispersed WAF as the former is widely considered to represent the most acutely toxic bioavailable fraction of crude oil and the latter has potentially greater toxic component concentrations due to the effect of the dispersant in stabilising large numbers of microscopic oil droplets within the water column. Preliminary tests were conducted using *C. volutator* with endpoints of survivorship, growth rate and reproductive success. This study has been published (Scarlett *et al.*, 2007c) and will not be repeated herein, but the publication is available within the Appendices.

3.2.2 Baseline whole-sediment toxicity tests using weathered and biodegraded oils

Baseline acute and chronic sediment exposure tests were performed using sediments spiked with the three crude oils (see **Chapter 2** Section 2.7): additionally weathered ANS (weathered ANS from preliminary study further weathered to achieve a total of 36 % loss by mass achieved by gentle airflow until stable weight, as described in **Chapter 2**), Silkolene-150 (SLK, a lube oil comprised almost entirely of aliphatic UCM when analysed by gas chromatography- flame ionization detection) and Tia Juana Pesado, (TJP, a biodegraded crude oil comprised almost entirely of aliphatic and aromatic UCM). Further descriptions and gas chromatograms of the oils are available in **Chapter 2**. Baseline tests were conducted using juvenile *C. volutator* with endpoints of survivorship, growth rate and reproductive success. This study has also been published (Scarlett *et al.*, 2007b).

Supply and maintenance of organisms during acclimation.

Sediment was collected from an intertidal area of the Avon estuary near Aveton Gifford, south Devon UK as detailed within **Chapter 2**. Amphipods were unavailable from this location at the time; consequentially, juvenile *C. volutator* were supplied by Guernsey Sea Farms (Vale, Guernsey, UK) and maintained as previously described. Test conditions and acceptability requirements are given in Table 2.3.

Reference toxicity test

In order to test that the supplied *C. volutator* population was consistent with the local *C. volutator* population from Aveton Gifford used previously, the test organisms were exposed to $CdCl_2$ using a method described by Ciarelli *et al.* (1997) and their sensitivity to a standard toxicant compared with published data (Ciarelli *et al.*, 1997) and that obtained during previous reference toxicity testing. Procedural details are provided in **Chapter 2** and (Scarlett *et al.*, 2007b).

Additional artificial weathering of ANS oil

The slightly weathered (*ca* 20 %) ANS[i] crude oil as produced for the preliminary tests was further weathered under conditions as described previously until a stable weight was achieved (72 h). The weathered oil was then rotary evaporated at 40 °C for 30 min to ensure loss of volatile components (no further loss was observed). Once weathered the oil was stored in completely filled amber glass bottles at 4°C until use.

Chemical analyses of oils and sediment

The three oils were dissolved in DCM and analysed by GC-MS. Full details are provided within **Chapter 2**.

Spiking of sediments

Sediments were spiked with ANS, TJP and SLK to give nominal concentrations of 1000 $\mu g\ g^{-1}$ (dry wt.) for acute tests and 500 $\mu g\ g^{-1}$ (dry wt.) for chronic tests. The spiking method was based on that of Roddie and Thain (2001) and as detailed within **Chapter 2** and Scarlett *et al.* (2007b).

Acute sediment toxicity test

Acute sediment tests were similar to that described for the preliminary acute test. Juvenile *C. volutator* (20 individuals per vessel) with a mean length 3.8 mm (SE = 0.01 mm) were exposed to a nominal concentration of 1000 $\mu g \cdot g^{-1}$ (dry wt. oil). Test conditions were as described for preliminary acute tests. Five replicate vessels were used for each of the five treatments; a total of 100 amphipods per exposure treatment.

Chronic sediment toxicity test

Chronic tests were similar to those described for the preliminary study but the test was shortened by initiating the test with juvenile amphipods. Juvenile *C. volutator* (20 amphipods per vessel, mean length = 3.8 mm, SE = 0.01 mm, mean wet weight = 1.580 mg, mean dry weight = 0.198 mg) were exposed to a nominal oil concentration of 500 $\mu g\ g^{-1}$ (dry wt). Five replicates for each of five treatments were used to assess the chronic toxicity of the oils; a total of 100 amphipods per treatment. The test was terminated after 35 days exposure when reproduction was apparent in all replicates of the control treatment. Further details are provided in **Chapter 2**.

3.2.3 Baseline aqueous toxicity tests of weathered and biodegraded oils

Collection and maintenance of Mytilus edulis

Mussels were collected and maintained as reported previously (Scarlett *et al.*, 2005). The details are provided within **Chapter 2**. The mean shell length of mussels used in the baseline tests was 21.5 mm (SE = 0.15 mm, n = 45).

Semi-static 48 h exposure tests were similar to the hydrocarbon tests described by Donkin *et al.* (1991), as detailed in **Chapter 2**. Due to the poor solubility of the oils, especially TJP, the oils were measured into preweighed 7 mL vials, dissolved into 1 mL of DCM and 5 mL of acetone added. The dissolved oils were then reduced under a gentle stream of nitrogen to 3 mL then made up to 5 mL with acetone. Test solutions of whole oils were prepared by injecting 5 mL of the acetone solution of the test compound into 10 L of filtered seawater held at 15 °C in a glass aspirator (i.e. acetone conc. 0.05 % v/v). The test solution was vortex mixed for 24 h prior to use. The stirring was stopped for five minutes prior to removal of the test solutions to allow non-dissolved or non-accommodated components to rise to the surface. Subsamples (*ca.* 20 mL) of the test solutions were taken for use with the bioluminescence inhibition assay. Test solutions were added to the mussel exposure vessels and replaced after 24 h. The maximum nominal concentrations of oils in seawater were 30.9, 30.8 and 27.7 mg L^{-1} for SLK, TJP and ANS respectively. Due to the poor solubility of the fractions in seawater, as evidenced by the formation of oily slicks on the surface of the test solutions and adsorbed onto the glass surface of the aspirators, the true aqueous concentrations of the fractions would have been considerably lower than the nominal concentrations.

Measurement of clearance rate of M. edulis

The clearance rate assay was adapted from Donkin *et al.* (1991; 1989) and as reported by Scarlett *et al.* (2005). Details of the protocol are provided in **Chapter 2**.

Bioluminescence inhibition tests

The bioluminescence inhibition assay was performed using the standard 'comparison of estuarine and coastal samples' protocol (MicrotoxOmni™, 2005). Details of the test protocol are provided in **Chapter 2**. Test solutions were prepared from whole oils and were as used for the 48 h mussel exposure test. The effects of the test solutions were compared to the solvent control solution (0.05 % acetone, as described above).

3.3 Results

3.3.1 Preliminary sediment tests using lightly weathered ANS crude oil

Results of the preliminary tests are presented in Scarlett *et al.* (2007c).

3.3.2 Baseline Sediment Toxicity Tests using weathered and biodegraded oils

Reference toxicity test

Mortality of *C. volutator* exposed to 7 mg L^{-1} $CdCl_2$ was consistent with the previously derived LC_{50} value for the local population used during preliminary oil-spiked sediment exposure tests and with 72 h LC_{50} literature values (Ciarelli *et al.*, 1997). The result corroborates the assumption that *C. volutator* populations from relatively unpolluted areas have similar sensitivities to toxicants.

Acute sediment toxicity test

Sediment tests showed no acute mortality within any treatment with mean survivorship > 90 %.

Chronic sediment toxicity test

Water quality measurements were well within acceptable limits (Table 2.3) and remained consistent throughout the exposure period. Growth rates based on dry weight of amphipods showed a very similar pattern to that based on wet weights (i.e. used previously during preliminary tests). Juvenile *C. volutator* exposed to nominal oil concentrations of 500 µg g^{-1} for 35 days had slightly lower growth rates than the negative control organisms but only the weathered ANS was significantly less ($P \leq 0.05$) than the solvent control (Fig. 3.1). The lower growth rates of the ANS exposed amphipods resulted in a significantly lower ($P \leq 0.05$) number of mature adults. Reproduction was significantly reduced ($P \leq 0.05$) in all oil exposures although this was more pronounced in the ANS treatment (Table.

3.2). There was no significant difference in the male:female ratios or the number of gravid females per survivor (Table 3.2)

Table 3.2 Effect of weathered Alaskan North Slope (ANS), Tia Juana Pesado (TJP) and Silkolene-150 spiked sediment on acute survival (sediment oil concentration 1000 μg g^{-1}) and chronic endpoints (sediment oil concentration 500 μg g^{-1})

	Acute		*Chronic*		
Treatment	% 10-day survival	% 35-day survival	Offspring/ Survivor	Offspring/ female	Gravid females/ survivor
Control	90 (2.9)	95 (3.2)	2.26 (0.23)	3.76 (0.35)	0.42 (0.07)
Solvent	95 (5.0)	92 (3.7)	2.52 (0.61)	4.26 (0.86)	0.45 (0.06)
ANS	90 (2.9)	92 (4.4)	0.67 (0.21)	1.56 (0.48)	0.33 (0.12)
TJP	90 5.0)	96 (2.3)	1.16 (0.35)	2.19 (0.59)	0.33 (0.04)
SLK	85 (5.8)	90 (3.2)	0.98 (0.29)	1.82 (0.56)	0.45 (0.08)

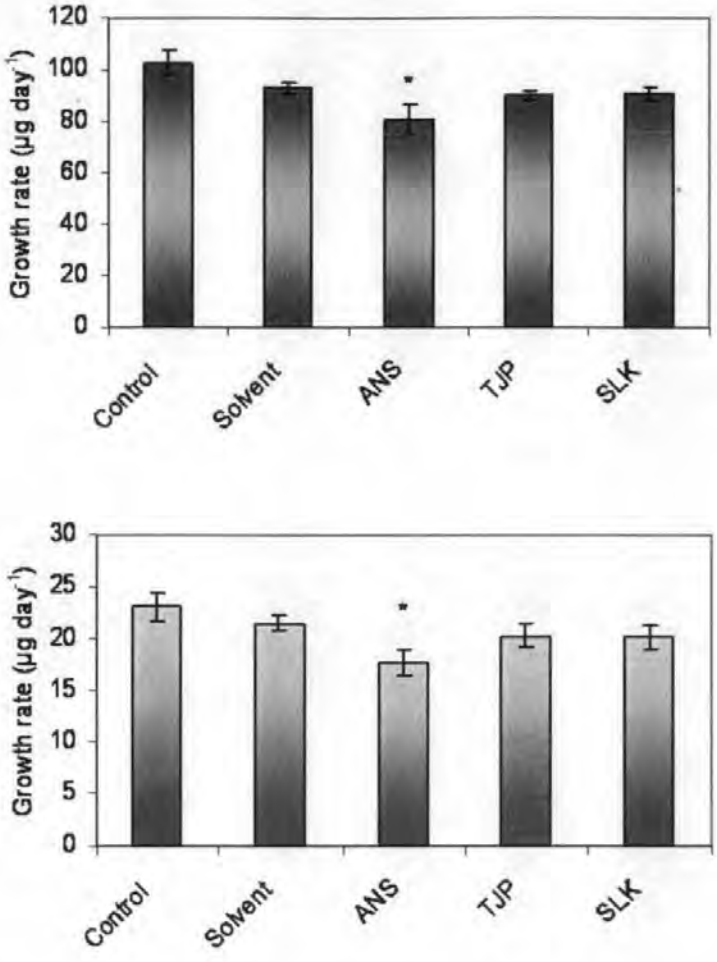


Figure 3.1 Mean growth rates based on (a) wet weights and (b) dry weights of amphipods *C. volutator* exposed to 35 days of sediment spiked with 3 UCM-dominated oils (ANS = weathered Alaskan North Slope, TJP = Tia Juana Pesado, SLK = Silkolene-150. Nominal concentrations were 500 µg g^{-1} (dry sediment weight). Tests used 20 juvenile *C. volutator* per vessel with 5 replicate vessels per treatment, error bars = 1 standard error, * donates significant difference ($P \leq 0.05$) from control/solvent values.

3.2.3 Baseline aqueous tests of biodegraded and weathered oils

Mytilus edulis

No significant differences ($P = 0.22$) were found between the clearance rates of the oil-exposed mussels and that of either the seawater or solvent control mussels; the possibility of a hormesis effect was however suggested by the response of the SLK exposed mussels (Fig 3.2).

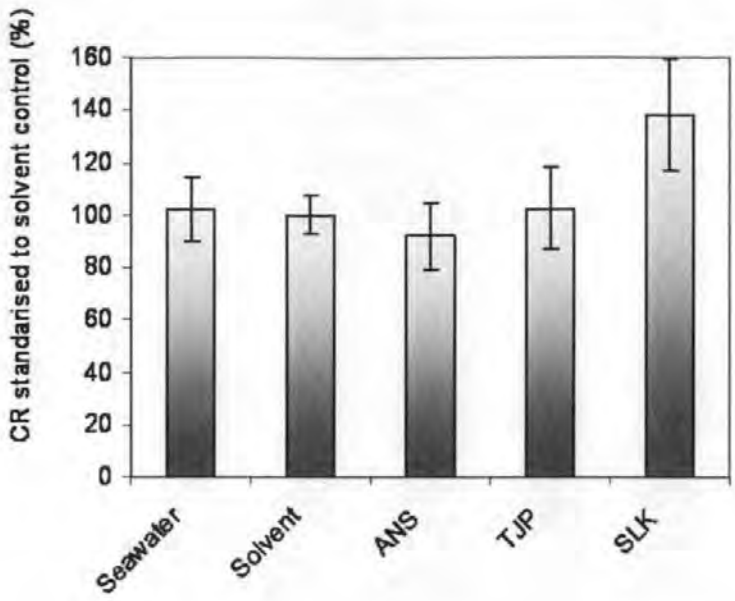


Figure 3.2 Mean clearance rates standardised to solvent control mussels *M. edulis* (n = 9 mussels per treatment) following 48 h semi-static aqueous exposure to Silkolene-150 (SLK), Tia Juana Pesado (TJP) and weathered Alaskan North Slope (ANS) oils. Errors bars = 1 standard error.

Bioluminescence inhibition test

The effects upon bioluminescence of test solutions prepared from whole oils were compared to that of the solution prepared from the solvent carrier. No significant effects ($P > 0.05$) on bioluminescence inhibition were found arising from 5 or 15 min exposure to any of the oils (Table 3.3).

Table 3.3 Test statistic *t* and probability *P* for differences in bioluminescence inhibition after 5 and 15 min exposure to test solutions of whole oils (maximum nominal ~30 mg L^{-1}) compared to solvent control solutions

	5 min		*15 min*	
	t	*P*	*t*	*P*
ANS	0.83	0.43	1.11	0.30
TJP	0.39	0.70	0.95	0.37
SLK	0.34	0.74	0.43	0.68

3.4 Discussion

3.4.1 Preliminary whole sediment toxicity test

Discussion related purely to this test is provided within Scarlett *et al.* (2007c). A comparison of the results of this test with those of the baseline tests for the UCM oils is discussed below.

3.4.2 Baseline whole sediment toxicity test using weathered and biodegraded oils

The chronic baseline whole-sediment toxicity tests were performed using a shortened protocol based on the life-cycle amphipod test established during preliminary studies (Scarlett *et al.*, 2007c). The test maintained exposure to early life stages of organisms which are thought to be more sensitive than adults (van den Heuvel-Greve *et al.*, 2007). Exposure to aromatic hydrocarbons has been hypothesised to disrupt the moulting process (Olmstead & Leblanc, 2005) by which growth occurs in *C. volutator*.

Although growth rate of amphipods was not significantly affected at the exposure concentrations of UCM-dominated oils tested, reproduction of amphipods was found to be significantly reduced ($P \leq 0.05$) in all oil treatments. The possible reasons for the reduced reproductive success are: 1) this was a secondary effect due to reduced growth and hence reproduction was delayed or, 2) the reproductive process was disrupted by the exposure. If the former scenario was correct, it would be expected that the proportion of gravid females would be higher within the oil-exposed treatments as the amphipods merely had delayed reproduction due to insufficient growth. The proportion of gravid females was found not to be significantly different ($P > 0.05$) between treatments and the lowest ratios were evident within the ANS and TJP treatments. This implies that the reproductive process was disrupted by the UCM hydrocarbons. In the wild, *C. volutator* reproduce in synchronicity with spring tides immediately following moulting of the females (Borowsky,

1991) and there is only a small time window of opportunity for reproduction. Hence, if reproduction is delayed, it may be for a period of weeks rather than days. The possibility that a small reduction in growth rate could substantially delay reproduction and therefore not result in a higher proportion of gravid females in oil-exposed amphipods cannot be ruled out. Any disruption or delay in reproduction may however have serious repercussions for natural populations of sediment-dwelling organisms that are exposed to UCM hydrocarbons. Additionally, for species such as *C. volutator* which do not possess planktonic offspring, recovery from population disturbance may be very slow as immigration from non-affected populations will be minimal.

The effect of SLK upon the reproductive success of amphipods was unexpected. This lube oil is dominated by aliphatic compounds that had previously been found not to elicit sublethal effects on mussels (Thomas, 1995). Therefore it was not predicted to have an effect similar to the more aromatic hydrocarbon -containing TJP oil. The long-term effects of exposure to aliphatic compounds are not well studied but it is possible that the small proportion of aromatic hydrocarbons in the SLK oil was responsible for the observed effects (see **Chapter 4**). The importance of the aromatic fraction in determining the toxicity of petroleum hydrocarbons was questioned by Barron *et al.* (1999). In a study of the toxicity of three environmentally-weathered middle distillate oils differing in aromatic content, it was demonstrated that the oil with the lowest aromatic content had the greatest toxicity. Silkolene-150 was found to have a similar effect to the TJP and both of these UCM-dominated oils were found to have less effect than the weathered ANS which contained resolved hydrocarbons as well as a pronounced UCM hump. This suggests that components within the UCM contributed to the observed toxicity but some of the compounds resolved by GC within the ANS oil also contributed. This is consistent with the known toxicity of aromatic hydrocarbons (Capuzzo *et al.*, 1988; Peterson, 1994).

Comparing the relative reduction in growth rates of the more weathered ANS exposed amphipods during the baseline test with that of the preliminary test with less weathered ANS[i] (Scarlett *et al.*, 2007c), it was observed that organisms exposed to the oil with enhanced weathering had relatively less reduction in their growth rates (Fig. 3.3). These results suggest that the loss of volatile components from within the slightly weathered ANS reduced its toxicity. Smith *et al.* (2006), using Iatroscan TLC-FID (thin layer chromatography-flame ionisation detection) analysis showed that lightly weathered ANS[i] retained some BTEX hydrocarbons which are known to be toxic (Ritchie *et al.*, 2001) and hence the observed reduction in toxicity is consistent with the loss of volatile components within the ANS[i] oil. However, oil spiking methods were not the same in Smith *et al.* (2006) which may have resulted in the more highly weathered oil being more strongly adsorbed onto the sediment and hence less bioavailable. Bobra (1983) stated that the effect of weathering on toxicity was dependent upon the definition of toxicity as although weathering can result in aqueous solutions that give lower LC_{50} values (i.e. more toxic), the weathering process may also reduce the aqueous solubility of the oil and thus fresh oils can generate aqueous solutions which are more toxic though at a higher concentration. Heintz *et al.* (1999) reported that highly weathered ANS from the *Exxon Valdez* oil spill was more toxic to pink salmon embryos than the unweathered oil which the authors attributed to elevated concentrations of larger PAHs in the weathered oil.

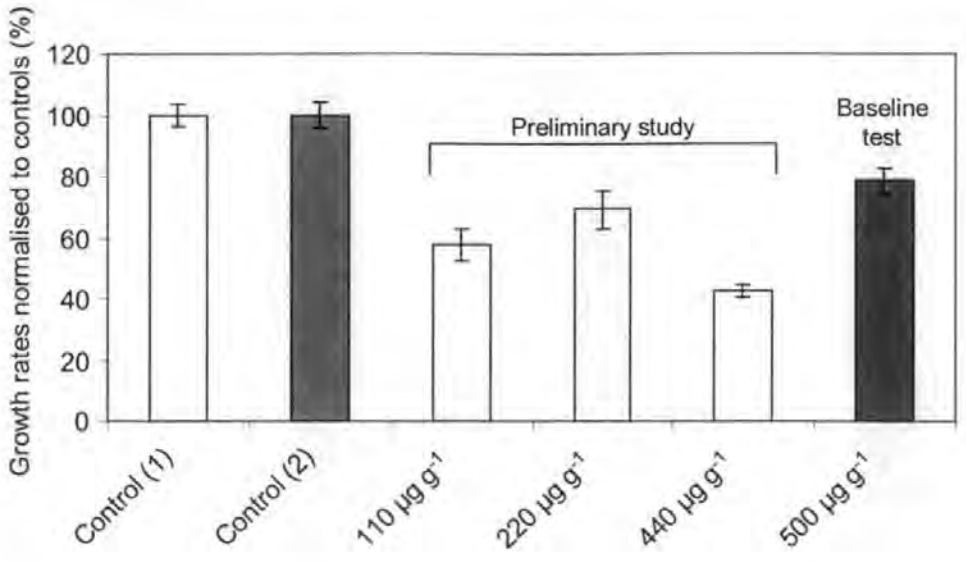


Figure 3.3 Comparison of mean (± SE) growth rates normalised to control values (%) of amphipods exposed nominal concentration (oil dry weight sediment^{-1}) of lightly weathered (20 % evaporative loss) ANS (preliminary study, normalised to control 1) and more weathered (36 % evaporative loss) ANS (baseline test, standardised to control 2). Preliminary study data available within Scarlett *et al.* (2007c).

3.4.3 Baseline aqueous tests

Mytilus edulis

No significant effects on mussel clearance rates were observed at the nominal aqueous concentration of *ca.* 30 mg L^{-1} for any of the oils tested (Fig.3.2). Smith *et al.* (2006) reported that a WAF derived from slightly weathered ANS (i.e. ANS^{i} as used in preliminary sediment exposures reported herein), containing *ca.* 3-4 mg L^{-1}, reduced mussel clearance rates by around 50 %. The mussels exposed to the ANS^{i} WAF were either subject to higher concentrations of bioavailable hydrocarbons and/or more toxic components of the oil. Following spillage of in-reservoir degraded oils in which large volumes of oil may be dispersed in the water column, very high aqueous concentrations of UCM hydrocarbons may be achieved e.g. TJP was spilled from a pipeline into the Mersey, UK in 1989 (Davies & Wolff, 1990). However, short-term exposure to high concentrations of UCM hydrocarbons is unlikely to be a common occurrence and a more

likely scenario is long-term exposure to low concentrations of environmentally degraded oils.

The low bioavailability of many of the UCM components suggests that chronic exposure is more environmentally realistic and acute exposure to these complex mixtures does not allow sufficient time for compounds to accumulate within the tissues of the mussels. This is explored in **Chapter 5**. The presence of dissimilar compounds within a mixture reduces the solubility of individual hydrocarbons in accordance with Raoult's law (Page *et al.*, 2000) and therefore high concentrations of UCM hydrocarbons may be less bioavailable than when highly dispersed. Booth *et al.* (2004) attempted to establish the solubility of the TJP but found this to be problematic: fractionation of the mixture altered the mole fraction of the components which enhanced the dissolution of the compounds compared to the original mixture leading to abnormally high solubility values when fractions rather than whole mixtures were analysed. Exposure to fractions of the oils that are more bioavailable to the mussels may therefore replicate bioaccumulation following long-term exposure better than acute exposure to whole oil at high concentration and hence greater environmental reality. Another consequence of the poor solubility of the whole oils was the necessity to increase the percentage of carrier solvent to dissolve/disperse the oils into the seawater which can potentially alter the toxicity of the contaminant as well as causing direct effects.

Bioluminescence inhibition

Johnson & Long (1998) reported bioluminescence inhibition EC_{50} values of 60, 400 and 1000 mg L^{-1} for Fuel Oil no. 2, crude oil and recycled motor oil respectively. Given that the reported EC_{50} values of the crude and recycled motor oils are considerably higher than the concentrations tested within the baseline study, it is perhaps not surprising that no

significant effect on bioluminescence inhibition was detected (Table 3.3). Hokstad *et al.* (2007) reported that exposure to the WSF of UCM-rich oil from the Troll oilfield in the North Sea caused bioluminescence inhibition to be greater (EC_{50} = 300 ppb, i.e. 300 μg L^{-1}) than to the UCM-poor oil Statfjord oil WSF (EC_{50} = 470 ppb). The WSFs were generated from oil loadings of 10 g L^{-1} and 25 g L^{-1} for the Troll and Statfjord respectively. Due to the restricted quantities of oils available during the current research, it was not possible to replicate the conditions reported by Hokstad (2007). However, bioluminescence inhibition testing of reduced scale WSFs created from SLK and ANS (unreported data) suggested that these oils were much less toxic than the Troll and Statfjord oils. Recent communication with SINTEF (Booth, Pers. Comm.) has cast doubt on the EC_{50} data reported by Hokstad *et al.* (2007) and suggested that the units stated should have been ppm not ppb as stated in the report but this has yet to be confirmed.

It is possible that the effect of the interaction between the solvent and the hydrocarbons may have lowered the toxicity of oils. Johnson and Long (1998) reported that acetone as a carrier solvent altered the EC_{50} of phenol from 5.5 mg L^{-1} to 21.0 mg L^{-1} i.e. the phenol became less toxic in the presence of acetone. The authors did not state the concentration of acetone, only that it did not exceed 5 % of the test solution. It is also possible that masking effects from the numerous hydrocarbons within the complex mixture may affect the toxicity as reported by Grote *et al.* (2005b). A further discussion relating to mussel exposure tests is given above.

3.4.4 General discussion

Taking the results as a whole, it is clear that the UCM-rich oils were not acutely toxic at environmentally realistic conditions. Although under spill conditions, concentrations higher than that used in the baseline tests are possible, damage to organisms is more likely

to arise *via* physical effects, such as clogging of gills, rather than toxic effects arising from exposure to UCM hydrocarbons. Elgershuizen & deKruijf (1976) reported that an oil-water dispersion of TJP was more toxic to the hermatypic coral *Madracis mirabilis* than a WSF, although neither produced lethal effects; this suggested that adverse effects were mainly related to exposure to oil droplets rather than dissolved TJP hydrocarbons. Bak and Elgershuizen (1976) studied the abilities and patterns of 19 species of hermatypic corals to reject, or cleanse themselves, of TJP-oiled sediment. The authors found no evidence of oil adsorption to coral tissues, and no sign of active ingestion of oil droplets. Indeed, oil introduced into and onto the corals was actively cleared by the colonies.

No literature could be found regarding long-term environmental exposure to TJP or UCM-dominated oils but from the sediment tests it is evident that long-term exposures to the UCM-dominated oils can cause effects at the population level. Although the effect upon growth rates and reproductive success was relatively small, and hence difficult to assess in terms of ecological consequences, *C. volutator* may not be as sensitive to hydrocarbons as other species of amphipod. For example, a study into the effects of the *World-Prodigy* oil spill in Narragansett Bay, Rhode-island (Widbom & Oviatt, 1994) reported that abundance of amphipods of the genus *Ampelisca* were reduced but not the genus *Corophium*. This differential sensitivity was also found in comparative laboratory studies with the gammarid amphipod *G. locusta* (Neuparth, personal communication). Large subtidal populations of *Ampelisca* amphipods (also a member of the suborder GAMMARIDEA) were eliminated following the Amoco Cadiz spill in 1978 and were slow to recover (Dauvin & Gentil, 1990; Gesteira & Dauvin, 2000). The reasons for this apparent difference in sensitivity are unclear. One possibility is the difference in habitat preferences: the *Ampelisca* amphipods live within sediment with a greater sand content than the *Corophium* which have a preference for mud (Connor *et al.*, 2004). Hydrophobic hydrocarbons may be less

bioavailable due to adsorption to the larger number of fine particles within the muddy sediment occupied by *Corophium*.

3.5 Conclusions

3.5.1 Preliminary whole-sediment toxicity test

The sediment chronic test has shown that sediment that was not evidently toxic during 10-day acute tests could have population-level effects on sediment-dwelling amphipods. The chronic test protocol and acceptability limits proved to be robust, and the test was determined to be sufficiently sensitive to detect effects on growth rate and reproduction. The very long time-frame for the whole life-cycle test has significant costs and therefore a partial life-cycle test may be preferably as many of the benefits of long-term exposure are retained but with reduced costs in terms of time and resources (Scarlett *et al.*, 2007c).

3.5.2 Baseline whole sediment toxicity test using weathered and biodegraded oils

Sediment-bound UCM hydrocarbons did not elicit acute effects in terms of survivorship on *C. volutator* but environmentally realistic concentrations of UCM hydrocarbon contaminated sediments significantly reduced reproductive success following long-term exposure. If the aromatic fractions of the oils were responsible for the observed toxicity, the components are either not all contributing or are not doing so uniformly. Fractionation of all three oils followed by biotesting of the fractions is therefore required to further elucidate the toxic nature of the UCM hydrocarbon components.

3.5.3 Baseline aqueous tests using weathered and biodegraded oils

The model UCM-dominated oils did not elicit any effects upon either the clearance rates of the mussel *M. edulis* or the bioluminescence of the bacterium *V. fischeri* during acute aqueous exposure. Such conditions are not environmentally realistic and further study is required. It is theoretically possible that fractions of the oils may elicit effects at the equivalent whole oil concentration.

3.5.4 General conclusions

Testing of complex mixtures using the EDA approach requires that the material under investigation is fractionated and subject to further biotesting only if a toxic effect is found during baseline tests. All of the UCM-dominated oils affected the amphipod *C. volutator* as a result of chronic exposure to contaminated sediment but no acute effects were observed either during sediment or aqueous exposures. Testing of oil fractions is therefore required for sediment exposures and, due to the theoretical possibility that the oil fractions may have greater bioavailability than the whole oils, it is prudent to carry out further aqueous exposures.

Chapter 4

Fractionation studies

Having established the baseline toxicity of UCM-dominated oils, the possible differential toxicity of the aliphatic and aromatic fractions of the oils is explored herein. Primary fractionation was carried out using open column chromatography and three fractions from each of the three oils previously investigated (**Chapter 3**) were subject to further biotesting including acute aqueous tests and chronic whole sediment exposure tests. Uptake and depuration of UCM compounds by *M. edulis* were investigated using both conventional GC-MS and GC×GC-ToF-MS. Some of the results reported herein have been published (Scarlett *et al.*, 2007b).

4.1 Introduction

4.1.1 General introduction

Following on from baseline toxicity tests, TIE/EDA studies typically proceed to the biotesting of fractionated samples. In order to fractionate environmental samples it is necessary to extract the potential toxicants from the relevant matrix. Extraction procedures are selective and therefore certain components remain in the sample and are not analysed. An efficient extraction method for the collection of non-polar organics will not also be efficient at collection of highly polar compounds. The method chosen for extraction is therefore dependent upon what is assumed to be the causative agent of toxicity. For environmental samples, if the initial TIE Phase I manipulations have been performed, this would help provide direction as to what chemical extraction method and fractionation procedure would be most relevant but otherwise the type of toxicant may be suspected based on additional information. For the investigation into the toxicity of UCM hydrocarbons, the extraction, fractionation and chemical analyses methods can be tailored to petrogenic hydrocarbons. Previous EDA studies relevant to an investigation of potential toxicity of petrogenic hydrocarbons are summarised in **Chapter 3,** Table 3.1. A summary review of fractionation procedures and biotests used within EDA studies is presented below. For the purposes of the current study, it was important to ensure good separation between aliphatic and aromatic compounds. It was therefore beneficial to adapt published methods to achieve an aliphatic fraction with no monoaromatic compounds as this may confound results of toxicity tests. During method development it was found that alkylbenzenes tended to split between the aliphatic and aromatic fractions but by small alterations to the deactivation of the alumina it was possible to achieve complete separation.

4.1.2 Biotests used in TIE and EDA studies

The toxicity of a sample and the fractions thereof determine further fractionation and chemical analysis and hence the selection of biotests affect which toxicants are finally identified. Biotests must be reproducible, sensitive, provide quantitative results, and discriminate between toxic and non-toxic fractions but the selection of biotests for the testing of fractions is limited by the requirement for small sample volumes, high throughput and rapidity (Brack, 2003). With these limitations it is apparent that biotests suitable for rapid screening of fractions may not be ecologically relevant compared to chronic studies with environmentally realistic concentrations.

During the late 1980s and early 1990s EDAs were mainly based on invertebrate bioassays such as those employing *Daphnia magna*, *Daphnia pulex*, *Ceriodaphnia dubia* and fish such as the fathead minnow *Pimephales promelas* (Lukasewycz & Durhan, 1992). More recently, the acute bioluminescence inhibition of the bacterium *V. fischeri* has become the predominant biotest (Castillo & Barcelo, 1999; Castillo & Barcelo, 2001; Reemtsma *et al.*, 1999a; Spiegel *et al.*, 2005; Svenson *et al.*, 1996a; Svenson & Hynning, 1997; Svenson *et al.*, 1996b). The bioluminescence inhibition test is normally performed by the Microtox test assay but may also be advanced to a high throughput system by using microtitre plates (Reemtsma *et al.*, 1999a) or direct combination with thin layer chromatography (TLC) (Reemtsma *et al.*, 1999b). The Microtox assay has several attributes that make it ideal for screening of fractions: it is highly reproducible, rapid and only requires small volumes. It does however have certain shortcomings (see **Chapter 2**) so should be used in conjunction with other assays to identify a wide range of toxicants (Bombardier & Bermingham, 1999). In addition to *V. fischeri*, biotests such as the oyster embryo assay with *Crassostrea gigas* Thunberg (Thomas *et al.*, 1999b), the acute toxicity tests with the marine copepod *Tisbe battagliai* (Thomas *et al.*, 1999a; Thomas *et al.*, 1999b) and the cell multiplication inhibition of the green algae

Scenedesmus vacuolatus (Altenburger *et al.*, 2004; Brack *et al.*, 1999) have successfully been applied to recent EDA investigations. However, none of these tests address the possible chronic effects of contaminants with low bioavailability.

4.1.3 Fractionation, testing and analysis of UCM-dominated oils

For the testing of primary fractions, the quantity of fractions is sufficient to examine the effects of long-term exposure to sediment-bound contaminants, thus providing direct comparison with baseline tests. Although baseline tests of aqueous exposure to the oils did not produce any significant effect upon mussel clearances rates or bacterial bioluminescence inhibition, it is possible that toxic effects arising from some compounds could have been masked by non-toxic compounds within the complex mixture. For example, in an EDA of contaminated sediment by Grote *et al.* (2005b), the toxicity of the primary fraction was found to be greater than that the whole extract. The authors suggested that this may be due to antagonism between different components and/or modification of the physical or physiochemical properties such as solubility. It was therefore prudent to test the effects of aqueous exposures as well as sediment exposures of fractions of the UCM-dominated oils. In addition, the characterisation and quantitation of UCM hydrocarbons within the tissues of mussels exposed to the oil fractions would be of benefit, especially for comparison with field-contaminated mussels.

Although GC-MS can be used to estimate the concentration of bioaccumulated UCM hydrocarbons, it provides little information on the character of the compounds due to the lack of resolution by conventional GC. Utilisation of GC×GC-ToF-MS to compare compounds found within the tissues of UCM hydrocarbon-exposed organisms with those characterised from oil fractions provides a far greater ability to separate and identify compounds than the use of conventional GC-MS. Data processing of GC×GC-ToF-MS analyses is, however,

labour intensive, as a vast quantity of data is generated. It is therefore beneficial to concentrate efforts on relatively few numbers of samples. This method is therefore well suited to EDA studies in which the chemical analysis is directed only at fractions found to cause deleterious effects on organisms.

4.1.4 Aims of the present study

Having established the baseline toxicity of the three oils, the study presented in this chapter is concerned with the comparative toxicity of the aliphatic and aromatic fractions of the oils. By comparing the toxicity of the fractions in terms of acute aqueous toxicity and chronic sediment toxicity, information could be gleaned concerning which broad spectrum of compound groups was responsible for any observed toxicity. Previous studies into UCM hydrocarbon toxicity (Donkin *et al.*, 2003; Rowland *et al.*, 2001; Smith *et al.*, 2001; Thomas *et al.*, 1995) have suggested that the aromatic fraction was solely responsible for adverse effects on *M. edulis*; the studies presented in this chapter were designed to test this hypothesis in the context of both aqueous and sediment exposure, as well as acute and chronic exposure. Aims were:

1. To develop a large scale method for the primary fractionation of the oils to separate aliphatic, relatively non-polar aromatic and polar aromatic compounds.
2. To establish and compare primary fraction toxicity for the three oils when associated with sediment.
3. To establish and compare primary fraction toxicity for the three oils when in the aqueous phase i.e. dissolved or accommodated within seawater.
4. To compare the results of 3 and 4 to derive information concerning routes of uptake of UCM hydrocarbons.
5. To characterise and quantify hydrocarbons accumulated by *M. edulis* following exposure to toxic fractions.

6. To characterise and quantify hydrocarbons accumulated by *M. edulis* following depuration of toxic fractions.
7. To compare the results of 5 and 6 to derive information concerning uptake and elimination of toxic components.

4.2 Methodology

4.2.1 Preparation of primary fractions

Full details of method development for the primary fractionation of the oils are provided within **Chapter 2**. The oils were fractionated by open column chromatography using increasingly polar solvents. Subsamples of fractions F1, F2 and F3 of all oils from each column run were analysed by GC-MS. The F4 fractions were retained but were not analysed by GC-MS. Gas chromatograms of replicated fractions appeared identical and were all dominated by UCM hydrocarbons with very little apparent resolved petrogenic hydrocarbons.

4.2.2 Chronic whole sediment test with *Corophium volutator*

Collection and maintenance of organisms during acclimation

Sediment and *C. volutator* were collected from an intertidal area of the Avon estuary near Aveton Gifford, south Devon UK at the same location as that used for preliminary tests (**Chapter 3**). Juvenile amphipods were collected and maintained as described in **Chapter 2**.

Reference toxicity test

Amphipods *C. volutator* collected from Aveton Gifford were exposed to a standard toxicant $CdCl_2$ (Ciarelli *et al.*, 1997) to ensure consistency of sensitivity with previous populations from Aveton Gifford and commercial suppliers used previously as described in **Chapter 2**.

Spiking of sediments

Sediments were spiked with fractions F1, F2 and F3 of ANS, TJP and SLK oils to give nominal concentrations of 500 $\mu g\ g^{-1}$ (dry wt.) of whole oil equivalents. The spiking method was based on that of Roddie and Thain (2001) as described for baseline whole sediment toxicity tests (**Chapter 3**). The nominal concentrations of fractions in sediment are given

below Ttable 4.1). Additional replicates were created for calculation of dry:wet ratios and behavioural tests.

Table 4.1 Nominal concentrations of oil fractions in sediment (μg g^{-1} dry weight) used for the spiked-sediment chronic exposure tests with *C. volutator*

	Concentrations of fractions in sediment (μg g^{-1} dry wt.), equivalent to 500 μg g^{-1} (dry wt) of whole oil		
Oil	F1	F2	F3
SLK	417	77	np
TJP	133	141	100
ANS	234	145	60

SLK =Silkolene-150, TJP = Tia Juana Pesado, ANS = weathered Alaskan North Slope, np = not performed.

Chronic whole sediment tests

Chronic tests were conducted as described for the baseline study except that slightly smaller amphipods were used. Juvenile *C. volutator* (mean length = 3.2 mm, SE = 0.02 mm, mean wet weight = 1.580 mg, mean dry weight = 0.129 mg) were exposed to a nominal oil concentrations as given in Table 4.1. The test was initiated at the beginning of July 2006.

The test was terminated after 60 days exposure when reproduction was apparent in all replicates of the control treatment. Survivorship, wet weights, dry weights and lengths of organisms were recorded as described for the baseline study. Details of the sediment exposure tests are provided in Scarlett *et al.* (2007b).

4.3.3 Behavioural tests with *Corophium volutator*

The behavioural responses of *C. volutator* in response to sediments spiked with oil fractions, as used in the chronic exposure tests, were examined using methods detailed in Scarlett *et al.* (2007a).

4.3.4 Aqueous exposure

Collection and maintenance of mussels Mytilus edulis

Mussels were collected and maintained as reported previously (Scarlett *et al.*, 2005) and as described within **Chapter 2**. Mussels with a mean shell length of 20.5 mm (SE = 0.07 mm, n = 180) were used for aqueous exposure tests.

Exposure tests with Mytilus edulis

Semi-static 48 h exposure tests were similar to the hydrocarbon tests described by Donkin *et al.* (1991) and were identical to these used for the baseline study as described in **Chapter 2**. Test solutions of primary oil fractions: F1, F2 and F3 of ANS and TJP, and F1 and F2 Silkolene-150, were prepared using a carrier solvent as described in **Chapter 3**. Subsamples (*ca.* 20 mL) of the test solutions were taken for use with the bioluminescence inhibition assay. Test solutions were added to the mussel exposure vessels and replaced after 24 h. The maximum nominal concentrations of oil fractions in seawater are given in Table 4.2 below. Due to the poor solubility of the fractions in seawater, as evidenced by the formation of oily slicks on the surface of the test solutions within the aspirators, the true aqueous concentrations of the fractions would have been considerably lower than the nominal concentrations.

Oil fractions that had a significant ($P \leq 0.05$ %) deleterious effect on the clearance rate of the mussels were subject to repeat tests using a smaller spiking volume of 1 mL with a corresponding solvent control of 0.01 % solvent (fractions were dissolved in 1 mL

DCM/acetone as previously described but reduced to 1 mL under nitrogen to remove the DCM. Although the clearance rate of mussels exposed to the F2 fraction of Silkolene-150 was not significantly less than the solvent control, it was significantly less than the negative control and clearance rates were reduced by a similar degree as the F2 fractions of the other oils and therefore this oil fraction was also repeated. Nominal concentrations of the repeated test solutions are given below (Table 4.3). Following measurement of clearance rates after 48 h exposure, the mussels were placed in clean seawater for five days with daily water exchanges. The clearance rates of the mussels were re-measured to ascertain the degree of recovery following depuration.

Table 4.2 Nominal concentrations of oil fractions in seawater (mg L^{-1}) used for the initial aqueous exposure tests with *M. edulis* and the bioluminescence inhibition test with *V. fischeri*

	Concentrations of fractions in seawater and whole oil equivalents (mg L^{-1}) in parenthesis		
Oil	F1	F2	F3
SLK	26.0 (31)	4.8 (32)	np
TJP	7.3 (27)	8.6 (38)	6.7 (34)
ANS	9.9 (21)	3.9 (13)	2.9 (24)

SLK =Silkolene-150, TJP = Tia Juana Pesado, ANS = weathered Alaskan North Slope, np = not performed.

Table 4.3 Nominal concentrations of oil fractions in seawater (mg L^{-1}) used for the repeated aqueous exposure tests with *M. edulis*

	Concentrations of fractions in seawater (mg L^{-1}) and whole oil equivalents (mg L^{-1}) in parenthesis		
Oil	F1	F2	F3
SLK	np	9.0 (60)	np
TJP	np	10.6 (23)	np
ANS	np	4.9 (17)	np

SLK =Silkolene-150, TJP = Tia Juana Pesado, ANS = weathered Alaskan North Slope, np = not performed.

Measurement of Mytilus edulis clearance rate

The clearance rate assay was adapted from Donkin *et al.* (1991; 1989) and described within **Chapter 2**.

Extraction of petrogenic compounds in Mytilus edulis tissues by GC-MS

Whole mussel tissues from each ANS and TJP aromatic F2 exposure and recovery treatment were extracted after addition of phenanthrene d_{10} as internal standard by an alkaline

saponification method adapted from Kelly *et al.* (2000) and described in **Chapter 2**. Estimates of dry tissue weights were obtained from dry/wet measurements of mussel tissues not used for extraction.

Characterisation and quantitation of bioaccumulated hydrocarbons using GC-MS

Compounds resolved by GC-MS and representing ≥1 % of area were compared to library data (NIST NBS54K) and identified where possible: only library matches ≥80 % were accepted. Quantitation by GC-MS was carried out by comparison of F2-exposed and solvent-exposed tissue extracts. The tissue concentrations of total petroleum hydrocarbons (TPH) and UCM were calculated based on standard curves for each oil F2 fraction and corrected for losses during extraction and clean-up based on measurement of the internal standard. The unresolved components were calculated using the Areasum function of MS Data Analysis and subtracting the sum of the integrated areas of resolved compounds from the total area. Full GC-MS method conditions are provided in **Chapter 2**.

Characterisation and quantitation of bioaccumulated hydrocarbons using GC×GC-ToF-MS

Tissue extracts of mussels that had suffered reduced clearances rates following exposure to oil fractions (i.e. aromatic F2 fractions of ANS and TJP only), were analysed by GC×GC-ToF-MS as this provides much greater separation and identification of hydrocarbons within complex mixtures than conventional GC-MS. Extracts from mussels given a period of recovery and control organisms were also analysed and compared to pure F2 oil fractions of both ANS and TJP. Chromatographic conditions, mass spectral analysis and data processing methods are detailed in **Chapter 2**. Compound groups were characterised by selected ions to encompass linear and branched homologues (Table 4.4) based on Booth (2004) and reports cited therein.

Table 4.4 Ions used to identify compound groups from oil fractions and *M. edulis* tissue extracts analysed by GC×GC-ToF-MS

Compound group	Selected Ions (*m/z*)							
Benzenes	91	92	105	119	133			
Indenes	116	129	143	157				
Tetralins/indans	118	131	145	159				
Naphthalenes	128	141	142	155	156	169	170	
Biphenyls	153	154	167	168	181	182	195	209
Fluorenes	165	179	180	193	221			
Phenanthrenes	178	191	192	205	206	219	220	
Dibenzothiophenes	184	198	212					

Vibrio fischeri bioluminescence inhibition

The bioluminescence inhibition assay was performed using the standard 'comparison of estuarine and coastal samples' protocol (MicrotoxOmni™, 2005). In brief: (i) the bacteria *V. fischeri* were exposed to five replicates of test solutions (fractionated oils dissolved in solvent and spiked into seawater) for 5 and 15 minute periods and their response compared to that of the reference solution (solvent control in seawater at equivalent solvent concentration). Method details are given in **Chapter 2.**

4.4 Results

4.4.1 Sediment exposure tests

Reference toxicity test

Mortality of *C. volutator* exposed to 7 mg L^{-1} $CdCl_2$ was consistent with the previously derived LC_{50} value for the local population used during preliminary oil-spiked sediment exposure tests and with 72 h LC_{50} literature values (Ciarelli *et al.*, 1997).

Chronic sediment toxicity test

The sediment exposure test was terminated after 60 days when it was apparent that reproduction had occurred within all control vessels. Most of the control vessels had shown evidence of reproduction earlier but in one replicate this was not clear and hence test termination was postponed. Full results of the chronic sediment toxicity tests are provided in Scarlett *et al.* (2007b).

4.4.2 Amphipod behaviour tests

No clear behavioural patterns were observed and no particular fraction-spiked sediment appeared to stress the amphipods. Mean initial time to burial was highest within the F1 fraction of ANS treatment and also elevated for amphipods exposed to ANS F2 spiked sediment (Fig 1a). Only one amphipod failed to burrow and there was very little re-emergence from the sediments (Fig1 b) and hence final time to burial was similar to initial time (Fig 1a). The one amphipod that failed to burrow within the specified time limit (exposed to TJP F2) was observed to spend all the time partly buried and constructed a burrow within 10 minutes of the time limit. The low levels of re-emergence and

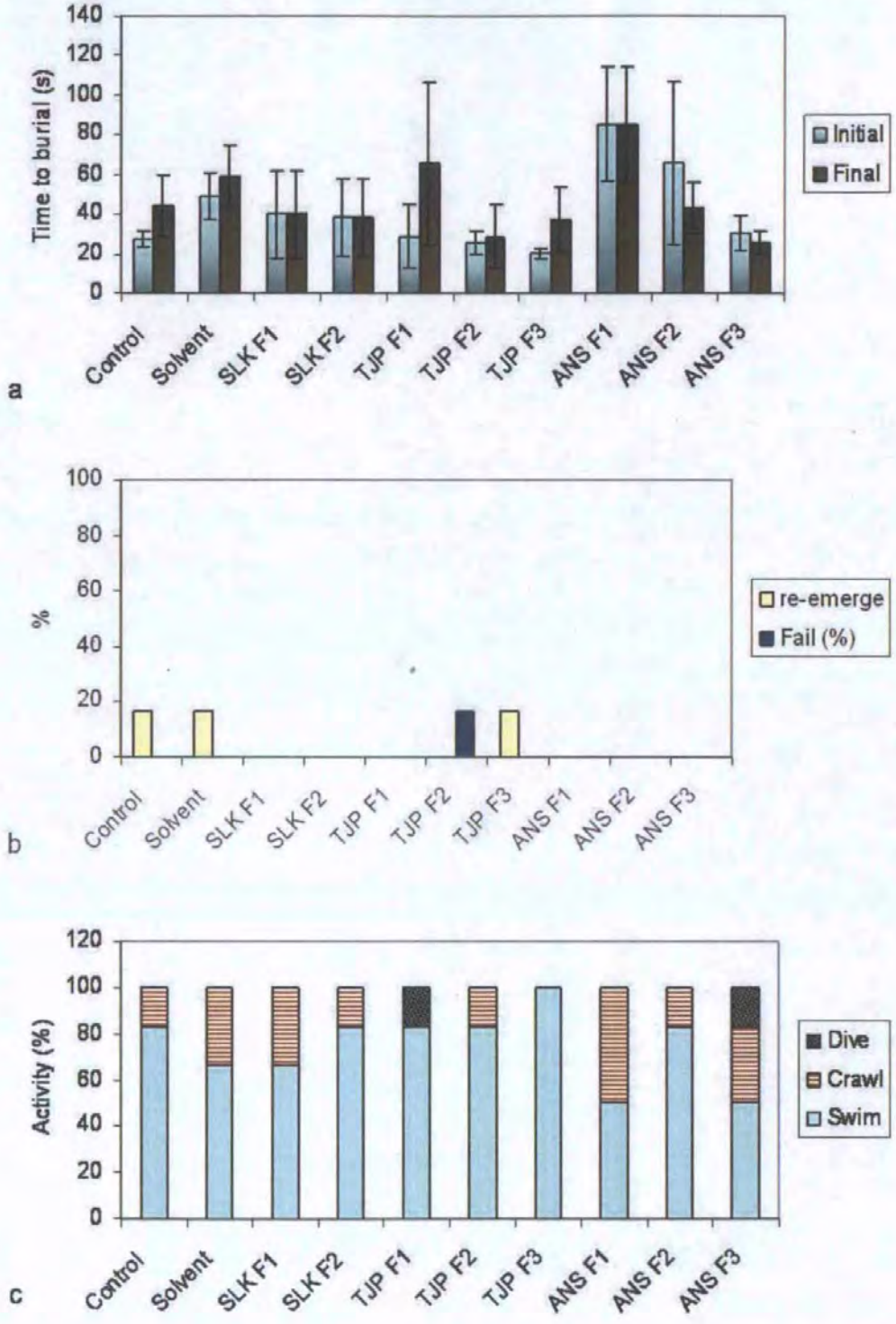


Figure 4.1 (a) Mean time for amphipods to initially and finally burrow within sediments spiked with 3 oils (ANS = weathered Alaskan North Slope, TJP = Tia Juana Pesado, SLK = Silkolene-150; (b) percentages of amphipods re-emerging from the sediment following initial burrowing and those failing to burrow within 5 minutes of test initiation; (c) majority activity (%) prior to initial amphipod burrowing, classified as swimming, crawling or diving directly to the sediment surface. Tests used 6 individual *C. volutator* per treatment, error bars = 1 standard error.

failure to burrow do not support the prediction of chronic toxic effects. Activity prior to burrowing was dominated by swimming with minimal diving directly to the sediment that had been associated with acute stress in previous tests. Overall, the results of the behaviour tests did not suggest sub-lethal effects, consistent with a previous report (Scarlett *et al.*, 2007a).

4.4.3 Aqueous exposure tests

M. edulis *clearance rates*

Following the initial 48 h semi-static exposure to the primary oil fractions dispersed in seawater, the clearance rates of the mussels were assessed using the mussel clearance rate assay. Only the F2 fractions of both TJP and ANS significantly ($P \leq 0.05$) reduced the clearance rate of the mussels when compared with the solvent control although it appeared that the SLK F2 fraction may also have been affecting the mussels but the effect may have been masked by the effect of the solvent (Fig. 4.2).

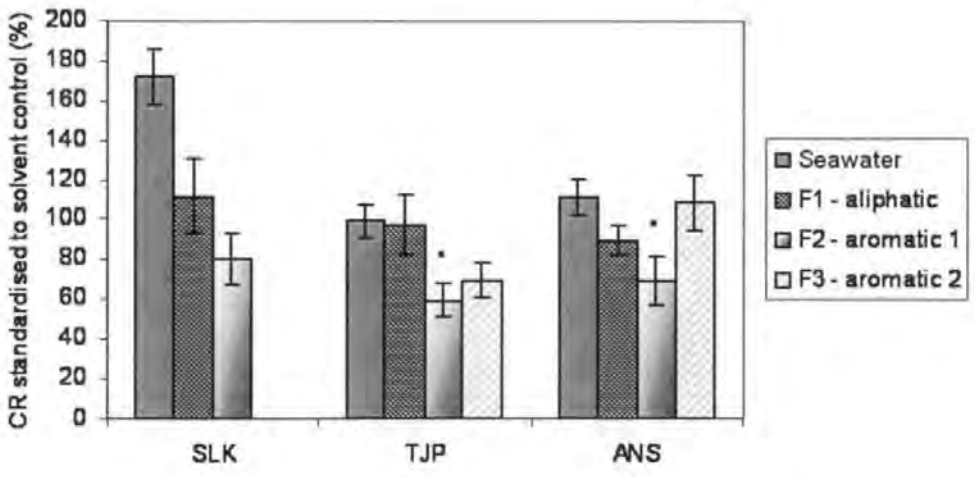


Figure 4.2 Mean clearance rates (CR) standardised to solvent control mussels *M. edulis* (n = 9 mussels per treatment) following 48 h semi-static exposure to fractions of Silkolene-150 (SLK), Tia Juana Pesado (TJP) and weathered Alaskan North Slope (ANS). Errors bars = 1 standard error, * = significantly less than solvent control.

Repeat tests of toxic fractions

Repeat 48 h semi-static exposure tests confirmed the toxicity of the F2 fractions of both ANS and TJP (Fig. 4.3). Although the clearance rates of mussels exposed to the F2 fraction of Silkolene-150 was not significantly ($P > 0.05$) different from that of the solvent exposed mussels, the clearance rates were similar to that of the other F2 exposed organisms (Fig. 4.2). Following five days recovery within clean seawater, all mussels, including those within the seawater and solvent control, achieved higher clearance rates than previously measured. The ANS and TJP exposed mussels recovered to the extent that their clearance rates matched that of the solvent control and the Silkolene-150 exposed mussels achieved a similar mean rate as that of the seawater controls (Fig. 4.3).

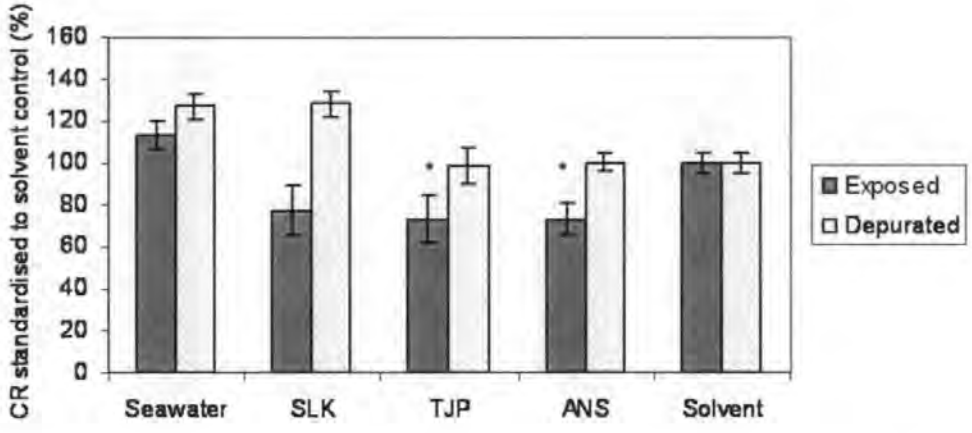


Figure 4.3 Mean clearance rates (CR) standardised to solvent control mussels *M. edulis* (n = 9 mussels per treatment) following 48 h semi-static exposure and five days recovery in clean seawater to fractions of Silkolene-150 (SLK), Tia Juana Pesado (TJP) and weathered Alaskan North Slope (ANS). Errors bars = 1 standard error, * = significantly less than solvent control.

4.4.4 Characterisation and quantitation of bioaccumulated compounds using GC-MS

The concentrations of F2 fractions bioaccumulated by the mussels were quantified in terms of total petroleum hydrocarbons (TPH) and UCM (Fig. 4.4) by interpolation from standard curves of TPH and UCM of the F2 fractions of both oils. The highest concentration of 1809 $\mu g\ g^{-1}$ TPH (tissue dry wt.) was found within ANS-exposed organisms, of this about 83 %

was due to unresolved compounds (Fig. 4.4). The concentration of total hydrocarbons from TJP-exposed mussels was about 50 % of that in the ANS tissue extracts i.e. 901 $\mu g\ g^{-1}$ (tissue dry wt.) but over 93 % of this was due to UCM compounds. Extracts from mussels that had experienced a period of depuration/recovery contained relatively small concentrations of hydrocarbons. Mussels recovering from the ANS F2 exposure depurated over 90 % of their total hydrocarbon burden and those recovering from TJP F2 exposure had only background concentrations remaining in their tissues (Fig 4.4).

