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Environmental Toxicology and Chemistry, Vol. 18, No. 10, pp. 2123–2130, 1999

Printed in the USA
0730-7268/99 $9.00 + .00

Environmental Chemistry

EXPOSURE OF NORTHERN LEOPARD FROGS IN THE GREEN BAY ECOSYSTEM TO POLYCHLORINATED BIPHENYLS, POLYCHLORINATED DIBENZO-*P*-DIOXINS, AND POLYCHLORINATED DIBENZOFURANS IS MEASURED BY DIRECT CHEMISTRY BUT NOT HEPATIC ETHOXYRESORUFIN-*O*-DEETHYLASE ACTIVITY

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(*Received* 20 *February*1998; *Accepted* 23 *December*1998)

Abstract—We measured concentrations of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) in northern leopard frogs collected from the Green Bay ecosystem and explored the catalytic activity of hepatic cytochrome P450-associated monooxygenase (P450 enzyme) as a biomarker for exposure to aryl hydrocarbon receptor (AhR) agonists. The two hypotheses tested were PCH concentrations in northern leopard frogs would be positively correlated with sediment polychlorinated hydrocarbon (PCH) levels in wetland habitats along a contamination gradient and hepatic ethoxyresorufin-*O*-deethylase (EROD) activity of northern leopard frogs, which is presumably mediated by aryl hydrocarbon receptor (AhR), would be positively correlated with PCH concentrations in frog carcasses (whole body minus liver) from different collection sites. In 1994 to 1995, frogs from seven sites along the lower Fox River and Green Bay, USA, were assayed for hepatic EROD activities and whole carcass concentrations of PCBs, PCDDs, and PCDFs. Tissue total PCB concentrations ranging from 3 to 154 ng/g were significantly correlated with sediment PCB levels. Only one PCDD and two PCDFs at concentrations of 6 to 8 pg/g were found in the frogs collected from one of the sites. The EROD activity in frogs ranging from 186 to 270 pmol/min/mg protein was not significantly correlated with frog body weight and was similar among sites except for Peter's Marsh. No significant correlation was found between EROD activity and carcass PCB concentration. This result was consistent with the fact that the frogs collected from the Green Bay ecosystem had relatively low PCB concentrations compared with what was required for induction in the laboratory (ED50 for EROD is between 700 and 2,300 ng/g).

Keywords—Tissue toxicant residues Ethoxyresorufin-*O*-deethylase activity Biomarkers Northern leopard frogs

INTRODUCTION

Green Bay and the lower Fox River, USA, are known to have been heavily contaminated with polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) from industries including kraft bleaching papermills [1,2], pesticides, herbicides, and agricultural and residential runoff [3]. There is a distinct gradient in sediment levels of these pollutants from highest concentrations at upstream sites along the Fox River to lowest concentrations at sites in Green Bay [4]. These pollutants have had adverse impacts on wildlife inhabiting that ecosystem. For instance, embryotoxicity and reproductive abnormality imposed by these polychlorinated hydrocarbons (PCHs) in a variety of wildlife have been previously reported [5–7]. Several reports proposed that amphibians might be good bioindicators of environmental pollution due to their complex life cycles, rapid larval growth rates, trophic position, poikilothermy, and permeable eggs, gills, and skin [8]. However, relatively little is known about PCH (e.g., PCBs, PCDDs, and PCDFs) bioaccumulation in or possible effects on amphibians. Korfmacher et al. [9] reported 2,3,7,8-tetrachlorodibenzo-*p*-dioxin concentrations in American bullfrogs (*Rana catesbeiana*) collected at a polluted site in Arkansas, and Dowd et al. [10] reported nondetectable concentrations of PCBs in a *Rana* spp. in Louisiana watersheds. American bullfrogs appear much less sensitive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) than guinea pigs and other mammals [9,11].

While comprehensive chemical analysis for contaminants is costly and does not provide information about biological effects, several physiological and biochemical parameters, such as hepatic cytochrome P450-associated monooxygenase (P450 enzyme), deoxyribonucleic acid adducts, thyroid function, and acetylcholinesterase, have been proposed as alternative approaches of assessing organismal exposure to environmental toxicants [12]. Among those biomarkers, the induction of hepatic cytochrome P450-associated monooxygenase activity (e.g., ethoxyresorufin-*O*-deethylase [EROD]) has been shown to be a promising, early warning system in vertebrates [13–15]. To our knowledge, there is only one recent report regarding the utility of amphibian P450 enzymes as a bioindicator for exposure to toxicants [16]. There has been no study on tissue toxicant residues of adult amphibians in the Green Bay ecosystem nor on the use of P450 enzymes as a measure of toxicant exposure. In the past, we have used leopard frogs to establish a dose–response relationship on P450 induction by a coplanar PCB, 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) [17]. In 1993, we conducted a pilot survey on tissue

