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ECOTOXICOLOGY OF AMPHIBIANS IN GREEN BAY AND THE FOX RIVER

by

MICHÈLE LAURA ROSENSHIELD

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE
(ZOOLOGY)

at the
UNIVERSITY OF WISCONSIN-MADISON

1999

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CHAPTER I

EFFECTS OF PCB 126 ON GREEN FROG (*Rana clamitans*) AND LEOPARD FROG (*Rana pipiens*) HATCHING SUCCESS, DEVELOPMENT, AND METAMORPHOSIS

Abstract. Although increasing evidence links planar chlorinated hydrocarbons, such as polychlorinated biphenyls (PCBs), to decreases in survival and reproduction of fish, mammals, and birds near Green Bay, Wisconsin and the Great Lakes, relatively little is known of their bioaccumulation or possible effects in amphibians. We exposed embryos and larvae of two ranid species commonly occurring in the Green Bay ecosystem, the green frog (*Rana clamitans*) and the leopard frog (*Rana pipiens*), to PCB 126, a model coplanar PCB compound. Nominal concentrations ranged from 0.005 $\mu\text{g/L}$ to 50 $\mu\text{g/L}$ and exposure lasted through metamorphosis. Tissue concentrations of PCB 126 in tadpoles that did not metamorphose by the end of the experiment ranged from 1.2 to 9600 ng/g wet mass. No significant mortality of embryos occurred before hatching, however survival of larvae was significantly reduced at the highest concentration for both species. Few deformities were observed, but the incidence of edema was significantly higher in tadpoles exposed to 50 $\mu\text{g/L}$. Swimming speed and growth of tadpoles was also significantly reduced in this treatment. The percent of tadpoles that reached metamorphosis was significantly lower in green frogs at the highest concentration and no leopard frogs survived past day 47 of the experiment in this treatment. At high concentrations, PCB 126 affected both ranid species, however sublethal effects were not apparent for the parameters we measured at concentrations that occur in water in the Green Bay ecosystem.

INTRODUCTION

The Green Bay watershed in Wisconsin is polluted with polychlorinated biphenyls (PCBs), dioxins, heavy metals, and over 100 organic contaminants [1, 2, 3]. PCB contamination of water and sediments in this ecosystem has been linked to industrial processes by paper and pulp mills that line the Fox River, the main tributary to Green Bay. Industries released PCBs into the Fox River and Green Bay from 1957 until 1972, after which time production of PCBs was officially banned in the United States. Although they are no longer being released into the watershed, PCBs persist in the Green Bay ecosystem due to slow biodegradation, sediment contamination, continued atmospheric deposition, and bioaccumulation up the food chain.

Most commercial PCB products are mixtures of different chlorinated biphenyl molecules, or congeners. Each PCB congener differs from the others by the substitution configuration of the chlorine atoms on the two phenyl rings. Coplanar PCBs and mono-ortho derivatives are particularly toxic congeners. These chemicals have been shown to bind a cytosolic *Ah* (aryl hydrocarbon) receptor [4], forming an activated complex that can move into the nucleus of the cell. Once inside the nucleus, nuclear receptor complexes are formed that can induce cytochrome P4501A1 activity (detoxification enzyme activity) or can bind to specific sequences of DNA known as dioxin-response elements, which mediate the cell's toxic response. Toxic responses in animals may include body weight loss, thymic atrophy, edema, teratogenesis, carcinogenesis, decreased immune function, hepatotoxicity and porphyria, and reproductive toxicity [5]. PCB 126 (3,3',4,4',5-pentachlorobiphenyl), the congener we used in this study, was chosen because it has a high affinity for the *Ah* receptor,

and is therefore considered a good model for the class of coplanar PCBs known to exhibit the greatest toxicity [6]. PCB 126 induces P4501A1 activity in both leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) [7] (Y. W. Huang, personal communication), the two species used in this study.

PCBs are extremely lipophilic and tend to partition out of the water column, where they are released by mobilization of sediment, and into biologic tissue. PCB accumulation in tissue has been linked to decreases in survival and reproduction of fish, mammals, and birds in Green Bay and the Great Lakes [8, 9, 10, 11, 12]. However, relatively little is known of their bioaccumulation in or possible effects on amphibians [13,14].

A recent study [15] (R. E. Jung and W. H. Karasov, unpublished data) determined the percent hatching success of green frog and leopard frog embryos in enclosures located at sites situated along a PCB-gradient in the Fox River and Green Bay. This field study found a significant negative correlation of percent hatching success with sediment PCB concentrations. We were interested in conducting a laboratory study where confounding environmental factors present in the field would be minimized and a cause-effect relationship between PCBs and anuran hatching success, growth, and metamorphosis could be measured.

Our study of acute and chronic exposures of anurans to PCB 126 addresses several questions. 1) To what extent will waterborne PCB 126 bioconcentrate in anuran larvae? 2) How will toxic effects in anurans compare with those in other animal groups that have been studied? PCBs have been correlated with depressed hatching success of bird and fish embryos [11, 12, 16], depressed survival and growth of fish fry and young mammals [17,

18], and increased edema in bird hatchlings and fish fry [19, 20], as well as other toxic effects. 3) Will PCB 126 negatively affect anurans at ecologically relevant concentrations? 4) Will PCB exposure provide us with a biological marker of toxicity that can be used with anurans in the field?

We studied the green frog (*Rana clamitans*) and the northern leopard frog (*Rana pipiens*) because they are common residents of the Green Bay ecosystem and their populations may be impacted by pollutants in the field. We exposed animals in a static-renewal experiment to increasing concentrations of PCB 126, as well as negative and vehicle controls, from an early egg stage until metamorphosis. We chose to study eggs and larvae because amphibian early life stages are thought to be more sensitive to waterborne pollutants than adults [13, 21]. We assessed PCB 126's effects on embryo hatchability and tadpole survivorship, deformities, edema, growth, swimming performance, and metamorphosis. Swimming performance may act as an indicator of underlying physiological stress and has been shown to decrease with exposure to certain toxicants [22]. It is also an ecologically relevant parameter because tadpoles that swim slowly may be more susceptible to predation [22, 23]. We also measured bioconcentration of PCB 126 in tissues of tadpoles that did not metamorphose by the end of the experiment. Overall, this study is the first to look at sensitivity of North American anuran amphibians to a model coplanar PCB compound and provides range-finding data for planning future PCB dose-response experiments.

METHODS

Study organisms

Two green frog egg clutches (approximately 1000 eggs/clutch) were identified [24] and collected by netting in a pond near Deerfield, Dane County, WI. Egg clutches were held in plastic containers and transported to the Water Science and Engineering Laboratory at the University of Wisconsin--Madison. Two leopard frog egg clutches (approximately 300 eggs/ clutch) were purchased from NASCO (Fort Atkinson, WI). Egg clutches were fertilized and then transported in plastic containers to the laboratory in Madison. Embryos and tadpoles were staged during the experiment following the table proposed by Gosner [25].

Exposure of eggs

Green frog eggs were in the neurula stage (stages 15 to 16) in clutch 1 and in the mid-gastrula stage (stage 11) in clutch 2 when placed into treatment solutions for the hatchability study. Both leopard frog egg clutches were in the 4 to 16 cell stages (stages 4 to 6) at the beginning of exposure. Clutches of eggs (eggs released by one female and fertilized by one male) were kept separate throughout the experiment, however there were no replicate treatments within clutches. Eggs were exposed to four or five levels of PCB 126 (Ultra Scientific and Accu Standard, Inc.): 0.005 (green frog only), 0.05, 0.5, 5, and 50 $\mu\text{g/L}$, and two control treatments. The first control (C+) contained water plus 0.08% acetone (99.9+% pure, HPLC grade, Sigma Chemical Co.) as carrier for the PCB and the second control (C-) contained only water. Two hundred and ten randomly drawn eggs from each

clutch were subdivided into groups of thirty eggs and each of these groups was exposed to 70mL of one treatment solution in 100x20mm glass petri dishes. Petri dishes were placed into a 23°C incubator on a 14:10 light:dark cycle. Treatment solutions were changed every 24 hours (static renewal system) and were prepared with dechlorinated, charcoal filtered water (pH 8.2, hardness 324 mg/L as CaCO₃, dissolved oxygen 11.5 µg/L).

Green frog embryos were exposed to treatments for 5 days. Leopard frog embryos were exposed to treatments for 6 days. On the day embryos hatched, hatching success, deformities (categorized as “bent or asymmetric tails” or “grossly deformed”), edema (distension of the body with fluid) and abnormal swimming performance were recorded.

Exposure of tadpoles

After all embryos had hatched, 20 surviving tadpoles (green frog) or 11 to 28 surviving tadpoles (leopard frog) from each petri dish were transferred to tanks containing 8 liters (green frog) or 6 liters (leopard frog) of the same treatment solutions described in the egg experiment. A higher number of tadpoles was used in the leopard frog experiment (up to 28) due to lower survival of animals purchased from NASCO (leopard frogs) as compared to animals collected in the field (green frogs). Clutches were kept separate throughout the experiment, however there were no replicate treatments within clutches. Tanks (two for each species/treatment) were placed in a thermoregulated water bath kept at 23 to 24°C and a 14:10 light:dark cycle. Water treatments in tanks were changed and tadpoles were fed every three days. Tadpole food consisted of boiled romaine lettuce blended into a puree and combined with a 3:1 Rabbit Chow:Tetra Min mixture (LM Animal Farms, Pleasant Plain,

OH; TetraMin Flake Food, TetraSales, Blacksburg, VA) . When the front legs of a tadpole emerged, the animal was measured and transferred to a tilted plastic tub containing 1 liter of treatment solution. The tilted tubs provided tadpoles with both dry and wet surfaces until they completed metamorphosis. Once placed in the tubs, tadpoles were not fed (metamorphosing tadpoles live off fat stored in the tail) and treatment solutions were changed every three days.

Green frog tadpoles were exposed to treatments for 125 days. Leopard frog tadpoles were exposed to treatments for 104 days. Tanks were checked every day for mortality and all dead tadpoles were removed and preserved in 10% formalin. Any deformities or abnormal swimming behaviors were recorded daily (green frog) or every three days (leopard frog). Snout-vent length (SVL), which is the total tadpole length minus tail length, (from day 23 after hatch) and total length (from day 50 after hatch) of ten green frog tadpoles chosen randomly from each tank, were measured every nine days. Total length of six leopard frog tadpoles chosen randomly from each tank (from day 13 after hatch) was measured every six days and then every nine days (from day 50 after hatch). We began length measurements of leopard frogs 10 days earlier than green frogs because leopard frogs metamorphose much faster than green frogs [26]. At day 50 after hatch, five green frog tadpoles chosen randomly from each tank were tested for swimming performance by chasing them in a 25cm plastic swimming channel with a paintbrush as described by Jung and Jagoe [23].

