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HYDROCARBON HUMPS IN THE MARINE ENVIRONMENT: SYNTHESIS, TOXICITY, AND AQUEOUS SOLUBILITY OF MONOAROMATIC COMPOUNDS

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Abstract—A recent study has shown that some monoaromatic hydrocarbon constituents of the so-called unresolved complex mixtures (UCMs) or gas chromatographic humps, which are widespread in the marine environment, are toxic to the mussel *Mytilus edulis*. Here we describe the synthesis and toxicological assessment of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, and 7-cyclohexyl-1-*n*-propyltetralin, which contain structural features consistent with some monoaromatic UCM hydrocarbons. The compounds were all toxic to *M. edulis* when measured in the assay used previously to determine the toxicity of a monoaromatic UCM. The aqueous solubilities of the hydrocarbons in fresh and seawater at different temperatures were determined and found to range from about 10 to 110 μg/L (10–60 μg/L in seawater at 15°C). Further studies of the aromatic UCM composition of a wide range of oils and oil residues are required to determine whether such alkylated compounds as 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-*n*-propyltetralin or their analogues are widespread in oils. If these aromatic compounds prove to be important in UCMs, toxicity experiments should be conducted with other biological end points and monitoring studies of pollutant hydrocarbons should probably include measurement of aromatic UCM hydrocarbons.

Keywords—Hump Hydrocarbons Solubility Alkyltetralins Unresolved complex mixtures

INTRODUCTION

Unresolved complex mixtures of hydrocarbons dominate the gas chromatograms of the weathered residues of petroleum. Such residues occur widely in the marine environment [1], yet only a few studies of the toxicity of unresolved complex mixtures (UCMs) appear to have been published [2,3]. Although nonaromatic UCM hydrocarbons appear to be nontoxic or to contribute in only a minor way to the toxic hydrocarbon burden of the mussel *Mytilus edulis* [2], we were recently able to demonstrate that some monoaromatic UCM components were toxic to mussels [4]. Furthermore, coastal mussels from the United Kingdom with impaired health, as measured by Scope for Growth, had substantial aromatic UCM burdens [4; Fig. 1]. Clearly, further studies of this pollutant burden are required and the identities of the toxic components need to be established.

Since the pioneering attempts of American Petroleum Institute scientists to unravel the composition of Ponca City, Oklahoma, USA, crude oil in the 1950s, comparatively few studies of the major unresolved hydrocarbons of crude oil have in fact been published [e.g., 1,5–10]. Instead, a detailed knowledge of the chromatographically resolved hydrocarbons of petroleum has been accrued [11]. Most recent studies of the unresolved hydrocarbon composition have been based on the principle of oxidative or pyrolytic degradation of UCM hydrocarbons followed by deuteration or derivatization of the resulting UCM fragments and gas chromatography-mass spectroscopy [1,7] or ion cyclotron resonance-mass spectroscopy [10] analysis of the resulting products. Many of these studies have shown that, of the aromatic UCM compounds, the monoaromatics are quantitatively important [5–7,9,10]. This also appears to be the case for pollutant aromatic UCMs in mussels [4]. So-called retrostructural analysis of UCM composition has suggested that, among these monoaromatics, alkyl-substituted alkyltetralins may be important [7,9,10]. Thus, ruthenium tetroxide oxidation of monoaromatic UCMs from several crude oils produced carbon dioxide from unsubstituted ipso carbons, straight chain carboxylic acids from *n*-alkyl substituents, and branched and cyclic carboxylic acids from naphthenic rings and branched chain substituents [5,7,9,10,12]. These data suggest that monoaromatic rings with straight chain, branched, and cyclic substituents are important in monoaromatic UCMs, and alkyltetralins are an example of possible UCM compounds. Since a recent study also indicated that the hydrocarbons of a monoaromatic UCM isolated from a crude oil were somewhat toxic to mussels, leading to a >40% decrease in filtering rate over 24-h accumulation of a UCM [4], we became interested in the toxicity of alkyltetralins. In the present study, we synthesized 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, and 7-cyclohexyl-1-*n*-propyltetralin by Haworth-type reactions of phenylcyclohexane and tested their toxicity to the mussel *Mytilus edulis.* The final hydrocarbon products were isolated pure (Fig. 1) and the structures were confirmed by spectroscopy. Although these compounds have yet to be identified as hydrocarbon entities of UCMs, they appear at least to contain structural features consistent with the limited known composition of monoaromatic UCM components [5–10,12]. The compounds were toxic to *M. edulis,* reducing the filtering rate substantially compared with untreated controls. The aqueous solubilities of the hydrocarbons ranged from about 10 to 110 μg/L, depending on molecular weight, temperature, and salinity. The concentrations of the bioaccumulated monoaromatic hydrocarbon residues giving rise to these toxic effects were within the range of aromatic UCM concentrations detected in mussels from polluted environments. These data fur-

