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Aromatic Hydrocarbon "Humps" in the Marine Environment: Unrecognized Toxins?

STEVEN ROWLAND,* PETER DONKIN, EMMA SMITH, AND EMMA WRAIGE#

Petroleum and Environmental Geochemistry Group, Department of Environmental Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA United Kingdom

Gas chromatographic analysis of the hydrocarbons of environmental samples often reveals that unresolved complex mixtures (UCMs) or gas chromatographic "humps" of aliphatic and aromatic hydrocarbons are most abundant—yet little consideration seems to have been given to the possible toxicological impacts of hydrocarbon "humps". Here we show, using a well-accepted bioassay, that monoaromatic components of a UCM of hydrocarbons from a crude oil elicit a sublethal toxic response in a typical marine pollution indicator organism (the mussel, *Mytilus edulis*). Furthermore, coastal U.K. mussels shown previously to have unexplained impaired health contained high concentrations of UCMs, including monoaromatic UCMs. These findings may have important implications for our understanding of the toxicological sublethal effects of oil residues in the environment. Given the relatively resistant nature of UCM hydrocarbons, the effects of both acute oil spills and more chronic discharges may need further consideration.

Introduction

An estimated 2.4 million tonnes of petroleum and petroleum fractions are introduced into the marine environment every year (*1*). Most research into the environmental effects of these oil residues has concentrated on those hydrocarbons which are resolvable by high performance liquid chromatography (HPLC) and gas chromatography (GC) and identifiable by GC-mass spectrometry (GC-MS), even though unresolved complex mixtures (UCMs) of hydrocarbons clearly dominate gas chromatograms of weathered petroleum (*2*−*4*). In fact, as a group, the UCM hydrocarbons are more abundant than the resolved hydrocarbons, even in unweathered oils, though this is seldom realized due to the normalization of most chromatograms on the resolved peaks (*5*). The limited data available suggests that aliphatic UCM hydrocarbons may not be toxicologically significant or may contribute in only a minor way to the toxic hydrocarbon burden (e.g. refs *6* and *7*). However the known toxicity of many resolved aromatic hydrocarbons (e.g. ref *8*) suggests the toxicity of the corresponding aromatic UCMs should be studied.

Aromatic UCM hydrocarbons appear to be widespread in the environment (e.g. refs *9* and *10*) but owing to legal requirements for measurements of resolved aromatic priority pollutants, workers employing GC-MS methods most routinely use selected ion monitoring (SIM) procedures which measure only designated resolved polyaromatic hydrocarbons (PAH (*11*)) and effectively "filter" out the aromatic UCM burdens. Also, to increase sample throughput and to keep costs low, toxicological studies of indicator organisms, such as mussels, have routinely employed the less specific HPLC determination methods for aromatic hydrocarbons (e.g. ref *12*). Thus the unresolved components have been largely ignored. The results of the present study show that some monoaromatic UCM components *are* toxic to a typical marine-monitoring organism, the mussel *Mytilus edulis*. Furthermore we show that coastal mussels from the U.K. with impaired health, as measured by scope for growth (S*f*G (*12*)), have substantial monoaromatic UCM burdens. Clearly, further studies of this previously unrecognized pollutant burden are required.

* Corresponding author phone: +44 1752 233013; fax: +44 1752 233035; e-mail: srowland@plym.ac.uk.

Present address: Ecotoxicology and Hazardous Substances National Centre, Environment Agency, Howbery Park, Wallingford, Oxon., OX10 8BD United Kingdom.

