

Cytochrome P450 Activity in Green Frogs (*Rana clamitans melanota*) Exposed to Water and Sediments in the Fox River and Green Bay, Wisconsin, USA

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The Fox River and Green Bay in Wisconsin are polluted with planar halogenated hydrocarbons (PHHs) such as coplanar polychlorinated biphenyls (PCBs) and 2,3,7,8-substituted dibenzo-*p*-dioxins (Sullivan and Delfino 1982). Past recycling of carbonless copy paper at paper and pulp mills contaminated the Fox River, and PCBs persisted in water and sediments of the River and Bay because of sediment contamination, slow biodegradation, continued atmospheric deposition, and food web bioaccumulation (Ankley et al. 1993). PHHs have been linked to decreased survival and reproduction of fish and fish-eating birds in this area (Mac et al. 1993; Custer et al. 1999). PHHs exert effects through initial binding to a cytosolic aryl hydrocarbon (Ah) receptor (Hahn et al. 1994). Upon binding to the Ah receptor, the activated complex moves to the nucleus and binds to specific sequences of DNA, inducing enzymes such as cytochrome P450s (cyt P450; Safe 1990). Multiple toxic effects can result, including weight loss, edema, decreased immune function, teratogenesis, and reproductive toxicity (Safe 1990).

Cyt P450 1A (CYP1A) enzymes are induced by exposure to PHHs and have been used as biomarkers for PHH exposure as a cost-effective measure compared to analyzing tissues for PHHs (Whyte et al. 2000). CYP1A enzymes, found primarily in the liver, transform PHHs into more excretable and less toxic forms, though in some cases toxicity is enhanced. CYP1A enzyme activity is typically measured using the 7-ethoxyresorufin-*O*-deethylase (EROD) assay, in which resorufin is produced from the deethylation of 7-ethoxyresorufin (Melancon 1996). EROD is well-established for fish (Whyte et al. 2000), but less is known of its value for determining PHH exposure in amphibians. Higher EROD induction in amphibians after exposure to PHHs compared to other CYP1A substrates such as methoxy-, benzyloxy-, and pentyloxy-ROD has been reported (Marty et al. 1992; Huang et al. 1998). Adult amphibians were shown to exhibit EROD induction after laboratory exposure to 3-methylcholanthrene (newt, *Pleurodeles waltl*; Marty et al. 1992) and PCB 126 (northern leopard frogs, *Rana pipiens*; Huang et al. 1998).

Only recently were EROD levels studied in amphibians collected from the wild or exposed to field-collected water or sediments (Harris et al. 1998; Huang et al.

1999; Gauthier et al. 2004). In the Fox River and Green Bay ecosystem, adult leopard frogs were studied for PHH exposure and EROD induction (Huang et al. 1999). Our study examined EROD levels in pre-adult stages of a different species, the green frog (*Rana clamitans melanota*). Differences in EROD induction within a taxonomic family of species and among life stages is well-documented in fish (Whyte et al. 2000). Huang et al. (1999) collected adult leopard frogs from some of the same sites used in our study (and the PCB levels in the frogs were related to the sediment PCB levels at those sites), but the individuals collected for that study may not have originated or been restricted to the site at which they were captured, as wild adult leopard frogs can disperse up to 8 km (Seburn et al. 1997). In our study, tadpoles and metamorphs were exposed to PHHs under controlled conditions, either to sediment and water within field enclosures or to field-collected water in the laboratory. We tested the hypothesis that EROD activity is higher (induced) in green frog tadpoles and metamorphs exposed to: 1) Fox River water compared to dilutions with tap water, and 2) water and sediments in field enclosures at sites with high and low sediment PCB concentrations.