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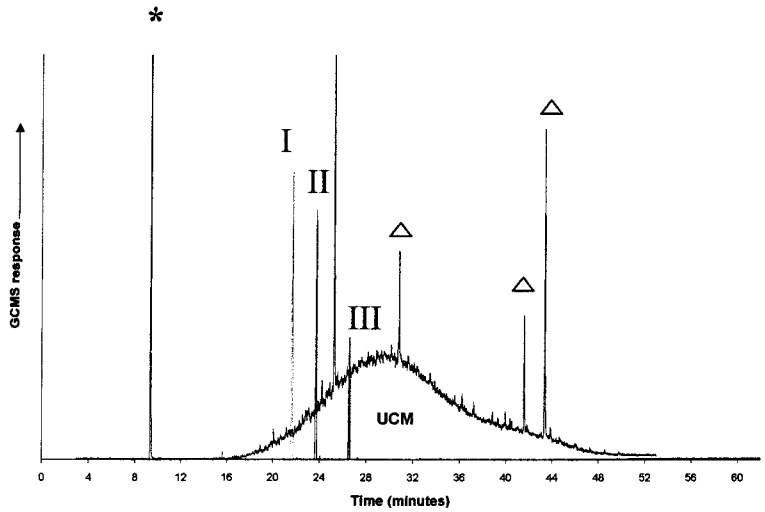

Fig. 1. Gas chromatograms showing a typical unresolved complex mixture (UCM) of monoaromatic (four double bond equivalents) hydrocarbons isolated from environmental mussels collected from Whitby, United Kingdom [13], and the three synthetic tetralins (I–III). * = internal standard d_{12} tetralin, Δ = biogenic tetraenes. Gas chromatography conditions are cited in the text and in Wraige [13].

ther suggest that monoaromatic UCM hydrocarbons may have been overlooked as a pollutant class.

MATERIALS AND METHODS

Synthesis and characterization of hydrocarbons

The 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, and 7-cyclohexyl-1-*n*-propyltetralin (I–III) were synthesized by a Haworth-type reaction scheme (Fig. 2). Intermediates and final products were characterized by mass spectrometry (MS), ^{1}H and ^{13}C nuclear magnetic resonance spectrometry (NMR), and infrared spectroscopy. Purity was measured by capillary gas chromatography (GC). The data for the intermediates are given elsewhere [13]. Data for the hydrocarbons are summarized in the Appendix.

Toxicological tests

A widely used, previously published mussel filtering-rate assay (15°C) was employed [13–16]. Briefly, toxicant solutions

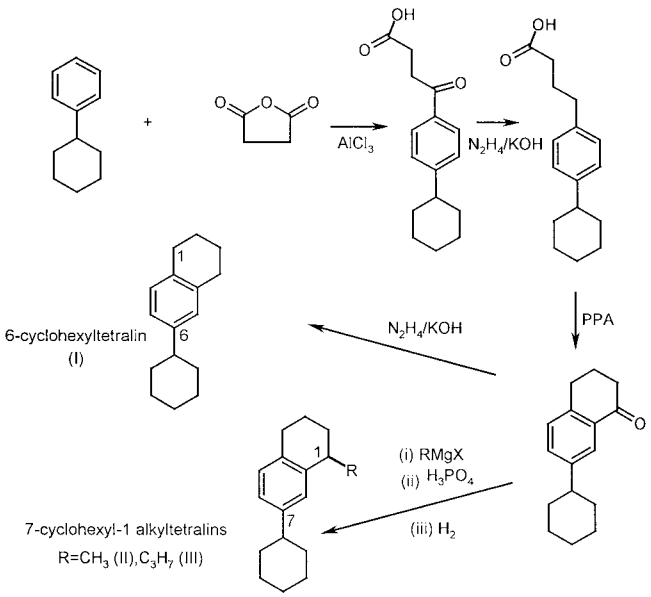

Fig. 2. Reaction scheme for synthesis of monoaromatic hydrocarbons (polyphosphoric acid [PPA]).

