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Toxicity of Chlorypyrifos to *Rana pipiens* Embryos

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Amphibians control many insect populations and serve as a connection between aquatic and terrestrial food webs. In order to ensure the continuity between the webs, it is important to protect amphibians from decline. Within the last two decades the frequency of mass incidences of frog deformities has increased and population declines have been observed. The reasons for these events are not thoroughly understood. Several causes that have been postulated for the deformities and population declines are ultraviolet radiation (Blaustein et al. 1997), parasites (Netting 1999), and agrochemicals, especially pesticides (Ouellet et al. 1997). Many amphibians lay their eggs in surface waters that pesticides can enter from runoff, overspray during application, or careless disposal of pesticide containers. Amphibians can absorb pesticides via the skin, gills or by ingestion.

Organophosphate pesticides (OP) are preferred over many other pesticides because they are less persistent in the environment. However, OP are acetylcholinesterase inhibitors and adversely affect the nervous system (Barron and Woodburn 1995). Concerns about malformations and population declines have led researchers to investigate the toxicity of OP to amphibian embryos. Malathion (5 – 10 ppm) caused gross morphological changes in dejellied embryos of the frog *Microhyla ornata* (Pawar et al. 1983). Phenylsaliginen cyclic phosphate, another OP, was found to be toxic to Southern Leopard frog embryos at concentrations $\geq$ 500 ppb (Fulton and Chambers 1985).

In June 2000, the EPA ordered an elimination of the production of chlorpyrifos (CPF), an OP insecticide, in order to protect the health and environment of all Americans, especially children (U.S. EPA 2000a). CPF is one of the most heavily used insecticides in the U.S., ranking 4th in usage in the Mid-Atlantic Region and 1st in Maryland (Martin and Denardo 1996; Ferrari et al. 1997). CPF has been detected in surface waters as well as attached to aquatic sediments (Giesy et al. 1999). A positive correlation between CPF sediment concentration and leg malformation in *Hyla chrysocelis* has been reported (Britson and Threlkeld 1998). CPF in combination with fenubucarb caused 100% mortality in *Bufo marinus* frogs (Calumpang et al. 1997). Information on the effect of CPF on frog embryos is not available in the literature. The median lethal concentration LC_{50}) for *Rana pipiens* tadpoles with hindlegs was reported to be 3000 ppb and for adult *R. pipiens* 30,000 ppb (U.S. EPA 2000b). Amphibian embryos can be

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very sensitive to environmental stressors, (e. g., enzyme inhibitors), because many complex processes are occurring during cellular differentiation. Our objective was to study the toxicity of CPF to *Rana pipiens* embryos, and to compare these results with those using *Vibrio fischeri*, *Daphnia magna*, and other OP amphibian data.

MATERIALS AND METHODS

CPF stock solution was prepared by dissolving 1 mg of 99.2 % pure CPF (Chem Services, West Chester, PA), in 500 mL of spring water and stirring continuously for 24 hr. No carrier solvent was used to eliminate the possibility of any effect from the solvent. Test concentrations were made by diluting the stock solution with either Evian® (*R. pipiens* tests) or Great Value® spring water (*D. magna* tests). *R. pipiens* test concentrations were 0, 10, 50, 100 and 200 ppb, and *D. magna* test concentrations were 0, 250, 500, 1000 and 2000 ppt.

Rana pipiens embryos were purchased from NASCO, (Fort Atkinson, WI). The embryos were kept in a temperature controlled environmental chamber at 19.5 $\pm$ 1°C, with a 14:10 L:D photoperiod. Shipping water and test water temperature, pH, dissolved oxygen, conductivity and hardness were recorded (Table 1). Embryos were acclimated at the test temperature for 24 hr in 105 mm x 44mm glass finger bowls. Groups of 10 *R. pipiens* embryos were then separated from the original large egg clutch and each group was transferred to a finger bowl containing 250 mL of one of the CPF test concentrations. Embryos were at Gosner stage 12 (Gosner 1960) at the time of exposure. All tests were run in triplicate. Hatching time, hatching success and occurrence of any malformation were visually monitored for 4 d. All hatched embryos were transferred to clean water and observed for 5 additional d.

Acute toxicity tests using *Daphnia magna* were conducted to determine the 48-hr EC_{50} (effective concentration value) (Clesceri et al. 1989). *Daphnia magna* (Carolina Biological Supply Company, Burlington, NC) were cultured and neonates were collected within 24 hr of release. Ten neonates were transferred to each finger bowl containing 100 mL of a CPF solution of the desired concentration. Neonates were monitored for 48-hr with immobilization as the endpoint. CPF toxicity was also measured using the Microtox® bioassay procedure for the Organic Solvent Solubilization of Sample (Azur Environmental 1995).

