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

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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 μg 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 μg 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 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.

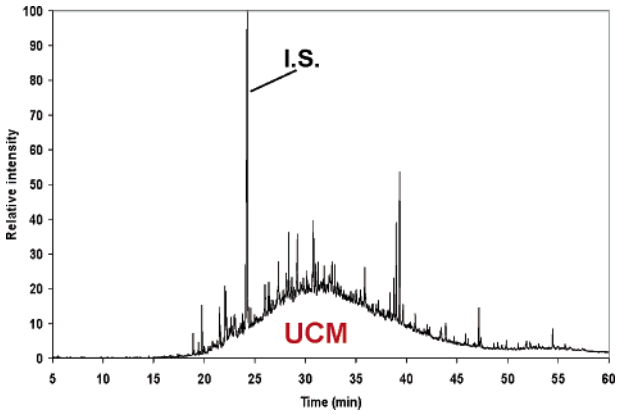

FIGURE 1. Total ion current gas chromatogram of the aromatic hydrocarbons extracted from mussels, *Mytilus edulis* sampled from Southend, U.K. I.S. = internal standard, d_{10}-phenanthrene, 5 μg g dry mussel tissue^{-1}; UCM = unresolved complex mixture; ca. 100 μg g dry mussel tissue^{-1}.

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

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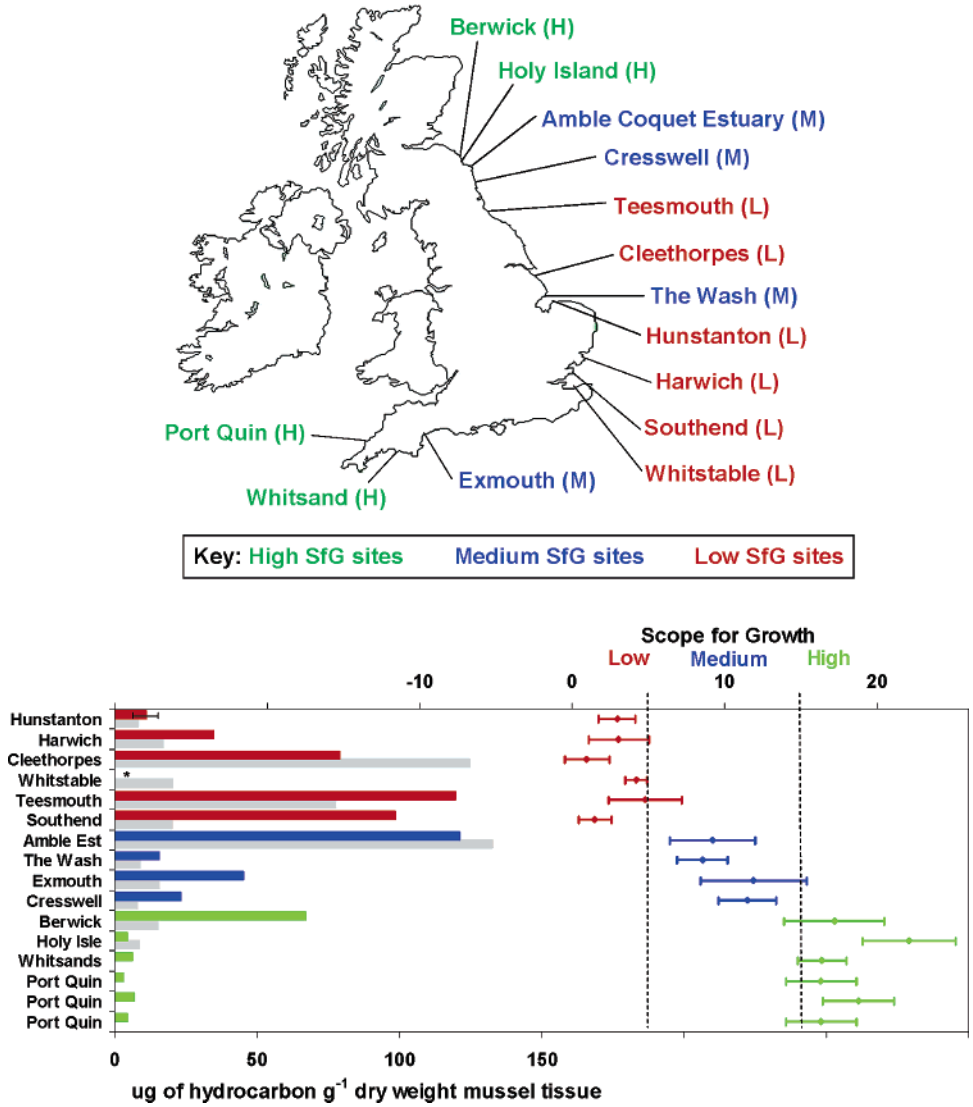

