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Effects of PCB 126 and Ammonia, Alone and in Combination, on Green Frog (*Rana clamitans*) and Leopard Frog (*R. pipiens*) Hatching Success, Development, and Metamorphosis

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The Green Bay watershed in Wisconsin is polluted with polychlorinated biphenyls (PCBs), dioxin, heavy metals, ammonia, and over 100 organic contaminants. In this study 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 (*R. pipiens*), to PCB 126 (3,3', 4,4', 5-Pentachlorobiphenyl, nominal concentrations 0–50 µg/l, two control treatments: water plus 0.08% acetone as carrier for the PCB, water alone), unionized ammonia (0–2 mg/l), and mixtures of both contaminants. Exposure to PCB 126 did not cause significant mortality of embryos before hatching. However, exposure to unionized ammonia (NH₃) concentrations in excess of 0.6 mg/l (green frogs) or 1.5 mg/l (leopard frogs) caused a decline in hatching success and an increase in prevalence of deformities. PCB 126 and NH₃ in combination had a significant negative effect on hatching success. Survival of larvae was significantly reduced at the highest PCB concentration (50 mg/l) for both species. Few skeletal deformities were observed in tadpoles at this concentration, but the incidence of edema was significantly increased. A slowing of growth was also observed in anuran tadpoles exposed to PCB 126. NH₃ exposure caused a decrease in the survival and growth of green frog tadpoles. When exposed to mixtures of both chemicals, green frog tadpoles showed a decrease in survival. However, growth was not affected. Fewer tadpoles metamorphosed with increasing PCB 126 and NH₃ concentrations. In tadpoles exposed to PCB 126, tissue concentrations of PCB 126 at the end of the experiment increased with increasing nominal concentrations, ranging from 1.2–9600 ng/g wet weight. Our data indicate that anurans may not be particularly sensitive to NH₃ as compared to many fish species, and that water quality criteria determined using data collected on fish species will be protective for many anuran amphibians. At high concentrations, PCB 126 and unionized NH₃ affected both ranid species. However, no sublethal effects were apparent at water concentrations that occur in the Green Bay ecosystem.

INDEX DESCRIPTORS: PCBs, ammonia, amphibians, survival, development, Green Bay.

The Green Bay watershed in Wisconsin is polluted with polychlorinated biphenyls (PCBs), dioxin, heavy metals, ammonia (NH₃) and over 100 organic contaminants (Harris 1990, Sullivan and Del-fino 1982, Swackhamer and Armstrong 1987). High levels of NH₃ in this ecosystem are caused by the decomposition of organic matter by heterotrophic bacteria and the release of sewage and discharge from industries lining the river (National Research Council 1979). In aqueous solution, ammonia assumes equilibrium between unionized (NH₃) and ionized (NH₄⁺) chemical species. Temperature and pH principally influence the equilibrium levels of ammonia as with higher values of both favoring NH₃, which can be toxic at high levels. NH₃ concentrations calculated from total ammonia concentrations at ambient pH and temperature exceed 0.04 mg/l in water (Harris and Kraft 1993, Jung 1996) and 1 mg/l in sediment pore water (Ankley et al. 1990) in the Green Bay ecosystem. Little is known about the possible effects of NH₃ at these levels on amphibians.

PCB contamination of water and sediments in the Green Bay 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, the main tributary to Green Bay. Although PCBs are no longer being released into the watershed, they 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. Some congeners have been shown to bind a cytosolic *Ah* (aryl hydrocarbon) receptor (Poland and Knutson 1982), forming an activated complex that can move into the nucleus of the cell where they form complexes with nuclear receptors and induce cytochrome P4501A1 activity (detoxification enzyme activity). These complexes can also bind to dioxin-response elements in the DNA, which are believed to 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 (Safe 1990). 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 (Safe 1984). PCB 126 induces P4501A1 activity in both leopard frogs (*Rana pipiens*) and green frogs (*R. clamitans*), the two species used in this study (Huang et al. 1998, Huang pers. comm.).

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 (Aulerich and Ringer 1977, Gilbertson and Fox 1977, Kubiak et al. 1989, Mac et al. 1985, Tillitt et al. 1992).

However, relatively little is known of their bioaccumulation in or possible effects on amphibians (Birge et al. 1978, Niethammer et al. 1984). The direct toxic effects of high environmental NH_3 on anurans are also little understood. There are few data available to determine whether anurans are adequately protected by the U.S. water quality criterion of 0.02 mg NH_3/l that has been established to protect aquatic life (US EPA 1977).

A recent study (Jung 1996, Jung and 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 negative correlation of percent hatching success with PCB concentrations in sediment and un-ionized ammonia concentrations in water.

To test whether this correlation might reflect a cause-effect linkage we exposed amphibian embryos and tadpoles in a static-renewal experiment to increasing concentrations of (1) PCB 126, (2) NH_3 , and (3) PCB 126 and NH_3 in combination. We studied the green frog (*Rana clamitans*) and the northern leopard frog (*R. pipiens*) because they are common residents of the Green Bay ecosystem and pollutants in the field may impact their populations. We assessed the effects of PCB 126, ammonia, and PCB 126 + ammonia on embryos (hatching success and deformities), tadpoles (survivorship, deformities, edema, growth), and metamorphosis (percent metamorphosis). We also measured bioconcentration of PCB 126 in tadpole tissues.

Overall, this study is the first to look at sensitivity of North American anuran amphibians to a model coplanar PCB compound and it doubles the information available on the sensitivity of North American anuran amphibians to NH_3 . It is also the first study of the combined effect of ammonia and PCB 126 and provides range-finding data for planning future PCB and ammonia dose-response experiments.

METHODS

Study Organisms

Embryos and tadpoles were staged during the experiment following the table proposed by Gosner (1960). Experiments were carried out at the Water Science and Engineering Laboratory at the University of Wisconsin-Madison.