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toxicant residues of adult northern leopards and green frogs (*Rana clamitans*) in the Green Bay ecosystem. Our data indicated that cis- and transchlordane, cis- and transnonachlor, dieldrin, aldrin, methoxychlor, *p,p′*-dichlorodiphenyltrichloroethane (*p,p′*-DDT), *o,p*-DDT, *p,p′*-dichlorodiphenyldichloroethane (*p,p′*-DDD), *o,p*-DDD, dichlorodiphenyldichloroethylene (*o,p*-DDE), lindane (γ-BHC), α-BHC, and hexachlorobenzene were below detection levels (0.01–0.05 μg/g) in both species (Huang and Karasov, unpublished data), but PCBs in frogs were measurable. Therefore, in the present study, we focused on PCBs, PCDDs, and PCDFs, which have been shown to be common contaminants present in the studied ecosystem [18] and are known to be monooxygenase inducers and toxicants in fish [19].

We tested the hypotheses that PCH concentrations in northern leopard frogs would be positively correlated with sediment PCH levels in wetland habitats along a contamination gradient and that hepatic EROD activity of northern leopard frogs, which is presumably mediated by aryl hydrocarbon receptor (AhR), would be positively correlated with PCH concentrations in frog carcasses (whole body minus liver) from different collection sites. Northern leopard frogs inhabit wetlands in the lower Fox River and Green Bay ecosystem in Wisconsin. They could have been exposed to PCBs, PCDDs, and PCDFs by consumption of toxicant-laden algae and invertebrates and therefore are a good animal model for evaluating the utility of cytochrome P450-associated monooxygenase as a biomarker of contaminant exposure. To test our hypotheses, we collected frogs from seven sites along the lower Fox River and Green Bay. Frogs were assayed for hepatic EROD activities and carcass (whole body minus liver) concentrations of PCBs, PCDDs, and PCDFs. As an index of sediment PCH levels, we used the total sediment PCB levels at several collection sites that were summarized from the Green Bay/Fox River mass balance study [4] or measured as part of our laboratory analysis.

MATERIALS AND METHODS

Collection of frogs and habitat description

From May to September in 1994 and 1995, we surveyed more than 20 possible marsh habitats for leopard frogs. However, due to the difficulty of collection or scarcity of frogs, only seven collection sites were selected for subsequent study, including three wetlands on Green Bay (Little Tail Point, South Sensiba, Pete's Marsh) and three wetlands on the Fox River (Railroad Museum, Strobe Island, and Deposit C) (Fig. 1). Barkhausen (1 km west of the bay) was classified as a reference site because of its isolation from Green Bay. We assumed initially that it would have the lowest PCH level among sites. Sites were chosen to represent a PCH contamination gradient based on data from a previous study [4] or from Steve Jaeger of Wisconsin Department of Natural Resources.

Microsomal preparation and enzymatic assay

The frogs were collected at night, then transported back to the University of Wisconsin–Madison campus. After weighing and determining their sex, frogs were euthanized and decapitated within 44 h postcapture. Each liver was excised, placed in a cryotube with glycerol to cover the whole tissue (M. J. Melancon, Patuxent Research Center, Laurel, MD, USA, personal communication). The liver then was immediately stored at −110°C and held for 2 months in liquid nitrogen until enzyme assay.

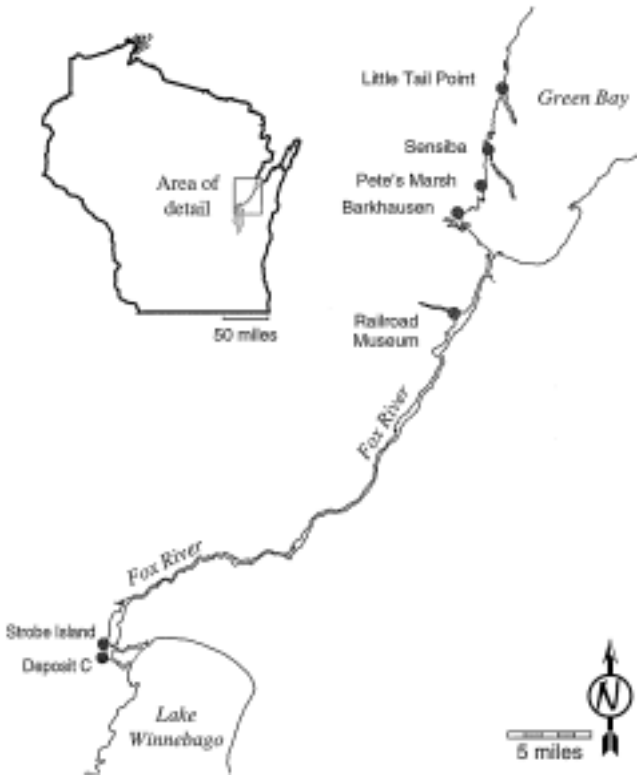

Fig. 1. Map of Green Bay/Fox River field site locations. Little Tail Point (T25/26N R21E S31) is located in Oconto County, Wisconsin, USA. Barkhausen (T24N R20E S1/2), South Sensiba (T25N R20E S25), Railroad Museum (T23N R20E S10/11), and Pete's Marsh (T24/25N R20E S1/36) are located in Brown County, Wisconsin, USA. Strobe Island (T20N R17E S3) and Deposit C (T20N R17E S14) are located in Winnebago County, Wisconsin, USA.

