

RESPONSE SPECTRUM OF FLUORANTHENE AND PENTACHLOROBENZENE FOR
THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)LANCE J. SCHULER,[†] PETER F. LANDRUM,^{*‡} and MICHAEL J. LYDY[†][†]Fisheries and Illinois Aquaculture Center and Department of Zoology, Southern Illinois University Carbondale,
Carbondale, Illinois 62901, USA[‡]Great Lakes Environmental Research Laboratory, National Oceanic and Atmospheric Administration, Ann Arbor, Michigan 48105, USA

(Received 4 April 2006; Accepted 31 July 2006)

Abstract—Internal body residue has been recognized as a potential dose metric for toxicological assessments. This relationship between body residue and biological effects, including both lethal and sublethal effects, is critically important for determining environmental quality in risk assessments. The present study identified the toxic equivalent body residues for fluoranthene (FLU) and pentachlorobenzene (PCBz) associated with mortality, reduced growth, and decreased hatchability in the fathead minnow. The toxic equivalent body residue was defined as the total of the parent compound and the organically extractable metabolites for FLU and of the parent compound only for PCBz, because no biotransformation was measurable. The lethal body residues corresponding to 50% mortality were 0.80 and 1.26 $\mu\text{mol/g}$ wet weight for FLU and PCBz, respectively. As expected, residues associated with sublethal effects generally are 2- to 40-fold lower than the lethal residues for FLU and PCBz. Juvenile fish growth was the most sensitive endpoint examined for both compounds. The maximum allowable toxicant residues were 0.02 and 0.43 $\mu\text{mol/g}$ wet weight for FLU and PCBz, respectively. The information collected from the present study will permit a greater understanding of residue–response relationships, which will be useful in risk assessments.

Keywords—Fathead minnow Fluoranthene Pentachlorobenzene Whole-body residue Sublethal effects

INTRODUCTION

Persistent organic contaminants are distributed throughout the aquatic environment and have been linked to a wide spectrum of adverse biological effects ranging from lethality to sublethal impairments, including reduced growth and reproduction [1–3]. Because of the potential for adverse effects, environmental managers, scientists, and assessors are challenged with the task of evaluating and protecting the environment from a multitude of chemical stressors. These environmental assessments commonly use tools such as biological monitoring and surveys of biota, sediment, and water contaminant concentrations to assess the level of environmental damage. Additional information, such as toxicity data, are collected using standardized test species, such as fathead minnow (*Pimephales promelas*), which frequently is used as a surrogate species to provide results that will be protective of more sensitive fish species [4].

One potential limitation of performing these types of assessments using external environmental concentrations is the assumption that these concentrations are proportional to the concentration at the target site within the exposed organism. It is well known that chemical bioavailability, or the fraction of the total chemical in the system that is available for uptake, complicates this use of external media concentrations as a surrogate of the dose and muddles interpretation of the analytical results [5,6]. Several approaches have been established to overcome these limitations for assessing chemical hazards; such approaches include equilibrium–partitioning theory [7] and the use of various semipermeable membrane devices [8], C-18 fiber, and polymer resin technologies [9–11] that attempt

to recover only the bioavailable fraction of the contaminants. At times, however, these methodologies are ineffective in predicting bioaccumulation and toxic effects [12,13]. Thus, a better dose metric is desirable for predicting biological effects. As such, the whole-body residue of an organism has been proposed as a more direct measure of the concentration at the target site. Use of whole-body residues in organisms rather than environmental concentrations provides a better surrogate measure for the concentrations of contaminants at the target site and should allow more accurate predictions of effects.

The objectives of the present work were to identify whole-body contaminant residues of fluoranthene (FLU) and pentachlorobenzene (PCBz) associated with adverse biological effects on the fathead minnow, including lethality, reduced growth, and decreased reproduction (i.e., egg hatchability, embryo survival, and embryo growth). The data obtained were used to develop a response spectrum that will allow better interpretation of biomonitoring activities relating contaminant residues to effects.

MATERIALS AND METHODS

Organism

The fathead minnow (*Pimephales promelas*) was selected as the vertebrate model for the present study because of its previous use for body residue testing, which allowed direct comparisons with other compounds. Organisms were cultured at Southern Illinois University Carbondale in accordance with U.S. Environmental Protection Agency (U.S. EPA) methods [14]. Original stocks were obtained from wild stocks at Logan Hollow Fish Farm (Gorham, IL, USA). All experiments were conducted following institutional animal care and use committee protocol 01-039.

* To whom correspondence may be addressed
(peter.landrum@noaa.gov).

Chemicals

Radiolabeled [^{14}C]PCBz and [^3H]FLU were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nonradiolabeled compounds (^{12}C) were obtained from Sigma-Aldrich with a minimum purity of 98%, as indicated by the manufacturer. Radiolabeled compounds were tested for purity using a high-pressure liquid chromatograph (model 1100; Agilent, Atlanta, GA, USA) and liquid scintillation counting (LSC; model 1900TR; Packard Instrument Company, Downers Grove, IL, USA). Stock solutions were prepared by adding known quantities of radiolabeled compound to known amounts of nonradiolabeled compound using acetone as a carrier. The specific activities were recalculated by adjusting for the isotopic dilution.

Exposure media

Reconstituted moderately hard exposure water was prepared by adding the necessary salts to deionized water and then allowing the water to mix overnight via aeration to ensure the required water quality. The spiking procedure consisted of adding the predetermined amount of contaminant (radiolabeled and nonradiolabeled) to a bulk aliquot of water using acetone as a carrier solvent. The volume of carrier was equal across all exposures, including controls that received acetone only.

Lethal bioassays

The toxicokinetics of FLU and PCBz were conducted at multiple concentrations to determine if toxicokinetic parameters were affected by dose. The time-dependent acute toxicity was examined over a 28-d exposure. The analysis of the lethal body residues at 12 and 28 d was performed both as total equivalents, which includes the parent compound and all metabolites, and as toxic equivalent fractions, which include the parent compound and the organically extractable fraction and allows better interpretation of the differences in residue effects data [15]. All body residue interpretations are based on the toxic equivalent fraction unless otherwise specified. The organically extractable fraction contains the parent compound and phase-1, but not phase-2, biotransformation products. Phase-1 metabolites are expected to contribute directly to toxicity, because the addition of functional groups (e.g., -OH) do not significantly change the lipophilicity of the molecule, whereas the addition of large polar groups, such as glucuronides, in phase-2 biotransformation makes the compound much more hydrophilic, reducing its ability to act as a nonpolar narcotic.

All experiments were conducted in environmental chambers (Precision Scientific, Chicago, IL, USA) maintained at 25°C with a 16:8-h light:dark photoperiod. Experiments were conducted using gold fluorescent light to reduce phototoxicity. Juvenile fish (mean \pm standard deviation, 6.26 \pm 0.52 mg wet wt) were exposed to a series of contaminant concentrations that were predetermined from range-finding experiments. Exposures consisting of five replicates per concentration and 10 fish per replicate were conducted in 1-L beakers containing 1,000 ml of water. Three-quarters of the treatment water was exchanged daily, and *P. promelas* were fed 0.2 ml of brine shrimp daily. Mortality was assessed daily over the course of the 28-d exposures. Accumulation kinetics for each compound were determined in concurrent experiments by sampling at 0, 1, 2, 4, and 10 d of exposure. The exposure concentrations were identical to those used in the lethal bioassays. At each

sampling time, two replicates were taken randomly from each concentration. From these replicates, live organisms were removed from the water and then killed using tricaine methane sulfonate (MS-222), a fish anesthetic, in accordance with the procedures described in our animal care and use protocol. Fish samples were then rinsed, blotted dry, and weighed to the nearest 0.01 mg using an analytical balance (Mettler, Toledo, OH, USA). Total equivalent residues were determined by placing five organisms directly into 10 ml of scintillation cocktail and then grinding thoroughly using a tissue homogenizer until no tissue was visible. The homogenized samples and an additional 5 ml from a rinse of the tissue homogenizer were transferred to a scintillation vial and counted by LSC. Before counting via LSC, samples were stored in darkness for at least 24 h to aid in the final extraction of the radiolabeled compounds and to minimize chemiluminescence. Samples were corrected for background and quench using the external standards ratio method.