MATERIALS AND METHODS

On 11 June 1994, we collected two green frog egg masses from the University of Wisconsin-Green Bay (UWGB) Prairie Pond (Figure 1), considered a reference site with no known toxicant inputs. Six hundred eggs from one egg mass were treated in the laboratory at UWGB and the remaining egg masses were treated in the field as described below. From 11 June to 20 October, we exposed green frogs from egg to 131 days of age to four treatments: Fox River water, a 2:1 dilution of Fox River water with Green Bay tap water, a 1:2 dilution, and tap water. Each treatment had five replicates for a total of 20 aquaria (41 x 20 x 25 cm), and 30 eggs were placed in each replicate. Using a static renewal system, we provided fresh aerated water and food (amount of boiled Romaine lettuce and a 3:1 Rabbit Chow:TetraMin mixture based on 15% of tadpole mass in tanks) every two days. Photoperiod was set at 14 hr:10 hr light:dark. We used Green Bay tap water, which originates as Lake Michigan surface water collected 21 km north of Kewaunee, Wisconsin. For the Fox River treatments, we collected unfiltered surface water from the mouth of the Fox River at the end of Pier One. PCB levels in Lake Michigan source water for Green Bay tap water are much lower (0.2 ± 0.02 ng/L at site 110) than PCB levels at the mouth of the Fox River (85 ± 9.01 ng/L at site 4085138) (data from June-August 1994, $n = 9$, US EPA 2002).

From 11 June to 26 September 1994, we exposed green frogs from egg to 108 days of age to water and sediments in field enclosures at five sites along the Fox River and Green Bay (Figure 1; see Jung 1996 for details). We placed 70 eggs from each egg mass into separate field enclosures at each site. The number of enclosures varied from 2-8, with one to four clutch replicates per site. Sediment PCB levels (mg/kg dry wt) at the enclosures were < 0.025 at South and North Sensiba, 0.07 at UWGB Bay Pond, 0.12 at Railroad Museum, and 3.20 at Deposit X (Jung 1996).

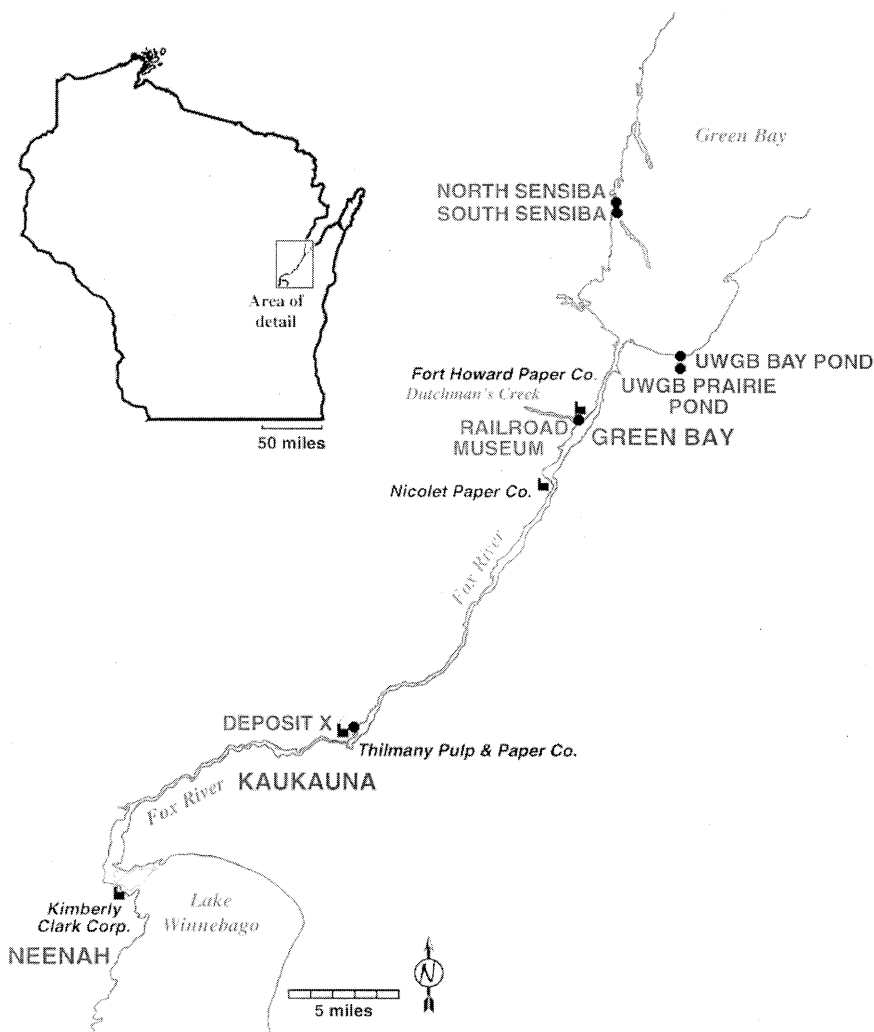


Figure 1. Location of six field sites (darkened circles, names in capital letters) and four potential sources of industrial contamination (rectangular symbols, italicized names in bold) along the Fox River and Green Bay, Wisconsin.

