

# Time-Dependent Toxicity of Fluoranthene to Freshwater Invertebrates and the Role of Biotransformation on Lethal Body Residues

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The time-dependent toxicity of fluoranthene was examined for *Hyalella azteca*, *Chironomus tentans*, and *Diporeia* spp. *C. tentans* appeared to be the most sensitive species, and *Diporeia* was the least sensitive. Incipient LC<sub>50</sub> values, the concentration at which the LC<sub>50</sub> reaches an asymptote and does not change with increasing duration of exposure, for *H. azteca* and *C. tentans* were approximately 60 and 40  $\mu\text{g}\cdot\text{L}^{-1}$ , respectively. Incipient levels were not reached for *Diporeia*; however, the 28-d LC<sub>50</sub> concentration was 95.5  $\mu\text{g}\cdot\text{L}^{-1}$ . There was a temporal relationship with respect to lethal body residues for each of the test species. For *H. azteca*, the LR<sub>50</sub>, the median lethal residue at an identified exposure time required to cause 50% mortality, based on total fluoranthene equivalents (parent + metabolite compounds) decreased from 3.19  $\mu\text{mol}\cdot\text{g}^{-1}$  at 5 d to 0.80  $\mu\text{mol}\cdot\text{g}^{-1}$  at 28 d. For *C. tentans*, the LR<sub>50</sub> decreased from 0.43 to 0.17  $\mu\text{mol}\cdot\text{g}^{-1}$  from 2 to 10 d. The 10-d LR<sub>50</sub> for *Diporeia* was 9.97  $\mu\text{mol}\cdot\text{g}^{-1}$ , and the 28-d value was 3.67  $\mu\text{mol}\cdot\text{g}^{-1}$ . The toxicokinetics are not sufficient to address the temporal changes in LR<sub>50</sub> values. Thus, the data were fit to a Damage Assessment Model that also accounts for toxicodynamic processes. This analysis provides estimates of the incipient lethal residues for *H. azteca*, *C. tentans*, and *Diporeia*: 0.84, 0.21, and 3.00  $\mu\text{mol}\cdot\text{g}^{-1}$ , respectively. When comparing the relative sensitivity among species using lethal body residues, special attention should be given to ensure that comparisons are made at a common point in relation to exposure duration (i.e., time to steady state,  $T_{\text{SS}}$ ). When the LR<sub>50(lipid)</sub> values among the three species were compared at steady state, *C. tentans* is more sensitive than *H. azteca* and *Diporeia* spp.; however, there are no significant differences between the amphipod species. The greater sensitivity of *C. tentans* to fluoranthene as compared to the amphipods may be due, in part, to a potential toxic metabolite.

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## Introduction

Approximately 60% of industrial chemicals present in the environment cause baseline or narcotic toxicity (1). Narcotic chemicals, for the purposes of this study, are defined as anesthetics that are hypothesized to have nonspecific and reversible binding and are assumed to elicit their toxic effects by disrupting the lipid bilayer of cells, resulting in loss of selective permeability and ultimately the death of the organism. Chemicals that have been classified as narcotics include chlorinated benzenes, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (see ref 2 for a review). In addition to death, exposure to narcotic chemicals also may affect a variety of sublethal processes including growth, reproductive capacity, and developmental time (3–5).

In assessing the risk of narcotic contaminants to aquatic biota, external contaminant concentrations in various environmental media (e.g., water, sediment, and food) have traditionally been used as a surrogate for the internal target site concentration that is required to produce an effect. As such, external concentrations of narcotics required to produce acute or chronic toxicity can vary by orders of magnitude (6). This variability occurs because estimates based on environmental concentrations describe the intrinsic toxicity as well as bioaccumulation potential of the contaminant, which is influenced by various biotic and abiotic factors (7). Furthermore, the use of external concentrations to describe exposure generally limits the assessment to a single dominant exposure route. Thus, it is desirable to establish a method to reduce the variability and uncertainty associated with measuring and reporting toxicity.

McCarty and Mackay (7) proposed the use of internal body residues for examining the relationship between bioaccumulation and subsequent effects on biota, suggesting that whole-body residues are reasonable first approximations to the amount of chemical at the site of toxic action. The advantages of this approach over the traditional environmental media-based dose are the implicit consideration of both contaminant bioavailability and multiple exposure routes. On the basis of empirical and theoretical studies, McCarty et al. (8) developed the critical body residue (CBR) approach, which has been defined as the internal residue concentration that produces 50% mortality. According to the “narcosis hypothesis” for poorly metabolized nonpolar organic compounds, an approximately constant toxic threshold exists independent of exposure concentration, exposure conditions, and time (9). Toxicity based on internal residue concentrations has been shown to be relatively constant for acute lethal narcosis in fish ranging from 2 and 8  $\text{mmol}\cdot\text{kg}^{-1}$ . Despite the apparent threshold suggested from acute mortality studies, chronic mortality has been determined to be approximately an order of magnitude less (6, 10, 11).

Despite the implicit assumption of a threshold resulting in a constant body residue for nonpolar narcosis (9, 12), recent work has demonstrated that, even for compounds with a narcotic mode of action, the body residue responsible for 50% mortality can be temporally variant for fish (13, 14), crabs (15), and amphipods (16, 17). This variation appears to result from a combination of toxicokinetic and toxicodynamic processes (18). For longer exposures, there is an apparent buildup of damage with constant exposure that is not addressed by the toxicokinetics alone. Thus, defining the temporal dependency of lethal body residues should improve the utility and application of the body residue as a dose metric for adverse effects in aquatic biota.

For compounds that are not biotransformed, the evaluation of body residue response relationships can be relatively straightforward. However, for polycyclic aromatic hydrocarbons (PAHs), the potential for biotransformation could be a complicating factor in assessing the role of body residues as a dose metric. The use of body residues could fail because the potential contribution of metabolites to the body residue response relationship was not determined. Conversely, the prevalent use of radiotracers in toxicity and bioaccumulation tests often results in body burdens being expressed as total radioactivity (parent compound plus metabolites). This may result in an over estimation of the active toxic compounds from the inclusion of essentially nontoxic metabolites, such as conjugated forms of phase I biotransformation products. For PAHs, the parent compound plus phase I metabolites are considered the likely contributors to nonpolar narcosis (19). In a previous study with *H. azteca*, the metabolites of naphthalene were suggested to be less toxic than the parent compound, but there was no definitive analysis of the extent of biotransformation (16). For *Daphnia*, the 9-hydroxyfluoranthene was found to be similarly toxic compared to the parent compound, while more degraded and more water-soluble fluoranthene metabolites were less toxic (20).

The objectives of this study were (i) to compare lethal body residues of fluoranthene for three species of freshwater invertebrates, (ii) to evaluate the temporal component of lethal body residues at differing exposure intervals up to 28 d, and (iii) to assess the potential impact of biotransformation on the body residue response relationship.