Fluoranthene biotransformation was further examined using a modified lipid extraction technique [16], which allows determination of FLU residues based on their affinity for the organic or aqueous fraction of the extract [17]. The organic fraction is expected to contain the toxic components (i.e., parent compound plus phase-1 metabolites, mainly hydroxy metabolites [18]), whereas the aqueous fraction should contain the nontoxic, water-soluble, phase-2 metabolites. Fluoranthene biotransformation was quantified at steady state (10 d). These data were used to calculate the toxic equivalent fraction of FLU residues by correcting the total equivalent concentrations (parent compound plus all metabolites) as determined from LSC by the toxic fraction (parent compound and phase-1 metabolites) to determine their potential for further reducing the variability of body residues among experiments. Normalizing the total equivalent residues measured in the fish to the toxic equivalent residue allows more appropriate comparisons to be made between compounds that differ in their capacity for biotransformation. Preliminary experiments determined that PCBz was not biotransformed by the fish; therefore, the toxic equivalent fraction of PCBz is equal to the total equivalent fraction.

Analysis of toxicokinetic data

Assuming that the percentage of metabolite determined at 10 d is constant throughout the exposure, the toxicokinetic data were fit by performing an iterative least-squares fit to the following differential equations using the fourth-order Runge-Kutta approach in the Scientist software package (MicroMath, St. Louis, MO, USA) [5]:

$$\frac{dC_{\text{tot}}}{dt} = (k_u C_w) - (k_{e,\text{toxic}} C_{\text{toxic}}) - (k_{e,\text{aq}} C_{\text{aq}}) \quad (1)$$

$$\frac{dC_{\text{toxic}}}{dt} = (k_u C_w) - (k_m C_{\text{toxic}}) - (k_{e,\text{toxic}} C_{\text{toxic}}) \quad (2)$$

$$\frac{dC_{\text{aq}}}{dt} = (k_m C_{\text{toxic}}) - (k_{e,\text{aq}} C_{\text{aq}}) \quad (3)$$

where C_{tot} is the concentration of total compound in the animal ($\mu\text{mol/g}$), C_{toxic} is the concentration of toxic equivalent compounds in the animal ($\mu\text{mol/g}$), C_{aq} is the concentration of aqueous metabolites in the animal ($\mu\text{mol/g}$), k_u is the uptake clearance coefficient (ml/g/h), C_w is the time-weighted average contaminant concentration in water ($\mu\text{mol/ml}$), $k_{e,\text{toxic}}$ is the toxic equivalent compound elimination rate constant (1/h), k_m

is the metabolite formation constant (1/h), $k_{e,eq}$ is the metabolite depuration rate constant (1/h), and t is time (h).

Analysis of lethal body residues

Mortality data at 12 and 28 d were analyzed using probit analysis in the SAS software package (SAS Institute, Cary, NC, USA) to predict the lethal body residue values corresponding to 50% mortality (LR50) based on residues determined from live organisms. The 12-d endpoint was selected because it corresponded to the time at which adequate mortality (>50%) was first observed in the FLU exposures. Residue levels for FLU and PCBz within the organisms were determined as toxic equivalents.

The 12-d LR50s were calculated from the lethal concentration producing 50% mortality and toxicokinetic data using the following equation [19]:

$$LR50_t = \frac{k_u}{k_{e,toxic} + k_m} LC50, \quad (4)$$

where t is time. The 28-d LR50s were determined from residues of surviving organisms at the end of the exposures. Although previous studies have shown that lethal body residues are similar for live and dead organisms [15,17], live animals were used in the present study for consistency between the 12- and 28-d sampling times.

Sublethal bioassays

Growth. Growth of juvenile fish (age, ~20 d) was determined by exposing the fish individually to each compound in 1-L beakers containing five organisms each and with five replicates per treatment. The exposure concentrations for FLU were 0, 6, 12.5, 25, and 50 $\mu\text{g/L}$, whereas the exposure concentrations for PCBz were 0, 0.1, 1, 10, 50, and 100 $\mu\text{g/L}$. Three-quarters of the treatment water was changed daily, and fish were fed brine shrimp daily. At the end of the 28-d experiments, fish were removed from the beakers, killed as described previously, rinsed, and weighed, and the body residues were analyzed using the methods described for the lethal bioassays. The daily growth rate was estimated using the following growth equation:

$$GR = \frac{W_f - W_i}{d} \quad (5)$$

where GR is growth rate, W_f is the final weight, W_i is the initial weight, and d is time (d). Wet weights of pooled fish were determined to the nearest 0.1 mg using an analytical balance (Mettler). Estimates of the initial mass were obtained from five replicates of 15 randomly selected organisms from the starting fish cultures.

Reproduction. The reproductive endpoints examined in the present study were 5-d hatching success, 7-d survival, and 7-d growth of *P. promelas*. The 5-d cumulative hatching success of *P. promelas* was determined by exposing fertilized eggs (age, <24 h) to a series of six exposure concentrations using 50-ml culture tubes. The exposure concentrations for FLU were 0, 15, 32, 62, 125, and 250 $\mu\text{g/L}$. The exposure concentrations for PCBz were 0, 25, 50, 100, 200, and 300 $\mu\text{g/L}$. Before exposure, fertilized eggs were immersed in a solution containing methylene blue for approximately 30 min to reduce fungal contamination. Each tube contained 10 fertilized eggs, with five replicates per treatment. Approximately 90% of the exposure water was exchanged daily for a period of 5 d. Tubes were checked daily for hatched eggs, and any larval fish were

removed. The majority of the larval fish hatched on the fourth day. These successfully hatched fish (fourth-day only) were then transferred to clean water, and survival was monitored for an additional 7 d. In addition to 7-d survival, the growth of surviving fish was evaluated at 7 d posthatch. To maintain consistency with established U.S. EPA methods [14], growth was determined as dry weight by drying the fish to a constant weight for 2 d at 90°C. Weights were determined to the nearest 0.01 mg using a microbalance (Cahn Instruments, Cerritos, CA, USA). Because of the variability of eggs hatching following a 4-d exposure, hatchability was determined as the cumulative hatchability over 5 d. Any tubes containing eggs with obvious fungal contamination were omitted from the analysis. Data from the sublethal exposures were compared using one-way analysis of variance and Dunnett's multiple-comparison test in SAS. Data were checked for normality and homogeneity of variances before analysis.

Chemical residue in the fertilized eggs following a 4-d exposure was chosen as the dose metric for the reproductive endpoints. Both FLU and PCBz residues were determined from an additional three replicates for each compound from a simultaneous exposure. Unhatched eggs were removed from the tubes, rinsed, blotted dry, and weighed to the nearest 0.001 mg using a Cahn microbalance. For analysis, eggs were placed directly into scintillation cocktail, sonicated (High-Intensity Ultrasonic Processor; Tekmar, Solon, OH, USA), and counted using LSC. The assessment of biotransformation was obtained from a separate exposure in which five replicates containing 25 eggs each were exposed as described above. Following the 4-d exposure, the fractions of parent compounds and metabolites were determined from the unhatched eggs using a modified lipid extraction technique [16] as detailed for the lethal bioassays.