Compounds resolved by GC-MS and representing ≥1 % of area were compared to library data (NIST NBS54K) and identified where possible: only library matches ≥80 % were accepted (Table 4.5). Other than the internal standard, phenanthrene d_{10}, none of compounds listed in Table 4.5 were found in the system blank. Biogenic compounds were found in all mussel extracts including the hydrocarbon squalene. This biogenic compound was also reported in mussel tissue extracts by Donkin *et al.* (2003) and found not to cause deleterious effects upon mussel clearance rates, but may also originate from contamination by contact with skin. No petrogenic compounds meeting the criteria outlined above were found in seawater or solvent control mussel extracts. Petrogenic compounds identified in the extracts of tissues from mussels following 48 h exposure to the F2 fractions were di and poly aromatic, typically alkylnaphthalenes and alkylphenanthrenes; these were largely found to be eliminated or severely depleted in extracts from mussels that had received a period of recovery in clean seawater (Table 4.5).

Extracted ion chromatograms (*m/z* 91 and 119) revealed only a minor presence of alkylbenzenes (Killops & Readman, 1985a) in tissue extracts from exposed mussels. Some of the compounds with *m/z* 91 base ion were also present within control mussel extracts and possessed mass spectra consistent with LABs. Alkylnaphthalenes, mono, di and tri-

substituted, were more evident (*m/z* 128, 141, 155, 169) representing <0.8 % and <0.1 % of the total integrated area of the ANS and TJP tissue extracts respectively but these were eliminated or highly depleted to background levels within extracts from recovery mussels. Alkylphenanthrenes (*m/z* 178, 191) represented about 0.5 % and 0.1 % of the total integrated area of the ANS and TJP mussel tissue extracts respectively and these were also much depleted following recovery. Alkyltetralins were detected in the ANS exposed mussel tissues but were not present in mussels exposed to the TJP F2 fraction. Alkylnaphthalenes and alkylphenanthrenes were undetectable within seawater and solvent control mussel extracts.

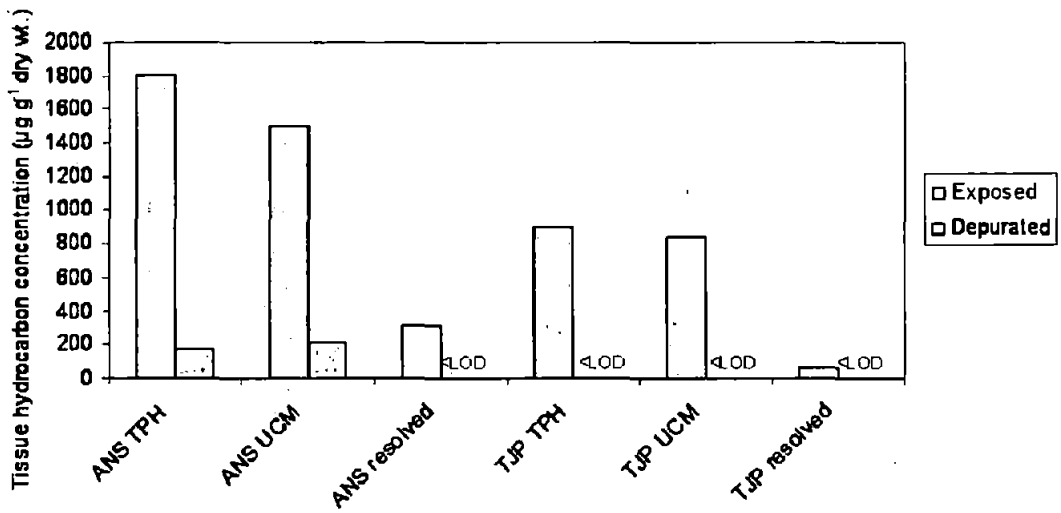


Figure 4.4 Concentrations of petroleum hydrocarbons, quantified by GC-MS, bioaccumulated in the tissues of *M. edulis* following 48 h exposure to aromatic fractions (F2) of ANS and TJP crude oils plus a five day recovery period in clean seawater. LOD = Limit of Detection.

Table 4.3 Major compounds resolved by GC-MS (≥ 1 % area in any extract) and identified by ≥ 80 % match in NIST library NBS54K found in tissue extracts of mussels *Mytilus edulis* following 48 h exposure (Exp) to aromatic fractions of ANS and TJP crude oils and a five day recovery period (Rec) in clean seawater

Origin of compound	Chemical name	Alternative name	Chemical formula	Present with mussel extract					Notes	Reference
				ANS		TJP		Controls		
				Exp	Rec	Exp	Rec			
Biogenic	2-Pentadecanone, 6,10,14-trimethyl			✓	✓	✓	✓	✓		
	7-Hexadecenoic acid		$C_{17}H_{32}O_2$	✓	✓	✓	✓	✓		
	Pentadecanoic acid, 14-methyl-, methyl ester		$C_{17}H_{34}O_2$	✓	✓	✓	✓	✓		
	Octadecanal		$C_{18}H_{36}O$	✓	✓	✓	✓	✓		
	Methyleicosa-5,8,11,14,17-pentaenoate		$C_{21}H_{32}O_2$	✓	✓	✓	✓	✓		
	Octadecenoic acid, methyl ester		$C_{19}H_{36}O_2$	✓	✓	✓	✓	✓		

Table 4.5 continued

	Cyclopropanenonanoic acid, methyl ester		$C_{21}H_{38}O_2$	✓	✓	✓	✓	✓		
	Cholesta-3,5-dien-7-one		$C_{27}H_{42}O$	✓	✓	✓	✓	✓	Co-eluting	
	Desmosterol		$C_{27}H_{44}O$	✓	✓	✓	✓	✓	Co-eluting	
Contaminants	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	Squalene	$C_{30}H_{50}$	✓	✓	✓	✓	✓	Natural compound in mussels but may also arise from contamination from skin contact	
	Hexanedioic acid, dioctylester	Dioctyl adipate	$C_{22}H_{42}O_4$	✓					Plasticizer	(Webb *et al.*, 1999)
Internal standard	Phenanthrene-d_{10}		$C_{14}D_{10}$	✓	✓	✓	✓	✓		

Table 4.5 continued

Petrogenic	9H Fluorene, 2-methyl-	$C_{14}H_{12}$	✓		✓			
	9H Fluorene, 1-methyl-	$C_{14}H_{12}$	✓		✓			
	Methyldibenzothiophene or dibenzothiophene, 3-methyl-	$C_{13}H_{10}S$	✓		✓		Organic sulphur contaminant found in petroleum	(Ichinose *et al.*, 1999)
	Methyldibenzothiophene or dibenzothiophene, 3-methyl-	$C_{13}H_{10}S$	✓		✓			
	Phenanthrene, 4-methyl-	$C_{15}H_{12}$	✓	✓	✓	✓	Compound much reduced in both recovery extracts	
	1H-Indene, 1-phenyl- or Phenanthrene, 1-methyl-	$C_{15}H_{12}$	✓	✓	✓		Co-eluting. Match for phenanthrene in ANS recovery but much reduced	

Table 4.5 continued

Anthracene, 2-methyl- or Phenanthrene, 4-methyl-	$C_{15}H_{12}$	✓	✓	✓		Co-eluting. Match for phenanthrene in ANS recovery but much reduced
Phenanthrene, 2,5-dimethyl-	$C_{16}H_{14}$	✓	✓	✓	✓	Other isomers likely. Compound much reduced in both recovery extracts

✓ = present; ✓ = present but relatively low.

4.4.5 Characterisation and quantitation of bioaccumulated compounds using GC×GC-ToF-MS

Examination of the three dimensional (3-D) plots of the 2-D chromatograms, generated by the GC×GC-ToF-MS Pegasus software, of the F2 aromatic fractions of the TJP and ANS (Fig. 4.5a and b) clearly shows the complexity of the fraction components. Although the plot of the ANS aromatic fraction (Fig. 4.5b) is dominated by a relatively few number of large peaks, the TJP aromatic fraction (Fig. 4.5a) can be seen to possess a considerable number of substantial peaks eluting across both the primary (apolar column) axis and the secondary (polar column) axis. Although visually useful, the 3-D plots are not suitable for viewing the large number of small peaks that make up a large component of UCM-dominated oils; peak marker plots were therefore generated. After removal of known column bleed peaks (*c.f.* Booth, 2004), peak marker plots of the oil fractions show the distribution of peaks: each peak representing a separate deconvoluted mass spectrum (Fig. 4.6). From this complex array of data it was possible to identify compound groups of mainly alkylated homologues of common oil components (Fig. 4.7). The same compound groups were identified in the aromatic fractions of both oils but the elution range along the primary axis was greater for the ANS F2 and a larger number of peaks were identified (Fig. 4.7). Many of the peaks with 2^{nd} dimension elution time above about 2000 s within the ANS oil were very small and minor changes to instrument sensitivity may have caused such small peaks to disappear within the baseline of the TJP oil; note that this region of the TJP 2-D chromatogram is devoid of peaks in the total peak marker plot (Fig. 4.6a).

All of the compound groups identified within the F2 aromatic fraction were also found within the tissues of the exposed mussels (Fig. 4.8). A total of 1046 peaks were identified belonging to the compounds groups found within the TJP F2 exposed mussels (Fig. 4.8a). Of these, the largest groups (by number of peaks) were the biphenyls, tetralins, and

naphthalenes, with 200, 199 and 151 peaks respectively. Within the extracts of the ANS F2 exposed mussels, only 681 peaks were identified (Fig. 4.8b). Of these, the biphenyls and naphthalenes were the largest groups with 153 and 132 peaks respectively. The number of peaks corresponding to tetralins, indans and indenes were far fewer within the ANS exposed mussel tissues and were largely absent above 1400 s primary elution time, although these compounds were much in evidence within this region for the TJP exposed mussels (Fig. 4.8). Many peaks from the TJP exposed mussel extracts remained unidentified but this was less pronounced for the ANS extracts (Fig. 4.9). Following depuration of the mussels, the vast majority of the peaks associated with the compound groups accumulated during the exposure were found to be absent (Fig. 4.10). Only 43 peaks were found within extracts from TJP treated mussels, these corresponded to alkylated benzenes, phenanthrenes and dibenzothiophenes (Fig. 4.10a). A few more peaks (157) were present within ANS depurated mussel tissue extracts; these were mainly alkylated benzenes and phenanthrenes (Fig. 4.10b). As with the TJP extracts, the tetralins, indans and indenes were completely removed.

The numbers of peaks present within an extract provide information on the numbers of compounds accumulated within the tissues but as some individual peaks are much larger than others it is also useful to look at the relative abundance of the compound groups. Figure 4.11 shows the percentage contribution that each of the compound groups represent following standardisation of peak areas relative to phenanthrene d_{10}. For the ANS treated mussels, the naphthalenes are the largest group (Fig. 4.11b), a similar percentage to that found in the fraction standard, whereas the unidentified peaks represent the majority within the TJP treated mussels (Fig. 4.11a). In accordance with the number of peaks, the percentage contribution by area of the tetralins, indans and indenes was low for ANS exposed mussels. However, the low contribution by area of these groups was in contrast to

the relative abundance of peaks within the TJP exposed mussel extracts. Following depuration, the relative contribution of compound groups was appreciably altered: for both ANS and TJP treated mussels, the compound groups remaining were dominated by the benzenes and phenanthrenes, although for the former, the unidentified peaks represented the largest group.

An attempt was made to quantify the compound groups in terms of $\mu g\ g^{-1}$ of dry weight tissue. However, it was not possible to derive standard curves for the fractions and therefore concentrations had to be calculated with reference to the compound groups present within a single standard concentration for each oil fraction. The calculated tissue concentrations must therefore be treated with caution. The highest concentration for TJP treated mussels was the group of unidentified peaks at around 1000 $\mu g\ g^{-1}$ with indenes having the lowest concentration (Fig. 4.12a). The highest concentration for ANS treated mussels was the naphthalenes at around 750 $\mu g\ g^{-1}$ with indenes having the lowest concentration (Fig. 4.12b). Following depuration, the vast bulk of the tissue burden was eliminated, although appreciable concentrations of alkylated benzenes and phenanthrenes remained in mussels from both treatments (Fig. 4.12). The unidentified compounds were eliminated from the ANS treated mussel tissues but remained within the TJP treated mussel tissues.

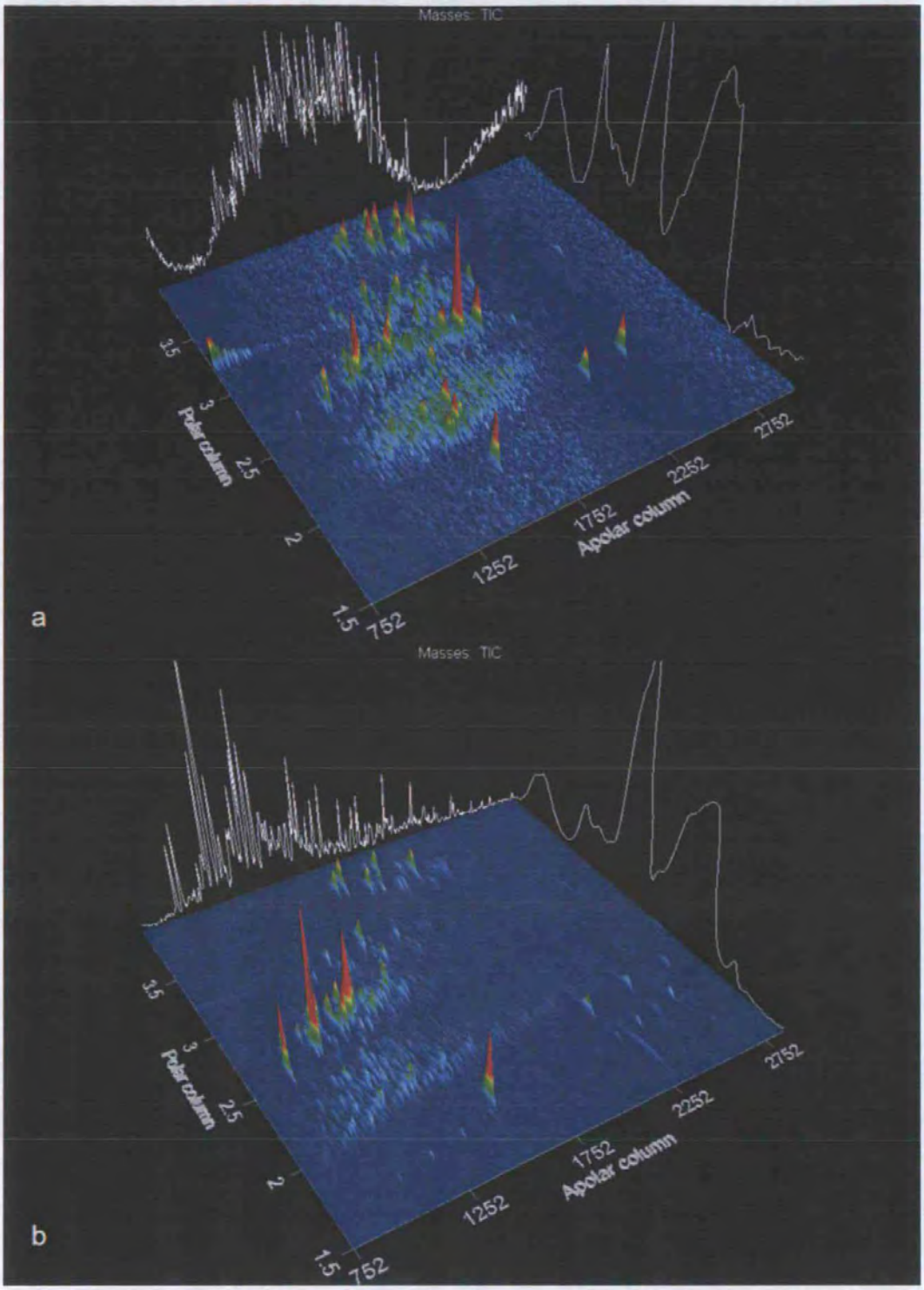
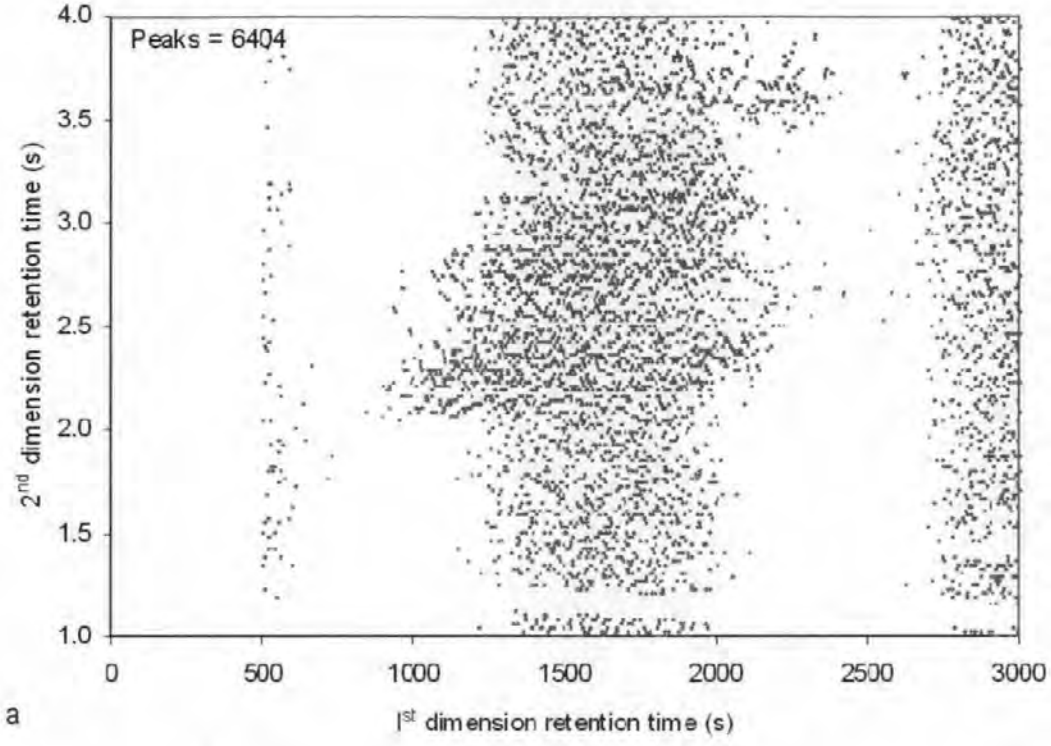
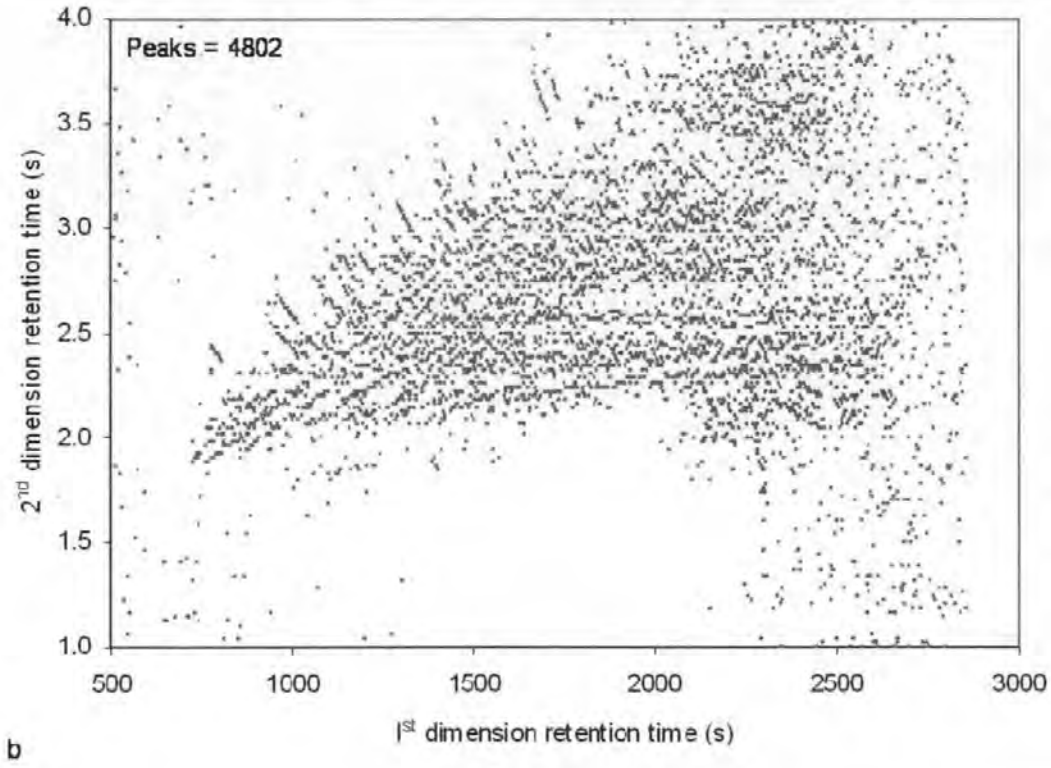


Figure 4.5 GC×GC-ToF-MS 3-D plots of F2 aromatic fractions of TJP (a) and ANS (b) oils. The chromatogram along the primary dimension axis (apolar) shows the typically UCM "hump" of conventional gas chromatograms.



a



b

Figure 4.6 Plot of the peak markers assigned by Pegasus software to the F2 aromatic fractions of TJP (a) and ANS (b) oils.

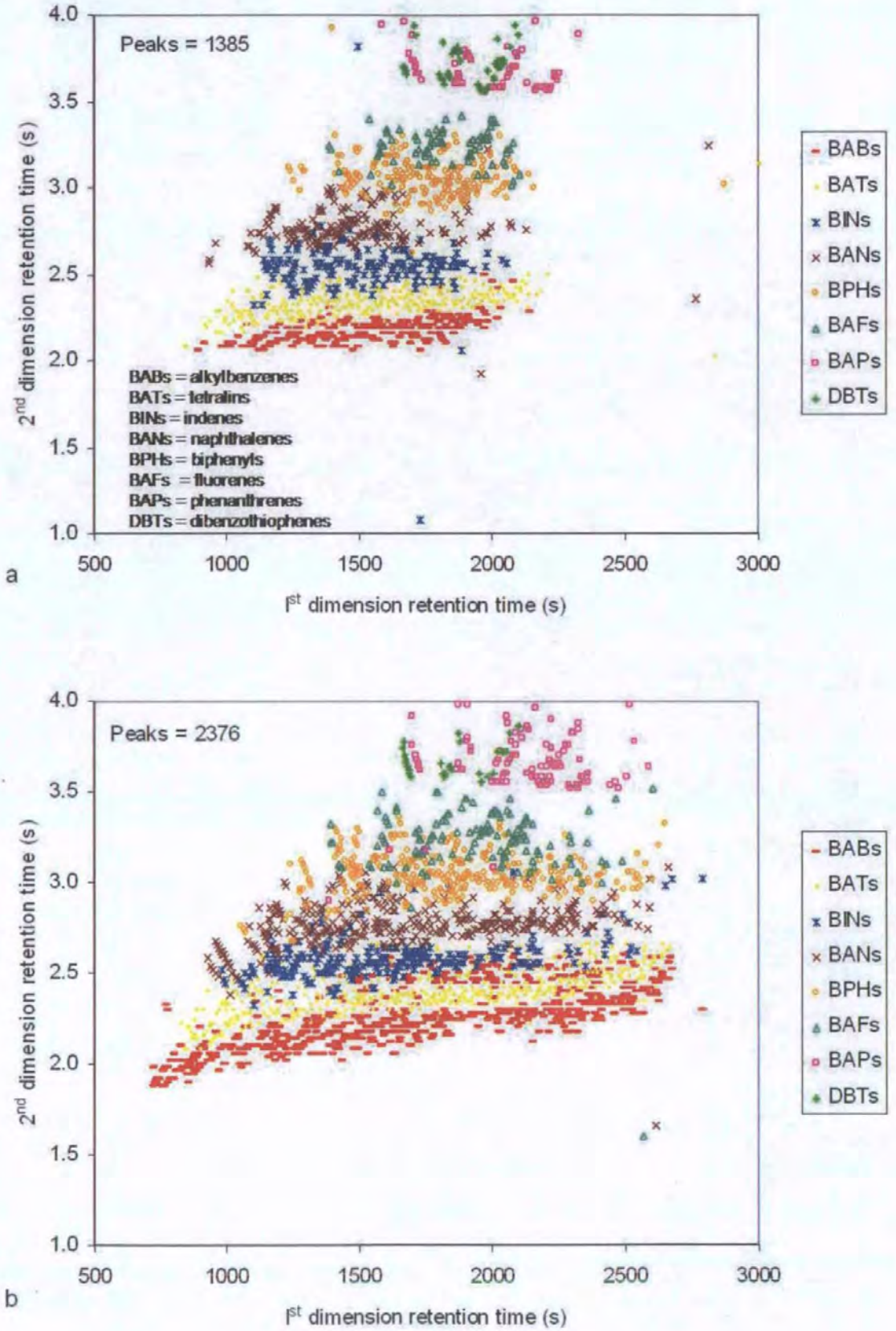


Figure 4.7 Plot of the peak markers of identified compound groups within F2 aromatic fractions of TJP (a) and ANS (b) oils.

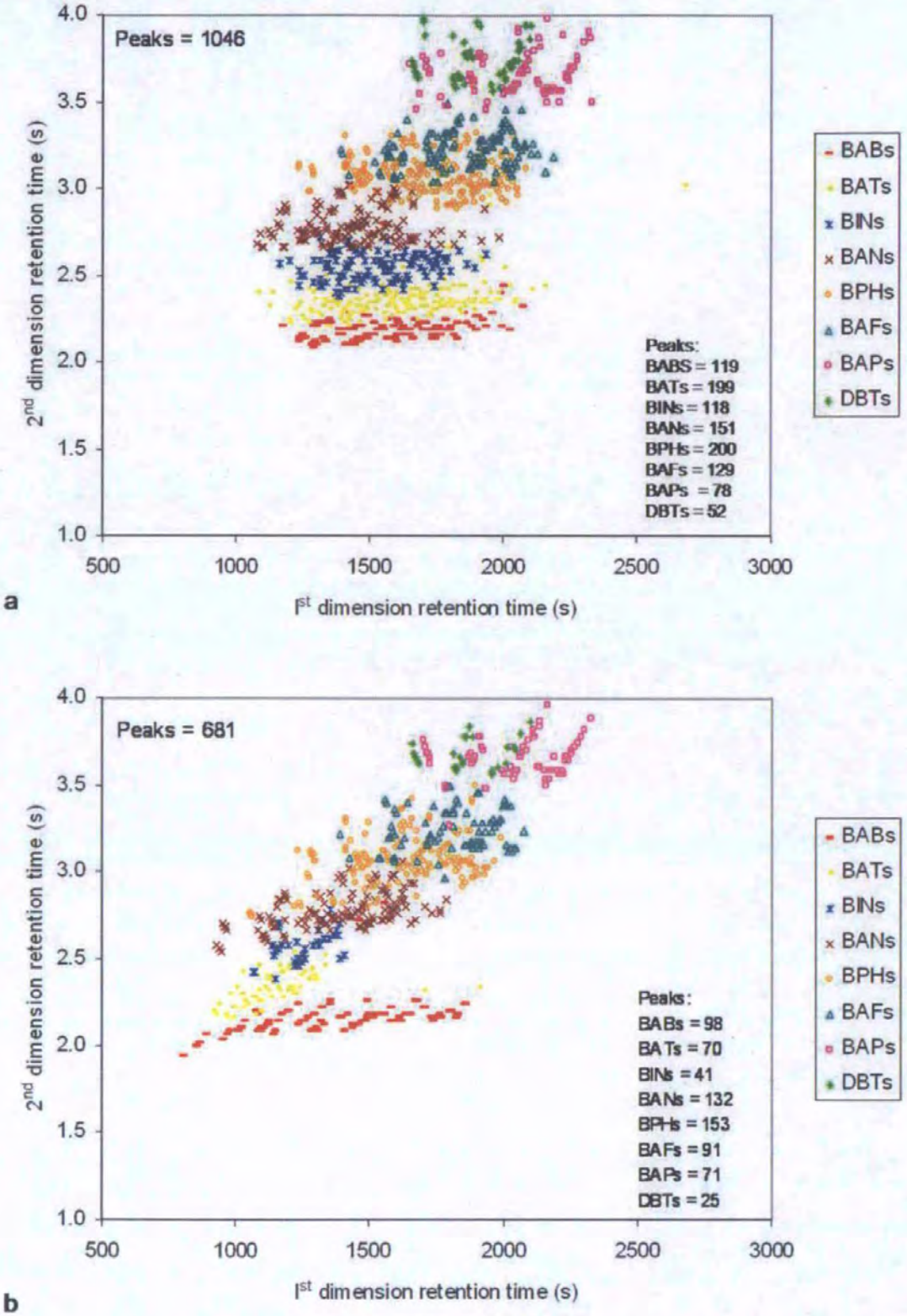


Figure 4.8 Plot of the peak markers of compound groups identified within tissues of mussels exposed to F2 aromatic fractions of TJP (a) and ANS (b). The number of individual peaks assigned to compound groups is also shown. See Fig. 4.7 for key.

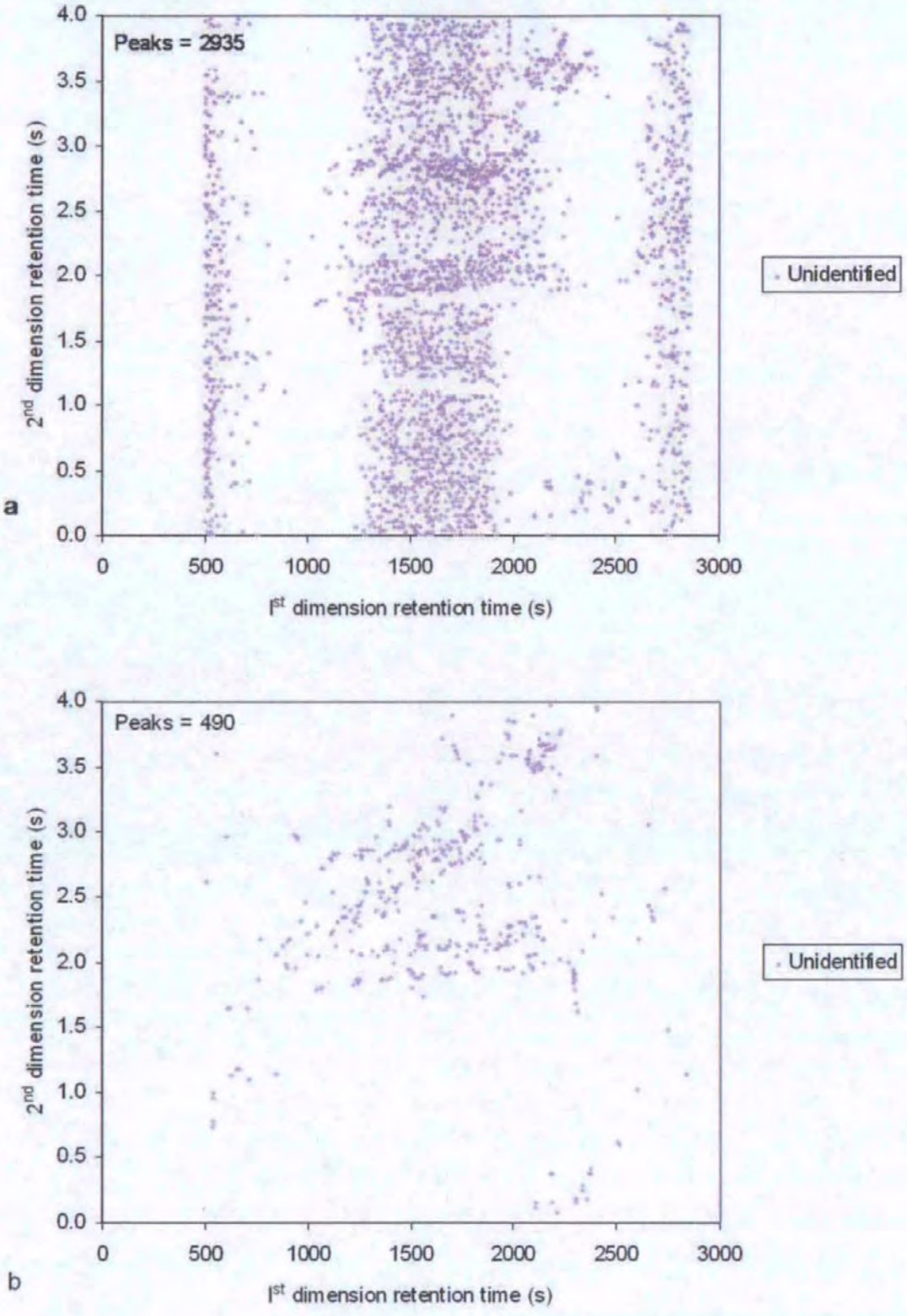


Figure 4.9 Plot of the peak markers of unidentified compounds within tissues of mussels exposed to F2 aromatic fractions of TJP (a) and ANS (b).

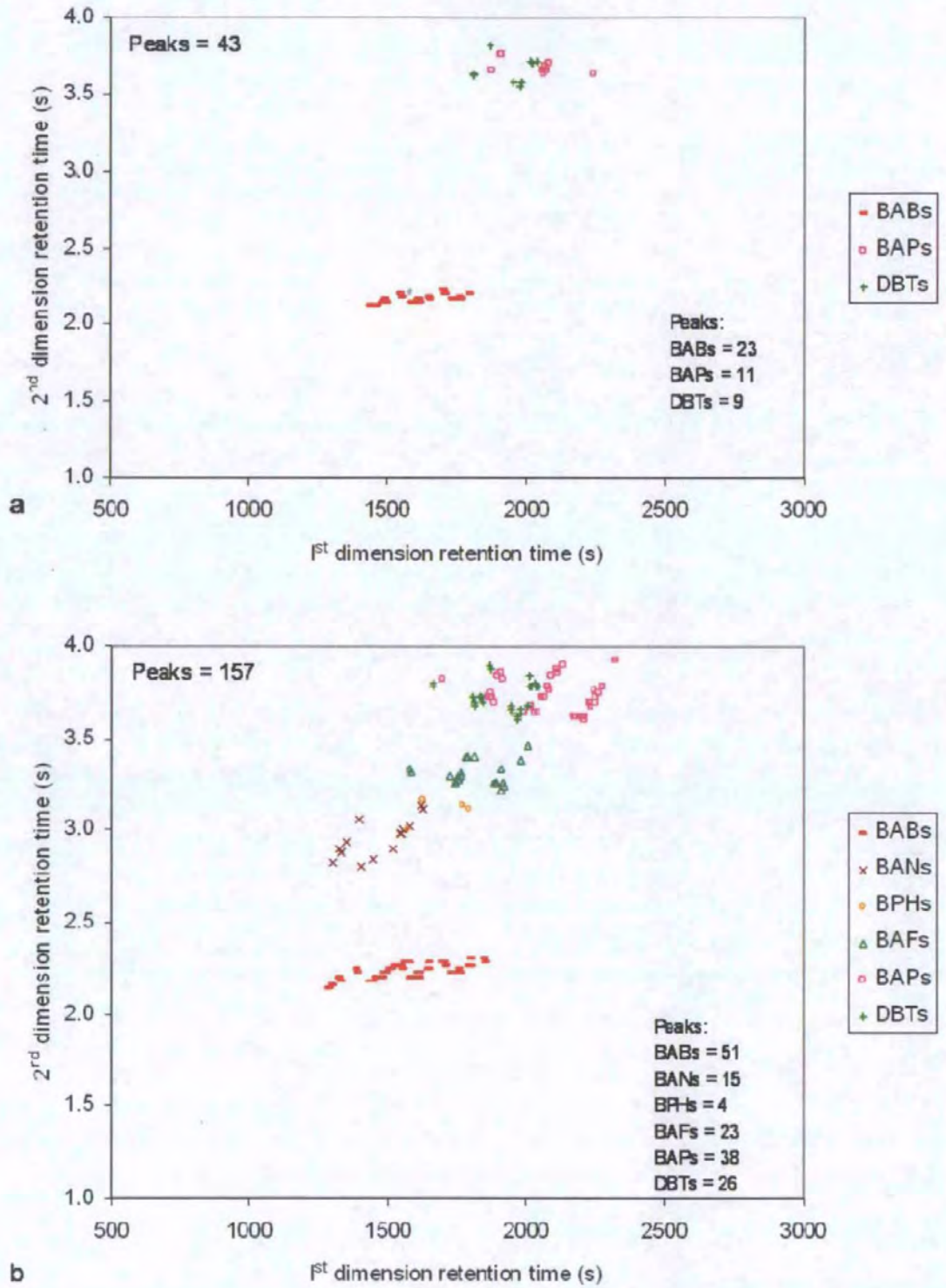


Figure 4.10 Plot of the peak markers of compound groups identified within tissues of depurated mussels following exposure to aromatic fractions of TJP (a) and ANS (b) and a further 5 d depuration.

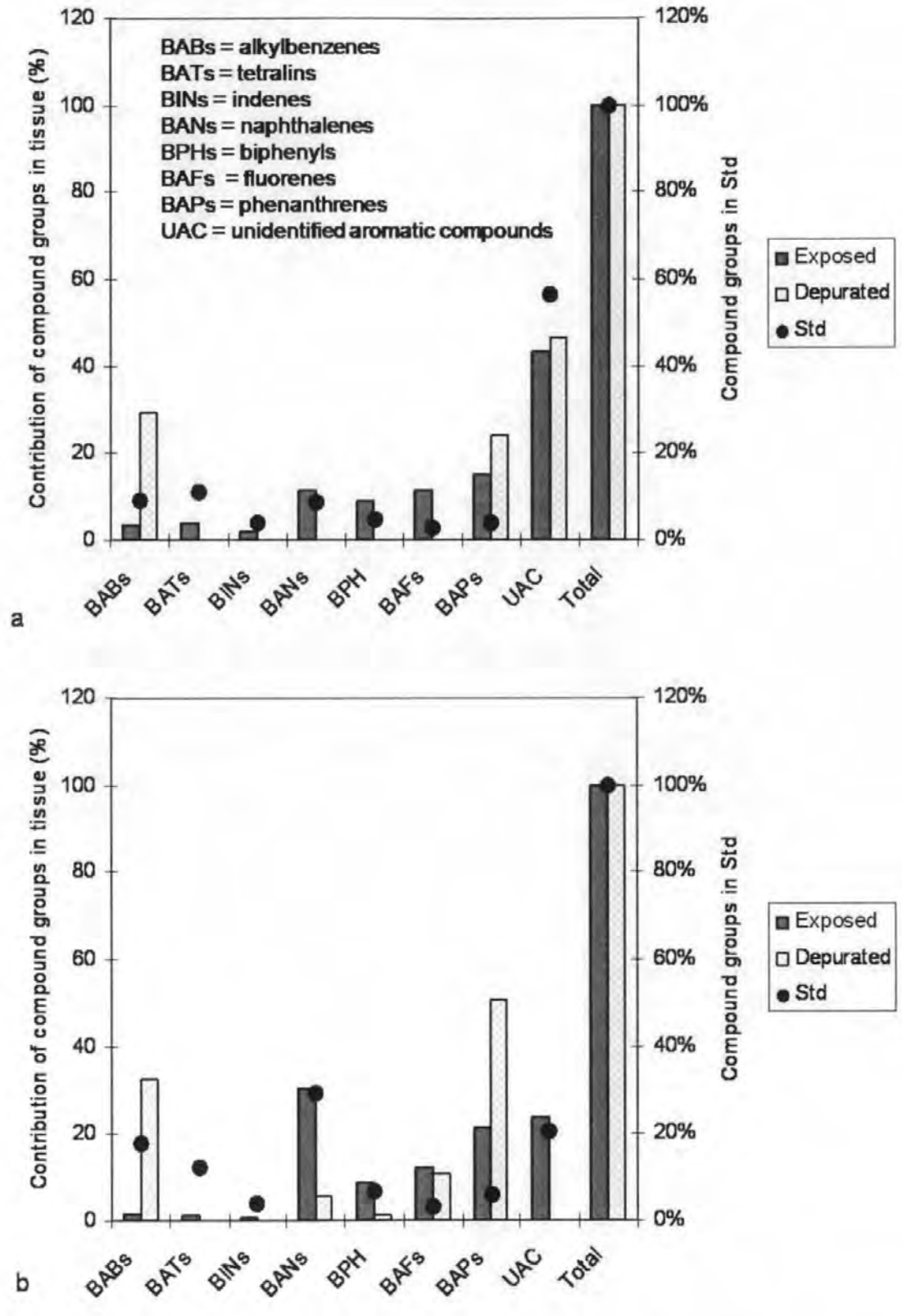


Figure 4.11 Percentage of compound groups identified by GC×GC-ToF-MS analysis of tissues extracts from exposed and depurated mussels compared to TJP (a) and ANS (b) F2 standards.

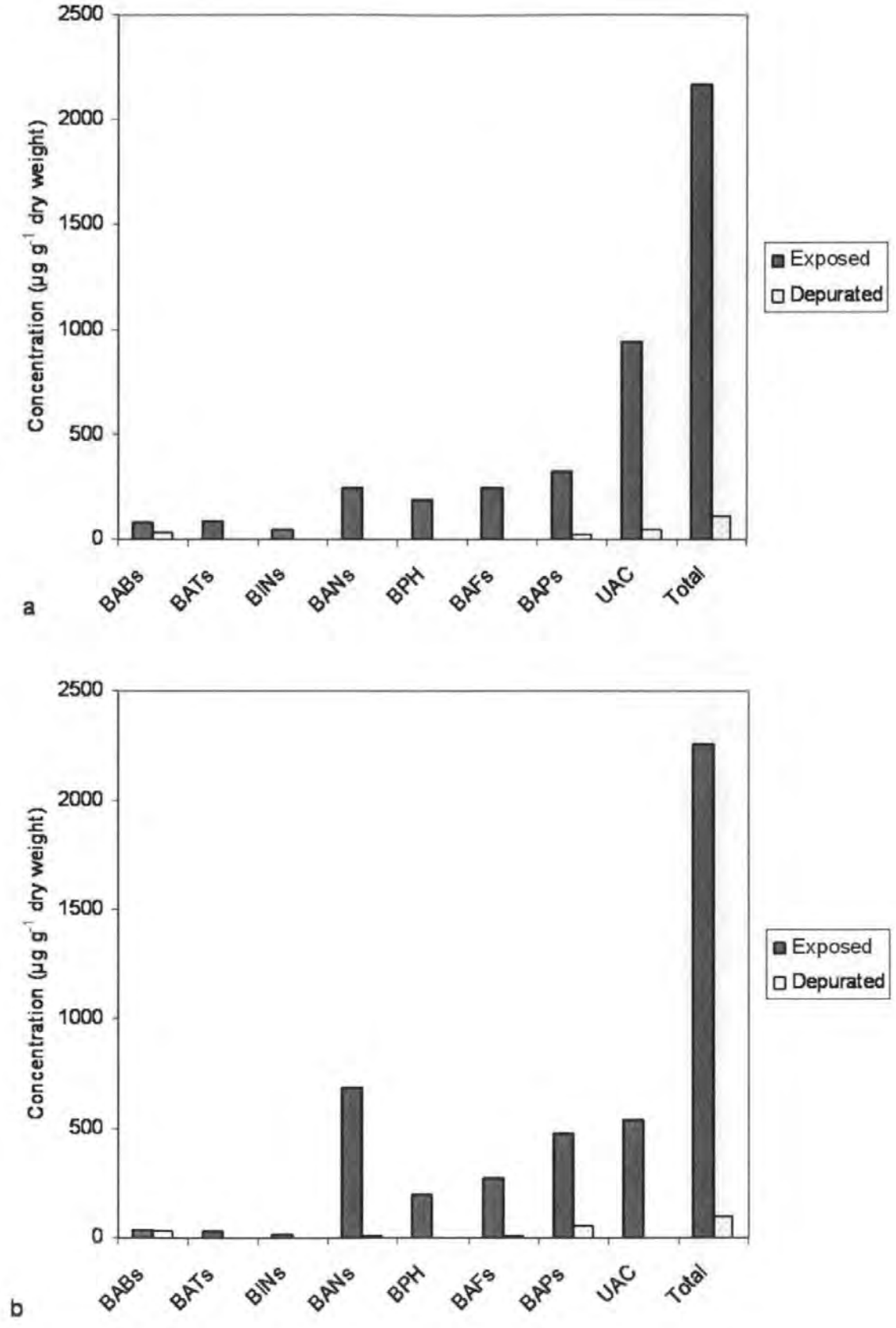


Figure 4.12 Concentrations (µg g^{-1} dry weight) of compound groups identified by GC×GC-ToF-MS analysis of tissues extracts from exposed and depurated mussels compared to TJP (a) and ANS (b) F2 standards.

4.4.6 Bioluminescence inhibition

The bioluminescence inhibition assay was performed using the standard 'comparison of estuarine and coastal samples' protocol (MicrotoxOmni™, 2005) to assess the acute toxicity of the aqueous test solutions used in the mussel exposure tests. Laboratory seawater was found to have a significant ($P \leq 0.01$) hormesis effect after 15 min compared to the reference solvent control water and hence the bacteria were affected by the 0.05 % solution of acetone in seawater. The effect on the solvent was less pronounced after only 5 min exposure ($P = 0.08$). All Microtox and statistical tests were performed comparing oil fraction treatments with the solvent control. Only the F2 fractions of both ANS and TJP were found to significantly inhibit the bioluminescence of the bacteria but some fractions had a significant hormesis effect (Table 4.6).

Table 4.6 Effect of 5 and 15 minute exposures of aqueous solutions of oil fractions (nominal concentrations given within Table 4.2) on the bioluminescence of the bacterium *V. fischeri* using the comparison test of estuarine and coastal samples (MicrotoxOmni™, 2005). np = test not performed, ns = no significant effect ($P > 0.05$)

	Oil fraction					
	F1		*F2*		*F3*	
Oil	*5 min*	*15 min*	*5 min*	*15 min*	*5 min*	*15 min*
SLK	ns	ns	ns	ns	np	np
TJP	Hormesis ($P \leq 0.01$)	Hormesis ($P \leq 0.01$)	Inhibition ($P \leq 0.05$)	ns	ns	ns
ANS	ns	ns	Inhibition ($P \leq 0.001$)	Inhibition ($P \leq 0.001$)	Hormesis ($P \leq 0.01$)	Hormesis ($P \leq 0.05$)

4.5 Discussion

Baseline toxicity tests had revealed that sediment containing UCM-dominated oils could have population level effects on the estuarine amphipod by reducing their reproductive success. By fractionating the oils into aliphatic and two aromatic fractions by open column chromatography, it was possible to investigate which broad category of hydrocarbons were responsible for the observed toxic effects. The use of acute testing of aqueous solutions of the fractions using the luminescent bacteria *V. fischeri* and the mussel, *M. edulis*, together with spiked whole sediment chronic exposures with *C. volutator*, provided insight into the short and long-term nature of the toxic effects, the bioavailability and the mode of action of the UCM hydrocarbons.

Despite the lack of observed toxicity at similar whole oil equivalent concentrations during baseline tests, acute aqueous exposures of fractions showed that the F2 fractions of the oils could significantly reduce the clearance rates of *M. edulis* (Fig. 4.2). The lack of acute toxicity of the F1 aqueous fraction was consistent with the results of a study by Thomas (1995) which showed that an aliphatic UCM isolated from Silkolene-150 had no effect up to a concentration of 1 mg L^{-1}. Smith (2002) reported that 24 h exposures of a monoaromatic fraction derived from the UCM-dominated Gullfaks (North Sea) crude oil at an aqueous concentration of 200 μg L^{-1} caused a significant (*ca.* 40 %) reduction in mussel clearance rates. A similar reduction in clearance rates (compared to solvent control) of F2 fractions were found in the current study (Figs. 4.2 and 4.3), although nominal concentrations were considerably higher (Tables 4.2 and 4.3). Reineke *et al.* (2006) reported that the aromatic fraction of a sulphur-rich (3.1%) degraded crude oil from Monterey California reduced the clearance rate of mussels by *ca.* 35 % after accumulating *ca.* 300 μg g^{-1} (dry weight) within their tissues following 24h exposure whereas a

characteristically similar biodegraded oil with a low sulphur content (0.2 %) caused no consistent effect on clearance rate despite similar accumulations of the oil fraction within their tissues. The degraded Venezuelan crude oil TJP also possesses a high sulphur content of 2.7 % (anon, 2006) but no major differences were observed between the effect of TJP F2 fraction and that of the other oils tested. Although the aqueous exposures were clearly not environmentally realistic, it has been reported that the concentrations of hydrocarbons accumulated by bivalves exposed to dispersed petroleum was dose-time dependent i.e. a six hour exposure at 5 ppm achieved a similar body burden as a 14 day exposure of 0.05 ppm (Engelhardt *et al.*, 1985). The improved clearance rates of the F2 fraction exposed mussels following five days depuration in clean seawater suggests that the organisms are able to rapidly recover from acute exposure (Fig. 4.3). Rapid recovery following exposure is consistent with a non-specific narcosis mode of action (van Wezel & Opperhuizen, 1995). Although not significant ($P > 0.05$) when compared with the solvent control, the F3 fraction of the TJP did appear to cause considerable reduction in clearance rates and therefore these more polar aromatics deserve further investigation.

Analyses by GC-MS of tissue extracts from mussels exposed to aromatic F2 fractions followed by depuration/recovery showed that only a small fraction of the nominal concentrations of both ANS and TJP were bioaccumulated during the 48 h exposure period. Despite a lower nominal aqueous concentration (~50 %), a greater concentration of hydrocarbons was found in the tissues of the ANS F2 exposed mussels (Fig. 4.4) reflecting a probable greater solubility of components from the less weathered oil. Lethality has been reported to occur at a critical membrane concentration of *ca* 40 – 160 mmol kg^{-1} lipid which is equivalent to 2 – 8 mmol kg^{-1} tissue wet weight in a range of aquatic species (McCarty & Mackay, 1993; van Wezel & Opperhuizen, 1995). The tissue burden of the mussels exposed to the F2 fractions were approximately 1 mmol kg^{-1} and 0.5 mmol kg^{-1}

tissue wet weight for ANS and TJP respectively (based on a mean molecular weight of C_{25} compounds) and therefore just below the critical lethal membrane concentration. Donkin *et al.* (1991; 1989) reported that tissue burdens of mussels exposed to a range of aliphatic and aromatic hydrocarbons were in the range 0.1 – 0.5 mmol kg^{-1} associated with reductions in clearance rates of 50 %.

Due to the limitations of conventional GC-MS, only a few petrogenic compounds were identifiable and examination of the mass spectra of even the well resolved peaks with good library matches (>90 %) suggested that their structures were probably branched isomers of the library identified compounds. The combined sum of naphthalenes and phenanthrenes represented only about 1.3 % and 0.2 % of the total integrated areas of ANS and TJP F2 mussel extracts respectively which suggests that resolved PAHs were not solely responsible for the observed reduction in mussel clearance rates. Donkin *et al.* (1991) estimated a 50 % reduction in mussel clearance rates (TEC_{50}) based on bioaccumulation of both naphthalene and phenanthrene of 31 μg g^{-1} (wet tissue weight, ~200 μg g^{-1} dry weight) and Widdows *et al.* (1987) reported a reduction in clearance rates of about 50 % following an eight month chronic exposure of mussels to WAF derived from diesel oil resulting in the bioaccumulation of two and three ring aromatic hydrocarbons of 49 μg g^{-1} (wet tissue weight, ~350 μg g^{-1} dry weight). Hence, the observed sublethal toxicity resulting from exposure to aromatic fractions of ANS and TJP is inconsistent with the bioaccumulation of resolved PAHs but is consistent with numerous unidentified compounds from within the UCM contributing to the toxic effect.

Analyses of the tissue extracts GC×GC-ToF-MS revealed that all the major compound groups identified within the aromatic F2 fractions of the oils were also bioaccumulated by the mussels (Figs. 4.7 and 4.8). The vast majority of the compounds were alkylated

homologues of the parent compounds that were not resolved by conventional GC; hence a far greater understanding of hydrocarbon bioaccumulation was achieved. The profile of the compound groups accumulated in the ANS and TJP exposed mussels differed considerably from that of the wild North Sea mussels (Fig. 1.4) previously found to possess high concentrations of UCM hydrocarbons (Booth *et al.*, 2006). The North Sea mussels with low S/G and moderate to high UCM concentrations had profiles dominated by monoaromatic groups, mainly branched alkylated benzenes (BABs), indans and indenes (BINs) and tetralins (BATs). The acutely exposed mussels in the present study possessed only relatively small quantities (based on integrated area) of these groups although the TJP exposed mussels did possess a large number of peaks consistent with BATs. In contrast with the North Sea mussels, the laboratory exposed mussels accumulated a relatively large concentration of alkylnaphthalenes (BANs). Interestingly, following depuration, the profile of compounds within the exposed mussels altered considerably (Fig. 4.10): the BANs were mostly lost whereas the BABs were resistant to depuration (see also **Chapter 5**) and contributed a substantial proportion of the tissue burden. Hence, the compound group profile of the depurated mussels had a greater similarity to the UCM contaminated wild mussels than the acutely exposed mussels. However, the small concentrations of BATs and BINs accumulated during the 48 h exposure were also lost during depuration (Figs. 4.10 and 4.11). The rapid accumulation and subsequent loss of the BANs is consistent with the greater polarity of these compounds compared to the BABs. The BATs and BINs also elute later on the polar column. A loss of aromatic hydrocarbons coupled with recovery of mussel clearance rates was reported by Widdows *et al.* (1987) although depuration of the hydrocarbons was slower following chronic exposure to low aqueous concentrations.

Mussels in the wild may experience pulses of contaminated water interspersed with clean water, especially in tidal estuaries, and therefore may be able to depurate accumulated hydrocarbons. Under such circumstances, the compounds that are most resistant to depuration will accumulate to a greater extent. The sampling and analysis of wild mussels will therefore reflect the long term accumulation and depuration of compounds together with recent accumulation of compounds that may be readily depurated. Such a scenario largely explains the profile of the North Sea mussels; however, alkylphenanthrenes were not a major constituent of the wild North Sea mussels but were prevalent in both the acutely exposed mussel tissues and that of the depurated mussels (Figs. 1.4, 4.8 and 4.10). Baussant *et al.* (2001b) studied uptake and elimination rates of PAHs by exposing mussels (*M. edulis*) to North Sea crude oil for eight days and then allowing them to depurate hydrocarbons for nine days. The uptake of PAHs into the mussel tissue and subsequent elimination was consistent with first order kinetics, hence the more lipophilic compounds (high log K_{ow}) were found to have a greater tendency to accumulate within the tissues of the mussels. This pattern is consistent with the results of the present study but does not explain the relatively low occurrence of PAHs within the North Sea mussels. Mussels are known to metabolise PAHs by enzymatic processes involving cytochrome P450 type oxidation reactions, but the efficiency of this degradation pathway is thought to be less than that of vertebrates (Stegeman, 1985), hence it may be possible for mussels to eliminate some PAHs by metabolic pathways if they are not subject to continuous exposure. Another large group of PAHs to resist depuration was the dibenzothiophenes (Fig. 4.10). These compounds are commonly reported in the tissues of oil contaminated organisms, such as following the *Prestige* oil spill (Diez *et al.*, 2007), and have been reported to be a strong inducer of blue sac disease in the early life stages of Japanese medaka, *Oryzias latipes*, leading to reduced hatching success (Rhodes *et al.*, 2005).

The near complete recovery of the mussels in the present study implies that the remaining compounds were not present at sufficient concentrations to cause adverse effects. The calculated tissue concentrations of BABs within both the TJP and ANS exposed mussels following depuration were about 30 $\mu g\ g^{-1}$ (Fig. 4.12). This tissue concentration should be sufficient to reduce mussel clearance rates (see **Chapter 5**). Although it is possible that different isomers were present from that of the commercial mixture of BABs (**Chapter 5**) which were less toxic, it is more likely that the tissue concentrations were overestimated due to the lack of standard curves for the compound groups.

The bioluminescence inhibition test carried out on aqueous test solutions used for the mussel exposure, showed that only the F2 fractions of TJP and ANS caused a significant inhibition ($P \leq 0.05$ and $P \leq 0.001$ respectively) of the bacterial luminescence (Table 4.6). It was however interesting to note that, as with the mussel exposure, the baseline test showed no apparent toxicity of the whole oils. In an EDA of contaminated sediment by Grote *et al.* (2005b), the toxicity of the primary fraction was greater than the whole extract. The authors suggested that this may be due to antagonism between different components and/or modification of the physical or physiochemical properties (e.g. solubility). Either of these explanations is possible for the observed increased toxicity of the F2 fractions as hormesis was present following exposure to some fractions and solubility was clearly improved within the fractionated oils. The results of the bioluminescence inhibition tests were consistent with that of the mussel clearance rate assay and suggest a mode of action common to a diverse group of organisms. The *V. fischeri* bioluminescence inhibition test is known to detect log K_{ow}-dependent non-specific effects including narcosis (Hermens *et al.*, 1985a) and therefore a narcosis mode of action of the aqueous aromatic components of the UCM is supported by the results of the *M. edulis* and *V. fischeri* exposures.

The behavioural tests conducted using *C. volutator* from the same population as used for the chronic exposure did not suggest any of the fractions would cause either acute of chronic effects based on patterns of behaviour observed during previous behaviour and chronic tests (Scarlett *et al.*, 2007a). Due to the lack of significant endpoints (Fig. 4.1) derived for the behavioural tests and the small levels of effects found during the primary fraction sediment exposure test, it is unclear as to whether the behavioural tests gave a reasonable prediction of no acute or chronic effects or, if the behaviour test is flawed and no association exists between the amphipods' behaviour on encountering the contaminated sediment and deleterious effects upon *C. volutator*. Given that the behaviour test requires minimal additional investment in time and resources when conducting a chronic test and that the test did not give rise to Type I errors (false positives), it remains a potentially usefully tool for identifying sediments likely to cause sublethal effects to chronically exposed organisms.

