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Unresolved Complex Mixtures (UCMs) of Aromatic Hydrocarbons: Branched Alkyl Indanes and Branched Alkyl Tetralins are present in UCMs and accumulated by and toxic to, the mussel *Mytilus edulis*

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Previously, comprehensive two-dimensional gas chromatography-time of flight-mass-spectrometry (GCxGC-ToF-MS) revealed that the unresolved complex mixtures (UCMs) of contaminant hydrocarbons accumulated by health-affected mussels *Mytilus edulis* (up to 125 μg g dry weight^{-1}) collected from around UK coasts, included many isomeric branched alkyl benzenes (BABs). A commercial mixture of BABs (C_{12}-C_{14}) was toxic to mussels in laboratory tests (tissue effective concentration EC_{20}10.5 μg g dry tissue^{-1}). Branched alkyl indanes (BINs) and branched alkyl tetralins (BATs) were also tentatively identified in the wild mussels, but no commercial sources of BINs or BATs were available for compound confirmation or toxicity testing. In the present study, we synthesized 14 isomeric BINs and BATs, investigated their chromatographic and mass spectral properties and measured their toxicity to mussels (*Mytilus edulis*). Comparison of the results of GCxGC-ToF-MS analysis of the synthesized compounds with those of complex mixtures of BINs and BATs in wild mussels confirmed the previous tentative identifications. Toxicity assays showed that in 72 h exposures, each of the synthetic BINs and BATs and a mixture of all were toxic to mussels at concentrations comparable to the BABs investigated previously (EC_{20}13 μg g dry tissue^{-1}). A further 5 day recovery period in clean water resulted in incomplete depuration of the accumulated body burden of BINs and BATs by the mussels. We suggest that monitoring of hydrocarbon contaminants in mussels should include an assessment of the concentrations of aromatic UCMs and ideally identification and measurement of the concentrations of BABs, BINs, and BATs and other toxic UCM components in order that the effects of these toxicants are not overlooked.

Introduction

Recently, we described the use of comprehensive two-dimensional gas chromatography-time of flight-mass-spectrometry (GCxGC-ToF-MS) to resolve, and tentatively identify, groups of contaminant alkylaromatic hydrocarbons comprising thousands of previously unidentified compounds in wild UK mussels with measurable differences in health (*1*). These mixtures, which amounted to 125 μg g dry weight^{-1} in some mussels, included structural isomers of branched alkylbenzenes (BABs) identified from mass spectra and comparison with a commercial mixture of BABs. We also showed that this mixture was toxic to mussels in laboratory tests, significantly reducing clearance rates compared with untreated control organisms. Such contaminants, usually referred to as unresolved complex mixtures (UCMs) of aromatic hydrocarbons, are widespread in many environmental compartments contaminated with petroleum-derived hydrocarbons and are distributed worldwide (e.g., reviewed in refs *2*–*5*). From their mass spectra and GCxGC behavior, groups of isomeric aromatic hydrocarbons other than BABs in the contaminated UK mussels could only be tentatively identified as branched alkyl indanes (BINs) and branched alkyl tetralins (BATs); reference hydrocarbons were not available from commercial sources, either for confirmation of the results of GCxGC-ToF-MS analysis or for toxicity testing. Since they have not hitherto been studied in this context, it was therefore not possible to establish what contribution, if any, BINs and BATs made to the depression of the health of the mussels and thus whether such compounds, like the BABs, should be considered toxic as well as persistent and bioaccumulative. Here, we describe the synthesis of six isomeric BINs and eight isomeric BATs and an assessment of their toxicity to mussels in a well-accepted clearance rate assay. Comparison of the results of GCxGC-ToF-MS analysis of the fourteen synthesized compounds with those of wild mussels confirmed the presence of similar BINs and BATs in the contaminated mussels, and the toxicity measurements showed the compounds are indeed toxic members of aromatic UCMs. A 5-day period in clean water failed to produce a complete reduction in the BINs and BATs accumulated by the mussels in the laboratory experiments.