## Materials and Methods

**Organisms.** Three invertebrate species were selected for use and included the amphipods *Hyalella azteca* (juvenile) and *Diporeia* spp. (juvenile) and the midge, *Chironomus tentans* (third instar). *H. azteca* and *C. tentans* were chosen because they have been recommended by the U.S. Environmental Protection Agency (EPA) for sediment toxicity testing (21, 22) and because of their ecological importance and geographical distribution. The amphipod, *Diporeia* spp., was selected because the organism is an important component of the Great Lakes food webs and its biology differs from that of the other two species, in that *Diporeia* has minimal ability to biotransform PAHs (23). *H. azteca* and *C. tentans* are currently being cultured at Southern Illinois University—Carbondale (SIUC), Carbondale, IL, in accordance with EPA methods (21). *Diporeia* spp. were field-collected using a ponar grab from an 80-m deep station on Lake Michigan (43°11.747' N, 86°28.957' W), which has been shown to have low sediment contaminant concentrations (24). Organisms were kept cool on ice during transport from the field to the Great Lakes Environmental Research Laboratory, Ann Arbor, MI, and then on to SIUC. Upon arriving at SIUC, organisms were placed into shallow aquaria containing 2–3 cm of sediment and 10 cm of water from Lake Michigan at 4 °C for 1 week to eliminate organisms possibly injured during collection and transport. At the end of 1 week, the temperature was increased 1 °C per day to a final temperature of 7 °C for testing.

**Chemicals.** <sup>14</sup>C-Radiolabeled fluoranthene (specific activity 44 mCi mmol<sup>-1</sup>) was purchased from Sigma-Aldrich (St. Louis, MO). The nonradiolabeled compound was obtained from Chemservice (West Chester, PA). The radiolabeled compound was tested for purity using a combination of high-pressure liquid chromatography (HPLC, Agilent model 1100, Atlanta, GA) followed by liquid scintillation counting (LSC) using a Packard 1900TR Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL). The HPLC separations were performed with a Zorbax SB-C18 column (5 μm, 4.6 × 150 mm) and separate fractions were collected using a Foxy Junior fraction collector (Isco, Lincoln, NE). Samples were counted on the LSC for 20 min per vial using

automatic quench control. Sample counts were corrected for background and quench using the external standards ratio method. The LSC was calibrated prior to each use, and the <sup>14</sup>C counting efficiency was found to be in excess of 96%. Isotope purity was determined to be >95%. Stock solutions were prepared by adding known quantities of radiolabeled compound to known amounts of nonradiolabeled compound using acetone as a carrier. Stock concentrations were verified using HPLC and LSC. The specific activity was recalculated by adjusting for the isotopic dilution.

**Exposure Media.** All experiments employed an aqueous exposure route. Moderately hard exposure water (MHW) was prepared by adding the necessary salts to deionized water and then allowing the water to mix overnight to ensure the required water quality (25). *Diporeia* spp. exposures utilized natural Lake Michigan water collected from the same site as the organisms. The lake water was filtered through a 0.45-μm glass fiber filter prior to spiking. The spiking procedure consisted of adding predetermined amounts of fluoranthene stocks to a bulk aliquot of water. The volume of the acetone carrier was the same for each exposure concentration and was less than 100 μL·L<sup>-1</sup> in all exposures. Solvent controls received the same amount of acetone as the fluoranthene-spiked treatments. Exposure media was prepared daily.

**Experimental Design.** The time-dependent acute toxicity for each species was examined using 10-d (*C. tentans*) and 28-d exposures (*H. azteca* and *Diporeia*). Experiments involving *H. azteca* and *C. tentans* were conducted in a Precision Scientific Environmental Chamber (Chicago, IL) maintained at 23 °C with a 16-h light:8-h dark photoperiod using gold fluorescent light to minimize photodegradation and photoinduced fluoranthene toxicity. *Diporeia* were exposed at 7 °C with no light to approximate their natural habitat. All organisms were exposed to a series of five contaminant concentrations, predetermined from range-finding experiments. *H. azteca* and *Diporeia* exposures were conducted using 500-mL beakers containing 200 mL of water. A total of 26 replicates and 10 organisms per replicate were used in each experiment, with five beakers remaining at 28 d. *C. tentans* were exposed individually in 25-mL beakers containing a substrate of 1.5 g of quartz sand using 100 replicates per concentration at the beginning of each experiment with 50 beakers remaining at the end of the experiment. *C. tentans* were exposed individually due to their cannibalistic nature and propensity to construct tubes from the available substrate, which makes assessing daily mortality difficult.

Exposure water concentrations were monitored before and after each daily water change by removing a 2-mL water sample and counting via LSC. *H. azteca* and *C. tentans* were fed 100 μL of a 40 g·L<sup>-1</sup> ground Tetramin daily (note that route of exposure is not important since all measurements will be linked to body residue levels). Mortality was assessed daily, and the remaining organisms at the end of the 28-d exposures, live and dead, were removed and body residues measured using LSC. Detailed methodology on the chemical analysis is described below.

The accumulation kinetics were determined at exposure times of 0.5, 1, 2, 4, 7, 10, and 28 d for *H. azteca* and 0.5, 1, 2, 4, and 10 d for *C. tentans*. Accumulation was determined for *Diporeia* spp. at 1, 2, 4, 8, 16, and 28 d. At each sampling time, two replicates were removed randomly from each concentration. Live organisms were removed from the water, rinsed, blotted dry, and weighed to the nearest 0.01 mg using a Mettler analytical balance (Toledo, OH). The organisms were then analyzed by placing them directly into scintillation cocktail, sonicated for 60 s (Tekmar High-Intensity Ultrasonic Processor, Tekmar Corp., Solon, OH) and then counted using LSC. Sample blanks were included in each analysis to track any potential contamination. The analysis of the lethal body residues as total fluoranthene equivalents and on a parent-

only basis allows for better interpretation of the differences in residue effects data. Therefore, biotransformation was assessed after 48-h exposure by a modification of a standard lipid extraction method (26). Extracts were ground using a tissue homogenizer in a 2:1 (v:v) chloroform/methanol and 0.88% KCl solution adjusted to pH 8. Using this extraction technique, organic, aqueous, and bound residue phases were obtained. The organic phase (i.e., chloroform) contained the parent compound and the organic-extractable polar metabolites. The aqueous phase (i.e., methanol/water) contained the water-soluble metabolites, and the residual tissue contained the bound (unextractable) fraction. The organic phase was separated from the total extract by aspiration, and parent compound and organic-extractable polar metabolites were separated using a HPLC attached to a fraction collector. The collected samples were quantified using LSC. The aqueous extract was filtered through a 45- $\mu$ m glass fiber filter, and the resulting water-soluble fraction was sub-sampled and radioactivity determined by LSC. The water-soluble fraction was assumed to contain mostly conjugates of phase I fluoranthene metabolites. This assumption was verified by digesting a sample of the water-soluble fraction with sulfatase at 37 °C. Following the 2-h incubation, extracts were extracted with chloroform and analyzed by LSC. Quantification of biotransformation allows for quantification of body residues as parent, metabolites, or total fluoranthene equivalent concentrations.

Lipid content of live animals was measured at the beginning and end of each experiment. Toxicokinetics and body residue analyses were a priority over lipid determinations at these time points. Total lipids were measured using the colorimetric method described in Van Handel (27). The results of the body residues, reported as wet weights, were lipid normalized to determine the potential for further reducing the variability of body residues.