Comparing the effects of aqueous exposure (Figs. 4.2, 4.3) to that of sediment-bound UCM hydrocarbons (Scarlett *et al.*, 2007b) it was evident that the sediment associated hydrocarbons were less bioavailable than the dissolved and water-accommodated oil components. Despite the lack of acute toxic effects arising from sediment exposure, chronic exposure did however induce adverse effects that could affect populations (Scarlett *et al.*, 2007b). *Corophium volutator* are closely associated with the sediments in which they live and are reported to deposit feed as well as to filter feed (Moller & Riisgard, 2006). These amphipods therefore interact with contaminants adsorbed to particulates *via* feeding as well as *via* the dissolved phase. Feeding may therefore have been the primary route of uptake which caused contaminants to accumulate within tissues resulting in reduced growth and reproduction (Scarlett *et al.*, 2007b). Although there appeared to be some differences between how the fractions affected the different organisms, it was

apparent that the relatively non-polar aromatic fraction of the oils was the most important toxic component. Recent research by Hokstad *et al.* (2007) used an EDA approach to investigate the toxicity of unresolved complex mixture (UCM) of petrogenic oils in the marine water column. This identified a number of effects arising from acute exposure to the water-soluble-fraction (WSF) of UCM-rich oil from the Troll oilfield in the North Sea. A fraction containing non-polar compounds was found to cause metabolic inhibition within the rainbow trout *O. mykiss* hepatocytes, however, the main fraction associated effects arising from a battery of toxicity test endpoints was a polar fraction containing mostly UCM hydrocarbons. Analysis of this fraction by GC×GC-ToF-MS identified sulfoxides as a notable group of compounds (Booth, personal communication). Only tissues of mussels exposed to the relatively non-polar F2 fraction were analysed by GC×GC-ToF-MS in the present study and no sulfoxides were apparent within the extracts.

4.6 Conclusions

The results of acute 48 h aqueous exposure tests with mussels *M. edulis* were consistent with a narcosis mode of toxic action following bioaccumulation of components from the aromatic F2 fraction of all three oils. This was supported by the bioluminescence inhibition tests with *V. fischeri*. The reductions in mussel clearance rates were similar for the F2 fractions of all three oils and therefore effects cannot be solely attributable to resolved components; this was supported by GC-MS analyses of mussel tissue extracts. Analyses of tissue extracts by GC×GC-ToF-MS revealed that an array of compound groups were accumulated by the mussels and that the alkylated benzenes, phenanthrenes and dibenzothiophenes were resistant to depuration and therefore likely to accumulate within wild mussels subject to pulses of weathered oils contaminated waters. Chronic spiked-sediment exposure tests identified the aromatic fractions as the most toxic but it appeared the all fractions contributed towards the effects of reduced growth rates and reproductive success and hence no specific mode of action is supported by the observed data. In agreement with mussel tests, the resolved hydrocarbons could not be solely responsible for the toxic effects measured following exposure of amphipods to spiked sediment.

As all the fractions contributed towards the observed effects related to sediment exposure of the UCM hydrocarbons, further fractionation and subsequent biotesting was not justified. Although the non-polar aromatic fraction was identified based on the aqueous exposures, problems with solvent toxicity, especially with the bioluminescence inhibition test which is most suited to low volumes of test sample, further fractionation and subsequent biotesting was unlikely to clearly identify specific UCM components within a reasonable time span. The use of GC×GC-ToF-MS to identify groups of compounds

previously unresolved by GC-MS within the tissues of mussels with poor health status has now opened up the possibility of directing research at target compound groups. This approach has been used in research reported within subsequent **Chapters 5 and 6**.

Chapter 5

Assessment of bioaccumulation, depuration and toxicity of branched alkyl benzenes – a principal component of unresolved complex mixtures bioaccumulated in mussels

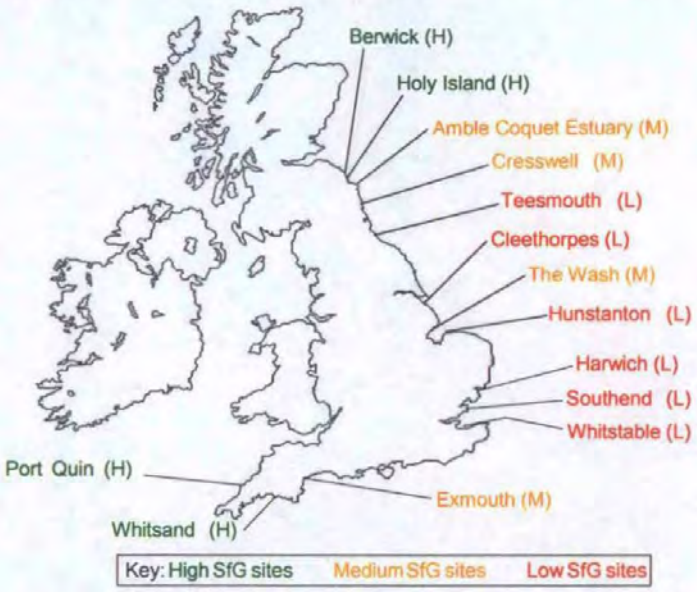
Recent research using GC×GC-ToF-MS has identified branched alkylbenzenes (BABs) as a principal component of some unresolved complex mixtures of hydrocarbons bioaccumulated in the tissues of N. Sea mussels, *M. edulis*. The potential for these compounds to accumulate in mussel tissues and their effect upon clearance rates was explored using a commercially available mixture of $C_{12\text{-}14}$ BABs. Tissue extracts were analysed and characterised by both GC-MS and GC×GC-ToF-MS. Some results from this study have been published (Booth *et al.*, 2007). Results relating to chronic exposure to BABs and analyses of mussel tissue extracts by GC×GC-ToF-MS have been accepted for publication (Scarlett *et al.*, In press).

5.1 Introduction

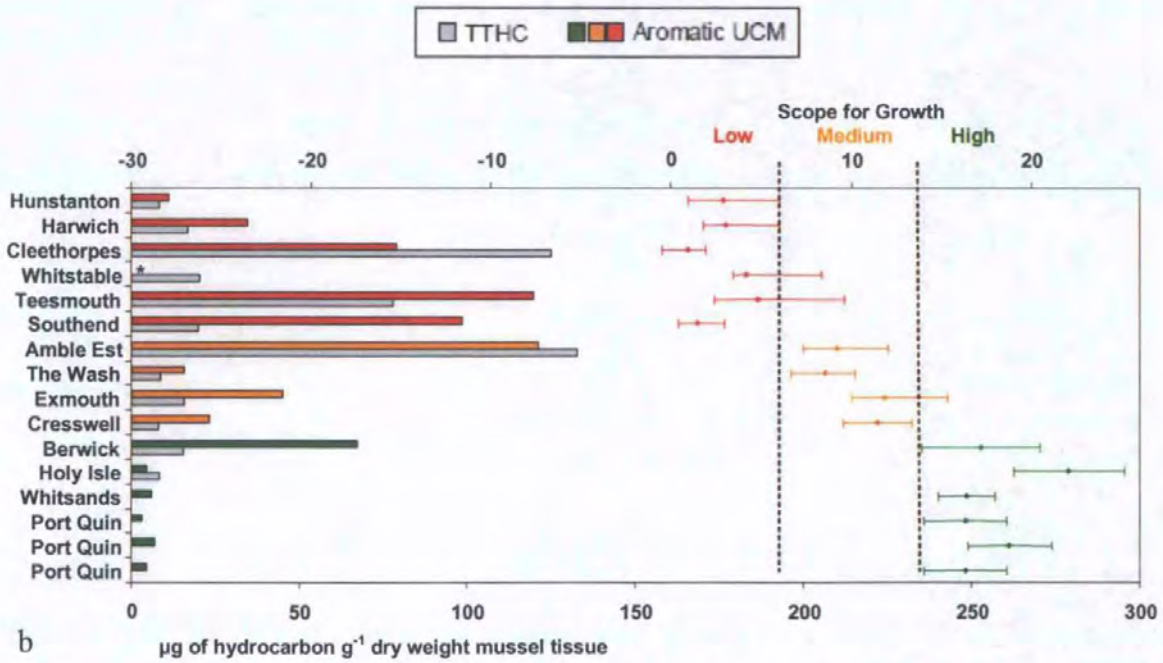
5.1.1 Background

Analysis by GC×GC-ToF-MS of UCMs extracted from the tissues of environmentally contaminated mussels (*M. edulis*) has shown that they contain a vast array of both known and unknown hydrocarbon compounds (Booth *et al.*, 2006). The comparative analysis of UCM hydrocarbons extracted from mussels known to possess high, moderate and low S*f*G (Widdows *et al.*, 1995), see Figs. 5.1 and 5.2, revealed large numbers of alkylated aromatic compounds of which the alkylbenzenes represented the largest structural class within the UCM of mussels with low S*f*G (Figure 5.2). The numbers of compounds within the alkylbenzene class far exceeded the numbers of possible linear alkylbenzenes (LABs, Fig. 5.3) that are often found in environmental samples polluted with detergent residues (Eganhouse *et al.*, 1988). Further analysis revealed the compounds to be a complex mixture of branched alkylbenzenes (BABs). In addition to the BABs, branched alkyltetralins (BATs), and branched alkylindans/alkylindenes (BINs) were also absent in mussels with high S*f*G but were prominent in the stressed mussels (Fig. 5.2). From the molecular weight ranges of the compounds revealed by the GC×GC-ToF-MS data, Booth *et al.* (2007) calculated that thousands of compounds are theoretically possible and thus the apparent complexity of the two-dimensional gas chromatograms.

Linear alkylbenzenes (LABs) are a common contaminant in the marine environment as they often occur along with linear alkylbenzene sulphonate (LAS) which are major ingredients of synthetic surfactants and are used worldwide in households, agricultural, biotechnological industries and cosmetic applications (Reich & Robbins, 1993; Riechers *et al.*, 1995) and hence are often present within sewage and industrial

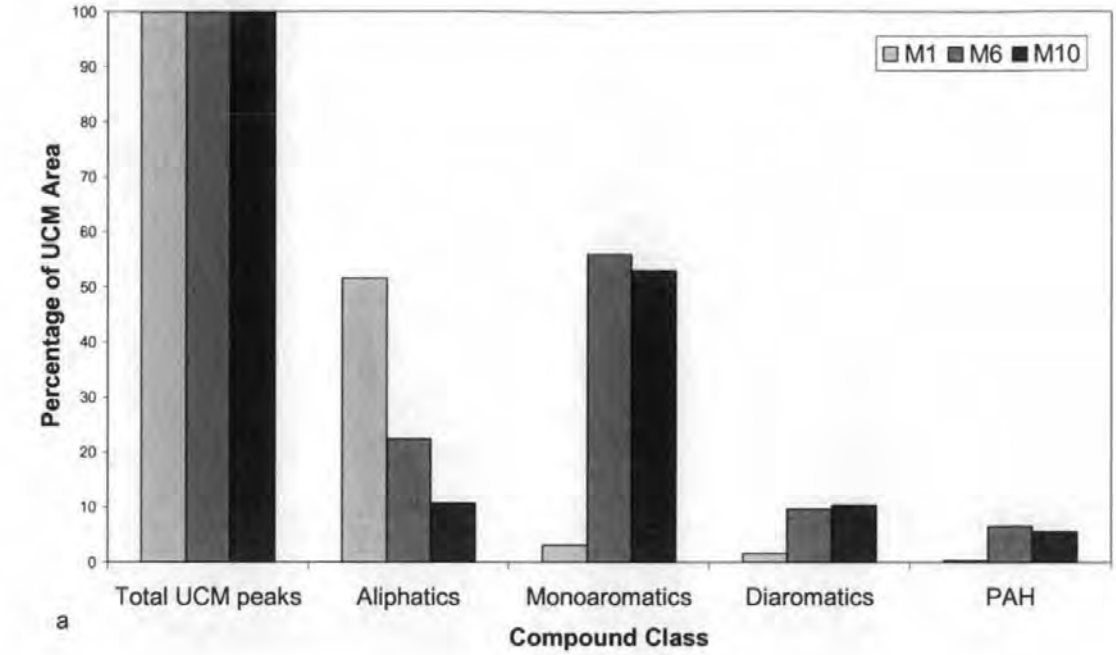


a



b

Figure 5.1 (a) Location of sampling stations and (b) comparison of the amount of aromatic UCM from each of the mussel tissue extracts with both the total toxic hydrocarbon (TTHC) and Scope for Growth (S*f*G) values determined by Widdows *et al.* (1995). Graphs reproduced from Booth *et al.* (2006).



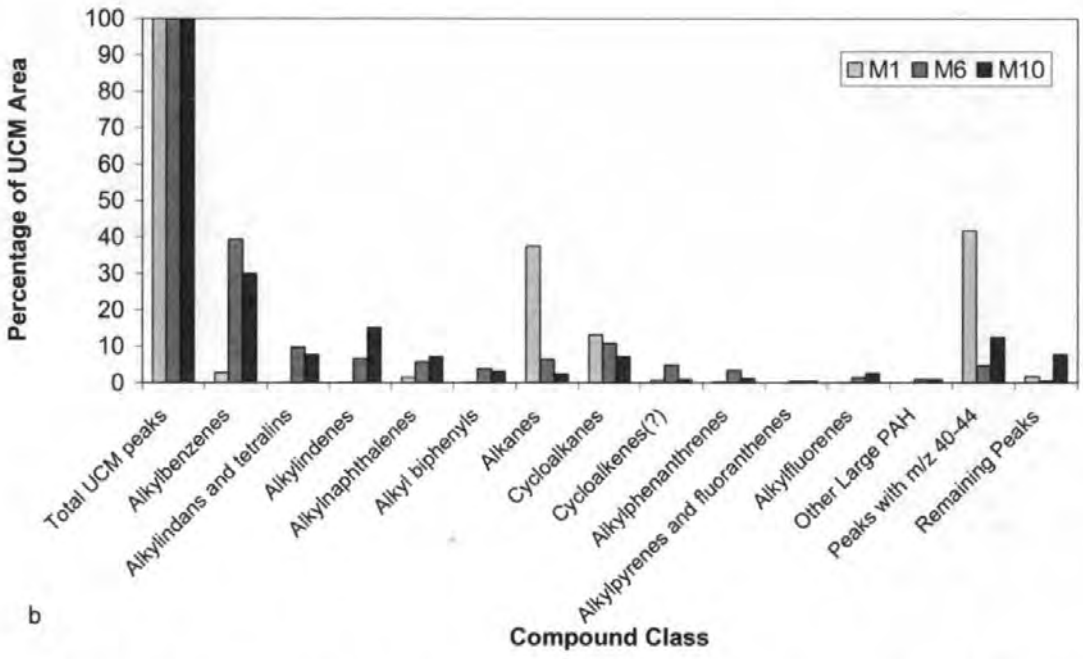


Figure 5.2 Structural classes of hydrocarbons derived by tandem gas chromatography analysis of three UCMs that elicit high (M1, Port Quin), medium (M6, Able Estuary) and low (M10, Southend) Scope for Growth in *Mytilus edulis*, (a) broad classes, (b) detailed classes. Graphs and data from Booth (2006).

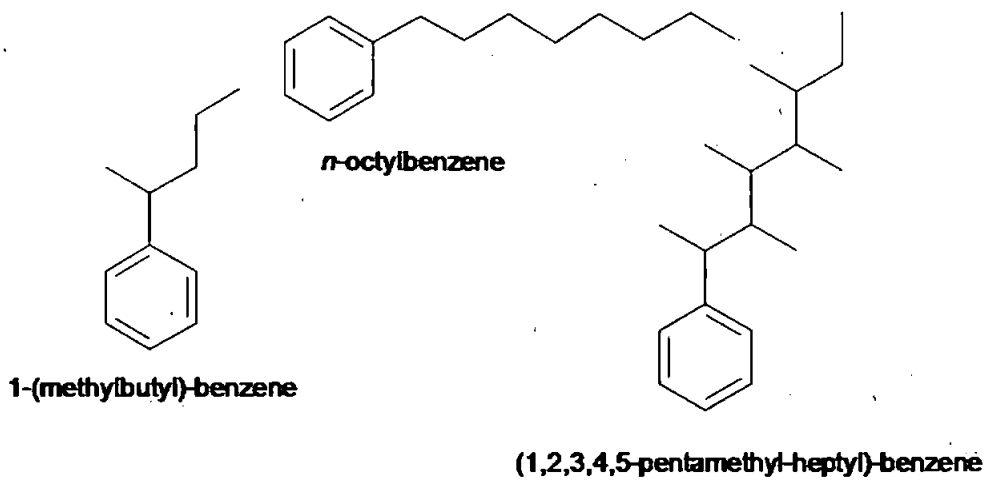


Figure 5.3 Examples of structures of linear alkylbenzenes 1-(methylbutyl)-benzene (C_5), *n*-octylbenzene (C_8 1-phenyloctane) and a branched alkylbenzene (1,2,3,4,5-pentamethyl-heptyl)-benzene (C_{12}).

wastes. The commercial LAB product mainly consists of a complex mixture of various homologues and isomers representing different alkyl chain lengths, typically between 10 and 14 carbon atoms (Abu-Hassan *et al.*, 2006), i.e. C_4 to C_8 alkyl chains bonded to a benzene ring. The lower molecular weight LABs were found to be toxic to mussels (*M. edulis*) up to a so-called toxicity 'cut-off' threshold of C_{10} alkyl chain (Donkin *et al.*, 1991; Widdows *et al.*, 1995). Fernandez *et al.* (2002) also reported that LABs caused a narcotic effect to the crustacean *Daphnia magna* Straus, 1820 and that the effect was additive. Crude oil contains both LABs and BABs (Booth, 2004; Golovko *et al.*, 2000; Vella & Holzer, 1992) but degradation by bacteria results in a relative concentration of the BABs as they are more resistant to microbial attack than the LABs (George *et al.*, 2002). Scott & Jones (2000) reported degradation of LABs of up to 99 %.

There is a paucity of literature concerning the toxicity of BABs. However, there is no *de facto* reason why they should be less toxic than the LABs. Indeed, it has been observed that branching can increase the solubility of hydrocarbons (George *et al.*, 2002; Silla *et al.*, 1992; Tolls *et al.*, 2002), which is usually associated with an increase in the bioavailability of compounds. Donkin *et al.* (1991) exposed mussels, *M. edulis*, to linear alkylbenzenes

and tested their toxicity using clearance rate as a measure of sublethal effects. It was observed that toxicity increased with increasing molecular weight up to 1-phenyloctane (C_8 alkyl chain) but 1-phenyldecane (C_{10} alkyl chain) was not toxic even when exposed at concentrations above the solubility limit. To determine the toxicity of BABs, a commercial mixture of predominantly C_{12} alkyl chain BABs was investigated using a mussel exposure test protocol similar to that previously described by Donkin *et al.* (1991), refer to **Chapter 2**. The C_{12-14} BABs mixture is described by the manufacturers (Chevon Oronite S.A., Levallois-Perret Cedex, France) as a clear and limpid liquid derived from petroleum hydrocarbons by distillation at 275 °C (5 %) and 293 °C (95%) with an average molecular weight of 241 Daltons (http://www.chevron.com/products/oronite/products/pdfs/bab.pdf) isolated from crude oil, and comprising components both resolved and unresolved by GC-MS. To relate the results to those previously obtained for the LABs, 1-phenyloctane was selected as a reference toxicant. The sublethal toxicity of the BABs was determined both in terms of nominal aqueous exposure and tissue concentrations as quantified by GC-MS. McCarty & Mackay (1993) argue that the linking of tissue concentration or critical body residue (CBR) with adverse biological effects has several advantages over aqueous concentration, including:

- Bioavailability is explicitly considered.
- Accumulation kinetics are considered, which reduces the confounding effect of organism exposure duration when interpreting results.
- Uptake from food (as distinct from water) is explicitly considered.
- Toxic potencies are expressed in a less ambiguous manner, facilitating identification and investigation of different modes of action.
- Effects of metabolism on accumulation are considered.
- Mixture toxicity may be more readily assessed.
- Experimental verification can be readily sought in the laboratory and the field.

Although the CBR approach is not universally appropriate, for example, biological effects may arise from readily metabolised compounds, it is appropriate for mixtures of non-polar narcotic compounds. Mixture toxicity is often found to be additive and the larger the number of compounds in the mixture the better the approximation to concentration addition; this has been found even when compounds with dissimilar modes of action are present and for both acute and chronic endpoints (Hermens *et al.*, 1984a; Hermens *et al.*, 1985b). It is possible that compounds that act by a specific mode of action when present in a mixture at low concentrations, below the threshold value for the specific mode of action, contribute to the narcotic activity of the mixture (McCarty & Mackay, 1993).

Additional analysis of mussel tissues by the GC×GC-ToF-MS analytical technique has been shown to provide superior separation and more accurate peak assignments than previously possible (Booth *et al.*, 2006; Shellie *et al.*, 2001) and hence offers a greater potential for resolving the BABs compounds accumulated by the mussels. In addition to the mussel clearance rate assay, the toxicity of the test solutions was also investigated using the bacterial bioluminescence inhibition assays based on *V. fischeri* (Microtox). This test has been shown to possess good correlation with numerous other toxicity bioassays (Kaiser & McKinnon, 1992; Kaiser & Palabrica, 1991; Leftley, 2000) and provides a rapid screening technique for contaminated marine and estuarine waters.

5.1.2 Aims of study

The main aims of the study were:

1. Compare the toxicity of the $C_{12\text{-}14}$ BABs mixture to that of the reference linear alkylbenzene 1-phenyloctane under similar conditions as that previously used by Donkin *et al.* (1991).

2. Establish meaningful aqueous and tissue effect concentrations for the C_{12-14} BABs mixture.
3. Test the hypothesis that long-term exposure to a low concentration of BABs can produce similar effects as acute exposure to higher concentrations.
4. Assess the ability of mussels to depurate and recover from exposure to BABs;
5. Assess possible differential accumulation/depuration of BABs compounds in terms of volatility and polarity using GC×GC-ToF-MS analysis.
6. Quantify mussel tissue concentration of BABs by GC×GC-ToF-MS and compare with GC-MS.

5.2 Methodology

5.2.1 Exposure tests using *Mytilus edulis*

Collection and maintenance of organisms

Mussels were collected and maintained as reported previously (Scarlett *et al.*, 2005) and detailed within **Chapter 2**.

Acute and chronic exposures

Semi-static acute (72 h) and chronic (14 day) exposure tests were performed as detailed within **Chapter 2**. Some of the acute tests were repeated to check for reproducibility and the chronic exposures were conducted in triplicate at the single nominal concentration of 5 $\mu g\ L^{-1}$. Test solutions (1-phenyloctane 42 $\mu g\ L^{-1}$ and BABs 5, 10, 20, 41 $\mu g\ L^{-1}$) were prepared using acetone as a carrier solvent (0.005 % v/v). Subsamples (*ca.* 20 mL) of the test solutions were taken for use with the bioluminescence inhibition (Microtox) assay. The mean shell lengths for initial acute tests and chronic/recovery tests were 47.2 mm (SE = 0.31 mm) and 44.2 mm (SE = 0.11) respectively. At the end of the chronic (14 day) exposures, one third of the mussels were examined for haemolymph cellular viability (Neutral Red dye retention assay) and one third randomly allocated to clean tanks containing clean filtered seawater to assess their ability to recover after a five day depuration period (water replaced every 24 h). Mussels were fed and aerated continuously, and water quality assessed as detailed in **Chapter 2**.

Measurement of Mytilus edulis clearance rate

The clearance rate was performed as detailed within **Chapter 2**.

Extraction, characterisation and quantification of alkylbenzenes

The extraction of hydrocarbons from mussel tissues was by alkaline saponification as described in **Chapter 2**. Water samples (100 mL) from the chronic exposure, nominal concentration 5 $\mu g\ L^{-1}$ were solvent extracted into DCM (100 mL), spiked with phenanthrene d_{10}, concentrated to 100 µL and analysed by GC-MS. In addition, $C_{12\text{-}14}$ BABs spiked water (10 mg L^{-1}) was DCM-extracted and analysed by GC-MS to verify that a broad range of compounds from the BABs mixture was entering the dissolved phase. Resolved peaks within the gas chromatogram of the BABs mixture were characterised in terms of their molecular ion and fragments ions. From their mass spectra, the resolved compounds were classified by the number of carbon atoms in their alkyl group and where possible, potential alkyl group structures assigned. Details of the GC×GC-ToF-MS are provided within **Chapter 2**.

Determination of cellular viability

Cellular viability of *M. edulis* haemolymph cells was assessed by measuring their absorbance following 3 h incubation with Neutral Red. Haemolymph was extracted from the posterior abductor muscle of BABs-exposed and solvent control mussels (8 mussels per treatment. The Neutral Red retention was then calculated per unit of protein. Full details are provided in **Chapter 2**.

Bioluminescence inhibition assay

The bioluminescence inhibition assay was performed using two standard protocols (MicrotoxOmni™, 2005): (i) the 'comparison test for marine and estuarine sample' and, (ii) the 'basic test'. Due to the low response observed, a second acute test was performed using increased concentrations of both 1-phenyloctane and BABs i.e. above solubility limit of 1-phenyloctane. In brief (ii), test solutions of 420 $\mu g\ L^{-1}$ and 410 $\mu g\ L^{-1}$ were prepared from stock solutions of 1-phenyloctane and BABs respectively (acetone concentration 0.05

%), the solutions were subject to serial dilutions and the concentration at which bioluminescence was inhibited by 50 % (IC_{50}) calculated. Further details are provided in **Chapter 2**.

5.3 Results

5.3.1 Characterisation of branched alkylbenzene mixture

GC-MS

When analysed by GC-MS, the C_{12-14} BABs formed a complex mixture of both resolved and unresolved compounds (Fig. 5.4). Alkylbenzenes were identified by the presence of fragment ions *m/z* 91, *m/z* 105 and *m/z* 119. The retention times, percentage area, molecular ions, major fragment ions and deduced fragment alkyl groups of resolved compounds are presented in Table 5.1. The percentage of compounds resolved by GC-MS (integrated by MS data analysis) of the total area (AREASUM function) was calculated to be 48 %. As no examples of branched alkylbenzenes exist within the library (nbs54k) used by the MSD it was not possible to provide definitive identification of compounds within the BABs mixture but where possible, potential alkyl fragment groups based on the fragment ions have been suggested (Table 5.1). A mass difference of 14 Daltons was common, consistent with branching along the alkyl chain. A large number of the resolved compounds possessed the base ion *m/z* 119 with a minority possessing *m/z* 105 or *m/z* 91 (Table 1). The vast majority of the compounds were found to have alkyl chains with 12 carbon atoms, with minority fractions of C_{11}, C_{13} and C_{14} but there were also a few smaller groups ranging from C_7 to C_{10} and odd larger compounds (Fig. 5.5). The resolved or partially resolved (many compounds were co-eluting but identifiable as BABs) compounds comprised about 85 % of the total area of the gas chromatogram with phthalates accounting for a further 8 %. The remaining 7 % contained completely unresolved compounds.

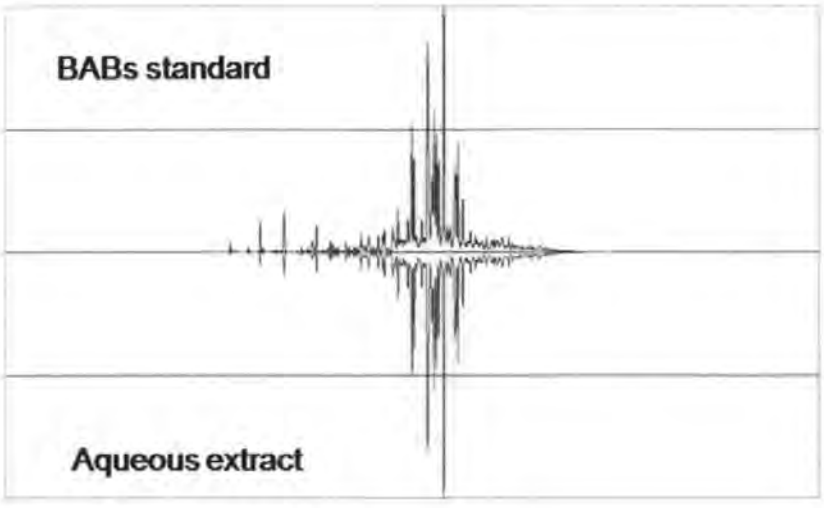


Figure 5.4 Comparison of a TIC of C_{12-14} BABs standard (1.0 mg mL^{-1}, top) with TIC of aqueous extract at equivalent concentration (mirror image below).

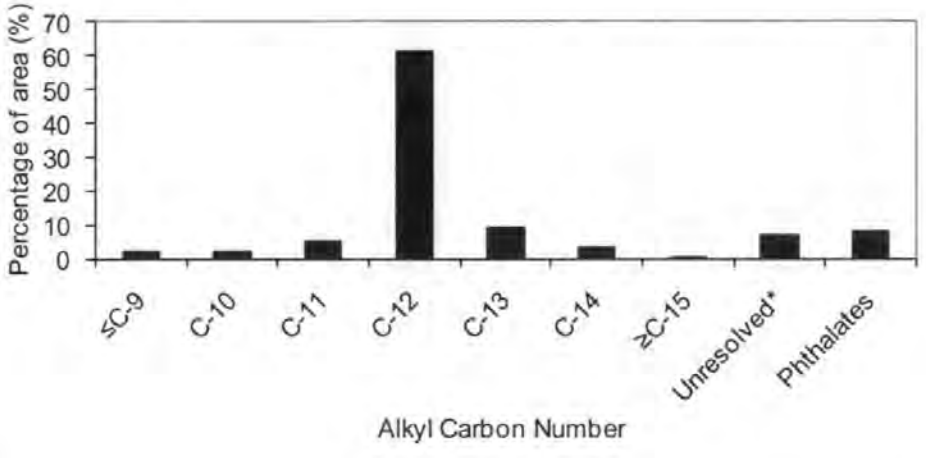


Figure 5.5 Characterisation of branched alkylbenzenes mixture with regard to proportions of compounds with different alkyl group sizes as identified by GC-MS. * unresolved compounds include compounds with ions consistent with monoaromatics substituted with alkyl groups.

GC×GC-ToF-MS

Analysis by GC×GC enabled much better resolution of individual compounds and the alkylbenzenes formed a distinct band within the 2-dimensional peak marker plot (Fig. 5.6). Although the BABs are very similar in structure and therefore do not undergo major polar chromatographic separation, it was possible to resolve many compounds that co-eluted with conventional GC. In addition, the ToF-MS provided improved mass spectral analysis

although the molecular ion was sometimes less apparent than when analysed by the quadrupole mass analyser. The chromatographic method differed from that used by Booth *et al.* (2007), see **Chapter 2**; thus, the results obtained from the mussel exposure study cannot be directly compared, in terms of retention times, with the results obtained from the analysis of tissue extractions of wild mussel populations. The majority of the resolved compounds were present at low concentrations so that 3-D representation was dominated by a relatively few large peaks originating from C_{12} branched alkylbenzenes.

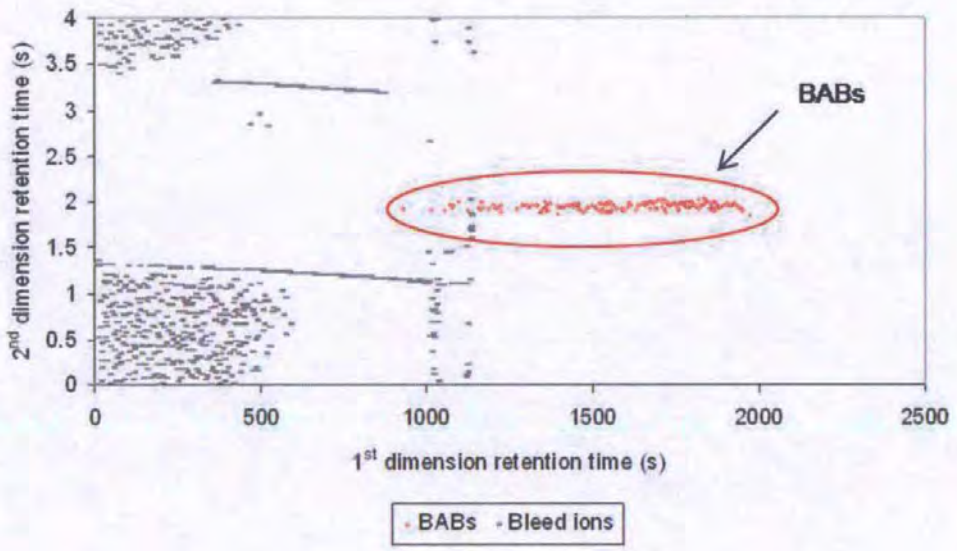


Figure 5.6 Chromatographic peak marker plot derived by GC×GC-ToF-MS analysis of a complex mixture of C_{12-14} branched alkylbenzenes (BABs, 0.1 mg mL^{-1}) showing band of alkylbenzenes distinct from the column bleed compounds.

Alkylbenzenes eluted across the 1st dimension (volatility) range from about 1100 s to 1900 s, although the majority eluted between 1600 s and 1750 s. The 2nd dimension (polarity) range was relatively narrow: from about 1.82 s to 2.06 s with the majority of compounds eluting between 1.92 s and 2.00 s. As many of the compounds were only present at low concentrations, several of the peaks identified from the BABs mixture at 0.50 mg mL^{-1} (~360) peaks were not present at 0.01 mg mL^{-1} (~30 peaks); this was especially true of the minority of compounds that eluted at the extreme ends of the primary dimension range.

Table 5.1 Characterisation of commercial mixture of branched alkylbenzene compounds by GC-MS

Retention Time (min)	Area (%)	M^+ ion (*m/z*)	Alkyl carbons	Base ion (*m/z*)	Major fragment ions (*m/z*) in order of abundance								Alkyl fragments		
7.9	0.4	176	7	119	91	105	120	176	79	78	77	51	C_4H_9		
8.7	1.0	190	8	119	91	105	120	118	79	190	77	78	C_5H_{11}		
10.1	0.9	204	9	119	91	120	105	55	77	79	78	103	C_6H_{13}		
10.4	0.6	218	10	119	91	105	133	120	57	79	78	77	C_6H_{13}		
10.8	0.4	218	10	119	91	105	120	218	55	79	77		C_7H_{15}		
11.2	0.8	218	10	119	91	105	120	218	77	79	133	55	C_7H_{15}	C_6H_{13}	
11.3	0.4	218	10	119	91	105	120	218	77	79	57		C_7H_{15}		
11.4	0.7	232	11	105	91	119	147	57	189	106	77	133	C_3H_7	C_6H_{13}	C_7H_{15}
11.7	0.6	232	11	119	91	105	120	232	79	78	55	133	C_7H_{15}		
11.7	0.9	232	11	119	91	120	105	57	79	77	55	232	C_8H_{17}		
11.9	1.2	232	11	119	91	105	133	118	120	55	71	203	C_7H_{15}	C_5H_{11}	
12.1	2.2	232	11	119	91	105	57	120	79	147	176	232	C_6H_{13}	C_2H_5	
12.2	1.9	246	12	91	105	119	161	57	104	118	246	147	C_6H_{13}	C_7H_{15}	C_2H_5
12.3	2.7	246	12	119	91	105	120	133	57	161	55	77	C_6H_{13}	C_7H_{15}	C_2H_5
12.4	4.5	246	12	105	91	57	161	119	189	106	117	118	C_4H_9	C_8H_{17}	
12.5	2.9	246	12	105	91	161	57	119	189	106	117	246	C_4H_9	C_7H_{15}	C_2H_5
12.6	1.0	246	12	105	91	119	106	175	120	57	55	117	C_5H_{11}		
12.7	2.4	246	12	119	91	105	120	57	117	246	55	175	C_9H_{19}	C_5H_{11}	
12.8	11.2	246	12	119	91	105	133	57	77	217	147	203	C_3H_7	C_2H_5	C_7H_{15}
12.9	4.0	246	12	119	91	105	120	57	54	133	246	217	C_2H_5		
13.0	6.7	246	12	119	91	105	120	77	55	53	246	147	C_7H_{15}		

Table 5.1 continued

Retention Time (min)	Area (%)	M^+ ion (*m/z*)	Alkyl carbons	Base Ion (*m/z*)	Major fragment ions (*m/z*) in order of abundance								Alkyl fragments		
13.0	7.4	246	12	119	91	105	133	55	217	71	120	246	C_2H_5	C_6H_{13}	
13.2	9.7	246	12	119	91	105	133	57	217	120	55	246	C_2H_5	C_6H_{13}	C_8H_{17}
13.3	0.9	246	12	105	119	91	133	118	57	147	246	203	C_3H_7	C_2H_5	C_5H_{11}
13.4	1.2	260	13	119	91	105	57	120	79	55	106	133	C_8H_{17}		
13.5	4.8	260	13	119	91	105	57	120	77	55	260	79	CH_2	C_4H_9	C_7H_{15}
13.5	4.2	246	12	119	91	105	120	55	77	246	77	175	C_5H_{11}		
13.6	2.0	246	12	119	91	105	120	246	55	77	79	57			
13.8	0.8	274	14	91	105	119	57	133	161	71	217	203	C_4H_9		
13.9	1.5	260	13	119	91	105	79	57	55	120	260	147	C_8H_{17}		
14.0	1.1	260	13	119	105	91	133	147	57	106	120	119	C_3H_7		
14.1	0.8	260	13	119	105	91	57	189	118	133	106	55	C_5H_{11}		
14.2	0.8	331	17	119	91	105	120	260	55	103	231	253	C_5H_{11}		
14.5	0.5	274	14	119	105	91	133	57	120	55	56	231	C_3H_7		
14.7	1.3	274	14	119	91	105	57	120	274	55	79	161	C_2H_5		
14.8	0.7	274	14	119	91	105	133	71	57	120	274	189	C_6H_{13}	C_7H_{15}	$C_{10}H_{21}$

5.3.2 Reference toxicant

Tissue concentration

A standard curve for the reference toxicant 1-phenyloctane was produced for the concentration range 0.5 – 50 mg mL^{-1} based on the TIC peak areas. The TIC for the 1-phenyloctane standard and mussel tissue extract are shown in Fig. 5.7 and the corresponding mass spectra in Fig. 5.8. The curve was linear, as described by equation 5.1. The limit of detection of the instrument, as defined by 3 × the signal at the intercept of the slope, corresponded to a concentration of 0.0008 mg mL^{-1}. The minimum signal for the mussel samples analysed was greater than twice the limit of detection.

$$\text{Peak area} = 2.292 \times 10^6 + 5.440 \times 10^9 \text{ OB} \qquad r = 0.9995 \ (P \leq 0.0001)$$

Equation 5.1 Relationship between TIC peak area and concentration of 1-phenyloctane concentration (OB, mg mL^{-1}).

From the retention time and presence/ratios of molecular ion *m/z* 190 and fragment ions, *m/z* 91, *m/z* 92, *m/z* 57, *m/z* 105 and *m/z* 133, and following correction of the peak areas using the internal standard phenanthrene d_{10} (mean recovery of phenanthrene d_{10} was 57.5 % (CV = 9.5 %, n = 16)), the concentration of 1-phenyloctane in the mussel extracts was determined from equation 5.1. Estimated 1-phenyloctane tissue concentrations were 2.9 and 6.5 μg g^{-1} (dry wt.). Assuming equilibrium between the aqueous phase and the mussel tissue had been achieved (not determined), this represents a log bioconcentration factor (BCF) of 2.0.

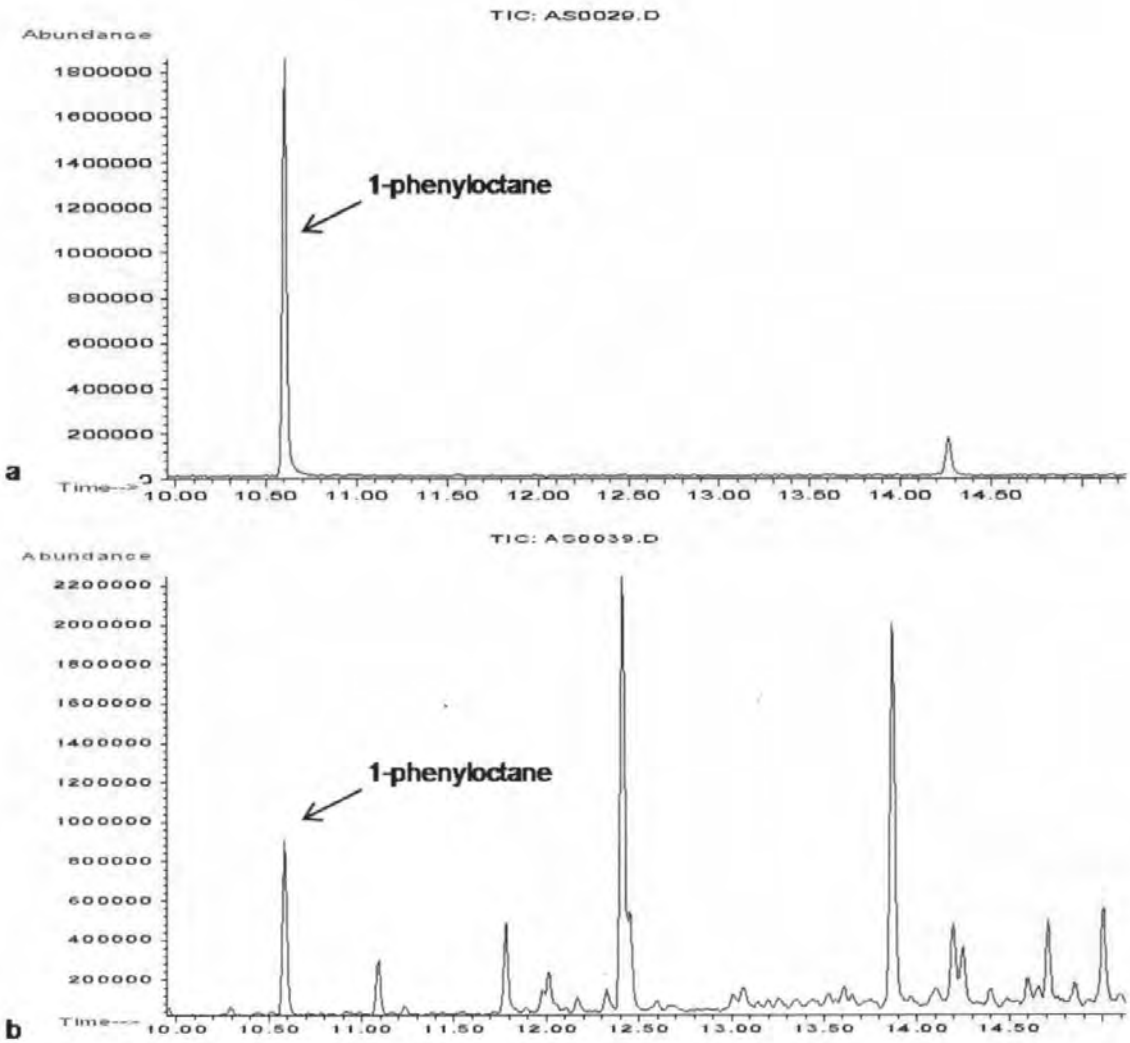


Figure 5.7 Total Ion Chromatograms of (a) 1-phenyloctane standard (0.005 mg L^{-1}) and (b) extract from mussels exposed to 41 µg L^{-1} 1-phenyloctane.

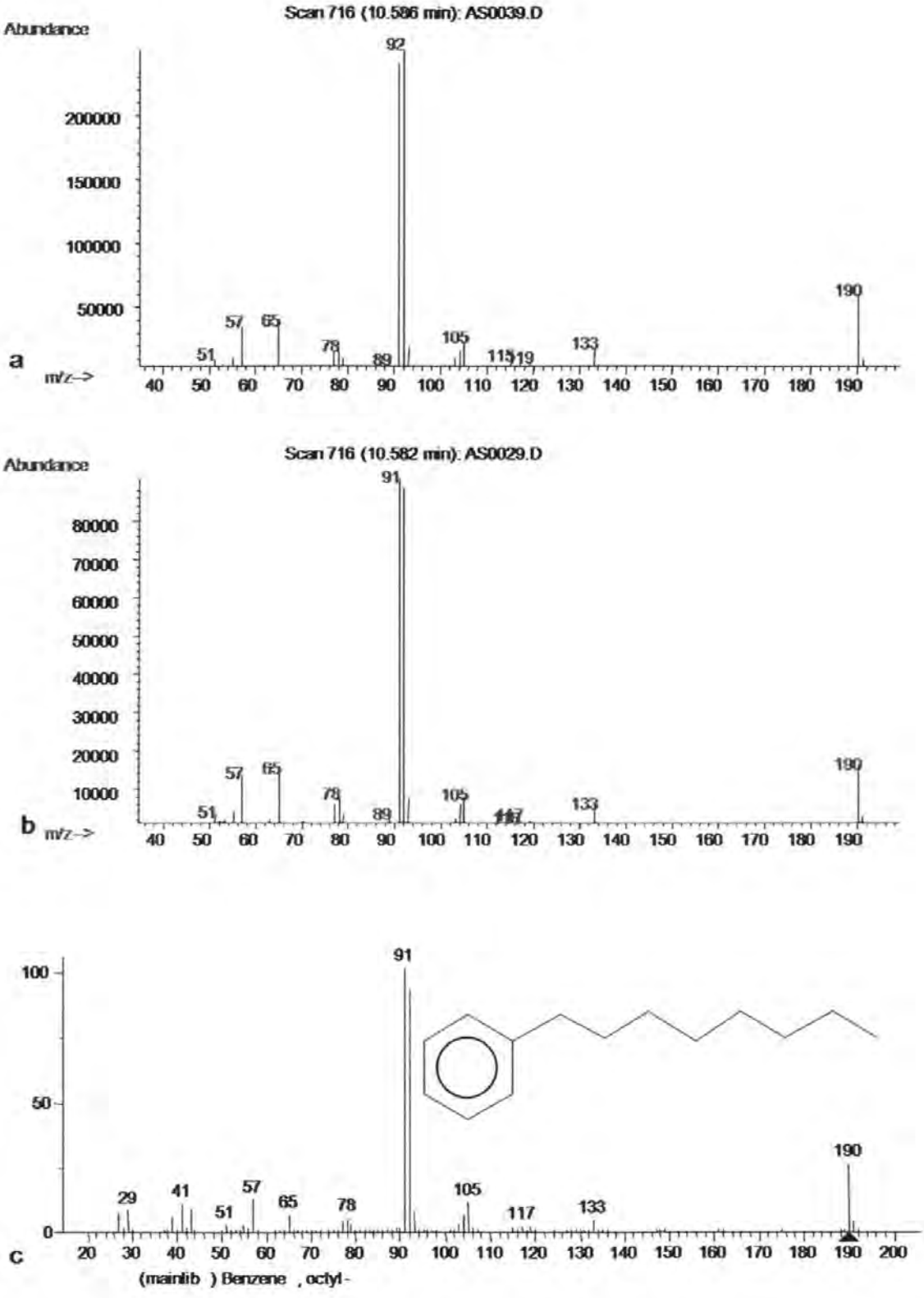


Figure 5.8 Mass spectra of peak at 10.6 min (a) 1-phenyloctane standard (0.005 mg mL^{-1}), (b) extract from mussels exposed to 41 µg L^{-1} 1-phenyloctane and (c) NIST library 1-phenyloctane.

Mussel clearance rates

No significant differences ($P > 0.05$) were found between the mussels exposed to seawater and acetone controls. The mussels exposed to 1-phenyloctane at a nominal aqueous concentration of 42 μg L^{-1} showed significant ($P \leq 0.05$) reductions in their clearance rates compared to seawater and acetone control organisms. Duplicate exposure tests showed that clearance rates were reduced by a mean 32 % with very little variation between tests (CV = 0.7 %).

Bioluminescence inhibition

No significant effect was observed between the 1-phenyloctane test solution used in the mussel exposures and that of the acetone control solutions. A small hormesis effect was observed but this was not significant ($P > 0.05$). Testing the solution at above its solubility limit at a concentration of 420 μg L^{-1} produced a 5 minute IC_{50} value of 650 μg L^{-1}. Confidence limits could not be calculated as too few of the dilutions produced an effect.

5.3.3 Branched alkylbenzenes

Quantitation of BABs by GC-MS

A standard curve for the BAB mixture was produced for the concentration range 0.01 – 1.0 mg mL^{-1} based on the total ion chromatogram of the C_{12} alkylbenzene eluted at a retention time of 12.8 min (molecular ion *m/z* 246, fragment ions *m/z* 119, *m/z* 91, *m/z* 105, *m/z* 133 and *m/z* 57) using GC-MS. The extracted ion chromatogram (*m/z* 119) for the BABs standard and mussel tissue extract showing the peak selected for quantitation is shown in Fig. 5.9 with the corresponding mass spectra in Fig. 5.10. The curve was linear, as described by equation 5.2. The limit of detection of the instrument, as defined by 3 × the signal at the intercept of the slope, corresponded to a concentration of 17.8 μg $sample^{-1}$. The minimum signal for the mussel samples analysed was just less than the limit of

detection and therefore the tissue concentration for the mussels exposed to 5 μg L^{-1} BABs should be viewed with caution.

$$\text{Peak area} = 6.0866 \times 10^6 + 6.84915 \times 10^8 \text{ BABs} \qquad r = 0.9998 \ (P \leq 0.0001)$$

Equation 5.2 Relationship between TIC peak area and concentration (mg mL^{-1}) of branched alkylbenzene mixture, BABs, based on major resolved C_{12} alkylbenzene.

Concentrations of the BABs within the mussel tissues were quantified based on the peak areas of the selected C_{12} alkylbenzene using the characteristic ions identified from the TICs of the BAB's standard to confirm identity. The ratios of the six most prominent peaks, confirmed as alkylbenzenes, from the mussel tissue extracts, relative to the C_{12} compound used for quantification were compared with the ratios observed in the BABs standard. The ratios of the compounds within the mussel tissues were found to be similar to those in the BABs standards and were not statistically different ($P = 0.28$). The main difference being that the C_{12} compound eluting at 13.0 min was relatively more abundant and the C_{12} compound eluting at 13.2 min was relatively less abundant. Many of the compounds present in small quantities were not identified by the autointegration function of the MSD data analysis and some were often masked by larger biogenic compounds. Because co-elution of compounds was commonly observed, quantitation was also determined on the basis of the molecular ion *m/z* 246 peak area for confirmation purposes (equation 5.3).

$$\text{Peak area} = 1.917 \times 10^5 + 8.387 \times 10^6 \text{ BABs} \qquad r = 0.9971 \ (P \leq 0.0001)$$

Equation 5.3 Relationship between molecular ion *m/z* 246 peak area and concentration (mg mL^{-1}) of branched alkylbenzene mixture, BABs, based on C_{12} alkylbenzene.

Following corrections for losses based on peak areas of phenanthrene d_{10}, concentrations of BABs within mussel tissues were determined to be in the range 11 – 56 μg g^{-1} for nominal aqueous exposure concentrations between 5 – 41 μg L^{-1}. The relationship between tissue concentration and nominal aqueous concentration (Fig 5.11) was best described by the natural logarithmic equation below (equation 5.4), giving a log BCF of 3.3 (assuming equilibrium between aqueous and tissues phases).

$$TC = 126.8 + 22.45\text{Ln.AqC.} \qquad r = 0.9885\ (P \leq 0.01)$$

Equation 5.4 Natural logarithmic relationship between branched alkylbenzene mixture tissue concentration, TC (μg g^{-1}) and aqueous concentration, AqC, (μg L^{-1}).

It was not possible to measure the aqueous concentration of the BABs mixture in seawater at the nominal concentration 5 μg L^{-1} as no alkylbenzenes were detected by GC-MS but analysis of spiked water produced a profile near identical to that of the BABs mixture standard at equivalent concentration (Fig. 5.4).

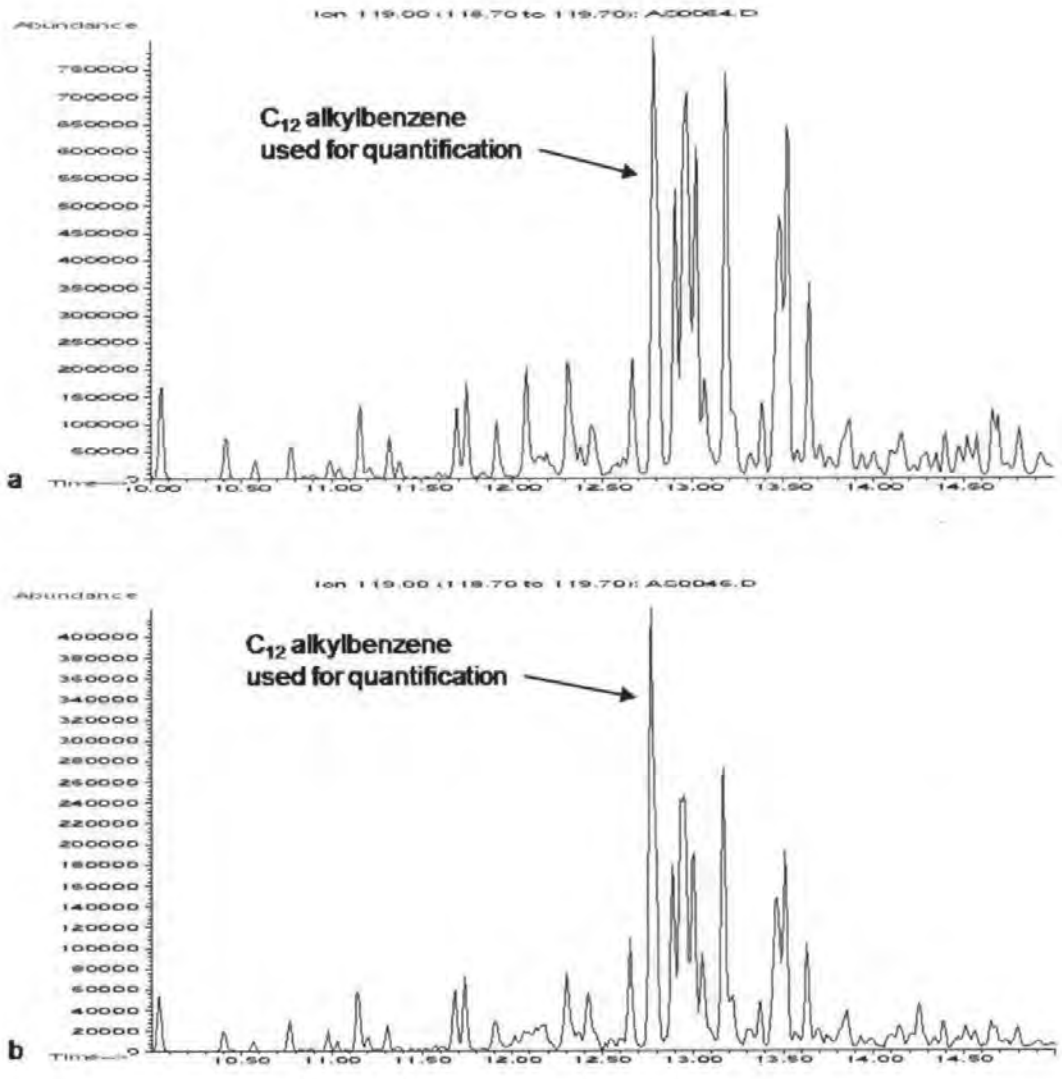


Figure 5.9 Chromatograms of common fragment ion *m/z* 119 of (a) branched alkylbenzenes mixture of compounds standard (0.1 mg mL^{-1}) and (b) extract of tissues from mussels exposed to 41 µg L^{-1}.

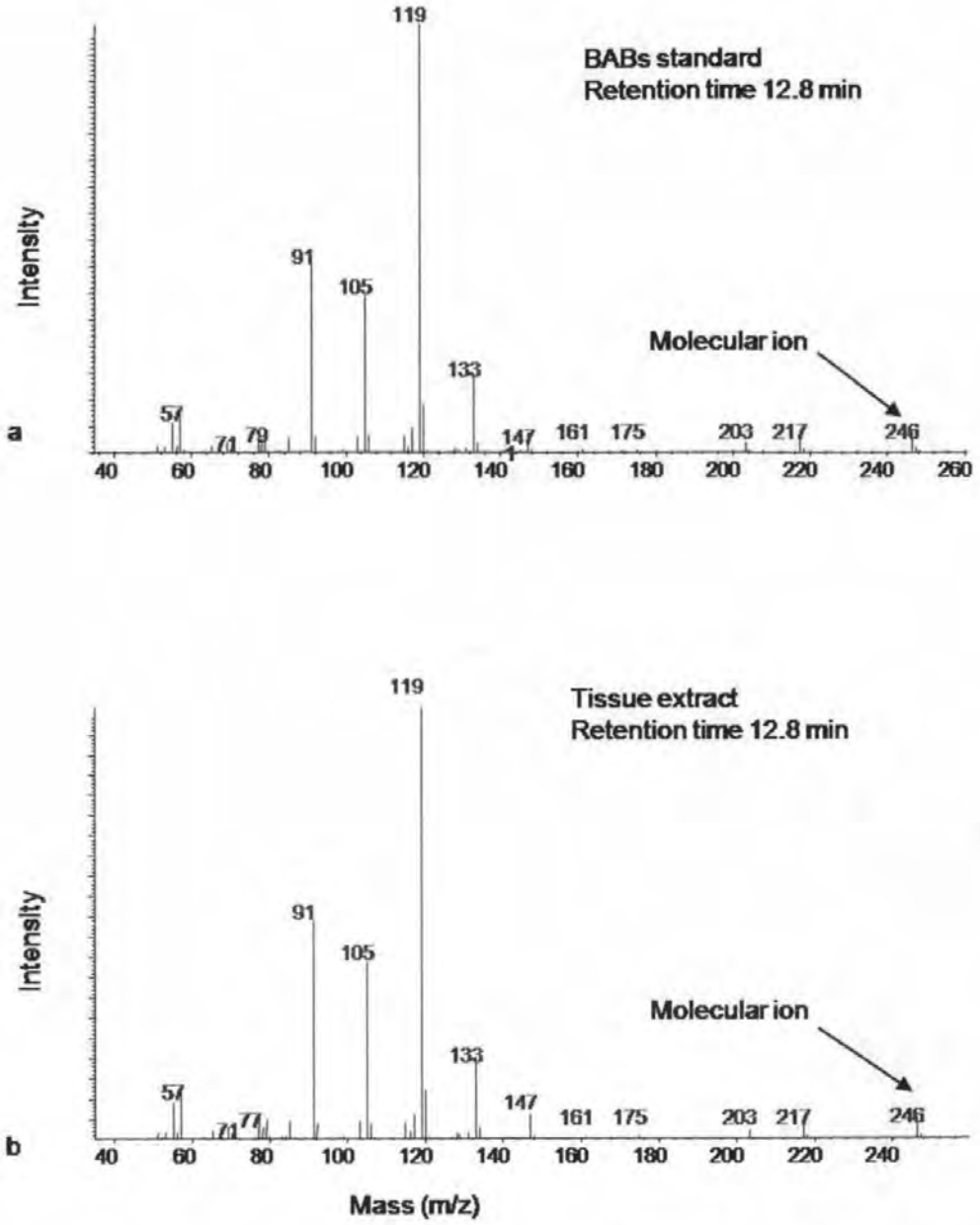


Figure 5.10 Mass spectra of peak at 12.8 min used for quantification of concentrations in mussel tissue extracts: (a) branched alkylbenzenes mixture of compounds standard (0.1 mg mL^{-1}) and, (b) extract of tissues from mussels exposed to 41 µg L^{-1}.