RESULTS

Biotransformation

To determine the toxic fraction of the fish extracts (parent compound plus phase-1 metabolites), the biotransformation capacity of *P. promelas* was evaluated for juvenile fish and fertilized eggs. Preliminary testing showed that *P. promelas* possesses limited ability to biotransform PCBz; therefore, biotransformation of PCBz was not assessed during the definitive tests. Using the differential extraction technique outlined in *Materials and Methods*, fish samples spiked with FLU yielded total compound recoveries ranging from 93 to 104% ($n = 3$).

Following a 10-d exposure, FLU was readily biotransformed by juvenile fish. The toxic fraction of FLU in the fish tissues was approximately 20%, and the remaining 80% were aqueous metabolites (Fig. 1). No significant differences were detected in biotransformation capacity among exposure concentrations ($p = 0.798$). The ability of the fertilized eggs to biotransform FLU was significantly less than that of the juvenile fish. Following the 4-d exposure, the toxic fraction of the total egg residue was approximately 95% (Fig. 1). Similar to juvenile fish, no differences in biotransformation by fertilized eggs were detected among treatments ($p = 0.701$).

Calculated bioconcentration factors, estimated 12-d whole-body residues associated with each treatment, and 28-d lethal residues determined from surviving organisms were similar across all doses for each compound (Table 1).

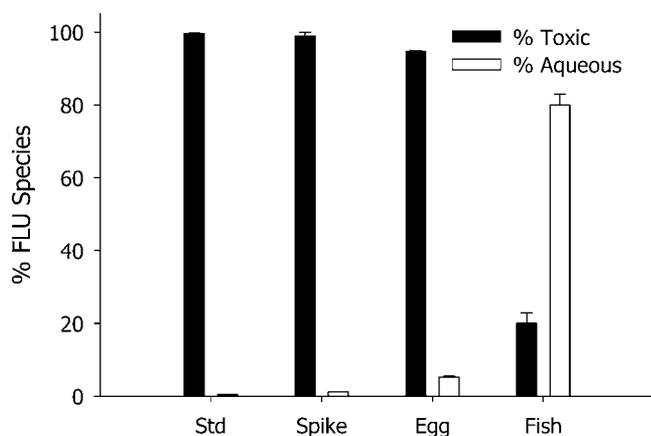


Fig. 1. Biotransformation capacity (mean \pm standard error) for *Pimphales promelas* following a 10-d exposure to fluoranthene (FLU). The toxic fraction is defined as both the parent compound and the phase-1 metabolites. The aqueous fraction is expected to contain the phase-2 biotransformation products. The standard (Std) and the spiked data are included to validate the extraction procedure with respect to the parent compound.

Mortality

Mortality of *P. promelas* was monitored over a 28-d period. No significant differences were detected between mortality from 12 d of exposure and that from 28 d of exposure to PCBz. The toxicity of FLU was significantly different between 12 and 28 d; however, the actual difference ($\sim 8\%$) was not biologically significant. On a total equivalent basis (parent compound plus all metabolites), the 12- and 28-d toxicities of PCBz were significantly more toxic to *P. promelas* compared to those of FLU. The 12- and 28-d LR50s (mean value [95% confidence interval]) for PCBz were 1.19 (1.16–1.22) and 1.26 (1.06–1.38) $\mu\text{mol/g}$ wet weight, whereas the 12- and 28-d values for FLU were 3.67 (3.23–3.80) and 4.00 (3.90–4.15) $\mu\text{mol/g}$ wet weight, respectively. Normalizing the residues for the toxic fraction of the contaminants, the difference between the LR50s remained significant, but the variation was reduced to less than a factor of two at 28 d, with FLU as the more toxic compound (Fig. 2).

Growth

Significant differences in growth were determined as a function of the daily growth rate from the bulk weight of juvenile *P. promelas* following 28-d exposures (Fig. 3). Whole-body residues of *P. promelas* were determined following the 28-d exposure. In each exposure, the weights of the control fish were statistically compared to those in treatments using Dunnett's test to determine significance. Control fish were consistently heavier than treatment fish. Growth for *P. promelas* exposed to FLU ranged from 1.03 to 0.66 mg/fish/d. Growth for *P. promelas* exposed to PCBz ranged from 0.98 to 0.56 mg/fish/d. The lowest-observed-effect residues (LOERs) for reduced growth in the juvenile fish were 0.016 ± 0.002 and 0.66 ± 0.15 $\mu\text{mol/g}$ wet weight for FLU and PCBz, respectively. The disparity between LOERs suggests that PCBz is acting via a narcotic mechanism whereas FLU is acting via a more specific mechanism.

Reproduction

The reproductive endpoints used in the present study were hatching success, 7-d posthatch survival, and 7-d growth of

Table 1. Calculated and measured whole-body residues for *Pimphales promelas* exposed to fluoranthene (FLU) and pentachlorobenzene (PCBz)^a

Chemical treatment ($\mu\text{g/L}$)	k_u (ml/g/h)	$k_{e,\text{toxic}}$ (1/h)	k_m (1/h)	Log BCF	Body residue ($\mu\text{mol/g}$ wet wt toxic equivalents)	
					12-d	28-d
FLU						
50	42.1 (2.8) ^b	0.002 (0.007)	0.088 (0.003)	2.67	0.14	0.35
100	32.7 (2.9)	0.003 (0.010)	0.048 (0.002)	2.81	0.28	0.35
150	20.0 (0.5)	0.001 (0.003)	0.037 (0.001)	2.72	0.40	0.42
200	30.9 (0.6)	0.001 (0.002)	0.049 (0.002)	2.79	0.56	0.70
250	23.3 (2.8)	0.001 (0.001)	0.031 (0.001)	2.86	0.76	0.87
PCBz						
100	26.9 (1.1)	0.017 (0.002)		3.08	0.51	0.55
150	17.5 (1.9)	0.013 (0.001)		3.14	0.76	0.79
200	26.0 (2.6)	0.020 (0.001)		3.10	1.02	1.10
250	26.6 (3.0)	0.015 (0.001)		3.01	1.27	1.39
300	21.7 (1.3)	0.020 (0.002)		3.02	1.53	1.44

^a The bioconcentration factor (BCF) for FLU is based on wet wt calculated from toxicokinetic estimates using $\text{BCF} = k_u/k_{e,\text{toxic}} + k_m$ and has units of ml/g wet weight, where k_u is the uptake rate coefficient for parent compound, $k_{e,\text{toxic}}$ is the elimination rate constant for the parent compound and phase 1 metabolites, and k_m is the biotransformation rate constant. The BCF for PCBz were calculated from estimates using $\text{BCF} = k_u/k_{e,\text{toxic}}$. Because PCBz was not biotransformed, $k_{e,\text{toxic}}$ is equal to the parent compound.

^b Numbers in parentheses represent standard error from the curve fitting.

exposed fertilized eggs. The dose metric for the reproductive endpoints was the 4-d whole-egg residue. The hatching success of fish eggs was not a sensitive endpoint for FLU exposures. Whole-egg residues of 1.85 $\mu\text{mol/g}$ wet weight, which corresponded to the water-solubility limit of the compound, did not significantly reduce hatching success relative to that of the control treatment. Hatchability of FLU exposures ranged from approximately 70 to 85%. Hatching success for the PCBz exposures ranged from 93% in the controls to 0% at 0.84 $\mu\text{mol/g}$ wet weight (Fig. 4, bars).

Following the transfer of day-4 hatched eggs to uncontaminated water, the 7-d survival of the FLU- and PCBz-exposed larval fish was reduced (Fig. 4, line). Survival of the FLU-exposed eggs ranged from approximately 83% in the controls to approximately 28% at 1.03 $\mu\text{mol/g}$ wet weight. Similar to the FLU exposures, the 7-d survival of the PCBz-exposed eggs was reduced compared to the control survival. The controls had approximately 94% survival, and the PCBz-exposed eggs had only 35% survival at 0.66 $\mu\text{mol/g}$ wet weight.