Materials and Methods

Isolation of Monoaromatic UCM Hydrocarbons from a Gullfaks (North Sea) Crude Oil. A partially biodegraded Gullfaks (North Sea) produced crude oil (*13, 14*) was placed in a 50 mL round-bottomed flask and rotary evaporated at 50 °C (10 min). Removal of the volatile hydrocarbons was monitored by GC-MS. Fractionation of the residue into "aliphatic" and "aromatic" hydrocarbon fractions was carried out using open column chromatography. A 350 × 10 mm column containing 10 g of fully activated silica over 10 g of alumina (0.15% deactivated) and 100 mg of oil residue was eluted with 1.5 column volumes (45 mL) pentane to yield the aliphatic fraction, followed by two column volumes (60 mL) of *n*-pentane:DCM (1:1, v/v) to yield the aromatic fraction (ca. 35 mg; 35% of evaporated oil). Preparative normal phase HPLC (*15*) was carried out on <15 mg subsamples of the aromatic fraction utilizing three Hypersil 8 micron Hyperprep HSAPS columns and a guard column. The mobile phase/solvent gradient was 100% hexane from 0 to 40 min and 100% DCM from 40 to 41 min. UV detection was carried out at 254 nm. Fractions were collected at various time intervals corresponding to UV absorption minima in the HPLC chromatograms**.** The resulting fractions were examined by GC-MS. HPLC fraction 2 (ca. 8.5% of evaporated oil; 22−29 min retention time; >95% unresolved) was found to comprise the monoaromatic constituents as evidenced by the presence (GC-MS mass fragmentography) of monoaromatic steroids (*m*/*z* 253) and alkylbenzenes (*m*/*z* 91), as observed previously (*15*). (Later eluting fractions contained di- and triaromatics such as alkylnaphthalenes, biphenyls, alkylphenanthrenes, and triaromatic steroids, as monitored by GC-MS. These will be discussed elsewhere.)

Toxicological Tests. The method was essentially as described previously (*12*). Toxicant solutions of the monoaromatic UCM (0, 25, 50, 100, and 200 μg L^{-1}) were prepared using 45 μm filtered seawater. The monoaromatic UCM was introduced to the seawater using acetone (0.001%) as a carrier to aid dispersion. This was injected directly into a vortex created by a magnetic stirrer and solutions stirred for 2 h prior to use. Control solutions were prepared in a similar manner using acetone (0.001%) only, previously shown to have no effect on mussel feeding rate. Groups of seven mussels, shell length 12 mm, were exposed to 1.4 L toxicant or control solutions in glass beakers. Gentle water movement was maintained using a Teflon stirrer bar (10 mm) and care was taken to position the animals as far away from the stirrer bar as possible. The animals were fed with an algal culture (*Isochrysis galbana*) for the duration of the exposure period in order to ensure that valves remained open and that animals

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were filtering. Mussels were exposed to solutions for 24 h. Each exposure was performed in duplicate with control vessels assembled in parallel. For the purposes of determining feeding rate, animals were transferred from the exposure vessel into individual 250 mL glass beakers, each containing 200 mL toxicant solution at the same exposure concentration. The animals were allowed an acclimatization period of 30 min to open their valves and resume pumping prior to the addition of algae. Algal culture (volume predetermined to give a cell concentration of 12 000−15 000 cells mL^{-1}) was then added to each beaker, and the water gently stirred to ensure an even distribution of algae within the beaker. An aliquot (20 mL) of medium was then immediately taken from each beaker, and the cell count was determined in triplicate per aliquot using a Coulter Counter set to measure particles greater than 3 μm in diameter. A further aliquot was taken after 15 min and the decline in cell concentration over 15 min calculated (*7*, *12*).

Mussels were dissected, and the tissue was extracted by alkaline digestion (NaOH), followed by extraction into hexane (×3). Extracts were concentrated and analyzed by GCMS. The concentration of monoaromatic UCM hydrocarbons was determined by external quantitation.

Statgraphics 4 was used for all data analysis. Data were tested for normality using standardized skewness and standardized kurtosis. All data were found to be parametric, thus a *t*-test was used to compare means and ANOVA used to test the distribution of the data. In all statistical analyses differences at the <5% level were considered significant.

Collection, Extraction, and Analysis of U.K. Mussels. Hydrocarbon-impacted mussels (*Mytilus edulis*) were collected from Cleethorpes, Teesmouth, and Whitby on the east coast of the United Kingdom and from a nonimpacted site at Whitsand, Cornwall in the southwest of England, at sites described in detail previously (*12*). Mussels were wrapped in solvent rinsed aluminum foil and immediately transported back to the laboratory packed in dry ice. Soft tissue was then dissected from the shell over ice to minimize losses of volatile analytes, homogenized, and stored in clean, solvent rinsed glass jars at −20° C until required.