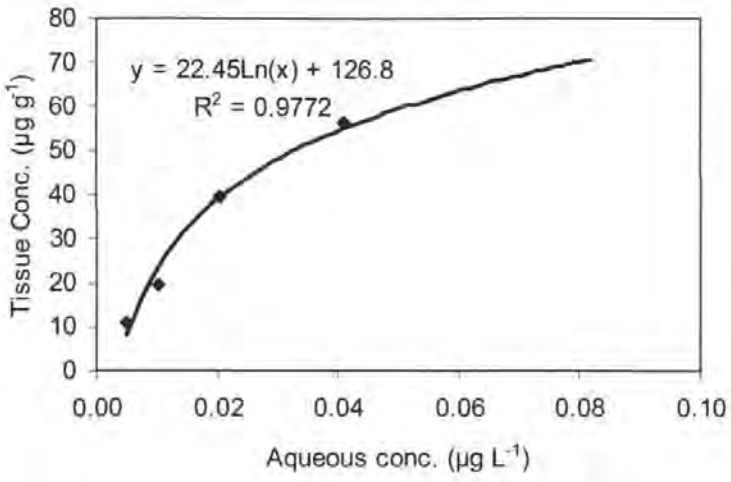


Figure 5.11 Relationship between mussel tissue concentrations (µg g^{-1}) and nominal aqueous concentrations (µg L^{-1}) of branched alkylbenzene mixture.

Tissue extracts from the 14 day exposure at 5 µg L^{-1} and the 5 day recovery period were analysed by GC-MS and quantified using the same C_{12} resolved compound as used for the analyses of acutely exposed mussels. This revealed that a mean tissue concentration of 46.7 µg g^{-1} dry weight (standard deviation (SD) = 0.8 µg g^{-1}) was accumulated after 14 days exposure compared to 11.1 µg g^{-1} dry weight following 3 days exposure. Following the 5 days recovery period, tissue concentrations had dropped to a mean of 20.7 µg g^{-1} dry weight (SD = 6.1 µg g^{-1}).

Characterisation by GC×GC-ToF-MS of BABs accumulated and depurated by M. edulis

The number of peaks identified as alkylbenzenes by GC×GC-ToF-MS in the tissue extracts of mussels exposed for 72 h to 41 µg L^{-1} was 111 (Fig. 5.12). This was reduced to 28 peaks for the exposure to BABs for 72 h 5 µg L^{-1} (acute exposure). As the exposure concentration reduced, peaks due to BABs were reduced in number from across both the 1st dimension (non-polar) and 2nd dimension (polar) GC retention time range but mostly at the earlier elution times of the 1st dimension. The peak marker distributions of the extracts from the 14-day 5 µg L^{-1} exposed mussels (136 – 166 peaks) were virtually identical to

those derived from the acute 41 μg L^{-1} exposed mussels (Fig. 5.12). The distributions of the peaks due to BABs from the depurated mussels (118 – 123 peaks) were also similar to those in the 14-day exposed mussels, with some peaks lost across the 1st dimension range including the later eluting peaks (Fig. 5.12). This small difference is consistent with the only partial recovery in clearance rates. A small number (9) of alkylbenzenes was detected in the control mussels exposed to solvent and seawater only. The mass spectra were consistent with those of 'linear' alkylbenzenes and only very small concentrations were present.

Quantitation of BABs by GC×GC-ToF-MS

The primary reason for analysing the tissue extracts by GC×GC-ToF-MS was to provide a clearer understanding of which compounds were accumulated and depurated by the mussels, an additional aim was to see if it was possible to use this technique for quantitation purposes. To this end, a number of peaks eluting over a range of volatilities and polarities from the BABs standards were examined. The majority of the peaks possessed very small areas and were highly variable between replicate analyses and were therefore unacceptable for quantitation purposes. Several major peaks were selected that gave reasonable reproducibility and linearity, and using these, estimates of tissue concentrations calculated.

Tissue concentrations of acutely exposed mussels obtained by GC×GC-ToF-MS were very similar to those obtained by GC-MS i.e. mussels exposed to 41 μg L^{-1} were estimated to have mean tissue burdens of 66 μg g^{-1} (SD = 4.6 μg g^{-1}) compared to 57 μg g^{-1} by GC-MS. Mussels chronically exposed to 5 μg L^{-1} were estimated to have tissue concentrations of 29 μg g^{-1} (SD = 7 μg g^{-1}) and 14 μg g^{-1} (SD = 3 μg g^{-1}) compared to 47 μg g^{-1} and 21 μg g^{-1} by GC-MS for the mussels sampled at the end of exposure and following depuration

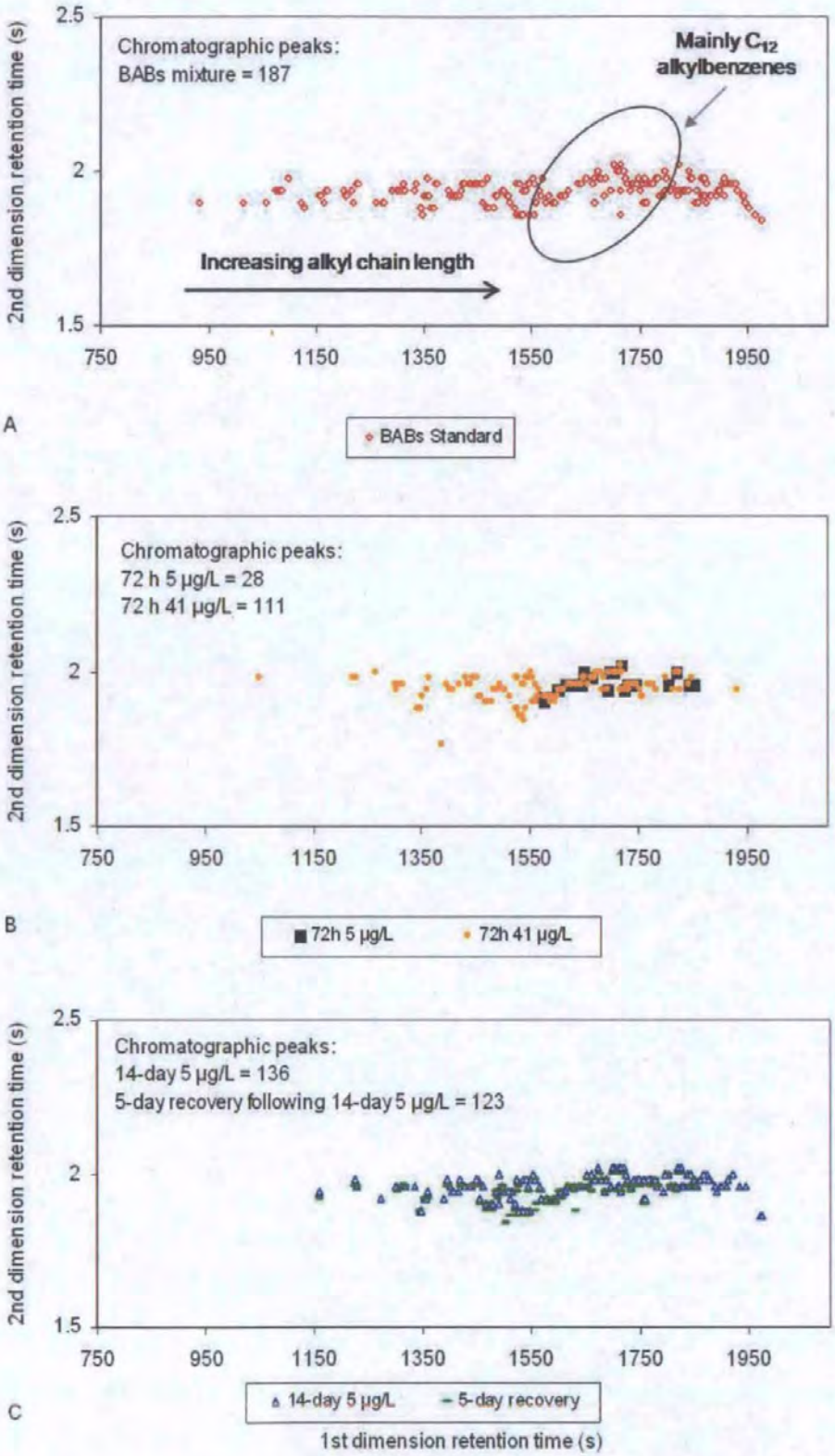


Figure 5.12 Chromatographic peak marker plots derived by comprehensive two-dimensional gas chromatography – time-of-flight–mass-spectrometry (GC×GC-ToF-MS) analysis of a complex mixture of C_{12-14} branched alkylbenzenes (BABs, A) and those accumulated in mussel tissues following 72 h exposure (B), and 14 days exposure plus 5 day depuration (C).

respectively. A small number (9) of alkylbenzenes was detected in the control mussels exposed to solvent and seawater only. The mass spectra were consistent with those of 'linear' alkylbenzenes and only very small concentrations were present. Mussels exposed to seawater or solvent had tissue concentrations of alkylbenzenes less than 1 $\mu g\ g^{-1}$ by either analysis method. These small amounts of LABs are probably due to traces of detergent residues from glassware washing (*c.f.* Eganhouse *et al.*, 1988).

Acute toxicity: Mussel clearance rates

Water quality measurements remained within acceptability limits throughout the tests: dissolved oxygen > 70 % saturation; pH 7.8 ± 0.2; temperature 15 °C ± 1 °C and salinity 34 psu ± 1 psu. No significant differences ($P > 0.05$) were found between the mussels exposed to seawater and acetone controls. The mussels exposed to the BABs mixture over the nominal aqueous concentration range of 5 - 41 $\mu g\ L^{-1}$ showed significant ($P \leq 0.05$) reductions in their clearance rates at all exposure concentrations compared to seawater and acetone control organisms. Clearance rates were reduced between 13 - 38 % and an EC_{50} of 131 $\mu g\ L^{-1}$ (95 % confidence limits of 74 – 299 $\mu g\ L^{-1}$) was extrapolated from the natural logarithmic relationship (Fig. 5.13) between reduction in clearance rate and aqueous concentration (equation 5.5). A parameter that is perhaps more meaningful in terms of environmental concentrations, the aqueous EC_{20} was interpolated to be 7 $\mu g\ L^{-1}$.

$$\text{CR reduction (\%)} = 0.28 + 10.19\text{Ln. AqC} \qquad (r = 0.9919, P \leq 0.01)$$

Equation 5.5 Natural logarithmic relationship between reduction in mussel clearance rates (CR, %) and branched alkylbenzene mixture aqueous concentrations, AqC ($\mu g\ L^{-1}$).

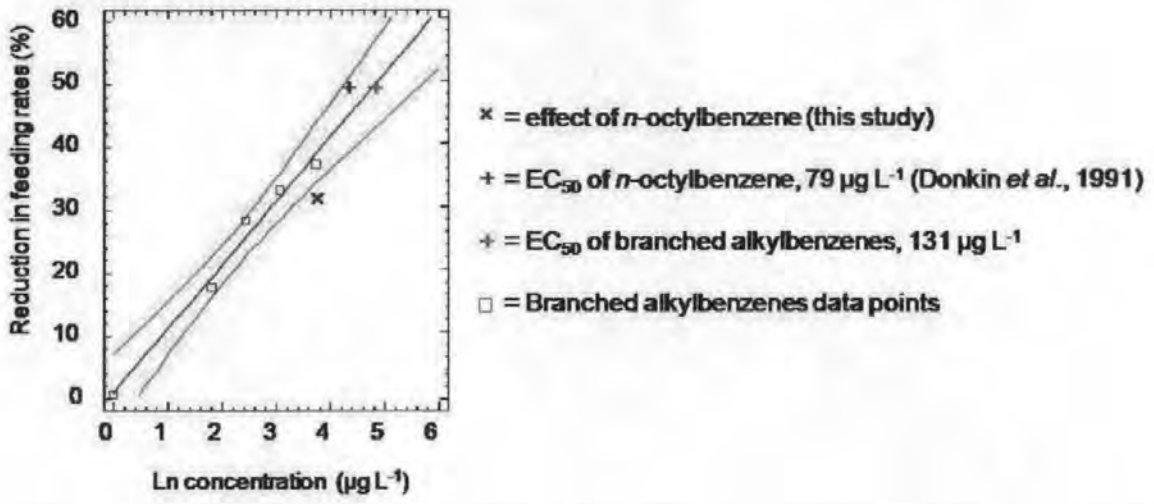


Figure 5.13 Concentration response relationship between branched alkylbenzenes (nominal aqueous concentration range 0 – 41 µg L^{-1}) and reduction in clearance rates (%) of mussels *M. edulis*. Curved lines = 95% confidence limits.

The relationship between reduction in mussel clearance rates and tissue concentrations of BABs (Fig. 5.14) was best described by equation 5.6 below. From equation 5.6 a tissue EC_{50} of 268 µg g^{-1} (dry weight, 95% confidence limits of 67 – 1064 µg g^{-1}) was extrapolated. The more environmentally meaningful tissue EC_{20} was determined to be 10.5 µg g^{-1} dry weight).

$$\text{CR reduction (\%)} = -1.22 + 9.03\text{Ln TC} \quad (r = 0.9865, P \leq 0.01)$$

Equation 5.6 Natural logarithmic relationship between reduction in mussel clearance rates (CR, %) and branched alkylbenzene mixture tissue concentrations, TC (µg g^{-1} dry weight).

Chronic toxicity and recovery: mussel clearance rates

Water quality measurements remained within acceptability limits throughout the tests although it was noted that a biofilm had developed on the surfaces of the glass in all exposure vessels with the exception of the seawater vessels where this was less prevalent. The clearance rates of the acetone exposed mussels were lower than the seawater exposed organisms but this was not found to be significant ($P > 0.05$). Over the course of the test,

the clearance rates of the seawater exposed mussels increased by about 30 %, the solvent exposed mussels by about 20 % and the BABs exposed organisms by about 7 %. Despite the improvement in clearance rates of the BABs exposed mussels, the reduction in mean rate relative to both that of the solvent and seawater controls approximately doubled to about 20 % and 30 % respectively (Fig 5.15). Following a five day depuration/recovery period in clean seawater, the clearance rates of the BABs exposed mussels improved but did not achieve that of either the solvent or seawater exposed mussels (Fig. 5.15): clearance rates were 13 and 21 % reduced relative to the solvent or seawater control mussels. From equation 5.6, the predicted reduction in clearance rates associated with tissue concentrations of 46.7 $\mu g\ g^{-1}$ and 20.7 $\mu g\ g^{-1}$ dry weight are 33 % and 26 % respectively which is in good agreement with measured rates.

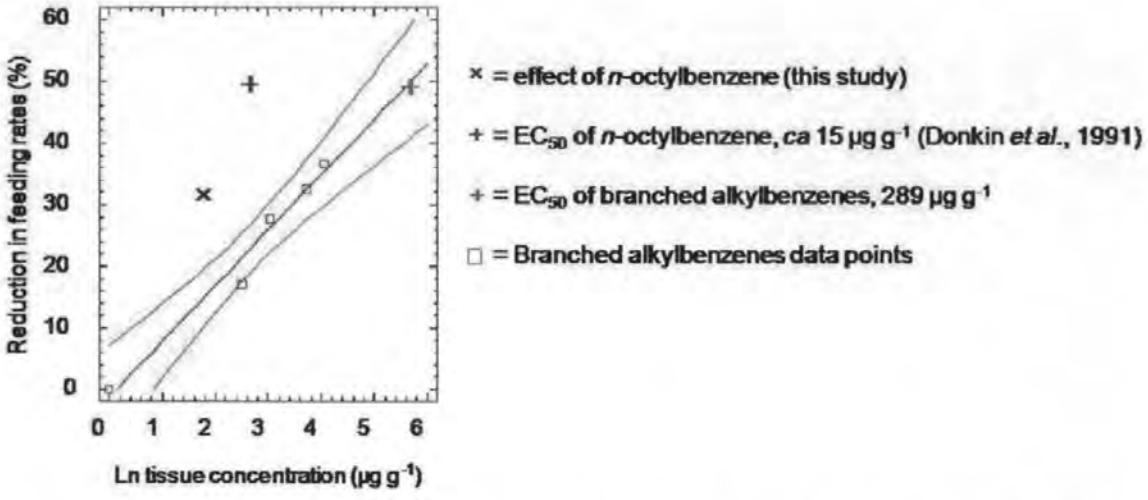


Figure 5.14 Concentration response relationship based on tissue concentrations between branched alkylbenzenes (nominal aqueous concentration range 0 – 41 $\mu g\ L^{-1}$) and reduction in clearance rates (%) of mussels *M. edulis*. Curved lines = 95 % confidence limits.

Chronic toxicity: cellular viability

No significant differences were found in haemolymph cellular viability. Neutral Red retention was very similar for both BABs-exposed and control organisms.

Acute toxicity: bioluminescence inhibition

No significant effect was observed between the BABs test solutions used in the mussel exposures and that of the acetone control solutions. A small hormesis effect was observed at a concentration of 41 μg L^{-1} but this was not significant ($P > 0.05$). Testing the solution at above its solubility limit at a concentration of 410 μg L^{-1} produced a 5 minute IC_{50} value of 423 μg L^{-1}. Confidence limits could not be calculated as too few of the dilutions produced an effect. The test was repeated and the IC_{50} value was within 10 % of the above value.

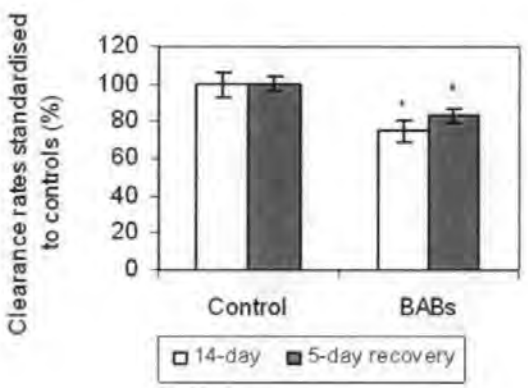


Figure 5.15 Effect of 14 days exposure to a complex mixture of C_{12-14} branched alkylbenzenes (BABs) of aqueous concentration 5 μg L^{-1} and recovery following 5 days depuration in clean seawater. Treatments significantly different from control ($P \leq 0.05$) are signified by *

5.4 Discussion

The sublethal toxicity of the reference toxicant 1-phenyloctane as determined by the mussel clearance rate assay was found to be similar to that reported previously (Donkin *et al.*, 1991). An aqueous EC_{50} of 79 μg L^{-1} was reported by Donkin *et al.* (1991) but this value must have been extrapolated as the authors had stated that the maximum exposure concentration was within the solubility limits of the test compounds which for 1-phenyloctane under test conditions is about 40 μg L^{-1} (66 μg L^{-1} at 25 °C, Wasik *et al.* (1981), cited by EPI Suite™ (2000). Therefore there are likely to be reasonably large confidence limits on the EC_{50} value although this was not reported by the authors. The clearance rates of mussels exposed to 42 μg L^{-1} 1-phenyloctane in this study were found to be 68 % (CV = 0.7 %) of control animals. Donkin *et al.* (1991) also reported an estimated tissue EC_{50} of 82 μg g^{-1} (wet wt.) but tissue concentrations of 1-phenyloctane in this study were only found to be in the range 2.9 and 6.5 μg g^{-1} (dry wt., *ca* 0.4 – 0.9 μg g^{-1} wet wt.). Mussel tissue samples were extracted using a steam distillation technique during the previous study which may provide superior extraction and/or fewer losses of alkylbenzenes than the alkaline saponification method (Kelly *et al.*, 2000) used in the present. Analysis was previously performed using GC only which may also account for discrepancies between the estimated tissue EC_{50} reported by Donkin *et al.* (1991) and the lower values found in this study.

Clearance rates of mussels exposed to the $C_{12\text{-}14}$ BABs mixture were significantly ($P \leq 0.05$) reduced at all concentrations tested. The clearance rate reduction of the mussels exposed to 10 – 41 μg L^{-1} BABs was very similar to the reduction observed for the 1-phenyloctane exposure concentration of 42 μg L^{-1} (Fig. 5.13). Linear alkyl alkylbenzenes with alkyl chains > C_8 were previously found to be above the toxicity cut-off level but the effect of branching on the alkyl chain probably brought the BABs compounds to below the

cut-off point (Silla *et al.*, 1992; Tolls *et al.*, 2002). Donkin *et al.* (1991) suggested that the toxicity cut-off was due to the reduced solubility of compounds corresponding to increasing molecular weight and therefore aqueous solubility may be a better indicator of the cut-off point than log K_{ow}. The calculated log K_{ow} values for 1-phenyloctane (C_8), 1-phenyldecane (C_{10}) and 1-phenyldodecane (C_{12}) are 6.3, 7.11 and 8.65 respectively (Sangster, 1993) and thus aqueous solubility rapidly declines with increasing alkyl chain length. Tolls *et al.* (2002) reported that although water solubility decreased with increasing atomic number, molecular size or surface area may provide a more accurate descriptor of solubility. Silla *et al.* (1992) also reported that, for a large number of hydrocarbons, solubility was mainly determined by molecular surface area, and that branching reduced the surface area thus increasing the solubility. Because the BABs mixture contained a large number of similar compounds it is possible that solubility was further increased in accordance with Raoult's law. Sherblom *et al.* (1992) reported that the water solubility of two LABs significantly increased when the compounds were present as a mixture with other LABs. In the environment, as part of the UCM, BABs are present in conjunction with numerous other compounds with a range of characteristics and thus, also, in accordance with Raoult's law, solubility would be expected to decrease (Page *et al.*, 2000). From the curves of the graphs relating aqueous with tissue concentrations (Fig. 5.11) and that of clearance rate reduction with tissue concentration (Fig. 5.14), it would appear that the solubility limit of the BABs occurred between 20 – 41 $\mu g\ L^{-1}$. In the marine environment, the bioavailability of contaminants may not necessarily be restricted by their solubility in seawater. Ahrens *et al.* (2001) reported that deposit-feeding polychaete worms were capable of desorbing hydrophobic sediment-bound organic compounds (HOCs) using a gut fluid surfactant. *Nereis succinea* Frey and Leuchart, 1847 gut fluid rapidly (minutes) desorbed a similar amount of HOCs as a 1 % solution (*ca.* 3.5 mM) of the synthetic surfactant sodium dodecyl sulfate (SDS), leading to high absorption

efficiencies (Ahrens *et al.*, 2001) and thus even very hydrophobic compounds with aqueous solubility less than that of the compounds within the C_{12-14} BABs mixture may become bioavailable and enter the food web.

Tissue concentrations determined by GC-MS of BABs within mussel tissues were in the range 11 – 56 $\mu g\ g^{-1}$ (dry weight) for nominal aqueous exposure concentrations between 5 – 41 $\mu g\ L^{-1}$ (Fig. 5.11) and were thus an order of magnitude higher than for 1-phenyloctane tissue concentrations, indicative of increased bioavailability of the branched compounds. As toxicity was only found to be slightly higher for the BABs exposed mussels, it is possible that not all of the components within the BABs mixture were bioaccumulated equally or possess equivalent and/or additive toxicity. The ratios of the six most prominent hydrocarbons present within the mussel tissues were similar to those within the BABs standard (Table 5.1) which implies the bioavailability of the numerous BABs components were similar. Comparing the profile of the GC×GC-ToF-MS peak marker plots of the BABs compounds accumulated within the tissues of mussels exposed to the BABs mixture with that of the standard mixture, showed that compounds from across the range of volatility were accumulated (Fig. 5.12); this was in agreement with the profile obtained by GC-MS. In addition, compounds with differing polarities were accumulated by the mussels. An exception to this was a small group of compounds eluting at the extreme end of the volatility range (>1900 s) and relatively early in the polarity range (1.82 s) which were not identified within the mussel extracts. However, these were also not observed within the lower concentration BABs standards so it is probably that they were present at concentrations that were too low to be detectable within the mussel tissue. As whole tissues were extracted it was not possible to detect if any of the BABs components were differentially compartmentalised. Wraige (1997) found that gill tissue concentrations correlate better with reduction of clearance rate than total body burdens. The gills are

presumed the primary site of toxic action and have a limited capacity for lipophilic compounds; hence additional hydrocarbons may be accumulated elsewhere in the tissue without effect upon the mussel clearance rate.

For compounds with a common site/mode of action the concept of concentration addition (CA) is normally applicable and this should therefore apply to the alkylbenzenes. Concentration addition is expressed mathematically as:

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1$$

where n is the number of mixture components, ECx_i is the concentration of the ith mixture component that provides x% effect when applied singly and c_i is the concentration of the respective component in the mixture. Each fraction (c_i/ECx_i) represents the concentration of a mixture component scaled for its relative toxicity, normally termed the toxic unit (TU) of that component. Hence each compound in the mixture can be replaced by another without changing the overall toxicity as long as the sum of toxic units remains unaltered. If, at a total concentration of the mixture provoking x% effect, the sum of the toxic units equals one, CA holds. Bioassays using a range of species have shown that CA provides an effective predictor of the toxicity of mixtures of non-specifically acting substances, mainly organic chemicals with narcotic properties (Hermens *et al.*, 1985a; Hermens *et al.*, 1984a; Hermens *et al.*, 1984b; Konemann, 1980). It would be necessary to carry out toxicity tests on individual BAB components to test the validity of the CA model but taking into account the 95% confidence intervals of the extrapolated EC_{50} values in this study and the unknown confidence intervals of the values reported by Donkin *et al.* (1991), the observed differences in toxicity are insufficient to suggest the need for an alternative model such as 'independent action' to describe the toxic behaviour of BABs.

No specific mechanism of toxicity is known for alkylbenzenes and it is assumed that narcosis is responsible for the reduction in clearance rates due to accumulation of hydrophobic organic chemicals within lipid membranes. Lethality has been reported to occur at a critical membrane concentration of *ca* 40 – 160 mmol kg^{-1} lipid which is equivalent to 2 – 8 mmol kg^{-1} tissue wet weight in a range of aquatic species (McCarty & Mackay, 1993; van Wezel & Opperhuizen, 1995). The tissue burden of the mussels exposed to BABs was approximately 0.03 mmol kg^{-1} tissue wet weight (based on molecular weight of C_{12} alkylbenzenes) and therefore well below the critical lethal membrane concentration. There are few reports of body burdens of toxicants linked to sublethal endpoints. Donkin *et al.* (1991; 1989) reported that tissue burdens of mussels exposed to a range of aliphatic and aromatic hydrocarbons were in the range 0.1 – 0.5 mmol kg^{-1} associated with reductions in clearance rates of 50 %. It was also reported that a toxicity cut-off occurred with compounds with a log K_{ow} >5 despite efficient accumulation of compounds and hence it is possible that some of the BABs compounds that were accumulated by the mussels did not cause a toxic effect.

The mussels exposed to a low concentration (5 μg L^{-1}) over an extended period of 14 days were found to continue to accumulate BABs compounds such that their tissue concentrations were close to that observed within mussels exposed to 41 μg L^{-1} for three days. The calculated BCF based on the 14 day exposure was 3.97 compared to 3.33 for the acute exposure. This suggests that wild mussels will accumulate BABs from seawater contaminated with very low concentrations with consequential reduction in health status. Indeed, no alkylbenzenes were detected by GC-MS within extracts of the exposure media with a nominal concentration of 5 μg L^{-1} and low aqueous concentrations are unlikely to be detected during normal measurement of seawater contaminants. The failure to completely depurate the BABs within five days and the incomplete recovery in clearance rates implies

that wild mussels may not be able to recover from exposure even if they later experience periods of uncontaminated seawater.

The accumulation of the BABs did not result in loss of cell viability. Martins *et al.* (2005) suggested that reduction in membrane lysosomal stability (essentially a similar measure as cellular viability but with reduction in Neutral Red retention time as an endpoint) of bivalves from a petroleum hydrocarbon polluted bay in Brazil could, in part, be explained by the concentration of aromatic UCM hydrocarbons. The results of this study do not suggest that toxic effects arising from accumulation of alkylbenzenes contribute to the loss of haemolymph lysosomal stability. Hokstad *et al.* (2007) reported that the C_{12-14} BABs mixture failed to cause cytotoxicity to rainbow trout hepatocytes when assessed as metabolic inhibition and loss of membrane integrity, whereas tetralins, aromatic and cyclic naphthenic acids plus fractions isolated from a WSF of UCM-dominated oil, all resulted in damage to the hepatocytes.

No examples of highly branched alkylbenzenes exist within the NIST library and therefore providing definitive identification of BABs within environmental samples is problematical. However, Frysinger *et al.* (2003) identified a series of alkylbenzenes with $C_8 - C_{14}$ alkyl chains in a monoaromatic UCM using GC×GC analysis and the authors noted that a large number of isomers were apparent. Booth (2006; 2007) using GC×GC-ToF-MS to analyse UCMs extracted from the tissues of mussels *M. edulis* found large numbers of compounds with mass spectra and GC×GC properties consistent with those of highly branched alkylbenzenes. Furthermore, the alkylbenzenes represented the largest class of compounds within the aromatic UCM of mussels with low S*f*G (Widdows *et al.*, 1995). The total concentration of UCM hydrocarbons within tissues of mussels with low S*f*G from Southend (Fig. 5.1 and 5.2) was ~150 $\mu g\ g^{-1}$ (dry weight), of which ~100 $\mu g\ g^{-1}$ was

aromatic with an alkylbenzene component of ~45 $\mu g\ g^{-1}$, similar to that found accumulated in the tissues of the BABs-exposed mussels reported herein. The wild mussels from Southend contained a broader range of alkylbenzenes, including higher molecular weight compounds, than found within the commercial mixture used to conduct the toxicity tests reported herein. It is evident that a large number of alkylbenzenes are present in the environment and are bioavailable to organisms. Widdows *et al.* (1995) were unable to explain the sublethal effects of the mussels from Southend in terms of 'toxic concentrations' based on 2 and 3 ring aromatics but the presence of alkylbenzenes within the mussels' tissues at concentrations found to significantly reduce the clearance rate of mussels reported herein, may better explain the poor health status of the animals. Although direct comparison between the compounds found within wild mussels and those within the laboratory-exposed mussels is difficult due to differences in chromatography, Booth *et al.* (2007) reported that the C_{12-14} BABs eluted at similar retention times as the BABs within the wild mussels' tissue and possessed similar mass spectra. Similarly, mass spectra obtained from compounds, resolved by GC×GC-ToF-MS, within the BABs exposed mussels closely matched the mass spectra of the C_{12-14} BABs (Fig. 5.16). Hellou *et al.* (1994) reported alkylbenzenes in the tissues of winter flounder *Pseudopleuronectes americanus* Walbaum,1792 exposed to Hibernia crude oil and these included compounds with molecular weights of 232 Daltons, 246 Daltons and 260 Daltons with fragment ions of *m/z* 91 and *m/z* 105, i.e. similar to some of the compounds present within the BABs mixture. It would appear therefore that BABs are present within crude oil (see **Chapter 4**) and are sufficiently soluble in the environment to be accumulated within the tissues of biota and implicated with sublethal toxic effects. The present study has confirmed that BABs are indeed bioaccumulated, resulting in depressed clearance rates of mussels.

There are few reports of toxicity of alkylbenzenes using the bioluminescence inhibition test, Microtox. Indeed, a literature search of the keywords 'toxicity' and 'alkylbenzene' using the 'Web of Science' database (The Thomson Corporation, http://portal.isiknowledge.com/portal.cgi, accessed on 02.04.07) found that of the first 100 papers, 99 were concerned with LABs or LASs. A review of Microtox toxicity data by Kaiser and Palabrica (1991) included reports of IC_{50} values for toluene in the range 20000-50000 $\mu g\ L^{-1}$ which suggests that this assay is considerably less sensitive than the mussel clearance rate assay for which an EC_{50} value of 2347 $\mu g\ L^{-1}$ was reported for toluene by Donkin *et al.* (1989). The 'comparison test for marine and estuarine samples' revealed no significant differences between any of the alkylbenzene solutions used in the mussel exposure test and the corresponding acetone controls. From the 'basic test' using concentrations above the solubility limits, it appeared that no effect would be apparent below ~100 $\mu g\ L^{-1}$ either for 1-phenyloctane or BABs. Although the results of the basic tests should be treated with extreme caution due to the extrapolation from only two usable data points, the results do suggest that the BABs mixture maybe a little more toxic than 1-phenyloctane, which is in agreement with the mussel clearance rate assay.

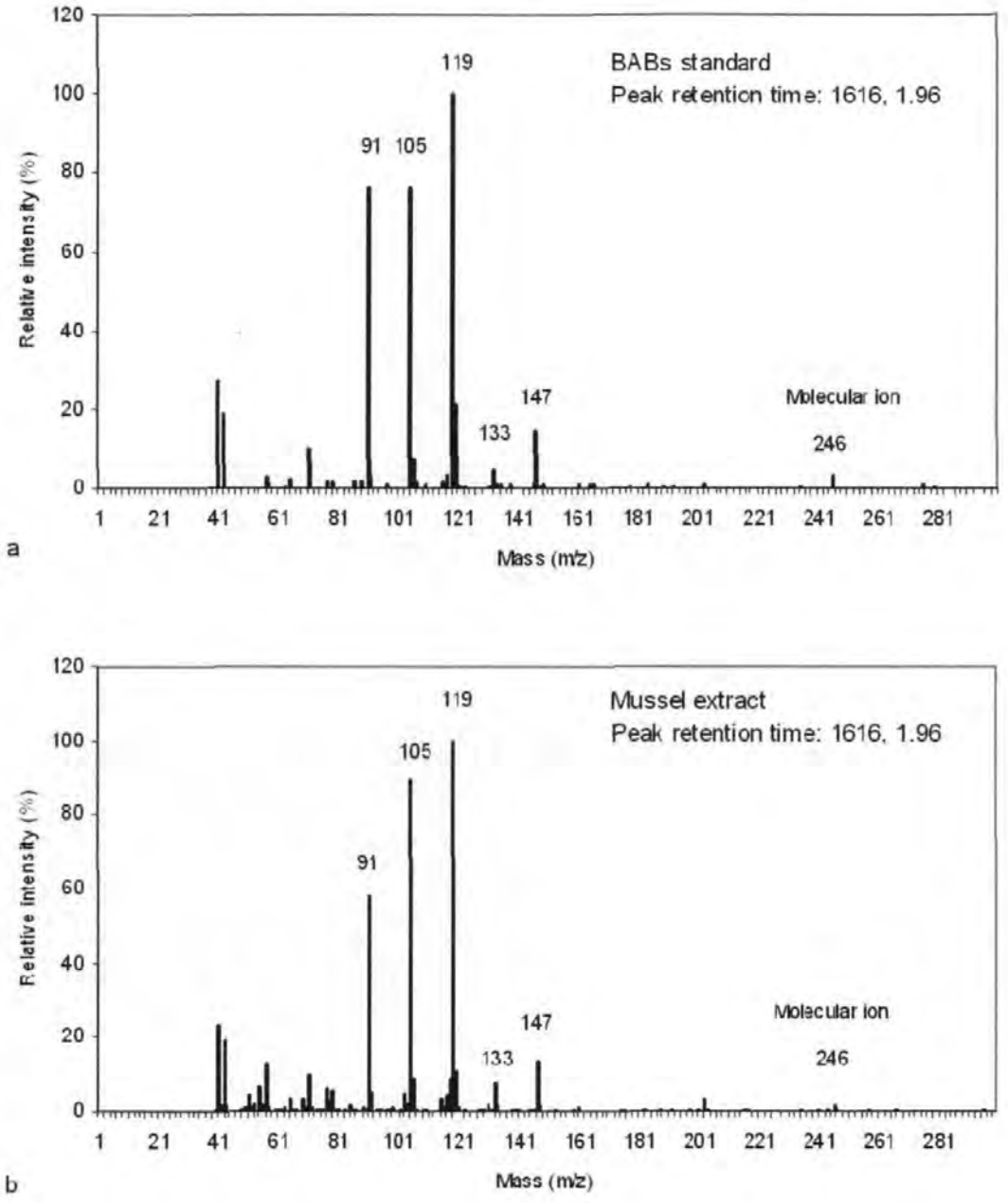


Figure 5.16 Comparison of mass spectra of a selected peak resolved by GC×GC-ToF-MS analysis of (a) the commercial BABs mixture and (b) from extract of tissue from mussels exposed to 5 μg L^{-1} BABs for 14 days.

5.5 Conclusions

Branched alkylbenzenes have been shown to be major constituents of the UCM accumulated in the tissues of mussels with poor health status, as measured by S*f*G (Booth *et al.*, 2007). Sublethal toxicity studies using a commercial mixture of BABs have now shown that these compounds can reduce the clearance rates (a crucial determinant of S*f*G) of mussels and that this is governed by the concentration of BABs accumulated within the tissues. Long-term exposure to low aqueous concentrations of BABs therefore results in accumulation of the contaminants within the mussels' tissue until sufficient concentration exists for deleterious effects upon the health of the organisms. Furthermore, depuration of the BABs following bioaccumulation is relatively slow (*ca.* 50 % in five days) and therefore mussels may not be able to depurate the contaminants during periods when not exposed. Analyses of mussel tissue extracts by GC×GC-ToF-MS show that the compounds accumulated during exposure to the commercial mixture BABs have similar retention times and mass spectra to many of the BABs accumulated in the wild population. Consequently, the results of this study are highly consistent with BABs as a contributor to the poor health status of mussels that possess high tissue concentrations of UCM hydrocarbons with a significant alkylbenzene component. As not all UCMs found in mussels with poor health status possess significant quantities of BABs, this group of compounds cannot be solely responsible for all the observed toxic effects.

Chapter 6

Trophic transfer of branched alkylbenzenes: physiological and behavioural implications for the shore crab *Carcinus maenas*

Having established the toxicity of a branched alkylbenzene fraction (BABs) of some UCMs, the potential for these compounds to transfer from contaminated mussels to a common predator species, the shore crab *C. maenas*, was assessed. The cellular, physiological and behavioural effects upon the crabs were measured and tissue concentrations quantified by GC-MS. The possibility of metabolism and excretion of the BABs was explored using UVF analysis of crab urine.

6.1 Introduction

Mussels are sessile organisms that are unable to escape from contaminated waters and they therefore accumulate hydrophobic compounds, such as hydrocarbon contaminants, from the water column into their tissues. For this reason mussels have been used as a bioindicator species for pollution (vanGestel & vanBrummelen, 1996). However, the effect of such accumulations in mussels on the wider community, including predatory organisms such as crabs, is open to question. The shore crab *C. maenas* is a mobile estuarine species that moves up and down estuaries with both diurnal and seasonal cycles (Naylor, 1962; Vernberg & Vernberg, 1983) and may only be subject to contaminated waters for short periods. However, *C. maenas* predates upon bivalve molluscs (Vernberg & Vernberg, 1983) and may therefore be exposed to hydrocarbon contaminants through feeding on contaminated mussels. Booth *et al.* (2006; 2007) reported that some wild N. Sea *M. edulis* populations with high UCM hydrocarbon tissue burdens and poor health status contained substantial concentrations of BABs. In **Chapter 4** it was demonstrated that mussels rapidly accumulated BABs from UCM-dominated oils but were slow to depurate these compounds. In **Chapter 5** it was shown that aqueous exposure to a complex mixture $C_{12\text{-}14}$ BABs resulted in accumulation of the full range of these compounds leading to a reduction in mussel clearance rates. Consumption of BABs-contaminated mussels may result in the transfer of the BABs to other species including *C. maenas* which may subsequently affect their health.

Bioaccumulation *via* trophic transfer is thought to be of limited importance for PAHs and biomagnification does not appear to occur due to the ability of higher organisms to metabolise and excrete the compounds (Bierman, 1990; NRC, 2003; Wan *et al.*, 2007). Wan *et al.* (2007) analysed a range of PAHs present within the tissues of species from a number of trophic levels. The results indicated that PAHs undergo trophic dilution in the

marine food web; the authors suggested that this was likely due to low assimilation efficiencies and efficient metabolic transformation at higher trophic levels. In contrast, the results of a pilot study by Rice *et al.* (2000) suggested that uptake of hydrocarbons *via* the food web may be important. In this study, polychaete worms were exposed for 28 days to sediments spiked with benzo(a)pyrene (BaP) and to field sediments contaminated predominantly with PAHs and chlorinated compounds. Exposed worms were then fed live to predatory juvenile flatfish, English sole (*P. vetulus)* for 10 or 12 days. The fish showed reduced growth and increased expression of CYP1A. Fish exposed to BaP-exposed worms also showed clear evidence of hepatic PAH-DNA adducts. Highly lipophilic compounds (e.g. organochlorines) with log $K_{ow} > 6.3$ were found by Russell *et al.* (1999) to strongly biomagnify and compounds with $K_{ow} > 5.5 < 6.3$ moderately so. The calculated log K_{ow} values for C_{10} and C_{12} LABs are reported to be 7.11 and 8.65 respectively (Sangster, 1993). No log K_{ow} values are available for the $C_{12\text{-}14}$ BABs but an estimated value based on BCF (**Chapter 5**) suggested a much lower value of 3.3. The highly branched structures of compounds within the BABs mixture may be less readily metabolised and excreted than the PAHs and hence may transfer to higher trophic levels. There is a paucity of literature concerning trophic transfer of hydrocarbons, and limited reports of UCMs within biota. The capacity for UCM hydrocarbons to transfer to higher trophic levels, and possibly biomagnify through the food web, cannot therefore be assessed without experimentation.

Crabs are known to biotransform PAHs into more hydrophilic compounds prior to excretion (Fillmann *et al.*, 2004; Sole & Livingstone, 2005; Watson *et al.*, 2004b) but their ability to transform and excrete highly branched alkylated monoaromatic compounds is unknown. Research by Dam *et al.* (2006) into the biotransformation of the PAH pyrene by cytochrome P450 enzymes (CYP) within red and green *C. maenas* revealed a reduced capacity of the hepatopancreas (aka midgut gland) within the red crabs to convert pyrene

into its initial phase I metabolite, 1-hydroxypyrene. Although it is possible that the transformation was carried out elsewhere within the tissues and then transported to the midgut gland, this is not supported by research using radiolabelled compounds (James *et al.*, 1989 cited by Dam *et al.*, 2006). The crabs were exposed to the pyrene by direct injection into the haemolymph; this may be more comparable to the food ingestion route of uptake compared to exposure to contaminated water which is likely to result in greater accumulation within the gills. The midgut gland serves as the principal storage organ of lipids (Mantel, 1983) and therefore would be expected to accumulate ingested lipophilic compounds such as BABs. The midgut gland also functions as the primary site for possible biotransformation and excretion (Mantel, 1983). Analysis by UVF of crab urine has been used to detect PAHs (Dissanayake & Galloway, 2004; Galloway *et al.*, 2004b; Watson *et al.*, 2004a; Watson *et al.*, 2004b) and therefore adaptation of this analytical method may provide a useful means for rapid screening of BABs although the presence of metabolites from such a complex mixture of compounds may prove problematical.

From the studies reported in **Chapter 5**, the mechanism of toxicity of the BABs compounds is consistent with non-specific narcosis. If ingestion of BABs-contaminated mussels were to have adverse effects upon the health of the crabs, it is therefore possible that this would be manifested as behavioural abnormalities such as their ability to detect and locate food. Culbertson *et al.* (2007) reported that both the behaviour and feeding rate of fiddler crabs *U. pugnax,* resident in marsh sediments contaminated with oil from the Florida barge spill in 1969, were affected by exposure to the contaminated sediment. Although the biological effects were reported in terms of TPH sediment concentrations, the vast majority of this was UCM hydrocarbons when analysed by conventional GC (Reddy *et al.*, 2002). The altered behaviour, which makes the crabs more vulnerable to predation, together with the lowered physiological condition, may have been responsible for their

lower densities of crabs found within the UCM hydrocarbon contaminated site (Culbertson *et al.*, 2007).

Accumulation of the BABs by *M. edulis* resulted in physiological impairment, so this may also be detectable within the crabs. The monitoring of heart rates has previously been used to measure physiological stress within *Carcinus* (Brown *et al.*, 2004; Camus *et al.*, 2004; Fossi *et al.*, 2000) and the use of the CAPMON system, which was originally described in detail by Depledge and Anderson (1990), provides a means by which physiological status can be measured simultaneously with behavioural monitoring (Spooner *et al.*, 2007). No significant effect on cellular viability was apparent within BABs-exposed mussels but PAHs have been shown to effect cellular viability in *C. maenas* (Dissanayake *et al.*, 2006; Dissanayake *et al.*, 2007) and therefore this is worthy of investigation.

Aims

The aims of this study were:

1. To determine if BABs could be transferred from mussel tissue to crab tissue *via* ingestion of contaminated mussels.
2. To determine if BABs or metabolites thereof could be detected within crab urine using UVF analysis.
3. To assess the effect of ingestion of BABs-contaminated mussels in terms of cellular, physiological and behaviour endpoints.

6.2 Methods

6.2.1 Overview

Common shore crabs, *C. maenas*, were fed with mussels contaminated by BABs to test for transfer of the contaminants from one trophic level to another. Due to logistical constraints it was not possible to conduct the experiment with both solvent-exposed and seawater – exposed control mussels. Previous exposures (**Chapter 5**) had revealed no differences, in terms of effect on clearance rates, between solvent- and seawater-exposed mussels; the trophic transfer tests were therefore performed using solvent-exposed mussels only. Following a seven day laboratory exposure, during which male crabs were fed two mussels per day, the health of the crabs was assessed in terms of cellular biomarkers, physiology, behaviour, and midgut gland tissue BABs concentration. Observation and quantitation of behavioural response was aided by digital video recordings. Crab urine was also analysed for the presence of BABs or their metabolites. Full details of the methodology are described within **Chapter 2**.

6.2.2 Semi-static aqueous exposure of *Mytilus edulis* to C_{12-14} BABs

Water spiking and mussel exposure conditions were as described previously (Section 2.7) except that 10 mussels were exposed in 10 L of test water. Mean length was 40.6 mm (SE = 0.13mm, CV = 5.0 %, n = 240). The mussels were exposed sequentially to provide two contaminated mussels per crab per day (i.e. one mussel from each of two exposure vessels). The remaining mussel from each exposure vessel was retained and stored frozen at -80 °C. Based on previous experiments (see **Chapter 5**), the crabs were exposed to ~15 µg BABs per day, i.e. a total maximum exposure of about 100 µg from their diet of contaminated mussels.

6.2.3 Exposure of *Carcinus maenas* to BABs-contaminated mussels

Crabs (n = 9 per treatment, mean carapace width = 65.7 mm (S.E = 0.87, CV = 5.6% ; mean weight = 73.4g, SE = 2.7 g, CV = 15.8 %) were each fed two exposed mussels per day for seven days with seawater exchanged every 48 h. Following exposure to the mussels, the crabs were not fed for two days prior to behaviour and physiological tests. After testing, the crabs were weighed and measured, then stored frozen at -80 °C prior to chemical extraction and analysis of tissues.

6.2.4 Behavioural and physiological responses

The tests were carried out in specifically designed glass aquaria. Crabs were fitted with heart rate transmitter/detectors and allowed to acclimate within the aquaria for 10 min prior to commencement of the tests. Effects on behaviour were assessed by measurement of the time taken to achieve specified actions associated with feeding upon a cockle. Simultaneous measurements of heart rate using the CAPMON system were made during the behavioural tests. Time points for behaviour and heart rate were:

1. Time taken to engage and break into the cockle; and,
2. Time taken to eat the cockle.

6.2.5 Cellular biomarkers of exposure and stress

Following the behaviour and physiological tests, the crabs were placed in clean seawater overnight and their behaviour monitored. The following day, three days after the crabs were last fed contaminated mussels, urine and haemolymph were removed from the organisms as described by Watson *et al.* (2004b). Haemolymph samples and crabs were stored at −80 °C until analysis. The crabs were subsequently thawed and dissected to remove the whole of the midgut gland (hepatopancreas). The tissues were weighed and stored frozen at −80 °C until further analysis. Fluorescence from crab urine dissolved in

ethanol (1:20) was used as a biomarker of exposure to BABs. Fluorescence was measured against a standard curve of the C_{12-14} BABs mixture dissolved in ethanol (0 – 5 mg mL^{-1}). Excitation λ was 273 nm with emission λ of 290 – 305 nm. Biomarkers of stress used were Neutral Red retention (haemolymph cellular viability) and phagocytosis inhibition (impairment to immune response). Both assays were described by Pipe *et al.* (1999), adapted for *C. maenas* by Brown *et al.* (2004), and are detailed within **Chapter 2**.

6.2.6 Chemical extraction and analysis of midgut gland tissue

The combined tissues from eight crabs from each treatment were split to give two replicates per treatment and placed in preweighed amber glass jars and reweighed to establish tissue wet weight (~14 g per replicate). An internal standard (phenanthrene d_{10}) was added to the tissues which were then extracted by alkaline saponification (see section 2.3). Following clean up, the extracts were analysed by GC-MS. The tissue concentrations were quantified by reference to two relatively resolved peaks. Full details of the methodology are provided within **Chapter 2**.

6.3 Results

6.3.1 General observations

During the first two days exposure to the BABs-contaminated mussels, the crabs appeared well, behaved normally and ate all the food presented to them. On day three, it was noted that some of the BABs-exposed crabs appeared slow to respond to stimuli and three crabs failed to open the mussels presented but consumed the food after the mussel shell was opened fully for them. As the exposure progressed, some BABs-exposed crabs continued to behave abnormally: mussels were not opened and crabs tended to be much less aggressive compared to control organisms. During water exchanges, control crabs typically attacked the siphon tube (some retreated) whereas BABs-exposed crabs typically were immobile. By day six of the exposure, six out of the nine BABs-exposed crabs failed to open one or both of the mussels presented, whereas all the control crabs continued to rapidly break open and consume the mussels presented. One BABs-exposed crab appeared to be dead but close examination revealed some slight movement of its antennae. However, 24 h later at the end of the 7-day exposure this crab was found to be dead and was removed from the vessel and frozen at -80 °C. On day seven of the exposure, the mussels were placed in the exposure vessels as far from the crabs as possible and their time to respond to, and open, one of the mussels was recorded to the nearest five seconds. Not all the crabs were located at one end of the vessel, therefore only six crabs from each treatment were in similar positions relative to the mussels. The median response times (Fig. 6.1) for the BABs-exposed crabs was 40.0 s (interquartile range = 36.25 – 43.75 s), significantly greater ($P \leq 0.01$) than control crabs with median response time of only 12.5 s (interquartile range = 10 – 15 s). Of the eight surviving BABs-exposed crabs, three failed to open and consume their mussels.

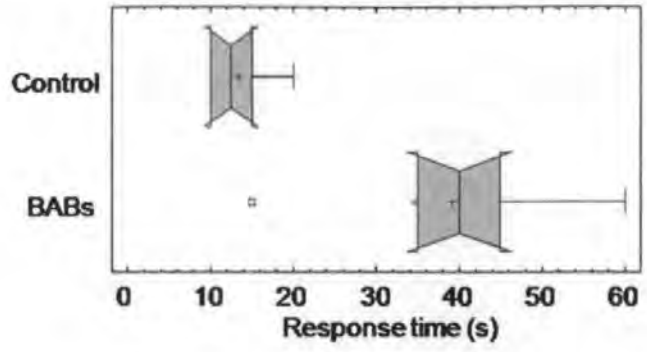


Figure 6.1 Response times of crabs *C. maenas* fed on a diet of mussels contaminated with BABs for 7 days compared to crabs fed on a diet of carrier solvent (acetone) exposed mussels.

6.3.2 Standardised behavioural tests

Although the general observations strongly suggested that the crabs exposed to BABs-contaminated mussels were behaving abnormally, such observations were not conducted under strictly controlled conditions. The video-filmed behaviour tests were used to generate fully quantifiable data. One of the crabs fed on a diet of BABs-contaminated mussels failed to respond at all to a cockle presented to it and appeared lethargic. One of the control crabs also showed no interest in the cockle presented but spent all its time attempting to escape from the test vessel. Both of these crabs failed to open their cockles within the 15 min time allocation and were excluded from statistical comparison. Six crabs from both treatments successfully engaged and broke into the cockles within the specified time (Fig. 6.2a). A comparison of the median times taken showed that crabs fed on a diet of BABs-contaminated mussels took significantly ($P \leq 0.05$) longer, 8 min (interquartile range = 6.5 – 9.5 min), than those fed on acetone-exposed mussels, 4 min (interquartile range = 1.5 – 6.5 min).

Once a crab had successfully opened a cockle, the time taken for it to consume the cockle was recorded. The 'eating time' for crabs fed on a diet of BABs-contaminated mussels was highly variable but median times were only slightly longer than control crabs (Fig.

2b). Median eating times for BABs-exposed crabs were not significantly longer than unexposed animals ($P = 0.21$). Two of the BABs-exposed crabs did not consume all of the cockles: one abandoned the cockle after 6 min and did not return within the 15 min allocation time (eating time recorded = 6 min) and one ate very slowly and did not complete the task within the time.

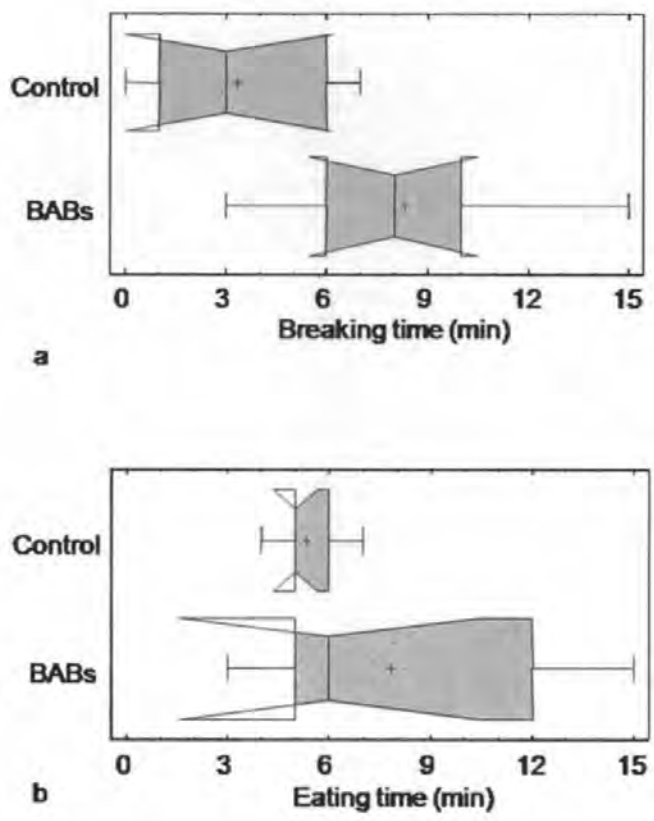


Figure 6.2 Time taken for crabs *C. maenas* fed on a diet of mussels contaminated with BABs for 7 days to break into (a) and consume (b) cockles compared to crabs fed on a diet of carrier solvent (acetone) exposed mussels.

6.3.3 Physiological tests

In conjunction with the behavioural tests, simultaneous measurements of heart rates were recorded using the CAPMON system (Fig. 6.3). Crabs fed on a diet of BABs-contaminated mussels had mean heart rates during the time spent breaking into the cockles of 111.8 beats min^{-1} compared to crabs fed on uncontaminated mussels of 94.9 beats min^{-1} but this was not significantly higher at the 95 % confidence level ($P = 0.11$). Heart rates

for BABs-exposed crabs were only slightly higher to control animals during the consumption of the cockles i.e. not significantly higher ($P = 0.31$).

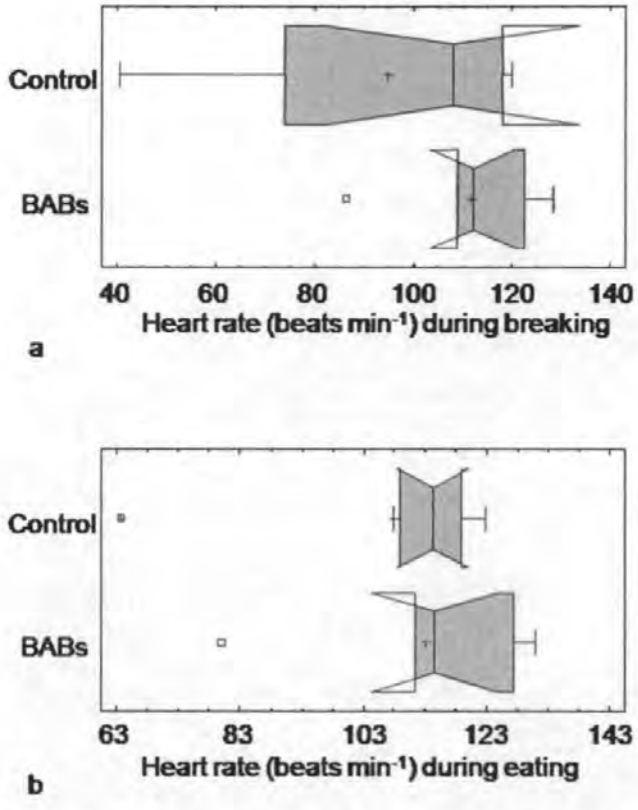


Figure 6.3 Heart rates of crabs *C. maenas* fed on a diet of mussels contaminated with BABs for 7 days during breaking into (a) and consumption of (b) cockles compared to crabs fed on a diet of carrier solvent (acetone) exposed mussels.