were prepared by adding different concentrations of the toxicant in 0.001% of acetone to filtered seawater (0, 12.5, 25, 50, and 100 μg/L toxicant). Solutions were stirred for 2 h prior to use. Controls comprising filtered seawater and acetone only (0.001% v/v) were also prepared. Groups of seven mussels, shell length 12 to 14 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 were filtering. Mussels were exposed to toxicant for 24 h. Two vessels were prepared for each toxicant concentration. 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) 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 determined in triplicate per aliquot using a Coulter Counter (Coulter Electronics, Toronto, ON, Canada) 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 was calculated.

Solubility measurements

Solubility measurements were made according to the following method, which is similar to a literature method [17]. Anthracene was purchased from Aldrich (Poole, UK) with a purity of >99% (as determined by GC). The model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7 cyclohexyl-1-methyltetralin, and 7-cyclohexyl-1-propyltetralin, were synthesized in good yield and with purities as described above. Each generator column consisted of a high-pressure liquid chromatography stainless steel column 30 cm × 0.46-mm i.d. with 2 μm stainless steel frits at either end, dry packed with

glass beads 60 to 80 mesh coated with test compound. Test compounds (35 mg) were dissolved in 50 ml of hexane to which 7 g of beads were added. The solvent was removed by rotary evaporation followed by a gentle stream of nitrogen to give a 0.005% coating. Once packed, the generator column was attached to a pump by means of Teflon tubing and attachments, supplied by a water reservoir. Temperature control (15 and 25°C) was by means of a water bath in which the generator column and water reservoir were immersed. A 3-L beaker containing either MilliQ water (Bedford, MA, USA) or seawater (salinity 33‰, to which was added mercuric chloride for sterilization, acted as a water reservoir.

The generator column was flushed with 500 ml water to allow equilibration of the system before measurement. Water was pumped through the column at a rate of 1 ml/min. Effluent from the column was collected directly into a 100-ml separating funnel containing 25 ml dichloromethane. Once the sample had been collected, an internal standard (I for the determination of II and III and II for the determination of I) was spiked into the water in 100 μl acetone and the funnel stoppered. Each funnel was shaken for 5 min with care so as not to form an emulsion. The dichloromethane (DCM) fraction was separated off and the water sample further extracted with another 25 ml DCM. The DCM extracts were combined and passed through an anhydrous sodium sulfate column. Analysis was performed using gas chromatography/mass spectrometry (GC-MS) operated in single ion monitoring (SIM) mode.

The effect of salinity on solubility was determined by carrying out the above procedure but replacing MilliQ water with seawater. The effect of temperature was determined by carrying out the above measurements at 25 and 15°C by using a dip chiller unit along with the water bath to achieve the required temperature. Temperature and salinity were monitored throughout the experiment.

Instrumental conditions

Gas chromatography-mass spectrometry 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, Avondale, PA, USA). 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 and held at 300°C for 10 min. Mass spectrometer operating conditions were ion source temperature 250°C, ionization energy 70 eV, mass range 35 to 600 Daltons, and selected ion monitoring.