At metamorphosis (tail length ≤ 2 mm), frogs were weighed, measured for snout-vent length, and euthanized. Frogs were then dissected to determine masses (± 0.001 g) of liver,

kidneys plus gonads, and fat bodies. Time to metamorphosis for each frog was recorded. Tadpoles that failed to metamorphose by the end of the experiment were weighed, measured for total length, staged, and euthanized by immersion in a MS222 solution (3-aminobenzoic acid ethyl ester: 0.05% solution, Sigma Chemical Co.). After euthanization, tadpoles were frozen for contaminant analysis (leopard frogs: 14 tadpoles from 0+, 15 from .05 $\mu\text{g/L}$, 8 from 0.5 $\mu\text{g/L}$, 10 from 5 $\mu\text{g/L}$; green frogs: 20 tadpoles from 0.5 $\mu\text{g/L}$, 23 from 5 $\mu\text{g/L}$, and 6 from 50 $\mu\text{g/L}$. One pool of tadpoles per treatment was used for both species).

Tadpoles were analyzed for PCB contaminant levels at the Wisconsin State Lab of Hygiene (WSLH), University of Wisconsin-Madison. Levels of six PCB congeners (#77, #123, #105, #156, #157, and #169) as well as PCB 126 were analyzed. Organic analyses followed procedures described in "Methods for Organic Analysis" [27].

Statistical analysis

Percent hatching, survival, edema, deformities, and metamorphosis were transformed to logit values (using one value per tank) because proportions tend not to be normally distributed. These logit values were used to create nine models conditional on three explanatory variables: species, clutch nested within species, and log [PCB concentration]. We used Mallows' C_p [28] as the basis for selecting the most appropriate model to explain variation in the data. The F and p values for log [PCB concentration] are given even when log [PCB concentration] is not included in the best model. The values of total length for tadpoles were compared between treatments using two-way ANOVA with treatment and clutch as factors keeping species separate. When ANOVA results were significant, Tukey's

honestly significant difference test (HSD) for multiple comparisons was used. Swimming speed in green frog tadpoles was compared using ANCOVA with clutch as factor and log [PCB concentration] and total length as covariates. Body mass, SVL, logit-transformed percent edema, and time to metamorphosis in metamorphosed frogs were also compared by creating nine models conditional on species, clutch nested within species, and log [PCB concentration]. The procedures recommended by Mallows [28] were followed for selecting the most appropriate model. Models use positive (C+), but not negative (C-) controls in the analyses. Organ masses for metamorphosed frogs were compared using ANCOVA with species as factor and log [PCB concentration] and SVL as covariates. Linear regression was used to relate concentration of PCB 126 in tissues of tadpoles that failed to metamorphose by the end of the experiment to nominal concentration of treatment water. P -values < 0.05 for main effects and < 0.1 for interaction terms were considered statistically significant. P -values for main effects < 0.10 and > 0.05 were considered to reflect trends.

RESULTS

Egg exposure

Treatment was not included in the best model to explain hatching success data (Fig. 1A). Hatchability was significantly different between species ($F_{1,18} = 269$, $p < 0.001$), with green frogs eggs hatching at higher percentages than leopard frog eggs. Green frog eggs were collected in the field, whereas leopard frog eggs were bought from NASCO, a biological supply company. The percent fertilization of leopard frog eggs from NASCO is approximately 30% lower than that of wild-caught leopard frog eggs (personal observations,

K. Loeffler and M. Rosenshield). Hatching success of clutches within each species was not significantly different ($F_{2,18} = 2.82, p = 0.086$). If treatment is added to our model with species and clutch, treatment is not significant ($F_{1,17} = 1.66, p = 0.22$). Hence, PCB exposure concentration had no significant effect on hatchability.

Tadpole survival

Survival of tadpoles evaluated at the end of the experiment was significantly lower in both species at the highest PCB concentration ($F_{1,19} = 20.6, p < 0.001$) (Fig. 1B). Survival also differed between species ($F_{1,19} = 5.81, p = 0.026$). For the green frog, 6 tadpoles out of 30 survived after 125 days of exposure to the highest PCB 126 concentration of 50 $\mu\text{g/L}$ (all in Gosner stages 30 to 39). Ten green frog tadpoles were lost during the experiment from the highest concentration treatment due to flooding of one tank (therefore, the $n = 30$ for both clutches). For the leopard frog, no tadpoles survived after 47 days of exposure to 50 $\mu\text{g/L}$ PCB 126.

Edema and deformities

Incidence of edema increased significantly in both species at high PCB concentrations ($F_{1,20} = 11.373, p = 0.003$) (Fig. 1C): 100 % of leopard frog tadpoles and 77 % of green frog tadpoles exposed to 50 $\mu\text{g/L}$ PCB 126 exhibited edema at some point during the experiment.

The incidence of deformities (bent, kinked, or asymmetric tails or asymmetric bodies) in tadpoles of both species exposed to PCB 126 was relatively low, never exceeding

10% in any treatment. Treatment was not included in the best model to explain incidence of deformities. There was no significant difference in deformities between leopard frogs and green frogs ($F_{1,18} = 0.48, p = 0.50$), however clutches within each species had a significantly different incidence of deformities ($F_{2,18} = 3.725, p = 0.044$). When treatment was included in the model with species and clutch, it was not significant ($F_{1,17} = 0.19, p = 0.67$). For green frogs in clutch 1, one tadpole in the C- treatment had a right back leg with only three toes, an undeveloped left back leg reduced to a gelatinous mass, and an asymmetric tail. In clutch 2, one tadpole in the 0.5 $\mu\text{g/L}$ treatment had a bent tail and asymmetric body with the right side less developed than the left (front right leg was developed but never emerged). This tadpole died before completing metamorphosis. Two tadpoles from clutch 2 exposed to the 50 $\mu\text{g/L}$ treatment showed large protuberances on the head between the eyes at the end of the experiment.

Only six leopard frog tadpoles exhibited deformities during the experiment. Three tadpoles from clutch 2 in the 0.05 $\mu\text{g/L}$ treatment exhibited asymmetric bodies on day 40 after hatch. One tadpole from clutch 2 in the 5 $\mu\text{g/L}$ treatment exhibited an asymmetric body on the last day of the experiment (day 104 after hatch). Also in clutch 2, one tadpole from the C- treatment and one tadpole from the C+ treatment had asymmetric tails.

Swimming performance

There was a significant decrease in swimming speed (cm/sec) in green frogs on day 50 after hatch at the highest concentration of PCB 126 ($F_{1,167} = 9.69, p < 0.05$) (Fig. 1D). The difference between clutches in swimming speed was significant ($F_{1,167} = 8.57, p < 0.05$)

as was the interaction between clutch and total length ($F_{6,167} = 7.56, p < 0.05$). Tadpole total length was a significant covariate for swimming speed ($F_{1,167} = 5.57, p < 0.05$).

Total length

Green frog tadpoles exposed to 50 $\mu\text{g/L}$ were smaller than tadpoles exposed to all other treatments on all dates measured ($n = 12$ dates), but the difference was significant only on six dates (days 50, 56, 62, 99, 105, and 117 after hatch, all $p < 0.05$) (Fig. 2A). The effect of clutch and interaction of treatment and clutch were not significant on any of the dates tadpoles were measured (all $p < 0.064$). Leopard frog tadpoles exposed to 50 $\mu\text{g/L}$ were significantly smaller than tadpoles exposed to all other treatments on all dates that both clutches were alive in this treatment (days 13, 19, and 25 after hatch, all $p < 0.002$) (Fig. 2B). There were no significant differences in total length of tadpoles in the five remaining treatments throughout the rest of the experiment (days 31, 37, 43, 50, 59, 68, 77, 86, and 95 after hatch; all $p < 0.272$). The effect of clutch and interaction of treatment and clutch were not significant on any of the dates tadpoles were measured (all $p < 0.082$) except the first date when interaction of treatment and clutch was significant ($F_{5,60} = 3.54, p = 0.007$).

Percent metamorphosis

There was a trend for decreased percent metamorphosis in tadpoles at the highest concentration of PCB 126 ($F_{1,20} = 3.97, p = 0.052$) (Fig. 3A). When percent metamorphosis was analyzed without the 50 $\mu\text{g/L}$ group, there was a significant increase in percent metamorphosis with increased concentration of PCB 126 ($F_{1,13} = 7.77, p = 0.015$). A higher

percentage of leopard frogs metamorphosed than green frogs ($F_{1,13} = 36.7, p < 0.001$) and clutches within species had significantly different numbers of tadpoles that metamorphosed ($F_{2,13} = 18, p < 0.001$).

Four green frog tadpoles died during the period of tail resorption just before completing metamorphosis (stages 41 to 45; 2 exposed to 0.5 $\mu\text{g/L}$ and 2 exposed to 5 $\mu\text{g/L}$) and nine leopard frog tadpoles died during this period (1 exposed to C+, 3 exposed to 0.05 $\mu\text{g/L}$, 1 exposed to 0.5 $\mu\text{g/L}$, and 4 exposed to 5 $\mu\text{g/L}$).

We observed edema in leopard frog metamorphs but not green frog metamorphs ($F_{1,12} = 11.156, p = 0.006$) (Fig.3C). When species were analyzed together, the effect of treatment and the treatment \times species interaction were not significant (respectively, $F_{1,12} = 1.426, p = 0.255$; $F_{1,12} = 1.426, p = 0.255$). Within leopard frogs alone, the incidence of edema was significantly higher in the 5 $\mu\text{g/L}$ treatment as compared to all other treatments combined (chi-square = 8.74, $df = 1, p < 0.01$). Two metamorphs in the 5 $\mu\text{g/L}$ treatment exhibited such severe edema in the head, legs, and body that they were unable to leap.

Time to metamorphosis

The effect of treatment was not included in the best model to explain time to metamorphosis in either species. However, species differences were important because leopard frogs metamorphosed significantly sooner than green frogs ($F_{1,123} = 146, p < 0.001$) (Fig. 3B). When treatment was included in the model with species to explain variation in time to metamorphosis, it was not significant ($F_{1,122} = 0.296, p = 0.59$). Therefore, exposure to PCB 126 did not significantly affect the time at which tadpoles metamorphosed.

Body mass and snout vent length (SVL) at metamorphosis

Treatment was not included in the best model to explain differences in body mass between metamorphs, however there was a trend for green frogs to be larger than leopard frogs ($F_{1,123} = 3.71, p = 0.056$) (Fig. 3D) which is typical for these species. When treatment was added to the model with species to explain body mass data, it was not significant ($F_{1,122} = 0.04, p = 0.84$). SVL was not significantly different between treatments for both species ($F_{1,118} = 1.92, p = 0.169$), but green frogs ($19.906 \pm 0.198\text{mm}$) were significantly longer than leopard frogs (19.3 ± 0.158) ($F_{1,118} = 10.2, p = 0.002$). Clutches were significantly different within each species ($F_{2,118} = 5.28, p = 0.006$), and treatment and clutch interactions were significant as well ($F_{2,118} = 3.58, p = 0.031$).