6.3.4 Cellular biomarkers

Immunosuppression response of haemolymph cells, as measured by the phagocytosis inhibition assay, was similar for both groups of crabs (Fig. 6.4a). Cellular viability, as measured by Neutral Red retention, was reduced by 38 % (by mean, n = 9) in crabs exposed to BABs-contaminated mussels compared to acetone-exposed crabs (Fig. 6.4b). However, this reduced cellular viability was not significant at the 95 % confidence level ($P = 0.09$).

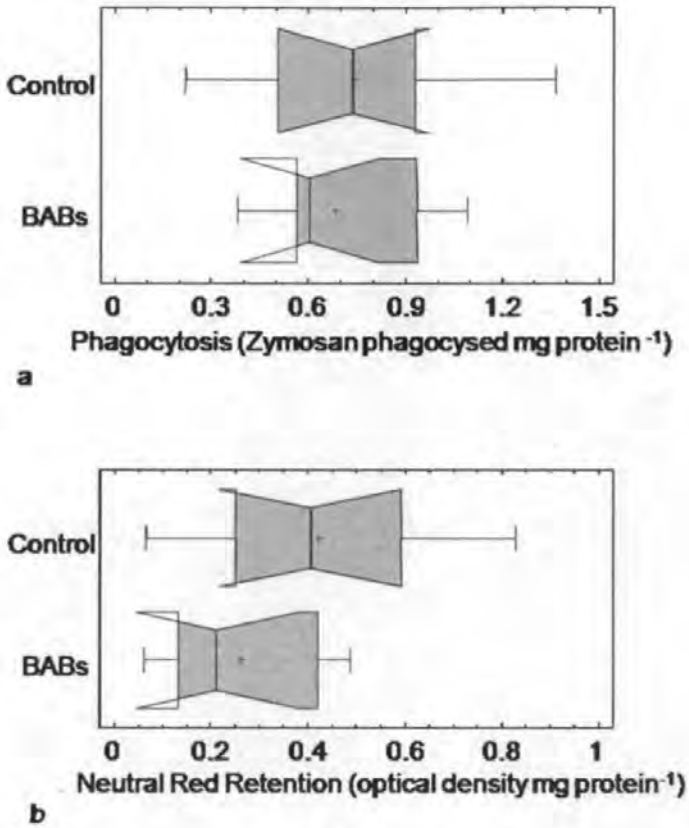


Figure 6.4 Phagocytic activity (a) and cellular viability (b) of crabs *C. maenas* fed on a diet of mussels contaminated with BABs for 7 days compared to crabs fed on a diet of carrier solvent (acetone) exposed mussels.

6.3.5 Urine analysis

The $C_{12\text{-}14}$ BABs mixture produced a broad emission fluorescence peak at λ 290 – 305 nm. A linear relationship between fluorescence area and concentration was established (r^2 = 0.976, P = 0002). Fluorescence area from the crab urine was within the range of the standard curve. The maximum fluorescence peak was shifted from λ 292 nm in the BABs standards to λ 296 nm in the crab urine samples. Triplicate analysis of crab urine showed a high degree of precision (CV = 2.3 % typical). The mean fluorescence emitted from urine of the crabs fed to the $C_{12\text{-}14}$ BABs-exposed mussels was found to be higher (13 %) than that from crabs fed on acetone-exposed mussels BABs but this was not significant at the 95 % confidence level (P = 0.11). The fluorescence area arising from the ethanol was only about 25 % of that from the crab urine in ethanol dilution. Hence, the majority of the

fluorescence was due to compounds within the crab urine. The C_{12-14} BABs present within the urine of the crabs exposed to BABs-contaminated mussels were quantified by the comparison of peak areas of the exposed and control organisms and interpolation from the standard curve. The concentration of BABs in the crab urine was estimated to be 204 μg mL^{-1}. However, given the CV between fluorescence measurements of crab urine samples (21 % and 17 % for BABs-exposed and control crabs respectively) this estimate is only approximate.

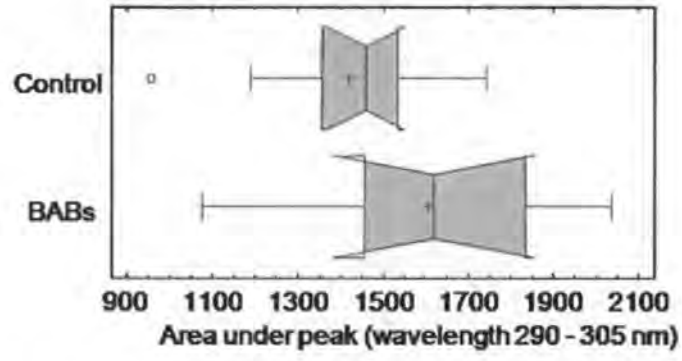


Figure 6.5 Area under fluorescence emission peak (λ 290 – 305 nm) produced by excitation at λ 273 nm of urine diluted in ethanol from crabs fed on BABs-exposed mussels and control organisms fed on acetone-exposed mussels.

6.3.6 Tissue analysis

Following extraction of the midgut glands by alkaline saponification, the extracts were analysed by GC-MS in full scan mode. This failed to show the typical chromatographic profile of the C_{12-14} BABs mixture. Extracted ion chromatograms using fragment ions *m/z* 91, *m/z* 105 and *m/z* 119 suggested that alkylbenzenes were present but at very small quantities. The extracts were therefore reduced in volume (×10) and re-analysed using SIM mode with fragment ions as above and the C_{12} BAB molecular ion *m/z* 246. This showed that alkylbenzenes consistent with that of the C_{12-14} BABs mixture were present in the midgut gland tissue extract. For confirmation purposes and to lower the limit of detection, the extracts were further analysed on a more sensitive GC-MSD. This analysis

confirmed the presence of some C_{12-14} BABs mixture compounds by similarity of retention times and mass spectra (Fig. 6.6 and 6.7).

Due to the low abundance of C_{12-14} BABs compounds and the presence of closely eluting compounds, quantitation was only approximated. Midgut gland tissue concentrations of C_{12-14} BABs were estimated based on comparison of two peaks from the C_{12-14} BABs mixture (Fig. 6.6) and the tissue extracts (Fig. 6.7). Concentrations of BABs within midgut gland tissue were 946 - 1257 ng g^{-1} and 1413 - 1910 ng g^{-1} (dry weight) for replicates 1 and 2 respectively (Fig. 6.8); the latter contained the extract from the crab that had died at the end of the 7-day exposure period. No peaks with similar mass spectra to the selected BABs at the same retention times were detected within blanks, system blank or controls. A very small peak with base ion *m/z* 91 was detected at peak 1 and a limit of quantitation (based on 10× standard deviation of the blanks) was calculated to be 5 ng g^{-1}.

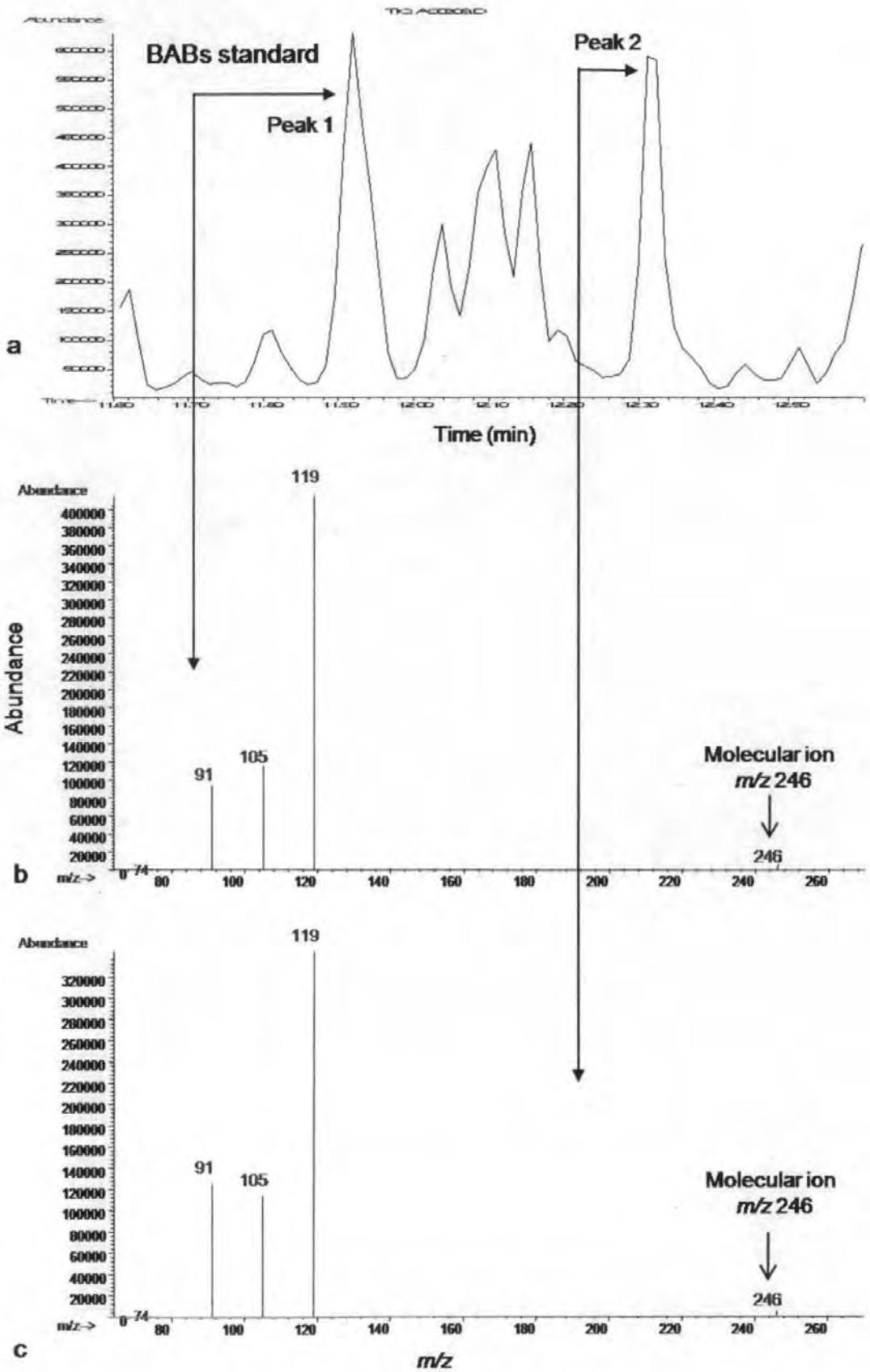


Figure 6.6 Gas chromatogram of C_{12-14} BABs mixture standard (a), showing peaks used for identification and quantitation of BABs in crab tissue, with mass spectra of the compounds shown below (b and c).

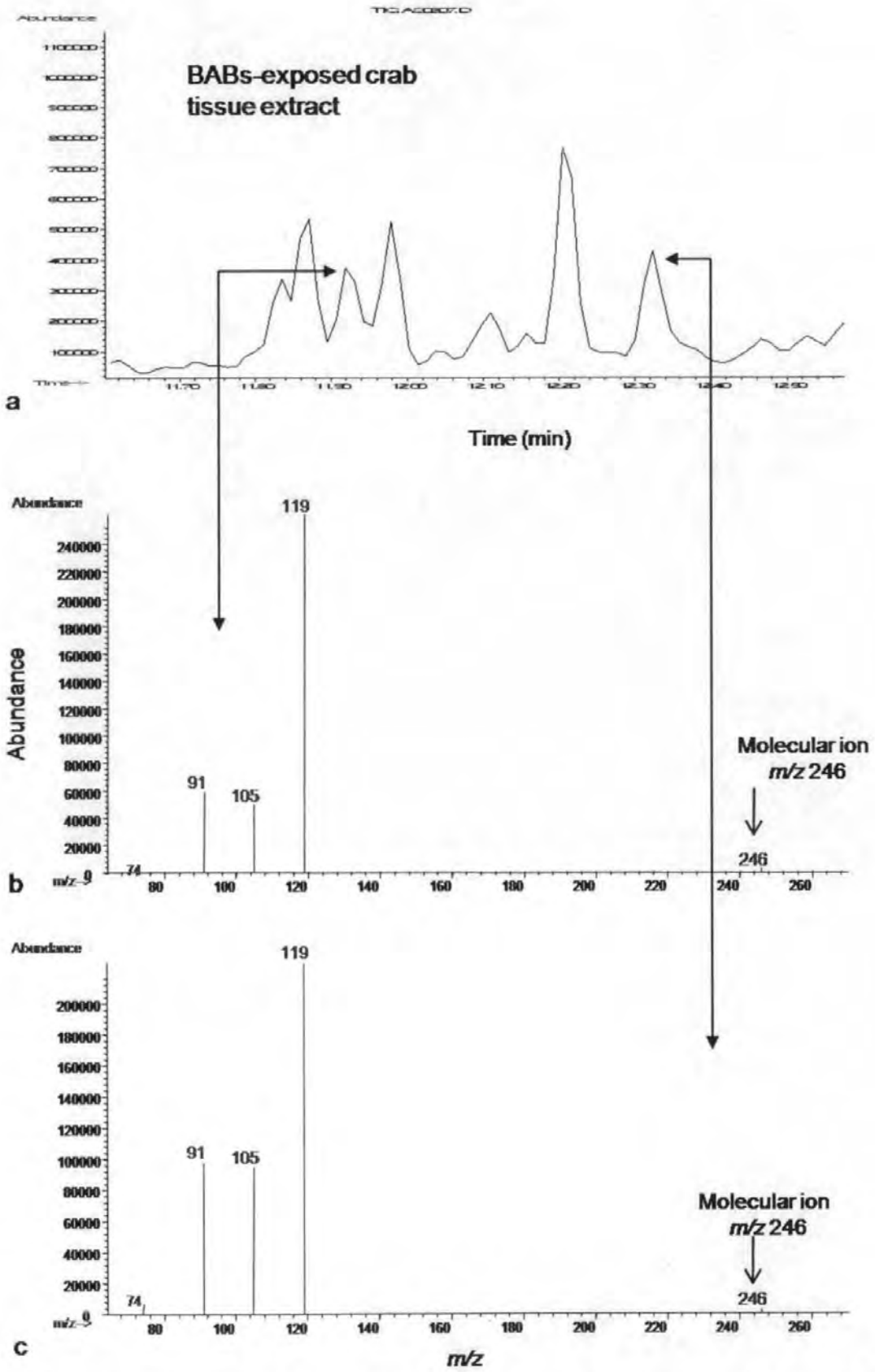


Figure 6.7 Gas chromatogram of extract from crab *C. maenas* midgut gland tissue (a), showing peaks used for identification and quantitation of BABs, with mass spectra of the compounds shown below (b and c).

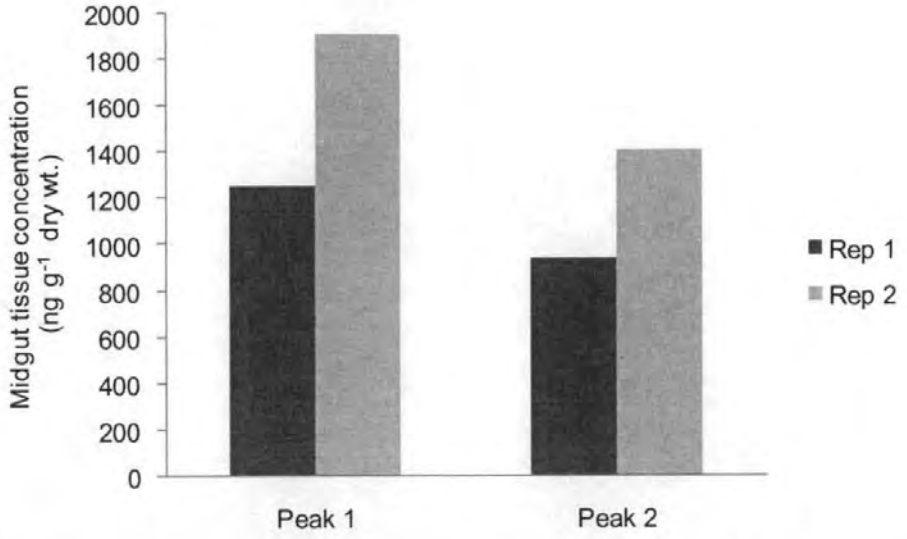


Figure 6.8 Estimated crab midgut gland tissues concentrations of C_{12-14} BABs based on comparison of gas chromatograph peak areas.

6.4 Discussion

The trophic transfer of contaminants from sessile to mobile organisms is not well reported within the scientific literature. For PAHs, biomagnification does not appear to occur due to the ability of higher organisms to metabolise and excrete the compounds and bioaccumulation *via* trophic transfer is thought to be of limited importance (Bierman, 1990; NRC, 2003; Wan *et al.*, 2007). However, a study by Rice *et al.* (2000) suggested that uptake of hydrocarbons *via* the food web was important in reducing the growth rate of juvenile fish. Uptake of the monoaromatic BABs into the tissues of mussels was found to rapidly occur but the compounds were only slowly lost when the organisms were placed in clean seawater (**Chapters 4** and **5**) and therefore theoretically BABs have the potential to accumulate to high concentrations as reported by Booth *et al.* (2007) for N. Sea mussels. The capability of the $C_{12\text{-}14}$ BABs mixture of compounds to transfer from contaminated mussels to the predator species *C. maenas* was therefore explored by feeding BABs-contaminated mussels to the crabs and measuring the concentration of the BABs within the midgut gland tissue and observing effects in terms of crab behaviour, cellular biomarkers and physiology. This research has shown that the BABs were indeed transferred to the midgut gland tissue (Fig. 6.8) and that the behaviour of the crabs was affected (Fig. 6.1 and 6.2).

Culbertson *et al.* (2007) reported that the behaviour and feeding rates of fiddler crabs *U. pugnax* were affected by the high concentrations of UCM hydrocarbons within marsh sediments contaminated with oil from the Florida barge spill in 1969. Although the authors related biological effects to sediment concentrations in terms of TPH, the vast majority of this was UCM hydrocarbons when analysed by conventional GC (Reddy *et al.*, 2002). It was reported that crabs from oiled sites avoided burrowing into oiled layers, had delayed escape responses and reduced feeding rates (Culbertson *et al.*, 2007). The failure

to burrow deep through the sediment may have made the crabs more vulnerable to predation. The fiddler crabs also were found to possess lowered physiological condition, and the authors speculated that this, together with altered behaviour, may have been responsible for the lower densities of crabs found within the UCM hydrocarbon contaminated site. The sediment concentrations were very high i.e. in the mg g^{-1} range and therefore the behavioural responses could have resulted from a reaction to the physical nature of the oiled sediment rather than a chemical effect upon the functioning of the organism. However, the crabs' feeding rate was also affected, which infers that the functioning of the crabs was impaired. Frysinger *et al.* (2003), using GC×GC-MS, reported that the oil from the Florida barge spill contained isomers of alkylbenzenes but Culbertson *et al.* (2007) did not report the concentrations of hydrocarbons within the crabs or provide any characterisation of compounds that may have been bioaccumulated by the crabs; therefore direct cause and effect remain open to question. Fiddler crabs *U. pugnax* are deposit feeding that sieve the sediment (Rosenberg, 2001) and are therefore in direct contact with contaminated sediment but may also ingest contaminated algae, fungus or decaying material. The shore crab *C. maenas* is a predatory species (Vernberg & Vernberg, 1983) and therefore more likely to be indirectly exposed to toxicants *via* contaminated prey as well as direct exposure to dissolved contaminants.

The midgut gland tissue of crabs, *C. maenas*, that had been fed BABs-contaminated mussels was found to possess alkylbenzenes with similar retention times and mass spectra to C_{12-14} BABs (Fig. 6.6 and 6.7). Tissue concentrations, measured by GC-MS analyses were low: < 2 μg g^{-1} (dry weight; Fig. 6.8). The crabs had three days in clean seawater without consuming contaminated mussels but were fed an uncontaminated cockle during behaviour/physiological tests following their exposure to BABs-contaminated mussels. Digestion and absorption are reported to take place 12 h after feeding in *C. maenas*

followed by an excretory phase of 12 – 48 h after feeding (Hopkin & Nott, 1980); the crabs therefore had ample time to excrete consumed tissues. The low concentrations found within the midgut gland may have resulted from: (i) insubstantial transfer of the BABs from the mussel to the crab tissue; (ii) transfer to tissues other than the midgut gland; or, (iii) rapid metabolism/removal from the midgut gland. Without further thorough investigations it is not possible to answer which (or whether any or all) of these three scenarios exists. The midgut gland serves as the principal storage organ of lipids (Mantel, 1983) and therefore would be expected to accumulate lipophilic compounds such as the alkylbenzenes. The midgut gland is also associated with metabolism and excretion of toxins (Mantel, 1983). Interestingly, analysis of the pooled sample of midgut gland tissue that contained the crab that died at the end of the exposure, and therefore which did not experience the three days without being fed contaminated mussels, was found to have a higher concentration of BABs (Fig. 6.8). This suggests that rapid removal of the BABs may have occurred. If so, it would be expected that the crab urine would contain BABs or their metabolites. Although not statistically significant at the 95 % confidence level (P = 0.11), the fluorescence corresponding to the wavelength of the $C_{12\text{-}14}$ BABs from the BABs-exposed crabs' urine was found to be higher than that from the control organisms' urine (Fig. 6.5). The $C_{12\text{-}14}$ BABs mixture produced a broad emission fluorescence peak at λ 290 – 305 nm, presumably due to the complex nature of the large number of isomers present. The wavelength at which maximum fluorescence was emitted from the BABs-exposed crab urine was a little longer (296 nm) compared to that observed for $C_{12\text{-}14}$ BABs standard (292 nm). The longer (red shift) maximum fluorescence wavelength observed for the urine samples may be indicative of metabolites, although Watson *et al.* (2004b) reported a 5 nm blue shift for metabolites of pyrene within crab urine.

Differences in the behaviour of the BABs-exposed crabs compared to that of the control organisms were apparent within three days of the commencement of the feeding exposure. As the exposure progressed this abnormal behaviour became more widespread. However, observations relating to apparent lethargy or aggression are difficult to quantify. The timing of the crabs' responses to mussels placed in their tanks at the end of the exposure period did reveal a significant increase ($P \leq 0.05$) in response time (Fig. 6.1) and throughout the test several crabs failed to open the BABs-contaminated mussels. Observation of the crabs' handling procedure for opening the mussels showed that BABs-exposed crabs often failed to orientate the mussels correctly to allow insertion of their pincers into the slightly gaping mussel shells; on occasions, the crabs would miss the mussel completely, reminiscent of 'drunk' behaviour, a term used by Culbertson *et al.* (2007) to describe the behaviour of the fiddler crabs exposed to UCM-contaminated sediment (see above). The abnormal response to stimuli and handling of prey appeared less pronounced during the standardised behaviour tests although response time was still significantly longer ($P \leq 0.05$) for crabs that had been fed BABs-contaminated mussels (Fig. 6.2). No significant differences ($P = 0.21$) were observed for median eating times for BABs-exposed crabs compared to unexposed crabs but variation was considerable greater i.e. CV was 59.0 % for BABs-exposed crabs compared to 19.4 % for unexposed crabs. Forbes and Depledge (1996) noted that variance was often greater within stressed populations. The apparent lethargy and passiveness of the BABs-exposed crabs was in contrast to observed behaviour of *C. maenas* exposed to pyrene (Dissanayake *et al.*, 2007) where it was found that the latter were more aggressive and more able to hold possession of their food item (cockle) than unexposed animals.

Simultaneous measurements of heart rates during the behaviour test did not reveal any significant differences between treatments, although mean rates were higher for BABs-

exposed crabs during breaking ($P = 0.11$). Fossi *et al.* (2000) reported elevated heart rates in the Mediterranean crab *Carcinus aestuarii* Nordo 1847, an organism similar to *C. maenas*, when exposed to an aqueous concentration of 1 mg L^{-1} of B(a)P. Measurement of the heart rate of *C. maenas* has been used previously as part of suite of biomarkers to assess the health of marine environments contaminated with PAHs. Galloway *et al.* (2004b) reported that although heart rates were highest in crabs collected in the vicinity of the Esso terminal at Cadland creek, Southampton water, UK (total PAHs in sediment 24092 μg g^{-1} dry weight), this was not significantly different from heart rates of crabs at other sampling stations (total PAHs in sediment 17 - 2646 μg g^{-1}). The PAHs in the sediment may not have been bioavailable to the crabs but an aqueous total hydrocarbon concentration of 48 μg L^{-1} was previously reported for this site (Kirby *et al.*, 1998) which suggests that crabs were probably subject to chronic petroleum hydrocarbon exposure. Galloway *et al.* (2004b) did not report any tissue concentrations but crab urine was found to possess significant PAH metabolites.

Cellular viability of crab haemolymph was not found to be significantly affected ($P = 0.09$) by consumption of BABs-contaminated mussels (Fig. 6.4). A small reduction was apparent and the relatively low P value suggested that cellular viability may have started to become compromised. Mussel haemolymph was not found to be significantly affected by chronic exposure to 5 μg L^{-1} C_{12-14} BABs (**Chapter 5**) and no suggestion of damage was apparent. Galloway *et al.* (2004b) reported that cellular viability was not significantly affected in *C. maenas* from sites in the Solent (UK) heavily contaminated by petroleum hydrocarbons but a population of the filter-feeding bivalve mollusc *C. edule* (cockle) collected in the upper estuary at Crackmore Hard did have significantly different cellular viability from other sites in Southampton water. Interestingly, although the Crackmore Hard sediments had relatively high total PAH concentrations (2646 μg g^{-1} dry weight)

compared to most of the sites (17 – 329 μg g^{-1} dry weight), this was still an order of magnitude less than that reported for the Esso terminal site (24092 μg g^{-1} dry weight). The Esso terminal PAH sediment concentration was originally reported by Rogers (2002); the concentrations were derived by GC-MS analysis in SIM mode and no information concerning TPH, UCM or monoaromatic hydrocarbon concentrations was provided.

Environmental contaminants, including hydrocarbons, have been implicated with reduced immune function in invertebrates (reviewed by Galloway & Depledge, 2001). The review gave examples of research that showed PAHs and sediments contaminated with complex mixtures of hydrocarbons had been implicated with immune suppression, but no reports were given for *C. maenas*. One measure of immunotoxicity in invertebrates is phagocytosis within haemolymph cells. Dissanayake *et al.* (2007), investigated the effects of pyrene exposure to *C. maenas,* and assessed its effect upon phagocytosis within haemolymph cells, but did not report any significant differences between exposed and unexposed organisms. The immune function of the crabs in the present study was unaffected by exposure to BABs-contaminated mussels. It is possible that longer exposure is required to trigger the immune response (Coles *et al.*, 1995; cited by Galloway & Depledge, 2001).

In a marine environment contaminated by UCM hydrocarbons, crabs may be expected to feed upon mussels and other organisms with tissue burdens of a range of petroleum hydrocarbons. Research by Booth *et al.* (2007) and studies reported herein (**Chapter 4 and 5**) show that BABs are among the most prominent compounds within the UCM accumulated by mussels. The present study has shown that if crabs feed upon BABs-contaminated mussels they will accrue BABs but it would seem that these compounds are rapidly metabolised and/or excreted and therefore the BABs are unlikely to biomagnify in

the food chain to fish, seabirds or mammals. Consumption of the BABs-contaminated mussels resulted in abnormal behaviour which may reduce the crabs' health status or make them more vulnerable to predation, especially if consumption continued over a long period. The diurnal and seasonal migration cycles for *C. maenas* (Naylor, 1962; Vernberg & Vernberg, 1983) may allow individuals not continually exposed to aqueous BABs to accumulate these toxic compounds by trophic transfer *via* consumption of prey resident in contaminated water. Sites with high petrochemical contamination still provide habitats for *C. maenas* (Galloway *et al.*, 2004b) and therefore either direct exposure, from aqueous contamination, or indirect exposure, *via* consumption of contaminated food, does not eliminate the population. Crabs living in highly contaminated areas may have acclimated or adapted to such conditions. Crowe *et al.* (2004) reported that juvenile *C. maenas* were abundant within mussel beds contaminated with UCM hydrocarbons. It is possible that other species that predate filter-feeding bivalve molluscs, such as starfish, may also accumulate BABs with resultant impact on their behaviour.

6.5 Conclusions

Analysis of crab midgut gland tissue revealed that C_{12-14} BABs were transferred from the mussel tissues to the crabs *via* consumption of contaminated mussels. Tissue concentrations were very low but crabs fed on BABs-exposed mussels had elevated BABs-associated fluorescence in their urine; this suggested that the compounds were metabolised and /or excreted. The results do not support the hypothesis that BABs are likely to biomagnify in the marine environment. Consumption of BABs-exposed mussels did result in significant abnormal behaviour which, in the wild, may affect the crabs' feeding ability and make them more vulnerable to predation. No evidence for impairment to immune function was found but the exposure period may have been too short to affect the phagocytosis response.

Chapter 7
Conclusions, implications and further research

In this chapter, the main findings of the research presented herein and within peer-reviewed publications arising from this research (Booth *et al.*, 2007; Scarlett *et al.*, 2007a; Scarlett *et al.*, 2007b; Scarlett *et al.*, 2007c; Scarlett *et al.*, In press) are summarised and conclusions drawn. Implications for the marine environment and the monitoring of petrogenic contamination are discussed. Further topics of research are also identified.

7.1 Summary

The review of literature concerned with the potential toxicity of UCM hydrocarbons in the marine environment (**Chapter 1**) identified a number of gaps in our current knowledge. Primarily, previous laboratory-based toxicology studies had been performed using acute aqueous exposures to *M. edulis*. No information was therefore available with regard to: exposure to UCM-contaminated sediment; effects from chronic exposure; or, the effect of UCM hydrocarbons upon organisms with routes of uptake different from that of *M. edulis*. Sub-lethal effects of UCM-hydrocarbons upon *M. edulis* may not necessarily affect populations and communities, although a study by Crowe *et al.* (2004) strongly implied that communities within mussel beds contaminated with UCM hydrocarbons were affected and not just the health of the individual mussels. Secondly, although the monoaromatic fraction of the UCM had been highlighted as containing compounds toxic to mussels, with compelling evidence from Donkin *et al.* (2003), attempts to identify possible structures have not been successful. For example, Smith *et al.* (2001) proposed cyclic alkyltetralins but these proved not to be present within environmental UCMs examined to date (Booth, 2004). Prior to commencement of the research reported herein, there was also little research into the more polar water soluble fraction of the UCM. However, an EDA approach to identify toxic compounds within WSFs of N. Sea oils was known to be in progress elsewhere (Rowland, pers. comm.) and therefore research subjects were selected herein to complement rather than to replicate this area of research. The research reported herein therefore concentrated on:

1. The effects of chronic exposure to UCM-contaminated sediment, using the amphipod *C. volutator* as a test species, with particular emphasis on population-level effects (**Chapter 3**).

2. Identification of fractions responsible for sub-lethal effects arising from both aqueous and sediment exposure (**Chapter 4**).

3. Testing the hypothesis that BABs could, in part, be responsible for the low S*f*G observed in some *M. edulis* populations contaminated with high concentrations of UCM hydrocarbons (**Chapter 5**).

4. Examination of the wider implications of contamination of *M. edulis* by BABs including the potential for trophic transfer and biomagnification (**Chapter 6**).

To carry out this research, a preliminary study had to be performed to develop a chronic sediment exposure test using *C. volutator*. This study, now published (Scarlett *et al.*, 2007c), reported that *C. volutator* was acceptable as a test species to observe the long-term implications of exposure to oil-contaminated sediment; it also served as a reference to compare the effects of UCM hydrocarbons (**Chapter 3**). The study revealed that population-level effects, in terms of reduction in growth rate and reproductive success, could occur at sediment concentrations that did not cause lethal effects during standard acute exposure tests. As an adjunct to the chronic sediment tests, behavioural tests were developed with the aim of linking the initial response of amphipods to contaminated sediment with that of long-term effects arising from chronic exposure. The results of the first two behaviour tests were inconclusive but suggested a possible link between amphipod burrowing behaviour and the toxicity of the sediment. This study was also published (Scarlett *et al.*, 2007a) and complemented another recently published behavioural study that used sediment choice tests with *C. volutator* (Hellou *et al.*, 2005). The behaviour test was further used to attempt to predict the effects of sediment contaminated with fractions of the UCM-rich oils (**Chapter 4**). This behaviour test suggested that the sediments would not be toxic to the amphipods but some small effects upon growth and reproduction were found. The value of behaviour tests remains open to question but they require relatively little additional work when preparing a sediment test and the results of the studies by Hellou (2005) give further weight to the hypothesis that

the behaviour of *C. volutator* is affected by sublethal concentrations of hydrocarbon contaminants within sediments.

Using an EDA approach, the toxicity of the UCM-rich oils was first tested to establish baseline toxicity, both within aqueous exposures and sediment exposures (**Chapter 3**). These studies used a partial life-cycle chronic exposure test developed from the preliminary studies and showed that all the model oils caused population-level effects when exposed to amphipods *via* chronic spiked sediment tests. Aqueous exposures of the whole oils did not cause significant effects either to the clearance rates of *M. edulis* or to bioluminescence inhibition of *V. fischeri*. In work described in **Chapter 4**, the oils were fractionated by open column chromatography and care taken to ensure that the alkylbenzenes were eluted within the non-polar aromatic (F2) fraction (it had been found during method development that some compounds eluted within the aliphatic (F1) fraction using published methods). Tests using the aliphatic (F1) and two aromatic fractions (F2 and F3) of the oils showed that all fractions contributed to the effects upon growth and reproduction of amphipods when exposed *via* spiked sediment. The studies were carried out using whole oil equivalent concentrations so although the fractions contributed more or less equally on this basis, the F2 fractions were more toxic using a $\mu g\ g^{-1}$ sediment concentration comparison. SLK oil contained over 80 % aliphatic (F1) compounds but the effect upon growth and reproduction was similar for both the F1 (nominal concentration (nominal concentration ~400 $\mu g\ g^{-1}$ dry weight) and F2 (nominal concentration ~100 $\mu g\ g^{-1}$ dry weight) fractions. Results from both the whole-oil and oil-fraction sediment exposure studies were published (Scarlett *et al.*, 2007b).

Aqueous exposures to the oil fractions using clearance rates of *M. edulis* and bioluminescence inhibition of *V. fischeri* revealed that only the F2 fraction caused

significant effects, although there was a strong suggestion that compounds in the F3 fraction of TJP were also toxic to mussels. Further studies involving the uptake and depuration of the F2 fractions with *M. edulis*, coupled to tissue analyses with GC-MS and GC×GC-ToF-MS, found that although a vast array of compounds was rapidly accumulated into the tissues, leading to reduced clearance rates, most of these compounds were readily depurated with a concomitant recovery by the mussels. The GC×GC-ToF-MS analysis was able to show that acute exposure to the oil fractions did not produce a profile of accumulated compounds similar to that found in UCM-contaminated wild mussels. Some groups of compounds were found to be resistant to depuration e.g. the BABs, and therefore were more likely to accumulate in wild mussels *via* long-term intermittent exposure leading to a profile of compound groups similar to that reported for the wild mussels.

Booth *et al.* (2006; 2007) had shown that BABs were prevalent within the tissues of UCM-contaminated mussels. It was thus hypothesised herein that these compounds could, at least in part, be responsible for the observed poor S*f*G that had been measured in some wild mussel populations contaminated by UCM hydrocarbons. Rather than pursue the EDA approach to identifying the toxic components of the UCM, which lacked a single toxic fraction to continue the iterative process, the toxic BABs hypothesis was tested. This more targeted approach was made possible by the availability of a commercial mixture of $C_{12\text{-}14}$ BABs and access to state-of -the-art GC×GC-ToF-MS. The $C_{12\text{-}14}$ BABs mixture contained many unresolved and co-eluting compounds (when analysed by conventional GC) so was effectively a partially resolved UCM, albeit much simplified compared to typical environmental UCMs. The use of GC×GC-ToF-MS analysis enabled near full resolution of the $C_{12\text{-}14}$ BABs mixture. Aqueous exposures of the $C_{12\text{-}14}$ BABs mixture to *M. edulis* (**Chapter 5**) showed that these branched compounds were similar in toxicity to the smaller linear C_8 alkylbenzene, 1-phenyloctane. Toxicity tests in conjunction with

tissue extraction and quantitation by GC-MS allowed a concentration-response relationship to be established and both 72 h aqueous and tissue EC_{20} values derived. This research formed part of a study published as Booth *et al.* (2007). Further research with *M. edulis*, involving longer-term exposure and depuration studies using C_{12-14} BABs, confirmed that these compounds can accumulate from low aqueous concentrations into the tissues of mussels until body burdens are sufficient to cause harm and that they are not readily depurated (**Chapter 5**). These studies, recently accepted for publication (Scarlett *et al.*, In press), also revealed that quantitation of tissue extracts using GC×GC-ToF-MS produced very similar results to those derived by GC-MS.

The research summarised above has shown that sediment-dwelling amphipods that graze the biofilm on the sediment surface as well as filter feed were affected by exposure to UCM hydrocarbons. It has also shown that the bacteria *V. fischeri* was affected by aqueous exposure of oil fractions in a similar pattern as *M. edulis*. To extend this exploration of effects relating to route of uptake, a study involving the trophic transfer of BABs from mussels *M. edulis* to crabs *C. maenas* was performed (**Chapter 6**). This study revealed that the consumption of BABs-contaminated mussels by *C. maenas* resulted in low concentrations of BABs accumulated in the lipid-rich midgut gland of the crabs. Consumption of BABs-contaminated mussels resulted in abnormal behaviour by the crabs that affected their ability to feed upon their natural prey organisms. Differences between exposed and unexposed crabs in terms of cellular biomarkers and physiological measurements were not statistically significant at the 95 % level but suggested that longer term exposure may cause reduction in health status. There was also suggestion that the BABs were metabolised and/or excreted into the crab urine, but these would require confirmation by further research.

7.2 Conclusions and implications

The research has shown that exposure to UCM hydrocarbons within sediments can result in population-level effects. The concentrations used were high but within reported environmental concentrations (Table 1.1) and therefore wild sediment-dwelling organisms are likely to be affected if exposed to high levels of UCM hydrocarbon contamination. The estuarine sediment used for the toxicity studies possessed relatively high OC content, as preferred by the test organism *C. volutator*, and therefore is likely to strongly adsorb lipophilic compounds perhaps reducing their bioavailability (Ditoro *et al.*, 1991). Furthermore, *C. volutator* is a relatively robust species compared to other genera of amphipods and therefore it would be expected that more sensitive organisms would be affected at lower concentrations especially in sediment with lower OC content. There are few reports of aromatic UCM concentrations in sediments but the research reported herein shows that the aromatic fraction of UCM hydrocarbons was the most toxic and therefore likely to contribute to toxicity arising from other contaminants such as PAHs. The research has also shown that the aliphatic fraction of the UCM may contribute towards toxicity and from the limited reports of sediment UCM concentrations (examples within Table 1.1) it would appear that high concentrations of aliphatic UCM hydrocarbons are more common than high aromatic UCM hydrocarbons. Marine environment monitoring and regulatory bodies may wish to consider the concentrations of aliphatic and aromatic UCM hydrocarbons within sediments.

Tests involving the aqueous exposure of UCM hydrocarbons showed that the UCM-dominated oils were not acutely toxic but the aromatic fractions did reduce mussel clearance rates at equivalent nominal concentrations. These results suggest the bioavailability and solubility of UCM hydrocarbons are major factors when considering toxicity. According to Raoult's Law, the solubility of compounds is, in part, influenced by

the other compounds present i.e. the more similar the compounds within the matrix the more soluble they become. Short-term exposures with high aqueous concentrations do not replicate conditions in the marine environment where organisms are more likely to experience low, and possibly episodic, concentrations over a long period. The solubility and bioavailability of UCMs within estuarine and coastal waters is therefore extremely complex and relating effects arising from acute exposure to nominal aqueous concentrations is perhaps not terribly useful. From studies with wild mussel populations (Booth *et al.*, 2007; Widdows *et al.*, 1995) it is known that mussels can accumulate high concentrations of UCM hydrocarbons (Table 1.2) and therefore the compounds accumulated within the tissues were evidently bioavailable. Comparing the profile of the compounds accumulated by the wild mussels with that of the laboratory exposed mussels; analyses by GC×GC-ToF-MS revealed that tissue extracts from depurated mussels most closely resembled that of the wild mussels. Marine organisms are most likely subject to pulses of contamination and therefore able to depurate some accumulated compounds when contamination is reduced. The studies involving the non-polar aromatic fractions (F2) of the UCM-dominated oils (**Chapter 4**) showed that naphthalenes were rapidly accumulated but also readily depurated whereas BABs and some alkylated PAHs were more resistant to depuration. To date, the monitoring of hydrocarbons in marine biota has been focused mainly on PAHs (NRC, 2003) and the presence of other contaminants, such as BABs, may be completely overlooked due to the common use of GC-MS SIM analysis.

Evidence from research presented in **Chapter 4** and that of compound groups accumulated in wild mussels with high UCM concentrations and low S*f*G (Booth *et al.*, 2006; Booth *et al.*, 2007) suggested that BABs may, in part, be responsible for the poor health status of some mussel populations. Acute tests using the commercially available $C_{12\text{-}14}$ BABs mixtures (**Chapter 5**) confirmed that these compounds were toxic to mussels and 72 h

aqueous and tissue EC_{20} values of 7 μg L^{-1} and 10.5 μg g^{-1} (dry weight) respectively were derived. It was also confirmed that longer-term exposure at a low concentration (5 μg L^{-1}) resulted in mussel tissue concentrations similar to that for acute exposure at higher concentration (41 μg L^{-1}) and a five day period in clean seawater was insufficient to depurate all the BABs with concomitant failure to recover fully. Analysis of tissue extracts by GC×GC-ToF-MS showed that there was no evidence for preferential uptake or depuration of the different isomers present within the C_{12-14} BABs mixture and Booth *et al.* (2007) reported that a much larger range of BABs was accumulated within the wild mussels. Marine environment monitoring and regulatory bodies should therefore be able to take into account the entire tissue concentration of BABs when assessing potential biological effects arising from contamination by hydrocarbons. Some fractionation methods may not record the presence of alkylbenzenes within the aromatic fraction and poor resolution by conventional GC may miss BABs within the UCM. As the use of GC×GC-ToF-MS becomes more widely used it will become easier to detect and quantify BABs.

Consumption of mussels contaminated with BABs resulted in significant abnormal behavioural effects in the shore crab *C. maenas* (**Chapter 6**). In the environment, such abnormal behaviour may reduce crab feeding efficiency and make the animals more vulnerable to predation. The crabs only consumed a maximum of 14 contaminated mussels and wild crabs resident in a contaminated estuary are likely to consume many more mussels than this. Crabs would however, also consume prey items that may be less contaminated. Wild crabs may also be subject to direct contamination from aqueous exposure which may add to the adverse effects. No significant effects were found for cellular or physiological measurements. This may have been due to the crabs' ability to metabolise and/or excrete the BABs or, the length of exposure may not have been

sufficient to affect these parameters at a measurable level. The results generated by the trophic transfer test do not support the hypothesis that BABs are likely to biomagnify within the marine food web, but further work is required to confirm this.

7.3 Further work

Some of N. Sea mussel populations that were found to have low S/G but high UCM hydrocarbon tissue concentrations possessed groups of branched alkylindans/alkylindenes (BINs) and branched alkyltetralins (BATs) as well as the predominant BABs (Booth *et al.*, 2006). These compounds were also accumulated by mussels exposed to aromatic fractions of TJP and ANS, although they were largely depurated within five days (**Chapter 4**). When analysed by GC×GC-ToF-MS these compounds eluted a little later in the 2nd GC dimension than the BABs. Smith *et al.* (2001) reported that some synthesised disubstituted cyclic alkyltetralins were toxic to mussels at more or less equivalent concentrations as the BABs and it is hypothesised that bioaccumulated BATs and BINs should contribute to narcotic effects within the exposed organisms. No BATs and BINs are currently commercially available, but a group of BATs has been synthesised and is available for testing (Booth, pers. comm.).

Analysis by GC×GC-ToF-MS of tissue extracts of mussels exposed to an aromatic fraction of TJP showed that a considerable number of unidentified compounds remained in the tissues following five days depuration (**Chapter 4**). These compounds were not found in the tissues of depurated mussels exposed to ANS oil. It would be interesting to identify these compounds and to compare them to compounds within wild mussel tissues. It is possible that a number of the unidentified compounds contain sulphur as TJP has relatively high sulphur content (anon, 2006). Dibenzothiophenes were found within the tissues of both TJP and ANS exposed mussels, and these were not depurated within five days. Dibenzothiophenes are common contaminants in marine biota (Diez *et al.*, 2007; Frysinger & Gaines, 2001; Marvin *et al.*, 2000b; Wang & Fingas, 1995) and are reported to be cause adverse effects (Incardona *et al.*, 2004; Rhodes *et al.*, 2005; Seymour *et al.*, 1997; Wassenberg *et al.*, 2005). The extent to which other sulphur-containing compounds may

be present within environmental UCMs will depend on the source oil(s) of the contamination.

Perhaps the biggest remaining question in this area of research is: how does contamination by UCM hydrocarbons affect communities and ecosystems? The chronic exposure of amphipods to the oils and oil fractions (**Chapters 3 and 4**) showed that population-level effects, in terms of reduced growth rates and reproductive success, occurred. Whether exposure to similar sediment concentrations would actually affect wild populations and the wider community is difficult to predict. It was observed that there was great variation in the effect upon individual amphipods: some even appeared to benefit from the exposure. If oil-tolerant individuals are able to grow faster and reproduce successfully, it is possible that oil-tolerance would be genetically selected for and populations of oil tolerant amphipods would develop. It is also possible that the amphipods that are unaffected by, or benefit from, exposure to hydrocarbons, may have a greater ability to acclimate to a range of conditions and tolerance to oil is not selected for. Weak individuals unlikely to survive in the wild, even within uncontaminated sediment, may be those individuals that are most affected in laboratory toxicity tests. The selection for oil-tolerance could be tested by carrying out toxicity studies on the offspring of oil-exposed amphipods in conjunction with analysis of gene regulation. Mesocosm experiments could perhaps be utilised to explore community-level effects but, for *C. volutator*, predatory birds play an important role as preferential predation upon different-sized individuals occur (Hilton *et al.*, 2002; Wilson & Parker, 1996). Parasites can also play a crucial role in *C. volutator* population dynamics (Damsgaard *et al.*, 2005; Mouritsen & Jensen, 1997; Mouritsen *et al.*, 2005). How exposure to hydrocarbons affects host-parasite interactions within amphipods is not known but could clearly have enormous influence on the functioning of estuarine communities.

The study into the possible trophic transfer of BABs from contaminated mussels to crabs (**Chapter 6**) generated many questions. Crucially, where are most of the BABs if they are only present in the midgut tissue at low concentrations? One possible way of answering this would be to repeat the tests using radiolabeled compounds. Radiolabeled BABs are not available but could be synthesised or, tests could be conducted using commercially available radiolabeled LABs to provide an indication of transfer within tissues, and metabolic and excretion pathways. The 7-day tests suggested that cellular and physiological effects may have started to occur, so longer-term exposures could be used to investigate this further. The 7-day tests proved to be logistically challenging, so longer tests would require a dedicated team of researchers. Additionally, the tissues of crabs from areas previously found to have high UCM hydrocarbon contamination e.g. Southend UK (Booth *et al.*, 2006; Booth *et al.*, 2007) could be analysed. Relatively large numbers of crabs could be sampled allowing sufficient tissue samples for analysis. Ideally, the tissue extracts should be analysed by GC×GC-ToF-MS which provides greater resolution of compounds. This would of course not show trophic transfer as BABs may be accumulated directly from the water column but tissue concentrations in excess of that found in mussels would indicate additional accumulation *via* the food uptake route.

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Appendix A

Publications within peer-reviewed journals arising from the studies reported herein

Scarlett, A., Rowland, S.J., Galloway, T.S., Lewis, A.C., Booth, A.M., (In press). Chronic sublethal effects associated with branched alkylbenzenes bioaccumulated by mussels. *Environmental Toxicology & Chem*istry.

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CHRONIC SUBLETHAL EFFECTS ASSOCIATED WITH BRANCHED ALKYLBENZENES BIOACCUMULATED BY MUSSELS

Alan Scarlett†, Steven J Rowland†*, Tamara S Galloway‡, Ally C Lewis§, And Andy M Booth†

† School of Earth, Ocean & Environmental Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA United Kingdom
‡ School of Biosciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter EX4 4PS United Kingdom
§ Department of Chemistry, University of York, Heslington, Yorkshire, YO10 5DD United Kingdom
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Abstract

Crude oils are complex mixtures of many thousands of compounds, both resolved and unresolved by conventional gas chromatography (GC). Recent research using comprehensive two-dimensional gas chromatography – time-of-flight–mass-spectrometry (GC×GC-ToF-MS) identified branched alkylbenzenes (BABs) as a major component of some unresolved complex mixtures of hydrocarbons (UCMs) bioaccumulated in the tissues of North Sea mussels, *Mytilus edulis*, previously found to have poor health status. Here the effect of long term exposure to low aqueous concentration of BABs, and their ability to recover, was determined. Mussels were exposed to 5 μg l^{-1} of a complex mixture of C_{12-14} BABs for 14 d. Feeding rates and the viability of haemocytes were measured immediately after exposure and again after 5 d depuration. Tissues were extracted, analysed and alkylbenzenes quantified by both GC-MS and GC×GC-ToF-MS. Mussel extracts from previous acute tests were also re-analysed and quantified using GC×GC-ToF-MS. Mussels exposed to 5 μg l^{-1} BABs for 14 d accumulated 46 to 47 μg g^{-1} dry weight alkylbenzenes; this was similar to tissue concentrations of mussels exposed to 41 μg l^{-1} for 72h. Feeding rates were significantly reduced ($p \leq 0.05$) and were dependent upon tissue concentration. Cellular viability was not significantly affected. Following 5 d in clean seawater, the BABs were only partially depurated and feeding rates failed to fully recover. The use of GC×GC-ToF-MS in the present study has shown that mussel tissue concentrations of complex mixtures of alkylbenzenes, and their corresponding effects, are consistent with reported concentrations within UCM-contaminated wild mussel populations with poor health status.

Keywords: Comprehensive two-dimensional gas chromatography; Time of flight – mass spectrometry; Petroleum hydrocarbons; Unresolved complex mixture; Mytilus edulis

INTRODUCTION

It is estimated that 2.5 million tonnes of crude and refined petroleum products enter the marine environment each year [1]. A common feature of the tissue extracts of biota contaminated by petroleum hydrocarbons when they are analysed by gas chromatography (GC), is the presence of unresolved complex mixtures (UCMs), the concentrations of which often far exceed the concentrations of many individually resolved priority pollutants [2, 3]. A small number of studies have demonstrated that bioaccumulated UCM hydrocarbons can affect the health of mussels, *Mytilus edulis* L [4-8] and chronic exposure to UCM-contaminated sediment can have population-level effects, e.g., on the amphipod *Corophium volutator* Pallas [9]. Other studies have inferred the effects of UCM contamination. Martins et al. [10] reported that the reduction in membrane lysosomal stability, a measure of immunity impairment [11], of bivalves from a petroleum hydrocarbon polluted bay in Brazil was greater than expected from the measured polycyclic aromatic hydrocarbons but could be explained if the concentration of aromatic UCM hydrocarbons was considered.

Comprehensive two-dimensional gas chromatography – time-of-flight –mass-spectrometry (GC×GC-ToF-MS) provides superior chromatographic separation and more accurate peak assignments from mass spectra than previously possible [12], therefore offering a greater potential for resolving extremely complex mixtures of compounds. Recent research using GC×GC-ToF-MS has identified branched alkylbenzenes (BABs) as a ubiquitous component of UCMs bioaccumulated in the tissues of mussels, *M. edulis* [4] found to have poor health status [13]. Booth et al. [4] reported that the number of chromatographic peaks attributed to the alkylbenzenes far exceeded the number of possible simple so-called linear alkylbenzenes (LABs) found previously in some environmental samples [14]. It was suggested that this complexity was due to the occurrence of thousands of BABs, which were likely more resistant to environmental weathering and biodegradation than the known linear homologues. From the molecular weight ranges of the compounds revealed by the clarity of the ToF-MS, it could be calculated that thousands of compounds are theoretically possible. Furthermore, Booth et al. [4] were also able to demonstrate that a commercially available complex mixture of C_{12-14} BABs, containing over 340 compounds, possessed a similar toxicity in terms of mussel clearance rates (also known as feeding rates, a major component of the Scope for Growth health index), to that of the linear C_8 alkylbenzene, 1-phenyloctane.

The C_{12-14} BABs mixture is described by the manufacturers (Chevron Oronite, Levallois-Perret Cedex, France) as a clear and limpid liquid derived from petroleum hydrocarbons by distillation at 275 °C (5 %) and 293 °C (95%) with an average molecular weight of 241 amu (http://www.chevron.com/products/oronite/products/pdfs/bab.pdf). The mixture is mainly composed of compounds with C_{12} alkyl chains (64 % by GC peak area: unpublished data) and does not contain more than trace quantities of LABs or other hydrocarbons. A comparison of the mass spectra of some of the C_{12-14} BABs in this mixture of compounds with that of BABs from North Sea mussels revealed a high degree of similarity [4]. Highly branched hydrocarbons have a reduced molecular surface area thus increasing their aqueous solubility [15]. These highly branched alkylbenzenes may therefore be more bioavailable than linear isomers of the same molecular weight. Acute aqueous toxicity tests [4] established a 72 h aqueous C_{12-14} BAB EC20 (concentration required to induce a 20 % effect) of 0.007 mg l^{-1} and a tissue C_{12-14} BAB EC20 of 10.5 μg g^{-1} dry weight. The 72 h tissue concentration EC20 determined by Booth et al. [4] was derived by analyses of tissue extracts using GC-MS.

The C_{12-14} BABs compounds bioaccumulated by mussels in their laboratory tests were not analysed by GC×GC-ToF-MS, and hence could not be directly compared to the distribution of BABs accumulated by the field mussels. Re-analysis of these tissue extracts by GC×GC-ToF-MS would provide additional information concerning the uptake of alkylbenzenes and would also permit comparison of GC-MS and GC×GC-ToF-MS as quantitation methods. In particular, separation on the 2nd GC dimension may reveal information about the polarity of accumulated compounds that cannot be seen by conventional GC due to co-elution. Research concerned with more highly complex samples, such as UCMs, which require GC×GC-ToF-MS analysis in order to resolve the compounds, would benefit from direct quantitation of extracts. Therefore the present study will help enable future research into the concentrations of individual (now resolved) components of complex mixtures in the environment.

Wild mussels, even in highly contaminated waters, are typically only exposed to low aqueous concentrations of hydrocarbons and also tend to be exposed to pulses of contaminated and relatively clean seawater, allowing them the opportunity to depurate compounds that have accumulated in their tissues. Here we reproduce these environmental conditions in the laboratory and investigate the uptake of the C_{12-14} BABs mixture at a low aqueous concentration over a 14-d semi-static exposure period followed by a 5-d depuration period. Clearance rates were measured and cell viability determined using a microtitre plate neutral red retention assay [16]. Tissue extracts were analysed by both GC-MS and GC×GC-ToF-MS. In addition, tissue extracts from the previous acute exposures [4] were re-analysed by GC×GC-ToF-MS and the concentrations of BABs quantified, thus allowing direct comparison between acute and longer term exposure conditions, as well as to compounds accumulated by wild mussels. To our knowledge, this is the first reported use of GC×GC-ToF-MS

for quantitation of hydrocarbon compound classes within previously unresolved complex mixtures accumulated in biota.

MATERIALS AND METHODS

Alkylbenzene exposure tests

Acute (72 h) semi-static exposure tests at a C_{12-14} BABs concentration of 5 μg l^{-1} were performed for comparative purposes, as described by Booth et al. [4]. Longer-term (14 d) exposures were extended versions of the acute test and were conducted in triplicate. Mussels were collected and maintained as reported previously [4, 17]. The mean shell lengths were 44.2 mm (standard error = 0.11 mm), similar to mussels used by Booth et al. [4] for previous acute 72 h tests (mean = 47.2 mm, standard error = 0.31 mm). Mussel tissues from the collection site at Port Quin, on the north Cornwall coast, United Kingdom (ordnance survey grid reference: SW 972 905) have been reported to contain negligible or no UCM or aromatic hydrocarbons [8, 13]. Mussels were maintained in filtered seawater at 15 °C (± 1°C), 35 practical salinity units (± 2 psu), with a 12:12 h light:dark cycle for a minimum of one week prior to exposure tests.

The test solutions were prepared by injecting 0.5 ml of an acetone solution of the test compound into 10 L of filtered seawater held at 15 °C in a 10 L glass aspirator (i.e., acetone concentration 0.005% v/v) and vortex mixed for a minimum of 2 h prior to use. The test solutions were added to the mussel exposure vessels and replaced every 24 h. Groups of nine mussels were exposed in 9 L of test compound. Mussels were fed continuously with *Isochrysis galbana* (Reed Mariculture,Campbell, CA, USA, 0.11 – 0.15 mg dry wt ml^{-1}) delivered via glass Pasteur pipettes by means of a peristaltic pump at a rate of approximately 20 ml h^{-1}. Aeration was supplied via glass Pasteur pipettes which also aided dispersion of *Isochrysis*. Water quality measurements of dissolved oxygen, pH, salinity and temperature were recorded daily prior to water exchange.