Materials and Methods

Synthesis of branched alkyl tetralins (BATs) and alkyl indanes (BINs). 3,7-dimethyloctylmagnesium bromide solution (1.0 M in diethyl ether), cerium III chloride heptahydrate (99.999%), 1-indanone (>99%), 4-methyl-1-tetralone (97%), 6-methyl-1-indanone (>99%), 3-methyl-1-indanone (>99%), and α-tetralone (97%) were supplied by Aldrich (UK). All solvents were supplied by Rathburns (UK). Palladium on carbon catalyst (5%) was supplied by BDH. The core synthetic method used was the same for all compounds and based upon a cerium chloride promoted Grignard addition. As an example, in the preparation of 1-(3′,7′-dimethyloctyl)indane (I; Figure 1), water of crystallization was removed from cerium III chloride heptahydrate (1.30 g; vacuum pistol 0.01 mBar, 150 °C, 1 h) and an argon atmosphere introduced. Diethyl ether (20 mL, redistilled from $LiAH_4$) was added. 3,7-dimethyloctylmagnesium bromide (4.8 mmol) was added and stirred (1 h). 1-indanone (3.2 mmol) in diethyl ether (5 mL) was added under Ar pressure dropwise and the mixture stirred (1 h). Saturated ammonium chloride solution (NH_4Cl; 10 mL) and distilled water (10 mL) were added and the mixture stirred (1 h), then the organic layer was collected. The extract was washed (saturated $NaHCO_3$ (30 mL), NaCl (30 mL), distilled water (20 mL)) before drying (anhyd. Na_2SO_4). Solvent was removed (N_2). Crude product was purified by open column chromatography (Al_2O_3, 40 g, Fisher, England; grade 1, neutral, 150 mesh; deactivated 4.5% above SiO_2, 40 g, Aldrich, grade

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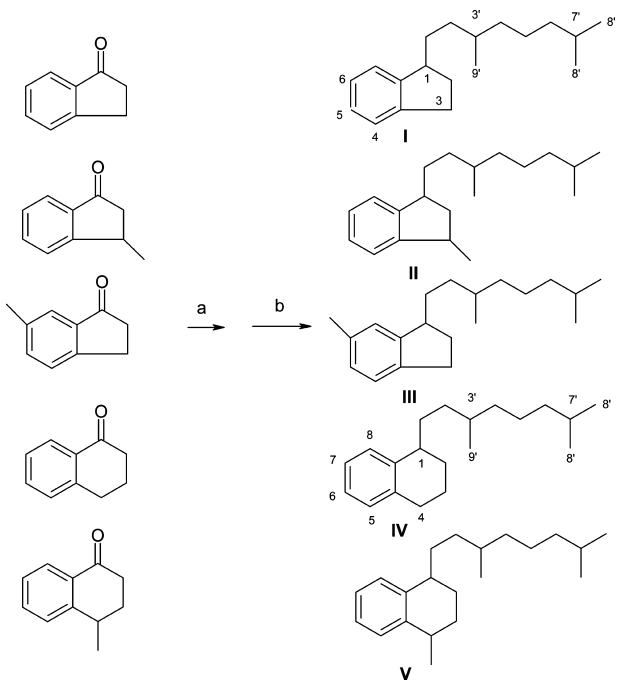

FIGURE 1. Synthetic scheme and structures of synthesized BINs and BATs. a. Grignard reaction with 3,7-dimethyloctylmagnesium bromide. b. Hydrogenolysis Pd/C (5%), AcOH, H_2.

645, 60−100 mesh). Sequential elution was by hexane, hexane (90%)/DCM(10%), DCM and methanol. Combined hydrogenation and hydrogenolysis of the DCM fraction completed the synthesis of I (H_2, 30 mL of hexane, 20 mg of palladium on carbon catalyst (5%) and 10 drops of glacial acetic acid; 3 h). Confirmation of the structures of each of the synthetic BATs and BINs (I−V; Figure 1) was provided by GC-MS, infrared and ^{1}H and ^{13}C NMR spectroscopy (e.g., Supporting Information Table S1).

Toxicology. The preparation of BIN/BAT solutions, exposure conditions and measurement of mussel clearance rates was similar to the methods described previously (*1*). The toxicity of the individual BINs, BATs and of a mixture of all fourteen isomers was tested.