The hydrocarbon extraction method used was a modification of that described by Rhead et al. (*16*). Wet mussel tissue (ca. 40 g) was acidified to pH 1 (concentrated HCl) and 15 mL of a mixture of *n*-pentane:2-propanol (1:4, v/v, added). The resulting mixture was sonicated for 40 min. A further 120 mL of *n*-pentane and 117 mL of pre-extracted water were then added. The mixture was gently shaken (5 min) followed by centrifugation at 2000 rpm (20 min). The upper (*n*-pentane) layer was transferred to a stoppered conical flask, and the lower aqueous layer was decanted and retained. The procedure was repeated (×2), and the *n*-pentane layers were combined and dried over anhydrous sodium sulfate (18 h). The sample was concentrated to 1 mL by controlled evaporation and analyzed by GC-MS.

To measure hydrocarbon recoveries of the authentic compounds from the mussel tissue homogenate using the extraction method, authentic reference compounds dissolved in acetone were spiked into wet mussel homogenate. Authentic 5-ethyltetralin, 2-ethylnaphthalene, and 1,3-diphenylhexane were provided by Dr. M. Hodges (BP Research and Engineering). Samples of *n*-phenyldecane, d_{12}-tetralin, 4-pentylbiphenyl, phenanthrene, and pyrene were purchased from Aldrich Ltd. The purity of all compounds was greater than 98% (determined by GC). The tissue was then mixed thoroughly and left at 4 °C for 4 h to allow partitioning of the analytes into the mussel tissue, which was then extracted as above.

The total organic extracts from both spiked and unspiked mussels were fractionated into aliphatic, aromatic, and "polar" fractions using a glass column (700 mm × 20 mm) packed with a *n*-pentane slurry of silica (60−100 mesh; fully activated, 20 g) under aluminum oxide (grade 1, neutral, 1.5% deactivated, 20 g). A sample-to-adsorbent ratio of 1:200 (w/w) was employed. The sample was applied to the top of the column and sequential elution of the column with solvents of increasing polarity yielded the desired fractions: F_1 (aliphatic) 1.5 column volumes *n*-pentane; F_2 (aromatic) two column volumes *n*-pentane:DCM (1:1, v/v); F_3 (polar) two column volumes DCM; F_4 (polar) two column volumes methanol. Results of the analysis of fractions $F_{1,3,4}$ will be discussed elsewhere. Fractions F_2 were concentrated by controlled evaporation and analyzed by GC and GC-MS. A ring size separation of the aromatic fraction obtained by open column chromatography was obtained using normal phase HPLC with a cyano/amino bonded phase essentially as described above for Gullfaks oil and according to a slight modification of the method of Killops and Readman (*15*). A mixture of authentic aromatic hydrocarbons (5-ethyltetralin, 2-ethylnaphthalene, and phenanthrene) was examined daily to monitor HPLC fractionation reproducibility. Each fraction was concentrated under a gentle stream of nitrogen to approximately 100 μL and analyzed by GC-MS.

Quantification of authentic compounds for method validation was performed using either GC or GC-MS. The percentage recovery of authentic compounds spiked into mussel tissue was calculated from external standard calibration graphs. A standard mixture containing the relevant authentic compounds was prepared. Aliquots of this standard mixture were removed and accurately diluted to produce a concentration range covering that necessary for analysis of the "spiked" samples. The peak areas obtained for each compound, using a Shimadzu CR4-A recording integrator, were plotted against the known concentration in the mixture to produce a calibration graph for each compound ($R^2 \geq 0.998$). The peak area of the spiked authentic compound was then measured, and the concentration was read from the relevant calibration graph. Recoveries of 1−3 μg authentic aromatic hydrocarbon spiked into 50 g wet mussel tissue were as follows: (mean ± standard deviation, $n = 6$) 71 ± 2% phenyldecane; 75 ± 2%, 5-ethyltetralin; 75 ± 2%, 2-ethylnaphthalene; 89 ± 2%, 1,3-diphenylhexane; 88 ± 2%, 4-pentylbiphenyl; 91 ± 2%, phenanthrene; 94 ± 4%, pyrene. Samples were then analyzed by GC-MS and quantification, including integration of the proportion of UCM compounds, performed using the Chemstation (Hewlett-Packard) software. The total resolved peaks were subtracted from the total hydrocarbons to give a value for the area of the unresolved component. Concentrations of resolved and unresolved hydrocarbons were calculated using an average response factor, calculated from the internal standards over the appropriate molecular weight range. Reproducibility of resolved peak measurement was 0.7% relative standard deviation ($n = 5$ (*7*)) and of UCM measurement was 0.6% relative standard deviation ($n = 5$ (*7*)).