A subset of laboratory tadpoles from 20 October and those that metamorphosed (tail length ≤ 2 mm) prior to that date and field tadpoles collected from enclosures on 27 September were weighed, measured (snout-vent or total length), and pithed. Livers were excised, weighed, immediately placed in cryotubes with glycerol, and frozen at -80°C until assay. Remaining tadpoles not used for EROD assays were measured, anesthetized in MS-222 (3-amino-benzoic acid ethyl ester: 0.05% solution), and frozen for PCB analyses. Differential mortality during the course of the study precluded large sample sizes in some treatments.

Laboratory tadpole samples were analyzed for total Aroclor (commercial mixtures of PCBs; limit of detection was 40 ng/g wet wt) using packed column gas chromatography with electron capture detection (GC-ECD) at the Wisconsin State Laboratory of Hygiene (WSLH 1994). Briefly, anhydrous Na_2SO_4 was added to 10 g of wet tissue and extracted with dichloromethane. The extract was run through automated gel permeation chromatography to separate PCBs from lipid, and PCBs were further fractionated using Florisil and silica gel chromatography prior to GC-ECD. Fish tissue spiked with Aroclor 1260 (99.2% recovery) was analyzed with the tadpole samples for quality control. Field tadpole samples were analyzed for routine (83 congeners) and coplanar non- (77, 81, 126, 169) and mono-*ortho* (105, 114, 118, 123, 156, 157, 167, 189) PCB congeners at the Geochemical and Environmental Research Group at Texas A&M University following high resolution gas chromatography-electron capture detection methods described in Sericano et al. (1994). Congener detection limits ranged from 0.01-4.0 ng/g, and recoveries in matrix spikes averaged $78 \pm 3.5\%$. The final range (maximum minus minimum values) of measured PCB values (258 ng/g wet wt) was nearly two orders of magnitude larger than the standard deviation of a replicated sample (SD of North Sensiba sample = 3.6).

We performed the EROD assay using the method of Burke and Mayer (1983) as modified by Melancon (1996) for 96-well plates using a computer-coupled fluorescence microwell plate reader (Fluoroskan II, Titertek). The optimal reaction conditions were determined using various combinations of substrate and NADPH concentrations. Assays were run at ambient temperature (26-28°C) at pH 7.4 with 5 μM substrate, microsomes derived from 16.7 mg of liver, and 0.25 mM nicotinamide adenine dinucleotide phosphate (NADPH). The optimal reaction solution, for each 260 μl reaction, consisted of 50 μl 66 mM Tris buffer solution, 150 μl substrate, 50 μl microsome suspension and 10 μl reduced NADPH. Tadpole livers were large enough to analyze individually but metamorph livers were small and 2-5 individuals per tank had to be pooled. EROD activities were calculated from the change in fluorescence over time as compared to fluorescence of known amounts of product added to a series of wells with all components present except NADPH. Activities were expressed as picomoles of resorufin produced per minute per mg of microsomal protein. Microsomal protein concentrations were measured according to Lowry et al. (1951) using crystalline bovine plasma albumin as standard. Spearman rank correlations and ANOVAs were performed using SPSS version 7.5, with significance judged at $\alpha = 0.05$.

RESULTS AND DISCUSSION

In the laboratory, PCB concentrations in green frog tadpoles increased with percent Fox River water exposure ($r_s = 0.99$, $p = 0.004$), but tadpoles ($F_{3,29} = 0.40$, $p = 0.75$) and metamorphs ($F_{3,15} = 0.40$, $p = 0.76$) from the treatments did not differ in EROD activity (Table 1). Green frog tadpoles from the field enclosure sites also differed in PCB concentrations, but not in EROD activity ($F_{4,59} = 0.68$, $p = 0.61$;

Table 1). Tadpole PCB body burden concentrations were correlated with field site sediment PCB concentrations ($r_s=0.98$, $n=5$, $p=0.005$). Tadpole EROD levels did not differ between the laboratory and field studies ($F_{1,95}=0.80$, $p=0.37$). Liver and body masses were not significant covariates for EROD activity.