**Data Analysis.** Toxicokinetic parameters were determined using the time-weighted average of the fluoranthene concentration in the water by fitting accumulation data to the following first-order two-compartment model (eq 1) using a fourth-order Runge-Kutta approach in the software package *Scientist*, version 2.01 (MicroMath, St. Louis, MO):

$$\frac{dC_a}{dt} = (k_u C_w) - (k_e C_a) \quad (1)$$

where  $C_a$  is the concentration of total compound in the animal ( $\mu\text{mol}\cdot\text{g}^{-1}$ ),  $C_w$  is the concentration of the chemical in water ( $\mu\text{mol}\cdot\text{mL}^{-1}$ ),  $k_u$  is the uptake clearance coefficient ( $\text{mL}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ),  $k_e$  is the total compound elimination rate constant ( $\text{h}^{-1}$ ), and  $t$  is the time (h).

Mortality data was analyzed using Probit Analysis with SAS (Cary, NC) to estimate  $\text{LC}_{50}$  values based on time-weighted average water concentrations and lethal body residue values ( $\text{LR}_{50(t)}$ ) corresponding to 50% mortality based on residues determined from dead organisms.

The time dependence of the lethal residue data was examined by fitting the data to the following damage assessment model (DAM) proposed by Lee et al. (18):

$$\text{LR}_{50(t)} = \frac{D_L/k_a}{\frac{1}{(1 - e^{-k_e t})} \left( \frac{e^{-k_e t} - e^{-k_r t}}{k_r - k_e} + \frac{1 - e^{-k_r t}}{k_r} \right)} \quad (2)$$

where  $\text{LR}_{50(t)}$  is the time-dependent lethal residue ( $\mu\text{mol}\cdot\text{g}^{-1}$ ),  $D_L/k_a$  is the toxic damage level required to cause 50% mortality ( $\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}$ ), and  $k_r$  is the first-order rate constant for damage recovery ( $\text{h}^{-1}$ ).

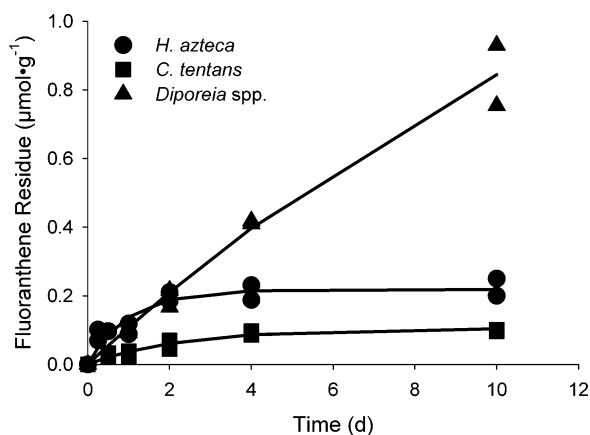
## Results

**Toxicokinetics.** The uptake clearance rate, elimination rate, and bioconcentration factor (BCF) were estimated for the

**TABLE 1. Estimated Toxicokinetic Parameters for Fluoranthene in *H. azteca*, *C. tentans*, and *Diporeia* spp.**

organism	exposure concn ( $\mu\text{g}\cdot\text{L}^{-1}$ )	$k_u$ ( $\text{mL}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ )	SD	$k_e$ ( $\text{h}^{-1}$ )	SD	log BCF <sup>a</sup>	$t_{1/2}$ <sup>b</sup> (d)
<i>H. azteca</i>	15.6	82.6	16.5	0.069	0.062	3.08	0.4
	31.3	162.9	54.4	0.049	0.020	3.52	0.6
	62.5	169.4	25.6	0.040	0.007	3.63	0.7
	125.0	124.1	8.5	0.023	0.003	3.73	1.3
	250.0	140.0	8.8	0.049	0.003	3.46	0.6
<i>C. tentans</i>	15.6	35.8	23.9	0.022	0.040	3.21	1.3
	31.3	29.5	9.1	0.020	0.013	3.17	1.4
	62.5	53.6	6.8	0.032	0.009	3.22	0.9
	125.0	32.5	2.7	0.013	0.004	3.40	2.2
	250.0	40.2	2.0	0.045	0.004	2.95	0.6
<i>Diporeia</i> spp.	15.6	52.8	5.4	0.001	0.001	4.77	28.9
	31.3	60.7	6.6	0.004	0.003	4.18	7.2
	62.5	76.8	10.2	0.002	0.001	4.58	14.4
	125.0	79.6	5.2	0.003	0.001	4.42	9.6
	250.0	61.5	5.7	0.004	0.001	4.19	7.2

<sup>a</sup> BCF based on wet weight calculated from kinetic estimates using  $\text{BCF} = (k_u/k_e)$  and has units of  $\text{mL}\cdot\text{g}^{-1}$  wet wt. <sup>b</sup> Half-lives ( $t_{1/2}$ ) were calculated from  $t = (0.693/k_e)$ .



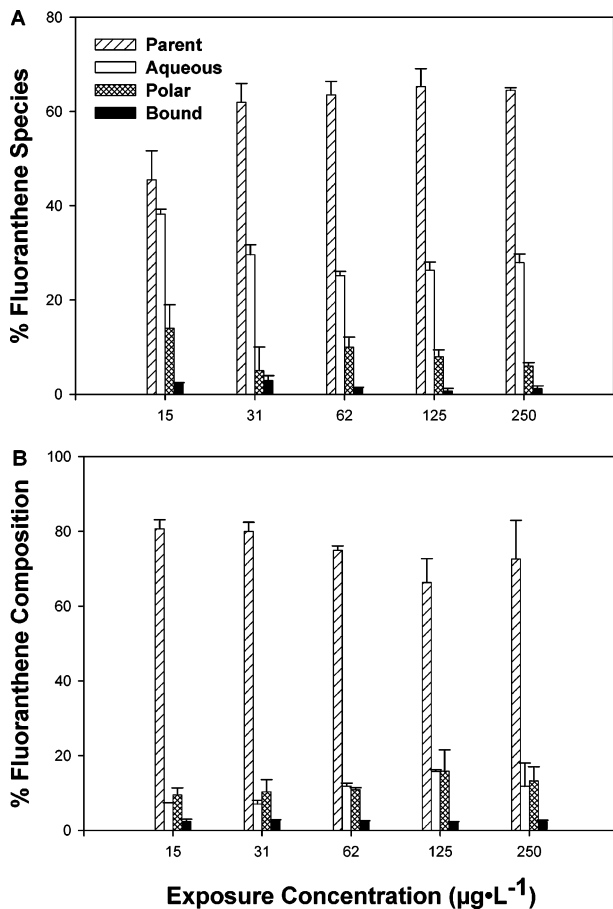
**FIGURE 1. Accumulation of total fluoranthene equivalents by *C. tentans*, *H. azteca*, and *Diporeia* spp. from water-only exposures.**

three organisms at each exposure concentration (Table 1). The toxicokinetics were different for the three species with *Diporeia* exhibiting the longest time to reach steady state (Figure 1). The uptake rate coefficients ( $k_u$ ) varied by test species. *H. azteca* had the fastest uptake clearance rates, ranging from 82.6 to 169.4  $\text{mL}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , followed by *Diporeia* and *C. tentans*, with values ranging from 52.8 to 79.6 and from 29.5 to 53.6  $\text{mL}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , respectively.