No significant differences were detected in the 7-d post-hatch weight of the surviving larval fish that hatched from FLU-exposed eggs at egg residues of 1.03 $\mu\text{mol/g}$ wet weight (Fig. 5). From the surviving larval fish in the PCBz exposures, the 7-d posthatch weight was significantly reduced at 0.66 $\mu\text{mol/g}$ wet weight (Fig. 5).

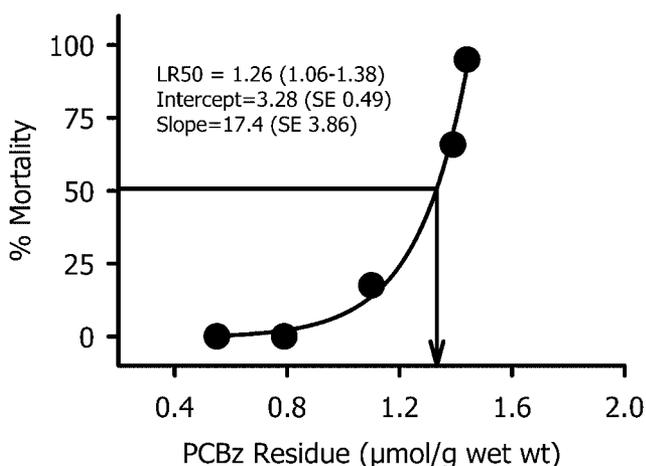
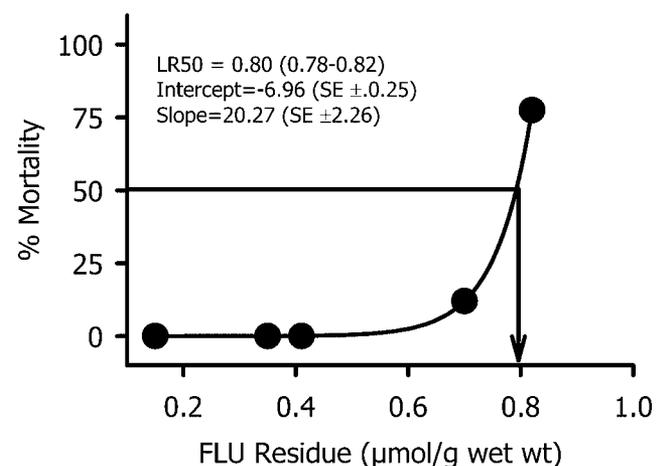


Fig. 2. The 28-d dose-response curves for *Pimephales promelas* generated from whole-body toxic equivalent residues and percentage mortality for fluoranthene (FLU) and pentachlorobenzene (PCBz), respectively. Lethal residues (median lethal residue \pm 95% confidence interval [CI]) were determined from probit analysis. SE = standard error.

Response spectrum

The overall goal of the present study was to develop a response spectrum of adverse biological effects from the two model contaminants (Fig. 6). As expected, relative to the sublethal effects, lethal residues generally required greater whole-body residues within the fish. In the FLU exposures, the no-observed-effect residue (NOER) and the LOER for lethality at 28 d were 0.44 ± 0.01 and 0.63 ± 0.09 $\mu\text{mol/g}$ wet weight, whereas the NOER and LOER for PCBz were 0.79 ± 0.10 and 1.10 ± 0.19 $\mu\text{mol/g}$ wet weight, respectively. Growth of juvenile fish was a particularly sensitive endpoint for the FLU exposures, with a NOER and LOER of 0.016 ± 0.002 and 0.023 ± 0.007 $\mu\text{mol/g}$ wet weight, respectively. The NOER and LOER for growth of PCBz exposures were 0.20 ± 0.03 and 0.65 ± 0.15 $\mu\text{mol/g}$ wet weight, respectively. Contaminant residues in fertilized eggs were used as the dose metric for all reproductive endpoints. For FLU treatments, no significant dif-

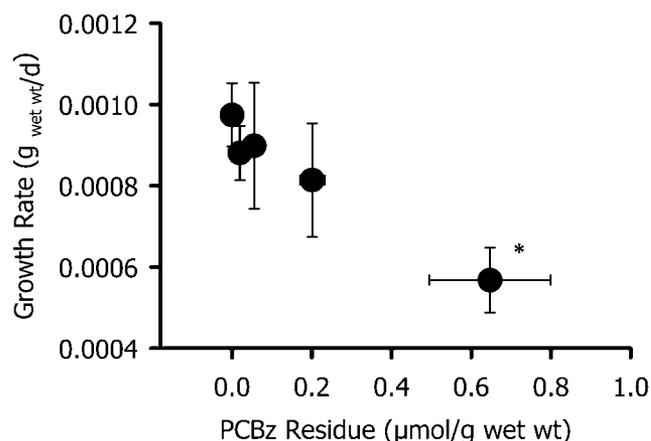
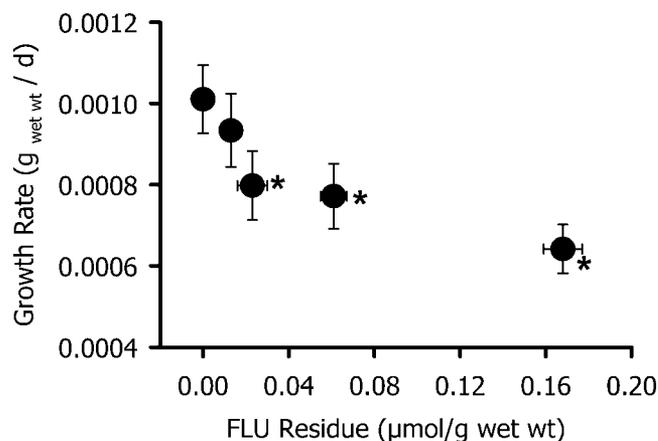


Fig. 3. Mean growth rates corresponding to 28-d whole-body toxic equivalent residues. Error bars represent the standard deviation, and asterisks denote significant differences from the control using Dunnett's test ($p < 0.05$). FLU = fluoranthene; PCBz = pentachlorobenzene.

ferences were detected in hatching success after a 4-d exposure; however, the 7-d survival of hatched fish was reduced at egg residues of 1.03 ± 0.02 $\mu\text{mol/g}$ wet weight. The growth of the surviving posthatch larval fish was not significantly affected by egg residues of less than 1 $\mu\text{mol/g}$ wet weight. The NOER and LOER for the cumulative hatching success, 7-d survival, and 7-d posthatch growth for fish exposed to PCBz relative to the control values were 0.47 ± 0.03 and 0.67 ± 0.11 $\mu\text{mol/g}$ wet weight, respectively.

DISCUSSION

Much bioaccumulation data have been collected with the assumption that elevated residues are related to adverse effects in biota. Therefore, the overall objective of the present study was to supplement these bioaccumulation data from various biomonitoring activities by relating contaminant residues in biota to the associated biological effects. The focus of the present research was to identify specific adverse effects on the fathead minnow for two model nonpolar organic contaminants.