Seven leopard frog egg clutches were purchased from NASCO (Fort Atkinson, Wisconsin). These egg clutches were fertilized the night before or the same morning they were transported to the laboratory in Madison. Three green frog egg clutches were collected by netting, in a pond near Deerfield, Dane County, Wisconsin and a pond near Stoughton, Dane County, Wisconsin. Clutches of eggs collected in the field were identified using the key from Watermolen (1995). Exposure began with embryos in the 4 to 16 cell stage (stages 4 to 6) and the neurula stage (stages 15 to 16). Eggs from the green frog clutches collected in Deerfield were used for the experiment of ammonia exposure in tadpoles. These embryos were raised to hatch in tap water and tadpoles at stage 24 to 26 (operculum development to early limb bud development) were exposed to treatments.

Exposure of Eggs

PCB 126 (Ultra Scientific and Accu Standard, Inc.) exposure levels were 0.005 (green frog only), 0.05, 0.5, 5, and 50 $\mu\text{g}/\text{l}$, and we had 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 egg masses of each species were exposed to PCB 126 for five (green frog) or six (leopard frog) days. For the ammonia exposure

experiments, five leopard frog egg masses were exposed for five days to four target concentrations of NH_3 (0, 0.5, 1, 2 mg/l). Green frog embryos (from one egg mass) were exposed for four days to five target concentrations of NH_3 (0, 0.1, 0.2, 0.5 mg/l). Ammonium chloride (NH_4Cl ; Sigma Chemical Co.) was used as the source of un-ionized ammonia (NH_3) and the concentrations were adjusted according to tables for aqueous ammonia equilibrium (Thurston et al. 1979) and our target values of NH_3 . In the combined exposure experiment, green frog eggs and tadpoles (from one clutch) were exposed to three PCB 126 concentrations (0, 0.1, 1 $\mu\text{g}/\text{l}$) and three ammonia concentrations (0, 0.1, 0.5 mg/l) arranged in a multifactorial design. All solutions were prepared with dechlorinated, charcoal filtered water (pH 8.0, hardness 324 mg/l as CaCO_3 , and dissolved oxygen 11.5 ppm.).

Thirty eggs from each clutch were exposed to 40–70 ml of each treatment solution in 100 × 20 mm glass petri dishes. Petri dishes were placed in a 23°C incubator on a 14:10 h light:dark cycle. Treatment solutions were changed every 24 h (static renewal system). In the ammonia and combined exposure experiments, water temperature ($\pm 1^\circ\text{C}$), pH (± 0.02 units), and total ammonia content by nesslerization (± 0.08 mg/l) were measured in each petri dish immediately before (final) and after (initial) the solution was renewed.

On the day embryos hatched, hatching success, deformities (bent or asymmetric tails), edema (distension of the body with fluid), and abnormal swimming performance were recorded.

Exposure of Tadpoles

The exposure of tadpoles was carried out in tanks containing 6.0 to 8.0 l of treatment solution. In the PCB 126 and the combined exposure experiments, between 11 and 28 tadpoles that survived after hatch from each petri dish were transferred to the tanks. In the ammonia experiment, 20 d after hatch tadpoles from each of two clutches hatched in tap water were transferred to tanks (clutches kept separate, 9 tadpoles in each tank) containing treatment solutions with 0, 0.01, 0.1, 1 mg/l NH_3 (target concentrations). Tanks were placed in a thermoregulated water bath kept at 23–24°C (14:10 h 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, Ohio; TetraMin Flake Food, TetraSales, Blacksburg, Virginia). When the front legs of a tadpole emerged, the animal was measured and transferred to a tilted plastic tub containing 1.0 l 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 3 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 every three days. Body length and/or total length were measured in a sample ($n = 5-10$) of tadpoles chosen randomly from each tank every six or nine days.

At metamorphosis (tail length = 2 mm), frogs were weighed, measured for snout-vent length (SVL), and euthanized. Frogs were then dissected to determine masses ($\pm 0.001\text{g}$) 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 MS-222 solution (3-aminobenzoic acid ethyl ester, 0.05% solution, Sigma Chemical Co.).

After euthanization, tadpoles from the PCB 126 exposure experiment were frozen for contaminant analysis. 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 (3,3',4,4'-tetrachlorobiphenyl), #123 (2',3,4,4',5-pentachlorobiphenyl), #105 (2,3,3',4,4'-pentachlorobiphenyl), #156 (2,3,3',4,4',5-hexachlorobiphenyl), #157 (2,3,3',4,4',5'-hexachlorobiphenyl) and, #169 (3,3',4,4',5,5'-hexachlorobiphenyl), as well as PCB #126 were analyzed. Organic analyses followed procedures described in "Methods for Organic Analysis" (1993).

Statistical Analyses

Logit values of percent hatching, survival, edema, deformities, and metamorphosis and raw data of tadpole total and body length (ammonia experiment), and body mass, SVL and time to metamorphosis in metamorphosed frogs were analyzed by ANCOVA. In the PCB 126 experiment the variation in these parameters was tested using nine models conditional on three explanatory variables: species, clutch nested within species, and log [PCB concentration]. Mallows' C_p (1973) was used as the basis for selecting the most appropriate model to explain variation in the data. In the ammonia experiment, we tested the effects of NH_3 (a covariate), species (a factor), and $\text{NH}_3 \times \text{species}$. In the combined exposure experiment, we tested for effects of NH_3 , PCB 126, and $\text{NH}_3 \times \text{PCB}$ 126 interactions (model: logit value = constant + NH_3 + PCB + $\text{NH}_3 \times \text{PCB}$). In all cases we used the general linear model in SYSTAT (Wilkinson 1992).

The values of total length for tadpoles were compared between treatments using two-way ANOVA with treatment and clutch (PCB experiment) or PCB 126 and NH_3 (combined experiment) as factors, keeping species separate. When ANOVA results were significant, Tukey's honestly significant difference test (HSD) for multiple comparisons was used. In the PCB experiment, organ masses for metamorphosed frogs were compared using ANCOVA with species as factor and log [PCB concentration] and SVL as covariates.