Each liver sample was thawed, blotted free of glycerol, and homogenized in a pH 7.25 buffer solution (0.25 M KPO_4, 0.15 M KCl, 10 mM EDTA) with 0.1 mM dithiol threitol (DTT) as antioxidant and 0.25 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. The homogenate was centrifuged at 9,000 *g* at 4°C, followed by centrifugation of the supernatant at 100,000 *g* at 4°C. To remove hemoglobin, the pellet was resuspended in a pH 7.25 washing buffer (0.1 M sodium pyrophosphate, 10 mM EDTA, 0.25 mM PMSF, 0.1 mM DTT) and recentrifuged. The final pellet was resuspended in a pH 7.25 dilution solution containing glycerol (0.1 M KPO_4, 10 mM EDTA, 0.25 mM PMSF, 0.1 mM DTT, 20% glycerol) and stored under liquid nitrogen.

The protocol for EROD fluorometric assay was slightly modified from Rabovsky et al. [20]. The optimal reaction conditions were determined using various combinations of time, temperature, substrate concentration, and nicotinamide adenine dinucleotide phosphate (NADPH) concentration. The optimal reaction solution, for each 1-ml reaction, contained 160 μg microsomal protein, 0.125 mM NADPH, and 5 μM ethoxyresorufin in a buffer (0.1 M KPO_4 with 3 mM $MgCl_2$, pH 7.8). Buffer, substrate, and sample were preincubated at 28.5°C for 10 min, then NADPH was added to start the reaction. The reaction was run for 10 min, then stopped by adding 0.5 ml of ice cold 16 mM $CaCl_2$. The reaction mixture was centrifuged 5 min to pellet proteins and membranes. Finally, clear supernatant was removed and fluorescence was read at an excitation of 530 nm and an emission of 585 nm in a Perkin-Elmer fluorescence spectrophotometer (Norwalk, CT, USA). The

EROD activity is expressed as picomoles of product per minute per milligram of microsomal protein. The microsomal protein concentration was measured with the Pierce protein assay [21].

As a positive control for the EROD assay, we also included microsomal samples from our previous dose–response study that demonstrated induction of EROD activity by PCB 126 [17]. In that study, leopard frogs were injected ip with different doses of PCB 126 in corn oil, and livers (minus gall bladder) were removed and frozen 7 d after treatment. Microsomes prepared from those livers were stored for 18 months in liquid nitrogen and were run concurrently with the field samples in this study. The EROD activity in those microsomes was correlated with cytochrome P4501 A (CYP1A) equivalents measured by Western blots [22].

Analyses of PCH concentrations in carcasses

Sample preparation. Each frog carcass (whole body minus liver) was individually ground in dry ice. The dry ice was sublimed gradually in a −20°C freezer. The ground carcasses were then proportionally mixed, based on their body weights, to create a pooled sample for a particular collection site. Each pooled sample had to be at least 10 g for analysis of contaminants. We chose to pool samples in each site because PCH analyses were costly.

PCB analyses. The protocol for tissue PCB and fat content analysis was according to method 1410 of the Organic Chemistry Section of the Wisconsin State Laboratory of Hygiene [23], which was used in the Green Bay/Fox River mass balance study [4]. The analysis was conducted by the Environmental Science Section of the Wisconsin State Laboratory of Hygiene. Detection limits for PCBs ranged from 0.6 ng/g to 4.0 ng/g (mostly ≤1.0 ng/g), depending on the congener. A pooled sample from each site was analyzed for 85 PCB congeners (hereafter, referred to as routine PCB congener analysis). For toxic PCB congener (coplanar and monoortho PCBs) analysis, we chose samples from Deposit C, Strobe Island, Little Tail Point, and Barkhausen because of their high, medium, and low total PCB concentrations in carcasses. For each site, we created two pools of frog carcasses, one containing the two frogs with the highest hepatic EROD and one containing the two frogs with the lowest hepatic EROD activity. The eight pools (two pools/site × four sites) were then analyzed for seven toxic PCB congeners, which were 77, 126, 169, 105, 156, 123, and 157. We selected frogs with the highest or lowest hepatic EROD, assuming that those with high hepatic EROD would have high PCB burdens and vice versa. Comparative toxic and biochemical potencies of these coplanar or monoortho PCBs have been widely studied in fish species and have been shown to have higher values of toxic equivalency factor (TEF) within this class of halogenated aromatics [19].