Organ masses

Log [PCB concentration] was not a significant covariate for liver mass ($F_{1,138} = 0.017, p = 0.898$). However, there was a significant effect of species on liver mass ($F_{1,138} = 5.87, p = 0.017$) and a significant effect of SVL as a covariate ($F_{1,138} = 112, p < 0.001$). The adjusted least square mean liver mass was $0.025 \pm 0.001\text{g}$ in green frogs and $0.022 \pm 0.001\text{g}$ in leopard frogs. Log [PCB concentration] as a covariate for kidney-gonad mass was not significant ($F_{1,135} = 0.777, p = 0.380$). However, effect of species was significant ($F_{1,135} = 35.9, p < 0.001$) and SVL was a significant covariate ($F_{1,135} = 53.5, p < 0.001$). The adjusted least square mean kidney-gonad mass was $0.013 \pm < 0.001\text{g}$ in green frogs and $0.009 \pm < 0.001\text{g}$ in leopard frogs. The effect of log [PCB concentration] as a covariate for fat body mass was not significant ($F_{1,137} = 1.63, p < 0.204$). However, there was a significant effect of

species on fat body mass ($F_{1,137} = 25.81, p < 0.001$) and a significant effect of SVL as a covariate ($F_{1,137} = 59.0, p < 0.001$). The adjusted least square mean fat body mass was 0.006 ± 0.001 g in green frogs and $0.003 \pm < 0.001$ g in leopard frogs.

Tadpoles that failed to metamorphose

A logistic regression showed no effect of treatment ($F_{1,20} = 0.34, p = 0.57$) or species ($F_{1,20} = 0.08, p = 0.78$) on the percent of tadpoles reaching the last stage before metamorphic climax (stage 40) by the last day of the experiment. Of 74 green frog tadpoles and 56 leopard frog tadpoles, 12.41% and 12.83% reached stage 40, respectively.

The concentration of PCB 126 in tissues of tadpoles living to the end of the experiment increased in relation to nominal concentration of treatment water (Fig. 4). The log [concentration of PCB 126 in treatment water] was a significant factor in determining log [concentration of PCB 126 in tadpole tissues] ($F_{1,3} = 116, p = 0.002$). The species term in the model was also significant ($F_{1,3} = 11.5, p = 0.043$), indicating that there was a difference between the two species in PCB bioconcentration. BCFs (bioconcentration factor = PCB 126 concentration in wet tadpole tissue/ PCB 126 concentration in treatment water) ranged from 22 to 28 in leopard frogs and 150 to 500 in green frogs. A significant interaction term of log [concentration of PCB 126 in treatment water] and species ($F_{1,3} = 6.92, p = 0.078$) indicated that the slopes of the regression lines for leopard frogs and green frogs were different. Control tadpoles did not have detectable concentrations of any PCB congener. No PCB congener other than #126 was detectable in treated animals, with the

exception of the 50 $\mu\text{g/L}$ green frog group, which had 5.3 ng/g PCB #77 and 1.1 ng/g PCB #169. These two congeners may have been impurities in the initial PCB stock solution.

DISCUSSION

Bioconcentration in tissue

PCB body burdens we determined from this study (0.0012 to 9.3 $\mu\text{g/g}$ wet mass) were comparable to or higher than body burdens recorded in other studies of anurans in the Green Bay ecosystem. Huang *et al.* [7] collected leopard frog adults from sites along the Fox River and Green Bay in the summers of 1994 and 1995. Adult frogs had total PCB levels between 0.0028 and 0.15 $\mu\text{g/g}$ wet mass (without livers). During the summers of 1994 and 1995, Jung *et al.* [15] (R. E. Jung and W. H. Karasov, unpublished data) raised green frogs and leopard frogs from the egg stage through metamorphosis in enclosures placed along a pollution gradient in the Fox River and Green Bay. Green frog tadpoles collected 106 days post-hatch had total PCB levels of 0.023 to 0.283 $\mu\text{g/g}$ wet mass (without livers) and leopard frog tadpoles and metamorphs (without livers) collected approximately 60 days post-hatch had total PCB levels of 0.020 to 2.74 $\mu\text{g/g}$ wet mass. Tetra-chlorinated biphenyls were the predominant homologs in tadpole tissue. Green frog tadpole and leopard frog tadpole and adult PCB body burdens were significantly correlated with sediment PCB levels in the river and the bay. Sediment PCB levels were also significantly correlated with decreased hatching success of embryos for both species. We did not observe decreased hatching success in this study, even though the tadpole PCB body burdens from the 50 $\mu\text{g/L}$ treatment were much higher than those from the field. However, it is important to consider

that there could be differences between the effect of exposure levels in a controlled, stable laboratory environment and exposure levels in a fluctuating field environment where disease, predation, inclement weather, and other stressors may act in concert with toxicants.

The PCB levels in tadpoles exposed to the high concentration treatments (0.14 and 0.75 $\mu\text{g/g}$ wet mass for 5 $\mu\text{g/L}$ and 9.3 $\mu\text{g/g}$ wet mass for 50 $\mu\text{g/L}$) were similar to levels reported for invertebrates, fish, and birds in the Green Bay watershed. Annelids and chironomids collected in the Fox River/Green Bay area had total PCB levels of 0.377 and 0.351 $\mu\text{g/g}$ wet mass, respectively [29]. Sullivan and Defino [30] reported total PCB concentrations in carp (fillets) captured in Little Lake Butte des Morts to be approximately 5 $\mu\text{g/g}$ wet mass. In 1988, Forster's tern and common tern chicks, tree swallow nestlings, and red-winged blackbird adults collected from colonies near the mouth of the Fox River [31] had total PCB concentrations ranging from 0.053 to 14.1 $\mu\text{g/g}$ wet mass. It is important to note that more than 100 PCB congeners are included in these "total PCB body burdens". In the case of frogs in the Green Bay ecosystem, PCB 126 and other coplanar congeners occurred at very low or undetectable levels. Furthermore, congeners vary greatly in their ability to cause deleterious effects in organisms. For example, PCB 126 is over 10,000 times more potent than 2,3',4,4',5,5'-hexachlorobiphenyl in a cytochrome P450 monooxygenase activity assay [32]. Therefore, the body burdens of PCB 126 recorded in this study would be expected to cause more toxicity than comparable *total* PCB body burdens reported for animals in the ecosystem.

Embryos and larvae

Newly hatched green frog and leopard frog tadpoles exhibited increased mortality as compared to embryos exposed to PCB 126. Hatching success of ranid embryos exposed to PCB 126 throughout the egg stage at concentrations up to 50 $\mu\text{g/L}$ was not significantly lower than controls. However, tadpoles in this group exposed during the egg and larval stages exhibited high mortality. All leopard frog tadpoles exposed during the egg and larval stages to 50 $\mu\text{g/L}$ ($n = 41$ combined for both clutches) were dead by day 47 of the experiment and only six green frog tadpoles ($n = 30$ combined for both clutches) survived to day 125. (The higher survival of green frogs was possibly due to the difference in developmental stage at which exposure was begun for the two species). Jung and Walker [15] also observed increased mortality of tadpoles compared to embryos when they exposed leopard frogs for 24 h during the egg stage to graded doses of waterborne 2,3',7,8-tetrachlorodibenzo-p-dioxin (TCDD), a highly toxic TCDD isomer stereochemically similar to PCB 126. TCDD binds the same receptor (*Ah*) as PCB 126 and is considered 10 to 10,000 times as potent, depending on the species tested. Jung did not see decreased hatching success at any concentration of TCDD tested. However, leopard frogs exposed for 24 h during the egg stage to 3 $\mu\text{g/L}$ TCDD (approximately equivalent to 30 $\mu\text{g/L}$ PCB 126 by conservative toxic equivalency factor [TEF] estimates) had significantly increased mortality as tadpoles when compared to controls. Therefore, it is possible that toxicity occurring during the embryo stage is not manifest until the eggs have hatched into larvae.

Our results are consistent with those found for other animal groups and other contaminants. Rainbow trout eggs injected with graded doses of PCB 126 at 24 to 50 h

post-fertilization [20] or exposed to waterborne TCDD for 48 h during the egg stage [33] also had high hatching success, but low survivorship of newly hatched fry. In an experiment with fathead minnows exposed to Aroclor 1242, a commercial PCB mixture, eggs maintained at high concentrations (15 to 51 $\mu\text{g/L}$) hatched with good success, but none of the fry survived [17]. Dial *et al.* [34] found leopard frog eggs to be very resistant to paraquat, a herbicide, until approximately 3 days post hatch when a significant increase in mortality was observed in all but one treatment group. Berrill *et al.* [35] also found in experimental exposures of leopard and green frogs to low concentrations of pyrethroid insecticides that "newly hatched tadpoles are considerably more sensitive than embryo stages".

Our results seemingly differ from those of Jung *et al.* [15] (R. E. Jung and W. H. Karasov, unpublished data) who found decreased hatching success of anuran eggs maintained in the field (Fox River and Green Bay) in water with concentrations of total PCBs as low as 0.12 $\mu\text{g/L}$. We suggest two hypotheses to explain these contrasting results. First, it remains a possibility that effects of PCB 126 on frogs may be different from effects of the PCB congeners found in higher concentrations in frogs in the field [7, 15], but which are supposedly less toxic to wildlife. Second, the possibility that environmental factors beside PCBs differentially influenced the field sites and therefore caused variation in hatching success is very likely. Wave action, extreme water level fluctuations, or unmeasured parameters caused by degradation of the Fox River sites that coincidentally have high PCB sediment levels are confounding factors that might influence hatching success of eggs in these environments. Rosenshield and Karasov (unpublished data)

performed a study to determine if the pattern of hatching success of anuran eggs exposed in the laboratory to water collected along the same pollution gradient in the Fox River would be different than the pattern of hatching success of eggs exposed in the field. Their study minimized the confounding environmental factors present in Jung *et al.*'s [15] field study. Rosenshield and Karasov found no significant differences in hatching success among sites or between sites and tap water controls in the lab experiment. Therefore, differences in hatching success between sites in the field study were likely due to factors other than toxicants in the water, including PCBs.

Growth of both green and leopard frog tadpoles was slowed at the highest concentration of PCB 126. By day 13 after hatch (the first day animals were measured), both clutches of leopard frog tadpoles exposed to the highest concentration were already significantly smaller in total length than tadpoles in the other treatments. In green frogs, body length of tadpoles exposed to the highest PCB concentration was also significantly smaller than tadpoles in all other treatments by day 20 after hatch. This suggests that effects of the contaminant on growth occur quite early in development. Perhaps contaminated larvae are already at a disadvantage at the time of hatching. Jung [15] found a negative correlation between tadpole total length and TCDD dose for green frogs 31 days after exposure for 24 hours during the egg stage. This retarded growth of newly hatched tadpoles exposed to TCDD, or coplanar PCBs that likewise bind the *Ah* receptor, could have detrimental effects on a frog population as a whole. Smaller tadpoles may take a longer period of time to reach metamorphosis than larger ones. Therefore, the time animals remain

in an aquatic environment is prolonged, leaving them vulnerable to predators and pond desiccation.