Elimination rates were approximately an order of magnitude lower for *Diporeia* (0.001–0.004  $\text{h}^{-1}$ ) as compared to both *H. azteca* (0.023–0.069  $\text{h}^{-1}$ ) and *C. tentans* (0.013–0.045  $\text{h}^{-1}$ ). The biological half-life of fluoranthene ranged from 0.4 to 1.3 d, from 0.6 to 2.2 d, and from 7.2 to 28.9 d for *H. azteca*, *C. tentans*, and *Diporeia*, respectively.

Overall, there was no major or systematic change in the toxicokinetics over the range of water concentrations employed. Thus, the log BCF for total fluoranthene equivalents was relatively constant across exposure concentrations for individual test species (Table 1). Log BCF values for *H. azteca* and *C. tentans* were similar ranging from 3.08 to 3.73 and from 2.95 to 3.40, respectively. Values for *Diporeia* were approximately an order of magnitude higher (4.18–4.77) reflecting its much higher lipid content, where lipid levels were 1, 2, and 6%<sub>ww</sub> for *C. tentans*, *H. azteca*, and *Diporeia*, respectively.

**Biotransformation.** *H. azteca* and *C. tentans* each possess a greater ability to biotransform fluoranthene than does *Diporeia*. The concentration of fluoranthene in the water



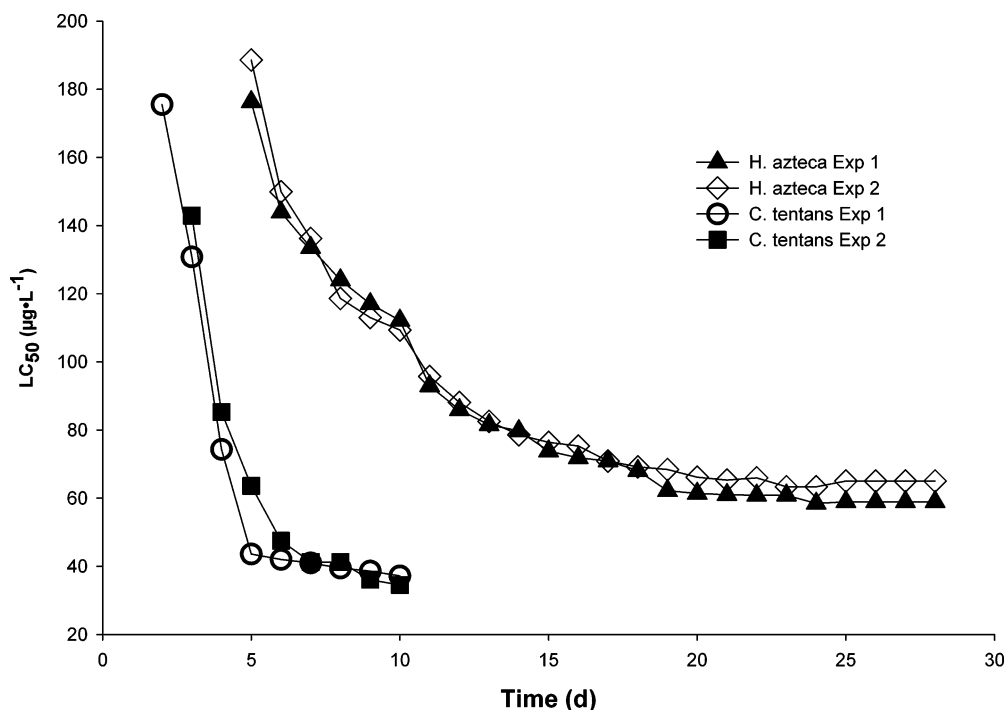
**FIGURE 2.** Percent fluoranthene composition for (A) *H. azteca* and (B) *C. tentans* following 48-h fluoranthene exposures.

seemed to have relatively little effect on the rate at which metabolites were formed in *H. azteca* and *C. tentans* (Figure 2). After 48 h of exposure, 45.5–65.1% of the total  $^{14}\text{C}$  activity in *H. azteca* was determined to be parent compound with

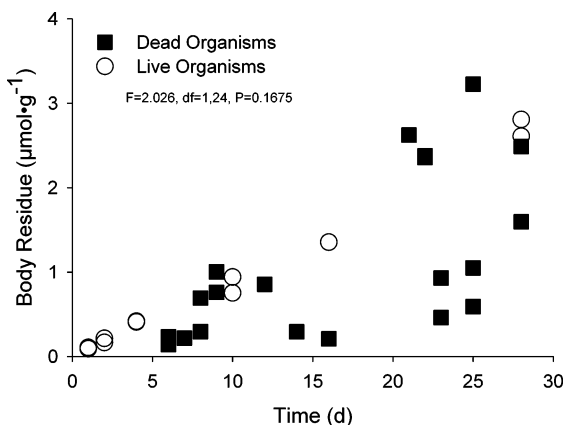
a mean of approximately 61.1% ( $\pm 6.2$ , SD) among all exposures. Of the remaining activity, 8.0% ( $\pm 2.9$ , SD) was present as organic extractable polar metabolites (hereafter called polar metabolites), 29.6% ( $\pm 5.4$ , SD) as aqueous metabolites, and 1.3% ( $\pm 0.4$ , SD) as bound material (Figure 2). The polar metabolites are assumed to be phase I metabolites, while the aqueous metabolites are assumed to be mostly phase II metabolites but may contain some very polar phase I compounds. The presence of phase II metabolites in the aqueous fraction was confirmed with enzymatic hydrolysis using sulfatase yielding the same compounds as the original organic extractable fraction. In the *C. tentans* exposures, the amount of parent compound in the organism ranged from 66.3 to 80.7% with a mean of 74.9% ( $\pm 5.9$ , SD) after 48 h. Of the remaining radioactivity, approximately 11.9% ( $\pm 2.6$ , SD), 10.8% ( $\pm 3.6$ , SD), and 2.4% ( $\pm 0.2$ , SD) was associated with polar metabolites, aqueous metabolites, and bound material, respectively (Figure 2). *Diporeia* has a limited ability to biotransform organic chemicals. Following 10-d exposure to fluoranthene, approximately 95% of the  $^{14}\text{C}$  activity was found as parent compound.

**Mortality  $\text{LC}_{50}$ .** Exposure and control waters were renewed daily, during which time mortality was evaluated and dead organisms removed. Estimated  $\text{LC}_{50}$  values were corrected for control mortality for all species. In *H. azteca* experiments, mortality was monitored for 28 d, and the controls in each experiment exhibited less than or equal to 6% mortality. *H. azteca* exhibited a strong dose–response relationship to increasing fluoranthene concentrations in the water (Tables 1A–3A in Supporting Information). Fluoranthene  $\text{LC}_{50}$  concentrations in *H. azteca* ranged from a 5-d  $\text{LC}_{50}$  of 188.6  $\mu\text{g}\cdot\text{L}^{-1}$  (174.6–203.7, 95% CI) to a 28-d  $\text{LC}_{50}$  of 59.0  $\mu\text{g}\cdot\text{L}^{-1}$  (51.0–68.2, 95% CI). After approximately 18 d of exposure to fluoranthene, *H. azteca* reached an incipient  $\text{LC}_{50}$  concentration of 66.1 and 60.1  $\mu\text{g}\cdot\text{L}^{-1}$  for experiments 1 and 2, respectively (Figure 3).