RESULTS AND DISCUSSION

The effects of the three tetralins on mussel filtering rates were determined using the assay employed previously to measure UCM toxicity and which is very widely accepted as a measure of sublethal narcotic toxicity [14–16]. A substantial decrease (over 60% reduction compared with controls) in mussel feeding was observed (Fig. 3). The relationship between exposure concentration and accumulated body burden after 24 h was linear (e.g. I, $R^2 = 0.998$; III, $R^2 = 0.986$). For 6-cyclohexyltetralin, the reduction in feeding rate was significantly different (*t* test, $p \leq 0.001$) from the controls at all exposure concentrations >12.5 μg/L (about 25 μg/g wet wt), and for 7-cyclohexyl-1-propyltetralin, the reduction in filtering rate was significantly different (*t* test, $p \leq 0.001$) from the controls at all exposure concentrations >25 μg/L (about 45 μg/g wet wt). Clearly, such hydrocarbons are toxic to mussels,

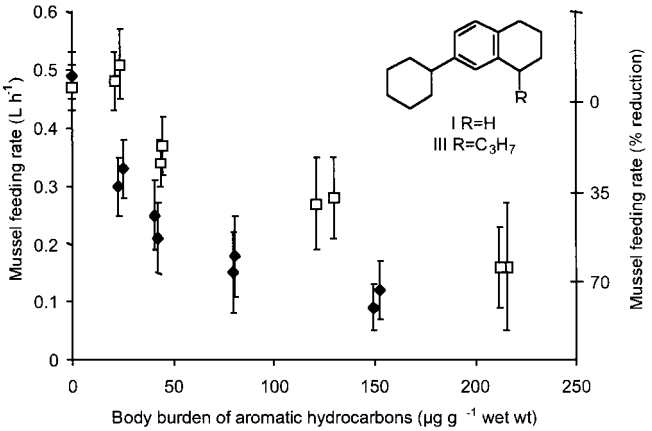

Fig. 3. Feeding rates and hydrocarbon body burdens of populations of laboratory mussels, *Mytilus edulis.* Untreated (controls, 0 μg/L, 0 μg/g accumulation) and treated (12–100 μg/L, ~25–225 μg/g accumulation) with 6-cyclohexyltetralin (I, ◆) and with 7-cyclohexyl-1-propyltetralin (III, ■) for 24 h. Values are means ($n = 7$) ± 1 standard deviation. (Data for 7-cyclohexyl-1-methyltetralin [II] are not shown in order to maintain the clarity of the diagram; values were intermediate to those for I and III).

and accumulation of up to 150 μg/g wet weight (average tissue effect concentrations [TEC50] 0.2 mmol/kg) of 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin and up to 220 μg/g wet weight (average TEC50 0.5 mmol/kg) of 7-cyclohexyl-1-*n*-propyltetralin occurred in 24 h. These correspond to a maximum of about 800 μg/g dry weight. The effects observed for the synthetic compounds in the laboratory are similar to those observed for other hydrocarbons with similar physicochemical properties [16].

Do these concentrations represent environmentally relevant values for accumulation of UCMs by mussels? The answer, we believe is, yes; while the aqueous concentrations of hydrocarbons used in our experiments may only be encountered rarely, such as during oil spills, mussels in the environment are able to accumulate high body burdens of UCMs by filtration. For example, about 100 to 500 μg/g dry weight aromatic UCM hydrocarbons were determined in toxicologically impacted (reduced scope for growth [16]) mussels from the east coast of the United Kingdom [4], and reductions of about 40% in filtering rates were observed when laboratory mussels accumulated 90 μg/g wet weight (~350 μg/g dry wt) monoaromatic UCM hydrocarbons [4]. The aromatic UCM concentrations and toxic effects on field mussels [4], aromatic UCM-treated laboratory mussels [4], and laboratory mussels treated with synthetic compounds herein therefore all suggest a previously unrecognized toxic effect of monoaromatic UCM hydrocarbons.

Although the mechanisms by which mussels accumulate organic pollutants have been quite extensively studied, to our knowledge, these studies have not included detailed investigations of the manner in which mussels accumulate monoaromatic UCM hydrocarbons. Presumably, whether the UCM hydrocarbons are present in dissolved, colloidal, or particle-sorbed phases may have important consequences for uptake and toxicity, and this will require further study. However, since aqueous solubility is often an important factor controlling the concentrations of hydrocarbons in solution, as an initial investigation into the physical characteristics of the synthetic tetralins, we determined the aqueous solubilities in freshwater and seawater at 15 and 25°C (Table 1) by a well-known gen-

Table 1. Solubilities of alkyltetralins I–III in fresh and seawater (μg/L; mean ± standard deviation, $n = 10$); data for anthracene are shown in parentheses for comparison of the method with literature values [17], ‰ = parts per thousand salinity