In our ammonia experiments we found that within petri dishes or tanks ammonia concentration varied significantly between water changes. Ammonia concentration rose during the 24–72 h in the solutions with low target NH_3 concentrations (0–0.2 mg/l), probably due to animal inputs, and fell in the solutions with higher target NH_3 concentrations (0.5–2 mg/l), perhaps due to evaporation.

For each petri dish or tank we calculated the initial and final NH_3 concentrations using the measured values for total ammonia, pH, and temperature, and then used the mean NH_3 concentration to characterize the exposure over the 24 or 72 h period. In the hatching success experiment, mean NH_3 concentrations for the two species for each of the target solution concentrations were 0.128 mg/l (for target of 0 mg/l), 0.173 (for target of 0.1), 0.347 (for target of 0.2), 0.597 (for target of 0.5), 0.808 (for target of 1.0), and 1.911 (for target of 2.0). In the chronic exposure experiments, NH_3 concentrations for each of the target concentrations were 0.125 (for target 0), 0.112 (for target of 0.01), 0.147 (for target of 0.1) and 1.171 (for target of 1).

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

Acute Exposure of Embryos

PCB 126.—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. 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

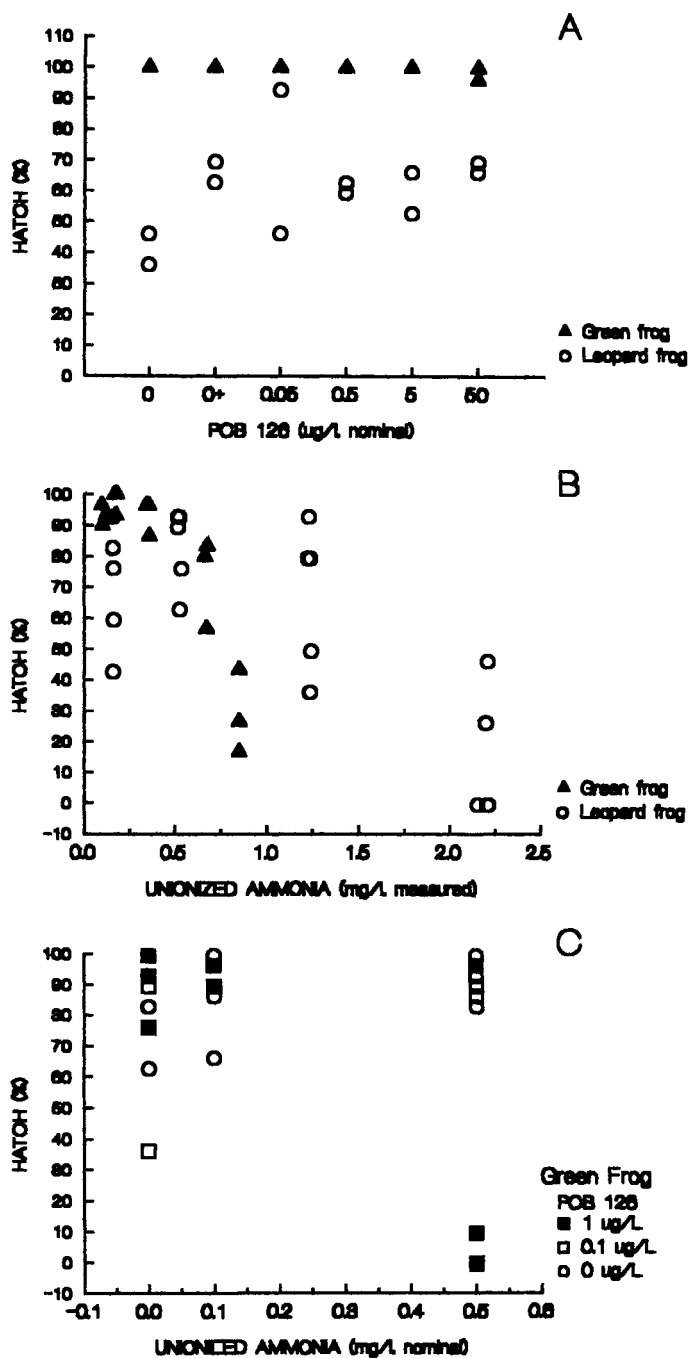


Fig. 1. Hatching success of Ranid embryos. (A) PCB 126: Leopard frogs and green frogs (two clutches of each species) were exposed to PCB 126 and controls with (0+) and without (0-) acetone vehicle. (B) Un-ionized ammonia: Three (green frog) and five (leopard frog) replicates, each containing 30 eggs, were exposed to the range of NH_3 concentrations. (C) Combined exposure: Three replicates from the same green frog egg clutch were exposed to each combination of PCB 126 and un-ionized ammonia.

($F_{1,17} = 1.66$, $P = 0.22$). Hence, PCB concentration had no significant effect on hatchability over the concentration range we tested. No deformities were observed in newly hatched tadpoles.