In the *C. tentans* exposures, mortality was monitored for 10 d. Control mortality was 3% and 10% at 10 d in experiments 1 and 2, respectively. The  $\text{LC}_{50}$  decreased rapidly from 2 to 6 d, after which the  $\text{LC}_{50}$  remained stable at approximately 40  $\mu\text{g}\cdot\text{L}^{-1}$  (Figure 3). The  $\text{LC}_{50}$  concentrations were in the



**FIGURE 3.** Calculated fluoranthene  $\text{LC}_{50}$  concentrations ( $\mu\text{g}\cdot\text{L}^{-1}$ ) in *H. azteca* and *C. tentans*.



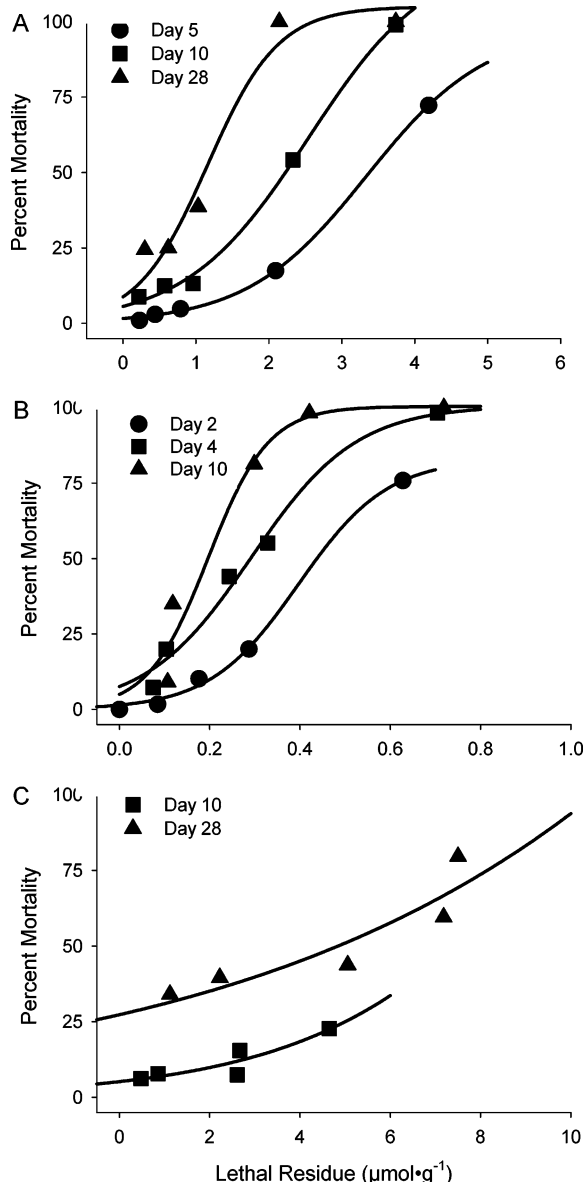
**FIGURE 4.** Typical relationship in total fluoranthene equivalent residues ( $\mu\text{mol}\cdot\text{g}^{-1}$ ) between live and dead organisms (*Diporeia* data). The concentrations in live organisms are data from two replicates of pooled animals, while the data from the dead organisms reflect the natural variability of individual measurements.

range of  $175.5 \mu\text{g}\cdot\text{L}^{-1}$  at 2 d,  $74.3\text{--}85.3 \mu\text{g}\cdot\text{L}^{-1}$  at 4 d, and  $34.5\text{--}37.2 \mu\text{g}\cdot\text{L}^{-1}$  at 10 d.

Control mortality in *Diporeia* experiments ranged from 7% in 10-d exposures to 13% in 28-d exposures. There was not sufficient mortality at 10 d to obtain an  $\text{LC}_{50}$  estimate; however, the 28-d  $\text{LC}_{50}$  concentration was  $95.5 \mu\text{g}\cdot\text{L}^{-1}$  (68.9–126.8, 95% CI). Incipient lethal levels were not achieved following 28 d of exposure.

**Mortality  $\text{LR}_{50}$ .** The  $\text{LR}_{50}$  concentrations were calculated from the mean lethal residue of individual dead organisms in each exposure concentration (Table 1A–3A in Supporting Information). Generally, there were no statistical differences detected in body residue levels using analysis of covariance between live and dead organisms (Figure 4). Replicate beakers were nondestructively sampled at each time point, thus allowing  $\text{LR}_{50}$  concentrations to be determined at multiple times during the 28-d exposures for each toxicant. Sufficient mortality did not occur until 2, 5, and 10 d to obtain  $\text{LR}_{50}$  estimates for *C. tentans*, *H. azteca*, and *Diporeia*, respectively (Figure 5). Due to limited mortality, the 10-d  $\text{LR}_{50}$  estimate for *Diporeia* was extrapolated from the mortality data. The  $\text{LR}_{50}$  values as total fluoranthene equivalents significantly declined with time for each species.  $\text{LR}_{50}$  values for *C. tentans* ranged from  $0.43 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 2 d to  $0.17 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 10 d.  $\text{LR}_{50}$  values for *H. azteca* ranged from  $3.19 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 5 d to  $0.80 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 28 d.  $\text{LR}_{50}$  values for *Diporeia* ranged from  $9.97 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 10 d to  $3.67 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 28 d (Table 2). Applying the values determined in the metabolite analysis the  $\text{LR}_{50}$  values expressed as parent compound only for *C. tentans* ranged from  $0.32 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 2 d to  $0.13 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 10 d.  $\text{LR}_{50}$  values for *H. azteca* ranged from  $1.95 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 5 d to  $0.49 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 28 d. Metabolite concentrations are assumed to be at steady state for this correction particularly for *H. azteca* based on the toxicokinetics of fluoranthene biotransformation (28). In addition to the parent-only fraction, the toxic equivalent fraction (that fraction organically extractable) can be obtained by subtracting the aqueous and bound fractions from the total. This corresponds to the assumption of Di Toro and McGrath (19) that the parent and phase I metabolites of PAHs contribute to the narcotic body residue. The toxic equivalent  $\text{LR}_{50}$  values for *C. tentans* ranged from  $0.38 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 2 d to  $0.15 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 10 d.  $\text{LR}_{50}$  values for *H. azteca* ranged from  $2.23 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 5 d to  $0.56 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  at 28 d.