PCDDs and PCDFs analyses

Assuming that PCDD/PCDF concentrations would be positively correlated with PCB concentrations and that hepatic EROD activity would positively correlate with body PCDD/PCDF burden, we selected one pooled sample from Deposit C (three ♂ and two ♀) and one pooled sample from Pete's Marsh (12 ♂ and 6 ♀). These sites were chosen because they had, respectively, the highest and lowest PCB levels in the sediment of all our collection sites analyzed by the Green Bay/Fox River mass balance study [4] or by our laboratory analysis [24]. The samples were shipped at −30°C to the Texas A&M University's Geochemical and Environmental Research Group (GERG) laboratory, Bryan, Texas, USA. The chemical analysis followed their standard operating procedures, namely, 9618, Procedures for the Extraction of Tissues and Purification of Extracts for Analysis of Polychlorinated Dibenzo-*p*-dioxins and Polychlorinated Dibenzofurans, and 9621, Quantitative Determination of Tetra- through Octa-Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans by Isotope Dilution High Resolution Gas Chromatography/High Resolution Mass Spectrometry. Isotopic ratios were within the method's specified quality control limits. Internal standard isotope recoveries ranged from 60 to 98%. Detection limits for these chemicals ranged from 2.1 pg/g to 16.8 pg/g, depending on the congener. The 17 PCDDs and PCDFs analyzed were 2,3,4,7,8-PeCDF, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,7,8,9-HxCDF, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,7,8,9-HpCDF, OCDF, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, and OCDD. All of the PCB, PCDD, and PCDF concentrations reported are based on wet body weight.

Sources of sediment PCB data

PCB sediment data of wetland and close-to-wetland habitats were summarized from our laboratory analysis [24] or the Green Bay/Fox River mass balance study [4]. The mass balance study was intended to monitor the sources, transport routes, and fates of pollutants in the Great Lakes ecosystem. All sediment data reported are based on dry weight.

Statistical analyses

The variation of whole-body weight among sites was analyzed using analysis of variance (ANOVA). Analysis of covariance (ANCOVA) models were used for detecting variation in EROD activity among sites, with body weight and sex as covariates. A Tukey's test was used to isolate significantly different mean values. The correlations between total PCB concentrations in frog carcasses (without liver) and either average EROD activity or sediment PCB concentrations were analyzed using Spearman correlation, which we chose because it makes no assumptions about the normality of the data's distribution and because we had to estimate some PCB concentrations when they were below the detection level. Statistical significance was set at $\alpha = 0.05$.

RESULTS

Sex, body weight, fat content, and liver appearance

Our study included males and females from each collection site except for Railroad Museum; analyses were not segregated by sex. The average body weight differed among sites ($F_{6,65} = 4.82$, $p < 0.001$; Table 1), with the lowest at Little Tail Point and the highest at Deposit C. Whole-body fat contents (without liver) ranged from 1.4 to 2.4%. In two of the pools, water content was 75 ± 2%. Liver appearance seemed normal by gross inspection.

Hypothesis I: PCH concentrations in northern leopard frogs would be positively correlated with sediment PCH levels in wetland habitats along the contamination gradient

No PCBs were detected in the sediment of two sites and, for the correlation test, these sites were assigned a level of 0.5 ng/g (50% of detection limit). The PCBs were above detection limit in the frog carcass pools from all the sites and varied from lowest to highest by 50 times (Table 1). Carcass PCB

Table 1. Sex, lipid (%, wet weight), body weight (g), hepatic ethoxyresorufin-*O*-deethylase (EROD) activity (pmol/min/mg), sediment PCB levels (μg/g dry basis), toxic PCB congener concentrations, and routine PCB congener concentrations (ng/g wet body weight) in carcasses from Green Bay/lower Fox River ecosystem; data expressed as mean ± 1 SEM

	Sediment[a]	Frogs					
Collection site	Routine[b] PCBs	Routine[b] PCBs	Toxic[b] PCBs	EROD	Lipid content	Body weight	♀, ♂
Deposit C	16.68 ± 11.93 (4)	151.9	2.2	240 ± 39	2.1	26.8 ± 2.4	2, 3
Strobe Island	0.88 ± 0.774 (5)	21.6	ND	229 ± 41	2.4	20.6 ± 3.2	3, 2
Railroad Museum	1.4 (1)	38.1	—	239 ± 69	2.4	19.2 ± 5.7	0, 3
Barkhausen	0.0005	11.1	ND	256 ± 24	1.8	15.7 ± 2.0	6, 4
Pete's Marsh	0.04	2.8	—	186 ± 18	1.4	11.5 ± 1.0	6, 12
Sensiba	0.0005	15.6	—	208 ± 12	1.8	15.1 ± 2.0	5, 13
Little Tail Point	—	16.0	ND	271 ± 27	2.2	10.6 ± 2.2	7, 6