Preparation of Test Solutions and Exposure Conditions. Mussels were collected from Trebarwith Sands on the northern coast of Cornwall, UK, (N50° 38.720′, W004°45.652′) and maintained at 15 °C. Mean length was 37.2 mm (standard deviation = 0.92 mm, n = 243). 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 concn. 0.005% v/v, nontoxic). The test solution was vortex mixed for a minimum of 2 h prior to use. The mussels were exposed to either solutions of single synthesized compounds or mixtures of all five compounds. The test solutions (1 L) 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 ca. 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 clearance rate assay was adapted from methods reported previously (*6, 7*). 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 ca. 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 30 min. Algal particles (3−10 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 clearance rates of the mussels were determined. Mussels were stored at −80 °C prior to extraction and quantitation of hydrocarbons.

Extraction of Mussel Tissues. Tissue samples were extracted using an alkaline saponification method adapted from previously reported methods (*8, 9*). In brief, frozen 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 (×3) in preweighed foil dishes then reweighing after drying at 105 °C for 16 h. Phenanthrene-d_{10} was added to provide an internal reference standard. Approximately 12 g of wet tissue samples were digested with potassium hydroxide pellets (12 g) and methanol (100 mL), under reflux for 2 h. The digests were filtered (Whatman 113v), extracted with n-hexane (2 × 50 mL) and dried with anhydrous sodium sulfate. Extracts were reduced in volume by rotary evaporation then further gently reduced under nitrogen and transferred to vials to provide a final volume of 1 mL.

GC-MS Analysis. The aromatic hydrocarbon mussel tissue extracts and synthesized BINs and BATs were examined on a Hewlett-Packard GC-MSD. This comprised a HP5890 series II gas chromatograph fitted with a Hewlett-Packard HP7673 autosampler and a HP5970 quadrupole mass selective detector. The column was a HP1-MS fused silica capillary column (30 m × 0.25 mm i.d. × 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 at 10 °C min^{-1} and held for 10 min. Quantitation was by the internal standard method. All samples were analyzed in selected ion monitoring mode using ions m/z 118, 131, 145. Standard curves of each compound were linear in the range 0−10 μg mL^{-1} ($r^2 < 0.998$, $p \leq 0.001$). Data and chromatograms were monitored and recorded using ChemStation (version B.02.05) software.

GCxGC-ToF-MS Analysis. The BINs and BATs were analyzed individually, as a simple mixture and with coinjection with randomly selected aromatic hydrocarbon mussel tissue extracts from a previous study (*1*). Samples 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, U.S.). Differences to the method previously reported (*1*) are outlined here. The first-dimension column was a 5% phenyl- 95% methyl-polysiloxane 30 m × 320 μm i.d. × 0.25 μm film thickness DB-5 (J&W Agilent), and the second-dimension column was a 50%-phenyl-methylpolysiloxane 1.9 m × 100 μm × 0.1 μm DB-17 (J&W Agilent). The first-dimension oven was held at 70 °C for 0.2 min, then raised from 70 to 120 at 10 °C min^{-1}, then raised from 120 to 270 at 4 °C min^{-1} and held at this temperature for 10 min. The second-dimension oven was held at 85 °C for 0.2 min, then raised from 85 to 135 at 10 °C min^{-1}, then raised from 135−285 at 4 °C min^{-1} and held at this temperature for 10 min. A second dimension modulation period of 4 s was employed. One μL (2 × 1 μL for dual injection analysis) of the sample was injected (splitless) into the GCxGC-ToF-MS system via a Gerstel multipurpose sampler (MPS 2). The inlet temperature was 300 °C. The time of flight mass spectrometer (ToF-MS) was operated at a spectrum storage rate of 50 Hz

(50 spectra s^{-1}) based on 5 kHz transients. The mass range monitored was from 50−550 Daltons.