Green frog tadpoles exposed to the highest concentration of PCB 126 swam significantly slower on day 50 post-hatch than animals from other treatment groups. This result differs from those of Jung [15] who found that swimming speed of American toad and green frog tadpoles, measured on day 6 and 41 post hatch, respectively, was not affected after an acute 24 hour exposure to TCDD during the egg stage. Jung [15] also did not find an effect on swimming speed (on days 11, 32, and 131 post hatch) when green frog tadpoles were chronically exposed to water collected from the Fox River (total PCB concentration = 81 ± 20.9 ng/L). In this study, tadpoles that swam significantly slower in the high concentration treatment ($50 \mu\text{g/L}$) also had a significantly higher incidence of edema, which may have caused a physical constraint on swimming performance. Therefore, swimming performance in tadpoles may be indirectly negatively affected by PCB exposure.

Metamorphs

The observed trend of decreased percent metamorphosis with increasing PCB 126 concentrations is probably due to a lack of metamorphosis in green frogs exposed to the highest PCB concentration because no leopard frogs tadpoles survived in this treatment. Only 6 green frog tadpoles in the $50 \mu\text{g/L}$ treatment survived to the last day of the experiment, therefore we should be cautious in relating this effect to PCB toxicity. Once percent metamorphosis was analyzed without the highest concentration group, it increased with increasing concentration of PCB.

The differences between species in time to metamorphosis are explained by different developmental schedules in green frogs and leopard frogs. In the laboratory, the duration of the tadpole stage for *Rana pipiens* (2 to 2.5 months) is shorter than that for *Rana clamitans* (3 to 4 months) [26]. Also, depending on the habitat and weather conditions, green frog tadpoles hatched from egg masses laid late in summer (during and after July) may not metamorphose that year, but will overwinter as tadpoles to metamorphose the following summer [36]. This explains why our green frog study was extended to the end of October, whereas the leopard frog study was completed in July.

The edema observed in leopard frog tadpoles from the higher concentration treatment (5 $\mu\text{g/L}$) during the period of tail resorption of metamorphosis (stage 41 to 45) was an interesting result. These animals did not show signs of edema before metamorphic climax began (stage 40), however edema (and in two cases, severe edema) became apparent during the 4 to 6 days required for the tail to be resorbed to 2mm in length. We hypothesize that PCBs stored in the fat of the tadpole tail were released and mobilized into the systemic circulation during these last stages of metamorphosis, causing detrimental effects in some animals. Also, it has been shown that thyroid hormones amplify the toxicity of some chlorinated compounds, such as TCDD [37]. Thyroid hormone levels (T4 in particular) in tadpole blood and pericardial fluid are maximal at the time of metamorphic climax [38, 39], thereby increasing the susceptibility of metamorphosing tadpoles to the toxic effects of the chlorinated contaminant.

In this study, the effect of PCB treatment was not included in the models to explain differences in body masses or snout-vent lengths of metamorphs. We suspect that body

mass measurements were skewed by the presence of edema in metamorphs from the 5 µg/L treatment, possibly masking the effects PCBs had on growth. Therefore, we recommend the use of dry body mass measurements in future studies of this kind. Differences in body weights between species explain the interspecific differences in organ masses. Jung [15] found that in green frogs exposed to Fox River water, only kidney masses of metamorphs differed between treatments. In this study, the effect of treatment as a covariate was not significant for liver, kidney/gonad, or fat weights. However, because no animals metamorphosed in the highest concentration of 50 µg/L, we were unable to assess its effects on all metamorphic parameters.

Ecological significance

Leopard frogs and green frogs were negatively affected by waterborne PCB 126 at high concentrations (5 to 50 µg/L). However, sublethal effects were not apparent for the parameters we measured at concentrations that occur in water in the Green Bay ecosystem or at tissue residue levels that occur in wild frogs. Based on sediment PCB concentrations and using a sediment-water partition coefficient of 1.5×10^5 [40], Jung *et al.* [15] approximated total PCB concentrations at Deposit A and Deposit X, two highly contaminated sites in the Fox River, to be 0.147 and 0.021 µg/L, respectively. Therefore, the most contaminated Fox River site, Deposit A, had total PCB levels more than 1 order of magnitude lower than the concentration of PCB 126 that caused the lowest level of observable effects in this study (5 µg/L).

The prevalence of deformities in this study was quite low. The number of deformities we observed was no higher than those observed in enclosures in the field [15] (R. E. Jung and W. H. Karasov, unpublished data) and did not differ between PCB treatments and controls. Contaminants are one of many putative causes of frog malformations suggested by scientists working in the field [41]; however, our lab study does not support the hypothesis that coplanar PCBs or other contaminants acting via the *Ah* receptor may be the cause.

For both leopard frogs and green frogs, clutches of eggs (eggs released by one female and fertilized by one male) were separated throughout the experiment. For some of the parameters measured, such as swimming performance and percent metamorphosis of larvae and body mass and snout-vent length of metamorphs, we found significant differences between clutches within species. This indicates that there may be substantial inter-individual or genetic variation within populations in responses to PCB toxicity. This variation could have important implications for adaptation of anurans to degraded environments. In areas where habitat degradation and environmental pollution are an important problem, amphibians that are more able to cope with the onslaught of chemicals released around them will more likely survive to reproduce.

Biological marker of toxicity

The edematous response of larvae exposed to high concentrations of PCB 126 in this study was consistent with signs of toxicity seen in other vertebrate classes. Walker *et al.* [20] observed sac fry mortality preceded by hemorrhages and severe fluid accumulation

beneath the yolk sac epithelial membrane in rainbow trout injected with PCB 126 at 24 to 50 h post-fertilization. Edema formation was also a common toxic response in chicken embryos whose mothers were fed PCBs [42] or in cockerel chicks that were fed PCBs themselves [19]. Waterborne exposure to Aroclors 1016, 1242, and 1254 from fertilization to 4 days posthatching was shown to cause acute abdominal edema in leopard frogs, American toads, and Fowler's toads [13]. This edematous response may be caused by induction of cytochrome P4501A1 in the vascular endothelium resulting in changes in hemodynamic or vascular permeability [43], but this hypothesis has yet to be tested.

In this study, all leopard frog tadpoles exposed to the highest concentration of PCB 126 developed edema in the body (not the tail) within 16 days after hatch. Mortality followed severe edema (tadpole body bloated to a spherical shape) in every case. Not all green frog tadpoles in this highest concentration developed edema, but all those that were severely affected died. Those animals exhibiting milder signs of edema (some fluid accumulation in the body) in the 5 and 0.5 $\mu\text{g/L}$ treatments survived throughout the experiment. We suspect that this pathologic response may be useful as a biological marker of PCB contamination in amphibians and deserves further examination. If scientists can identify edema in tadpoles in the field, the response could be used to indicate anuran exposure to chemicals that work by the same *Ah*-receptor-mediated mechanism as PCB 126.

In summary, our research is the first to relate the concentration of a coplanar PCB contaminant in ravid larvae to toxicity manifested during development from egg to frog. The signs of toxicity we observed at high concentrations of PCB 126 (5 to 50 $\mu\text{g/L}$)

consisted of a decrease in tadpole survivorship, an increase in edema, decrease in growth and decrease in swimming performance of larvae, as well as an increase in edema of leopard frog tadpoles during metamorphic climax. Edema in tadpoles is a pathologic response that may be useful as a biological marker of PCB exposure in the field. Although both ranid species were affected by the PCB contaminant at high concentrations, no sublethal effects were apparent for the parameters we measured at ecologically relevant concentrations for the Green Bay ecosystem.

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Figure 1. (A) Percent hatching success of embryos, (B) survivorship of tadpoles, and (C) edema in tadpoles for leopard frogs and green frogs exposed to PCB 126 and controls with (0+) and without (0-) acetone vehicle. Two clutches of each species were exposed to the range of PCB concentrations. For the egg experiment, 30 eggs from each species/clutch were exposed to each treatment. For the tadpole experiment, 40 green frog tadpoles were exposed to each treatment, but numbers varied for leopard frogs as follows: 40 tadpoles in 0+, 25 in 0-, 42 in 0.05 $\mu\text{g/L}$, 37 in 0.5 $\mu\text{g/L}$, 36 in 5 $\mu\text{g/L}$, and 41 in 50 $\mu\text{g/L}$. (D) Swimming speed of green frog tadpoles on day 50 after hatch during exposure to PCB 126 ($n = 10$ tadpoles per treatment). Points represent mean speed (\pm SE) of tadpoles of two green frog clutches.

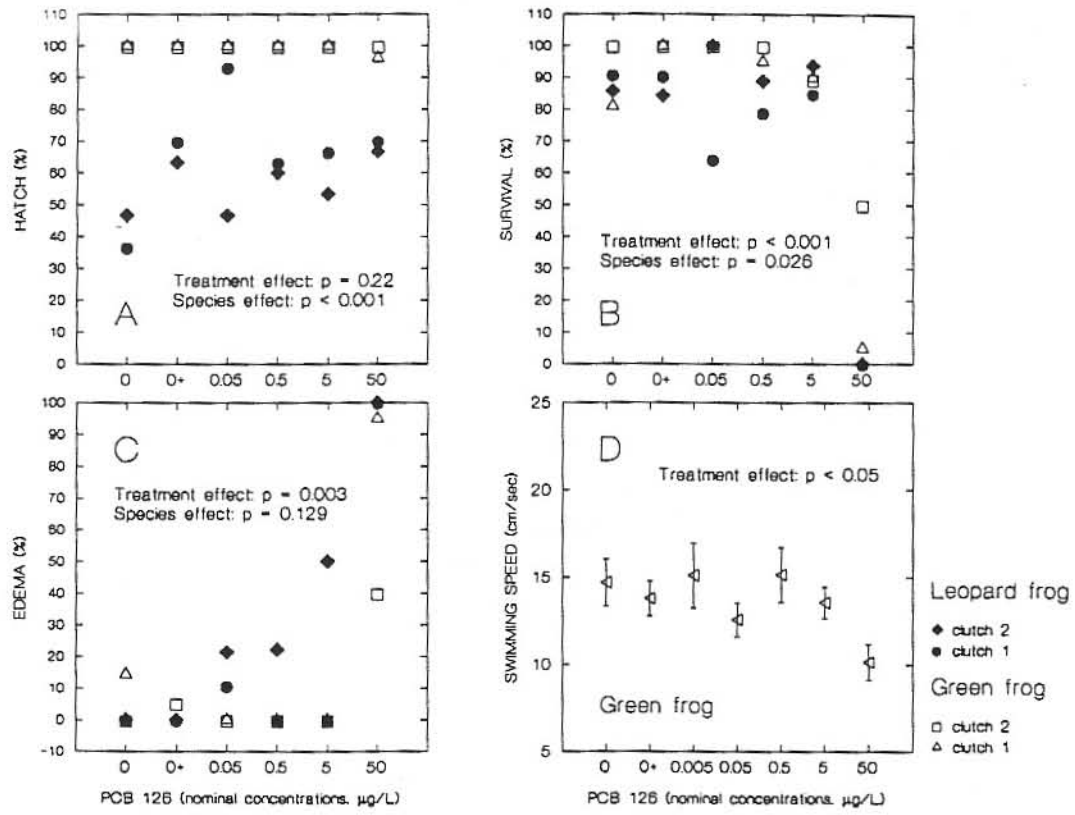


Figure 1

Figure 2. Growth of (A) leopard frog and (B) green frog tadpoles exposed to PCB 126 and controls with (0+) and without (0-) acetone vehicle. In these plots, the clutches (each a mean value from 6 [leopard frog] or 10 [green frog] tadpoles in a tank) are pooled within each exposure concentration (except for leopard frogs in the 50 $\mu\text{g/L}$ concentration). There were significant differences for lengths for the following species and days: leopard frogs; days 13, 19, and 25 after hatch, all $p \leq 0.002$; green frogs: days 50, 56, 62, 99, 105, and 117 after hatch, all $p \leq 0.05$.