Instrumental Conditions. GC was carried out on a Carlo Erba 5300 Mega series gas chromatograph fitted with a 25 m × 0.32 mm internal diameter DB-5 fused silica capillary column. Hydrogen was the carrier gas at 2 mL min^{-1} flow rate. Oven temperature was programmed from 40 to 300° at 5 °C min^{-1} and held at 300° for 10 min. All chromatograms were recorded using a Shimazu C−R4A chromatopac integrator. GC-MS was carried out with a Hewlett-Packard MSD GC-MS fitted with a HP-1 Ultra, fused silica column 12 m × 0.2 mm i.d. (Hewlett-Packard). Auto splitless injection (250 °C) was used. Helium was the carrier gas (40 kPa head pressure). Oven temperature was programmed from 40 to 300 °C at 5 °C min^{-1} and held at 300 °C for 10 min. Mass spectrometer operating conditions were as follows: ion source temperature 250 °C, ionization energy 70 eV, mass range 35−600 Daltons.

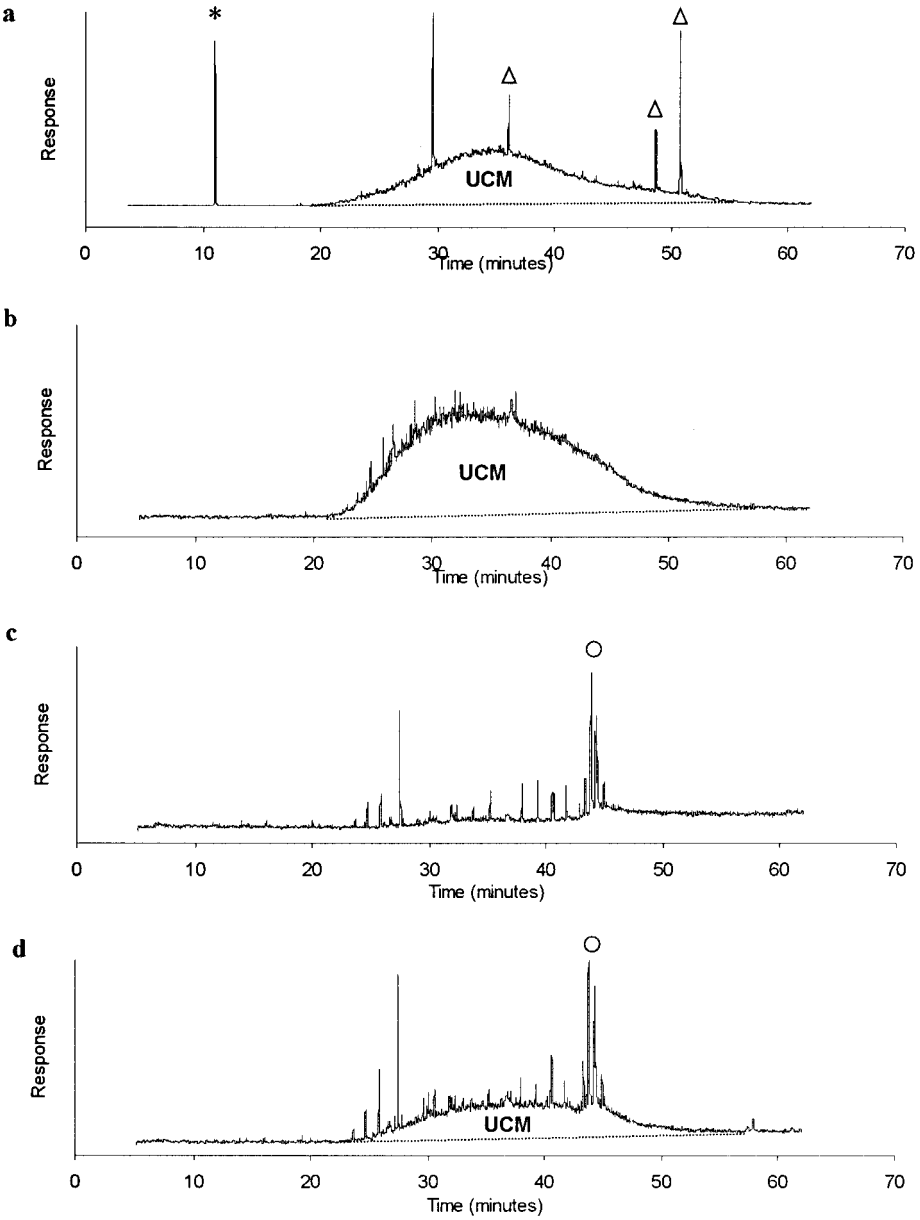

FIGURE 1. Gas chromatography−mass spectrometry total ion current chromatograms (Hewlett-Packard MSD 5890, 12 m HP-1 column, 40−300 °C min^{-1} at 5 °C min^{-1}). (a) A monoaromatic hydrocarbon fraction isolated from mussels (*M. edulis*) collected from Whitby, U.K. in May 1995. Resolved components are an internal standard (d_{12}-tetralin *) and biogenic alkenes (△). (b) A monoaromatic hydrocarbon fraction isolated from a Gullfaks North Sea crude oil. (c) Total organic extract of control mussels not treated with monoaromatic UCM fraction of Gullfaks crude. The additional resolved components in (c) and (d) are not hydrocarbons but are due to oxygenated lipids of *Isochrysis galbana* algal foodstock. Peaks marked (○) are found in the TOE of mussels from Whitby. (d) Total organic extract from mussels exposed for 24 h with monoaromatic UCM fraction of Gullfaks crude (see (b)).