[a] Sediment (top 5 cm) data are from three sites of one to five sampling points (parentheses) in Fox River [4]. Each sampling point chosen from the Green Bay/Fox River mass balance study was close to a frog collection site, with an average distance of 50 m from lakeshore or riverside. Pooled sediment (top 5 cm) samples around enclosures were taken from Pete's Marsh, Barkhusen, and Sensiba [33]. Enclosures were located near leopard frog collection sites. — = not analyzed; ND = not detected (below detection level).

[b] Routine and toxic PCB congener analyses were described in the Materials and Methods. Note that carcasses were analyzed without livers.

concentrations were significantly correlated with sediment PCB levels ($r_s = 0.812$, $p < 0.05$, $n = 6$) (Fig. 2). Also, the three highest carcass PCB concentrations were detected in pools of individuals with the highest average body weights.

Frogs from Deposit C, which had the highest PCB concentrations of all collection sites, had more highly chlorinated PCB congeners. Coplanar and monoortho PCB analyses showed that only the pooled sample of two frogs with higher hepatic EROD activity collected from Deposit C had detectable levels of PCB 105 and 156, at 1.0 and 1.2 ng/g, respectively. The other seven pooled samples from Strobe Island, Little Tail Point, Barkhausen, and the other sample from Deposit C (i.e., the two frogs with lower hepatic EROD activities) were below the minimum detection limit (1 ng/g).

Congeners 2,3,7,8-TCDF, 1,2,3,4,6,7,8-HpCDF, and 1,2,3,4,6,7,8-HpCDD were found in the pooled sample of frogs from Deposit C at concentrations of 26.21, 28.88, and 31.12 pg/g, respectively. No PCDDs and PCDFs were found in frog carcasses (without livers) collected from Pete's Marsh.

Hypothesis II: Hepatic EROD activities of northern leopard frogs would be positively correlated with PCH concentrations in frog carcass (whole body minus liver) from different collection sites

The mean EROD activities of frog livers from seven sites ranged from 186 ± 18 to 271 ± 27 pmol/min/mg protein, with the lowest at Pete's Marsh and the highest at Little Tail Point (Table 1). These means are based on frogs of both sexes collected in the months June through October, and EROD might vary with sex or season. Therefore, we performed two statistical analyses. In the first analysis, frogs collected in August through September (2 months postbreeding season) were analyzed because samples were available from all sites during that period. The EROD activity was not significantly correlated with frog body weight ($F_{1,51} = 1.03$, $p = 0.31$) and was similar among sites with one exception, where EROD activity of frogs collected from Pete's Marsh was lower than from Little Tail Point (Tukey, $p = 0.001$) (Fig. 3). In the second analysis, frogs collected in August through September in Barkhausen, Little Tail Point, and Pete's Marsh were analyzed because both frog sexes were present. The activity did not vary significantly with sex or body weight ($p > 0.30$), whereas EROD activity of frogs collected from Pete's Marsh was significant lower than from Little Tail Point (Tukey, $p = 0.001$).

Furthermore, no significant correlation was found between mean EROD activity and tissue PCB concentration ($r_s = 0.321$, $p > 0.5$, $n = 7$) (Fig. 4). We also tested for correlations with specific congeners. Congeners PCB 74, 99, and 118 were found in frogs from all sites, and coeluting congeners 66/95 and 132/153 were found in all sites except Pete's Marsh. Mean EROD activity was not significantly correlated with any of these specific congeners ($r_s = -0.200$–0.450, $p \geq 0.5$, $n = 6$–7).

The general lack of difference in EROD activity by site or sex was not due to insensitivity of the EROD assay, as shown by the highly significant variation in EROD activity among samples from the previous dose–response study that were run simultaneously. In that data set, there was clear induction of EROD activity in frogs in the two highest PCB 126 dose groups ($p < 0.05$, $n = 20$) (Fig. 4, inset). Note, however, that the concentrations of total PCBs in all field-collected frogs were below the level of PCB 126 that caused EROD induction in the laboratory. The EROD activity of field frogs was higher than EROD activity of control frogs in the laboratory but was well below the EROD activity of induced frogs in the two highest PCB 126 dose groups. Furthermore, the difference in EROD activity between field frogs and those induced frogs exposed for 1 week to PCB 126 in the laboratory is probably