Results and Discussion

Synthesis. The BATs and BINs (I−V; Figure 1) were synthesized by coupling a tetrahydrogeranyl (3,7-dimethyloctyl) moiety, as the alkylmagnesium bromide Grignard reagent, to a variety of tetralones and indanones. The resultant alcohols were hydrogenolysed to the final hydrocarbons (I−V) which were assigned by GC-MS and ^{1}H and ^{13}C NMR spectroscopy (Supporting Information Table S1). The monosubstituted indane (I) and tetralin (IV) and the indane substituted in the aromatic ring (III) all chromatographed (GC-MS) as doublets due to diastereoisomerism at C-1 and C-3′. The remaining compounds (II and V) chromatographed as quartets due to the one additional stereocenter in each case (at C-3 or C-4, respectively).

GC-MS and GCxGC-ToF-MS. Previously, GC-MS of aromatic hydrocarbon fractions isolated from mussels with low scope for growth (SfG; viz "unhealthy"), revealed mainly UCMs (*1*). Even GCxGC-ToF-MS only partially resolved the mixtures, but the computer algorithm used to process the GCxGC-ToF-MS data did reveal thousands of components now distinguishable by their ToF mass spectra containing molecular and characteristic base peak ions (*1*). When mass fragmentography was used to process these data, important compound classes in mussels with low SfG were shown to include branched alkyl benzenes (BABs), branched alkyl indanes (BINs), and branched alkyl tetralins (BATs). Although authenticated mixtures of BABs were available for comparison, examples of BINs and BATs were not and the available mass spectral library matches were not exact (*1*).

Herein, when the newly synthesized BINs and BATs were examined by GC-MS, the 14 individual isomers (excluding enantiomers) of a mixture of I−V could be discerned (Figure 2A). Two of the stereoisomers of the disubstituted tetralin (V) were barely resolved. GCxGC-ToF-MS of this mixture revealed no better separation of the stereoisomers of V (Figure 2B,C) but some additional separation of some of the synthetic compounds was observed in the second GC dimension (2nd dimension retention times ca. 2.3−2.6 s, Figure 2C). When examined under the same conditions as the aromatic UCMs of the wild mussels (cf (*1*)), GCxGC-ToF-MS revealed that the synthetic BATs and BINs did indeed elute in the expected regions of the 2-dimensional chromatograms (Figure 2B). Furthermore, the ToF mass spectra of the synthetic BATs and BINs were dominated by ions with *m*/*z* 117, 131, 145, as predicted previously (ref *1*; Supporting Information Figure S1 and Table S1), and ToF mass spectra of BINs and BATs in the mussel UCMs were much more clearly discerned than spectra from conventional GC-MS. Thus the tentative identification of BINs and BATs in contaminated mussels (*1*) seems well founded and is further supported by the results obtained here. However, the question remains: Are the BINs and BATs toxic to mussels and can they be concentrated by mussels to induce such effects?

Toxicity of BINs and BATs to mussels (*Mytilus edulis*). Previously, we investigated the effects of a commercial mixture of C_{12-14} BABs such as those which we found in the polluted coastal mussels, on the clearance rate of laboratory mussels (*1*). This mixture comprised components both resolved and unresolved by conventional GC-MS and when examined by GCxGC-ToF-MS, over 340 compounds were revealed, many with retention times similar to or the same as, those of the BABs in the polluted mussels. The toxicity of *n*-octylbenzene was found to be similar to that observed previously, thereby verifying the assays used (*1*). An aqueous EC_{20} of 0.007 mg L^{-1} and a tissue EC_{20} of 10.5 $\mu g\ g^{-1}$ dry weight was established for the BABs mixture (*1*). These findings confirmed that C_{12-14} BABs were indeed toxic to mussels and probably accounted for some of the depression in SfG observed in the contaminated mussels. However, there were insufficient BABs alone in the mussels to account for the observed depression in SfG; rather, the narcosis may be nonspecific and due to the additive effects of many UCM (and other) components. Indeed, the concentrations of total