The  $\text{LR}_{50}$  concentrations of total fluoranthene equivalents show a strong time dependence for *C. tentans*, *H. azteca*, and *Diporeia* with values dropping 2–4-fold over the time course of the exposures (Figures 5 and 6). *C. tentans* was sensitive



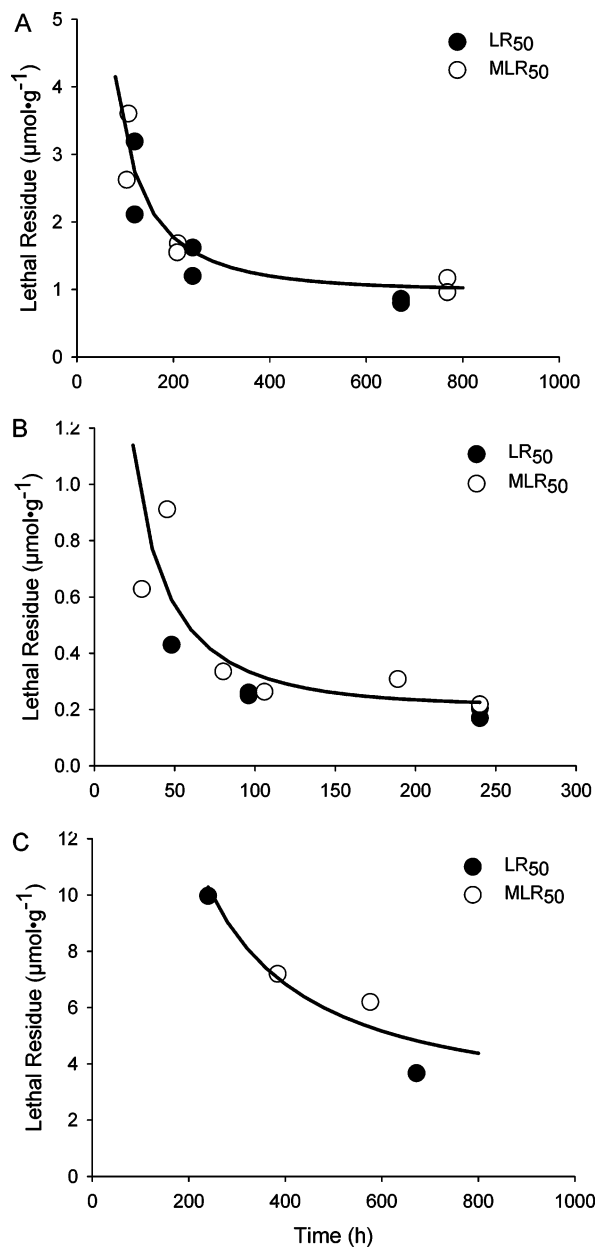
**FIGURE 5.** Typical dose–response curves for (A) *H. azteca*, (B) *C. tentans*, and (C) *Diporeia* spp. generated from total fluoranthene equivalent residues in dead organisms and percent mortality.

**TABLE 2.**  $\text{LR}_{50\text{ww}}$  and  $\text{LR}_{50\text{lipid}}$  Values ( $\mu\text{mol}\cdot\text{g}^{-1}$ ) as Total Fluoranthene Equivalents

organism	time (d)	$\text{LR}_{50}$ ( $\mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$ )	95% CI	$\text{LR}_{50}$ ( $\mu\text{mol}\cdot\text{g}^{-1}_{\text{lipid}}$ )	95% CI
<i>H. azteca</i> (exp 1)	5	3.19	2.81–3.69	128.5	118.9–138.8
	10	1.62	1.42–1.84	78.1	67.9–90.2
	28	0.86	0.75–0.98	45.5	36.6–56.1
<i>H. azteca</i> (exp 2)	5	2.11	1.86–2.24	105.1	84.6–130.6
	10	1.20	1.06–1.36	62.9	55.9–70.9
	28	0.80	0.71–0.91	45.3	39.9–51.4
<i>C. tentans</i> (exp 1)	2	0.43	0.38–0.49	33.4	30.0–38.0
	4	0.25	0.21–0.29	19.2	12.6–30.2
	10	0.17	0.15–0.19	13.8	8.8–19.9
<i>C. tentans</i> (exp 2)	2	0.26	0.20–0.31	19.0	12.6–30.1
	10	0.20	0.11–0.27	14.7	9.8–19.7
	10	9.97 <sup>a</sup>		213.5	
<i>Diporeia</i> spp.	10	9.97 <sup>a</sup>		213.5	
	28	3.67	2.32–5.80	85.3	65.5–111.3

<sup>a</sup> Estimate determined from extrapolating mortality data.

to fluoranthene, exhibiting initial  $\text{LR}_{50}$  residues as total toxic equivalents below  $0.5 \mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$  after which the residues



**FIGURE 6.** Plot of total fluoranthene equivalent lethal body residues producing 50% mortality over time for (A) *H. azteca*, (B) *C. tentans*, and (C) *Diporeia* spp.

declined to an asymptote or incipient LR<sub>50</sub> value of 0.21  $\mu\text{mol g}^{-1}_{\text{ww}}$ . *H. azteca* LR<sub>50</sub> values dropped rapidly for the first 10 d of exposure and then approached an incipient LR<sub>50</sub> value of approximately 0.84  $\mu\text{mol g}^{-1}_{\text{ww}}$ . *Diporeia* LR<sub>50</sub> values also dropped rapidly; however, no incipient value was obtained by the end of the 28-d exposure.

The time-dependent toxicity was determined using LR<sub>50</sub> values together with a second measure of toxicity, the mean lethal residue (MLR<sub>50</sub>, Table 4A in Supporting Information). The MLR<sub>50</sub> is defined as the mean residue of the dead organisms for a given treatment level, at the exposure duration corresponding to the lethal time at 50% mortality (LT<sub>50</sub>; 16). These data were modeled on a whole body residue basis using the DAM (Figure 6). The whole body residue is a reasonable surrogate for the site of toxic action (although lipid-normalized values could also have been used), since the bioaccumulation of nonpolar contaminants is dominated by partitioning to the lipid fraction and, for small organisms, the partitioning between storage and membrane lipids is

**TABLE 3.** Input and Output Parameters for Fluoranthene in *H. azteca*, *C. tentans*, and *Diporeia* spp. from the Damage Assessment Model

organism	$k_e$ (h <sup>-1</sup> )	$D_l/k_a$ ( $\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}$ )	$k_r$ (h <sup>-1</sup> )	LR <sub>50∞</sub> ( $\mu\text{mol}\cdot\text{g}^{-1}_{\text{ww}}$ )
<i>H. azteca</i>	0.040	208.7 (±20.7)	0.004 (±0.001)	0.84
<i>C. tentans</i>	0.032	13.5 (±2.6)	0.016 (±0.007)	0.21
<i>Diporeia</i> spp.	0.002	1053 (±228)	0.003 (±0.001)	3.00

essentially equal (29). The model input and output parameters are presented in Table 3 as well as the estimated LR<sub>50∞</sub> for total fluoranthene equivalents.

## Discussion

Given the complexities associated with multiple species comparisons and exposure differences, interpreting toxicity remains a challenge. Some of the factors that affect toxicity can be better identified by using body residue as a dose metric. This study focused on improving the utility and interpretation of this residue approach by examining differences in using external versus internal exposures, the impact of lipid content, the importance of biotransformation to improve species comparisons, and the importance of exposure duration and temporal response of organisms on a body residue basis.