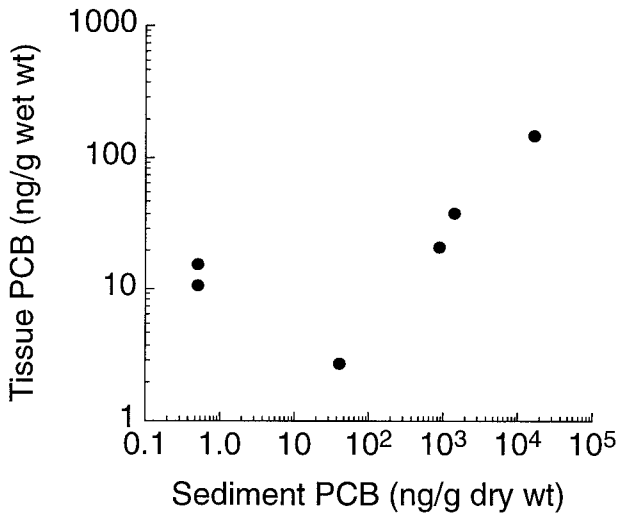

Fig. 2. Carcass PCB concentrations (without livers) in relation to sediment PCB levels ($r_s = 0.812$, $p < 0.05$, $n = 6$).

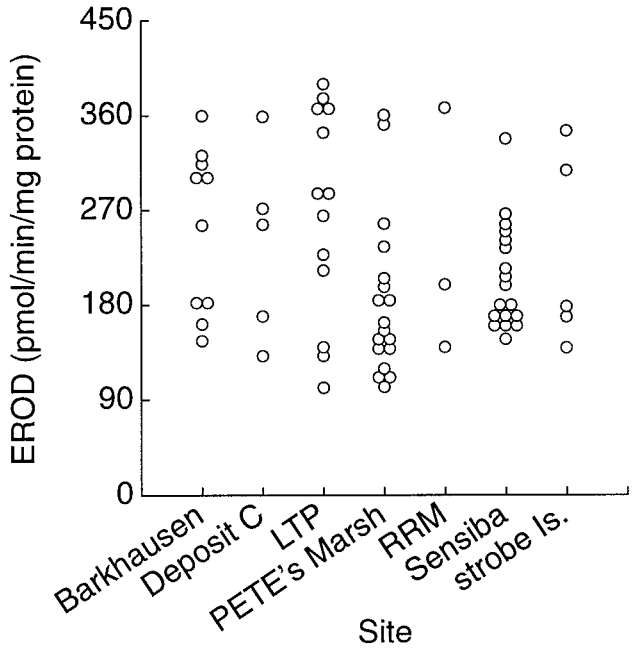

Fig. 3. The EROD activities of leopard frogs at seven sites in Green Bay/lower Fox River ecosystem.

even larger than indicated because frogs exposed for 2 weeks and longer in the laboratory had EROD values twice as high as those exposed for 1 week [17].

DISCUSSION

Hypothesis I

We found that total PCB concentrations in frog carcasses were significantly correlated with sediment PCB levels close to each study area. While such a correlation can be a useful addition to predictive ecotoxicology, we note two features of our data set that limit its utility in this regard. First, the three highest carcass PCB concentrations were detected in pools of individuals with the highest body weights, which is a possible indication of association with age confounding the apparent correlation with sediment PCH level. Second, our sample size is small. We do expect that the correlation with sediment PCH level will hold up, however, because in our laboratory's concurrent field study [24], the PCB concentrations of green frog tadpoles (23–283 ng/g wet weight) were significantly correlated with sediment PCB levels.

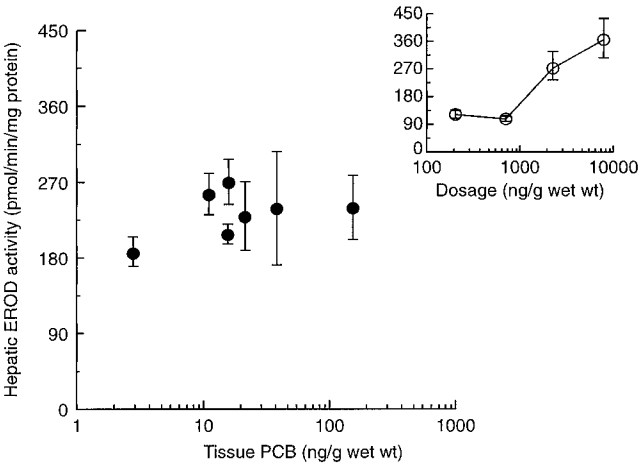

Fig. 4. Average hepatic EROD activity in relation to tissue PCB concentration (pools). The larger figure is for field-captured frogs ($r_s = 0.321$, $p > 0.5$, $n = 7$). The inset shows the significant ($p < 0.05$, $n = 20$) variation in hepatic EROD activity among frogs injected with PCB 126 in the laboratory whose liver microsomes were run simultaneously with those from the field study. Data were expressed as mean ± 1 SEM.