Measurement of clearance rate

The clearance rate assay was adapted from Donkin et al. [8] and as reported by Booth et al. [4]. In brief, mussels were placed individually in beakers containing clean seawater. After an acclimation period with slow vortex mixing, 500 μl of *Isochrysis* algal solution was added to give approximately 2×10^4 cells ml^{-1}. A 20 ml water sample was removed immediately from all the beakers upon the addition of the algae and retained in vials for enumeration of algal cells. Further samples were taken after 15 and 30 min. Algal particles (3 - 10 μm) were analysed using a Beckman Z2 Coulter particle count and size analyzer (Beckman Coulter, Wycombe, UK). From the loss of algal particles, the feeding rates of the mussels were determined. Mussels were stored at -80 °C prior to extraction and quantitation of alkylbenzenes by GC-MS and GC×GC-ToF-MS.

Extraction and quantitation of alkylbenzenes

The extraction of hydrocarbons from mussel tissues was by alkaline saponification, adapted from Kelly et al. [18] and as described by Booth et al. [4]. In brief, phenanthrene d_{10} (internal standard) was added to thawed mussel tissues (~15 g wet wt) and refluxed for 2 h with methanol and potassium hydroxide (~15 g), filtered, then solvent exchanged into hexane. Following reduction in volume and clean-up on 5% deactivated alumina, the extracts were analysed by GC-MS and GC×GC-ToF-MS. Dry weights were obtained from subsamples of wet mussel tissue followed drying at 60 °C for 24 h. To compare the profile of the dissolved phase with that of the BABs within the tissue, 1 L of water was spiked with 10 mg BABs and 100 ml aliquots (n = 3) solvent extracted (3 × 25 ml + 25 ml rinse with dichloromethane). Quantitation of BABs using GC-MS was by measurement of the major resolved component via integration of total ion current and m/z 246 ($M^{+\cdot}$) responses for which a linear calibration of GC-MS response was obtained (r^2 = 0.999; 0-0.06 mg ml^{-1} injected). Quantitation of BABs by GC×GC-ToF-MS was achieved by summation of all the resolved peak areas with fragment ions consistent with alkylbenzenes (m/z 91, 92, 105, 119 and 133). A linear calibration of the GC×GC-ToF-MS response was obtained (r^2 = 0.994; 0-0.01 mg ml^{-1} injected) for the C_{12-14} BABs mixture.

Determination of cell viability

Cell viability of haemolymph cells from mussels exposed to 5 μg l^{-1} for 14-d and from solvent control organisms was assessed based on methodology reported by Rickwood et al. [19]. Haemolymph was extracted by syringe from the posterior abductor muscle of BABs-exposed and solvent control mussels (eight mussels per treatment). Aliquots of haemolymph (50 μl) were incubated, in triplicate, in a flat-bottomed microtitre plate in order to allow a monolayer of cells to adhere to the wells. After 45 min, nonadhered cells were discarded and the plates washed with physiological saline. A solution of 0.004 % neutral red dye in physiological saline (200 μl) was added to each well and the cells incubated for 3 h at 15 °C. The wells were washed and an acidified solution of 1% acetic acid and 20% ethanol was added to resolubilize the dye. The plates were shaken for 10 min before reading the absorbance at 540 nm, in an Optimax tunable microplate reader (Molecular Devices, Sunnydale, Ca, USA)) using SoftMax Pro (Ver 2.4.1) software. Protein concentration was determined following the method of Bradford et al. [20]. Results were calculated as optical density per milligram of protein.

Gas chromatography - mass spectrometry

The aromatic hydrocarbon mussel tissue extracts and commercial BABs mixture were examined on a Hewlett Packard GC-mass spectrometry detector (Agilent Technologies, Wilmington, DE, USA) as described by Booth et al. [4]. This was comprised of a HP5890 Series II gas chromatograph fitted with a HP7673 auto-sampler and a HP5970 quadrupole mass selective detector. The column was a HP1-MS fused silica capillary column (30 m x 0.25 mm inner diameter × 0.25 μm film thickness). The carrier gas was helium at a constant flow of 1.0 ml min^{-1}. A 1.0 μl sample was injected into a 250 °C splitless injector. The oven temperature was programmed from 40 to 300 °C at 10 °C min^{-1} and held for 10 min. Data and chromatograms were monitored and recorded using ChemStation (Ver B.02.05) software (Agilent Technologies). The quadrupole mass spectrometer used ionisation energy of 70 eV and an ion source temperature of 280 °C, operated in full scan mode, with a mass range of 50 to 550 Daltons monitored.

Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry

The aromatic hydrocarbon mussel tissue extracts and commercial BABs were analysed on a Pegasus 4D (Leco®, St. Joseph, MI, USA) GC×GC-ToF-MS system, based on a Agilent 6890 gas chromatograph (Agilent Technologies) interfaced to a Pegasus III time-of-flight mass spectrometer (Leco). The system used the following parameters: injector 300 °C, transfer line 280°C. The first-dimension column was a 5 % phenyl- 95 % methyl-polysiloxane 28.9 m × 320 μm × 0.25 μm DB-5 (J&W Scientific, Wilmington, DE, USA), and the second-dimension column was a 50 % phenyl polysilphenylene-siloxane 2.0 m × 100 μm × 0.1 μm DPX-50 (SGE, Melbourne, Australia). The first-dimension oven was held at 70 °C for 0.2 min, then raised from 70 to 240 °C at 5 °C min^{-1} then raised to 270 °C at 20 °C min^{-1} and held at this temperature for 5 min. The second-dimension oven was held at 85 °C for 0.2 min, then raised from 85 to 245 °C at 5 °C min^{-1} then raised to 285 °C at 20 °C min^{-1} and held at this temperature for 5 min. A second dimension modulation period of 4 s was employed. The modulator hot temperature was offset 30 °C above secondary oven temperature with a hot pulse time of 1.0 s, and cool time between stages of 1.0 s; the cold temperature during trapping was estimated at -140 °C; electronic pressure control was used in constant flow mode at 1.5 ml min^{-1}. The carrier gas was helium 99.9999%. Sample was injected (1 μl, splitless) into the GC×GC-ToF-MS system via an Agilent Technologies 7863 series autosampler. A ToF-MS was used as the detector, and operated at a spectrum storage rate of 100 Hz (100 spectra s^{-1}) based on 5 kHz transients. The system used the following parameters: ion source 250 °C, EM Electron Multiplier 1750 V. The mass range monitored was from 40 to 500 Daltons. Automated data processing was achieved using Leco ChromaTof™ software (Ver 2.01, Leco). The software was used to complete a peak finding routine, the deconvolution of mass spectra from partially coeluting compounds and a preliminary National Institute of Standards and Technology (Gaithersburg, MD, USA) library search.

Statistical analyses

Statistical analyses of results were performed using Statgraphics Plus 5.1 (Herndon, Virginia, USA). Following checks for variance using Levene's test, data were analysed by analysis of variance. Where there was a significant difference ($p \leq 0.05$) of means, the data were further analysed by Student-Newman-Keuls test to determine significant differences ($p \leq 0.05$) between treatments. Comparisons between two treatments were performed using unpaired *t* tests following checks for standardized skewness and standardized kurtosis.

RESULTS

Water quality remained high throughout the tests and all mussels survived. Clearance rates of mussels exposed to 0.005 % solvent were not significantly different ($p > 0.05$) to the seawater control organisms' rates. Acute 72 h exposures to C_{12-14} BABs at 5 μg l^{-1} resulted in a small reduction in mussel mean clearance rates of 13 % compared to control values, confirming the reproducibility of the method (previous reported reduction [4] was also 13 % conducted a year earlier). The longer-term exposure of 14 d resulted in a significant ($p \leq 0.05$) relative decrease in clearance rates of 25 % (Fig. 1). Following the depuration period of 5 d, the mussels partially recovered but clearance rates were still significantly lower (17 %, $p \leq 0.05$) than control values (Fig. 1). No significant differences ($p > 0.05$) were found between the BABs-exposed mussels' cell viability and that of the control organisms.

Quantitation of alkylbenzenes by GC-MS based on the methodology as used by Booth et al. [4], gave values of 46 to 47 μg g^{-1} dry weight for the tissues of mussels exposed for 14 d and 16 to 25 μg g^{-1} dry weight for the tissues of mussels following depuration for 5 d in clean seawater (**Table 1**). Quantitation of alkylbenzenes by GC×GC-ToF-MS was determined by summation of the areas of all the alkylbenzene peaks in the tissue extracts and interpolation from the linear standard curve of the summed peaks of the C_{12-14} BABs mixture. This gave values for the 14-d exposed mussels of 31 μg g^{-1} dry weight and 11 to 15 μg g^{-1} dry weight for the depurated

mussels (Table 1). Assuming steady state conditions, a log bioconcentration factor (BCF) of 2.9 was estimated. Using the relationship between log BCF and the octanol-water co-efficient, log K_{OW}, given by van Gestel et al. [21], a log K_{OW} value of 4.2 is implied for the C_{12-14} BABs mixture. Re-analyses by GC×GC-ToF-MS of the mussel extracts examined by the GC-MS in the previous study [4], gave tissue concentration values from 7 to 27 μg g^{-1} dry weight for nominal aqueous exposure concentrations of 5 to 41 μg l^{-1} (Table 1). From these GC×GC-ToF-MS data, the tissue concentration EC20 was calculated to be 6 μg g^{-1} dry weight. This compares favourably to the value of 10.5 μg g^{-1} dry weight as determined previously using GC-MS [4].

The total ion chromatogram of the C_{12-14} BABs mixture (Fig. 2,) formed a UCM type profile that was clearly apparent within of the BABs spiked water and BABs-exposed mussels (Fig. 2). Although a few major peaks were resolved by conventional GC and produced mass spectra consistent with alkylbenzenes, the majority of peaks were unresolved and clear spectra unobtainable. Analysis by GC×GC enabled much better resolution and the alkylbenzenes formed a distinct band within the two-dimensional peak marker plot (Fig. 3). The number of peaks identified as alkylbenzenes by GC×GC-ToF-MS in the tissue extracts of mussels exposed for 72 h to 41 μg l^{-1} was 111 (Fig. 3). This was reduced to 28 peaks for the exposure to BABs for 72 h 5 μg l^{-1} (acute exposure). As the exposure concentration reduced, peaks due to BABs were reduced in number from across both the 1st dimension (nonpolar) and 2nd dimension (polar) GC retention time range but most apparently at the earlier elution times of the 1st dimension. The peak marker distributions of the extracts from the 14-d 5 μg l^{-1} exposed mussels (136 – 166 peaks) were virtually identical to those derived from the acute 41 μg l^{-1} exposed mussels (Fig. 3). The distributions of the peaks due to BABs from the depurated mussels (118 – 123 peaks) were also similar to those in the 14-d exposed mussels, with some peaks lost across the 1st dimension range including the later eluting peaks (Fig. 3). This small difference is consistent with the only partial recovery in clearance rates (Fig. 1). A small number (9) of alkylbenzenes was detected in the control mussels exposed to solvent and seawater only. The mass spectra were consistent with those of linear alkylbenzenes and only very small concentrations were present. Mussels exposed to seawater or solvent had tissue concentrations of alkylbenzenes less than 1 μg g^{-1} by either analysis method. These small amounts of LABs are probably due to traces of detergent residues from glassware washing, per Eganhouse et al. [14].

DISCUSSION

To our knowledge this is the first reported use of GC×GC-ToF-MS for quantitation of bioaccumulated (un)resolved complex mixtures of toxic hydrocarbons in biota. Most research into petrochemical hydrocarbons using GC×GC has utilised flame ionisation detection and although attempts were made to use GC×GC with quadrupole mass spectrum analysis [22, 23], the peaks eluting from the second dimension column were very narrow (typically 100–200 ms) whereas time-of-flight MS can deliver the high acquisition rates necessary for quantitative description of the peaks [24]. One of the main advantages of GC×GC-ToF-MS is the very high separation power making the technique ideal for unravelling the composition of complex mixtures [25]. Another important feature of GC×GC is that chemically related compounds show up as ordered structures within the chromatograms, i.e. isomers appear as distinct groups in the chromatogram as a result of their similar interaction with the second dimension column phase [26]. As a consequence the alkylbenzenes form a distinct band of peaks with compounds exhibiting greater alkylation eluting later in the 1st dimension (Fig. 3). The use of GC×GC-ToF-MS in the present study has shown with greater clarity than with GC-MS, that a broad range of C_{12-14} BABs are slowly accumulated by mussels to the point where their ability to feed is significantly reduced and that 5 d within clean seawater is insufficient to allow the mussels to recover fully.

Considering the complexity of the C_{12-14} BABs mixture, the calculated tissue concentrations were remarkably similar using the two analysis methods (Table 1). Indeed, the tissue concentration EC20 based on the GC×GC-ToF-MS measurement of 6 μg g^{-1} dry weight, was close to that calculated previously by GC-MS (10.5 μg g^{-1} dry wt) [4]. The tissue concentrations of the mussels exposed to 5 μg l^{-1} for 14 d were measured to be 46 to 47 μg g^{-1} dry weight by GC-MS; this was a little lower than that accumulated by the mussels exposed to 41 μg l^{-1} for 72 h (57 μg g^{-1} dry wt) and was consistent with the slightly lower effect upon the 14-d exposed mussels. Tissue concentration measurements of 14-d exposed mussels based on GC×GC-ToF-MS were slightly higher (31 μg g^{-1} dry wt) than those calculated for the 72 h, 41 μg l^{-1} exposed mussels (27 μg g^{-1} dry wt) but the measurements were in broad agreement and were certainly consistent with the observed biological effects (Table 1, Fig. 1).

Crude oil contains both LABs and BABs [27, 28] but environmental of spilled oil degradation by bacteria results in gradual removal of LABs and a relative increase in concentration of the BABs which are more resistant to microbial attack [29]. Indeed LABs have been shown to suffer up to 99% degradation under some conditions [30]. Mussels were found to accumulate BABs when exposed to an aqueous aromatic fraction of the biodegraded UCM-dominated Venezuelan crude oil, Tia Juana Pesado, for 48 h but failed to fully depurate these

compounds after 5 d in clean seawater, whereas other compound groups, such as the alkylnaphthalenes, were fully depurated [31]. Marine organisms are likely to experience pulses of hydrocarbon contamination and therefore may be able to depurate compounds during periods of clean seawater. The results of the present study are in agreement with that of Scarlett et al. [31] and show that alkylbenzenes accumulate in the tissues of mussels and are not always readily depurated, leading to this group of hydrocarbons becoming an ubiquitous component of UCMs in contaminated mussels, as identified in North Sea mussels by Booth et al. [4]. The log K_{ow} value of 4.2, derived from the estimated log BCF of 2.9, is considerably lower than that of the linear C_{12} alkylbenzene, decylbenzene (log K_{ow} 7.35, http://logkow.cisti.nrc.ca/logkow [32]), signifying that the branched isomers maybe more bioavailable but are still highly bioaccumulative. It should be noted that the exposures reported herein and Booth et al [4] were carried out in the presence of the alga *I. galbana*. This may have influenced the uptake of compounds as it has been reported that bioaccumulation of nonpolar hydrocarbons can be enhanced in the presence of algae [33]. However, this would also occur for wild mussels. The profile of the BABs aqueous phase extract matched that of the mussel tissue and therefore if adsorption to the algae did occur this did not result in biased uptake of specific compounds by the mussels. The results also demonstrate that the BABs will accumulate in the tissues at sufficient concentrations to cause sublethal effects and thus reduce the health status of exposed mussels. Wild mussels with low scope for growth, collected from Southend, UK, have been reported to contain around 100 $\mu g\ g^{-1}$ dry weight aromatic UCM [4] corresponding to an estimated alkylbenzene concentration of 45 $\mu g\ g^{-1}$ dry weight. Mass spectra, obtained by GC×GC-ToF-MS, of the BABs compounds closely match the alkylbenzenes found within the tissue extracts of wild mussels [4]. Thus the tissue concentrations and effects on clearance rates reported herein are consistent with data obtained from wild mussel populations. However, the accumulation of the BABs did not result in loss of cell viability. Martins et al. [10] suggested that reduction in membrane lysosomal stability (essentially a similar measure as loss of membrane lysosomal integrity but with reduction in neutral red retention time as an endpoint) of bivalves from a petroleum hydrocarbon polluted bay in Brazil could, in part, be explained by the concentration of aromatic UCM hydrocarbons. The results of the present study do not suggest that toxic effects arising from accumulation of alkylbenzenes contribute to the loss of haemolymph lysosomal stability.

CONCLUSIONS

Quantitation by GC×GC-ToF-MS of complex mixtures of alkylbenzenes accumulated in the tissues of *M. edulis* revealed that a broad range of alkylbenzene isomers was accumulated by *M. edulis*, resulting in reduced clearances rates. The accumulated compounds were not fully depurated within a 5 d period of exposure to clean seawater and the organisms failed to recover fully. Consequently, branched alkylbenzenes in the marine environment arising from petroleum hydrocarbon contamination, can bioaccumulate and persist as a common component of the UCM within tissues, where they can impact the health of exposed organisms. The mussel tissue concentrations of complex mixtures of alkylbenzenes and their corresponding effects reported herein are consistent with observations of wild mussel populations.

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Tables and Figures

Table 1. Comparison of tissue concentrations measured by gas chromatography – mass spectrometry (GC-MS) and comprehensive two-dimensional gas chromatography – time-of-flight–mass-spectrometry (GC×GC-ToF-MS) following acute (3-d), and long-term (14 d) exposure plus 5 d depuration in clean seawater, to a complex mixture of $C_{12\text{-}14}$ branched alkylbenzenes

Nominal aqueous exposure ($\mu g\ l^{-1}$)	Exposure length (d)	Tissue conc. by GC-MS ($\mu g\ g^{-1}$ dry wt)	Tissue conc. by GC×GC-ToF-MS ($\mu g\ g^{-1}$ dry wt)
05	3	11.1*	7.5
10	3	19.8*	11.6
20	3	39.5*	23.5
41	3	56.5*	27.0
05 (Replicate 1)	14	46.2	31.1
05 (Replicate 2)	14	47.3	31.4
Depuration (1)	5	16.4	11.3
Depuration (2)	5	25.0	15.3

*First reported by Booth et al. [4]

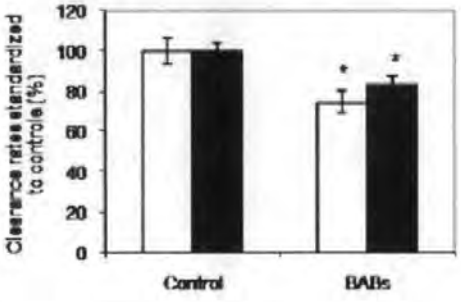

Fig. 1. Effect of 14 d exposure (□) to a complex mixture of $C_{12\text{-}14}$ branched alkylbenzenes (BABs) of aqueous concentration 5 $\mu g\ l^{-1}$ and recovery following 5 d depuration (■) in clean seawater. Treatments significantly different ($p \leq 0.05$) are signified by *.

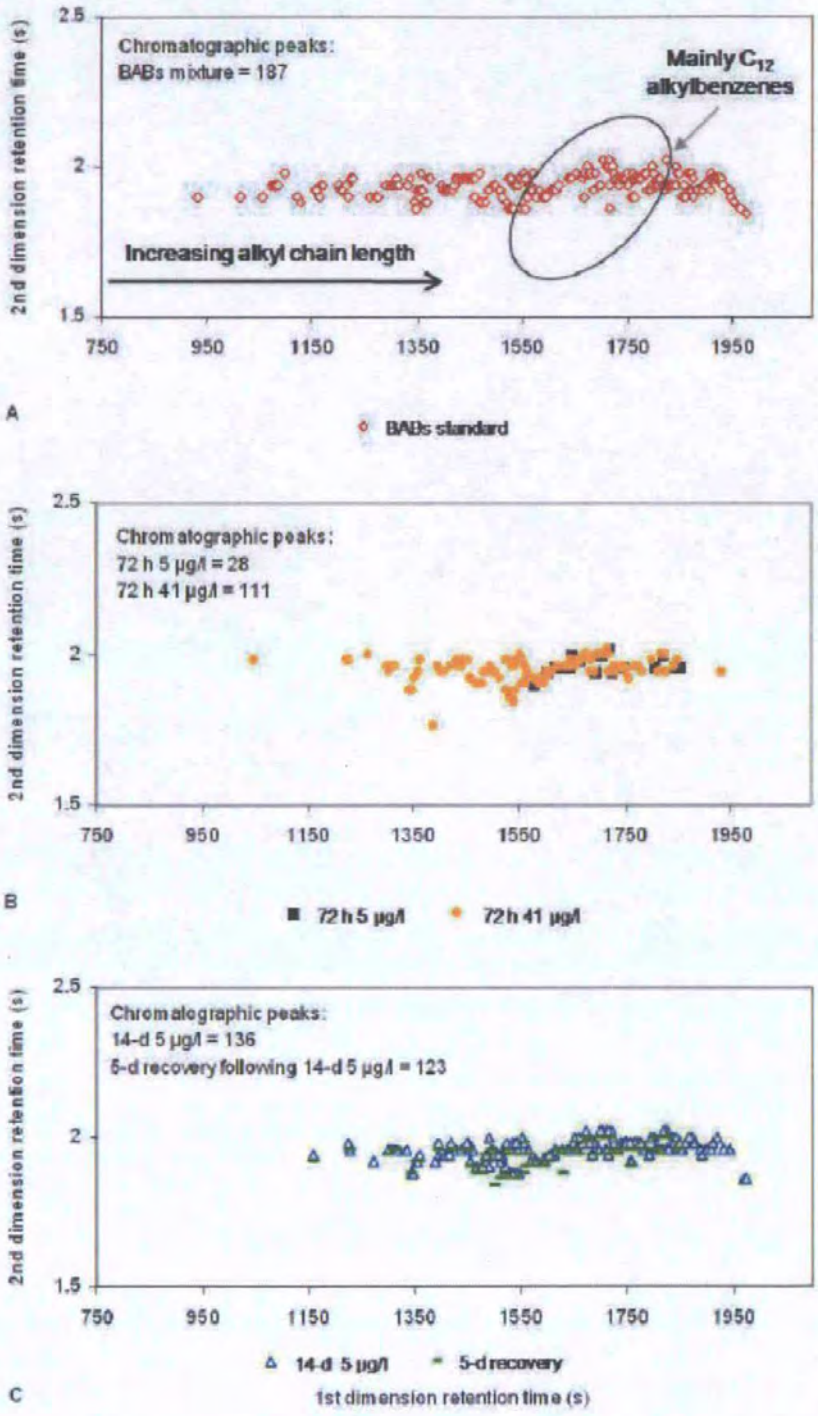

Fig. 3. Chromatographic peak marker plots derived by comprehensive two-dimensional gas chromatography – time-of-flight–mass-spectrometry (GC×GC-ToF-MS) analysis of a complex mixture of C_{12-14} branched alkylbenzenes (BABs, A) and those accumulated in mussel tissues following 72 h exposure (B), and 14 d exposure plus 5 d depuration (C).

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Unresolved Complex Mixtures of Aromatic Hydrocarbons: Thousands of Overlooked Persistent, Bioaccumulative, and Toxic Contaminants in Mussels

ANDY M. BOOTH,† PAUL A. SUTTON,† C. ANTHONY LEWIS,† ALASTAIR C. LEWIS,‡ ALAN SCARLETT,† WING CHAU,† JOHN WIDDOWS,§ AND STEVEN J. ROWLAND*,†

Petroleum & Environmental Geochemistry Group, University of Plymouth, Plymouth, PL4 8AA, Devon, U.K., Department of Chemistry, University of York, Heslington, YO10 5DD, Yorkshire, U.K., and Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, PL1 3DH, Devon, U.K.

Comprehensive two-dimensional gas chromatography–time-of-flight–mass-spectrometry can be used to resolve and identify individual petroleum-derived hydrocarbons in unresolved complex mixtures (UCMs), such as those accumulated by mussels (*Mytilus edulis*). Mussels exhibiting a range of scope for growth values were collected from sites around the UK coast. Tissue extracts from mussels exhibiting impaired health contained large amounts of aromatic hydrocarbon UCMs compared to the extracts from healthy mussels. The UCMs (up to 125 $\mu g\ g^{-1}$ dry tissue) contained thousands of previously unidentified branched alkyl homologues of known aromatic hydrocarbons such as branched alkylbenzenes (BABs), tetralins (BATs), and indanes and indenes (BINs). The toxicity of few such alkyl branched compounds has been investigated previously, but here we show that a commercial mixture of BABs (C_{12}–C_{14}) is toxic to mussels in laboratory tests (11–57 $\mu g\ g^{-1}$ dry tissue), reducing feeding rate by up to 40% in 72 h. Thus, some, if not all aromatic UCMs, apparently comprise potent mixtures of persistent, bioaccumulative and toxic compounds which have been overlooked previously.

Introduction

No feature of hydrocarbon-contaminated environmental samples can have been so commonly encountered by scientists as the unresolved complex mixture (UCM) (Figure 1). Yet most studies have done little more than record the presence of UCMs, with just a few authors even reporting concentrations of UCMs (which are typically in the high parts-per-million range and, therefore, exceed those of many individually resolved priority pollutants) (*1*, *2*). Previous attempts to identify UCM constituents have relied mainly on degradative methods (*2*–*4*), modeling approaches (*5*–*7*), or multistep chromatographic procedures (*8*). The latter

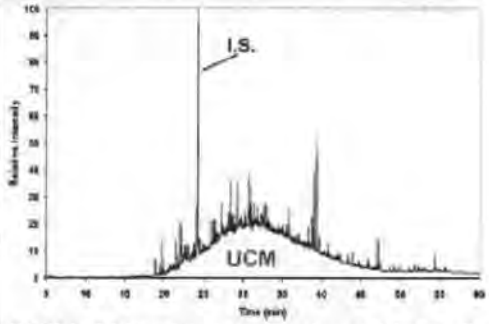

FIGURE 1. Total ion current gas chromatogram of the aromatic hydrocarbons extracted from mussels, *Mytilus edulis* sampled from Southend, U.K. I.S. = internal standard, d_{10}-phenanthrene, 5 μg g dry mussel tissue^{-1}; UCM = unresolved complex mixture; ca. 100 μg g dry mussel tissue^{-1}.

suggests that thousands of chemicals may be present in some aromatic UCM fractions. However, separation of this number of compounds represents a formidable chromatographic challenge.

Nonetheless, a growing body of evidence suggests that UCMs are important environmental toxicants (*9*). A "monoaromatic" UCM isolated from a Norwegian crude oil was rapidly accumulated by mussels, *M. edulis* and reduced feeding rate by over 40% in 24 h, illustrating a significant narcotic toxic response (*9*). Aromatic UCMs isolated from mussels collected from a polluted harbor also significantly reduced the feeding rates of clean laboratory mussels (*10*) with an operationally defined "monoaromatic" HPLC fraction exhibiting most toxicity. The retention time of this toxic UCM fraction corresponded to monoaromatic hydrocarbons in the range 4–6 double bond equivalents. Lowered scope for growth (SfG; a measure of mussel health in which feeding rate is a major component) and adverse population effects, also correlated with increased UCM concentrations in five out of six UK coastal sites (*11*). The "total toxic hydrocarbon" burden proposed to explain lowered SfG in mussels from a further four UK coastal sites correlated with the concentrations of aromatic UCMs in the same mussels (*9*).

Given the inadequacy of conventional gas chromatography (GC) methods to resolve UCMs, a number of studies oxidized UCMs, including aromatic fractions, and examined the partially resolved oxidation products by GC–mass spectrometry (GC–MS) and other conventional techniques (*2*–*4*, *12*). This led to postulations of a number of structural types for UCM hydrocarbons, including "T"-branched hydrocarbons (*2*–*4*) and alkylaromatics such as alkyltetralins (*12*). Three alkyltetralins were synthesized and tested for narcotic toxicity. They reduced the feeding rate of mussels by up to 70% in 24 h, again suggesting that aromatic UCM hydrocarbons are toxicologically important and require more rigorous identification (*13*). Resolution of these complex mixtures finally proved possible by means of comprehensive multidimensional GC, in which the individual chromatographic peak capacity of two GC columns is multiplied together (*14*–*17*). However, with a nonspecific flame ionization detector (FID), identification of the unknowns relied entirely on the chromatographic elution orders of the compounds. The least polar compounds (e.g., alkanes and cycloalkanes) have the shortest second dimension retention times with more polar compounds having longer retention

* Corresponding author phone: +44 1752 233 013; fax: +44 1752 233 035; e-mail: srowland@plym.ac.uk.
† University of Plymouth.
‡ University of York.
§ Plymouth Marine Laboratory.

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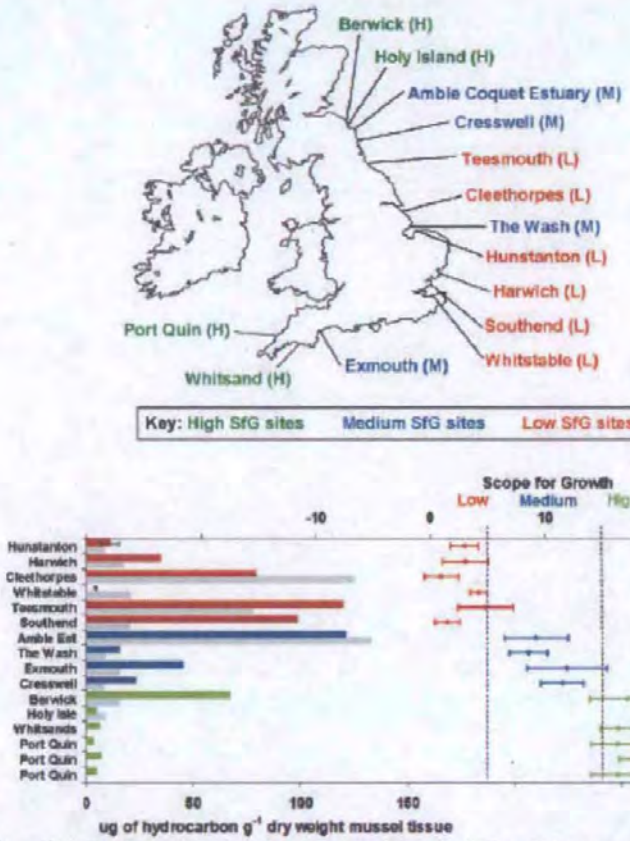

FIGURE 2. A. Map of U.K. showing locations at which populations of mussels, *Mytilus edulis* were sampled for analysis. Color code indicates Scope for Growth (health) of mussels (green = high; blue = medium, red = low) (*16*). B. Histogram and bar chart showing relationships between Scope for Growth (health) of mussels (Right: green = high; blue = medium; red = low) and concentrations of the total toxic hydrocarbons (*16*) (Left; gray) and aromatic UCM hydrocarbons (Left; red, blue, green) extracted from mussels, *Mytilus edulis* sampled from U.K. locations (n = 10–35 mussels per location). Mussel populations from Hunstanton (mean n = 21) were sampled and analyzed in duplicate such that the range of UCM values provides an estimate of the analytical error. Samples from Port Quin (mean n = 27) were sampled and analyzed in triplicate and separate SfG measurements were made for each population. Thus the range of values for Port Quin samples provides an estimate of the full procedural reproducibility (analytical error plus sample population differences). The aromatic UCM concentrations in mussels from Whitstable were not determined herein and the total toxic hydrocarbons in Port Quin mussels were not determined previously (*16*).

times (e.g., benzenes < naphthalenes < phenanthrenes). This ordered nature of two-dimensional chromatograms makes identification of compounds readily achievable. The use of known compounds can provide specific information, but this approach is not suitable for the analysis of complex mixtures (*14*). The recent coupling of comprehensive two-dimensional GC with time-of-flight–MS (ToF–MS), however, provides a further degree of specificity in which the molecular weights and mass spectra of the (now resolved) unknowns can be obtained. Due to its high data acquisition rate (up to 1000 full scan spectra s^{-1}), ToF–MS is the only mass spectrometric detector fast enough to handle the narrow chromatographic peaks (typically 150 ms) produced by the second dimension column. In addition, it has a high spectral deconvolution power. Here, we describe the use of two-dimensional gas chromatography–time-of-flight–mass-spectrometry (GCxGC–ToF–MS) to resolve and identify groups of alkylaromatic hydrocarbons comprising thousands of new compounds in mussels with measurable differences in SfG (from 14 UK sites, with analysis of replicate mussel populations from two sites). Furthermore, we show that a commercial complex mixture of alkylaromatics with similar GCxGC retention times and ToF mass spectra to those in some of the polluted mussels is toxic to mussels in laboratory tests, reducing feeding rate significantly compared with untreated control organisms.

Materials and Methods

Collection and Extraction of Mussel Tissue Samples. Mussels (*Mytilus edulis*), of shell length 3–4 cm, were collected from sites around the UK over the period 1995–2001 (Figure 2). Following storage at −20 °C, the mussels were defrosted and the tissue removed from the shells using a scalpel. Extraction of the mussel tissue was based on the method described by Wraige (*18*) who previously compared the recovery efficiencies of a range of aromatic hydrocarbons

from the different techniques available. The tissues were then homogenized using a hand-held blender. Authentic d_{10}-phenanthrene (Aldrich Chem. Co; purity >98%) was employed as an internal standard, and 50 μg was added in acetone to the wet tissue (~35 g) from each sample site and mixed thoroughly. The mussel tissue was ground with anhydrous sodium sulfate to remove water. The resulting mixture was transferred to cellulose thimbles and Soxhlet extracted with dichloromethane (DCM) for 24 h. Anhydrous sodium sulfate was added to the total organic extracts (TOEs) to remove any remaining moisture. The TOEs were concentrated using rotary evaporation (Büchi, 35 °C), transferred to 7 mL vials and the remaining solvent was removed under a gentle stream of nitrogen.

Open Column Chromatography. The column chromatography method used was that reported by Wraige (*18*). The TOEs were dissolved in hexane (1 mL) and transferred onto a sintered glass column (20 mm i.d. × 400 mm) packed with alumina on silica (1:1 w/w, 20 g each). The adsorbents were activated at 110 °C overnight prior to use. The silica gel (SiO_2, Aldrich, grade 645, 60–100 mesh) was employed in a fully activated state, with the alumina (Al_2O_3, BDH, England; grade 1, neutral, 150 mesh) being deactivated (1.5%) with Milli-Q water. The silica and alumina were each slurried using hexane and packed into the column, alumina above silica. The column was then sequentially eluted with solvents of increasing polarity to yield the desired fractions.

F_1 (aliphatic); 1.5 column volumes of *n*-hexane

F_2 (aromatic); 2 column volumes of *n*-hexane:DCM (1:1 v/v)

F_3 (polar1); 2 column volumes of DCM

F_4 (polar2); 2 column volumes of methanol

The fractions (F_1–F_4) were concentrated using rotary evaporation (Büchi, 35 °C), transferred to preweighed vials and gently blown to dryness (N_2). GC–MS analysis (Hewlett-Packard HP5890 series II fitted with a HP5970 mass selective detector; HP1-MS fused silica capillary column, 30 m × 0.25 mm i.d. × 0.25 μm film thickness) of the F_1 and F_2 (aliphatic and aromatic) fractions revealed the samples were dominated by large amounts of polar biogenic lipids from the mussel tissue (SI-1).

Alkaline Saponification. Alkaline saponification was used to saponify the biogenic lipids in the extract so that the hydrocarbons could be isolated. The method used is that described by Allard et al., (*19*). Potassium hydroxide (6.5 g) was dissolved in 100 mL of an 80% methanol : 20% water (Milli-Q) mixture. Excess (2–3 mL) KOH methanol/water solution was added to each sample and heated for 1 h at 80 °C. The remaining hydrocarbons were isolated using 3 × 2 mL hexane extractions, and the extracts were dried using anhydrous sodium sulfate. A final micro column chromatography "cleanup" step developed in-house was employed to isolate the pure "aliphatic" and "aromatic" fractions. Fully activated silica gel (SiO_2, Aldrich, grade 645, 60–100 mesh) was placed in a Pasteur pipet plugged with extracted cotton wool. The silica was wetted with 3–4 mL of hexane, and the aliphatic and aromatic tissue extracts were then transferred to the column in hexane. Each column was sequentially eluted with 6 mL of DCM to isolate the aliphatic or aromatic hydrocarbons.

Gas Chromatography–Mass Spectrometry (GC–MS). The aromatic hydrocarbon mussel tissue extracts and commercial BABs mixture were examined on a Hewlett-Packard GC–MSD. This comprised a HP5890 Series II gas chromatograph fitted with a Hewlett-Packard HP7673 auto-sampler and a HP5970 quadrupole mass selective detector. The column was a HP1-MS fused silica capillary column (30 m × 0.25 mm id × 0.25 μm film thickness). The carrier gas was helium at a constant flow of 1.0 mL min^{-1}. A 1.0 μL sample was injected into a 250 °C splitless injector. The oven temperature was programmed from 40 to 300 °C at 10 °C min^{-1} and held for 10 min. Data and chromatograms were monitored and recorded using ChemStation (version B.02.05) software. Since no standard method for quantification of the UCM is currently available, quantification of the aromatic UCMs was made by comparison of sample UCM areas with calibration data from known concentrations of an oil-derived aromatic UCM and corrected for internal standard recoveries (*20*). The quadrupole mass spectrometer used ionization energy of 70 eV and an ion source temperature of 280 °C. It was operated in full scan mode, with a mass range of 50–550 Daltons monitored.

Comprehensive GCxGC–ToF-MS. The aromatic hydrocarbon mussel tissue extracts and commercial BABs were analyzed on a Pegasus 4D (Leco Corporation, U.S.) GCxGC–ToF-MS system, based on a Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, DE) interfaced to a Pegasus III time-of-flight mass spectrometer (LECO, St Joseph, MI). The system used the following parameters: injector 300 °C; transfer line 280 °C. The first-dimension column was a 5% phenyl–95% methyl-polysiloxane 10 m × 180 μm × 0.25 μm HP-5 (J&W Scientific, Wilmington, DE), and the second-dimension column was a 14% cyanopropylphenyl-polysiloxane 1 m × 100 μm × 0.1 μm BP-10 (SGE, Melbourne, Australia). The first-dimension oven was held at 40 °C for 0.2 min, then raised from 40 to 160 °C at 10 °C min^{-1} and held at this temperature for 1 min, then raised from 160 to 270 °C at 3 °C min^{-1} and held at this temperature for 30 min. The second-dimension oven was held at 50 °C for 0.2 min, then raised from 50 to 170 °C at 10 °C min^{-1} and held at this temperature for 1 min, then raised from 170 to 280 °C at 3 °C min^{-1} and held at this temperature for 30 min. A second dimension modulation period of 4 s was employed. The modulator hot temperature was offset 30 °C above secondary oven temperature with a hot pulse time of 1.0 s, and cool time between stages of 1.0 s; the cold temperature during trapping was estimated at −140 °C; electronic pressure control was used in constant flow mode at 1.5 mL min^{-1}. The carrier gas was helium 99.9999%. One μL of the sample was injected (splitless) into the GCxGC–ToF-MS system via an Agilent Technologies 7863 Series autosampler. A time-of-flight–mass spectrometer (ToF–MS) was used as the detector, and operated at a spectrum storage rate of 100 Hz (100 spectra s^{-1}), based on 5 kHz transients. The system used the following parameters: ion source 250 °C, EM 1750 V. The mass range monitored was from 40 to 500 Daltons. The automated data processing was achieved using LECO ChromaToF software (version 2.01, Leco Inc., U.S.). The software was used to complete a peak finding routine, the deconvolution of mass spectra from partially coeluting compounds and a preliminary NIST library search.

Toxicity Assays

Test Chemicals. A mixture of C_{12}-$_{14}$ branched alkylbenzenes (BABs) was obtained from Chevron and *n*-octylbenzene (98% purity) from Sigma-Aldrich Chemie Gmbh (Munich, Germany).

Collection and Maintenance of Mussels, *Mytilus edulis*. Mussels were collected and maintained as reported previously (*21*) except that a slightly larger size range (47.2 mm, sem = 0.3 mm, n = 45) was used. Mussel tissues from the collection site at Port Quin, on the North Cornwall coast, UK (Ordnance Survey grid reference: SW 972 905) have been reported to contain negligible or no UCM or aromatic hydrocarbons (*10*, *22*). Mussels were maintained in filtered seawater at 15 °C (±1 °C), 35 psu (±2 psu), with a 12:12-h light:dark cycle for a minimum of one week prior to exposure tests.

Alkylbenzene Exposure Tests. Semi-static 72 h exposure tests were similar to the linear alkylbenzene tests described by Donkin et al., (*23*), except that groups of nine mussels

were exposed in 9 L of test compound instead of 16 mussels in 18 L as previously reported. Test solutions (*n*-octylbenzene 42 $\mu g\ L^{-1}$ and C_{12-14} branched alkylbenzenes 5, 10, 20, 41, 82 $\mu g\ L^{-1}$) were prepared by injecting 0.5 mL of an acetone solution of the test compound into 10 L of filtered seawater held at 15 °C in a glass aspirator (i.e., acetone conc. 0.005% v/v). The test solution was vortex mixed (magnetic system with Teflon-coated follower) for a minimum of 2 h prior to use. The test solutions were added to the mussel exposure vessels and replaced every 24 h. Mussels were fed continuously with *Isochrysis galbana* (Reed Mariculture Inc., Campbell, CA, 0.11−0.15 mg dry weight mL^{-1}) delivered via glass Pasteur pipettes by means of a peristaltic pump at a rate of ~20 mL h^{-1}. Aeration was supplied via glass Pasteur pipettes which also aided dispersion of the *Isochrysis*. Water quality measurements of dissolved oxygen, pH, salinity, and temperature were recorded daily prior to water exchange.

Measurement of Feeding Rate. The feeding rate assay was adapted from Donkin et al., (*23*, *24*) and as reported by Scarlett et al., (*21*). In brief: mussels were placed individually in 400-mL glass beakers containing 350 mL of clean seawater. After an acclimation period with slow vortex mixing, 500 μL of *Isochrysis* algal solution was added to give ~2 × 10^4 cells mL^{-1}. A 20 mL water sample was removed immediately from all the beakers upon the addition of the algae and retained in vials for algae enumeration. Further samples were taken after 30 min. Algal particles (3−10 μm) were analyzed using a Beckman Z2 Coulter particle count and size analyzer (Beckman Coulter, Wycombe, UK). From the loss of algal particles during the 30-min period, the feeding rates of the mussels were determined. Mussels were stored at −80 °C prior to extraction and quantification of alkylbenzenes by GC−MS.

Extraction and Quantification of Alkylbenzenes. The extraction of hydrocarbons from mussel tissues was by alkaline saponification as described by Kelly et al., (*25*) except that hexane was used in the solvent exchange step in preference to pentane. In brief: phenanthrene d_{10} (internal standard) was added to thawed mussel tissues (~15 g wet weight) and refluxed for 2 h with methanol and potassium hydroxide (~15 g), filtered, then solvent exchanged into hexane. Following reduction in volume and cleanup on 5% deactivated alumina, the extracts were analyzed by GC−MS. Dry weights were obtained from subsamples of wet mussel tissues followed drying at 60 °C for 24 h. BABs were quantified by measurement of the major resolved component *via* integration of total ion current (TIC) and *m/z* 246 (M^+) responses for which linear calibrations of GC-MS response were obtained ($R^2 \geq 0.999$; 0−0.06 mg mL^{-1} injected).

Results and Discussion

Gas Chromatography Mass Spectrometry. Mussels (*M. edulis*) were collected previously from fourteen UK sites (Figure 2A) and SfG, together with so-called "total toxic hydrocarbon" (TTHC) and concentrations of other selected pollutants, determined (*22*). The TTHC concentration is defined as primarily the aromatic hydrocarbons with log octanol−water partition coefficient ($\log K_{ow}$) values < 5.5 and measured using high performance liquid chromatography with ultraviolet fluorescence detection (*22*). The aromatic UCM concentrations were determined from GC−MS analysis of all the aromatic hydrocarbons in the mussel extract. A relative proportion of approximately 25% aromatic UCM to 75% nonaromatic UCM for polluted mussels collected at New Brighton, UK, has been reported (*10*). The relative proportions of the aromatic UCM in the current study ranged from 17−69% depending upon sample site and SfG (SI-2). GC−MS analysis of the tissue extracts indicated that mussels with low SfG (poor health) often, though not always, contained abundant aromatic hydrocarbon UCMs (e.g., Figure 1). A plot of aromatic UCM concentration ($\mu g\ g^{-1}$ dry weight) against SfG is shown in Figure 2B. In contrast, mussels exhibiting high SfG (good health) generally contained little or no aromatic UCM (the only exception being mussels from Berwick-on-Tweed where there was a discrepancy between the TTHC and UCM concentrations). In four samples examined previously (9), the TTHC concentrations measured by high performance liquid chromatography correlated with the aromatic UCM concentrations measured by GC−MS. This correlation was also found in the expanded data set herein ($R^2 = 0.918$; $n = 15$). Therefore, the reduction in SfG previously attributed to TTHC (*22*) can just as reasonably be ascribed to the effects of the aromatic UCM hydrocarbons in many cases (Figure 2B). Interestingly, mussels from Harwich and Hunstanton exhibited a comparably low SfG, yet contained less aromatic UCM than the other low SfG sites. Widdows et al., (*22*) also reported that the TTHC concentration was too low to solely account for the low SfG observed in mussels at these two sites. Neither metals (Cd, Cu, Hg, Pb, Zn) nor organotins (DBT and TBT), were responsible for the increased reduction of SfG. Instead Widdows et al., (*22*) concluded that this "unexplained component" could be due to the effects of industrial and agrochemical contaminants as chemical analysis indicated these mussels had accumulated significant levels of organochlorine compounds (e.g., dieldrin, DDTs, HCB, HCHs, and PCBs). However, at the time of the study no concentration−response data for mussels were available (*22*). In mussels from most of the other sites (Figure 2A), the observed toxicity is attributable to the aromatic hydrocarbons (Figure 2B). We have now shown that these are almost entirely present as high concentrations of unresolved complex mixtures (UCMs) as well as some well-known resolved compounds (Figures 1 and 2). Therefore the key question is this: what are the individual UCM hydrocarbons which produce these toxic effects in the mussels?

Comprehensive Two-Dimensional Gas Chromatography−Time-of-Flight−Mass Spectrometry. GCxGC−ToF−MS of the aromatic fractions isolated from mussels with high and low SfG illustrated the complexity of the UCMs in the latter (Figure 3). In a typical example, (Figure 3A; mussels from Southend, U.K.) the computer algorithm used to process the data points, revealed over 3400 individually distinguishable components. Even allowing for some degree of "double-counting" for components with closely similar GCxGC retention times, this complexity is unprecedented and the chromatographic resolution quite remarkable. A combination of the ordered nature of the two-dimensional chromatogram and the mass spectral information generated by the ToF−MS were used to study the composition of the aromatic UCMs. Mass fragmentography was used to process the MS data, in which ions typical of the structural features of numerous alkylaromatics were examined (Figure 3B) and mass spectra compared to library spectra. This revealed that important compound classes in mussels with low SfG included alkylbenzenes (ABs), alkyltetralins (ATs), and alkylindanes (INs) (e.g., Figure 4) and others. However, the mass spectral library matches were not exact (Figure 4) and the number of chromatographic peaks in each of these compound classes (Figure 3B) far exceeded the number of possible simple "linear" alkylaromatics such as the linear alkylbenzenes (LABs) found in some environmental samples (*26*, *27*). We suggest this complexity is due to the occurrence of thousands of branched alkylaromatics such as BABs, BATs, BINs, and others. Although the mass spectra of many UCM compounds are similar to those of known compounds, they also show differences attributable to the presence of branched alkyl substituents in the UCM constituents (Figure 4). From the molecular weight ranges of the compounds revealed by

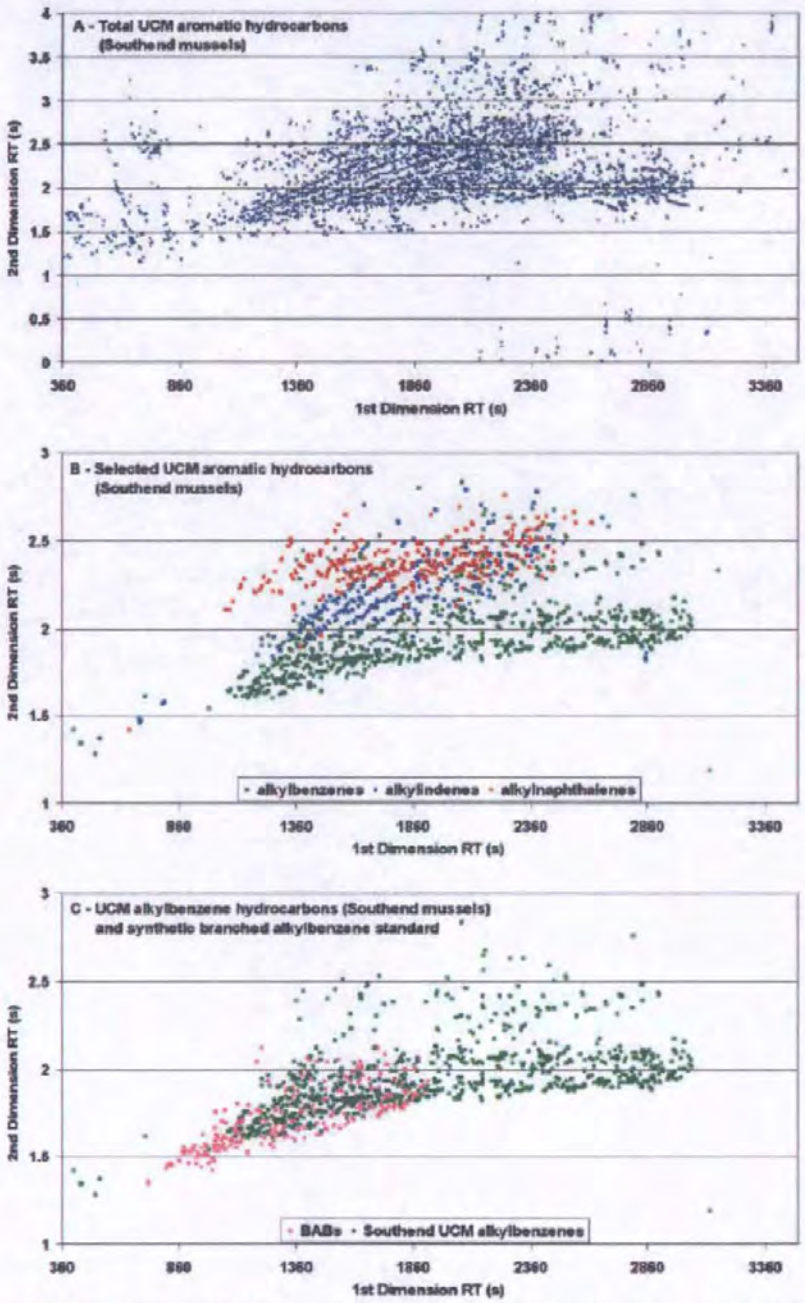

FIGURE 3. Comprehensive two-dimensional gas chromatography−time-of-flight−mass spectrometry two-dimensional chromatograms of aromatic hydrocarbon UCM fractions (cf., Figure 1) showing A, over 3400 peak marker identifiers of components in the complex mixture from mussels from Southend U.K. for which the computer algorithm was able to assign unique mass spectra. B, Peak marker identifiers for each component in the mixture for which the mass spectrum contained base peak ions with mass:charge (*m/z*) ratios 91, 105 (green; alkylbenzenes), 129, 143 (blue: alkylindenes), 141, 155 (red: alkylhaphthalenes). C, over 340 unique peak marker identifiers (pink) for components of a commercial (*26*) mixture of C_{12}−14 branched alkylbenzenes (BABs). Also shown are the peak markers (green) for the BABs in mussels from Southend, U.K. Clearly, many of the BABs isomers in the commercial mixture are also present or are similar to those in the mussels, but the mussels have an even greater number of BABs extending over a greater molecular weight range.

the clarity of the GCxGC−ToF−MS data (e.g., Figure 4), it can be calculated that thousands of compounds are theoretically possible. This explains the complexity of the two-dimensional chromatograms (Figure 3). Some of the theoretically possible isomers are of course unlikely, due to steric constraints, but many are present.

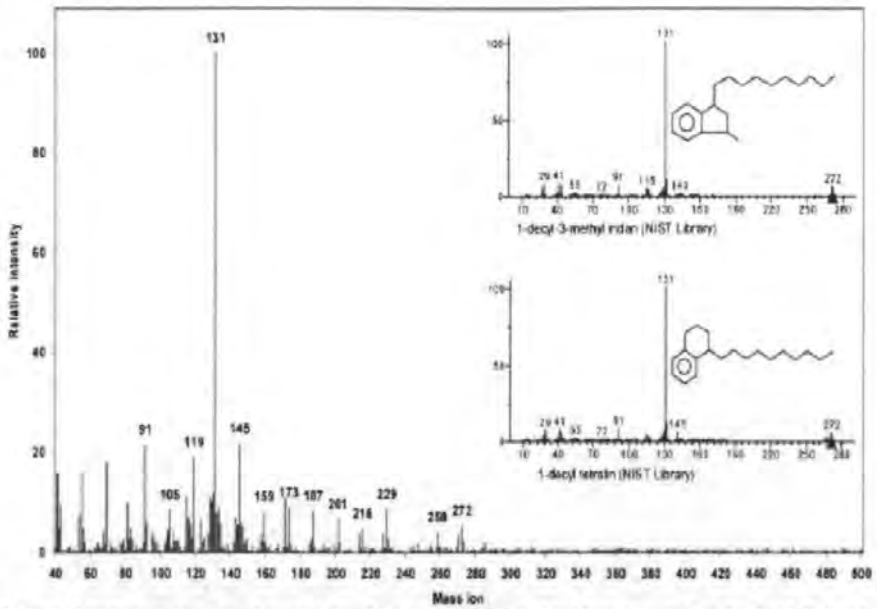

FIGURE 4. Time-of-flight mass spectrum of components of the aromatic UCM isolated from mussels from Southend, U. K. (cf. Figures 1 and 3) with GCxGC retention times 1836 × 1.960 s. The mass spectrum is assigned to a mixture of C_9 and C_{10} branched alkylindans (BINs) and/or branched alkyltetralins (BATs). Inset shows mass spectra of a C_{10} alkylindan (1-decyl-3-methylindan) and a C_{10} alkyltetralin (1-*n*-decyltetralin) from the NIST library of mass spectra. Note that the mass spectrum of the BINs/BATs in the aromatic UCM, while exhibiting the same base peak ion (*m/z* 131) as the library spectra, also show ions indicative of the branched alkyl chains (i.e. *m/z* 229, 215, 201, and so on). These latter are absent, or much reduced, in the library spectra of the known linear substituted compounds.

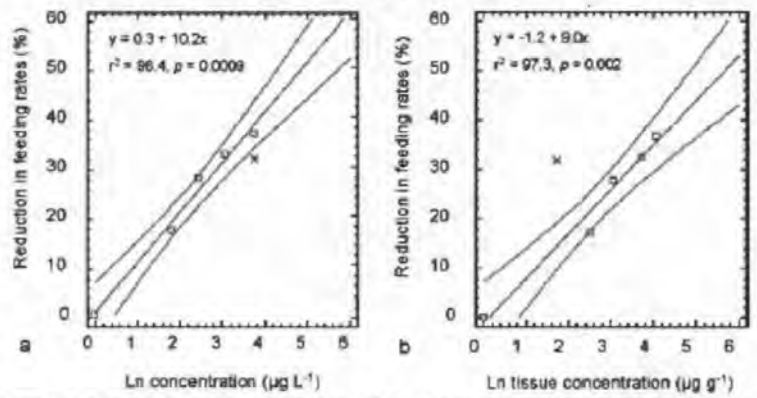

FIGURE 5. Concentration response relationships between branched alkylbenzenes and reduction in feeding rates of mussels *M. edulis*: (a) based on nominal aqueous exposure concentration range 0–41 μg L^{-1} (□) and (b) based on whole tissue concentrations (μg g^{-1} dry weight; □). Curved lines = 95% confidence limits, *x* = effect of reference toxicant *n*-octylbenzene at an aqueous concentration of 42 μg L^{-1}.

Although the UCMs of hydrocarbons are most obvious in chromatograms of biodegraded crude oils and refined fractions such as lubricating oils (*3*), they are present even in undegraded crude oils (*28*). Thus, the UCM hydrocarbons appear to be formed at the same time as the major resolved hydrocarbons of crude oils. We suggest they are comprised of small amounts of thousands of branched pseudo-homologues of the major linear alkylated compounds (e.g., BABs in addition to LABs, BATs in addition to LATs, branched alkylnaphthalenes in addition to linear alkylnaphthalenes, and so on). Since these compounds differ only in the positions of branching on the alkyl chains, they exhibit very similar chromatographic behavior to one another and form UCMs, which are difficult to resolve by conventional GC–MS (Figure 1). Subsequent to oil formation, anaerobic subsurface bacterial action (*29*) or aerobic biodegradation of spilled or refined crude oil in the environment (*1*, *2*) preferentially reduces the linear alkyl hydrocarbons. This results in accumulation of the persistent branched hydrocarbon UCMs over the linear forms in many environmental compartments.

Ecotoxicology. Branched alkylaromatic UCM components are expected to induce toxic effects on mussels because many linear alkylaromatics are already known to reduce the feeding rate of mussels by the mechanism of nonspecific narcosis

(22). For a given carbon number, branched hydrocarbons are generally more water-soluble and, therefore, more toxic than their linear homologues. We, therefore, investigated the effects of a commercial mixture of C_{12-14} BABs (*30*), on the feeding rate of laboratory mussels. This BABs mixture comprised components both resolved and unresolved by conventional GC–MS, but when examined by GCxGC–ToF–MS, over 340 compounds were revealed, many with retention times and mass spectra similar or the same as those of the BABs in the polluted mussels (Figure 3C; SI-3).