Ammonia.—Over the range of concentrations that we tested, NH_3

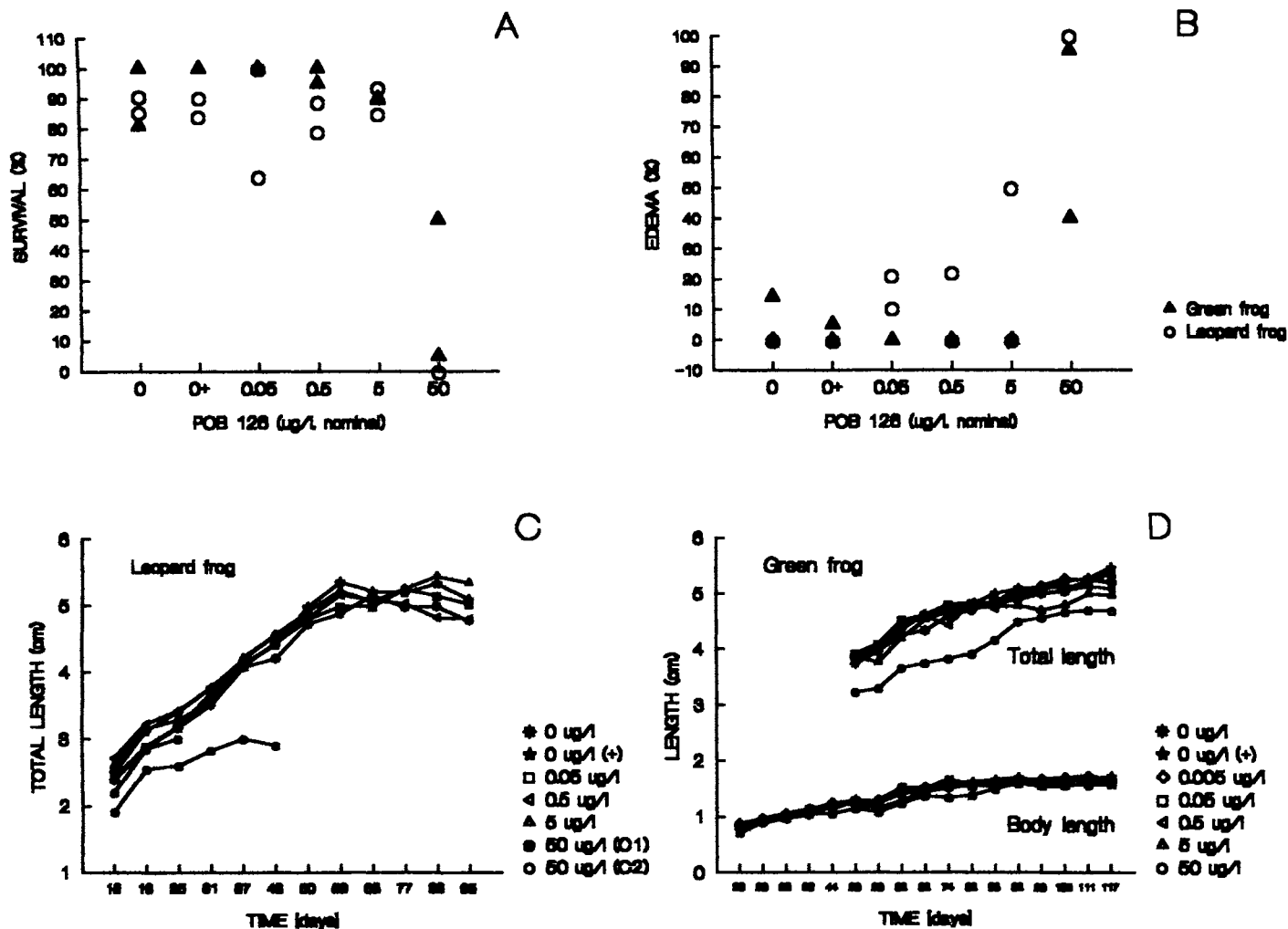


Fig. 2. Survival (A), edema (B) and growth (C, D) of tadpoles exposed to PCB 126. In (C) and (D) leopard frog (C) and green frog (D) clutches (each a mean value from six or ten tadpoles in a tank) are pooled within each exposure concentration (except for leopard frogs in the 50 µg/l concentration).

had a negative effect on hatching success ($F_{1,31} = 31.151$; $P < 0.001$) but this differed among species ($F_{1,31} = 4.463$; $P = 0.043$; Fig. 1B). Green frogs were affected at lower concentrations compared with leopard frogs. Un-ionized ammonia concentration was positively related to the percent deformities in newly hatched tadpoles ($F_{1,28} = 39.836$, $P < 0.001$), and this did not differ among species ($F_{1,28} = 0.332$; $P = 0.569$). The deformities observed were the same in the two species: body curled up or down, asymmetric body, curled spine, short tail, abnormal tail fins, and deformed tail.

Combined exposure.—PCB 126 and NH_3 (green frog only) had a significant effect on hatching success ($F_{3,23} = 3.898$, $P < 0.022$, $r^2 = 0.337$), but only when in combination ($F_{1,23} = 7.975$, $P < 0.01$). There was not a significant effect of either PCB ($F_{1,23} = 0.949$, $P = 0.340$) or NH_3 ($F_{1,23} = 0.450$, $P = 0.509$) alone. The combination of chemicals at the highest concentrations produced a decrease of around 60% (average) in hatching success (Fig. 1C). The percent deformities in newly hatched tadpoles was higher in embryos exposed to the highest ammonia concentration for both PCB concentrations (0.1 and 1 µg/l), but the effects of NH_3 , PCB 126, or the interaction were not significant ($F_{1,23} = 2.322$, $P = 0.541$; $F_{1,23} = 0.920$, $P = 0.347$ and $F_{1,23} = 1.496$, $P = 0.234$, respectively).

Tadpole Exposure

PCB 126.—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. 2A). Survival also differed between species ($F_{1,19} = 5.81$, $P = 0.026$). For the green frog, six tadpoles out of 30 survived after 125 days of exposure to the highest PCB 126 concentration of 50 µg/l. For the leopard frog, no tadpoles survived after 47 days of exposure to this concentration.

The incidence of edema increased significantly in both species at high PCB concentrations ($F_{1,20} = 11.373$, $P = 0.003$; Fig. 2B): 100% of the leopard frog tadpoles and 77% of the green frog tadpoles exposed to 50 µg/l PCB 126 exhibited edema at some point during the experiment.