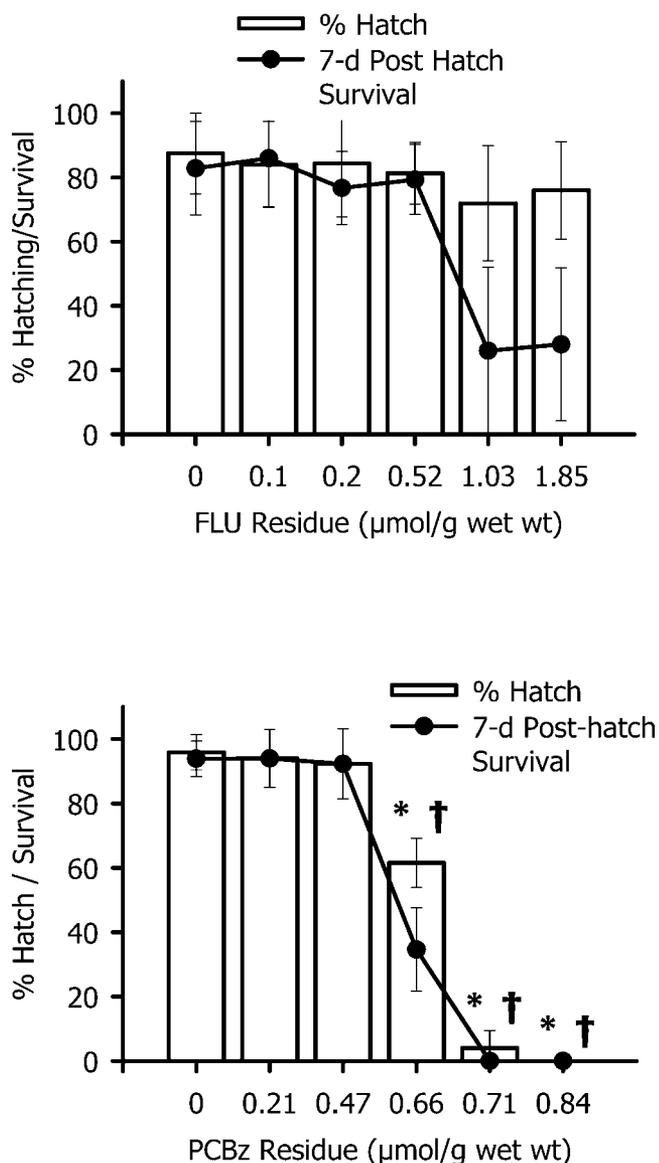


Fig. 4. Cumulative hatching success (vertical bars) of *Pimephales promelas* eggs following exposures to fluoranthene (FLU) and pentachlorobenzene (PCBz). Error bars represent the standard deviation. The mean residues are toxic equivalent concentrations from eggs at 4 d. Percentage survival of day 4 hatched fish (solid lines) followed a typical dose-response relationship. Percentage survival was calculated by counting live organisms after a 7-d monitoring period. Significant differences in percentage hatch and percentage survival using Dunnett's test ($p < 0.05$) are represented by asterisks and daggers, respectively.

Lethal endpoints

This work identified the acute and chronic lethal residues for fathead minnows over the course of a 28-d exposure. The associated lethal whole-body residues for FLU and PCBz were comparable to the range expected for compounds acting via nonpolar narcosis in fish. Previous studies have identified the LR50s for nonpolar narcotic contaminants as ranging from approximately 2 to 8 $\mu\text{mol/g wet weight}$ for fish in acute exposures and approximately an order of magnitude less for chronic exposures [20,21]. In the present study, acute mortality for FLU was limited; 50% mortality was not obtained until 12 d at concentrations equivalent to the water-solubility limits. Sufficient mortality was observed following a 4-d PCBz ex-

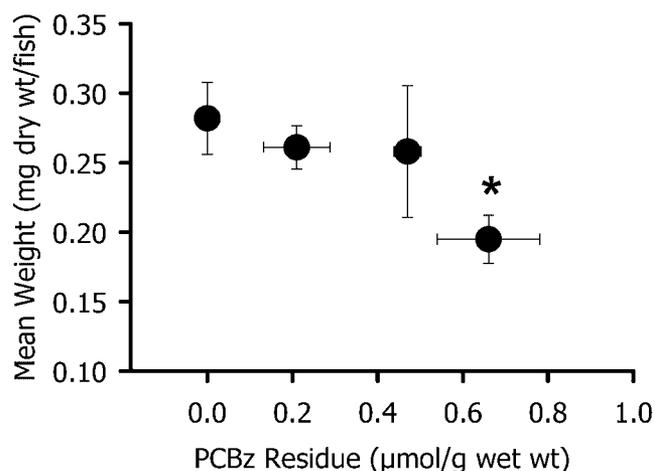
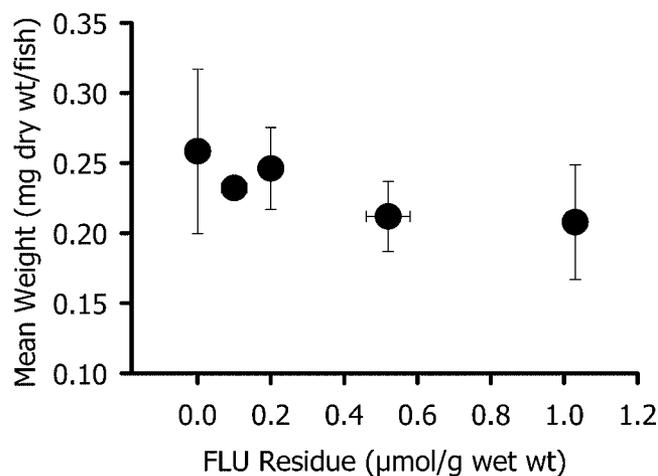


Fig. 5. Mean 7-d posthatch weight following 4-d egg exposure to fluoranthene (FLU) and pentachlorobenzene (PCBz). Residues correspond to the mean 4-d egg toxic residue. Error bars represent the standard deviation, and asterisks denote significant differences from the control using Dunnett's test ($p < 0.05$).

posure to the fish at the highest exposure concentrations to estimate a mean lethal residue. The 4-d mean lethal residue, defined as the mean lethal residue of organisms in a given treatment level at the exposure duration corresponding to the lethal time to 50% mortality, was 1.53 $\mu\text{mol/g wet weight}$. The LR50s determined for FLU and PCBz generally were within a factor of two of those determined in previous studies with fish. For example, the LR50s for a series of chlorobenzenes to the guppy (*Poecilia reticulata*) ranged from 2.1 to 2.5 $\mu\text{mol/g wet weight}$ [20], and the LR50s for mosquitofish (*Gambusia affinis*) exposed to PCBz ranged from 2.3 to 3.6 $\mu\text{mol/g wet weight}$, respectively [22].

In addition to comparing the lethal residues in the fathead minnow between chemicals, a secondary objective of the lethal exposures focused on time-dependent toxicity of the two test compounds to the fathead minnow. From the data collected in the present study, no time dependence of the lethal residues for PCBz was detected. In the case of FLU, the change in

