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Toxicity of Guthion® and Guthion® 2S to *Xenopus laevis* Embryos

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Abstract. The development of *Xenopus laevis* (African clawed frog) embryos exposed to the pesticide Guthion® (technical grade) and Guthion® 2S (commercial formulation) was evaluated in modified Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX) tests. The embryos were exposed to five or six increasing concentrations of pesticide in 10- and 100-ml exposure volumes of test solution for 96 h. Embryos exposed in 10-ml volumes of Guthion exhibited increased mortality, increased deformation, and decreased size as compared to those exposed in 100-ml volumes. LC50s for embryos exposed in the 10-ml Guthion tests ranged from 6.1 to 6.3 mg/L as compared to 10.6 to 11.9 mg/L for those in the 100-ml tests. The percentage of deformities at 3 mg/L Guthion in test survivors in 10-ml tests ranged from 73 to 89%, while in the 100-ml tests less than 2% were deformed at the same concentration. Mean control embryo lengths at test completion were 8.2 and 10.6 mm, respectively, for 10- and 100-ml tests. The LC50 for embryos in 100 ml Guthion 2S was 1.6 mg/L active ingredient, indicating a much greater toxicity of the commercial formulation. NOAEL (No Observed Adverse Effect Level) values for Guthion and Guthion 2S ranged from 0.48 to 7.96 mg/L, depending upon basis (length, deformity, mortality) and pesticide formulation, and were many times greater than the existing water quality criterion of 0.01 μg/L.

Differences in sensitivity to waterborne contaminants between wildlife species have prompted investigations on the sensitivity of amphibians to potentially hazardous environmental chemicals (Schuytema *et al.* 1991, 1993). The development of water quality criteria protective of amphibians in addition to reptiles, birds, and mammals (to be included in existing criteria for fish and other aquatic life) is being investigated by the U.S. Environmental Protection Agency. Only criteria for a few chemicals (DDT, PCBs, mercury, selenium) now include wildlife data (Williams *et al.* 1989).

Over 520,000 kg (active ingredient) of the organophosphate insecticide Guthion® (azinphosmethyl) were used in the United States in 1991 on fruit crops and cotton in the major producing states (USDA 1992a, 1992b). Organophosphates act by inhibiting acetylcholinesterase (AChE) essential to nerve transmission (USEPA 1986). A reduced affinity to AChE in frogs as compared to rats and chickens apparently decreases the sensitivity of amphibians to organophosphates (Anderson *et al.* 1977). Mulla *et al.* (1963) reported Guthion had no effect on *Bufo boreas* and *Scaphiopus hammondi* tadpoles at an application rate of 0.45 kg active ingredient (AI)/ha. Ninety-six hour LC50s of 0.109 to 0.13 mg/L Guthion have been reported for *Bufo woodhousii fowleri* tadpoles (Sanders 1970; Mayer and Ellersieck 1986) and 3.2 mg/L for *Pseudacris triseriata* tadpoles (Mayer and Ellersieck 1986).

Little evidence is available linking Guthion with adverse effects through the food chain (USEPA 1986a). However, the resistance of amphibians to cholinesterase inhibitors suggests that a number of organophosphate pesticides may be bioconcentrated to varying degrees and so may represent a hazard to amphibian predators (Hall and Kolbe 1980). Amphibians have the same potential for being adversely affected by direct application and associated run-off of Guthion as have other animals. For instance, commercially important wetland crayfish populations can be impacted by run-off from the large quantities of Guthion used in Louisiana sugar cane plantations; levels as low as 25 mg/kg in crayfish food can affect survival (Sklar 1985). Application rates range from 0.05 to 11.6 kg AI/ha (USEPA 1986b). The formulation of Guthion should be considered in the development of an amphibian water quality data base. Linder *et al.* (1990), for example, found significant differences in acute and sub-acute toxicity to technical grade and formulation grade paraquat for *Xenopus laevis* and *Rana pipiens*.

The relationship between amphibian toxicity bioassay results and the size of the exposure container suggests caution in interpreting data from different exposure volumes. Container volume is important since there should be sufficient test solution during a test to accurately characterize exposure concentrations, to provide sufficient dissolved oxygen, and to provide sufficient volume to compensate for test animal metabolic wastes, especially toxic pesticide metabolites. de Llamas (1985) noted that the ultimate size attained by a tadpole depends on temperature, population density and size and type of vessel. Schuytema *et al.* (1991) have noted that survivors with

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fewer animals per container tend to increase in size compared to controls in long-term tadpole toxicity tests.

The purpose of this study was (1) to evaluate mortality, teratogenesis, and growth in *Xenopus laevis* embryos exposed to Guthion®, a representative organophosphate pesticide, (2) to compare the effects of Guthion with effects of the field formulation Guthion 2S, and (3) to evaluate possible effects of test vessel size on pesticide effects.

Methods

Test Organisms

Xenopus laevis (Daudin) eggs were obtained from a breeding colony at the Environmental Research Laboratory—Corvallis from parents induced by injection of human chorionic gonadotropin (250 IU per female and 125 IU per male). Jellied fertilized eggs were tested at stage 10 to 11 (New 1966).