	Aqueous solubility (μg/L) (mean ± standard deviation)			
Hydrocarbon	25°C 0‰	15°C 0‰	25°C 35‰	15°C 35‰
Anthracene	45 ± 2 (44)	22 ± 1 (17–23)	29 ± 1 (32)	
6-Cyclohexyltetralin (I)	109 ± 6	75 ± 3	95 ± 5	56 ± 3
7-Cyclohexyl-1-methyltetralin (II)	45 ± 4	27 ± 3	40 ± 2	21 ± 2
7-Cyclohexyl-1-propyltetralin (III)	23 ± 3	13 ± 2	17 ± 2	9 ± 2

erator column method [17]. The method was calibrated by determination of the solubility of anthracene, for which literature data are available (Table 1). The solubilities were found to range from 10 to 110 μg/L. These solubility values extend the measured effects of soluble hydrocarbons on mussel feeding rate according to previously established quantitative structure–activity relationships for *M. edulis* [14–16].

Interestingly, Barron et al. [18] recently examined the water-accommodated aromatic hydrocarbon content of three environmentally weathered oils collected from underground plumes of spilled oil at a coastal California, USA, oil field by selected ion monitoring gas chromatography-mass spectroscopy and reported that, although the fractions were toxic to the mysid shrimp *Mysidopsis bahia*, the toxicity was, unexpectedly, not correlated with polycyclic aromatic hydrocarbons concentration. Although the actual toxic components were not identified, the total ion current chromatograms of the oils [18] show that the hydrocarbon fractions tested were dominated by aromatic UCMs. Given the solubility of the present synthetic compounds, it seems reasonable that a contribution of monoaromatic UCM hydrocarbons to mysid toxicity should be considered.

CONCLUSIONS

The experiments outlined above indicate that 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, and 7-cyclohexyl-1-*n*-propyltetralin are toxic to *M. edulis*. Further studies of the aromatic UCM composition of a wide range of oils and oil residues are required to determine whether such alkylated compounds as 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-*n*-propyltetralin or their analogues are widespread in oils, and these are underway in our laboratory. If these aromatic compounds prove to be important in UCMs, toxicity experiments should be conducted with other biological end points.

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APPENDIX

Spectroscopic and chromatographic data for the synthetic alkyltetralins.

6-Cyclohexyltetralin

I; 87% yield; >99% gas chromatography (GC) purity: characterized by mass spectrometry (MS), infrared (IR), and nuclear magnetic resonance (NMR). MS: *m*/*z* 214 ($M^{+\cdot}$, 100%);

171; 145; 158; 129. ^{13}C-NMR (ppm): 145.2, 136.8, 134.5, 129.0, 127.4, 124.0, 44.2, 34.6, 29.5, 26.9, 26.2, 23.3. ^{1}H-NMR (ppm): *quintet* 6.9; *d* 2.7; *br m* 2.4; *m* 1.7; *m* 1.4.

7-Cyclohexyl-1-methyltetralin

II; 75% yield; >90% GC purity: characterized MS, IR, and NMR. MS: *m*/*z* 145 (100%), 129 (64%), 228 (M^+, 61%), 213 (51%), 128 (26%), 131 (22%), 55 (17%). ^{13}C-NMR (ppm): 145, 142, 134, 129, 127, 125, 44, 33, 22. ^{1}H-NMR (ppm): *m* 7.0; *br m* 2.5, 2.8, 2.9; *br m* 1.3 to 1.9.

7-Cyclohexyl-1-propyltetralin

III; 91% yield; 97% GC purity: characterized MS and NMR (^{13}C, DEPT, ^{1}H). MS: *m*/*z* 256 ($M^{+\cdot}$, 12%), 213. ^{13}C-NMR (ppm): 145.2, 141.4, 134.4, 128.9, 127.0, 123.8, 44.3, 39.3, 37.4, 34.6, 34.5, 29.4, 27.4, 27.0, 26.2, 20.7, 19.8, 14.3. ^{1}H-NMR (ppm): *quintet* 6.9, *br m* 2.7, *br m* 2.4, *m* 1.3 to 1.7, *t* 0.9.