FIGURE 2. A. Map of U.K. showing locations at which populations of mussels, *Mytilus edulis* were sampled for analysis. Color code indicates Scope for Growth (health) of mussels (green = high; blue = medium, red = low) (*16*). B. Histogram and bar chart showing relationships between Scope for Growth (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 μg L^{-1} and C_{12-14} branched alkylbenzenes 5, 10, 20, 41, 82 μg 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 ($M^{+\cdot}$) 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 17−66% 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 (μg 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 1and 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

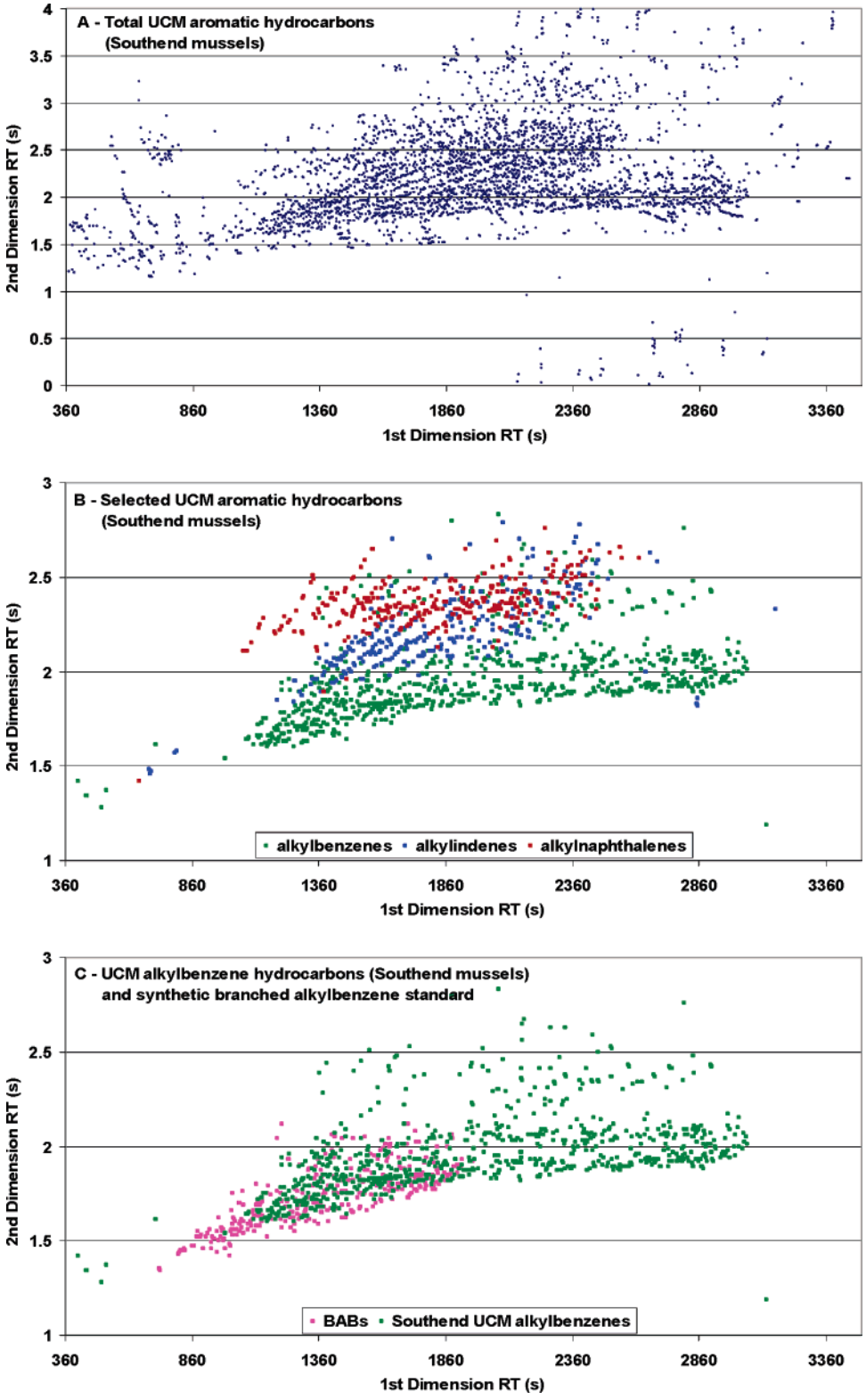

FIGURE 3. Comprehensive two-dimensional gas chromatography—time-of-flight—mass spectrometry two-dimensional chromatograms of aromatic hydrocarbon UCM fractions (cf., Figure 1) showing A, over 3400 peak marker identifiers of components in the complex mixture from mussels from Southend U.K. for which the 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.