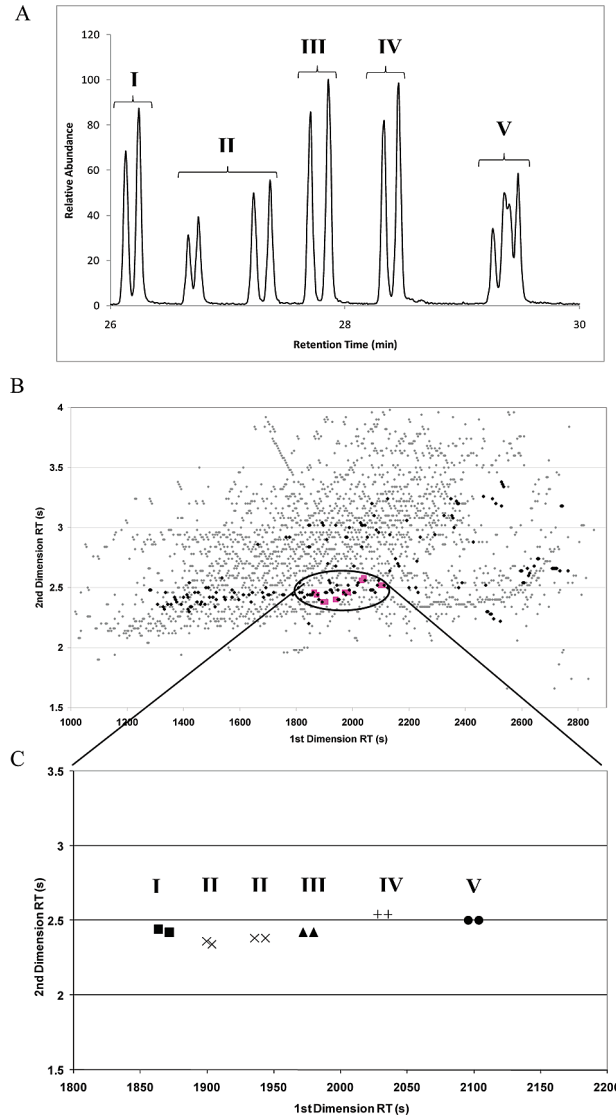

FIGURE 2. A. GC-MS total ion current chromatogram showing resolution of 14 isomeric BINs and BATs. B. GCxGC-ToF-MS two-dimensional chromatogram of aromatic hydrocarbon UCM fraction showing peak marker identifiers of thousands of components in the complex mixture in mussels collected from Southend U.K. for which the computer algorithm was able to assign unique mass spectra (*1*). Peak marker identifiers for the branched alkyl tetralin and alkyl indane components in the mixture, identified from mass spectra containing base peak ions with mass:charge (*m*/*z*) ratios 117, 131, 145, and 159 are highlighted in black points. The main "band" of peak markers corresponding to the tetralins and indanes is circled, and the retention times of coinjected synthetic BINs and BATs (I−V) are shown in pink. C. Detailed GCxGC-ToF-MS two-dimensional chromatogram of the 12 peak marker identifiers for components of the mixture of synthetic C_{19-21} BINs and BATs. The retention times of the BATs and BINs isomers in the synthetic mixture closely match some of those in the mussels and elute in the same region of the chromatogram but the mussels contain a much greater number of BATs/BINs extending over a much greater (predominantly lower) molecular weight range.

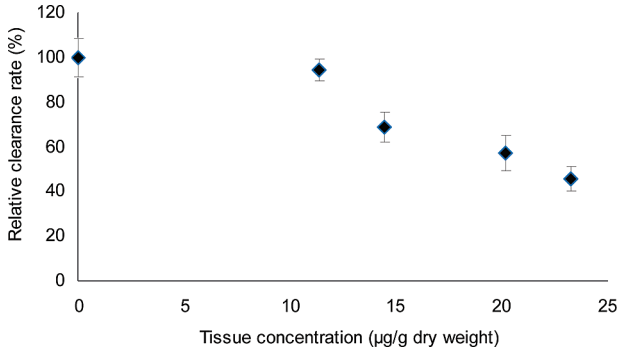

FIGURE 3. Effect of a mixture of BINs and BATs on the relative clearance rates of *M. edulis,* in terms of measured tissue concentration. Errors bars = 1 standard error.

aromatic UCM components (<10.5 μg g^{-1} dry weight) in mussels with low SfG, correlated fairly well with the measured EC_{20} for BABs, suggesting that the sum of the classes of toxic branched alkylaromatics in the UCMs is important (*1*). Clearly, at least some other UCM chemicals (including perhaps BINs and BATs) contribute to the toxic effects in contaminated mussels. The availability of the synthetic BINs and BATs now provided an opportunity to test this and to investigate whether BINs/BATs have similar, or more, or less toxic effects (EC_{20}) than BABs.