Our measures of PCB concentration in leopard frogs are slight underestimates because the carcasses lacked livers which, in a different whole-body distribution experiment, contained about 6.0 ± 0.8% of the total carcass PCB [22]. A similar underestimate is also expected for the tissue residues of PCDDs and PCDFs. According to our PCB data, bioaccumulation factors (ratios of PCBs in frogs/PCBs in environmental compartment) are in the range of 0.00025 to 0.0175 for frogs compared with sediment, 21.25 to 292 for frogs collected from sites in the Fox River compared with Fox River water (130 ng/L in 1987), and 17.5 to 135 for frogs collected from sites in Green Bay compared with Green Bay water (<40 ng/L in 1987) [25–27].

The tissue PCB residues in leopard frog adults and green frog metamorphs are similar but lower than those of omnivorous yellow perch and white perch (diet, e.g., microplankton, insect larvae, and small fishes) of the same age living in the same ecosystem (Table 2). Several factors might explain some of the difference. First, fish acquire PCBs through food and across skin and gills over their entire life, whereas leopard frogs and green frogs do not spend their whole lives in the water. After metamorphosis, they were not always exposed to the water and might or might not consume food that has PCBs from Green Bay and the Fox River. Second, if the fish consume other fish, which the tadpoles and adults of leopard frogs and green frogs most likely do not, they are effectively situated at a higher trophic level and thus there is more opportunity for biomagnification of PCB.

Our preliminary analysis of the likely PCB toxicokinetics in leopard frogs suggests that they do not eat the same contaminated foods as the fish species. Insects that emerged from Pete's Marsh had total PCBs ranging from 0.14 to 0.27 μg/g, wet weight based [28]. Insects removed from the stomachs of the tree swallow nestlings living in Green Bay and the Fox River in 1994 to 1995 had 0.22 to 0.70 μg/g of total PCBs [29]. If leopard frogs accumulate PCBs through consumption of invertebrates emerging from the Green Bay/Fox River ecosystem, they would have accumulated much (13 times) higher PCB concentrations in their bodies based on the slow elimination rate ($t_{1/2}$ = 763 d) in our toxicokinetics study [22]. Therefore, we suspect that they might feed on uncontaminated invertebrates in the woods and fields adjacent to the bay or in the nearby agricultural land instead of those emerging from Green Bay or Fox River. Low PCB concentrations in their carcasses might be residues from their early developmental stages when they fed exclusively within the aquatic food chain.

Hypothesis II

This is the first comprehensive field study to measure tissue residues of PCBs, PCDDs, and PCDFs as well as evaluate the catalytic activity of hepatic cytochrome P450-associated monooxygenase as a biomarker of exposure to AhR agonists in adult leopard frogs. We rejected the prediction that hepatic EROD activities of northern leopard frogs would be positively correlated with PCB concentrations in frog carcasses (whole body minus liver) from different collection sites along a con-

Table 2. The PCB concentrations in sediment, metamorphs, adult frogs, and fish in the Green Bay/Fox River ecosystem; sediment data expressed as μg/g dry weight; animal data expressed as μg/g wet weight; numbers in parentheses are lipid contents (%, wet weight)

	Location	
	Green Bay	Fox River
Sediment[a]	0.025–0.040	0.32–31.0
Frog[b]		
Leopard frog (adult)	0.003–0.016 (1.8%)	0.022–0.152 (2.3%)
Green frog (metamorph)	0.023–0.027 (2.3%)	0.156–0.283 (1.7%)
Fish[c]		
Yellow perch	0.16 (0.7%)	—
White perch	2.4 (6.3%)	2.3 (6.6%)

[a] Sediment analysis as indicated in footnote a of Table 1.
[b] Leopard frogs in Green Bay (three sites) and Fox River (three sites) from our collection as described in the Materials and Methods; green frog metamorphs from pooled samples of ≥10 individuals from each site from a 1994 enclosure study in Green Bay (three sites) or Fox River (two sites) [24].
[c] Fish data, collected during 1992 to 1994, are averages of 5 to 54 individuals per location and species (Amrhein, unpublished data). — = not analyzed.