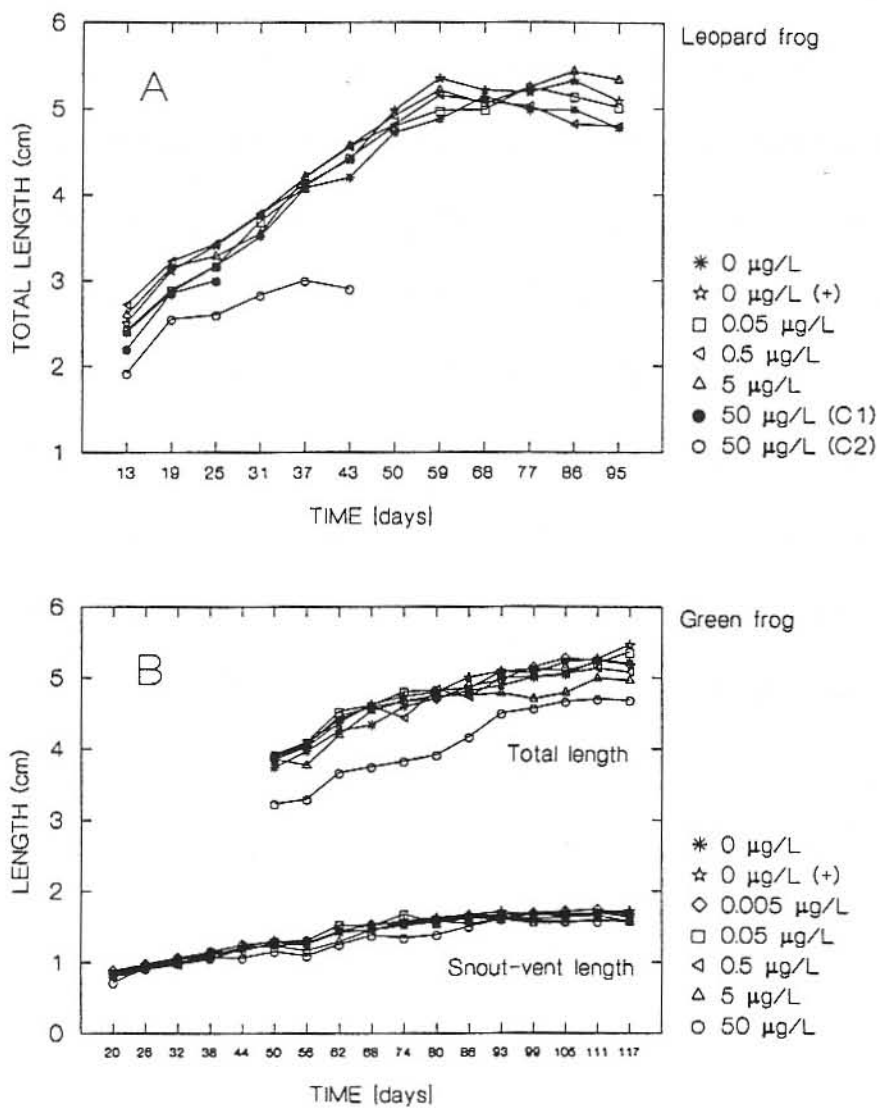


Figure 2

Figure 3. (A) Percent metamorphosis of leopard frog and green frog tadpoles exposed to PCB 126 and controls with (0+) and without (0-) acetone vehicle. Two clutches of each species were exposed to the range of PCB concentrations. The total number of leopard frog tadpoles that survived and were capable of metamorphosis were: 35 tadpoles in 0+, 22 in 0-, 32 in 0.05 $\mu\text{g/L}$, 31 in 0.5 $\mu\text{g/L}$, and 32 in 5 $\mu\text{g/L}$. There are no data for leopard frogs at 50 $\mu\text{g/L}$ because no animals survived at this concentration. The total number of green frog tadpoles that survived and were capable of metamorphosis were: 39 tadpoles in 0+, 37 in 0-, 39 in 0.05 $\mu\text{g/L}$, 38 in 0.5 $\mu\text{g/L}$, 35 in 5 $\mu\text{g/L}$, and 30 in 50 $\mu\text{g/L}$. Treatment effect: $p = 0.06$, species effect: $p < 0.001$. (B) Mean time to metamorphosis (\pm SE) of leopard frog and green frog tadpoles exposed to PCB 126. Sample sizes for panels B-D are as follows: leopard frogs; 20 metamorphs in 0+, 13 in 0-, 14 in 0.05 $\mu\text{g/L}$, 22 in 0.5 $\mu\text{g/L}$, and 18 in 5 $\mu\text{g/L}$; green frogs; 8 metamorphs in 0+, 4 in 0-, 14 in 0.05 $\mu\text{g/L}$, 17 in 0.5 $\mu\text{g/L}$, and 12 in 5 $\mu\text{g/L}$. (C) Percent edema and (D) mean body mass (\pm SE) of leopard frog and green frog metamorphs exposed to PCB126.

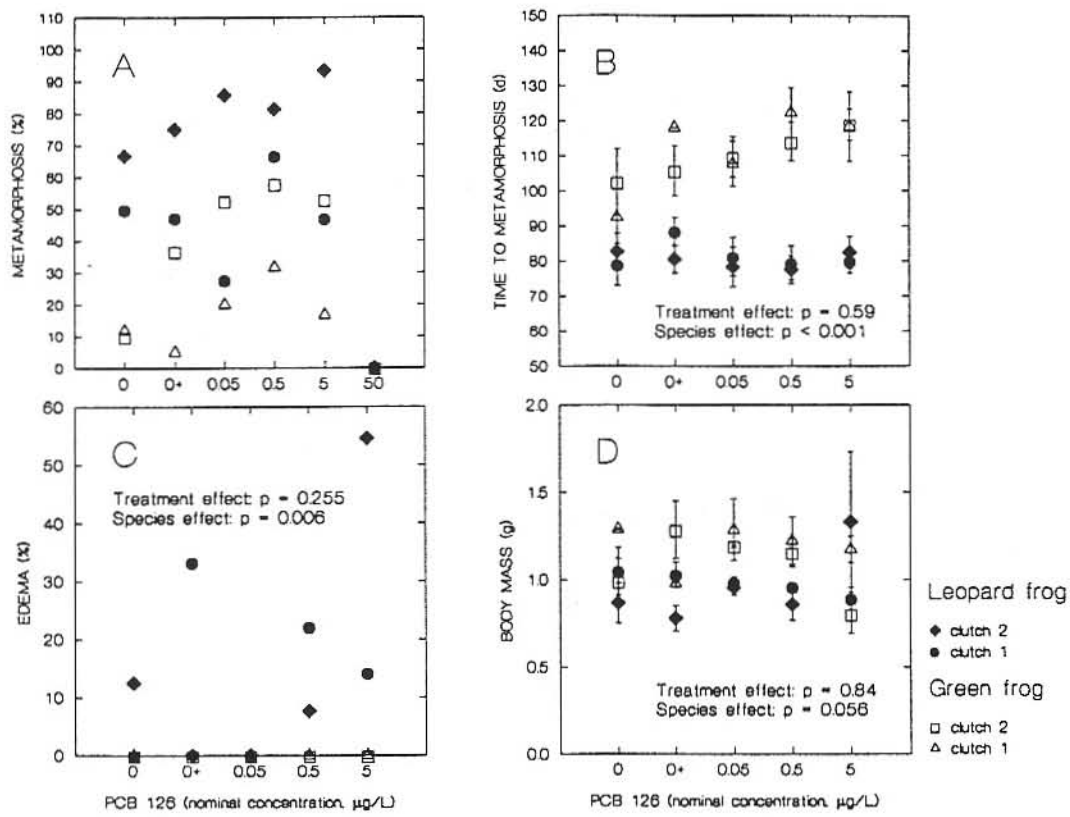


Figure 3

Figure 4. Relationship between nominal concentration of PCB 126 in treatment water ($\mu\text{g/L}$) and concentration of PCB 126 in tadpole tissue ($\mu\text{g/kg}$ wet mass). One pool per treatment containing the following numbers of individuals: leopard frogs, 14 tadpoles from 0+, 15 from 0.05 $\mu\text{g/L}$, 8 from 0.5 $\mu\text{g/L}$, and 10 from 5 $\mu\text{g/L}$; green frogs: 20 tadpoles from 0.5 $\mu\text{g/L}$, 23 from 5 $\mu\text{g/L}$, and 6 from 50 $\mu\text{g/L}$.