All tests waters were analyzed (pre and post test) to determine the actual CPF concentration, using a RaPID Assay kit (Strategic Diagnostics Inc., Newark, DE). A Chi-square test for independence, using a 2 x 5 contingency table, was performed on the amphibian morphological data. A one-way analysis of variance (ANOVA) was run to determine significance between amphibian hatching time for the control versus the CPF treatments and on the *Daphnia* data.

RESULTS AND DISCUSSION

The most prominent morphological observation was a downward curvature of the tail. The angle of curvature of each hatchling, after the 4 d treatment, was measured and compared with the tail angles of the hatchlings in the control. The results (Table 2), however, show that the degree of curvature was not correlated with the CPF treatments. No significant differences ($p>0.1$) were found between

Table 1. Mean water quality parameters for amphibian embryo tests

	Temperature (°C)	Dissolved Oxygen (mg/L)	Conductivity (μS)	Hardness (mg/L $CaCO_3$)	pH
Shipping Water	17.8	9.5	568.3	383.7	8.2
Evian Water	19.0	7.6	505.7	300.3	7.3

tail anglees in the control and treated groups. There was also no significant difference ($p>0.5$) in time to hatching in controls versus the treatments (Table 3).

The static acute toxicity value (EC_{50}) for *Daphnia magna* determined by probit analysis (Finney 1971) was 1074 ppt (Table 4). This value agrees with the reported value of 1000-2000 ppt (Kersting and van Wijngaarden 1992; U.S. EPA 2000a). Moore et al. (1998) reported the 48-hr LC_{50} for *D. magna* to Lorsban (CPF 44.9 % active ingredient) to be 0.6 ppb. Other *Daphnia magna* tests with OP compounds showed the EC_{50} for diazinon (>89% active ingredient) to be 0.83 ppb (U.S. EPA 2000c). Diazinon and CPF are in the same class of OPs and have low solubilites in water and similar metabolic activation pathways (Chambers and Levi 1992).

Table 2. Angle of tail curvature in newly hatched *Rana pipiens* embryos treated with chlorpyrifos; n=90

Concentration (μg/L)	Mean Angle	Std Dev.
0 (control)	9.6°	7.3°
10	9.6°	7.1°
50	8.5°	7.5°
100	10.2°	15.5°
200	8.9°	6.0°

CPF at the concentrations tested (0.1 ppb – 100 ppm) showed no toxicity to *Vibrio fischeri*, in the Microtox® assay, using 1% methanol (vol/vol) as the carrier solvent. Somasundaram et al. (1990) have reported the EC_{50} for CPF, parathion and diazinon to be 46.3, 8.5 and 10.3 ppm respectively, using 8% (methanol/DMSO) (vol/vol) as the carrier solvent. Parathion is in the same class as chlorpyrifos and diazinon (phosphorothionates), and has similar metabolic activation pathways (Chambers and Levi 1992).

Table 3. Mean percentage of *Rana pipiens* embryos hatched during 96 hr acute toxicity test with chlorpyrifos

CPF concentration (ppb)	Mean % hatched 48 hr	SD	72 hr	SD	96 hr	SD
0	27.8	2.1	94.5	1.2	100	1.0
10	26.7	0.9	91.2	0.4	100	1.3
50	26.7	1.2	96.7	1.2	100	0.6
100	23.3	0.3	94.5	0.5	100	0.2
200	22.2	0.8	66.6	3.9	100	3.4

SD: standard deviation values

Table 4. Chlorpyrifos toxicity results from *Daphnia magna* 48 hour acute tests

Concentration (ppt)	Mean number immobilized
0	0.0
250	0.0
500	0.3
1000	5.3
2000	10.0
LSD	2.13

Toxicity of the phosphorothionates is the result of metabolic activation by the P450 monooxygenase system, beginning with oxidative desulfuration (Chambers and Levi 1992). Studies show that most aquatic plants and microorganisms are relatively resistant to CPF, with LC_{50} or EC_{50} > 100 ppb; however, aquatic crustaceans and insect larvae are among the most sensitive to CPF (Barron and Woodburn 1995; Giesy 1999). According to Chambers and Levi (1992), the OP insecticides are chemicals that fail to show the same interspecies regression of acute toxicity exhibited by a wide range of pesticides and industrial chemicals. Our results reinforce the idea that it is difficult to extrapolate toxicity among species. The only organism in our tests to be adversely affected by CPF was *Daphnia magna*. Because no EC_{50} values were found for *Rana pipiens* or *Vibrio fischeri*, a toxicity comparison could not be made. Based on the results of this

study we conclude that CPF does not adversely affect *Rana pipiens* embryos morphologically, at concentration levels that are normally found in surface waters.

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