For reference, we also examined the toxicity of a C_8-LAB (*n*-octylbenzene), which has a known toxic effect (*23*). The *n*-octylbenzene (aqueous concentration 0.042 mg L^{-1}; accumulated body burden ca. 5 $\mu g\ g^{-1}$ dry weight) produced a 32% reduction in feeding rate over 72h which was consistent with an aqueous EC_{50} of 0.079 mg L^{-1} reported previously (*23*). By comparison, various nominal aqueous solutions of the BABs mixture (0.005–0.041 mg L^{-1}; accumulated body burdens ca. 11–57 $\mu g\ g^{-1}$ dry weight) produced 17–37% reductions in feeding rate compared with controls (Figure 5A and B). This established an aqueous BAB EC_{20} of 0.007 mg L^{-1} (Figure 5A) and a tissue BAB EC_{20} of 10.5 $\mu g\ g^{-1}$ dry weight (Figure 5B). The lowest observed internal total body concentration associated with adverse effects (critical body residue) was 11.1 $\mu g\ g^{-1}$ dry weight. A meaningful aqueous EC_{50} value could not be determined from the data as the decrease in feeding rate with increasing toxicant concentration appears to level off at approximately 40%, indicating the solubility limit of the BAB mixture. These findings confirm that C_{12-14} BABs are indeed toxic to mussels and probably account for some of the depression in SfG observed in the polluted mussels. Since narcosis is nonspecific and additive, the other UCM chemicals also contribute to the measured body burden (Figure 2B). Furthermore, it is likely that the narcotic toxicity arising from the UCM outweighs that of individual or summed PAHs (*9*). The amounts of aromatic UCM extracted from each mussel sample were compared to the tissue EC_{20} (10.5 $\mu g\ g^{-1}$ dry wt) determined for the BAB mixture. This indicated that samples exhibiting low or medium SfG values (with the exception of Berwick) contained aromatic UCM concentrations above the BAB mixture tissue EC_{20} (SI-4). Our previous studies have also demonstrated that alkyltetralins reduce mussel feeding rates (*13*). Alkyltetralins and naphthalenes with both cyclic and alkyl substituents are resistant to aerobic biodegradation, with those exhibiting branched substituents being most persistent (*31*), consistent with their identification in the UCMs of the mussels by GCxGC–ToF–MS (e.g., Figure 3B).

Thus, UCMs of aromatic hydrocarbons, which are widespread in the environment, are comprised of thousands of individual branched alkyl-substituted compounds. Although each individual compound occurs at low concentration, in summation the compounds constitute a high UCM concentration in environmental samples. Due to their overall structural similarities to the major resolved hydrocarbons the UCM compounds are able to produce nonspecific narcotic toxic effects in the mussel *Mytilus edulis*. Such aromatic UCMs appear to represent an important group of overlooked environmental pollutants.

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Supporting Information Available

Amounts of total organic and both aliphatic and aromatic UCM extracted from each mussel tissue sample, together with a summary comparison of the aromatic UCM and SfG to the determined EC_{20} value are presented. Also included are a selection of mass spectra and retention times for similar compounds found in both the mussel tissue sample from Southend and the BABs mixture. This material is available free of charge via the Internet at http://pubs.acs.org.

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Can Amphipod Behavior Help to Predict Chronic Toxicity of Sediments?

Alan Scarlett,[1] Martin N. Canty,[1] Emma L. Smith,[2] Steven J. Rowland,[2] and Tamara S. Galloway[1]
[1]School of Biological Sciences; and [2]School of Earth, Ocean, and Environmental Sciences, University of Plymouth, Drake Circus, Plymouth, UK

ABSTRACT

Amphipods are widely used in both acute and chronic (sub-lethal) sediment tests. Acute sediment tests provide relatively rapid results, but may fail to detect moderately toxic contaminants that are bound to the sediment, whereas chronic life-cycle tests are rarely performed as they are time consuming and expensive. Observations during chronic testing of oil-contaminated sediment suggested that there may be a link between the behavior of the marine amphipod *Corophium volutator* and reduction in growth rate. Behavior tests were performed with six individual amphipods per treatment using sediment spiked with weathered Forties oil with burrowing time, re-emergence from sediment, and activity prior to burrowing as endpoints. Further behavior tests were used to predict the chronic toxicity of sediments spiked with three crude oils each with a dominant unresolved complex mixture of hydrocarbons (UCM). The effect of sediment type on behavior was also investigated. The results suggested that although the behavior test could not be used alone as a viable alternative to sediment toxicity tests, it could prove useful as an adjunct to acute tests, and help select sediments that deserve further investigation.

Key Words: *Corophium*, behavior, sediment, oil, chronic, toxicity.

INTRODUCTION

Amphipods are commonly used as test organisms for standard acute sediment exposure bioassays (PARCOM 1993; USEPA 1994; Bat and Raffaelli 1998; ASTM 2000; Roddie and Thain 2001). However, the value of such acute tests is questionable as many sediment-bound toxicants are only bioavailable at concentrations that are moderately toxic and may only give rise to deleterious effects as a result of chronic exposure (USEPA 2001). The importance of detecting the effects of long-term exposure to toxicants was emphasized by Eggen *et al.* (2004) as one of the major challenges in ecotoxicology but chronic tests are time-consuming and expensive, and thus the

Address correspondence to Alan Scarlett, School of Biological Sciences, University of Plymouth, Drake Campus, Plymouth, PL4 8AA, UK. E-mail: ascarlett@plymouth.ac.uk

numbers of samples that can be tested are restricted. The selection of sediments for chronic testing is therefore important and behavioral tests are well suited to play a role in the sediment selection process as they are rapid, inexpensive, and potentially sensitive.

In order to provide information on sublethal effects of toxicants, a reburial test is sometimes included at the end of a standard 10-day acute test, that is, ability to re-burrow within one hour (PARCOM 1993; Bat and Raffaelli 1998). This can be useful in situations where the amphipods are alive but suffering from narcosis and unlikely to survive in the wild; hence an EC_{50} can be generated that more truly reflects the toxicity of the contaminant than a LC_{50} derived from mortality. However, reburial of surviving amphipods within an hour may not necessarily provide supplementary or more sensitive information (Marsden and Wong 2001). Studies of amphipod behavior have been used as an alternative to acute lethality tests (Poulton and Pascoe 1990; Pascoe *et al.* 1994; Bat *et al.* 1998; Kravitz *et al.* 1999; Watts *et al.* 2001; Briggs *et al.* 2003). When groups of *Corophium volutator* (Pallas) were given a choice between sediment spiked with metals and non-contaminated sediment and their burrowing behavior observed for up to 96 h, it was found that the amphipods avoided the metal-contaminated sediments and this appeared to be a more sensitive indicator of toxicity than mortality at low concentrations (Bat *et al.* 1998). Kravitz *et al.* (1999) exposed the amphipod *Eohaustorius estuarius* to PAH-contaminated field sediment and recorded their avoidance behavior; although this appeared to predict sediment toxicity there were confounding problems with sediment type preferences of the amphipods, that is, there was considerable variation in the grain size distribution between sediments and this may have been the primary reason for the avoidance response. Briggs *et al.* (2003) found a significant relationship between the turbidity caused by sediment re-suspended by the behavior of amphipods, *C. volutator*, and acute mortality caused by field sediments contaminated by hydrocarbons. Although useful, these tests were performed on highly contaminated sediments that were acutely toxic to the organisms and therefore did not address the problem of assessment of moderately contaminated sediments that do not cause significant mortality during acute sediment tests.

Abnormal amphipod behavior such as failure to burrow rapidly and re-emergence from the sediment has previously been associated in our laboratory with reduced growth rate in oil-exposed organisms (unpublished data). This raised the possibility that changes in behavior follow a characteristic and repeated pattern. The aim of this research was to investigate the use of *C. volutator* burrowing behavior as a predictor of chronic toxicity. The objectives of the study were to (i) quantify the behavior previously observed during initiation of chronic tests using weathered Forties crude oil; (ii) determine the effect of variation in sediment type as a potential confounding factor; and (iii) predict the chronic toxicity of sediment spiked with three crude oils, including two dominated by unresolved complex mixtures of hydrocarbons (UCM) not previously reported to be toxic. Weathered and biodegraded oils are common and persistent sediment contaminants that represent an ongoing threat to benthic organisms (Reddy *et al.* 2002). The value, possible uses and problems associated with the behavior test are discussed. The possible implications for benthic communities arising from the chronic exposure tests will be discussed elsewhere (Smith *et al.* 2006; Scarlett *et al.* 2007).

MATERIALS AND METHODS

Sediment Toxicity Tests

Details of the methodologies used for the chronic oil exposures are provided elsewhere (Smith *et al.* 2006; Scarlett *et al.* 2007). In brief, for the first chronic oil exposure test, sediments were spiked with lightly weathered Forties crude oil (artificially weathered by gentle airflow [Smith *et al.* 2006] to give a 28% loss in weight) with nominal sediment concentrations of 0, 110, 220 and 440 $\mu g\ g^{-1}$ (dry weight, 3 replicate vessels per treatment); measured concentrations, by ultraviolet fluorescence (UVF) were *ca* 25% of nominal values. Following aeration of spiked sediments for 24 h, 30 neonate *C. volutator* (mean length 1.4 mm, standard deviation (SD) 0.31 mm excluding antennae) were introduced into 2 L exposure vessels containing 160 mL of spiked sediment plus 1 L overlying water (25 psu). Test conditions were 15 ± 1°C, 25 ± 2 psu, pH 8.0 ± 0.3, 12:12 h dark:light period, 80% water exchange once per week 24 h following feeding, with continuous gentle aeration via glass Pasteur pipettes. The test was terminated after 28 days and the number of surviving amphipods and their growth recorded (based on increase of wet weight). Acute 10-day tests were also performed at nominal concentrations up to 880 $\mu g\ g^{-1}$ (3 replicates per treatment) using adult *C. volutator* under identical conditions except that amphipods were not fed. For the second chronic exposure test, sediments were spiked with three crude oils: weathered Alaskan North Slope (ANS, 36% loss by mass achieved by gentle airflow until stable weight), Silkolene-150 (SLK, a lube oil comprised almost entirely of aliphatic UCM when analysed by gas chromatography-flame ionization detection) and Tia Juana Pesada, (TJP, a biodegraded crude oil comprised almost entirely of aliphatic and aromatic UCM), all at a nominal concentration of 500 $\mu g\ g^{-1}$ (dry weight). The test was initiated using 20 juvenile *C. volutator* with mean length 3.8 mm (SD = 0.30 mm) and terminated upon reproduction in all control vessels (35 days exposure; 5 replicates per treatment).

Acute 10-day tests were also performed using adult amphipods at a nominal concentration of 1000 $\mu g\ g^{-1}$. Test conditions were as described earlier. Water quality measurements were recorded prior to commencement of all tests, on day 5 or 6 of acute tests, weekly during chronic tests, and at the end of all tests. Sediment for all tests was collected locally from an intertidal area of the Avon estuary near Aveton Gifford, South Devon, United Kingdom (ordinance survey grid reference: SX 683 467). *Corophium volutator* from this location were used for the sediment type and Forties behavior and toxicity tests; for the UCM tests *C. volutator* were supplied by Guernsey Sea Farms (Vale, Guernsey, UK).

Spiking of Sediments

Forties oil tests: acute, chronic and behavior

Direct spiking of (weathered) Forties oil was performed via micro-litre syringes into 160 mL aliquots of sieved wet sediment within 2 L Pyrex beakers. The oil and sediment were homogenized with 50 mL of 25 psu seawater for 60 s using a motorized hand blender to produce a slurry. The blender was rinsed into the vessel with an additional 50 mL of 25 psu seawater. The slurry was left to settle for 24 h, the supernatant poured off and the beakers refilled to the 1200 mL mark with 25 psu seawater.

An additional replicate for each treatment was produced for chemical analyses. The procedure was repeated to create sediments for use in behavioral tests.

UCM-dominated oils: acute, chronic and behavior

The spiking method was based on that of Roddie and Thain (2001). Oils were dissolved in dichloromethane (DCM) as they were not readily soluble in acetone or methanol and spiked (5 mL) onto 20 g aliquots of dry sediments. The spiked sediment was left overnight for the solvent to completely evaporate and then mixed with 320 mL aliquots of wet sediment and 100 mL of 25 psu seawater in wide neck 500 mL glass boules (Schott). The combined spiked sediments were shaken vigorously by hand, then by orbital shaker at 200 rpm for 3.5 h. The bottles were again vigorously shaken by hand and the slurry from each divided equally between two 2 L Pyrex beakers. Solvent controls were created using 5 mL of DCM as described earlier. Additional replicates were created for chemical analysis and behavioral tests.

Sediment Type Test

Wet sediment was mixed using a stainless steel spoon with "general purpose grade sand" (Fisher Scientific, Loughborough, UK) to give 0, 10, 20, 40, 80, and 100% sand (based on dry weights, calculated using dry:wet ratios of sediment subsamples). The mixed sediments were each left to settle for four hours, the overlying waters poured off and then divided into six 25 mL aliquots for behavior tests and two 160 mL aliquots for 10-day survival tests.

Behavioral Tests

Homogenized sediment was divided between 6 replicate 100 mL glass beakers (25 mL per beaker) with 75 mL of overlying water (25 psu seawater). Beakers were labelled with randomly generated numbers to eliminate bias when evaluating behavior. After settling, the water was aerated for 24 h. Amphipods were sorted from stock to give a size range of about 3–4 mm. Initial tests had indicated that large individuals, especially mature males, were more likely to fail to burrow in clean sediment and very small amphipods were difficult to observe. Individual amphipods were placed in 25 mL beakers with 25 psu seawater, then randomly allocated to test vessels. Aeration was stopped prior to an individual amphipod being transferred to the exposure vessel via a plastic Pasteur pipette with a widened neck. The time to initial burrowing, that is, complete burial of the entire organism, was recorded as was its activity prior to burrowing, classified as majority of time spent swimming, crawling or diving straight to the sediment surface. Following amphipod burrowing, the vessel was observed for a further 2 minutes to check for re-emergence from the sediment, that is, completely emerged and not simply partial emergence from the end of its U-shaped burrow. The timing of subsequent burrowing and any further re-emergence was recorded up to 5 min from initiation, at which point the time to final burrowing was recorded as >5 min. To reduce possible confounding factors such as time of day, the tests were carried out as rapidly as possible using several observers, but only one vessel was monitored by an observer at any one time. From the data, mean time to initial burrowing, mean time to final burrowing, percentage

emergence, percentage failing to burrow within 5 min, and percentage of dominant activity—swimming, crawling or diving—were calculated.

Statistical Analyses

Statistical analyses of results were performed using Statgraphics Plus 5.1. Proportional data were arcsine transformed prior to analysis. Following checks for variance using Bartlett's test, data were analyzed by Analysis of Variance (ANOVA). Where there was a significant difference ($p \leq 0.05$) of means, the data were further analysed by Fisher's LSD test to determine significant differences ($p \leq 0.05$) between treatments.

RESULTS

Sediment Type: Behavior and Acute Tests

Both initial and final time to burrow were unaffected by the addition of sand to sediment up to 80% by dry weight of total, but no burrowing occurred with 100% sand (Figure 1a). Re-emergence following initial burial only occurred with 80% sand (Figure 1b). Failure to burrow within 5 min was only significantly increased ($p \leq 0.05$) at or above 80% sand. Activity prior to initial burrowing was similar at low sand concentrations to that of the control but at 40% was dominated by diving directly to the sediment surface; with 100% sand, all organisms swam constantly (Figure 1c).

Acute 10-day exposure to the different substrates resulted in 100% mortality in the 100% sand treatment but no other sediment had any effect, with survivorship remaining >90%.

Forties Oil: Behavior, Acute and Chronic Tests

There were no significant differences in either initial or final time to burrow, but amphipods exposed to nominal weathered oil concentrations of 220 and 400 $\mu g\ g^{-1}$ (dry weight) did take longer to burrow and were more variable in their response (Figure 2a). Control organisms did not re-emerge following initial burrowing, and all successfully burrowed within 5 min; re-emergence and failure to burrow were highest within the 440 $\mu g\ g^{-1}$ treatment (Figure 2b). Activity prior to initial burrowing was similar at the lowest oil concentration to the control, but swimming was dominant with the 440 $\mu g\ g^{-1}$ treatment, and diving directly to the sediment surface was more prevalent at the highest oil concentration (Figure 2c).

Sediment tests showed that only the highest exposure of 880 $\mu g\ g^{-1}$ was acutely toxic to adults, causing 50% mortality. Chronic 28-day tests with neonates resulted in a significantly reduced ($p \leq 0.05$) growth rate of amphipods exposed to 220 $\mu g\ g^{-1}$ and no growth at all in amphipods exposed to 440 $\mu g\ g^{-1}$ (Figure 3).

UCM-Dominated Oils: Behavior, Acute and Chronic Tests

Initial and final times to burrowing were not significantly different between treatments, but were highest in the weathered ANS 500 $\mu g\ g^{-1}$ (dry weight) treatment (Figure 4a). Re-emergence was generally low but elevated in the 1000 $\mu g\ g^{-1}$

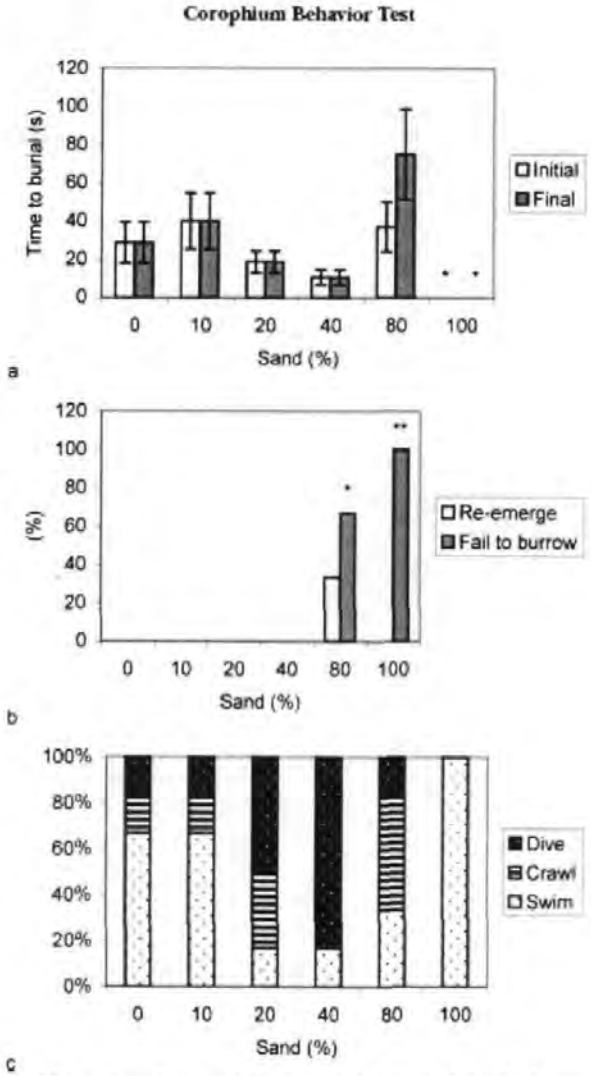

Figure 1. (a) Mean time for amphipods to initially and finally burrow (if within 5 minutes of test initiation) within sediments with increasing percentages of sand content; (b) percentages of amphipods re-emerging from the sediment following initial burrowing and those failing to burrow within 5 minutes of test initiation; (c) majority activity (%) prior to initial amphipod burrowing, classified as swimming, crawling or diving directly to the sediment surface. Tests used 6 individual *C. volutator* per treatment, error bars = 1 standard error, * and ** donate significant differences ($p \leq 0.05$) and ($p \leq 0.01$), respectively, from control values.

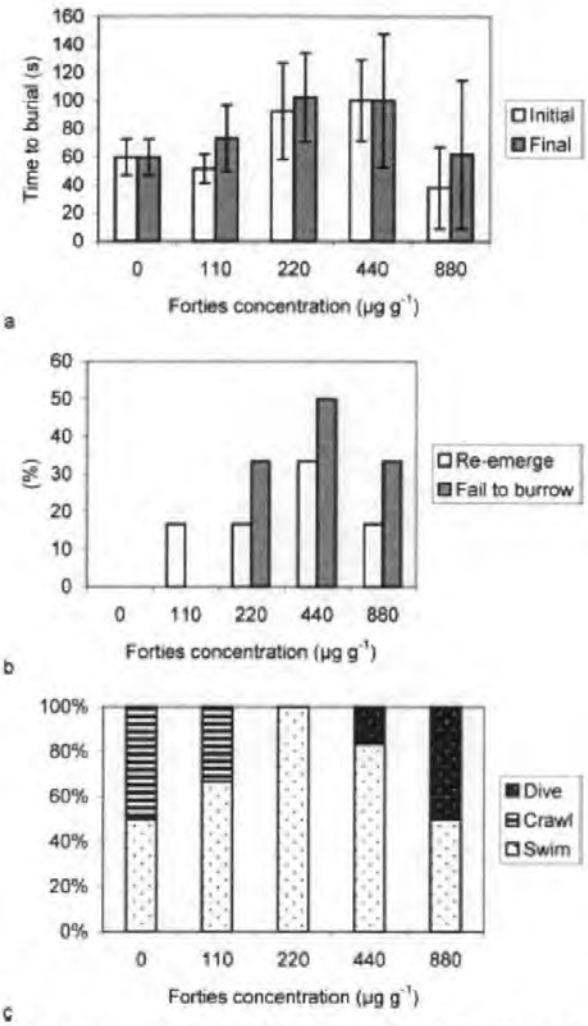

Figure 2. Same format as Figure 1 for sediments spiked with weathered Forties crude oil.

treatments of both Silkolene and ANS oils; failure to burrow within 5 min was significantly increased in the latter treatment (Figure 4b). Swimming was the dominant activity prior to initial burrowing for amphipods exposed to 1000 $\mu g\ g^{-1}$ ANS, but no trend was apparent (Figure 4c).

Sediment tests showed no acute mortality within any treatment with survivorship >90%. Juvenile *C. volutator* exposed to nominal oil concentrations of 500 $\mu g\ g^{-1}$ for 35 days had slightly lower growth rates than the negative control organisms but

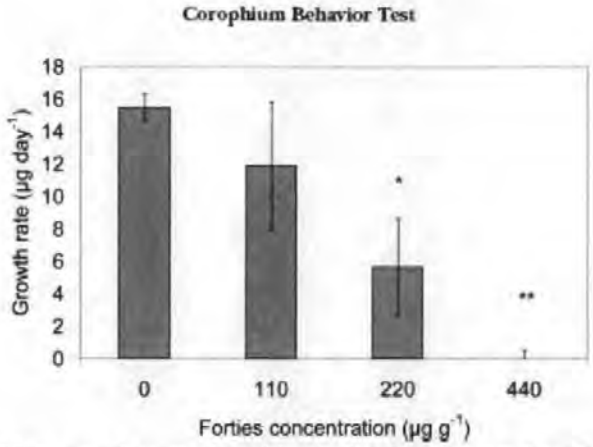

Figure 3. Mean growth rates of amphipods *C. volutator* exposed to 28 days of sediment spiked with weathered Forties crude oil. Tests used 30 neonate *C. volutator* per vessel with 3 replicate vessels per treatment, error bars = 1 standard error, * and ** donate significant differences ($p \leq 0.05$) and ($p \leq 0.01$), respectively, from control values.

only the weathered ANS was significantly less ($p \leq 0.05$) than the solvent control (Figure 5a). Reproduction was significantly reduced ($p \leq 0.05$) in all oil exposures (Figure 5b).

DISCUSSION

The aim of this research was to investigate the use of *C. volutator* burrowing behavior as a predictor of chronic toxicity. The objectives of the study were the quantification of behavior previously observed during initiation of chronic tests (unpublished data), to establish the effects of variation in sediment type, and to predict the chronic toxicity of oil-spiked sediments. From the results of the behavior tests it is clear that they are not a replacement for either acute or chronic sediment tests. However, there do appear to be certain trends in the data that suggest that useful information can be obtained from simple observations of *C. volutator* behavior.

There was a greater tendency to avoid burrowing and to re-emerge from sediments spiked with up to 440 $\mu g\ g^{-1}$ Forties oil (Figures 2a & b). This was in accordance with previous observations when initiating the chronic trial and corresponded with reduced growth rates during chronic exposure (Figure 3). This behavior did not, however, have a clear concentration-response as both re-emergence and failure to burrow declined at 880 $\mu g\ g^{-1}$, and hence it was not possible to derive median effect concentration values that are of great value for comparative and predictive purposes in risk assessment. With regard to the UCM-dominated oils, the amphipods exposed to weathered ANS at 500 $\mu g\ g^{-1}$ had the longest time to burrow (Figure 4a) corresponding to the lowest growth rate during chronic exposure (Figure 5a), which suggested a causal link. The quantification of behavior in terms of initial and final time to burrow or re-emergence from sediment did not produce significant differences

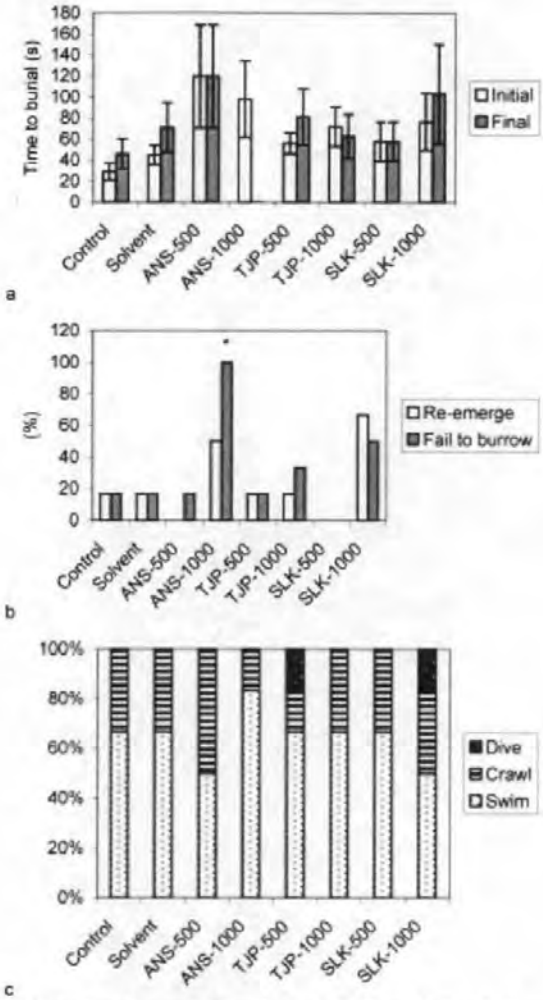

Figure 4. (a) Mean time for amphipods to initially and finally burrow (if within 5 minutes of test initiation) within sediments spiked with 3 UCM-dominated crude oils (ANS = weathered Alaskan North Slope, TJP = Tia Juana Pesada, SLK = Silkolene-150), nominal concentrations were 500 and 1000 $\mu g\, g^{-1}$ (dry weight); (b) percentages of amphipods re-emerging from the sediment following initial burrowing and those failing to burrow within 5 minutes of test initiation; (c) majority activity (%) prior to initial amphipod burrowing, classified as swimming, crawling or diving directly to the sediment surface. Tests used 6 individual *C. volutator* per treatment, error bars = 1 standard error, * donates significant difference ($p \leq 0.05$) from control/solvent values.

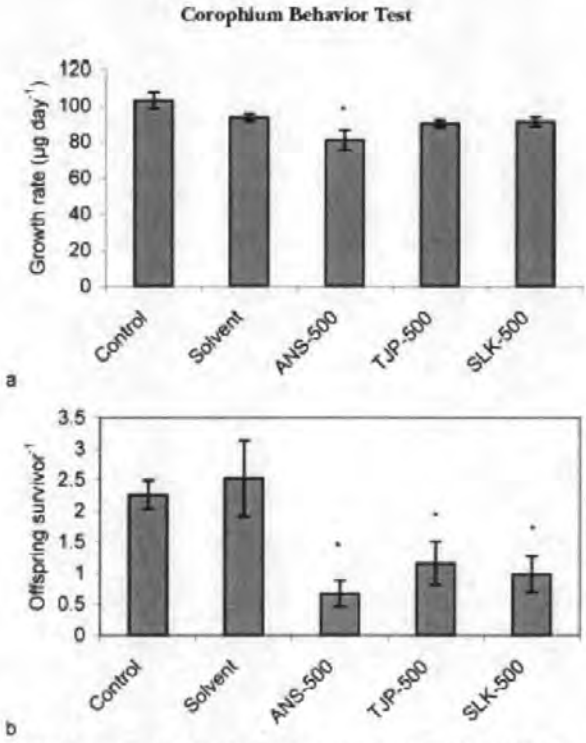

Figure 5. (a) Mean growth rates of *C. volutator* exposed to 35 days of sediment spiked with 3 UCM-dominated oils (ANS = weathered Alaskan North Slope, TJP = Tia Juana Pesada, SLK = Silkolene-150), nominal concentrations were 500 $\mu g\ g^{-1}$ (dry weight); (b) reproductive success, defined as the mean number of offspring per survivor. Tests used 20 juvenile *C. volutator* per vessel with 5 replicate vessels per treatment, error bars = 1 standard error, * donates significant difference ($p \leq 0.05$) from control/solvent values.

between oil-contaminated sediments. Only failure to burrow within 5 min gave a significant result (ANS 1000 $\mu g\ g^{-1}$, Figure 4b), and although a chronic exposure was not performed with this treatment, it can be assumed that it was chronically toxic as this oil at the lower concentration of 500 $\mu g\ g^{-1}$ significantly reduced both growth rate and reproduction (Figure 5).

From the activity prior to initial burrowing, it appeared from the Forties oil tests that an increased likelihood of diving directly to the sediment may provide an indication of acute toxicity but as there was neither increased diving activity nor acute toxicity within the UCM tests, this remains speculation. Amphipods that failed to burrow had a much greater tendency to swim (Figures 1, 2, & 4), and were apparently affected by water soluble compounds as little or no time was spent in contact with the sediment. Mechanisms of toxicity may include epithelial damage to sensory structures, neurotoxic effects on the nervous system and avoidance responses

(Boyd *et al.* 2002). The mode of action of hydrocarbon toxicity is through a non-specific mechanism causing anaesthetic effects (van Wezel and Opperhuizen 1995) and hence alteration of behavior. However, the UCM-dominated oils do not contain any volatile components and are very hydrophobic. The swimming behavior was also observed in the 100% sand-exposed amphipods and therefore the altered behavior cannot necessarily be explained by water-soluble hydrocarbons derived from the oils.

Burrowing is a complex behavioral pattern consisting of a chain of instinctive reflexes; any minor failure in reflexive response may lead to behavioral dysfunction (Pynnonen 1996). Previous attempts to use amphipod burrowing behavior as a surrogate for acute sediment toxicity tests have had limited success (Bat *et al.* 1998; Kravitz *et al.* 1999; Briggs *et al.* 2003). One particular problem that can arise when testing field-collected sediment is that the grain sizes can vary between sites thus confounding the results (Kravitz *et al.* 1999). Therefore a test was performed in an attempt to quantify the extent to which *C. volutator* behavior may be affected by sediment type. The preferred sediment type for *C. volutator* is mud, sandy mud, or muddy fine sand (Roddie and Thain 2001) so the test was conducted with increasing percentages of sand, and from this it was observed that there was little effect on burrowing time or re-emergence with up to 20% sand but there was a greater tendency to dive directly to the sediment at 40% sand and burrow a little faster (Figure 1). With 80% sand some amphipods were re-emerging from the sediment and failed to burrow within 5 min, however this did not impact their survival during 10-day sediment tests. Unfortunately it was not practically possible to perform chronic exposures with the sand to test if growth was affected, but it would be logical for sediments with a lower organic carbon content to have an impact on food availability. The results of the sediment type test suggest that every effort should be made to match test and reference sediments as closely as possible.

Had the UCM-dominated oil-spiked sediments been field-collected samples of unknown contamination, the acute tests would have proved negative and the sediments deemed to be safe. The behavior tests highlighted both of the ANS-spiked sediments and possibly the higher Silkolene-spiked sediment as potentially toxic (Figure 4). Chronic tests were only performed on the lower oil concentrations, as these were more environmentally relevant, which revealed that ANS significantly reduced growth at 500 $\mu g\, g^{-1}$ as suggested by the behavior test. However, all of the UCM-dominated oil-spiked sediments were shown to reduce reproductive success of the amphipods and therefore the behavior test failed to predict the long-term toxic effects of two of the sediments. Field sediments may also contain metals and pesticides. A 96 h behavioral study by Bat *et al.* (1998) gave *C. volutator* the choice of burrowing within sediments spiked with metals (copper, zinc, and cadmium) or clean sediment. The study showed that amphipods were not only capable of responding to lethal concentrations of metals but that the choice test was more sensitive. Pascoe *et al.* (1994) used the mate-guarding behavior of *Gammarus* spp. to detect sublethal toxicants including atrazine and lindane within freshwater sediments.

The complex mixtures of chemicals to which organisms are subject present a challenge in ecotoxicology (Eggen *et al.* 2004) and behavioral responses may have a role in exploring mechanisms of toxicity. The "Multispecies Freshwater Biomonitor" (MFB) is a sophisticated device that may help to explore mechanisms of toxicity

and has been used in recent behavioral studies (Gerhardt *et al.* 2002, 2005) to detect stress responses in a range of both water and sediment species including *C. volutator* (Kirkpatrick *et al.* 2006). An interesting result of the study by Kirkpatrick *et al.* (2006) was that both the locomotory and ventilation behavior of *C. volutator* exposed to acutely toxic concentrations of the biocide Bioban were similar to control animals, whereas sublethal Bioban-exposed animals showed reduced activity. The results from the present study are consistent with the Bioban study (Kirkpatrick *et al.* 2006) in that both suggest that sublethal toxicants can affect behavior and that this may be more apparent than the organisms' responses to acutely lethal concentrations.

CONCLUSIONS

The results of this preliminary study show that the behavior of *C. volutator* in terms of burrowing time and re-emergence cannot be used to replace acute or chronic tests; they do however suggest that that these rapid and simple tests may highlight sediments that deserve additional testing. We would therefore recommend that behavior tests be conducted as an adjunct to acute sediment tests, and that chronic tests be considered for sediments that cause *C. volutator* to fail to burrow within 5 min, burrow more slowly, or re-emerge more often than individuals provided with clean sediment of a similar type.

ACKNOWLEDGMENTS

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Sediments, Section 3: Sediment management at the river basin scale

Research Article

Chronic Toxicity of Unresolved Complex Mixtures (UCM) of Hydrocarbons in Marine Sediments *✧

Alan Scarlett[1], Tamara S Galloway[1]** and Steven J Rowland[2]

[1] School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, United Kingdom
[2] School of Earth, Ocean and Environmental Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, United Kingdom

** Corresponding author (tamara.galloway@plymouth.ac.uk)

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Abstract

Background, Aim and Scope. Unresolved complex mixtures (UCM) of hydrocarbons, containing many thousands of compounds which cannot be resolved by conventional gas chromatography (GC), are common contaminants of sediments but little is known of their potential to affect sediment-dwelling organisms. Evidence exists for reduced health status in mussels, arising from aqueous exposure to aromatic UCM components acting through a narcotic mode of action. However, UCM contaminants in sediments may not be sufficiently bioavailable to elicit toxic effects. The aim of our study was therefore to measure the sublethal effects of chronic exposure to model UCM-dominated oils at environmentally realistic concentrations and compare this to effects produced by a UCM containing weathered crude oil. A further aim was to determine which, if any, fractions of the oils were responsible for any observed toxicity.

Materials and Methods. Whole oils were spiked into estuarine sediment to give nominal concentrations of 500 $\mu g\,g^{-1}$ dry weight. Juveniles of the estuarine amphipod *Corophium volutator* were exposed to the contaminated sediment for 35 days and their survival, growth rate and reproductive success quantified. Using an effect-directed fractionation approach, the oils were fractionated into aliphatic and two aromatic fractions by open column chromatography and their toxicity assessed by further chronic exposures using juvenile *C. volutator*.

Results. The growth rates of amphipods were reduced following exposure to the oils although this was only statistically significant for the weathered oil; reproductive success was reduced by all oil exposures. Sediment spiked with UCM fractions also caused reduced growth and reproduction but no particular fraction was found to be responsible for the observed toxicity. Survivorship was not affected by any oil or fraction.

Discussion. The study showed that chronic exposure to sediments contaminated by UCM-dominated oils could have population level effects on amphipods. The observed effects could not be explained by hydrocarbons resolved by conventional GC and effects were similar for both UCM-dominated and weathered oils. All of the fractions appeared to contribute to the observed effects; this is in contrast to previous research which had shown that an aliphatic UCM did not cause adverse effects in mussels.

Conclusions. To our knowledge, this is the first study to demonstrate population-level effects arising from exposure to sediments contaminated by realistic environmental concentrations of UCM hydrocarbons. The results are consistent with many compounds, at very low individual concentrations, contributing towards the overall observed toxicity.

Recommendations. Risk assessments of contaminated sediments should take into account the contribution towards the potential for toxic effects from UCM hydrocarbons. Studies into sediment contamination should report both aliphatic and aromatic UCM concentrations to aid risk assessments.

Keywords: *Corophium volutator*; amphipods; UCM; hydrocarbons; toxicity; bioassay-directed fractionation; risk assessment; Tia Juana Pesada; Alaskan North Slope; weathered oil

Introduction

Despite the widespread occurrence of unresolved complex mixtures (UCM) of hydrocarbons in sediments and the fact that they often represent the majority of the petroleum hydrocarbons present (Table 1), very little is known about the potential for these sediments to affect sediment-dwelling organisms. A small number of studies have demonstrated that bioaccumulated UCM hydrocarbons can impact the health of mussels, *Mytilus edulis* L [1–5]. These studies suggest that components from the aqueous aromatic fraction, acting via a narcotic mode of action, are responsible for the observed reduction in health status. The UCM hydrocarbons within soils and sediments are resistant to biodegradation and bioremediation [6,7] and can therefore persist for decades [8]. Due to the relatively low bioavailability of sediment-bound UCM hydrocarbons, adverse effects on sediment-dwelling biota are likely to result from chronic exposure and therefore unlikely to be highlighted by standard

* ESS-Submission Editor: Henner Hollert (hollert@uni-heidelberg.de)
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Table 1: Examples of reported UCM and total hydrocarbon (H) concentrations in marine sediments

Location	UCM ($\mu g\ g^{-1}$)	H ($\mu g\ g^{-1}$)	Reference
Argentina – Rio de la Plata Estuary	0–1445[a] 0–206[b]	0–1532[a] 0–207[b]	[33]
Azerbaijan – S. Caspian Sea	36–310[a]	39–315[a]	[34]
Brazil – Todos os Santos Bay	32–156[a] 5–64[b]	33–185[a] 6–67[b]	[35]
Egypt – Alexandria, Eastern Harbour	54–1214[a]	61–1357[a]	[36]
France – Rhone prodelta	76–275[a]	86–302[a]	[37]
Hong Kong – coastline	6–1760[a]	6–1998[a]	[38]
Kuwait – Gulf	28–150[a] 6–80[b]	30–160[a] 7–80[b]	[39]
Spain – Barcelona	488[a]	906[a]	[37]
Spain – Ceuta harbour (N. African coast)	~400–4500	496–6972	[40]
Saudi Arabia Gulf	6–6300[a] 1–1400[b]	9–5500a 1–1400[b]	[39]
Turkey – Bosphorus, Black Sea	4–38[a] 3.6–30[b]	6–44a 6–44[b]	[41]
UK – Mersey estuary	104[a]	ns	[42]
Ukraine – Odessa, Black Sea	78–232[a] 25–63[b]	83–240[a] 30–66[b]	[41]
USA – West Falmouth, Massachusetts	~8000	~8000	[6]

[a] aliphatic UCM
[b] aromatic UCM
ns = not stated

acute sediment tests. In order to test the potential for UCM hydrocarbons to elicit toxic effects, it is preferable to expose sediment-dwelling organisms over a time span sufficient to register meaningful sublethal endpoints such as growth rate and reproductive success.

Corophium volutator Pallas is a sediment-dwelling amphipod that occurs widely in muddy sediments within estuaries of temperate Europe and the east coast of North America [9]. Used as a standard acute sediment toxicity test species in Europe [10], *C. volutator* has now been used to test the chronic toxicity of marine sediments [11,12]. The full life-cycle of *C. volutator* requires over two months to complete and therefore the tests reported herein used a partial life-cycle (≥ 35 days exposure) with sublethal endpoints of growth rate and reproductive success. Sediment contaminated with UCM hydrocarbons contain many thousands of hydrocarbons [13] and investigations into their potential toxic effects are therefore amenable to an effect-directed fractionation approach which aims to reduce the complexity by an iterative process of toxicity testing and fractionation and thus identification of the compounds responsible [14,15]. Sediments contaminated with UCM hydrocarbons often contain toxicants such as polycyclic aromatic hydrocarbons (PAHs), metals, and pesticides e.g. many of the studies cited in Table 1 also report a range of sediment contaminants, thus further complicating toxicity studies into UCM effects. Our study therefore tests the toxicity of two model UCM-dominated oils (Fig. 1): in reservoir biodegraded crude oil Tia Juana Pesada (TJP: 98% UCM) and a lube oil Silkolene-150 (SLK: 96% UCM), both of which have been used previously to model UCM contamination [16, 17]. In addition, a crude oil Alaskan North Slope (ANS) was evaporatively weathered and tested for comparative purposes; this also contained a substantial UCM of 87% (see Fig. 1). Further to baseline testing of the whole oils, aliphatic and two aromatic fractions derived from the oils were also subjected to toxicity testing.

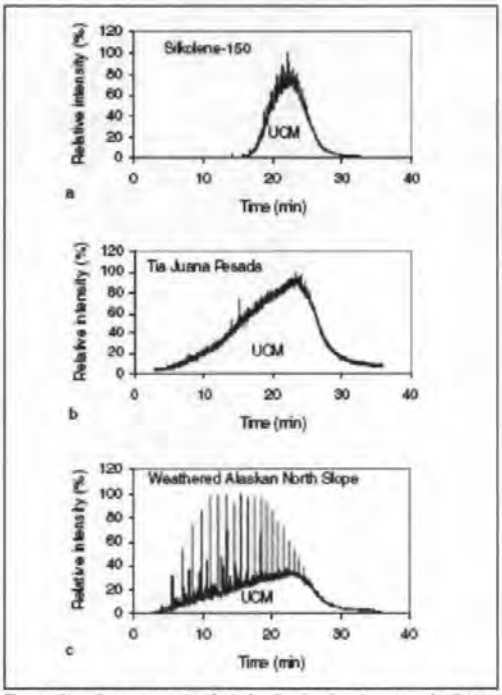

Fig. 1: Gas chromatograms of whole oils showing the unresolved complex mixture (UCM) of hydrocarbons

1 Materials and Methods

1.1 Oils and fractionation

Two UCM-dominated oils were chosen as model UCMs: TJP, a biodegraded crude oil comprised almost entirely of aliphatic and aromatic UCM; and SLK, a lube oil comprised almost entirely of aliphatic UCM when analysed by gas chromatography – flame ionization detection. For comparative purposes, a crude oil, ANS, was weathered by an evaporative method [18] to achieve a 36% loss of mass. The oils were fractionated by open column chromatography adapted from methods described by Sutton et al. [13] and Brack et al. [19]. Aliquots of ca. 1.5 g of oil were adsorbed onto ca. 10 g

of deactivated alumina (4.5% MilliQ water w/w) by rotary evaporation of the oil and alumina with hexane (20 mL) at 40°C until near dry. The column was packed with alumina (4.5% MilliQ water w/w) over activated silica (ratio 1:1 w/w) and eluted with increasingly polar solvents: 100% hexane (F1 – aliphatic), 90:10 (v/v) hexane:dichloromethane (dcm; F2 – aromatic 1), 100% dcm (F3 – aromatic 2) and 100% methanol (F4 – polar). Elution volumes (x column volumes) were 1.5x for F1 and 2x for F2, F3 & F4. Analysis by GC-MS of column extracts spiked with a mixture of known hydrocarbons showed that the F1 fraction contained only aliphatic compounds and that the F2 fraction contained aromatic hydrocarbons from alkylbenzenes to fluoranthene. Tests were not performed on the negligible F3 fraction of SLK or any of the polar F4 fractions.

1.2 Spiking of sediments

Sieved (300 µm) sediments were spiked with ANS, TJP and SLK to give nominal concentrations of 500 µg g^{-1} (dry wt.) for whole oils based on a method described by Roddie and Thain [10]. For fractionation tests whole oil equivalents were used based on gravimetric analysis of fractions (Table 2). Oils were dissolved in dcm as they were not readily soluble in acetone or methanol and spiked (5 mL) onto 20 g aliquots of dry sediments. The spiked sediment was left overnight for the solvent to completely evaporate and then mixed with 320 mL aliquots of wet sediment and 100 mL of 25 psu seawater in wide neck 500 mL glass bottles (Schott). The combined spiked sediments were shaken vigorously by hand, then by orbital shaker at 200 rpm for 3.5 h. The bottles were again vigorously shaken by hand and the slurry from each divided equally between two 2 L Pyrex beakers. Solvent controls were created using 5 mL of dcm as above. Aliquots of sediment were taken for dry:wet weight measurement.

Table 2: Nominal concentrations of whole oils and fractions in sediment (µg g^{-1} dry wt.) used for the spiked-sediment chronic exposure tests with *C. volutator*. Fraction concentrations correspond to 500 µg g^{-1} (dry wt) of whole oil based on gravimetric analysis

	Concentrations of fractions in sediment (µg g^{-1} dry wt.), and gravimetric analysis of fractions (%)			
Oil	Whole	F1	F2	F3
Silkolene 150	500 (100%)	417 (83%)	77 (15%)	np (<1%)
Tia Juana Pesada	500 (100%)	133 (27%)	141 (28%)	100 (20%)
Alaskan North Slope	500 (100%)	234 (47%)	145 (29%)	60 (12%)

np = not performed

1.3 Sediment Toxicity Tests

1.3.1 Sediment and test organisms

Sediment for all tests (grain size: 33% sand, 67% silt/clay; organic carbon (OC) 3.8%) was collected locally from an intertidal area of the Avon estuary near Aveton Gifford, South Devon, United Kingdom (ordnance survey grid reference: SX 683467). *Corophium volutator* from this location were used for the fractionated oil exposure tests and were supplied by Guernsey Sea Farms (Vale, Guernsey, UK) for the baseline tests with whole oils. Acute 72 h aqueous exposure to a standard toxicant ($CdCl_2$) had shown no differences between the two populations and similar sensitivities to that of reported *C. volutator* populations [20]. Organisms were acclimated to laboratory conditions for a minimum of a week as described by Scarlett et al. [12].

1.3.2 Chronic exposure tests

Chronic sediment exposure tests were performed using *C. volutator* in accordance with that described by Scarlett et al. [12] except that the tests were initiated using 20 juvenile rather than 30 neonate amphipods which allowed the tests to be conducted within a shorter period. In brief: tests were initiated using juvenile *C. volutator* with mean length (excluding antennae) 3.8 mm (standard deviation (sd) = 0.30 mm) and 3.2 mm (sd = 0.50 mm, mean wet weight = 1.580 mg, mean dry weight = 0.129 mg) for whole oil and fractionated oil tests respectively. Nominal oil concentrations are given in Table 2. Exposures were carried out using 160 mL of spiked sieved sediment with 1 L overlying water. Test conditions were 15 ± 1°C, 25 ± 2 psu, pH 8.0 ± 0.3, 12:12 h dark:light period, 80% water exchange once per week 24 h following feeding, with continuous gentle aeration via glass Pasteur pipettes. Five replicate exposures were performed on each of the test sediments including negative and solvent controls. The tests were terminated upon reproduction in all control vessels (35 days and 60 days exposure for whole oil and fractionated oil tests respectively) whereupon the adults and neonates were counted and the growth of adult amphipods measured. For whole oil tests, growth rate was quantified in terms of mean weights (µg day^{-1}) and size classes (subadults <5 mm, mature adults ≥5 mm). For fractionation tests, in addition to growth rate by weight, lengths of all adult amphipods were recorded to provide growth rate in terms of length (mm day^{-1}).

1.4 Statistical analyses

Statistical analyses of results were performed using Statgraphics Plus 5.1. Proportional data were arcsine transformed prior to analysis. Following checks for variance using Cochran's C test, data were analysed by one way analysis of variance (ANOVA). Where there was a significant difference ($P \leq 0.05$) of means, the data were further analysed by the Student-Newman-Keuls test to determine significant differences ($P \leq 0.05$) between treatments.

2 Results

2.1 Whole oil tests

The whole oil sediment exposure test was terminated after 35 days when it was apparent that reproduction had occurred within all control vessels. Throughout the exposure period, normal behaviour was observed within all vessels and water quality measurements were well within acceptable limits. No statistical differences were observed between negative and solvent control treatments for any of the mea-

Table 3: Effect of 35 days spiked sediment exposure at a nominal concentration of 500 µg g^{-1} dry weight to three oils Silkolene-150, Tia Juana Pesada (TJP) and weathered Alaskan North Slope (ANS) on the mean (standard error) survivorship, growth rate and reproductive success of the amphipod *C. volutator*. Mean effects compared with combined negative and solvent controls. Values significantly different from solvent control ($P \leq 0.05$) = *

Treatment	Survivorship (%)		Growth rate (µg day^{-1} dry weight)		Offspring/ Survivor		Offspring/ female	
Seawater control	95 (3.2)		23.0 (1.4)		2.26 (0.23)		3.76 (0.35)	
Solvent control	92 (3.7)		21.5 (0.7)		2.52 (0.61)		4.26 (0.86)	
Silkolene-150	90 (3.2)		20.1 (1.1)		0.98 (0.29)	*	1.62 (0.56)	*
Tia Juana Pesada	96 (2.3)		20.3 (1.2)		1.16 (0.35)	*	2.19 (0.59)	*
Alaskan North Slope	92 (4.4)		17.7 (1.3)	*	0.67 (0.21)	*	1.56 (0.48)	*

sured endpoints. Survivorship was very high (≥90%) within all treatments. Juvenile *C. volutator* exposed to all oil exposures had lower growth rates than the negative control organisms but only the weathered ANS was significantly less ($P \leq 0.05$) than the solvent control (Table 3). Reproduction was significantly reduced ($P \leq 0.05$) in all oil exposures although this was more pronounced in the ANS treatment (see Table 3).

2.2 Fractionated oils tests

The test was terminated after 60 days upon reproduction within all control vessels. Most of the control vessels had shown evidence of reproduction earlier but in one replicate this was not clear and hence the test was continued until reproduction was confirmed. Normal behaviour was observed within all vessels and water quality measurements were well within acceptable limits. No statistical differences were observed between negative and solvent control treatments for any of the measured endpoints Survivorship was high (mean >80%) within all treatments although one vessel from the ANS F2 treatment had only 65% survival from the starting population of 20 individuals. Growth rates were reduced for organisms exposed to most oil fractions but this was only significant ($P \leq 0.05$) for the TJP aromatic fraction exposed *Corophium* (Fig. 2). A summary of the growth rates and reproduction data is provided in the form of notched box and whisker plots (see Fig. 2). Comparisons of each of the oils (fractions grouped) with the controls showed that all three of the oil treatments reduced the growth rate and reproductive success of the amphipods, although the latter was not significant at the 5% level for ANS (Table 4).

3 Discussion and Conclusions

Toxicity studies of UCM hydrocarbons have previously been concerned with the short-term effects of an aqueous monoaromatic fraction [3], branched alkylbenzenes [1], alkyltetralins [2] and an extract from UCM-contaminated mussels [5], on the clearance rates of *M. edulis*. Bivalve molluscs are known to bioaccumulate lipophilic compounds as they have little ability to metabolise them [21]. Sediment-associated hydrocarbons on the other hand may be less bioavailable than dissolved compounds. *C. volutator* are closely associated with the sediments in which they live and are reported to deposit feed as well as to filter feed [22]. Hence they interact with contaminants adsorbed to particulates via feeding rather than via the dissolved phase.

The chronic tests revealed that UCM contaminated sediment at concentrations found in the marine environment can indeed elicit sublethal effects on the estuarine amphipod *C. volutator* without affecting survivorship (see Table 3). McGee et al. [23], using a chronic sediment toxicity test with the estuarine amphipod *Leptocheirus plumulosus*, reported that some moderately contaminated sediments from Chesapeake Bay, USA, significantly reduced growth rates and reproductive success of organisms but this was only true of those sediments that had also significantly affected survival. Similarly, Allen et al. [24] reported that a chronic sediment exposure with *C. volutator* did not show any greater sensitivity than the standard acute test. However, the amphipods were not fed during the 28 day exposure and this may have affected their growth in that study. Sediment spiked with oil fractions herein confirmed the baseline toxicity tests with whole oils, with deleterious effects on both growth rates

Table 4: Effect of 60 days spiked sediment exposure of three oils (combined aliphatic F1 and aromatic F2 & F3 fractions), Silkolene-150, Tia Juana Pesada (TJP) and weathered Alaskan North Slope (ANS) on the growth rate and reproductive success of the amphipod *C. volutator*. Mean effects compared with combined negative and solvent controls. Analysis of variance *P* values ≤0.05 = *, ≤0.01 = **

	Silkolene-150		Tia Juana Pesada		Alaskan North Slope	
Endpoint	Effect	*P* value	Effect	*P* value	Effect	*P* value
Growth rate (µg day^{-1} dry wt.)	reduction	0.108	Reduction **	0.002	Reduction *	0.024
Growth rate (mm day^{-1})	Reduction **	0.009	Reduction **	0.001	Reduction *	0.024
Offspring $survivor^{-1}$	Reduction **	0.004	Reduction *	0.025	reduction	0.131
Offspring $female^{-1}$	Reduction *	0.010	Reduction *	0.036	reduction	0.144

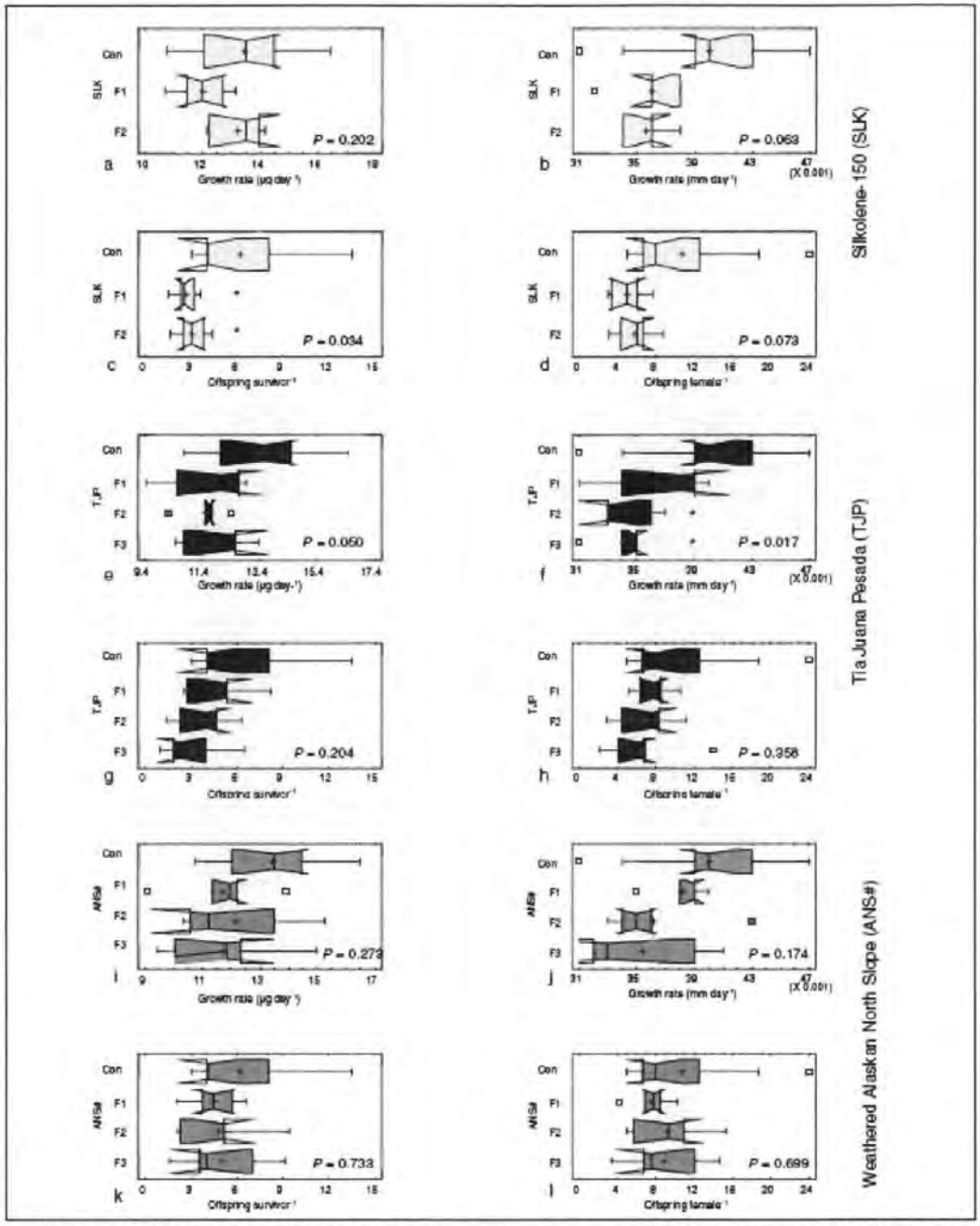

Fig. 2: Box and whisker plots comparing the effects of oil fractions upon growth and reproduction of *C. volutator*. Con = controls, F1 = aliphatic fraction, F2 = aromatic 1 fraction (elution range alkylbenzenes to fluoranthene) and F3 = aromatic 2 fraction. Effects of fractions significantly different ($P \leq 0.05$) to control values identified by *

and reproductive success (see Table 4). However, unlike the baseline tests where only ANS produced significant reduction in growth rate, all the oils significantly affected the growth of amphipods. Furthermore, significant reductions in reproductive success were not found for all oils as was the case for the baseline study. This was possibly due to the greater size variation within the starting population of the fractionation study.