The incidence of skeletal 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

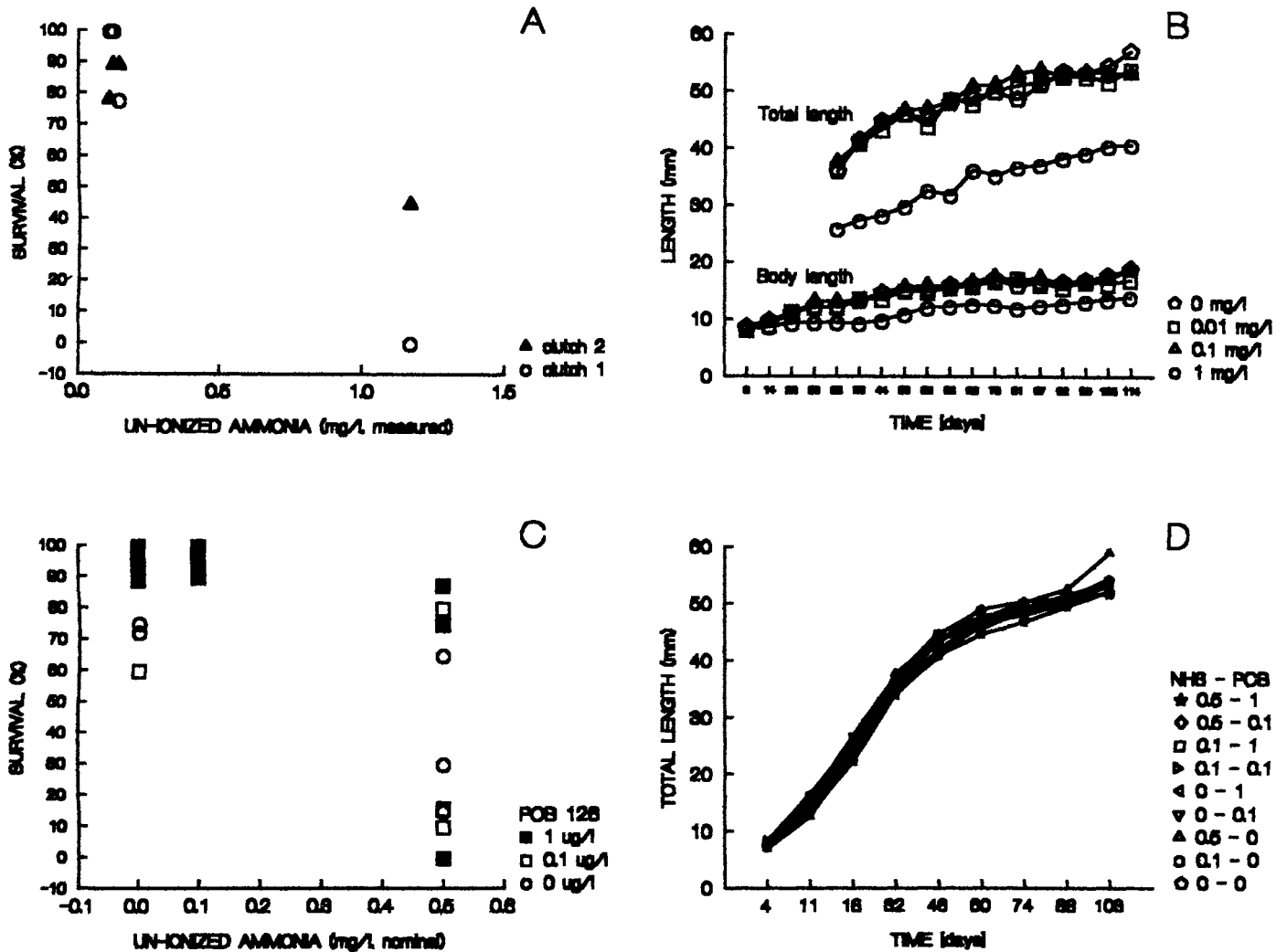


Fig. 3. Survival (A) and growth (B) of green frog tadpoles exposed to un-ionized ammonia. Two replicates (clutches) containing nine tadpoles each were exposed to the range of NH₃ concentrations. In (B) the replicates (each a mean value from 5 tadpoles in a tank) are pooled within each exposure concentration. Survival (C) and growth (D) of green frog tadpoles exposed to un-ionized ammonia and PCB 126 in combination. Three replicates from the same clutch containing 20 tadpoles each were exposed to the treatments. In (D) the replicates (each a mean value from 10 tadpoles in a tank) are pooled within each exposure concentration.

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 ($F_{1,17} = 0.19$, $P = 0.67$).

Leopard frog tadpoles exposed to 50 µg/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 $P_s > 0.002$; Fig. 2C). There were no significant differences in total length of tadpoles in the five remaining treatments throughout the rest of the experiment. Green frog tadpoles exposed to 50 µg/l were smaller than tadpoles exposed to all other treatments on all dates measured ($n = 12$), but the difference was significant only on six dates (days 50, 56, 62, 99, 105, and 117 after hatch, all $P_s < 0.05$; Fig. 2D).

Ammonia.—Tadpole survival (green frog only) evaluated on the last day of the experiment was significantly affected by NH₃ concentration ($F_{1,4} = 21.449$, $P < 0.05$). The effect of clutch was not significant ($F_{1,4} = 4.100$, $P = 0.113$), however the interaction between clutch and treatment was significant ($F_{1,4} = 8.130$, $P < 0.057$),

probably due to the large difference in survival between clutches at the highest concentration (0% survival in clutch 1 versus 44% in clutch 2; Fig. 3A). Deaths at the higher concentrations of NH₃ began after 20 days of exposure.

The prevalence of deformities in chronically exposed green frog tadpoles was low and there was no significant effect of NH₃ concentration ($F_{1,4} = 0.911$, $P = 0.394$).

Growth, as indexed by body length or total length (Fig. 3B), was slower for the highest NH₃ concentration compared with the lower concentrations. On the last day of the experiment, tadpoles exposed to the highest concentration had a significantly shorter body and total length than those exposed to the lower concentrations ($F_{1,19} = 6.451$; $P < 0.05$ and $F_{1,19} = 13.671$; $P < 0.05$, respectively).