Results and Discussion

To investigate whether aromatic UCM hydrocarbons exert a toxic effect on mussels, we isolated, by open column chromatography and HPLC, the monoaromatic UCM hydrocarbons from a Gullfaks (North Sea) produced crude and examined its effects on mussel feeding rate. Gullfaks produced crude (>84 000 tonnes) was spilled by the *Braer* in January 1993 into the marine environment around Shetland (*17*).

GC-MS analysis of the whole oil showed that volatile hydrocarbons such as toluene were present, in addition to a dominant UCM with resolved hydrocarbons limited to residues of acyclic isoprenoids such as farnesane, norpristane, pristane, and phytane which suggested that the produced oil was a blend of a light petroleum fraction with a biodegraded oil (cf. ref *14*). The isolation methods we used to obtain an unresolved aromatic hydrocarbon fraction therefore involved initial removal of the volatiles by evaporation, removal of the nonaromatics by column chromatography, and, finally, separation of the mono- from the di- and higher aromatic fractions by preparative HPLC. A hump or UCM dominated the gas chromatogram of the monoaromatic fraction (>95% unresolved, Figure 1b). The composition of the isolated monoaromatic fraction was confirmed by the presence of alkylbenzenes and C-ring monoaromatic steroids revealed by GC-MS mass fragmentography.