In contrast to previous studies that suggested only the aromatic fraction of the UCM have deleterious effects [1-5], all of the oil fractions appeared to contribute to the overall toxicity of the oils (see Fig. 2) resulting in very few significant differences ($P > 0.05$) when analysed by ANOVA. Significant reductions were found for both the F1 and F2 fractions of SLK which produced similar effects upon the reproductive success of the amphipods (see Fig 2. c and d). This similarity in effects was unexpected as the aliphatic F1 fraction of SLK had previously been found not to show toxicity in mussels *M. edulis* [25] and aliphatic hydrocarbons in the environment have been assumed to be of low toxicity and low bioavailability. For example, an oil toxicity model by French-McCay [26] was solely based on aromatic hydrocarbon toxicity. Barron et al. [27], in a study of short-term growth and survival of mysids *Mysidopsis bahia* exposed to three oil-derived WAFs, questioned that aromatic hydrocarbons were the primary determinant of toxicity as their results demonstrated that oils with low aromatic content had greater toxicity. One theory of the narcotic mechanism of toxicity suggests that all soluble components of oil bioaccumulated by organisms may contribute to toxicity [28]. Whilst the low solubility of the aliphatic compounds should render them unavailable to the amphipods by this route and therefore not contribute to the overall toxic effect, deposit feeding may provide an alternative route of uptake [22]. It should also be noted that the nominal sediment concentration of the SLK aliphatic fraction was about five times that of the aromatic fraction but produced similar toxic effects. The monitoring of sediment hydrocarbons should therefore consider the aromatic UCM concentration.

The monitoring of hydrocarbon contamination of sediments has routinely focused on resolved PAHs which are known to be highly toxic with mutagenic and carcinogenic effects [29]. The biodegraded crude oil TJP, used in this study, contains very few such resolved compounds (ca. 2%, see Fig. 1) which is similar to the hydrocarbons reported in many of the studies listed in Table 1, but had similar toxic effects (see Tables 3 and 4, Fig. 2) as the weathered ANS oil containing a larger resolved component (ca. 13%). Shelton et al. [30] reported that water-accommodated fractions of biodegraded weathered ANS were toxic to larvae of marine crustaceans whereas the unbiodegraded oil was not toxic, suggesting that degradation products of the hydrocarbons were the main contributors to the observed toxicity rather than the PAHs themselves. The concentration of compounds resolved by conventional GC in the TJP whole oil test sediment was ca. 10 $\mu g\ g^{-1}$ (dry wt.), equivalent to ca. 260 $\mu g\ g^{-1}$ OC, of which the PAHs represent a small fraction. Swartz [31] reported a consensus threshold effect concentration for total PAH of 290 $\mu g\ g^{-1}$ OC although this did not take into account the alkyl-substituted homologues which have been reported to more toxic [32]. Some UCMs accumulated in tissues of mussels with poor health status have now been shown to contain branched alkyl-substituted homologues of mono, di and polycyclic aromatic hydrocarbons [1] and it is therefore likely that all of these compounds contribute to the overall toxic effects.

The results of the sediment exposure tests show that reductions in growth rates and reproductive success of *C. volutator* cannot be attributable to resolved compounds alone or to components from any single fraction. To our knowledge, this is the first study to demonstrate population-level effects arising from exposure to UCM hydrocarbons. Risk assessments of contaminated sediments would benefit from the widespread reporting of both the aliphatic and aromatic UCM concentrations, and from further investigations into the potential sublethal toxic effects arising from exposure to these sediments.

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Method for assessing the chronic toxicity of marine and estuarine sediment-associated contaminants using the amphipod *Corophium volutator*

A. Scarlett [a], S.J. Rowland [b], M. Canty [a], E.L. Smith [b], T.S. Galloway [a,*]

[a] *School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, United Kingdom*
[b] *School of Earth, Ocean and Environmental Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, United Kingdom*

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Abstract

Acute sediment toxicity tests do not test key life stage events such as moulting and reproduction and therefore do not reveal the longer-term effects of contaminant exposure. A laboratory method is described for determining the chronic toxicity of contaminants associated with whole sediments. The test is conducted using neonates of the estuarine amphipod *Corophium volutator* at 15 °C, salinity 25 psu and a 12 h light:12 h dark photoperiod. The endpoints are survival and growth after 28 days and survival, growth and reproduction of amphipods upon termination of test i.e. reproduction within all control vessels (*ca* 75 days). The sediment chronic toxicity test was used to investigate the effects of sediments spiked with environmentally relevant preparations of slightly weathered Alaskan North Slope crude oil, including a water-accommodated-fraction (WAF) and a chemically-dispersed (Corexit 9527) WAF. Sediment oil concentrations were quantified using ultra-violet fluorescence. The amphipods exposed to chemically dispersed oil had higher mortality and lower growth rates than control-, Corexit 9527- and WAF-exposed organisms, resulting in reduced reproduction. The described method supplements the standard acute sediment test and would be particularly useful when long-term ecological effects are suspected but acute tests reveal no significant mortality. The sediment chronic test reported herein has shown that sediment that was not evidently toxic during 10-day acute tests could have population-level effects on sediment-dwelling amphipods.
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* Corresponding author. Tel.: +44 (0) 1752 232904; fax: +44 (0) 1752 232970.
E-mail address: tamara.galloway@plymouth.ac.uk (T.S. Galloway).

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1. Introduction

Acute tests are very useful for identifying highly toxic chemicals but do not test key life stage events such as moulting and reproduction during which sensitivity to toxicants may be increased. Also, marine and estuarine sediments are more likely to contain moderately toxic contaminants that fail to cause significant acute mortality. In response to these issues, the USEPA have developed a 28 day life-cycle test using the estuarine amphipod *Leptocheirus plumulosus* (USEPA, 2001) and this was recently used by McGee et al. (2004) to compare chronic with acute toxicity of sediment from Chesapeake Bay, USA. The *L. plumulosus* test is conducted at 25 °C and the organism's life-cycle is completed within the 28 day exposure period. A similar but slower growing amphipod test species in northern European waters is *Corophium volutator* Pallas, where it resides in muddy intertidal sediment with summer seawater temperatures typically around 15 °C. The species *C. volutator* is now a standard European test organism for acute sediment toxicity testing (PARCOM, 1993; Roddie and Thain, 2001) and has been used in many acute studies (Bat and Raffaelli, 1998; Briggs et al., 2003; Ciarelli et al., 1997). *C. volutator* has also been used in long-term life-cycle tests (Brown et al., 1999; Conradi and Depledge, 1998, 1999) but the amphipods were only exposed to aqueous toxicants and not contaminants associated with whole sediments. Peters and Ahlf (2005) have demonstrated that *C. volutator* can be successfully cultured in the laboratory and the authors recommended the use of this species for chronic exposure toxicity testing. A chronic sediment test using the amphipod *Gammarus locusta* L. has recently been described which is conducted at 20 °C in order to complete the life-cycle within 28 days (Costa et al., 2005) *C. volutator* occupies a different ecological niche to *G. locusta* (Connor et al., 2004) and therefore an additional chronic test to complement the standard acute test using *C. volutator* to evaluate the toxicity of whole sediments would be of benefit. In the test described herein endpoints are survival, growth and reproduction.

Oil contamination of marine sediments remains an on-going problem whether it originates from low-level discharge from refineries and drilling platforms or from more dramatic spills from tanker disasters. Spills such as the *Sea Empress* and *Prestige* disasters resulted in considerable contamination of marine and estuarine sediments (de la Huz et al., 2005; SEEEC, 1998), but the impact of oil contamination is difficult to predict due to its complex nature. Within just hours at sea after release from a damaged tanker, weathering processes substantially change the oil composition with a subsequent loss of many of the most toxic components (Riley et al., 1980). Addition of dispersants may result in large numbers of small oil droplets in the water column which may reach the benthos and enter the sediment, thus exposing sediment-dwelling organisms to hydrocarbon contamination (SEEEC, 1998). As the oil degrades, so its composition and toxicity changes further complicating an assessment of its potential impact upon biota.

This paper describes the use of 10-day and chronic whole sediment life-cycle tests using *C. volutator* (chronic being defined as the long-term sublethal effects of acute exposure (Connell and Miller, 1984)). To simulate environmental exposure following an oil spill, the long-term sublethal effects of exposure to sediment initially spiked with the oil

dispersant Corexit 9527, weathered Alaskan North Slope (ANS) crude oil, water accommodated fraction of ANS crude oil (WAF) and chemically dispersed (using Corexit 9527) WAF (DWAF) was investigated using both the acute 10-day test and the chronic whole sediment life-cycle test using *C. volutator*.

2. Material and methods

2.1. Collection and maintenance of organisms during acclimation

Sediment and *C. volutator* were collected from an intertidal area of the Avon estuary near Aveton Gifford, south Devon UK (ordnance survey grid reference: SX 683 467). Neonate amphipods were collected and separated from adults by sieving the upper 5 cm of sediment (neonates pass through a 500 μm sieve but are retained on a 300 μm sieve), transported back to the laboratory within one hour and placed in 5 L culture tanks lined with field-collected sieved (<300 μm) sediment. The tanks were filled with filtered seawater 25 ± 1 psu which was aerated and maintained at 15 ± 1 °C with a 12:12 h light/dark cycle. The animals were fed weekly with two drops of aquarium invertebrate food (Waterlife Invert Food, Waterlife Research Industries, Longford, UK; Liquifry Marine, Interpret Ltd., Dorking, UK; Roti-Rich, Florida Aqua Farms, Dade City, FL, USA; and dried algae) per litre of overlying water and the water replaced 24 h after feeding. Amphipods were maintained under the above conditions for 7–10 days after removal from the field to acclimate them to experimental conditions. Water quality measurements: dissolved oxygen, temperature, pH and salinity were measured prior to water changes. Test conditions and acceptability requirements are given in Table 1.

2.2. Reference toxicity test

In order to test that the selected *C. volutator* population was representative of the *C. volutator* populations in general, in terms of sensitivity to a standard toxicant, the test organisms were exposed to cadmium chloride ($CdCl_2$) using a method described by Ciarelli et al. (1997) and their sensitivity compared with published data (Ciarelli et al., 1997). In brief, static aqueous tests of 72 h exposure were performed in the absence of sediment. Twenty adult amphipods were placed in nominal $CdCl_2$ concentrations ranging from 0 to 14.0 mg L^{-1}, two replicates per treatment, with a salinity of 31 psu and gentle aeration *via* a glass Pasteur pipette. The organisms were monitored daily throughout the experiment and the number surviving and deceased recorded after 72 h. A 72 h LC_{50} value was derived using the trimmed Spearman–Kärber method and compared with literature values.

2.3. Artificial weathering of oil

The fresh ANS crude oil was artificially weathered in order to simulate evaporative losses of *ca.* 20% that typically occur during the first 2–3 h at sea following a spill; this represents the earliest optimal time that dispersants may be deployed at sea (Kevin Colcomb, maritime and coastguard agency (MCA), personal communication). In brief, approximately 1000 mL of oil was placed in a 2000 mL container on a top pan balance in a fume hood with a controlled airstream and the oil allowed to evaporate. The weight of the oil at the start of the experiment was noted. Triplicate density measurements were taken to

Table 1
Test conditions and acceptability limits for chronic sediment toxicity tests with *Corophium volutator*

1.	Test type	Whole sediment toxicity test, static-renewal
2.	Test sediment	Mud, sandy mud or muddy sand. Sieved through 300 μm
3.	Overlying water	Filtered seawater Salinity: daily limits: 25 psu ± 3 psu (*Mean = 25.2, CV = 0.4%*)
4.	Temperature	Daily limits 15 °C (±2 °C) (*Mean = 15.1 °C, CV = 1.4%*)
5.	Photoperiod	12 h light 12 h dark
6.	Test vessel	2-L glass Pyrex squat-form beaker
7.	Sediment volume	160 ml (15 mm depth)
8.	Overlying water volume	Fill to 1200 ml mark in test vessel (*ca.* 1000 ml)
9.	Water renewal	Once per week: siphon off and replace 800 ml
10.	Life stage and size of amphipod	Neonates: retained between 300 μm and 500 μm mesh screens
11.	Number test organisms/vessel	30
12.	Number of replicate vessels	9 for toxicity test (including 3 sacrificed at 28-day stage) 1 dummy vessels for chemical analyses
13.	Feeding	Standard aquarium invertebrate diet: 2 mL per vessel, once per week. (mix of Waterlife Invert Food, Liquifry Marine, Roti-Rich and dried algae)
14.	Aeration	Constant 1–2 bubbles/s via glass Pasteur pipette
15.	Dissolved oxygen (DO)	Daily limits >3.6 mg L^{-1} (*Mean = 6.73 mg L^{-1}, CV = 1.4%*)
16.	pH	Within 7.0–9.0 pH units (*Mean = 8.22, CV = 0.9%*)
17.	Test duration	Intermediate 28 days then when reproduction occurs in all control replicates
18.	Endpoints:	Survival and growth rate (μg individual^{-1} day^{-1}) at 28 days. Survival, growth rate and reproduction upon test termination
19.	Performance criteria:	Control survival at test termination ⩾ 70% (*Mean = 95%, CV = 9.0%*).

Water quality measurements and performance criteria achieved, mean and coefficient of variance (CV), given in italics.

calculate the initial volume of oil. Density measurements were made by weighing known volumes of the oil at the beginning, half way through, and at the end of the experiment. Density measurements were compared to published literature (Blenkinsopp et al., 1996). The weight of the oil was noted over the duration of the experiment and adjusted to volume using the density measurements. Temperature (18–20 °C) and air flow (~0.5 ms^{-1}) through the fumehood was noted during the experiment. The weathering process was halted after 24 h. The whole experiment was carried out three times to investigate whether the method was reproducible. The slightly 'weathered' oil (19.88 ± 0.63% evaporative loss) was used to produce the starting material for all experiments. Once weathered the oil was stored in completely filled amber glass bottles at 4 °C until use.

2.4. Chemical analyses of water and sediment

Water samples aliquots (100 mL) of WAF or DWAF were extracted into dichloromethane (DCM, 3 × 25 mL + 25 mL rinse of separating funnel) for quantification of hydrocarbon concentrations by UVF analysis. Sediment samples were extracted using an alkaline saponification method described by Kelly et al. (2000). In brief: frozen sediment samples were allowed to defrost at room temperature then mixed with a stainless steel spatula. The dry weight percentage of each sample was determined by weighing subsamples (×2) in pre-weighed foil dishes then re-weighing after drying at 105 °C for 16 h. Approximately 50 g of wet sediment samples were digested with potassium hydroxide pellets (5 g) and methanol (100 mL), under reflux for 2 h. When cool, the digests were filtered through solvent-rinsed filter papers (Whatman 113v) into 250 mL separating funnels. The digests were extracted with *n*-pentane (2 × 50 mL) and the combined extract dried with anhydrous sodium sulphate then transferred to 100 mL volumetrics and made up to volume. Fluorescence values were compared to a standard curve derived from weathered ANS oil dissolved in DCM (WAFs and DWAF) or *n*-pentane (sediment). Excitation wavelengths (λ) were 254 nm (DCM) 310 nm (*n*-pentane) and emission λ 360 nm.

2.5. Preparation of water-accommodated-fractions (WAFs) and chemically dispersed WAFs (DWAFs)

The apparatus was similar to that described by Ali et al. (1995). In brief, 25 mL of weathered ANS crude oil was slowly vortex mixed with 2475 mL of 25 psu seawater at a ratio of 1:99 for 24 h within a 5 L Pyrex bottle then left to re-equilibrate for 1 h. DWAF was produced as above but with the addition of Corexit with an oil:dispersant ratio of 25:1 premixed with the oil. After mixing, the solution of WAF or DWAF was carefully siphoned off under low nitrogen pressure. As it was desired that the WAF should contain the soluble component of the oil with minimal presence of droplets, the number of particles present in various size categories were counted using a Beckman Z2 Coulter particle count and size analyser (Beckman Coulter, Wycombe, UK), and compared with that of seawater. WAFs were considered acceptable if particle counts were <5× that of seawater.

2.6. Spiking of sediments

Aliquots of 160 mL of sieved sediment (grain size: 33% sand, 67% silt/clay; organic carbon 3.8%) were placed in wide neck glass 500 mL bottles (Schott). Aliquots of 320 mL WAF,

DWAF, Corexit and 25 psu seawater were added to the bottles and shaken at 15 °C for 3.5 h at 200 rpm on an orbital shaker. The slurry from each bottle was transferred to 2 L Pyrex beakers, allowed to settle for 16 h, the supernatant poured off and the beakers refilled to the 1200 mL mark with 25 psu seawater. Direct spiking of weathered ANS oil was performed *via* micro-litre syringes into 160 mL aliquots of sieved wet sediment within 2 L Pyrex beakers. The oil and sediment was homogenised with 50 mL of 25 psu seawater for 60 s using a motorised hand blender to produce a slurry. The blender was rinsed into the vessel with an additional 50 mL of 25 psu seawater. The slurry was left to settle for 24 h, the supernatant poured off and the beakers refilled to the 1200 mL mark with 25 psu seawater. All beakers were left to settle for a minimum of 20 h with gentle aeration before the addition of the amphipods. An additional replicate for each treatment was produced for chemical analyses.

2.7. Acute sediment toxicity test

Acute sediment tests were based on standard 10-day sediment toxicity tests (ASTM, 2000; Roddie and Thain, 2001; USEPA, 1994). A slight alteration to the standard protocol was made in order to give greater consistency with the chronic tests, i.e. a 12:12 h light:-dark regime was imposed in preference to continuous light. Adult *C. volutator* (size range 4–7 mm, $n = 20$) were exposed to nominal oil-spiked sediment concentrations of 0, 220, 440 and 880 μg g^{-1} dry wt. plus 100% WAF, DWAF and dispersant-only (400 mg L^{-1} Corexit) spiked sediment exposures (three replicates per exposure treatment and six negative control replicates). The animals were not fed during the test. At the end of the test the sediment was gently sieved (300 μm) and the number of alive, dead and missing amphipods in each vessel recorded. Water quality measurements were recorded prior to commencement of the test, on day 5 or 6, and at the end of the test.

2.8. Chronic sediment toxicity test

Chronic tests were based on the acute 10-day sediment test (Roddie and Thain, 2001) and the USEPA (2001) amphipod chronic sediment test. Neonate *C. volutator* (mean length 1.4 mm, SD 0.31 mm; mean weight 0.1 mg) were sorted from stock by carefully passing organisms through a 500 μm sieve to remove larger organisms. The sediment was then carefully re-sieved (300 μm) to collect the test organisms. Amphipods ($n = 30$) were transferred to 25 mL beakers *via* plastic Pasteur pipettes and then randomly allocated to exposure vessels. Care was taken to ensure that the amphipods were not trapped by the surface tension of the water. Two litre squat-form Pyrex beakers were used as exposure vessels as these provide a greater sediment surface area than the standard one litre beakers. Nominal sediment concentrations were 0, 110, 220, and 440 μg oil g^{-1} dry weight sediment. Control treatments were spiked with deionised water. Nine replicates for each of seven treatments were used to provide three replicates per treatment for evaluation after 28 days and the remaining six replicates for evaluation upon termination of the test. An additional set of control replicates were used to give a total of 72 exposure vessels. The animals were fed weekly with two drops of standard aquarium invertebrate food and the overlying water 80% replaced 24 h after feeding. Water quality measurements were measured before addition of amphipods and prior to water exchanges during the test. Test conditions, acceptability and water quality measurements achieved are given in Table 1. The test was initiated at the beginning of June 2004.

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

2.9. Test chemicals and solvents

All solvents were HPLC Grade from Rathburns Ltd. (Walkerburn, Scotland). Reference estuarine sediment and $CdCl_2$ was obtained from Sigma–Aldrich Company Ltd, Gillingham, UK and potassium hydroxide from Fisher Scientific (Loughborough, UK. Fresh Alaskan North Slope crude oil and Corexit 9527 was obtained gratis from minerals management service (US).

2.10. Quality assurance (QA) and data analyses

For QA purposes, $\geqslant$10% of all counts and measurements were repeated by a second operator. Statistical analyses of results were performed using Statgraphics Plus 5.1. Following checks for variance using Bartlett's test, data were analysed by one way analysis of variance (ANOVA). Where there was a significant difference ($P \leqslant 0.05$) of means, the data were further analysed by the Fisher's LSD test to determine significant differences ($P \leqslant 0.05$) between treatments. Non-parametric data were analysed by the Kruskal–Wallis test. Where there was a significant difference ($P \leqslant 0.05$) of medians, the data were further analysed by comparison of notched box-plots to determine significant differences ($P \leqslant 0.05$) between treatments.

3. Results

3.1. Reference toxicity test

No mortality was recorded within control vessels. The calculated 72 h LC_{50} for $CdCl_2$ was 7.45 mg L^{-1} (confidence limits 6.37–8.72 mg L^{-1}) which is within the reported LC_{50} value range of 2.7–9.9 mg L^{-1} (Ciarelli et al., 1997).

3.2. Chemical analyses of water and sediment

Total petroleum hydrocarbon (TPH) concentrations of ANS (oil:seawater ratio 1:99) WAF and DWAF were calculated to be 3 mg L^{-1} and 20 mg L^{-1}, respectively when quantified using DCM extractions with ANS oil as a calibration standard and analysis by UVF.

Analysis of the sediment using UVF found that after correction for background fluorescence, mean measured sediment concentrations of ANS oil were 19, 40, and 97 μg g^{-1} dry wt. for nominal spiked concentrations of 110, 220 and 440 μg g^{-1}, respectively. The ANS concentrations in the sediment from spiking with WAF and DWAF were 7 and 131 μg g^{-1} dry wt., respectively. Duplicate analyses of reference estuarine sediment gave a coefficient of variance (CV) of 0.7%.

3.3. Acute sediment toxicity test

Mortality of control organisms was low (mean = 5%). No significant mortality was exhibited at or below 440 μg g^{-1} nominal sediment or WAF exposure (Table 2). Although 20% mortality was observed in the DWAF exposure, this was not significantly different to the Corexit 9527 treatment.

3.4. Chronic sediment toxicity test

Water quality measurements during the first 28 days of the test were well within water quality criteria as defined in Table 1. Burrows were constructed in the sediment of all exposures and no abnormal behaviour was apparent other than a possible reluctance to burrow by some oil-exposed amphipods. After 28 days exposure, survivorship was over 90% for control, Corexit, WAF and 220 μg g^{-1} treatments and over 80% for other treatments with the exception of DWAF in which there was a significant ($P \leqslant 0.05$) mortality of 43%. Only organisms from the 110 μg g^{-1} and 440 μg g^{-1} treatments had significantly lower growth rates than control organisms but the growth rates of survivors from the DWAF-exposed sediment, 19 μg day^{-1}, were significantly less than that of both the Corexit, 41 μg day^{-1}, and WAF treatments, 36 μg day^{-1} (Fig. 1). The lowest growth rate of 7 μg day^{-1} was recorded within the highest nominal exposure of 440 μg g^{-1} (Fig. 1). Differences in median lengths of *C. volutator* (Fig. 2) showed a similar trend to that of the growth rates i.e. control-, Corexit- and WAF-exposed organisms were significantly longer than all other treatments. The largest individual at 4.6 mm occurred within the Corexit treatment and smallest at 1.2 mm occurred within both the 110 μg g^{-1} and 440 μg g^{-1} treatments.

Table 2
Mean (standard error) of *Corophium volutator* acute and chronic toxicity test results on sediment spiked with weathered Alaskan North Slope crude oil (nominal concentrations μg g^{-1} dry weight), water-accommodated fraction (WAF) and Corexit 9527 dispersed WAF (DWAF) and Corexit 9527

Treatment	Acute	Chronic			
	% 10-day survival	% 28-day survival	% 75-day survival	Offspring/survivor	Offspring/female
Control	95 (2.9)	93 (3.2)	95 (2.5)	1.3 (0.2)	2.6 (0.5)
Corexit	77 (1.7)*	100 (0.0)	91 (1.7)	1.7 (0.5)	4.2 (0.2)
WAF	100 (0.0)	100 (0.0)	98 (1.4)	2.0 (0.4)	5.7 (1.3)
DWAF	80 (2.9)*	58 (6.8)*	23 (3.5)*	0.3 (0.1)*	1.6 (1.0)*
110 μg g^{-1}	NP	86 (4.0)	84 (4.5)	0.4 (0.1)*	3.8 (1.0)*
220 μg g^{-1}	95 (2.9)	93 (3.3)	95 (5.0)	0.4 (0.1)*	2.0 (0.8)*
440 μg g^{-1}	93 (1.7)	88 (4.8)	89 (2.8)	<0.1*	0.8 (0.4)*
880 μg g^{-1}	75 (2.9)*	NP	NP	NP	NP

Asterisks indicate treatments significantly less than the control ($P \leqslant 0.05$). NP indicates test not performed.

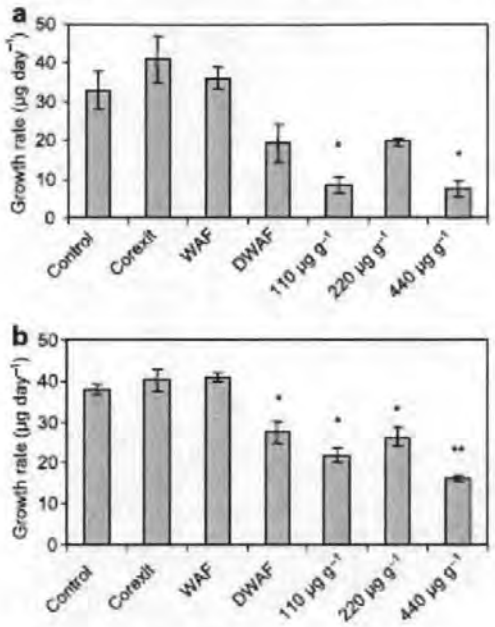

Fig. 1. Mean growth rates (µg day^{-1}) of *Corophium volutator* exposed to sediment spiked with weathered Alaskan North Slope crude oil (nominal concentrations µg g^{-1} dry wt.), water-accommodated fraction (WAF) and Corexit 9527 dispersed WAF (DWAF) and Corexit 9527 for 28 days (a) and 75 days (b). Error bars = standard error and * indicates treatments significantly less than the control ($P \leqslant 0.05$), ** indicates treatments significantly less than other treatments ($P \leqslant 0.05$).

Water quality measurements remained well within water quality criteria during the remainder of the test (Table 1). The chronic test was terminated after 75 days when it was observed that reproduction had occurred in all of the control vessels, evidenced by the presence of tiny burrows in the sediment and greater turbidity of the overlying water. Survivorship continued to be high (>80%) within all treatments except for DWAF-exposed amphipods in which there was a mean survivorship of only 23%, significantly lower ($P \leqslant 0.05$) than all other treatments. There was also an extremely high mortality (87%) in one replicate vessel of the Corexit treatment which also contained a single isopod, later identified as *Cyathura carinata* Krøyer, 1847. This organism was believed to be responsible for the high mortality of the amphipods within the test vessel. The results from the Corexit replicate containing the isopod were therefore omitted from statistical analyses.

The lowest mean growth rate of 16 µg day^{-1} again occurred within the highest nominal oil concentration treatment of 440 µg g^{-1}, this was significantly ($P \leqslant 0.05$) less than all other treatments. Organisms exposed to, 110 µg g^{-1}, 220 µg g^{-1} and DWAF-spiked sediment had mean growth rates significantly ($P \leqslant 0.05$) less than that of control, Corexit and WAF treatments. The highest mean growth rate of 41 µg day^{-1} was achieved by amphipods exposed to WAF-spiked sediment, a rate similar to that of the controls and Corexit exposed organisms. The measured lengths of amphipods (Fig. 2) reflected that of growth

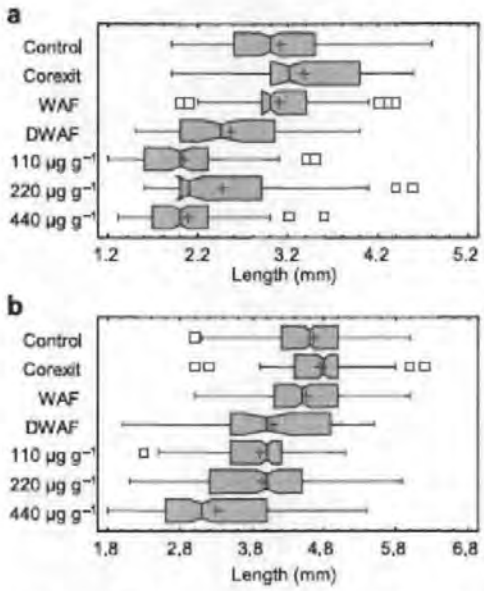

Fig. 2. Body lengths (excluding antenna) of *Corophium volutator* exposed to sediment spiked with weathered Alaskan North Slope crude oil (nominal concentrations $\mu g\,g^{-1}$ dry weight), water-accommodated fraction (WAF) and Corexit 9527 dispersed WAF (DWAF) and Corexit 9527 for 28 days (a) and 75 days (b). Notched boxes contain inter-quartile range with + denoting mean length.

rates by weight i.e. amphipods of control, Corexit and WAF treatment were significantly ($P \leqslant 0.05$) longer than all other treatments and 440 $\mu g\,g^{-1}$ exposed *Corophium* were significantly ($P \leqslant 0.05$) shorter than all others. The largest individuals of 6.2 mm were found within the Corexit treatment and the smallest of 2.0 mm were found in both the DWAF and 440 $\mu g\,g^{-1}$ treatments.

Reproduction occurred in all treatments, although three out of the six replicate vessels of both the DWAF and 440 $\mu g\,g^{-1}$ treatments contained no offspring as did two vessels of the 220 $\mu g\,g^{-1}$ treatment. Reproduction was greatest within the WAF-exposed organisms with a mean of 1.98 neonates per surviving adult (Table 2). The lowest mean offspring per survivor of 0.05 was recorded in the 440 $\mu g\,g^{-1}$ treatment. Reproduction within DWAF, 110 $\mu g\,g^{-1}$, 220 $\mu g\,g^{-1}$ and 440 $\mu g\,g^{-1}$ treatments were all significant less than that of Control, Corexit and WAF treatments (Table 2). No significant difference ($P > 0.05$) in sex ratio was observed, therefore the number of offspring per surviving female showed a similar trend to that of offspring per survivor (Table 2).

4. Discussion

4.1. Acute versus chronic endpoint sensitivity

The present study describes a method for conducting chronic whole sediment toxicity tests using the estuarine amphipod *C. volutator*. By continuing to monitor the

survivorship following an initial exposure to sediment spiked with slightly weathered ANS crude oil, we have shown that sediment that might not normally be considered toxic during a standard 10-day test could significantly reduce survivorship during the life-cycle of the organism (Table 2). In addition, it was also observed that non-acutely toxic sediment could significantly depress growth rates of amphipods leading to significantly reduced reproduction (Table 2). Although only one treatment i.e. the DWAF-spiked sediment, was found to cause significant mortality during the chronic test, all other oil-exposed organisms had reduced growth rates with the exception of the WAF-spiked sediment in which only a low concentration of ANS-derived hydrocarbons was found. This is consistent with Corexit 9527 dispersing and stabilising the oil as small droplets that then became associated with the sediment. Had these contaminated sediments been collected from the environment, chemically analysed and subject to the standard acute testing test (Roddie and Thain, 2001), the potential for population level effects would not have been observed.

4.2. Chronic test performance

The long-term nature of this test provides opportunities for systematic errors to amplify and it was therefore essential to keep variation in water quality parameters within strictly defined limits. During this test, the coefficients of variance for all water quality measurements were below 1.5% (Table 1). Mean survivorship of amphipods within control vessels was >90% with above 75% in all replicates. Variation within oil-exposed organisms was larger than that of controls, a phenomena that can be used as a stress indicator but also can be problematical for statistical analysis which assumes homogeneity of variances (Forbes and Depledge, 1996). The use of six replicates per treatment for the full life-cycle exposure was therefore desirable to enhance the power of the statistical analysis. One problem that did arise was the presence of an individual isopod, *C. carinata*, which presumably was mistakenly added to the vessel as a neonate whilst selecting neonate *C. volutator*. The isopod *C. carinata* resides in muddy intertidal sediments and often co-occurs with *C. volutator* (Connor et al., 2004). Although there is no literature on competition between these two species, our own tests (unpublished) suggest that when placed together within the restricted confines of an exposure vessel, *C. carinata* can eliminate *C. volutator*. The isopod has a slightly different motile action and therefore should be distinguished from the amphipods as they swim but errors are clearly possible when using large numbers of such small organisms. It is therefore important that test operators are familiar with a range of common sediment organisms as well the test species. The main limitation with the *C. volutator* chronic test is the length of time required for the test species to reach maturity and reproduce i.e. 75 days in this study compared with 28 days for both the *L. plumulosus* (USEPA, 2001) and the *G. locusta* (Neuparth et al., 2005) chronic tests. Despite the relatively short test period, the *G. locusta* test suffers from very low survivorship, 50–60%, of the control organisms, probably due to cannibalistic behaviour of the test species (Dick, 1995; Neuparth et al., 2005) compared to >90% control organism survivorship during the present study. As feeding behaviour is commonly altered by toxicants, cannibalism confounds the results thus making interpretation difficult. The additional costs in time and effort required for the *C. volutator* test is mitigated by the enhanced control organism survivorship. It may also prove useful to conduct partial lifecycle tests by initiating the

test using juvenile *C. volutator*, thus providing a shorter test but retaining reproduction as an endpoint. Growth rate based on wet weight of organisms was used in preference to dry weight, as used by the USEPA (2001) chronic test, as it permits chemical analysis of tissue should this be required. Previous comparisons of dry and wet weight measurements showed equivalence of results (correlation significance, $P \leqslant 0.01$) with a dry:wet ratio of 0.228 and a strong relationship between wet weight and body length (unpublished data), hence measurement of wet weight is an acceptable rapid means of assessing growth whilst retaining tissue for future analysis. The mean growth rate of control amphipods based on body length during the 75 days exposure was 0.07 mm day^{-1}; this value was also reported by Peters and Ahlf (2005) for *C. volutator* grown at 15 °C.

4.3. Ecological relevance

The current study was conducted during the summer (June–August) using neonates produced by amphipods that had over-wintered from the previous year. In their natural environment, *C. volutator* born at the beginning of the summer period can, if conditions are suitable, grow, mature and reproduce in the same year; individuals born later in the season grow more slowly and over-winter to reproduce the following year (Wilson and Herbert, 1989). Comparing the growth rates of control organisms in the present study with those from a preliminary test initiated the previous autumn, it would appear that the early summer cohort have a greater capacity for growth than the late summer/autumn cohort, i.e. despite identical laboratory conditions, the amphipods in the present study grew at a greater rate than those in the previous test (Scarlett et al., 2005). Studies conducted at different times of year may therefore not be directly comparable; this may have particular relevance to the testing of field-contaminated sediment. Sediment contaminants may have a longer period in which they can degrade during the life-cycle of late summer/autumn neonates thus allowing the organisms a greater time to recover in the absence of the toxicant. It is therefore recommended that chronic sediment tests be initiated in summer using the first generation cohort of neonate *C. volutator* or from cultured organisms, see Peters and Ahlf (2005). In terms of oil pollution, it is possible that a spill occurring in early summer will more severely affect amphipod growth rates than if it occurred later in the year.

The use of dispersants in the nearshore shallow water environment may result in surface oil being transported to the benthos leading to mortality of sensitive species; this is believed to have occurred during the Sea Empress spill off Milford haven SW Wales in 1996 where it was observed that amphipod populations disappeared following the spill (Nikitik and Robinson, 2003; SEEEC, 1998). In the present study the chemically dispersed oil treatment resulted in the highest concentration of hydrocarbons within the sediment and the greatest toxic effect in terms of survivorship. However, survivors of the DWAF exposure had greater mean growth rates than organisms exposed to the highest nominal whole oil-spiked sediment (Fig. 1) suggesting that oil-tolerant individuals can prosper following the elimination of more sensitive individuals, thus creating the potential for oil-tolerant populations to develop. The concentrations of crude oil within the sediment reported in this study are consistent with subtidal concentrations following real spills e.g. Sea Empress (Nikitik and Robinson, 2003; SEEEC, 1998) and experimental chemically dispersed spills (Boehm et al., 1987).

4.4. Conclusions

The sediment chronic test reported here has shown that sediment that was not evidently toxic during 10-day acute tests could have population-level effects on sediment-dwelling amphipods. The amphipods exposed to chemically-dispersed weathered crude oil had higher mortality and lower growth rates than control-, Corexit 9527- and WAF-exposed organisms, resulting in reduced reproduction. It was evident from the sediment concentrations and toxicological response of the amphipods that the presence of the dispersant Corexit 9527 increased the concentration of bioavailable toxic components of the weathered ANS oil with a consequential impact on sediment-dwelling organisms. The test protocol and acceptability limits proved to be robust but the test would benefit from further development by applying the test method to field-contaminated sediments.

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Appendix B

Publications in peer-reviewed journals prior to, and not directly connected with, the studies presented herein

Front pages only of the papers are included. Full papers available on request.

Scarlett, A., Donkin, M.E., Fileman, T.W., Donkin, P., (1997) Occurrence of the marine antifouling agent irgarol 1051 within the Plymouth Sound locality: Implications for the green macroalga *Enteromorpha intestinalis*. *Marine Pollution Bulletin*, 34(8), 645-651.

Scarlett, A., Donkin, P., Fileman, T.W., Evans, S.V., Donkin, M.E., (1999a) Risk posed by the antifouling agent Irgarol 1051 to the seagrass, *Zostera marina*. *Aquatic Toxicology*, 45(2-3), 159-170.

Scarlett, A., Donkin, P., Fileman, T.W., Morris, R.J., (1999b) Occurrence of the antifouling herbicide, Irgarol 1051, within coastal-water seagrasses from Queensland, Australia. *Marine Pollution Bulletin*, 38(8), 687-691.

Scarlett, A., Galloway, T.S., Canty, M., Smith, E.L., Nilsson, J., Rowland, S.J., (2005) Comparative toxicity of two oil dispersants, superdispersant-25 and corexit 9527, to a range of coastal species. *Environmental Toxicology and Chemistry*, 24(5), 1219-1227.

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Occurrence of the Marine Antifouling Agent Irgarol 1051 within the Plymouth Sound Locality: Implications for the Green Macroalga *Enteromorpha intestinalis*

A. SCARLETT*, M. E. DONKIN*‡, T. W. FILEMAN† and P. DONKIN†

**Plymouth Environmental Research Centre, University of Plymouth, Drake Circus, Plymouth, Devon PL4 8AA, UK*

†*Plymouth Marine Laboratory, West Hoe, Plymouth, Devon PL1 3DH, UK*

Water samples taken from the Plymouth Sound locality were analysed for the presence of the s-triazine herbicide Irgarol 1051, which is an ingredient of antifouling paints used on pleasure boats and ships. Irgarol 1051 was detected at all sampling sites within the Sound; the highest levels were found in close proximity to areas of high boat density, especially where water flow was restricted within marinas. Concentrations within the semi-enclosed Sutton Harbour were less than values predicted from leach rate data. The highest detected concentration of over 120 ng dm^{-3} significantly inhibited the growth of *Enteromorpha intestinalis* spores under laboratory conditions; the no effect concentration was 22 ng dm^{-3}. Photosynthetic efficiency in the adult frond of *E. intestinalis* from Sutton Harbour marina was inhibited by Irgarol 1051 in the laboratory with an EC 50 (72 h) of 2.5 μg dm^{-3}. A small adverse impact on *E. intestinalis* reproduction within the harbour is therefore likely. More polluted sites identified elsewhere in Europe will suffer proportionally greater impact. © 1997 Elsevier Science Ltd

Restrictions on the use of tri-*n*-butyl tin in marine antifouling paints has led to an increase in the number of formulations containing the herbicide 2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-s-triazine (brand name 'Irgarol 1051'). There are currently over 80 products containing Irgarol 1051 registered for use as antifouling paints (HMSO, 1994). The manufacturers of Irgarol 1051 report that it is effective against the main fouling algal species such as the green alga *Enteromorpha* spp. and the brown alga *Ectocarpus* spp. and quote a 72-h EC 50 for inhibition of the growth of unicellular green algae of 1.4 μg dm^{-3} (Ciba-Geigy, 1995a–c).

Irgarol 1051 is highly stable in the marine environment (Ciba-Geigy, 1995a), which explains why several workers have been able to detect the compound close to areas of intense boating activity. Aqueous concentrations as high as 1.7 μg dm^{-3} (Readman *et al.*, 1993) have been reported in marinas in the Côte D'Azur and concentrations exceeding 500 ng dm^{-3} have been detected in several Mediterranean locations and in Sussex, Hampshire and Hull in the UK (Readman *et al.*, 1993; Gough *et al.*, 1994; Zhou *et al.*, 1996; Tolosa *et al.*, 1996). The most important question raised by these observations is whether the reported levels of Irgarol 1051 either alone or in combination with other herbicidal contaminants can adversely influence indigenous algal populations. Dahl and Blanck (1996) demonstrated that Irgarol 1051 had adverse effects on the biomass, net photosynthesis and community structure of microalgal populations in coastal water microcosms. Similarly, Bester *et al.* (1995) observed that the related agrochemical triazine herbicide atrazine, had adverse effects on marine phytoplankton in mesocosms exposed to environmentally realistic levels of contamination. While this work has made a very valuable contribution to our understanding of the effects of triazines in the marine environment, there is at the moment no information available on sublethal effects on macroalgal communities.

The purpose of this study was: *1.* to determine the current concentrations of Irgarol 1051 dissolved in the Plymouth coastal waters and to compare this with predicted levels; *2.* to ascertain the concentrations at which Irgarol 1051 has an effect on a key reproductive stage (zoospores) of the early colonizing alga *Enteromorpha intestinalis* L. under laboratory conditions; *3.* to detect the level at which Irgarol 1051 affects the

‡Author for correspondence.

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AQUATIC TOXICOLOGY

Risk posed by the antifouling agent Irgarol 1051 to the seagrass, *Zostera marina*

A. Scarlett [a], P. Donkin [a,*], T.W. Fileman [a], S.V. Evans [a], M.E. Donkin [b]

[a] *Plymouth Marine Laboratory, West Hoe, Plymouth, Devon PL1 3DH, UK*
[b] *Plymouth Environmental Research Centre, University of Plymouth, Drake Circus, Plymouth, Devon PL4 8AA, UK*

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Abstract

Irgarol 1051 (2-(*tert*-butylamino)-4-cyclopropylamino)-6-(methylthio)-1,3,5-triazine) is a triazine herbicide that is increasingly being used to boost the effectiveness of antifouling paints. Estuarine plants, such as the marine angiosperm *Zostera marina* L. (eelgrass) may accumulate, and be affected by, Irgarol 1051, in locations with high boat densities. Bioconcentration of Irgarol 1051 within *Zostera* tissue was determined in field plants and laboratory semi-static exposure experiments. Effects of Irgarol 1051 upon the growth rate and photosystem II photosynthetic efficiency of *Zostera* were examined over a concentration range of 0 to 25 μg dm^{-3}. Growth rate was assessed by comparison of leaf specific biomass ratios, and was found to be reduced at and above an Irgarol 1051 concentration of 10 μg dm^{-3}. Photosynthetic efficiency was assessed using fluorescence induction kinetics: efficiency was significantly reduced at 0.18 μg dm^{-3} (0.4 μg g^{-1} dry weight leaf tissue) and a 10-day EC_{50} value of 2.5 μg dm^{-3} (1.1 μg g^{-1}) calculated. Longer-term exposure revealed a 36-day EC_{50} value of 0.2 μg dm^{-3}. Uptake of Irgarol 1051 was rapid within the *Zostera* leaves: tissue concentrations (dry weight basis) in excess of 300 times the water concentration were found within 2 days of exposure. Leaf concentrations in excess of 14 times root tissue concentration were found. Estuaries sampled in S.W. England had low aqueous Irgarol 1051 contamination, typically < 0.003 μg dm^{-3}, but *Zostera* leaf tissue concentrations (dry weight basis) were up to 25000 times the aqueous values; this was only 15 times below the 10-day EC_{50} value. The reported results will enable the level of risk to isolated *Zostera* meadows from Irgarol 1051 to be assessed based on leaf tissue concentration and also have implications for the siting of marinas.
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Keywords: Irgarol; Triazine; Bioconcentration; *Zostera*; Photosynthesis; Fluorescence

* Corresponding author. Tel.: +44 1752 633458; fax: +44 1752 633101; e-mail: p.donkin@pml.ac.uk

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Occurrence of the Antifouling Herbicide, Irgarol 1051, within Coastal-water Seagrasses from Queensland, Australia

A. SCARLETT†, P. DONKIN†*, T. W. FILEMAN† and R. J. MORRIS‡
†*Plymouth Marine Laboratory, West Hoe, Plymouth, Devon PL1 3DH, UK*
‡*Anatomy Dept., University of Queensland, Australia*

The *s*-triazine herbicide Irgarol 1051 is now widely distributed throughout European coastal waters. In Australia, the compound is not registered for use as a biocide in antifouling paints. To investigate contamination, seagrasses were sampled from the east coast of Queensland and within the Great Barrier Reef Marine Park. A green alga was also sampled from the Outer Barrier Reef. Tissues were analysed for the presence of Irgarol 1051 using solvent extraction followed by quantification with GC-MS and confirmation by GC-MS-MS. Irgarol 1051 was detected at nine of the ten locations sampled. Concentrations of up to 118 ng g^{-1} wet weight leaf tissue were recorded in samples from the Gold Coast (near Brisbane) which is the highest plant tissue concentration yet reported. Antifouling paint purchased within Australia was analysed by GC-MS (full scan) and found to contain Irgarol 1051. The concentrations of Irgarol 1051 reported within the Australian coastal environment are potentially toxic and the possible consequences for long-lived herbivores (such as the dugong) and for endosymbiotic algae of corals, are discussed. © **1999 Elsevier Science Ltd. All rights reserved**

Keywords: Irgarol 1051; antifouling substances; bioaccumulation; marine parks; Zostera; halodule.

Following the restriction of the use of tri-*n*-butyl tin in marine antifouling paints, there has been an increase in the number of formulations containing 'booster' herbicides. One of the booster herbicides, Irgarol 1051, 2-(*tert.*-butylamino)-4-cyclopropylamino)-6-(methylthio)-1,3,5-triazine, has been shown to be widely distributed in European estuarine and coastal waters and sediments (Readman *et al.*, 1993; Gough *et al.*, 1994; Tolosa *et al.*, 1996; Zhou *et al.*, 1996; Scarlett *et al.*, 1997), but prior to April 1998 it was not registered for use as an antifouling agent in the US (Swindell, US EPA, personal communication) or listed within the Australian National Registration Authority database. In Europe, aqueous Irgarol 1051 concentrations of up to 1.7 μg dm^{-3} have been reported (Readman *et al.*, 1993); outside of Europe, concentrations of up to 0.5 μg dm^{-3} have been found within Bermudan waters (Readman, personal communication).

Irgarol 1051 has a mode of action similar to other *s*-triazines such as atrazine and simazine, and is a particularly effective algicide. An EC_{50} value for reduction in photosynthetic activity of 0.205 μg dm^{-3} was reported by Dahl and Blanck (1996) for marine microalgae and EC_{50} values for reduction of photosystem II efficiency of 2.5 μg dm^{-3} have been calculated for marine macroalgae (Scarlett *et al.*, 1997) and the seagrass, *Zostera marina* L. (Scarlett *et al.*, in press). Accumulation of Irgarol 1051 has been shown to occur in freshwater macrophytes (Tóth et al., 1996) with bioconcentration factors (BCFs) of up to 30000×, and within marine macrophytes with BCFs of 25000× (Scarlett *et al.*, in press).

Seagrasses are marine angiosperms that grow in dense meadows in both temperate and tropical shallow-water coastal environments (den Hartog, 1977). Australia has the largest seagrass meadows, and largest number of species, in the world (Kuo and McComb, 1989). Seagrass meadows stabilise the seabed, creating habitats with high biodiversity and productivity (Edgar and Shaw, 1995) and their importance to fisheries is well documented (Bell and Pollard, 1989). Few animal species feed directly upon seagrasses, but two species that do depend upon it for a major part of their nourishment are the green turtle, *Chelonia mydas* L, and the dugong, *Dugong dugon* Müller (Marsh *et al.*, 1982; Great Barrier Reef Marine Park Authority, 1994). Worldwide, the former is listed as endangered, and the latter as vulnerable to extinction (The World Conservation Union, IUCN, 1990). In Australia, green turtle and dugong

*Corresponding author. Tel.: +44-01-752-633458; fax: +44-01-752-633101; e-mail: pdonkin@pml.ac.uk

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COMPARATIVE TOXICITY OF TWO OIL DISPERSANTS, SUPERDISPERSANT-25 AND COREXIT 9527, TO A RANGE OF COASTAL SPECIES

ALAN SCARLETT,† TAMARA S. GALLOWAY,*‡ MARTIN CANTY,‡ EMMA L. SMITH,† JOHANNA NILSSON,‡ and STEVEN J. ROWLAND†

†School of Earth, Ocean, and Environmental Sciences, and ‡School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA United Kingdom

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Abstract—The acute toxicity of the oil dispersant Corexit 9527 reported in the literature is highly variable. No peer-reviewed data exist for Superdispersant-25 (SD-25). This study compares the toxicity of the two dispersants to a range of marine species representing different phyla occupying a wide range of niches: The marine sediment-dwelling amphipod *Corophium volutator* (Pallas), the common mussel *Mytilus edulis* (L.), the symbiotic snakelocks anemone *Anemonia viridis* (Forskål), and the seagrass *Zostera marina* (L.). Organisms were exposed to static dispersant concentrations for 48-h and median lethal concentration (LC50), median effect concentration (EC50), and lowest-observable-effect concentration (LOEC) values obtained. The sublethal effects of 48-h exposures and the ability of species to recover for up to 72 h after exposure were quantified relative to the 48-h endpoints. Results indicated that the anemone lethality test was the most sensitive with LOECs of 20 ppm followed by mussel feeding rate, seagrass photosynthetic index and amphipod lethality, with mussel lethality being the least sensitive with LOECs of 250 ppm for both dispersants. The results were consistent with current theory that dispersants act physically and irreversibly on the respiratory organs and reversibly, depending on exposure time, on the nervous system. Superdispersant-25 was found overall to be less toxic than Corexit 9527 and its sublethal effects more likely to be reversible following short-term exposure.

Keywords—Dispersants *Anemonia viridis* *Corophium volutator* *Mytilus edulis* *Zostera marina*

INTRODUCTION

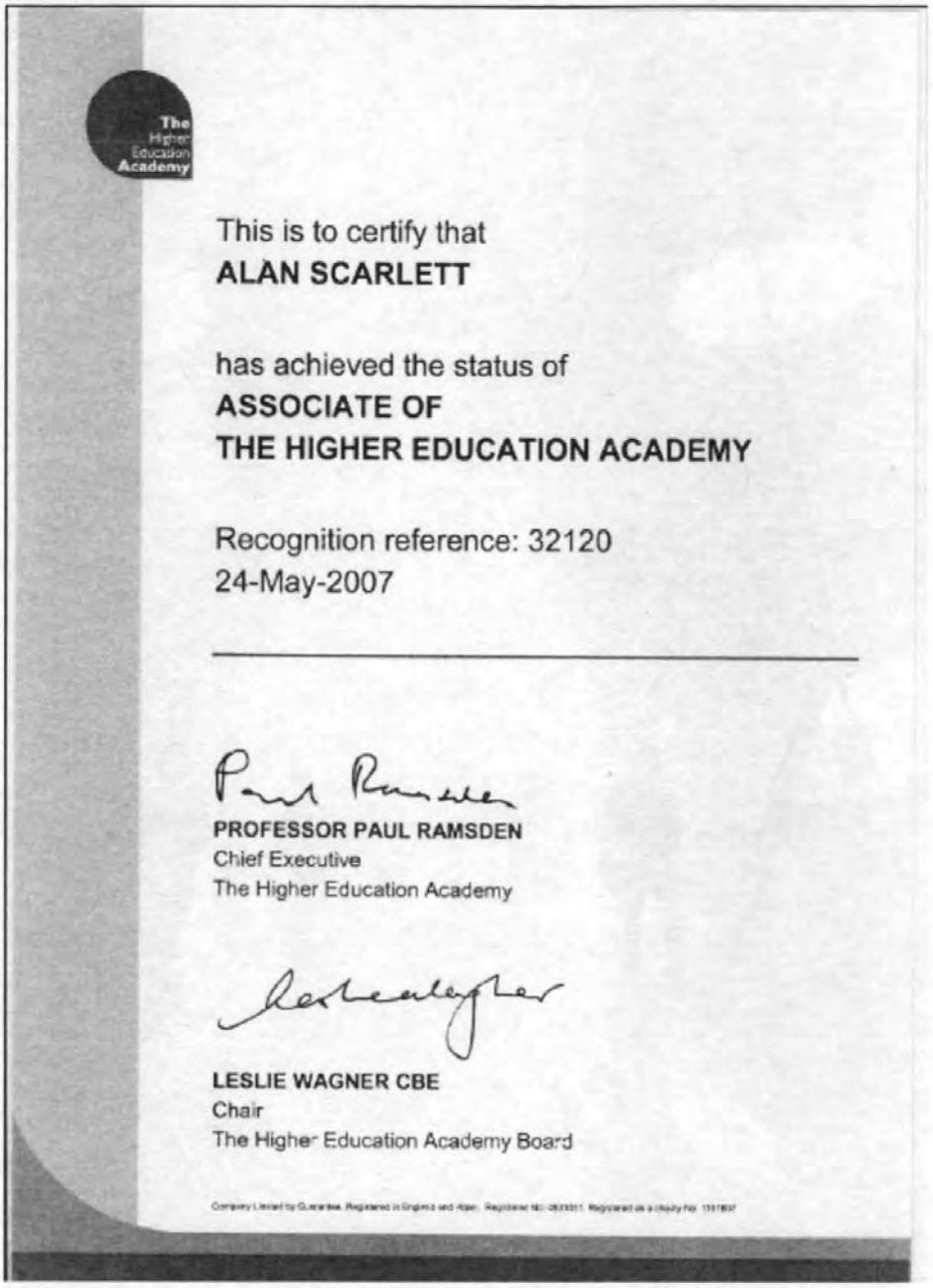
Faced with the prospect of an oil spill coming ashore or passing over reefs, decisions have to be made swiftly as to how best to deal with the situation. One option is to use chemical dispersants to break up the slick into a large number of small droplets. Once broken up, the slick poses less of a physical risk to seabirds or marine mammals but may transfer oil into the water column and possibly to the benthos. Within estuaries, inlets, enclosed bays, or shallow water reefs, the concentration of the dispersants alone may be sufficient to cause toxic effects. In the United Kingdom, dispersants cannot be used in water less than 20 m deep or within one nautical mile of such [1] without the permission of the Department for Environment Food and Rural Affairs; similar rules relating to sensitive habitats such as coral reefs and mangroves exist in tropical regions [2]. Hence, the option to use dispersants within estuaries, inlets, and shallow water does exist and it is in such circumstances that difficult decisions on how best to protect the environment and commercial operations have to be made. The handling of large volumes of dispersant under difficult conditions may result in accidental release of potentially toxic chemicals into the sea. Research into the toxicity of dispersants has been reported widely [3,4] and companies continue to improve the efficiency of the chemicals and reduce their toxicity. In the United Kingdom, the oil dispersant Superdispersant-25 (SD-25) is now the Maritime and Coastguard Agency's main stockpiled chemical for spraying onto oil slicks at sea. No data exist within peer-reviewed literature for SD-25. However, SD-25 in association with oil meets all the relevant clauses of Warren Spring Laboratory Specification LR 448(OP) and has been approved as a type-2, as well as a type-3, dispersant under test qualification CSP. 4600/8902798 (Oil Slick Dispersants Ltd. product profile, cited May 5, 2004; http://www.crofpark.co.uk/osd-products.html). Superdispersant-25 has been tested by the Center for Environment, Fisheries, and Aquaculture Science and has been found to be of low toxicity to *Crangon crangon* (brown shrimp) for use at sea and on beaches, and *Patella vulgata* (common limpet) for use on rocky shores; it is licensed under the Ministry of Agriculture, Fisheries, and Food, Food and Environment Protection Act 53/98 (Oil Slick Dispersants Ltd. product profile, cited May 5, 2004; www.crofpark.co.uk/osd-products.html). Corexit 9527 has been tested extensively in the laboratory and used on oil spills since 1978 [4]. A considerable number of toxicity reports exist concerning a wide variety of species, reviewed by George-Ares and Clark [3]. Thus Corexit 9527 provides a useful comparative toxicant for the study of SD-25.

The use of dispersants within enclosed bodies of water may pose a threat to a diverse range of species. This study compares the toxicity of SD-25 with that of Corexit 9527 to the marine sediment-dwelling amphipod *Corophium volutator* (Pallas), the blue mussel *Mytilus edulis* (L), the symbiotic snakelocks anemone *Anemonia viridis* (Forskål), and the seagrass *Zostera marina* (L.). The mudshrimp *C. volutator* is distributed widely around the coasts of western Europe and northeast America, and is significant in structuring and sustaining the ecology of near-shore sediment communities [5,6]. *Corophium volutator* is now used commonly as a European acute toxicity test organism [7–12]. Amphipods occupying a similar niche exist in other regions, e.g., *Ampelisca abdita* (Mills) also are used for toxicity testing. Blue mussels mainly occur on exposed rocky

* To whom correspondence may be addressed (tamara.galloway@plymouth.ac.uk).

Appendix C

Certificates of Education



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MR AG SCARLETT
7 UNDERWOOD ROAD
PLYMOUTH
PL7 1SY

Faculty of Education
University of Plymouth
Douglas Avenue
Exmouth
Devon EX8 2AT
United Kingdom
fax +44 (0)1395 255 303
www.plymouth.ac.uk

ALAN GEORGE SCARLETT

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