Test Water

High quality chlorine-free test water with low levels of dissolved constituents was obtained from wells near the Willamette River at Corvallis, OR. Temperatures were measured daily with a standardized mercury thermometer and continuously on a thermograph. Water quality was characterized prior to and during testing. Dissolved oxygen and pH were measured daily in the 100-ml exposure volumes by electrode. Total hardness (mg/L as $CaCO_3$), alkalinity, and conductivity were determined by USEPA Methods Nos. 130.2, 310.1, and 120.1, respectively, prior to the start of each test (USEPA 1979). Mean (± SE) water quality parameters during testing were: dissolved oxygen, 7.5 ± 1.0 mg/L; hardness, 46.0 ± 3.2 mg/L; alkalinity, 39.6 ± 2.0 mg/L; conductivity, 137.7 ± 8.9 μS; median pH 7.4 (range 7.1–7.7). Water temperature was maintained at 23 ± 1°C for Guthion Tests 1-3 and at 24 ± 1°C for Guthion Test 4 and the Guthion 2S test.

Test Procedures

Five static daily-renewal tests were conducted using the standard protocol for the Frog Embryo Teratogenesis Assay-*Xenopus* as a guideline (ASTM 1991); adaptations included the addition of 100-ml test volumes, the use of jellied eggs, and the use of well water instead of FETAX solution. Jellied eggs were exposed to five to six concentrations of Guthion and Guthion 2S (Table 1) in covered 60-mm glass Petri dishes containing 10 ml of test solution, or in covered 250-ml beakers (cut to 4.3 cm high) containing 100 ml of test solution. Guthion Tests 1 and 2 were conducted in 100-ml volumes; Guthion Tests 3 and 4 used both 10- and 100-ml test volumes. The Guthion 2S test was conducted in 100-ml volumes. There were 10 embryos per replicate and two replicates per concentration in Test 1. The remaining tests had 20 embryos per replicate and three replicates per concentration. The 100-ml test volumes allowed the collection of sufficient test solution for pesticide analysis. The tests were conducted in an environmental chamber and kept on a 16:8 light:dark schedule.

Dead organisms were removed daily and mortality was recorded. Gross terata in surviving animals were evaluated at test conclusion. Embryo length (taking into consideration observed curves or contours) was measured with an ocular micrometer and compared to the controls at the end of the test. Survivors were euthanized with MS-222 (Methane tricaine sulfonate) prior to evaluation and measurement.

Analytical Procedures

The percent purity of the technical grade Guthion (*O,O*-Dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4-H)-yl)methyl] phosphorodithioate) (Chem Service Inc[1], West Chester, PA) was 99%; the purity of the formulation grade Guthion 2S (Mobay Corp, Kansas City, MO) was 22%. Dimethyl formamide (DMF) was used as a carrier in preparation of the Guthion and Guthion 2S stocks. The concentration of DMF in all test solutions, including the control, was 100 ppm for Guthion Tests 1, 3, and 4 and the Guthion 2S test and was 65 ppm for Guthion Test 2. Each test was also run with a carrier-free control. Measured guthion concentrations were analyzed from a 110-ml pooled water sample comprised of equal volumes taken from each stock bottle or replicate 100-ml test vessel. The samples were extracted for Guthion with toluene via liquid-liquid extraction in a 100-ml serum bottle on a reciprocating shaker (Henderson *et al.* 1977). Samples were analyzed on a Model 5890 Hewlett-Packard high resolution gas chromatograph with a 25 meter SE-54 column. The GC was operated in the split mode with a nitrogen-phosphorus flame ionization gas detector. Approximately 10% of the samples were run in duplicate. Several standards were analyzed at the beginning of an analytical run, with a single standard analyzed after every five samples. Five to six standards (typically 0.48, 2.77, 5.51, 14.69, 24.43, and 34.14 ng Guthion) were used in the calculation of a standard curve. The slope, y-intercept, and correlation coefficient of a typical curve were 0.6094, −0.003, and 0.998, respectively. The detection limit for Guthion in water was 10 μg/L. The specific recovery of Guthion was 96.2% ± 1.0 (mean ± SE, n = 37). The mean coefficient of variation for paired duplicate samples was 3.7 (n = 36 pairs). The mean percent loss of Guthion in the test containers over each 24-h period was 4.2% ± 1.36 (mean ± SE, n = 103).

Calculations

Data from replicates were pooled prior to calculating LC50s (median lethal concentration), EC50s (median effective concentration based on malformation), and 95% confidence intervals by the trimmed Spearman-Karber method (Hamilton *et al.* 1977). The EC50s were based on the number of surviving embryos. The Teratogenic Index (TI) was derived by dividing the 96-h LC50 by the 96-h EC50 (malformation) (ASTM 1991). LOAEL (Lowest Observed Adverse Effects Level, the lowest concentration producing adverse effects significantly different from the controls) and NOAEL (No Observed Adverse Effects Level, the highest concentration producing no adverse effects significantly different from the controls) values were determined by Dunnett's multiple comparison procedure (Computer Sciences Corp. 1988). Percentage mortality and deformity data were adjusted with an arcsine square root transformation prior to calculating LOAELs and NOAELs. Carrier controls were used in all comparisons with different exposure concentrations of Guthion. Guthion and Guthion 2S water exposure concentrations were calculated as the mean of the total number of daily exposure values (a daily exposure value was the average of the measured stock solution at test start, and the measured test container sample at the end of each 24-h period).