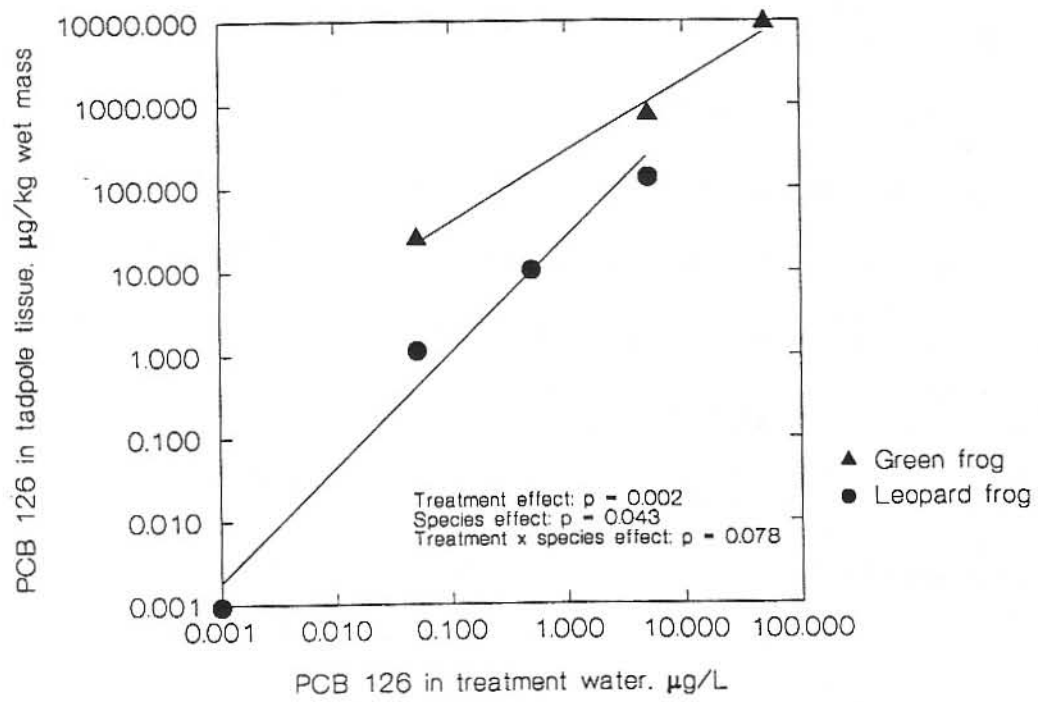


Figure 4

CHAPTER II

LABORATORY HATCHING SUCCESS OF GREEN FROG (*Rana clamitans*), LEOPARD FROG (*Rana pipiens*), AND AMERICAN TOAD (*Bufo americanus*) EMBRYOS EXPOSED TO WATER COLLECTED ALONG A POLLUTION GRADIENT IN THE FOX RIVER AND GREEN BAY, WISCONSIN

Abstract. In previous field studies by Jung [1] (RE Jung and WH Karasov, unpublished data), hatching success of anuran embryos varied between sites along a pollution gradient in the Fox River and Green Bay. Hatching success at these sites was negatively correlated with water quality factors such as ammonia and hardness and with contaminants such as sediment polychlorinated biphenyls (PCB) and several metals. The goal of this study was to replicate the Fox River/Green Bay field experiments in the laboratory by exposing anuran embryos to water collected along the same pollution gradient. Green frog, leopard frog, and American toad eggs were maintained in an incubator and exposed to water collected from six sites and a dechlorinated tap water control until hatch. We did not find significant differences in hatching success of embryos exposed to water collected at different sites along the Fox River, leading us to suspect that: 1) unmeasured environmental factors may have influenced hatching success in the field study, or 2) water quality parameters changed between the years that field and laboratory studies were conducted or between the field and laboratory conditions during each of our experiments.

INTRODUCTION

The Fox River, the main tributary to Green Bay, Wisconsin, and the Bay itself are polluted with polychlorinated biphenyls (PCBs), dioxins, heavy metals, and over 100 organic contaminants [2, 3]. PCB contamination of water and sediments in the ecosystem has been linked to industrial processes such as the recycling of carbonless copy paper by paper and pulp mills that line the Fox River. Industries released PCBs into the Fox River and Green Bay from 1957 until 1972, after which time production of PCBs was officially banned in the United States. Although they are no longer being released into the watershed, PCBs persist in the Green Bay ecosystem due to slow biodegradation, sediment contamination, atmospheric deposition, and bioaccumulation up the food chain.

The Fox River also suffers from poor water quality. Runoff of excess nutrients, particularly phosphorus, from nearby agricultural areas cause increased growth of algae which utilize large amounts of dissolved oxygen and leave less for other aquatic organisms [3]. Pulp wastes released by paper mills also reduce oxygen levels [3]. Elevated ammonia levels in the river caused by decomposition of algal blooms can be toxic to benthic organisms [4]. High loads of suspended solids and sediments from farm runoff impede aquatic macrophyte growth by reducing light penetration through surface water [5]. Finally, wetland loss and fragmentation [6], as well as water level changes caused by dams or seiches (R.E. Jung and T. Edblom, pers. comm.), can also negatively impact aquatic organisms in this ecosystem.

Heavy metals such as arsenic, cadmium, copper, lead, mercury, nickel, and selenium are present in the river [1, 2] and may adversely affect wildlife. Glass *et al.* [7] recorded

high concentrations of mercury immediately downstream from the DePere Dam originating from sediment sources and/or unidentified discharges. Elevated levels of mercury have stimulated restrictions on fish consumption on inland lakes throughout the states of Michigan, Minnesota, and Wisconsin and may become a problem in the Fox River as well [7].

A study by Jung [1] (RE Jung and WH Karasov, unpublished data) determined the percent hatching success of green frog and leopard frog embryos placed in enclosures located at sites along the Fox River and in Green Bay (Fig. 1). Water quality and contaminant data (i.e. dissolved oxygen, acidity, ammonia-nitrogen, metals, chlorinated pesticides, PCBs, suspended solids, chlorophyll a, etc.) were collected from each site. Jung found negative correlations of percent hatching success with log-transformed sediment PCB concentrations and several metals as well as with water quality factors such as ammonia and hardness. These correlations only reached significance ($p < 0.05$) for sediment arsenic and log-transformed sediment PCB concentrations.

Our goal was to determine if the pattern of hatching success of anuran eggs exposed to water collected along a pollution gradient in the Green Bay watershed and brought back to the laboratory would be different than the pattern of hatching success of eggs exposed to the same water in the field. We were interested in conducting a study where confounding environmental factors such as wave action present in the field would be minimized in a laboratory setting. We hypothesized that there would be no differences in hatching success among the sites or between sites and tap water controls, therefore suggesting that the

significant differences in hatching success between sites in the field study were due to factors other than toxicants in the water.

We studied the leopard frog, green frog, and American toad because: 1) two of these species were used in the field experiments, and 2) they are common residents of the Green Bay ecosystem and their populations may be impacted by pollutants in the field. Anuran embryos of all three species were collected from clean sites and exposed to water from six Green Bay/Fox River sites and a dechlorinated tap water control until hatch. After hatch, tadpoles from the green frog experiment were transferred to tanks with dechlorinated tap water and raised until metamorphosis to determine whether there were effects during the egg exposure that might not be apparent until later stages of development. Green frog larvae were assessed for effects on mortality, growth, development, percent metamorphosis, and time to metamorphosis.

METHODS

Acute exposure: study organisms.

On June 14, 1996, we collected four clutches of green frog eggs (*Rana clamitans*) from the University of Wisconsin-Green Bay (UWGB) Prairie Pond in the Cofrin Arboretum. On May 26, 1997, we collected three clutches of American toad eggs (*Bufo americanus*) from a marsh in Washburn County in northwestern Wisconsin. Three clutches of leopard frog eggs (*Rana pipiens*) were collected from Barkhausen Waterfowl Preserve in Green Bay, Wisconsin, on April 6, 1998.

Acute exposure: study sites.

On the same day that egg clutches were collected, we also collected water samples in 4 gallon carboys (Nalgene polypropylene) from six designated study sites along the Fox River and Green Bay. These study sites correspond to a pollution gradient, with sites along the Fox River having higher levels of toxicants and lower water quality than those in the Bay [2, 3]. Deposits A, C, X (DepA, DepC, DepX) and Bay Port (in Ken Euer Nature Area) are located in or adjacent to the Fox River (Fig. 1). These Fox River sites are found near paper mills, which have released PCBs among other organic and inorganic pollutants into the river [2]. Lineville (at the end of Lineville Road opposite Pete's Marsh) and Barkhausen (in Barkhausen Waterfowl Preserve) are located on or near Green Bay (Fig. 1).

Acute exposure: hatching success.

All frog egg clutches were transported in plastic containers to the Water Science and Engineering Laboratory at UW-Madison the morning after they were collected. Thirty eggs from each clutch (late gastrula through early neurula stages; stages 11-13 [8]) were placed in 100 x 20mm glass petri dishes. Eggs from each clutch were exposed to every one of six water treatments consisting of water samples from each of the six designated study sites and a dechlorinated, charcoal filtered water control (pH 8.2, hardness 324 mg/L as CaCO₃, dissolved oxygen 11.5 µg/L). Due to a loss of access to Deposit C, this treatment was eliminated from the American toad experiment. In the green frog experiment, two of the four clutches were duplicated, and in the American toad and leopard frog experiments, all three clutches were duplicated due to a large number of eggs in these clutches (Table 1).

Each petri dish was filled with 70mL of treatment water and placed into a 22°C incubator on a 14:10 light: dark cycle. We changed treatment water daily (static-renewal system) and collected new water from the six study sites every other day to minimize fungal contamination and buildup in water stored in carboys during the experiment. All viable eggs hatched by June 19, 1996 (green frog), May 30, 1997 (American toad), and April 9, 1998 (leopard frog). On these dates, all hatched eggs were counted and any observed deformities were recorded.

Field collected samples of water were analyzed with a Hach Test Kit FF-2 (Hach Co., Ames, IO) for acidity, alkalinity, ammonia, carbon dioxide, chloride, dissolved oxygen, hardness, nitrite, and pH every time new water was collected. Other studies have found water PCB concentrations to vary in relation to PCB contamination in sediments [9, 10]. Therefore, we assumed that water PCB concentrations were correlated with sediment concentrations and used sediment values as an approximate index of exposure to PCBs in collected water. It is highly likely that PCBs in the collected water entered the anuran embryos rather than residing in the jelly capsule surrounding the embryos. Jung and Walker [1] exposed leopard frog and green frog embryos for 24 hours to graded doses of waterborne 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a planar chlorinated hydrocarbon stereochemically and lipophilically similar to coplanar PCBs. They determined that only 1.2 - 3.7 % of the TCDD was found in the jelly capsule relative to the embryo.

We considered the possibility that PCBs may adhere to Nalgene carboys during the transport of water from the Fox River to the laboratory, causing PCBs to become unavailable for bioconcentration by frogs during the experiment. In an experiment by Jung [1], green

frog embryos and tadpoles were exposed to Fox River water brought back to the laboratory (in Nalgene carboys) and serially diluted with tap water. Jung showed that tadpole PCB body burdens increased with percent Fox River water exposure, assuring us that appreciable amounts of PCBs were available for bioconcentration by frogs. Also, due to the short amount of time water was stored in Nalgene containers (< 24 hours), we assumed that most PCBs would preferentially adhere to sediment in the collected water rather than the container walls (William Sonzogni, pers. comm.).

Chronic study: green frog.