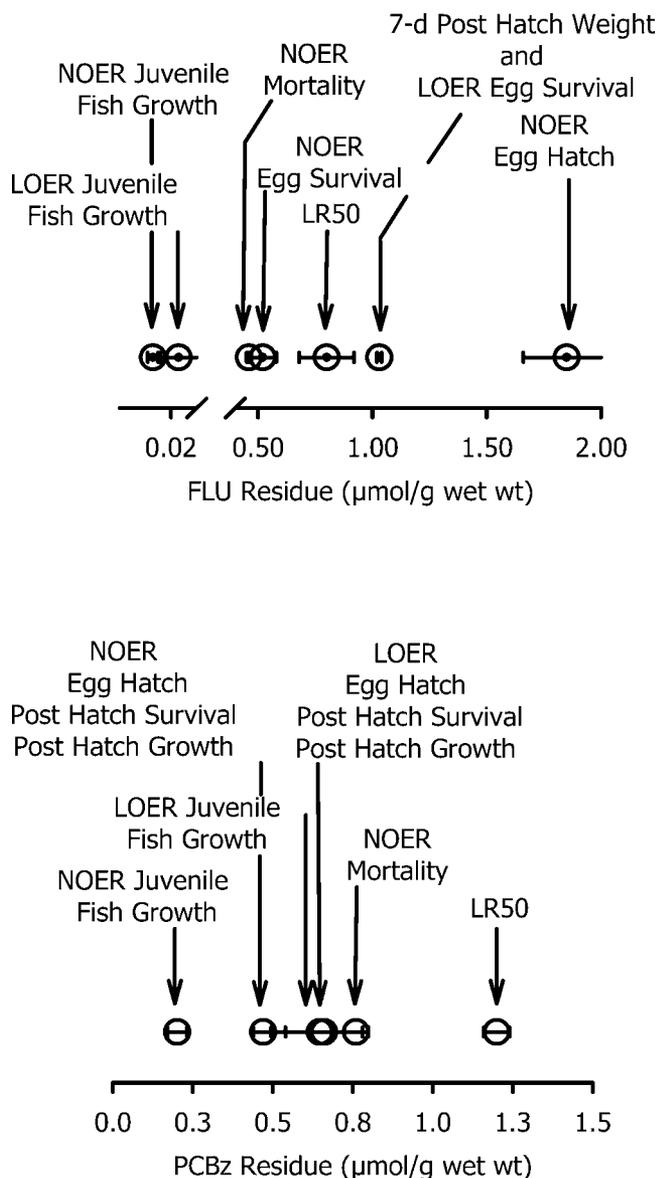


Fig. 6. Response spectrum of biological effects ranging from mortality to sublethal effects (e.g., growth and reproductive effects) on *Pimephales promelas* for fluoranthene (FLU) and pentachlorobenzene (PCBz). All residues are expressed as toxic equivalents. LOER = lowest-observed-effect residue; NOER = no-observed-effect residue.

LR50 was only 15% from 12 to 28 d. This finding was somewhat surprising, because earlier studies estimated chronic lethal residues to be approximately an order of magnitude lower than those determined in acute residues for fish [19]. In addition, several recent studies have reported the time-dependent toxicity of several fish species [22,23] as well as several invertebrate species [15,17,24–26] for many narcotic compounds, including those tested in the present study. However, a number of studies have shown that lethal body residues for a variety of organic compounds are independent of exposure time for fish [19,27,28]. These studies support earlier interpretations of critical body residues suggesting that a constant critical residue exists that is independent of time [21,29]. The variability in the response found during the present study with respect to lethal chronic residues, as well as those mentioned above, may simply be a result of the exposure time not being sufficient to elicit a chronic response.

The absence of time dependence for the lethal residues in this species compared to studies with other species for these compounds [15,17,26] most likely results from differences in the loading or influx of chemicals and, perhaps, the rate of distribution for this test organism. For example, under similar exposure conditions, the uptake rates for *Hyalella azteca* exposed to FLU [17] were greater by a factor of three compared to the uptake rates for *P. promelas*. The majority of previous studies describing the time-dependent toxicity of lethal body residues have demonstrated the greatest temporal change early in the time series (<96 h) [17,23,25,26,30], suggesting that a sufficient concentration existed at the site of toxic action with short exposure, causing enough damage to produce a response [25]. Additionally, most of the time-dependent responses reported for lethal residues have been observed for small invertebrates, in which the distribution of nonpolar organic contaminants is rapid and the internal lipid pools reach dynamic equilibrium with each other in less than 24 h [31]. This is not necessarily the case for larger invertebrates [31]. Whether complete distribution is required for the nonpolar narcotics to reach the site of action is not known. However, most of the time dependent data were found for smaller organisms; therefore, the loading and distribution of the contaminants into the fish appears to be the important factor limiting toxicity during the early portions of the time course for exposure. This illustrates the importance of toxicokinetics in assessing the temporal change in lethal body residues.

Growth and reproductive endpoints

Little data exist regarding egg residues and the corresponding effects from contamination of fish eggs and subsequent posthatch larvae. Some reproductive effects were noted following exposure to the two test compounds (Figs. 4 and 5). Egg hatchability was the most variable sublethal endpoint for *P. promelas*, in which PCBz egg residues were reduced compared to those required for the lethal exposures, whereas the egg seemed to be protective in FLU exposures, with no observed inhibition of hatchability. Egg hatchability as a toxicological endpoint was shown previously to be ineffective among chemicals and test species, and our data support this observation. For example, Elonen et al. [32] exposed seven different species of fish to a series of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) concentrations and found hatchability to be significantly reduced in only one species compared to controls. Similarly, Billiard et al. [33] did not observe significant differences in hatchability among rainbow trout (*Oncorhynchus mykiss*) or zebrafish (*Danio rerio*) eggs exposed to varying concentrations of retene, a polycyclic aromatic hydrocarbon (PAH). Additionally, Hannah et al. [34] did not observe significant differences in hatchability among rainbow trout eggs exposed to benzo[*a*]pyrene. In the present study, PCBz was directly toxic to the developing embryo when hatchability was significantly reduced at 0.66 µmol/g wet weight; however, hatchability was unaffected by FLU residues of approximately 1.85 µmol/g wet weight. The lack of response to FLU was surprising, because the residues in the egg were similar to those associated with the LR50s in juvenile fish and were threefold greater than the residues determined to be toxic in the PCBz exposures. One possible explanation for this variability in the hatchability data between test compounds may be the distribution of the chemicals within different parts of the egg. For example, *n*-hexadecane accumulated in the chorion, whereas naphthalene and bisphenol A accumulated to a

greater extent in the egg yolk [35,36]. This differential accumulation in the various tissues of the egg may affect the toxicity: Some of the tissues, such as the lipid-rich egg yolk, may act as a storage depot, exposing the developing embryo when used as a food source, whereas contaminant associated with the chorion would not likely be available to the embryos. Unfortunately, the small size of the eggs used in the present study prevented further manipulation and separation of the different tissues for separate analyses to explore this possibility. We can speculate, however, that FLU may have accumulated in the chorion, thus having a protective effect on the embryo. This assumption is based on the toxic fraction of FLU in the eggs, which was up to 1.85 $\mu\text{mol/g}$, substantially higher than the PCBz egg residues found to cause an effect and the residues determined to cause mortality in juvenile fish. This suggests that the embryo is not the primary site of accumulation, because the FLU egg residues would be expected to cause substantial mortality if accumulated by the embryo. Additionally, normalizing residues for the lipid content of eggs ($2.0\% \pm 0.2\%$) and juvenile fish ($4.3\% \pm 3.2\%$) does not account for the lack of sensitivity in the eggs.

Interestingly, following transfer of the newly hatched fish larvae to uncontaminated water, the 7-d posthatch survival of the FLU-exposed hatched eggs decreased in a dose-dependent manner (Fig. 4). Because the exposure to the treated water was removed, additional external loading to the organism cannot be implicated. The delayed mortality supports the possibility, in this early life stage, that FLU is acting by a specific mode of action, perhaps after the induction of P450 enzymes. The induction of P450 enzymes, specifically CYP1A, through the aryl hydrocarbon-receptor pathway is common to many PAHs, polychlorinated biphenyls, and dioxin-like compounds. This induction leads to enhanced metabolism and, potentially, to reactive metabolites, such as epoxides and diols, which can bind to macromolecules, or to other structures that yield toxicity. An alternative mechanism of toxicity for FLU to larval fish may be receptor-mediated toxicity, as has been reported for TCDD [32]. This mechanism has been implicated for PAH exposure to early life stages of fish, during which enzyme induction causes oxidative stress, leading to lipid peroxidation and damage to the vasculature supplying the yolk sac [33,37]. Although not quantified in the present study, the gross anomalies, including lack of pigment and edema, were consistent with developmental abnormalities seen in larval fish exposed to PAHs and TCDD [32–34].