tamination gradient. Admittedly, our use of chemical analyses conducted on pooled samples (necessary due to cost constraints) restricts the ability to apply and interpret correlations with biological (i.e., EROD) analyses conducted on individuals. Nonetheless, there was no hint of an association between level of exposure and EROD activity. The simplest explanation is that frogs in this ecosystem are exposed to levels of inducers of P450 enzymes that are too low to cause significant induction of EROD activity in leopard frogs. Routine PCB concentrations in carcasses (without liver) ranged from 2.8 to 151.9 ng/g. Only very low concentrations of PCB 105 and 156 (1.0 and 1.2 ng/g), two relatively potent inducers of PCBs in other vertebrates [30], were found in the frogs collected from Deposit C. From our previous study [17], EROD activity in leopard frogs was not significantly elevated above baseline at concentrations <0.7 μg/g dosage of PCB 126 (Fig. 4, inset), the most potent inducer of all of the PCB congeners in vertebrates [30]. Other chemicals in the Fox River/Green Bay ecosystem are also possible inducers, but exposure to these chemicals also seems low. Though we found congeners 2,3,7,8-TCDF, 1,2,3,4,6,7,8-HpCDF, and 1,2,3,4,6,7,8-HpCDD in the frogs collected from Deposit C, they are unlikely to induce EROD activity in wild leopard frogs considering their low tissue concentrations (all <35 pg/g) and their likely induction potency relative to PCB 126 [30,31]. Furthermore, 15 pesticides that are possible P450 inducers were not detectable in leopard frogs in our 1993 pilot survey (see Introduction).

We note that the EROD activities in the leopard frogs captured even at relatively clean sites were higher and more variable than EROD activities of control frogs in the dose–response experiment (Fig. 4, inset). Several factors might explain the differences. First, it has been reported that the use of protease can partially enhance activity recovery in the preparation of microsomes [32]. In the field study, DTT (an antioxidant) and PMSF (a protein inhibitor) were used in the preparation of microsomes to prevent oxidation and degradation of enzymes. These two reagents were not used during the preparation of microsomes in the dose–response study. Second, the frogs in this study were dissected after they were transported back to the laboratory while the frogs used in the dose–response study were acclimated in the laboratory for 4 months. A pattern we see in all of our studies on leopard frog monooxygenase biochemistry is a general decline in activity and reduction in variation with increasing time held in the laboratory (Huang, unpublished data). Some of these changes may relate to other endogenous and/or exogenous factors (e.g., ambient temperature, diet, reproductive state, physiological stress, age) that are known to influence monooxygenase activity in wild vertebrates [12] but are removed in the laboratory setting. While these factors may explain the differences in EROD activity between field-caught frogs and frogs in the dose–response study, we do not see how they can change the overall conclusion that EROD activity in wild leopard frogs in the Green Bay ecosystem was not correlated with PCB exposure.

Cytochrome P450-associated monooxygenase as a biomarker for exposure to AhR agonists

What, then, are the prospects for using specific activity of cytochrome P450-associated monooxygenase as a biomarker for exposure to AhR agonists in wild frogs? The methodology might work in ecosystems in certain situations. First, the method might work where exposure to enzyme inducers is much higher than in the Green Bay ecosystem. The method has worked for several wild bird species in the field [33], but tissue toxicant residues in those studies were much higher than measured in leopard frogs. Indeed, EROD activity was induced in captive leopard frogs with tissue residues as high as reported in those studies on wild birds (≫0.2 μg/g). However, wild frogs do not occupy the trophic level of the fish-eating birds and simply do not achieve such high body burdens of AhR agonists.

Second, EROD induction might be an effective biomarker for exposure to AhR agonists in amphibian species that are more sensitive than leopard frogs. However, the data available to date indicate that amphibians are relatively insensitive to AhR agonists. For example, EROD activity in green frog tadpoles from a mesocosm study did not differ along a range of total tissue PCBs between 23 and 283 ng/g wet weight [24]. In contrast, significant increases of hepatic EROD activity in rainbow trout and carp treated with PCB mixes (Aroclor 1254) were observed at the lowest dosages of 100 and 200 ng/g, respectively [34].

The species difference in sensitivity to AhR agonists has been known for years [35]. The possible mechanisms underlying the insensitivity or low inducibility of P450 enzymes in fish species by AhR agonists are proposed to be related to

fundamental differences in AhR signaling or AhR-CYP1A coupling or competition among chemicals for receptors [36,37]. Furthermore, induction of P450 enzyme has been shown to correlate with AhR-mediated toxicity [38,39]. Therefore, we would expect a lower sensitivity to AhR-mediated toxicity in *Rana* spp. compared to sensitive species such as rainbow trout and rats. The few data available to date are consistent with this. For example, anuran embryos exposed to 2,3,7,8-TCDD are much less sensitive than trout [40], and adult ranids tolerate very high 2,3,7,8-TCDD [41] and PCB 126 [17].

In the future, *Rana* spp. and other fish species that show low inducibility or insensitivity to AhR agonists might be used for elucidating the differential molecular mechanisms underlying differences in sensitivity across animal taxa. Such a comparative approach could increase understanding of the responses of animals to chronic exposure to AhR agonists as well as reveal the evolution of elements in the P450 induction pathway.

Acknowledgement—We thank Bruce Darken, John Cary, and David Alexander for their technical assistance. This study was funded by the University of Wisconsin Sea Grant Institute under grants from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, and from the State of Wisconsin grant NA46RG0481 and project R/MW-54.

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