The mussel bioassay was chosen from the many biological end-points available because it is widely used in the calculation of the so-called scope for growth, which has been shown consistently to be one of the most sensitive measures of pollution induced stress (ref *12* and references therein). At the highest nominal aqueous concentration of monoaromatic UCM tested (200 μg L^{-1}) when accumulation of up to

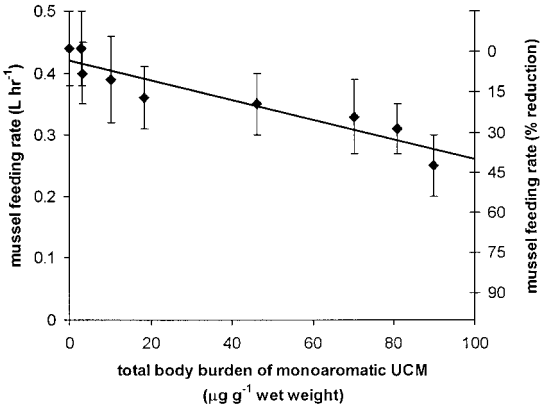

FIGURE 2. Concentration (tissue)-response for the effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate over 24 h. Feeding rates are expressed as mean ± standard deviation ($n = 7$) for exposure animals. Values for control animals (0 μg g^{-1}) were pooled ($n = 56$). Feeding rate is also expressed as a percent reduction of the control value (100%). Expressing the results in this manner effectively eliminates the influence of other environmental variables such that the results describe a reduction that is attributable to the test substrate. A linear regression line ($r^2 = 0.8715$) has been fitted to the data.

90 μg g^{-1} wet weight (approximately 350 μg g^1 dry weight) of the monoaromatic UCM hydrocarbons had occurred (Figure 2) mussel feeding rate had been reduced by over 40% in 24 h when compared to control (0 μg g^{-1} UCM) mussels (Figure 2). The UCM was clearly visible in the total organic extract of the exposed mussels (Figure 1d) when compared to untreated controls (Figure 1c). The feeding rate was statistically different ($p \leq 0.015$) from control data at all exposure concentrations of ≥ 50 μg L^{-1} corresponding to an accumulation of approximately ≥ 15 μg g^{-1} wet weight ($\sim$60 μg g^{-1} dry weight). Duplicate exposure data was not statistically different, and thus data were pooled and compared to control data. Clearly the reduction in feeding rate suggests that monoaromatic UCM hydrocarbons are toxic at sublethal concentrations to mussels.

It is important to note that the resolved monoaromatic compounds in the oil fraction (Figure 1b) cannot account for the observed decrease in mussel feeding rates. The tissue effective concentration of typical resolved monoaromatics required to reduce mussel feeding rates by 50% (TEC_{50}) ranges from 35 to 94 μg g^{-1} wet weight for 1-phenylpentane to 1-phenyldecane (*18*), whereas the concentration of resolved monoaromatics in the mussels exposed to the oil was less than 5 μg g^{-1} wet weight. (The additional resolved components present in the mussels after exposure were not hydrocarbons but due to lipids of the *Isochrysis galbana* culture, as can be seen from a comparison of parts c and d of Figure 1). The results have quite wide implications since mussel feeding rate is included in the calculation of the well-known scope for growth index, which is a widely used measure of mussel "health" (*12*).

To demonstrate the occurrence of UCM hydrocarbons in field samples of mussels, specimens of *Mytilus edulis* were collected from three sites on the east coast of the U.K. and from a reference site in Cornwall, southwest U.K. (Table 1). A hydrocarbon extraction method using an acidified mixture of *n*-pentane, propan-2-ol, and water was validated by measurement of the recoveries of added hydrocarbons and an oil UCM from unpolluted wet mussel tissue (*7*). The optimized extraction method was then used to examine the east coast U.K. and Cornish mussel samples. A full procedural blank was carried out in parallel with each extraction. Nonaromatic and aromatic hydrocarbons were isolated from total extracts by open column chromatography, and, in most cases, the aromatic fractions were further fractionated by normal phase HPLC (*15*) to obtain mono-, di-, and triaromatic fractions (Table 1). Each fraction was examined by GC-MS (e.g. Figure 1a).