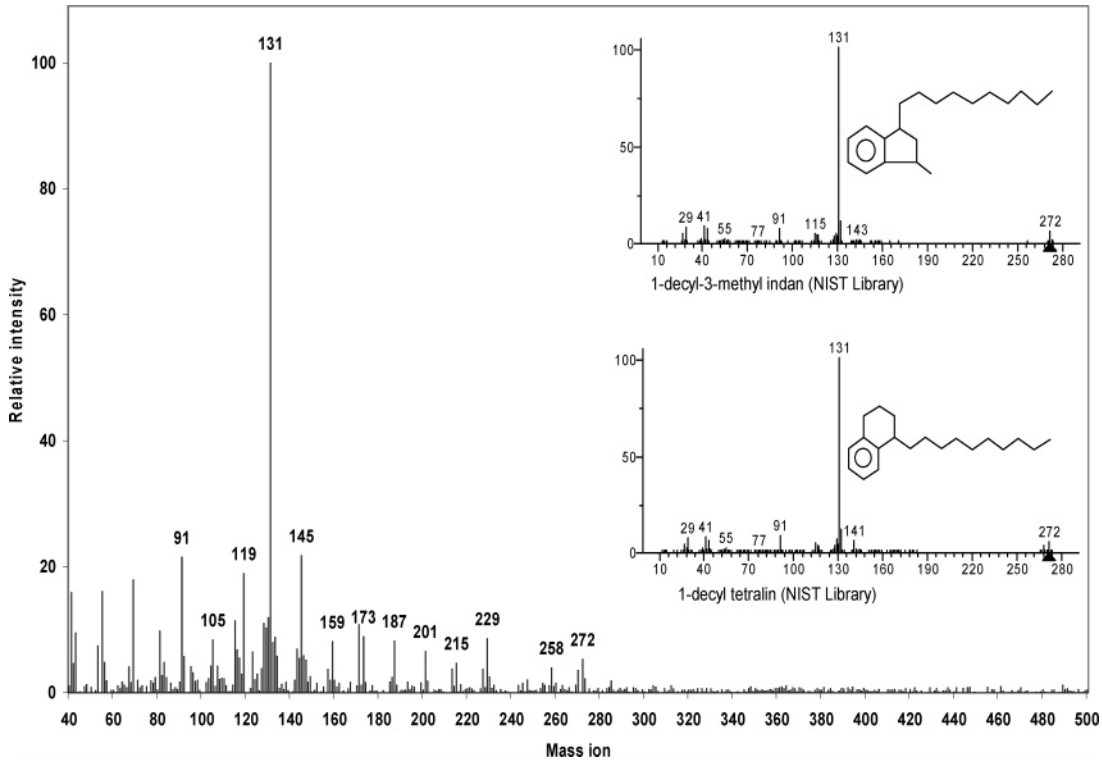

FIGURE 4. Time-of-flight mass spectrum of components of the aromatic UCM isolated from mussels from Southend, U. K. (cf. Figures 1 and 3) with GCxGC retention times 1836 × 1.960 s. The mass spectrum is assigned to a mixture of C_9 and C_{10} branched alkylindans (BINs) and/or branched alkyltetralins (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.

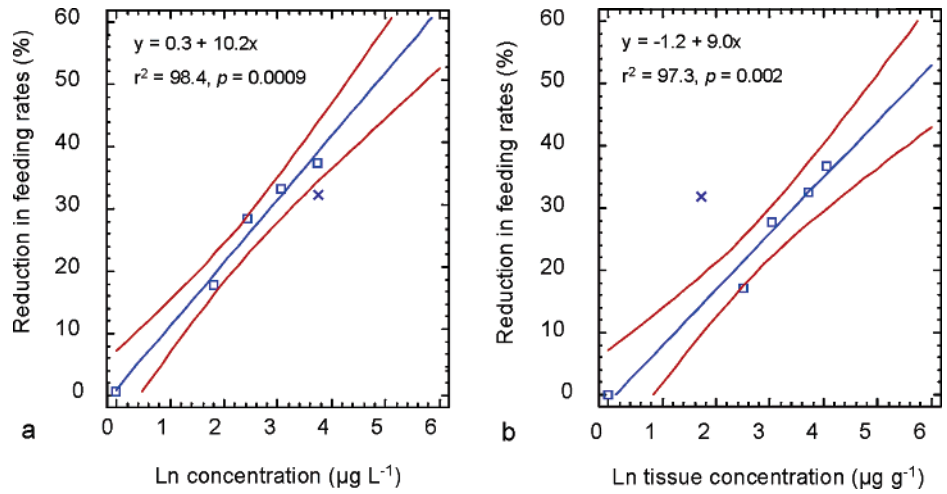

FIGURE 5. Concentration response relationships between branched alkylbenzenes and reduction in feeding rates of mussels *M. edulis*: (a) based on nominal aqueous exposure concentration range 0−41 μg L^{-1} (□) and, (b) based on whole tissue concentrations (μg g^{-1} dry weight; □). Curved lines = 95% 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 μg 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 μg 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 μg g^{-1} dry weight (Figure 5B). The lowest observed internal total body concentration associated with adverse effects (critical body residue) was 11.1 μg 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 μg 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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