Growth rates in fish have been established as a sensitive endpoint for use in evaluating the toxicity of contaminated waters [14], presumably as a result of direct/indirect effects of contaminants causing reduced energy allocation [38,39]. The present results support the assumption that sublethal effects are the more sensitive endpoints, because the whole-body toxic equivalent residues associated with sublethal adverse effects generally were lower than those measured for the lethal residues. Growth of juvenile fish was the most sensitive endpoint tested for both compounds. A reduction in growth has been reported with increasing contaminant residues in the tissues of various aquatic species. Based on growth, the maximum allowable toxicant residue (MATR) calculated for whole-body tissues residues from the mean of the NOER and LOER for FLU and PCBz was 0.02 and 0.43 $\mu\text{mol/g}$ wet weight, respectively (Fig. 3). Unfortunately, for comparative purposes, very little information is available in the primary literature regarding tissue residues and sublethal effects for FLU or the

PAH class of contaminants as a whole. However, benzo[*a*]pyrene was shown to significantly affect *O. mykiss* (alevin) growth at concentrations as low as 0.05 $\mu\text{mol/g}$ wet weight [34].

The literature is more substantial regarding sublethal effects in fish for chlorobenzenes. For example, Chaisuksant et al. [40] reported whole-body residues of *G. affinis* (recalculated assuming 4% lipid) of 0.2 to 1.2 $\mu\text{mol/g}$ wet weight as causing a 50% reduction in growth rate compared to acute lethal residues of 2.8 to 6.4 $\mu\text{mol/g}$ wet weight. Nebeker et al. [41] reported no effects on survival or growth at 0.16 $\mu\text{mol/g}$ wet weight for hexachlorobenzene. The MATR of 0.43 $\mu\text{mol/g}$ wet weight obtained for PCBz compares to fathead minnow data reported by Carlson and Kosian [42], who calculated the MATRs for 1,3-dichlorobenzene, 1,4-dichlorobenzene, and 1,2,3,4-tetrachlorobenzene as 0.98, 0.58, and 4.0 $\mu\text{mol/g}$ wet weight, respectively. In that same study, those authors also reported NOERs of 1.52 and 0.34 $\mu\text{mol/g}$ wet weight for PCBz and hexachlorobenzene, respectively. Growth rates of yellow perch and rainbow trout were not affected by hexachlorobiphenyl residues of 0.36 and 0.61 $\mu\text{mol/g}$ wet weight, respectively [43].

Growth of aquatic organisms decrease with increasing exposure to a variety of contaminants, likely because of the requirement that organisms must allocate resources to respond to the chemical insult rather than devote them to growth. Our data also suggest that this may be the case. In addition, comparing the growth data of the juvenile fish suggest that the difference in sensitivity between FLU and PCBz (~1.5 orders of magnitude) may be caused by different mechanisms of action between the two compounds. The small acute to chronic ratio (ACR) in whole-body residues of approximately two, as determined from the LR50 and LOER for reduced growth for PCBz, is suggestive of narcosis as the mechanism of action for both endpoints. Previous studies have determined that ACRs for nonpolar narcotic compounds range from 2.58 to 5.09 [18,44,45]. If other, more specific mechanisms of action occur, then the ACR probably would be larger. That said, the ACR for FLU was 40, which indicates that the mechanism of action for growth inhibition may not be narcosis. Deviations from a narcotic mechanism of action for PAHs has been identified in which biotransformation of FLU and fluorene has produced metabolites that are more toxic than the parent compounds to aquatic invertebrates [17,46]. The exact mechanism by which these toxic metabolites exert their toxicity has not yet been established; however, mechanisms other than the production of toxic metabolites has been reported for other PAHs, including oxyradical formation because of oxidative stress from prolonged induction of biotransformation enzymes [33].

CONCLUSIONS

The present study has established a causative relationship between whole-body residues and a spectrum of toxic effects for the fathead minnow exposed to FLU and PCBz. These compounds were shown to exert their toxicity to a variety of endpoints, ranging from lethality to reduced growth. The present study also established MATRs for reduced growth, the most sensitive endpoint, with values ranging from approximately 2- to 40-fold lower than residues associated with lethality (i.e., LR50s). These values compare relatively well with previously reported estimates based on water concentrations, in which the mean MATRs of fish exposed to narcotic chemicals were ap-

proximately 12- to 30-fold lower than short-term toxicity values [47].

Overall, because the whole-body residues, not the external media concentrations, are related to biological effects, the complexities associated with bioavailability and multiple exposure routes do not necessarily need to be defined. Therefore, these critical values can be more widely applied to values determined from field-collected organisms for use in risk assessments.

Acknowledgement—This work was supported by the Society of Environmental Toxicology and Chemistry Doctoral Fellowship, sponsored by the Proctor & Gamble Company, and by the Illinois Waste Management and Research Center (DNR Contract HWR04190). Great Lakes Environmental Research Laboratory contribution 1395.