When mussels, *M. edulis*, were exposed for 72 h to aqueous mixtures of all 14 BINs and BATs at nominal concentrations of 0−500 μg L^{-1}, their feeding rates were indeed reduced by 30% at the lowest, to over 50% at the highest concentrations (Supporting Information Figure S2) as observed for BABs. However, the known limited aqueous solubilities of C_{19+} hydrocarbons (<20 μg L^{-1} (*10*)) requires that the tissue body burden of the mussels should also be determined. This is because the actual uptake of hydrocarbons by the mussels governs toxicity, not the nominal concentrations "dissolved" in the water, only part of which may be bioavailable. Thus, the reduction in feeding rates by over 50% compared with the control mussels (Figure 3), produced by accumulation of a body burden of 23 μg g dry weight^{-1} total BINs/BATs is of more significance and again suggests toxic effects comparable to those of the BABs observed previously (*1*). Indeed, the tissue EC_{20} for the BINs/BATs mixture was 13 μg g^{-1} dry weight (95% confidence interval 11−16; EC_{50} 22 μg g^{-1} dry weight; 95% confidence interval 17−27), very close to that of the BABs mixture (EC_{20}10.5 μg g^{-1}). For further comparison, the TEC_{50} values reported for polycyclic aromatic hydrocarbons (PAH) such as phenanthrene and fluoranthene (*11*) were about 31 μg g^{-1} wet weight (95% confidence interval 26−37) and 627 μg g^{-1} wet weight (95% confidence interval 362−1083).

When the toxicities of each of the compounds (I−V, Figure 1) were assayed, clearance rates were reduced by 50−75% compared to control mussels exposed to solvent only (Figure 4) under conditions (nominal aqueous concentrations 500 μg L^{-1}) whereby exposure to the mixture also produced a reduction of 50%. The variability in the effects for the different compounds can probably be attributed mainly to the variability in uptake of each, rather than to major differences in toxicity of each, since they likely have the same nonspecific narcotic mode of action. The body burden was indeed determined for each experiment with each individual compound (Figure 5) and found to vary from over 23 μg g dry weight^{-1} for the C_{19} hydrocarbon (I) to less than 1 μg g dry weight^{-1} for the C_{21} hydrocarbon (V). These variable uptakes no doubt reflect the differences in true solubilities in seawater. Smith et al. (*10*), found that the measured aqueous solubility of a C_{19} cyclohexylalkyl tetralin was only 15 μg L^{-1}; the solubilities of C_{20} and C_{21} compounds would undoubtedly

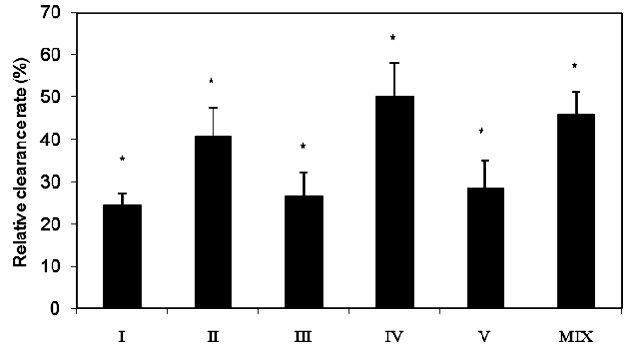

FIGURE 4. Comparison of *M. edulis* clearance rates relative to solvent control mussels when exposed to BINs and BATs individually and as a mixture. Nominal aqueous concentration was 500 μg L^{-1}. Error bars = 1 standard error. * Indicates significant reduction ($P = 0.05$).