Some newly hatched green frog tadpoles were raised to metamorphosis in clean water to determine whether there were effects during the egg exposure that might not be manifest until later stages of development. Eight newly hatched tadpoles from each clutch/treatment combination were pooled and transferred into 8L tanks, keeping tadpoles from the seven treatments separate for the chronic study. The chronic study consisted of raising the tadpoles from all seven treatments in dechlorinated, charcoal filtered, tap water (pH 8.2, hardness 324 mg/L as CaCO₃, dissolved oxygen 11.5 µg/L) with a static-renewal system. Thirty-two eggs were placed in each of twenty-one tanks representing seven treatments with three replicates per treatment. Tanks were maintained in a thermoregulated water bath system (24.1°C ±0.2) and water in the tanks was changed on Mondays, Wednesdays, and Fridays. A 14:10 light:dark cycle was maintained in the animal room. Tadpoles were fed boiled Romaine lettuce blended into a puree and a 3:1 Rabbit Chow: TetraMin mixture (LM Animal Farms, Pleasant Plain, OH; TetraMin Flake Food,

TetraSales, Blacksburg, VA). We provided animals with new food every time water in the tanks was changed. When the forelimbs of a tadpole emerged, we removed the animal from the tank and placed it into an inclined plastic tub with 1 L of water. The inclined tub provided access to a dry area in order to prevent drowning during metamorphosis.

Tadpoles were measured for total length ($n = 6$ per tank) on 15 occasions, staged (as determined by Gosner [8]) from day 64 on, and observed for mortality, deformities, and pigmentation approximately 3 times a week.

At metamorphosis (determined by tail length ≤ 2 mm), animals were weighed, measured for snout-vent length (SVL), and decapitated. Metamorphs were then dissected to determine masses of liver, kidneys plus gonads, and fat bodies. On October 21, 1996 (123 days after hatching), all tadpoles that had not yet metamorphosed were measured for total body length, weighed, staged, and euthanized with MS-222 (3-aminobenzoic acid ethyl ester: 0.05% solution, Sigma Chemical Co.).

Statistical Analysis.

We calculated hatching success (proportion of eggs that hatched) for each of the egg clutches raised in water collected at six sites and in tap water controls. The logit of hatchability ($= \log [(hatchability + 0.01)/(1-hatchability + 0.01)]$) was used in the following ANOVA model: $\text{logit hatchability} = \text{constant} + \text{species effect} + \text{clutch (nested within species) effect} + \text{site effect}$. The interaction term for site and species was not included in the model because one site was not available for one of the species. Logit transformation of

hatchabilities was used because hatchability proportions are sometimes not normally distributed.

Logit transformations were also used for percent survival and percent metamorphosis of green frog tadpoles raised beyond hatch. Sites were compared using one-way ANOVAs with one value per tank for each analysis. Days to emergence of front legs, days to metamorphosis (resorption of the tail to $< 2\text{mm}$), body mass, snout-vent length, liver mass and fat mass of metamorphs were compared between sites using one-way ANOVAs with one value per tank. Growth ($n = 15$ observations) and development ($n = 7$ observations) of tadpoles were compared between sites using one-way ANOVAs. When ANOVAs were significant, Tukey's multiple comparison test was used to determine differences between groups.

RESULTS

Hatching success.

Hatching success of anuran embryos did not significantly differ among sites or between sites and tap water controls ($F_{6,104} = 0.42, p = 0.86$) (Fig. 2). However, green frogs showed significantly lower hatching success than American toads or leopard frogs ($F_{2,104} = 22.8, p < 0.01$). There was also a significant difference in hatching success of clutches nested within species ($F_{7,104} = 3.58, p < 0.01$).

Water quality analysis.

We compared our water quality measures with those determined in the earlier field

studies [1] (RE Jung and WH Karasov, unpublished data). The constant temperatures maintained during each of the laboratory experiments fell above and below the temperatures recorded in the field for the leopard frog and green frog studies, respectively (Fig. 3). Un-ionized ammonia levels in the green frog field experiment were high compared to all other experiments due to high temperatures and slightly higher pHs in the field in June. Only this green frog field experiment showed significant variation in un-ionized ammonia levels between sites, whereas laboratory and field data for all other experiments showed little variation in un-ionized ammonia among sites. Dissolved oxygen levels were more variable in the green frog field experiment than the other experiments as well. Bay Port showed very low levels of dissolved oxygen in both the leopard frog and green frog field studies, but not in any other experiments. Nitrite levels were high in the green frog field and laboratory studies at Dep X and high in the green frog field and laboratory studies as well as the American toad laboratory study at Dep A. pH levels decreased from upstream on the Fox River down to Green Bay in all laboratory and field experiments.

Chronic study.

There was no significant difference between treatments for survival of green frog tadpoles to the end of the experiment ($F_{6,14} = 1.42, p = 0.276$) or for percent metamorphosis of tadpoles ($F_{6,14} = 1.31, p = 0.315$). Tadpoles measured on 15 occasions were not significantly different in total length between treatments (all $p > 0.15$) except on one occasion (day 46 after hatch; $F_{6,14} = 2.937, p = 0.045$). However, when a post hoc Tukey's significance test was prepared, there were no significant differences between any two sites

on day 46. Stage of development of tadpoles was recorded on 7 occasions and there were no significant differences between treatments on any of those dates (all $p > 0.17$).

The number of days until front legs of tadpoles emerged from the body was not significantly different between treatments ($F_{6,71} = 0.74$, $p = 0.62$), however the number of days to completed metamorphosis was significantly different ($F_{6,72} = 2.28$, $p = 0.045$). Tukey's multiple comparison test showed that metamorphosis was significantly earlier in animals raised to hatch in water from Deposit X (79.0 ± 7.25 , $n = 8$) than those raised to hatch in water from Bay Port (110.8 ± 6.83 , $n = 9$) or Lineville (108.0 ± 5.48 , $n = 14$) ($p = 0.033$ for both).

Body mass ($F_{6,72} = 1.0$, $p = 0.43$), snout-vent length ($F_{6,71} = 0.58$, $p = 0.74$), liver mass ($F_{6,66} = 0.569$, $p = 0.75$), and fat mass ($F_{6,69} = 0.83$, $p = 0.55$) of metamorphosed tadpoles were not significantly different between treatments.

DISCUSSION

Acute exposure.

The hatching success of anuran embryos exposed to field-collected water samples in the laboratory did not differ significantly between sites or between sites and controls. Our laboratory results did not show the same significant variation in hatching success between sites as seen in the field by Jung [1] (RE Jung and WH Karasov unpublished data). We evaluate three hypotheses to explain the different patterns of response among sites when studied with water in the laboratory vs. in situ: 1) some environmental factor that was not measured in the field caused the differences in hatching success at the field sites, 2) water

quality parameters changed significantly from the years field experiments were conducted (1994-1995) to the years laboratory studies were conducted (1996-1998), and 3) water quality parameters changed from the time water was collected in the field to the time animals were exposed in the laboratory during the course of each of our experiments.

1) Environmental factors may have differentially influenced field sites, and therefore hatching success, in the green and leopard frog field studies. For example, intermittent changes in water flow and/or level on the river were never measured during the field experiments. These water level fluctuations may have affected egg viability by altering the diffusion distance between sediments and eggs or the volume of water in which eggs were exposed to contaminants. Also, Fox River sites have turbulent waters whereas waters at the bay sites are always calm due to their protected location away from wind and large bodies of water. Therefore, factors such as water turbulence may have adversely influenced egg viability only at the Fox River sites during the field study.

2) The second hypothesis considers that water quality parameters potentially changed from the years field experiments were conducted to the years laboratory studies were conducted. Because no water PCB data are available for each of the years of the field and laboratory studies, it is difficult to conclude how this factor may have differentially influenced the experiments. However, water quality data collected during the field and laboratory experiments do show some differences. It appears that high ammonia levels at Deposit A, Deposit X, and Bay Port during the green frog field experiments may have adversely affected hatching success. Water quality measurements for the laboratory experiments did not show appreciable differences between sites. Therefore, the lack of

variation in hatching success between sites seems plausible since there was little variation in water quality factors between sites. Also, it is possible that spikes in contaminants occurred during high flow events on the river during the field experiments that did not occur during the laboratory experiments.

3) A third possibility to explain differences in hatching success between the field and laboratory studies involves a potential change in water quality parameters during the time of transit from field to laboratory. When water quality measurements were taken for the same experiment (leopard frog) in the field and then subsequently in the laboratory, very few differences were detected. Dissolved oxygen levels did decrease slightly between field and laboratory conditions. However, decreased dissolved oxygen levels would favor a decrease in hatching success in the laboratory as opposed to the field. As related in Methods, we expect that relative differences in water PCB levels between sites were not obscured by our water handling procedures.

Our hatching success results are supported by results of a recent study by Rosenshield *et al.* [11] that showed no effects of PCB 126, a particularly toxic PCB congener, on green frog or leopard frog hatching success in concentrations of up to 50 ppb. Based on sediment PCB concentrations and using a sediment-water partition coefficient of 1.5×10^5 [12], Jung [1] approximated total PCB concentrations at Deposit A in the Fox River, our most contaminated site, to be 0.147 ppb. Therefore, if PCBs were causing decreased hatching success in the Fox River, they must work by a different mechanism than that used by PCB 126 and other co-planar derivatives considered the most toxic to wildlife. The Fox River PCB congeners would have to cause decreased hatching success at

concentrations more than two levels of magnitude lower than concentrations of PCB 126 that did not adversely affect hatching in the laboratory.

Jofré *et al.* [13] determined the effects of un-ionized ammonia on green frog, leopard frog, and American toad hatching success. Jofré did not observe decreased hatching success of eggs incubated at ammonia concentrations found in the Fox River-Green Bay ecosystem (0.04 mg NH₃/L), further supporting our result that Fox River water alone was not detrimental to anuran hatching success.

Chronic study.

Exposure of green frog eggs to Fox River/Green Bay water until hatch (5 days) did not cause negative effects on tadpole survival, growth, or metamorphosis. The only significant developmental effect observed in the chronic study was an earlier metamorphosis in animals raised to hatch in water from Deposit X than those raised to hatch in water from Bay Port or Lineville. The reason for this shortened development time in frogs exposed to Deposit X water until hatch is not clear. Since Deposit X is not one of the most polluted sites in this study, it is unlikely that water contamination is the cause for decreased time to metamorphosis in these frogs.

Our results are similar to those of Jung [1] who exposed green frogs in the laboratory to Fox River water and to three serial dilutions of Fox River water with dechlorinated tap water for 131 days (egg to metamorphosis). Jung did have tadpoles that were shorter in the Fox River and 2:1 (Fox River water:tap water) treatments as compared to the 1:2 and tap water treatments on the last day of the experiment. However, Jung concluded that Fox River

water quality and toxicant levels "caused few or only subtle problems for green frog development."

In summary, this study tests for the effects of water collected from the Fox River/Green Bay watershed and brought back to the laboratory on anuran development from egg to frog. We did not observe variation in hatching success of embryos exposed to water from several sites along a contamination gradient in the Fox River and Green Bay. Jung [1] (RE Jung and WH Karasov, unpublished data) recorded variation between sites in hatching success of anuran embryos exposed to the same water in the field, which leads us to suspect that unmeasured environmental factors may have influenced hatching success in the field study or that water quality parameters changed between years or during each laboratory experiment. We also did not observe negative impacts on growth or metamorphosis in green frogs exposed to Fox River water until hatching and then raised in tap water until metamorphosis. The embryonic stages of three anuran species did not show lethal (green frog, leopard frog, American toad) or sublethal (green frog) effects for the parameters we measured from exposure to water from the Green Bay ecosystem.