TABLE 1. Concentrations of UCM Hydrocarbons in Mussels *Mytilus edulis* Collected from East Coast U.K. Sites in 1995 and in 1990[c]

	total UCM[a]	nonAr UCM[a]	Ar UCM[a]	monoAr UCM[b]	diAr UCM[b]	triAr UCM[b]
Cleethorpes	503	401	102	85	12	3
	306	170	136			
Teesmouth	271	188	83	74	18	8
	369	275	94			
Whitby	3975	3610	365	79	16	5
	3776	3280	496			
Whitby (1995)			369			
			408			
Whitsand	9	9	nd[d]	nd[d]	nd[d]	nd[d]
	5	5				

[a] μg g^{-1} dry weight. [b] Percent of aromatic UCM (mean of two analyses). [c] Shown to have reduced scope for growth (*12*). Two batches of mussels from each site were examined in each case (ca. 50 mussels total). Data for mussels collected in May 1995 from Whitsand Bay, Cornwall, which is an unpolluted site, are also shown for comparison. The differences in concentrations for duplicate populations are due to natural variability of the mussel populations. [d] Not detected, nd.

The burden of UCM hydrocarbons in the east coast U.K. mussels ranged from 271 to 3975 μg g^{-1} dry weight compared with a maximum of 9 μg g^{-1} in the mussels from an unpolluted site in Cornwall (Table 1). The aromatic UCMs in the east coast mussels ranged from 83 to 496 μg g^{-1} of which most was monoaromatic in character (mean 76%, Figure 1a). No aromatic UCM was detected in the Cornish mussels (Table 1). Concentrations of typical resolved priority pollutant PAH were low in comparison (e.g. the concentration of the most toxic narcotic resolved aromatics, the alkylnaphthalenes, in mussels from Whitby 1995 was only 0.53 μg g^{-1} dry weight (*7*)). When examined previously in 1990, the mussels collected from each of these three east coast sites exhibited impaired health (low scope for growth, S*f*G (*12*)). S*f*G correlated inversely with the tissue concentration of aromatic hydrocarbons as determined by a simple HPLC method (*12, 18*). However, the HPLC method did not differentiate resolved compounds such as PAHs from aromatic UCMs. In fact, others have noted that the concentrations of aromatic hydrocarbons measured by HPLC correlate well ($r = 0.99$, $n = 11$) with aromatic UCMs determined by GC in mussel tissue (*19*). Our reexamination of stored mussels collected from Whitby in 1990 (Table 1) by the more specific GC-MS method confirmed that high concentrations of aromatic UCMs were originally present in 1990 (mean 389 μg g^{-1} dry weight) and, indeed, remained high in 1995 (mean 431 μg g^{-1} dry weight). Furthermore, in mussels collected from the other east coast sites in 1995 (Table 1), the concentrations of aromatic UCMs determined by GC-MS also correlated well ($r^2 = 0.995$, $n = 3$) with those determined by HPLC in mussels from the same sites in 1990. Thus the effects on S*f*G originally ascribed to PAH determined by HPLC (*12*) were probably due in part, if not entirely, to aromatic UCMs. To date these have been ignored.

The experiments outlined above indicate that some components of the aromatic UCM from Gullfaks crude are toxic to *M. edulis*. Experiments should now be conducted with other UCMs and other biological end points. Two recent reports demonstrate that weathered or highly weathered crude oils characterized by UCMs are indeed toxic to other marine organisms. Barron et al. (*20*) examined the water-accommodated PAH content of three environmentally weathered oils collected from underground plumes of

spilled oil at a coastal California oilfield by SIM GC-MS and reported that toxicity to the mysid shrimp *Mysidopsis bahia* was, unexpectedly, not correlated with PAH concentration. The published total ion current GC-MS chromatograms of the oils show that the hydrocarbon fractions tested are dominated by aromatic UCMs. Similarly, in a widely publicized study, Heintz et al. (*21*) found that highly weathered oil from the *Exxon Valdez* spill was more toxic to pink salmon embryos than the unweathered oil. The authors attributed the enhancement of toxicity to elevated concentrations of larger PAH in the weathered oil. These were measured by SIM GC-MS, and a lowering of the recommended legal limits for PAH concentrations in seawater was suggested. We have no reason to question these findings but also suggest that the concentrations of aromatic UCM compounds in the weathered oil would have been elevated by weathering compared to the fresh oil, and that these may have contributed to the toxic effects.

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