Combined exposure.—The survival of green frog tadpoles after 123 days of joint exposure to PCB 126 and NH₃ was significantly reduced by NH₃ alone ($F_{1,22} = 8.248$, $P < 0.05$) but not affected by PCB 126 alone ($F_{1,22} = 0.904$, $P = 0.352$) and the interaction was not significant ($F_{1,23} = 0.336$, $P = 0.568$; Fig. 3C). There was

no effect of PCB 126 alone ($F_{1,22} = 3.240$, $P = 0.086$) or NH_3 alone ($F_{1,22} = 0.081$, $P = 0.778$) on the percent of deformities in tadpoles, and the interaction was also not significant ($F_{1,22} = 2.893$, $P = 0.103$). Growth as indexed by total length (Fig. 3D) was not different between treatments on all dates tadpoles were measured.

Metamorphs

PCB 126.—There was a trend for decreased percent metamorphosis in tadpoles at the highest concentration of PCB 126 ($F_{1,20} = 3.97$, $P = 0.06$) for both species. 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 and nine leopard frog tadpoles (exposed to 0+, 0.05, 0.5 and 5 $\mu\text{g/l}$) died during the period of tail resorption. Exposure to PCB 126 did not significantly affect the time at which tadpoles metamorphosed. There was a significantly higher incidence of edema in leopard frog metamorphs from the 5 $\mu\text{g/l}$ treatment.

For both species, Log [PCB concentration] was not a significant covariate for liver mass ($F_{1,138} = 0.017$, $P = 0.898$), kidney-gonad mass ($F_{1,135} = 0.777$, $P = 0.380$), or fat-body mass ($F_{1,137} = 1.63$, $P < 0.204$). There were significant differences between species in liver mass ($F_{1,138} = 5.87$, $P = 0.017$), kidney-gonad mass ($F_{1,135} = 35.9$, $P < 0.001$), and fat-body mass ($F_{1,137} = 25.81$, $P < 0.001$). The effect of SVL as a covariate was also significant for the three organ masses ($F_{1,138} = 112$, $P < 0.001$ for liver; $F_{1,135} = 53.5$, $P < 0.001$ for kidney-gonad; $F_{1,137} = 59.0$, $P < 0.001$ for fat body). The adjusted least squares mean masses for green frogs and leopard frogs respectively were: liver $0.025 \pm 0.001\text{g}$ and $0.022 \pm 0.001\text{g}$; for kidney-gonad $0.013 \pm 0.001\text{g}$ and $0.009 \pm 0.001\text{g}$; and fat-body mass $0.006 \pm 0.001\text{g}$ and $0.003 \pm 0.001\text{g}$.

Ammonia.—Metamorphosis of green frogs was first observed in a control tank 51 days after exposures began. By day 114 the percent metamorphosis observed in the tanks was 44% of tadpoles in control tanks (mean time to metamorphosis 104 ± 9 d, $n = 5$), 59% in 0.01 mg/L NH_3 (113 ± 6 d, $n = 10$), 50% in 0.1 mg/L NH_3 (104 ± 8 d, $n = 7$), and 0% in 1 mg/L NH_3 ($n =$ number of tadpoles that metamorphosed in each treatment). There was a trend for higher percent metamorphosis at lower ammonia concentrations ($F_{1,4} = 7.106$, $P = 0.056$). The effect of NH_3 concentrations on time to metamorphosis was not significant ($F_{1,18} = 1.084$, $P = 0.312$). There was a trend for smaller snout-vent length of metamorphs with increasing NH_3 concentrations ($F_{1,18} = 4.063$, $P = 0.059$). There was also a significant effect of clutch ($F_{1,18} = 8.144$, $P < 0.05$) on snout-vent length and the interaction between treatment and clutch was significant ($F_{1,18} = 4.522$, $P = 0.048$). From lowest to highest target NH_3 concentrations, body masses of metamorphs were 1.47 ± 0.12 g, 1.20 ± 0.09 g, and 1.16 ± 0.10 g ($F_{1,18} = 0.431$, $P = 0.52$).

Combined exposure.—Only six green frog tadpoles (1.2% of the 502 tadpoles that survived) metamorphosed in this experiment, therefore percent metamorphosis was not a parameter considered in the analysis.

Tadpoles That Failed to Metamorphose

PCB 126.—The concentration of PCB 126 in tissues of both green frog and leopard frog 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

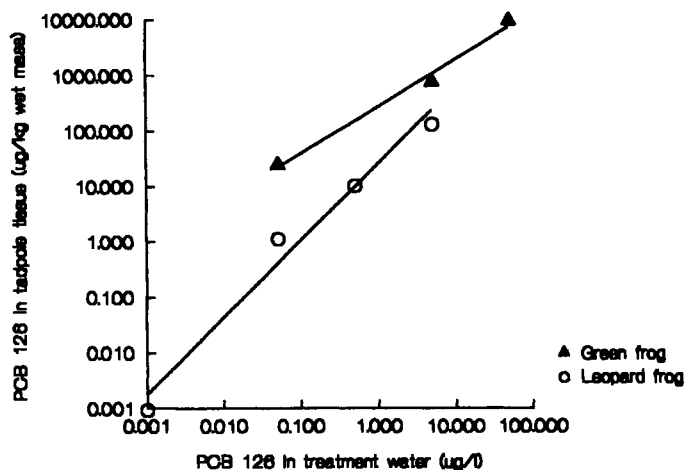


Fig. 4. Concentration of PCB 126 in treatment water ($\mu\text{g/l}$) vs. concentration of PCB 126 in tadpole tissue (mg/kg wet mass). One pool per treatment, leopard frogs: 14 tadpoles from 0+, 15 from 0.05 $\mu\text{g/l}$, eight from 0.5 $\mu\text{g/l}$, and ten from 5 $\mu\text{g/l}$, and green frogs: 20 tadpoles from 0.5 $\mu\text{g/l}$, 23 from 5 $\mu\text{g/l}$, and six from 50 $\mu\text{g/l}$.

tadpole tissues] ($F_{1,3} = 116$, $P < 0.01$). The species term and the interaction were also significant ($F_{1,3} = 11.5$, $P = 0.04$ and $F_{1,3} = 6.92$, $P = 0.08$, respectively). 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 very small concentrations of PCB #77 (5.3 ng/g, 0.05% of the total PCB body burden) and PCB #169 (1.1 ng/g, 0.01% of the total PCB body burden). These two congeners may have been impurities in the initial PCB stock solution.