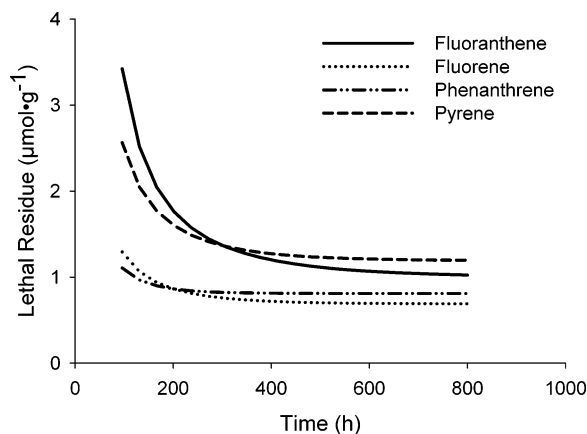
**Exposure Metric.** The toxicity of fluoranthene to *H. azteca*, *C. tentans*, and *Diporeia* spp. was evaluated up to 28 d. Based on external concentrations, *C. tentans* was somewhat more sensitive than *H. azteca* and approximately 2–3 times more sensitive than *Diporeia*. The experimentally derived incipient LC<sub>50</sub> values range from 39.6 to 40.5  $\mu\text{g}\cdot\text{L}^{-1}$  for *C. tentans* and range from 60.1 to 66.1  $\mu\text{g}\cdot\text{L}^{-1}$  for *H. azteca*, and a 28-d LC<sub>50</sub> of 95.6  $\mu\text{g}\cdot\text{L}^{-1}$  was determined for *Diporeia*. Our *H. azteca* results were similar to a previous study under yellow light, which reported 10-d LC<sub>50</sub> values of 97–114  $\mu\text{g}\cdot\text{L}^{-1}$  (30) but were somewhat higher than values collected under white light (31) and 45  $\mu\text{g}\cdot\text{L}^{-1}$  (32) where some photoinduced toxicity could have contributed to the lower values. For *C. tentans*, a LC<sub>50</sub> of 37.8  $\mu\text{g}\cdot\text{L}^{-1}$  (31) was very similar to our values despite the difference in light regime. Although, an incipient lethal concentration was not reached during the 28-d *Diporeia* experiment, a calculated LC<sub>50∞</sub> of approximately 37  $\mu\text{g}\cdot\text{L}^{-1}$  was determined using an equation describing the time-dependent median lethal concentration in Lee et al. (eq 14 in ref 18).

The acute LR<sub>50</sub> values for *C. tentans* were significantly lower than either *H. azteca* or *Diporeia*, suggesting that fluoranthene is much more toxic to the midge than the two amphipods. The 2-d LR<sub>50</sub> value for *C. tentans* was 5–7-fold lower than the 5-d *H. azteca* estimate and 23 times lower than the 10-d *Diporeia* estimate. The difference between *H. azteca* and *C. tentans* reflects the problem with using the external concentration as the dose metric. While the LC<sub>50</sub> values were not very different for these two organisms, the sensitivity based on body residue shows substantial species differences. Likewise, comparing the LC<sub>50</sub> values between *H. azteca* and *Diporeia* does not reflect the apparent difference in the sensitivity between the two species based on 28-d exposures. For *H. azteca*, the acute LR<sub>50</sub> values in the current exposures were 2.11 and 3.19  $\mu\text{mol}\cdot\text{g}^{-1}$  total fluoranthene equivalents. These values are somewhat lower than the 10-d LR<sub>50</sub> values of 5.6 and 3.6  $\mu\text{mol}\cdot\text{g}^{-1}$  for fluoranthene reported by Driscoll et al. (30). However, after accounting for biotransformation and evaluating the values on a parent-only or toxic equivalents basis, the values are still within the expected 2–8  $\mu\text{mol}\cdot\text{g}^{-1}$  range for acute mortality for narcotic

chemicals. The estimated LR<sub>50</sub> value of approximately 10  $\mu\text{mol}\cdot\text{g}^{-1}$  for *Diporeia* is higher than the expected residues for narcotic chemicals; however, this estimate agrees with a previous study where residues of 6.0  $\mu\text{mol}\cdot\text{g}^{-1}$  were associated with only 10% mortality (30) and studies with other PAHs showing that the 28-d LR<sub>50</sub> ranges from 5.8 to 12.3  $\mu\text{mol}\cdot\text{g}^{-1}$  for *Diporeia* (33). The differences among species on a body residue basis suggest that the lipid content of the organisms may be an important factor influencing the toxicity at a given body residue.

**Lipid Content.** Normalizing body residues to the lipid content of organisms is performed to correct for differences in the lipid content among species. The distribution of nonpolar contaminants in an organism will largely be determined by the partitioning into the lipid compartment. Previous work has shown that the partitioning between storage and membrane lipids is similar and that the two lipid pools come to a dynamic equilibrium with one another for small aquatic organisms, such as *Diporeia*, within 24–48 h (29). On the basis of the similarity of partitioning and the dominance of lipid for determining the bioaccumulation of nonpolar narcotics, normalizing to lipid content of an organism provides an estimate of the concentration at the site of toxic action, presumably the membrane lipid pool. Thus, the “fatter” the organism, the more chemical can be stored before a toxic effect is manifested (34, 35). This can be seen when comparing lipid levels among the three test species to differences in LR<sub>50ww</sub> values, where *C. tentans*, with the lowest lipid levels, was most sensitive and *Diporeia*, with the highest lipid levels, was the least sensitive to fluoranthene intoxication. Normalizing to lipid content removed the differences between replicated experiments for *C. tentans* or *H. azteca*, and the values are in agreement with lethal body residues in fish following lipid normalization (36). Normalizing for lipids also reduced the range of lethal residues among species by approximately an order of magnitude (Table 2). However, when comparing lipid-normalized LR<sub>50</sub> values at similar exposure times among species, there are still significant differences that are not explained by lipid content alone.

**Biotransformation.** LR<sub>50(lipid)</sub> values for narcosis have been previously shown to be approximately 50–100  $\mu\text{mol g}^{-1}\text{lipid}$  (37). The LR<sub>50(lipid)</sub> values for *H. azteca* and *Diporeia* range from 85 to 128  $\mu\text{mol g}^{-1}$ , suggesting that narcosis is the mode of toxic action, whereas the LR<sub>50(lipid)</sub> value of 33  $\mu\text{mol g}^{-1}$  for *C. tentans* is lower suggesting a mode of toxic action other than narcosis. The enhanced toxicity of fluoranthene to *C. tentans* as compared to the amphipods may be a result of the larger fraction of organically extractable polar metabolites in its tissues. The biotransformation of fluoranthene may have produced a toxic metabolite. Analysis of the midge extracts revealed that most of the polar metabolite fraction is comprised of a single compound. The residue data suggests that unlike *H. azteca*, this metabolite is causing the nonadditive toxicity in *C. tentans*. This nonadditive toxicity is not in agreement with earlier assumptions that narcotic chemicals exhibit strictly additive toxicity and that PAH metabolites can be assumed to be additive with parent compound (19). The only other study to directly assess the potential of PAH metabolites to affect body residue response for *H. azteca* found that naphthalene metabolites were apparently less toxic than the parent compound (16). A metabolite acting as a specific toxin is not new and is well-established for compounds such as organophosphate insecticides. However, finding a metabolite of fluoranthene that is apparently more toxic than the parent compound is unusual. Sepic et al. (20) did find differential toxicity of fluoranthene metabolites and parent compound in aquatic invertebrates. In general, metabolites were much less toxic, but for *D. magna*, 9-hydroxyfluorene was about equally toxic as fluoranthene



**FIGURE 7. Relative toxicity of fluoranthene compared to the other PAHs on a temporal basis to *H. azteca* using total PAH equivalents. Data for the other PAHs was taken from Lee et al. (16).**

with respect to water concentrations (20). Assuming the toxicokinetics are similar for fluoranthene and the 9-hydroxy metabolite, this follows the expectations of Di Toro and McGrath (19) in that phase I metabolites of PAHs should be equally toxic when compared to the parent compound. However, none of the metabolites were more toxic than fluoranthene, which is different than the findings for *C. tentans*. The data presented in this paper are only suggestive of a toxic metabolite, and the mechanism for the sensitivity of the midge remains to be confirmed with additional studies.