Table 1. Routine and coplanar PCB concentrations and EROD activities in green frog tadpoles and metamorphs from laboratory and field treatments.

Treatment	Tadpole PCB		Tadpole EROD ^b	n	Metamorph	
	Routine	Coplanar ^a			EROD	n
Laboratory						
Fox	110*	- ^c	0.2 ± 0.06	9	4.7 ± 0.91	7
2:1	87*	-	0.3 ± 0.06	9	6.2 ± 1.65	5
1:2	54*	-	0.4 ± 0.15	8	3.9 ± 1.01	3
Tap	<40*	-	0.3 ± 0.06	7	4.9 ± 2.11	4
Field						
Deposit X	279	33	0.2 ± 0.01	9	-	
Railroad Museum	144	27	0.3	1	-	
UWGB Bay Pond	24	6	0.5 ± 0.16	17	-	
North Sensiba	21 ± 1.6	5	0.5 ± 0.14	20	-	
South Sensiba	21	5	0.3 ± 0.08	17	-	

*Total Aroclor (ng/g wet wt)

^a Non- and mono-*ortho* PCBs (ng/g wet wt)

^b 7-Ethoxyresorufin-*O*-deethylase activity (pmol resorufin/min/mg microsomal protein) expressed as mean \pm standard error (n indicates sample size)

^c Data not available

Tadpoles (33 Fox, 43 2:1, 34 1:2, 35 Tap, 10 Deposit X, 5 Railroad Museum, 16 UWGB-Bay Pond, 20 North Sensiba and South Sensiba) were pooled into a single sample per site for Aroclor and PCB analyses; a duplicate was conducted for the North Sensiba sample

Combining laboratory and field treatments, we found no significant relationship between tadpole PCB and EROD levels ($r_s=0.08$, $p=0.83$). Given the relatively low total PCB concentrations in the Fox River (110 ng/g wet wt) and Deposit X (312 ng/g) tadpoles, it is reasonable that EROD was not induced. Huang et al. (1998) found that only environmentally unrealistic levels of toxic PCB exposure (≥ 2.3 mg/kg PCB 126) induced EROD activity in adult leopard frogs. EROD levels in adult leopard frogs from Fox River and Green Bay sites did not differ despite total PCB whole body concentrations ranging from 3 to 154 ng/g wet wt (Huang et al. 1999). Adult leopard frogs can disperse up to 8 km in the field (Seburn et al. 1997), making correlations between exposure and toxicant endpoints more tenuous. In our study, green frog tadpoles and metamorphs were raised under controlled conditions contained within laboratory tanks and field enclosures. The lack of elevated EROD levels in amphibians at higher PHH sites

contrasted with fish downstream from bleached kraft paper mills that showed elevated EROD levels (Soimasuo et al. 1995; van den Heuvel et al. 1995). Positive control experiments testing EROD levels in green frog tadpoles and metamorphs exposed to increasing levels of a known CYP1A1 inducer in the laboratory need to be conducted. However, we suggest that green frog tadpoles and metamorphs exposed to higher PHH levels under the study conditions did not show induced EROD activity and that EROD may not be useful as a biomarker for their exposure to field-relevant PHH concentrations in the Green Bay area.

We found higher average EROD levels in metamorphs (5 pmol/min/mg protein) than tadpoles (0.3 pmol/min/mg protein) in the laboratory ($F_{1,50}=82.1$, $p<0.001$; Table 1). South African clawed frogs (*Xenopus laevis*) also had low cyt P450 induction as tadpoles, a two-fold increase as two-week old metamorphs, and a 4-fold increase as adults (Doherty and Khan 1981). Our EROD levels were lower than those reported for adult green frogs (10-36 pmol/min/mg protein, Harris et al. 1998) and leopard frogs (186-271 pmol/min/mg protein, Huang et al. 1999), indicating that cyt P450 enzymes in amphibians are not fully developed until adulthood, which may affect sensitivity to PHHs in early development.

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