Ammonia.—Among green frog tadpoles that failed to metamorphose 100% in both the control and 0.01 mg/L tanks had passed stage 30 (toe development) whereas 87% in the 0.1 mg/L tank and 50% in the 1 mg/L tank had passed this stage. Thus, there was a tendency for slowing of development time as well as growth rate in tadpoles exposed to higher NH_3 concentrations.

Combined exposure.—All the green frog tadpoles that failed to metamorphose had passed stage 30 by the end of the experiment. There was no effect of NH_3 alone ($F_{2,212} = 2.200$, $P = 0.113$) or PCB 126 alone ($F_{2,212} = 1.682$, $P = 0.189$) on the total length of these tadpoles and the interaction of chemicals was not significant ($F_{4,212} = 2.115$; $P = 0.08$).

DISCUSSION

PCB 126 Exposure

Toxic effects in anurans.—Newly hatched green frog and leopard frog tadpoles were more susceptible than embryos to the toxic effects of PCB 126. Hatching success of rapid 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. Jung and Walker (1997) also observed increased mortality of tadpoles compared to embryos when they exposed leopard frogs during the egg stage to graded doses of waterborne 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a highly toxic dioxin isomer stereochemically similar to PCB 126. 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 (Berrill et al. 1993, Dial et

al. 1984, Nebeker et al. 1974, Walker and Peterson 1991, Walker et al. 1991).

Our results seemingly differ from those of Jung (1996, Jung and Karasov unpubl.) 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 of all, it is possible that effects of PCB 126 on frogs may be different from effects of the mixture of TCDD-like and non-TCDD-like total PCB congeners found in higher concentrations in frogs in the field (Huang et al. 1998, Jung 1996), but which are supposedly less toxic to wildlife. Secondly, the possibility that environmental factors besides PCBs differentially influenced the field sites and therefore caused variation in hatching success is very likely. Rosenshield and Karasov (unpubl.) 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 and Karasov (unpubl.) field study. Rosenshield and Karasov (unpubl.) 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. Another possible explanation is that the more important "toxic" fraction of PCBs may be those bound to sediments rather than those present in the water column.

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 the 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 (1996) found a negative correlation between tadpole total length and TCDD dose for green frog 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.

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 six 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.

Tadpoles that developed edema during the period of tail resorption of metamorphosis (stage 41 to 45) did not show signs of edema before metamorphic climax began (stage 40). 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 or, that the high levels of thyroid hormone, which are maximal at the time of metamorphic climax (Mondou and Kaltenbach 1979, Weil 1986), amplified the toxicity of PCB 126 (Rozman et al. 1984). The edematous response of larvae exposed to high concen-

trations of PCB 126 was consistent with signs of toxicity seen in other vertebrate classes (Birge et al. 1978, Cecil et al. 1974, Vos and Koeman 1970, Walker et al. 1991). The edematous response may be caused by induction of cytochrome P4501A1 in the vascular endothelium resulting in changes in hemodynamic or vascular permeability (Guiney et al. 1990), but this hypothesis has yet to be tested. We suspect that this pathologic response may be useful as a biological marker of PCB contamination in amphibians and deserves further examination.

Bioconcentration in tissue.—Leopard frog and green frog tadpoles bioconcentrated PCB 126 from treatment water over the course of the experiment. BCFs (bioconcentration factor = PCB 126 concentration in wet tadpole tissue/ nominal PCB 126 concentration in treatment water) ranged from 22 to 28 in leopard frogs and 150 to 500 in green frogs. The difference in bioconcentration between species might be explained by the difference in exposure time (leopard frog = 104 days, green frog = 125 days).

The PCB 126 body burdens we determined from this study (0.0012 to 9.3 $\mu\text{g/g}$ wet mass) are comparable to total PCB body burdens recorded in other studies of anurans in the Green Bay ecosystem (Huang et al. 1998, Jung 1996, Jung and Karasov, unpubl.). The PCB 126 levels in tadpoles exposed to the high concentration treatments (0.14 and 0.75 $\mu\text{g/g}$ wet mass for 5 $\mu\text{g/g}$ and 9.3 $\mu\text{g/g}$ wet mass for 50 $\mu\text{g/g}$) were similar to levels of total PCBs reported for invertebrates, fish, and birds in the Green Bay watershed (Ankley et al. 1993, Call et al. 1991, Sullivan and Defino 1983). 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 (Safe 1987). 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.

Ammonia Exposure

We found declines in embryo survival, increases in prevalence of deformities in newly hatched tadpoles, and a slowing of growth and development in anuran embryos and tadpoles exposed to NH_3 concentrations in excess of 0.6 mg/L (green frogs) or 1.5 mg/L (leopard frogs). Our findings are consistent with those of Diamond et al. (1993) who reported 96-h LC_{50} 's (pH 8, 20 °C) of 1.9 mg/L NH_3 for leopard frog embryos and >0.9 mg/L for spring peepers (*Hyla crucifer*). Many of the species of fish that have been tested appear to be more sensitive to NH_3 than these anuran amphibians. The highest concentration shown not to depress hatching in fathead minnows (*Pimephales promelas*) was 0.42 mg/L NH_3 (Swigert and Spacie 1983), and rainbow trout (*Salmo gairdneri*) embryos exhibited malformations when exposed to NH_3 concentrations between 0.01 to 0.2 mg/L (Cotta Ramusino 1980). Acute and chronic LC_{50} 's among fish species are reported to be 0.03 to 2.55 mg/L NH_3 and 0.3 to 2.7 mg/L NH_3 , respectively (Arthur et al. 1987, Colt and Tchobanoglous 1978, Diamond et al. 1993, Knoph 1992, Robinette 1976, Ball 1967, Swigert and Spacie 1983, Thurston et al. 1978, Thurston and Russo 1983).