REFERENCES

- Lotufo GR. 1998. Bioaccumulation of sediment-associated fluoranthene in benthic copepods: Uptake, elimination, and biotransformation. *Aquat Toxicol* 44:1–15.
- Fisher SW, Chordas SW, Landrum PF. 1999. Lethal and sublethal body residues for PCB intoxication in the oligochaete, *Lumbriculus variegatus*. *Aquat Toxicol* 45:115–126.
- Hwang H, Fisher SW, Landrum PF. 2001. Identifying body residues associated with 10-d mortality and partial life cycle effects in the midge, *Chironomus tentans*. *Aquat Toxicol* 52:251–267.
- Fairchild JF, Allert AL, Sappington LC, Waddell B. 2005. Chronic toxicity of un-ionized ammonia to early life-stage of endangered Colorado pikeminnow (*Ptychocheilus lucius*) and razorback sucker (*Xyrauchen texanus*) compared to the surrogate fathead minnow (*Pimephales promelas*). *Arch Environ Contam Toxicol* 49:378–384.
- Lydy MJ, Lasater JL, Landrum PF. 2000. Toxicokinetics of DDE and 2-chlorobiphenyl in *Chironomus tentans*. *Arch Environ Contam Toxicol* 38:163–168.
- Schuler LJ, Bailer AJ, Wheeler M, Lydy MJ. 2003. Toxicokinetics of sediment-sorbed BaP and HCBP using the freshwater invertebrates: *Hyalella azteca*, *Chironomus tentans*, and *Lumbriculus variegatus*. *Environ Toxicol Chem* 22:439–449.
- Di Toro DM, Zarba CS, Hansen DJ, Berry WJ, Swartz RC, Cowan CE, Pavlou SP, Allen HE, Thomas NA, Paquin PR. 1991. Technical basis for the equilibrium partitioning method for establishing sediment quality criteria. *Environ Toxicol Chem* 11:1541–1583.
- Huckins JN, Manuweera GK, Petty JD, Mackay D, Lebo JA. 1993. Lipid-containing semipermeable membrane devices for monitoring organic contaminants in water. *Environ Sci Technol* 27:2489–2496.
- van Loon WMGM, Wijnker FG, Verwoerd ME, Hermens JLM. 1996. Quantitative determination of total molar concentrations of bioaccumulatable organic micropollutants in water using C¹⁸ empore disk and molar detection techniques. *Anal Chem* 68:2916–2926.
- Leslie HA, Ter Laak TL, Busser FJM, Kraak MHS, Hermens JLM. 2002. Bioconcentration of organic chemicals: Is a solid-phase microextraction fiber a good surrogate for biota? *Environ Sci Technol* 36:5399–5404.
- Mayer P, Tolls J, Hermens JLM, Mackay D. 2003. Equilibrium sampling devices. *Environ Sci Technol* 37:184A–191A.
- Landrum PF, Dupuis WS, Kukkonen J. 1994. Toxicokinetics and toxicity of sediment-associated pyrene and phenanthrene in *Diporeia* spp.: Examination of equilibrium partitioning theory and residue-based effects for assessing hazard. *Environ Toxicol Chem* 13:1769–1780.
- Neff JM, Burns WA. 1996. Estimation of polycyclic aromatic hydrocarbon concentrations in the water column based on tissue residues in mussels and salmon: An equilibrium partitioning approach. *Environ Toxicol Chem* 15:2240–2253.
- U.S. Environmental Protection Agency. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 4th ed. EPA-821-R-02-013. Office of Water, Washington, DC.
- Schuler LJ, Landrum PF, Lydy MJ. 2006. Comparative toxicity of fluoranthene and pentachlorobenzene to three freshwater invertebrates. *Environ Toxicol Chem* 25:985–994.
- Bligh EG, Dryer WJ. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917.
- Schuler LJ, Landrum PF, Lydy MJ. 2004. Time-dependent toxicity of fluoranthene to freshwater invertebrates and the role of biotransformation on lethal body residues. *Environ Sci Technol* 38:6247–6255.
- Di Toro DM, McGrath JA, Hansen DJ. 2000. Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria. I. Water and tissue. *Environ Toxicol Chem* 19:1951–1970.
- McCarty LS, MacKay D, Smith D, Ozburn GW, Dixon DG. 1992. Residue-based interpretation of toxicity and bioconcentration QSARs from aquatic bioassays: Neutral narcotic organics. *Environ Toxicol Chem* 11:917–930.
- Van Hoogen G, Opperhuizen A. 1988. Toxicokinetics of chlorobenzenes in fish. *Environ Toxicol Chem* 7:213–219.
- McCarty LS, MacKay D. 1993. Enhancing ecotoxicological modeling and assessment. *Environ Sci Technol* 27:1719–1728.
- Chaisuksant Y, Yu Q, Connell D. 1997. Internal lethal concentrations of halobenzenes with fish (*Gambusia affinis*). *Ecotoxicol Environ Saf* 37:66–75.
- De Wolf W, Opperhuizen A, Seinen W, Hermens JLM. 1991. Influence of survival time on the lethal body burden of 2,3,4,5-tetrachloroaniline in the guppy, *Poecilia reticulata*. *Sci Total Environ* 109:457–459.
- Yu Q, Chaisuksant Y, Connell D. 1999. A model for nonspecific toxicity with aquatic organisms over relatively long periods of exposure time. *Ecotoxicol Environ Saf* 38:909–918.
- Lee J, Landrum P, Koh C. 2002. Prediction of time-dependent PAH toxicity in *Hyalella azteca* using a damage assessment model. *Environ Sci Technol* 36:3131–3138.
- Landrum PF, Steevens JA, Gossiaux DC, McElroy M, Robinson S, Begnoche L, Chernyak S, Hickey J. 2004. Time-dependent lethal body residues for the toxicity of pentachlorobenzene to *Hyalella azteca*. *Environ Toxicol Chem* 23:1335–1343.
- De Bruijn J, Yedema E, Seinen W, Hermens JLM. 1991. Lethal body burdens of four organophosphorous pesticides in the guppy (*Poecilia reticulata*). *Aquat Toxicol* 20:111–122.
- Van Wezel AP, Opperhuizen A. 1995. Narcosis due to environmental pollutants in aquatic organisms: Residue-based toxicity, mechanisms, and membrane burdens. *Crit Rev Toxicol* 25:255–279.
- McCarty LS. 1986. The relationship between aquatic toxicity QSARs and bioconcentration for some organic chemicals. *Environ Toxicol Chem* 5:1071–1080.
- Landrum PF, Steevens JA, McElroy M, Gossiaux DC, Lewis JS, Robinson SD. 2005. Time-dependent toxicity of dichlorodiphenyldichloroethylene to *Hyalella azteca*. *Environ Toxicol Chem* 24:211–218.
- Gardner WS, Landrum PF, Chandler JF. 1990. Lipid-partitioning and disposition of benzo[a]pyrene and hexachlorobiphenyl in Lake Michigan, *Pontoporeia hoyi*. *Environ Toxicol Chem* 9:1269–1278.
- Elonen GE, Spehar RL, Holcombe GW, Johnson RD, Fernandez JD, Erickson RJ, Tietge JE, Cook PM. 1998. Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin to seven freshwater fish species during early life-stage development. *Environ Toxicol Chem* 17:472–483.
- Billiard SM, Querbach K, Hodson PV. 1999. Toxicity of retene to early life stages of two freshwater fish species. *Environ Toxicol Chem* 18:2070–2077.
- Hannah JB, Hose JE, Landolt ML, Miller BS, Felton SP, Iwaoka WT. 1982. Benzo[a]pyrene-induced morphologic and developmental abnormalities in rainbow trout. *Arch Environ Contam Toxicol* 11:727–734.
- Kuhnhold WW, Busch F. 1978. On the uptake of three different types of hydrocarbons by salmon eggs (*Salmo salar* L.). *Meeresforsch* 26:50–59.
- Honkanen JO, Heinonen J, Kukkonen JVK. 2001. Toxicokinetics of waterborne bisphenol A in landlocked salmon (*Salmo salar*) eggs at various temperatures. *Environ Toxicol Chem* 20:2296–2302.
- Hawkins SA, Billiard SM, Tabash SP, Brown RS, Hodson PV. 2002. Altering cytochrome P4501A activity affects polycyclic aromatic hydrocarbon metabolism and toxicity in rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 21:1845–1853.
- Congdon JD, Dunham AE, Hopkins WA, Rowe CL, Hinton TG. 2001. Resource allocation-based life histories: A conceptual basis

- for studies of ecological toxicology. *Environ Toxicol Chem* 20: 1698–1703.
39. Smolders R, Bervoets L, De Boek G, Blust R. 2002. Integrated condition indices as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). *Environ Toxicol Chem* 21:87–93.
 40. Chaisuksant Y, Yu Q, Connell DW. 1998. Effects of halobenzenes on growth rates of fish (*Gambusia affinis*). *Ecotoxicol Environ Saf* 39:120–130.
 41. Nebeker AV, Griffis WL, Wise CM, Hopkins E, Barbitta JA. 1989. Survival, reproduction and bioconcentration in invertebrates and fish exposed to hexachlorobenzene. *Environ Toxicol Chem* 8: 601–611.
 42. Carlson AR, Kosian PA. 1987. Toxicity of chlorinated benzenes to fathead minnows, *Pimephales promelas*. *Arch Environ Contam Toxicol* 16:129–135.
 43. Dabrowska H, Fisher SW, Dabrowski K, Staubus AE. 1999. Dietary uptake efficiency of 2,2',4,4',5,5'-hexachlorobiphenyl in yellow perch and rainbow trout: Role of dietary and body lipids. *Environ Toxicol Chem* 18:938–945.
 44. Roex EWM, Van Gestel CAM, Van Wezel AP, Van Straalen NM. 2000. Ratios between acute aquatic toxicity and effects on population growth rates in relation to toxicant mode of action. *Environ Toxicol Chem* 19:685–693.
 45. Wong DCL, Ray AW, Rausina GA, Mancini ER, Steen AE. 2001. Development of a freshwater aquatic toxicity database for ambient water quality criteria for methyl tertiary-butyl ether. *Environ Toxicol Chem* 20:1125–1132.
 46. Lee JH, Landrum PF. 2006. Application of multicomponent damage assessment model (MDAM) for the toxicity of metabolized PAH in *Hyalella azteca*. *Environ Sci Technol* 40:1350–1357.
 47. Call DJ, Broke LT, Knuth ML, Poirier SH, Hoglund MD. 1985. Fish subchronic toxicity prediction model for industrial chemicals that produce narcosis. *Environ Toxicol Chem* 4:335–341.