**Toxicokinetic Status.** Another important consideration, when making interspecies comparisons of lethal residues, is the relation of the exposure duration to the time to steady state among species. Therefore, when making comparisons at predetermined times (e.g., 10 d), different species may be at different relative positions on the toxicokinetics curve. For example, it is evident that over 10 d of exposure *H. azteca* and *C. tentans* reached steady state in approximately 2–4 d, whereas *Diporeia* had not (Figure 1). Thus, it would be incorrect to make 10-d comparisons between *Diporeia* and the other species because the toxicity is being controlled by differing factors (38). The toxicity of *Diporeia* is being controlled more by the toxicokinetics (i.e., uptake and elimination) of fluoranthene, whereas for *H. azteca* and *C. tentans*, the 10-d toxicity is governed primarily by the toxicodynamics. Therefore, to make the appropriate LR<sub>50</sub> comparisons, values approximating each species time to steady state ( $T_{ss}$ ) should be compared, thereby normalizing the toxicity to the same kinetically defined time frame. Using the estimated elimination rates for *H. azteca* and *C. tentans*, the calculated time to steady state for both species is approximately 4 d. Assuming that *Diporeia* is approaching steady state at 28 d, when we compare the lipid-normalized LR<sub>50(lipid)</sub> values at  $T_{ss}$ , there is no difference in lethal residues between the two amphipods. However, LR<sub>50(lipid)</sub> values for *C. tentans* remain lower than that for *H. azteca* and *Diporeia*.

**Temporal Response.** The time-dependent toxicity of fluoranthene for the three invertebrate test species exhibited a 2–4-fold reduction in LR<sub>50</sub> values over the time course of the exposures. This time dependence is consistent with several studies. For instance, a temporal component in the lethal residue has been demonstrated for halobenzenes to fish (13), chlorobenzenes in the juvenile crab (15), and for several PAHs in *H. azteca* (18). In fact, when comparing the *H. azteca* toxicity data for total fluoranthene equivalents with the toxicity of the other PAHs from Lee et al. (18), the modeled toxicity data matches closely (Figure 7). Total fluoranthene equivalents were used for the temporal analysis to match the work of Lee et al. (16). The impact of using total fluoranthene equivalents is greatest for *H. azteca* among the

species examined in this study because these amphipods exhibited the largest fraction of aqueous and bound metabolites (approximately 30%), which are assumed not to contribute to the toxicity. Again, we assume that both the parent compound and the organically extractable metabolites contribute to the toxicity as was proposed by Di Toro and McGrath (19). Because biotransformation was only measured at 48 h, some of the observed temporal variation could have been due to a continued buildup of metabolites. Although biotransformation data are not available beyond the 48-h time point, our previous work estimates that fluoranthene metabolites are at steady state at approximately 48 h (28). Thus, this effect was considered negligible. Furthermore, there is no evidence of a toxic metabolite for *H. azteca*, so reductions in the LR<sub>50</sub> values are not attributed to this cause. In addition, similar temporal responses in LR<sub>50</sub> values were observed for *H. azteca* exposed to pentachlorobenzene, which is not biotransformed (17). Thus, the temporal variation observed for *H. azteca* is unlikely a buildup of metabolites but a response to long-term exposure and an accumulation of damage in the organism. For *C. tentans*, the temporal decline in toxicity could have some contribution from the buildup of a toxic metabolite, since at 48 h the organism was not quite at steady state and there was evidence of a toxic metabolite. For *Diporeia*, there was essentially no biotransformation occurring, thus the observed decline in LR<sub>50</sub> values could only have been due to buildup of damage. This is somewhat different from previous research where there was no evidence of a temporal response for *Diporeia* exposed to PAHs (33).

The temporal change was modeled using the DAM to account for both the influence of the toxicokinetics and more importantly the influence of the toxicodynamics (18). If the mechanism of action does not change, then the model clearly demonstrates the importance of the rate of damage formation ( $k_a C_a$ ) early in the time series, since there is little chance for damage repair with short exposure times. Likewise, the model shows the time dependence of the repair process as represented by  $k_r$  with longer-term exposures. In this model, the threshold for a toxic response is a buildup of a critical level of damage, designated  $D_L$  (the damage to cause 50% mortality). Thus, it is the damage producing mortality that is constant and not the body residue, as has been proposed for nonpolar narcosis (9, 12). The assumption of a first-order damage process is reasonable for nonpolar compounds that exert their influence by their concentration in the membrane. Even though the first-order assumption for damage repair may be simplistic and may need to be modified in the future, it still allows a first attempt to explain the time dependence of compounds that are presumably acting as nonpolar narcotics. Furthermore, the damage assessment model demonstrates that elimination is not the sole process for the recovery of organisms. This is reasonable, since even after compound is eliminated, cellular damage will need to be repaired before the cell will be fully functional. In the cases of *Diporeia* and *H. azteca*, the apparent damage occurs from nonpolar narcosis. For *Diporeia*, it is assumed that the mechanism of toxic action (narcosis) does not change since biotransformation has not been observed for this species (23). For *H. azteca*, biotransformation does take place, but there is no indication of a specific mode of toxicity that would suggest a mechanism of action other than nonpolar narcosis. This assumption for *H. azteca* is supported since the concentrations required to produce a toxic response are similar to the concentrations for acute and chronic toxicity in fish (7), adjusting for the lipid content among species. Thus, the damage assessment model likely addresses the repair process and buildup of damage for a single mode of toxic action for *Diporeia* and *H. azteca*. However, for the midge, the body residues required to produce mortality, even

after accounting for the lipid content, are much lower than for the two amphipod species, suggesting a specific mode of toxic action. Thus, some of the time dependence of the body residue data may reflect a shift in the mechanism of toxic action since an apparent specific-acting metabolite is formed. Overall, the observed temporal variability in lethal body residues is not apparently governed exclusively by toxicokinetic processes alone but rather in conjunction with toxicodynamic processes. This is an important consideration when examining data as part of a hazard assessment where long-term body residue data are needed. Furthermore, issues of differing lipid content, biotransformation, and toxicokinetic status should be considered for interspecies comparisons.

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## Supporting Information Available

Four tables showing data collected from toxicity test and MLR and LT<sub>50</sub> values. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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