Depressions of growth rate have been observed in fish exposed to un-ionized ammonia concentrations between 0.05 to 0.99 mg/L (Alderson 1979, Colt and Tchobanoglous 1978, Robinette 1976, Swigert and Spacie 1983). Some proposed mechanisms for the effect of un-ionized ammonia on growth in fish include reduction of oxygen uptake due to gill damage, imposition of additional energy demand caused by the use of alternative detoxification pathways, increased

loss of ions by increased urine flow, inhibition of sodium uptake and damage to various tissues (Colt and Tchobanoglous 1978).

Combined Exposure to PCB 126 and Unionized Ammonia

Aquatic organisms are usually exposed to a wide variety of toxicants (Cairns et al. 1990) which may interact with each other or with other environmental parameters and influence a number of animal responses (Voyer and Heltshe 1984).

In this experiment, the hatching success of green frog embryos was reduced and the percent of deformities in newly hatched tadpoles was increased (not significantly) for the combination of PCB and unionized ammonia at the highest concentrations. Interaction between chemicals may occur at different levels: in chemical and physico-chemical processes (make the chemical more or less available), in physiological processes (influence the quantity of chemical in the body), and in intoxication processes (affect the interaction with receptors at target sites) (Calamari and Alabaster 1980). PCB 126 and NH_3 are very different chemicals that produce toxic responses through different biochemical pathways. In this experiment we did not expect any interaction between the chemicals at the chemical or physico-chemical level. Nor did we expect any interaction between the chemicals at the level of cellular receptors. However, the observed effects of increased embryo mortality at the highest combination of both chemicals may be due to an interaction at the physiological level. The damage caused by one of the chemicals may be exacerbated by the other chemical through modifications in absorption, transport, distribution, transformation, accumulation or excretion of the toxicants.

Survival of tadpoles was significantly affected by the highest ammonia concentration independent of the PCB 126 exposure concentration. Therefore we can conclude that chronic exposure to un-ionized ammonia constitutes a harm in itself and that embryos may be more susceptible than tadpoles to the joint action of NH_3 and PCB. The decrease in survival observed in tadpoles exposed to ammonia and PCB 126 was comparable to that observed in tadpoles exposed to ammonia alone (see above). We must be cautious in drawing general conclusions from this combined exposure experiment because we exposed only one clutch of eggs to the two contaminants. However, we can consider these results as a basis for future research in this area. Presently, there is a lack of information concerning the effects of chemicals in combination on amphibians.

Ecological and Regulatory Implications

Waterborne PCB 126 and un-ionized ammonia at high concentrations negatively affected leopard frogs and green frogs, however no sublethal effects were apparent at concentrations that occur in water in the Green Bay ecosystem. Jung (1996) approximated total PCB concentrations at two highly contaminated sites in the Fox River to be 0.147 and 0.021 $\mu\text{g}/\text{l}$. Therefore, the most contaminated Fox River site 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 $\mu\text{g}/\text{l}$).

It appears from the few data available that anurans may not be particularly sensitive to NH_3 when compared with many fish species. The criterion established by U.S. EPA to protect fresh water aquatic life, 0.02 mg/l NH_3 (US EPA, 1977), is protective for embryos of the two ranid species we tested. Many natural waterways have NH_3 concentrations above this level, such as the Fox River-Green Bay ecosystem, which typically has NH_3 concentrations in excess of 0.04 mg/l (Harris and Kraft 1993, Jung 1996). Though this concentration appears from our data too low to affect anuran hatching success and development, higher concentrations (0.2 and 0.5 mg/l) have been observed in sites along the Fox River. At these sites, green frog

hatchability was reduced (Jung, pers. com.). We also speculate that under certain conditions native amphibians may be harmed by ambient NH_3 . In the Fox River-Green Bay area, sediment pore water contains much higher NH_3 concentrations (1.3 to 4.4 mg/l NH_3 at pH 8.2) than surface water (Ankley et al. 1990). Amphibian embryos and tadpoles could be negatively affected during episodic releases of ammonia from sediments (during resuspension events such as dredging or storms) or when, in conditions of low oxygen concentrations in sediment, ammonia is not adsorbed. Furthermore, adults of both ranid species we studied and tadpoles of green frogs hibernate in the winter buried in sediment, (Pinder et al. 1992) potentially exposed to hazardous levels of NH_3 .

The number of deformities observed in tadpoles was no higher than those observed in enclosures in the field (Jung 1996, Jung and Karasov, unpubl.) and did not differ between treatments and controls. Contaminants are one of many putative causes of frog malformations suggested by scientists working in the field. Our lab study does not support the hypothesis that coplanar PCBs or other contaminants acting via the *Ah* receptor may be the cause. More studies are necessary to determine whether deformities observed in newly hatched tadpoles after exposure to un-ionized ammonia persist during larval development.

For some of the parameters measured, we found significant differences between clutches within species. This indicates that there may be substantial sub-species variation in responses to PCB and ammonia toxicity and this variation could have important implications for adaptation of anurans to degraded environments.

In summary, our research is the first to relate the concentrations of a PCB compound and un-ionized ammonia alone and in combination to toxicity manifested during development from egg to frog. The signs of toxicity we observed consisted of a decrease in embryo and tadpole survivorship, an increase in edema, decrease in growth of larvae, as well as an increase in edema of tadpoles. PCB 126 and un-ionized ammonia affected both Ranid species, primarily at high concentrations. Though no sublethal effects were apparent at ecologically relevant concentrations of PCB126 and un-ionized ammonia for the Green Bay ecosystem, joint exposure to both chemicals may have a detrimental effect at lower concentrations.

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