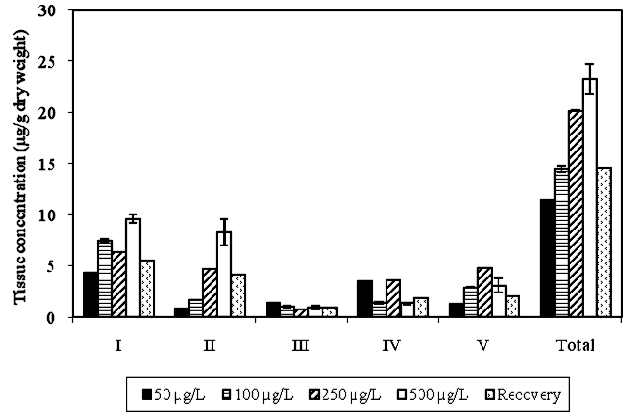

FIGURE 5. Comparison of concentrations of BINs and BATs in *M. edulis* tissues following 72 h exposures to a mixture of compounds with a nominal aqueous concentration range of 50−500 μg L^{-1} and 5 days recovery in clean seawater following 500 μg L^{-1} exposure. Error bars = 1 standard error.

be lower still. The effect is additionally illustrated by the measured uptake of different tissue body burdens (ca 2−10 μg g dry weight^{-1}) of each individual component even when mussels were exposed to an initially equimolar mixture and was observable at each nominal aqueous concentration between 50 and 500 μg L^{-1} (Figure 5). Importantly, the accumulated tissue burdens of the total BATs and BINs were the same, whether delivered individually or as a mixture to the mussels (Figure 5). Of all the BINs/BATs tested, only compound III appeared to have a somewhat greater toxicity than the others (cf. Figures 4 and 5) with the relatively low body burden of about 1 μg g^{-1} producing a 75% decrease in clearance rates. Interestingly, this was the only compound to be substituted with a methyl group in the aromatic ring (Figure 1). To determine whether this is significant would require further syntheses and toxicity measurements.

In the wild, sessile organisms such as mussels may be exposed to contaminants only periodically, while at intervening intervals, they may be able to filter uncontaminated seawater. It was thus to be expected that after a further 5 days in clean water following initial exposure to BINs and BATs, the tissue concentrations of the mussels were also reduced (Figure 5). However, no component was removed entirely and only 37% of the maximum accumulated burden of total hydrocarbons was depurated even after 5 days in clean water, suggesting longer lasting toxicological effects might be observed (Figure 6).

In conclusion, we believe these experiments demonstrate that numerous of the components of aromatic UCMs are toxic to mussels, now including not only BABs, but also BINs

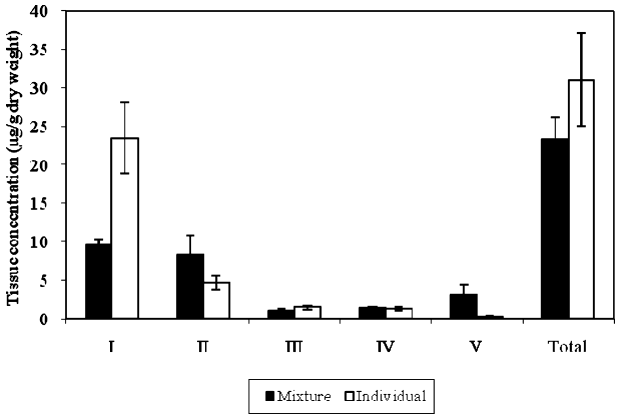

FIGURE 6. Comparison of tissue concentrations of *M. edulis* exposed to an aqueous concentration of 500 ug L^{-1} BINs and BATs, either as individual compounds or as a mixture. Error bars = 95% confidence limits.

and BATs. In all, hundreds of different compounds may be involved (e.g., refs *1, 4, 5*,). These compound classes can now be at least partially resolved, identified, and measured in mussels. We suggest that monitoring of hydrocarbon contaminants in mussels should include an assessment of the concentrations of aromatic UCMs and, where possible, identification and measurement of the concentrations of BABs, BINs, and BATs and other toxic UCM components in order that the effects of these toxicants are not overlooked.

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Supporting Information Available

The figures show time of flight mass spectra of an isomer of a synthetic BIN and of a component of the aromatic UCM isolated from mussels from Southend, U. K.; a graph showing the effects of BINs and BATs on the mussel clearance rates versus nominal aqueous concentrations; tabulated NMR and MS data for the synthetic BATs and BINs. This material is available free of charge via the Internet at http://pubs.acs.org.

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