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Table I. Experimental design for hatching success experiment. Treatments are water collected from six Green Bay/Fox River sites and a dechlorinated tap water control.

Species	Clutch	Replicates*	Treatments
Green frog	1	2	7
	2	2	7
	3	1	7
	4	1	7
American toad	1	2	6
	2	2	6
	3	2	6
Leopard frog	1	2	7
	2	2	7
	3	2	7

* 30 eggs / replicate

Figure 1. Map of all six water collection sites located on the Fox River and in Green Bay.

Township (T), Range (R), and Section (S) numbers and counties are given. Deposit A (T19N R16E S21) and Deposit C (T20N R17E S14) are located in Winnebago county. Deposit X (T21N R19E S18/19) is located in Outagamie county. Lineville (T24/25N R20E S1/36), Bay Port (T24N R20E S14), and Barkhausen (T24N R20E S2) are located in Brown county. Inset: Hatching success of leopard frogs (○) and green frogs (□) at 12 sites along the Fox River and Green Bay [1] (RE Jung and WH Karasov, unpublished data).

Figure 1

Upper Fox River Valley and Green Bay

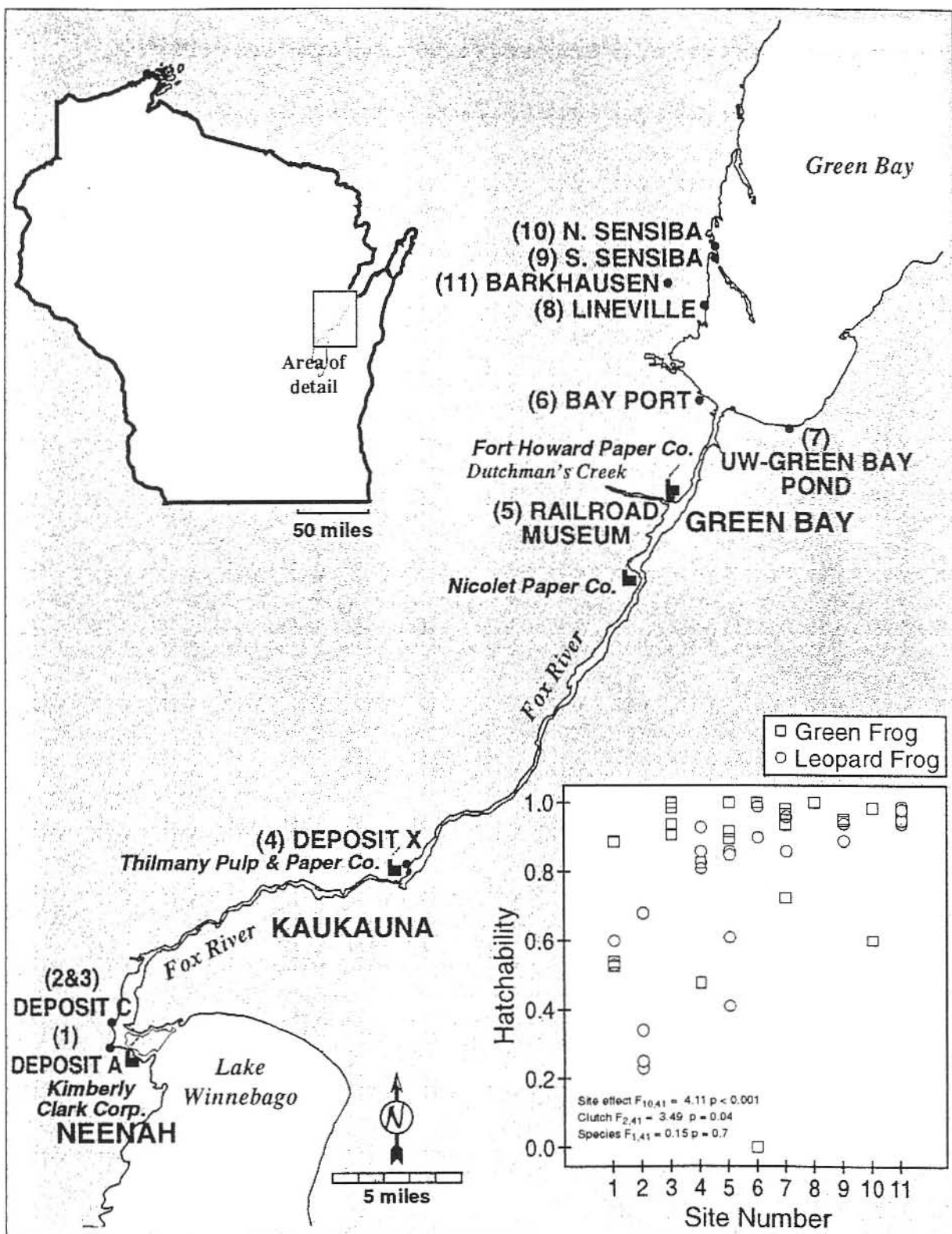


Figure 2. Percent hatching success of (A) American toad, (B) leopard frog, and (C) green frog eggs raised in water collected from 6 sites situated along a pollution gradient in the Fox River and Green Bay and a tap water control. American toad shows only five sites due to loss of access to DepC. All clutches and duplicates of clutches are shown for each species.

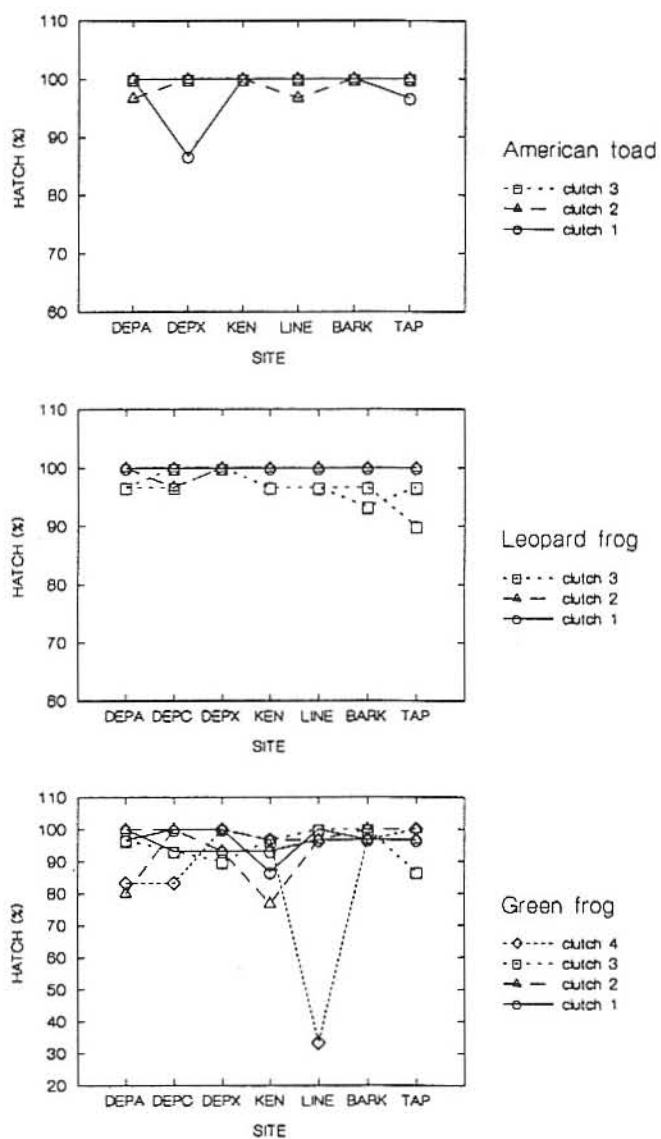


Figure 2

Figure 3. Water quality factors at 6 field sites in the Fox River and Green Bay. Data are given for water analyzed in the laboratory during this study (\blacktriangle , \bullet , \blacklozenge), in the field during this study (\diamond), and in the field during the Jung study [1] ($*$, \star). The units for temperature are $^{\circ}\text{C}$ and the units for non-ionized ammonia, dissolved oxygen, and nitrite-nitrogen are mg/L.

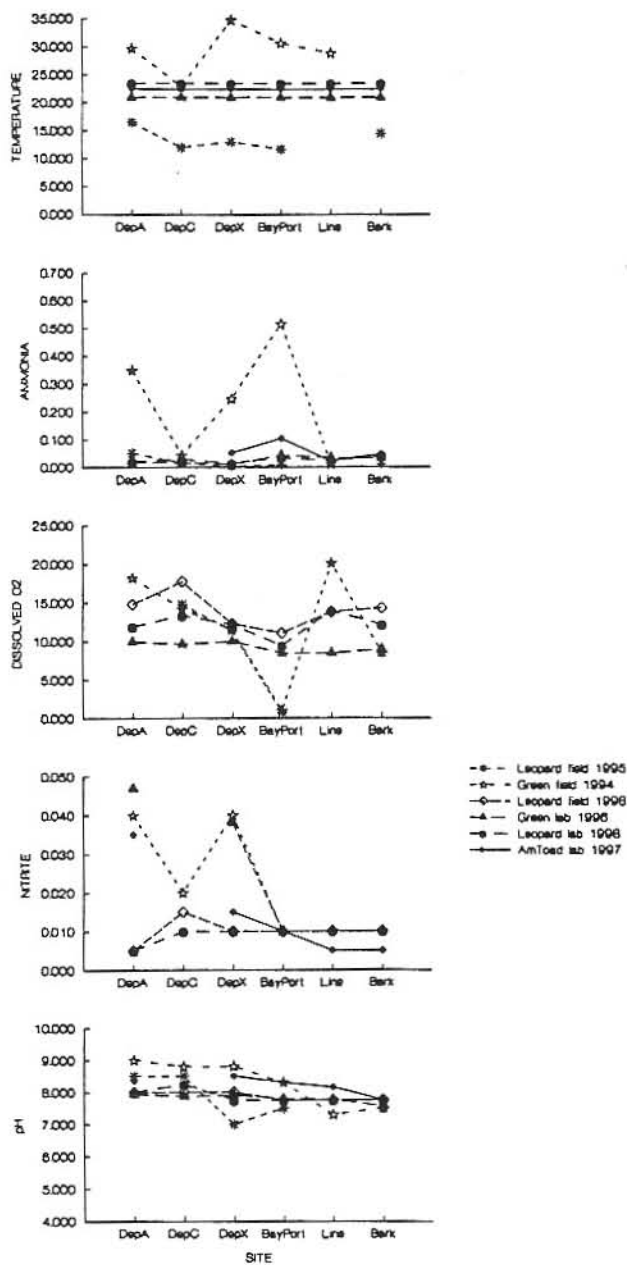


Figure 3

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Rosenshield, Michele
Ecotoxicology of
amphibians in Green
Bay and the Fox
River

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