Results and Discussion

Hatching begins in *X. laevis* at about age 48 h at 22–24°C (New 1966). Percent hatch in the Guthion tests, relative to carrier control hatch, was never less than 91% (Table 1). In the

[1]Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Table 1. Hatchability, mortality, deformity, and length in *Xenopus laevis* embryos exposed to Guthion® and Guthion® 2S for 96 h

Mean ± SE measured conc. (mg/L AI)	10 ml exposure volume of test solution				100 ml exposure volume of test solution			
	Percent[a] hatch	Percent mortality	Percent[b] deformed	Mean ± SE length (mm)	Percent[a] hatch	Percent mortality	Percent[b] deformed	Mean ± SE length (mm)
Guthion Test 1								
<0.01 CF[c]					100.0	5.0	0	10.8 ± 0.1
<0.01 CC[d]					100.0	10.0	0	10.8 ± 0.0
0.02 ± 0.0					100.0	0	0	10.8 ± 0.0
0.03 ± 0.0					96.7	25.0	0	10.9 ± 0.0
0.10 ± 0.0					91.4	20.0	0	10.8 ± 0.2
0.33 ± 0.01					100.0	5.0	5.2	10.9 ± 0.1
0.89 ± 0.05					100.0	10.0	0	10.8 ± 0.1
Guthion Test 2								
<0.01 CF[c]					100.0	3.3	3.4	10.7 ± 0.1
<0.01 CC[d]					100.0	10.0	0	10.7 ± 0.0
0.21 ± 0.01					95.0	1.7	1.7	10.6 ± 0.1
0.61 ± 0.01					100.0	8.3	1.8	10.7 ± 0.1
1.25 ± 0.02					100.0	3.3	1.7	10.5 ± 0.1
3.05 ± 0.10					100.0	1.7	3.4	9.6 ± 0.1[e]
7.62 ± 0.34					100.0	1.7	91.5[e]	7.5 ± 0.1[e]
Guthion Test 3								
<0.01 CF[c]	100.0	1.7	0	7.8 ± 0.0	100.0	5.0	1.8	10.6 ± 0.1
<0.01 CC[d]	100.0	5.0	5.3	7.9 ± 0.1	100.0	5.0	1.8	10.4 ± 0.1
0.51 ± 0.01	100.0	3.3	10.3	7.8 ± 0.1	100.0	3.3	0	10.5 ± 0.0
1.31 ± 0.02	100.0	5.0	24.6[e]	7.5 ± 0.2[e]	100.0	3.3	3.4	10.4 ± 0.1
3.20 ± 0.08	100.0	5.0	73.7[e]	6.8 ± 0.1[e]	100.0	1.7	1.7	9.4 ± 0.1[e]
7.96 ± 0.29	98.3	71.7[e]	100.0[e]	6.7 ± 0.1[e]	96.7	3.3	98.2[e]	7.4 ± 0.1[e]
18.57 ± 1.34	100.0	100.0[e]			100.0	100.0[e]		
Guthion Test 4								
<0.01 CF[c]	100.0	3.3	1.7	8.7 ± 0.2	100.0	3.3	0	10.6 ± 0.1
<0.01 CC[d]	100.0	0	1.7	8.6 ± 0.1	100.0	0	1.7	10.6 ± 0.1
0.82 ± 0.01	100.0	8.3	5.4[e]	8.4 ± 0.02	100.0	3.3	1.7	10.5 ± 0.2
3.08 ± 0.04	100.0	6.7	89.3[e]	7.6 ± 0.2[e]	100.0	3.3	1.7	9.7 ± 0.1[e]
6.36 ± 0.09	98.3	38.3[e]	100.0[e]	7.3 ± 0.2[e]	100.0	3.3	8.6[e]	8.5 ± 0.1[e]
9.56 ± 0.12	100.0	100.0[e]			100.0	18.3[e]	95.9[e]	6.9 ± 0.0[e]
13.15 ± 0.24	100.0	100.0[e]			100.0	100.0[e]		
15.68 ± 0.09	100.0	100.0[e]			100.0	100.0[e]		
Guthion 2S Test								
<0.01 CF[c]					95.0	5.0	8.8	10.2 ± 0.1
<0.01 CC[d]					100.0	6.6	1.8	10.4 ± 0.1
0.02 ± 0.0					98.3	6.6	3.6	10.2 ± 0.2
0.06 ± 0.0					98.3	6.6	3.6	10.3 ± 0.2
0.12 ± 0.0					100.0	5.0	5.3	10.4 ± 0.1
0.48 ± 0.01					98.3	13.3	3.8	10.1 ± 0.0
1.30 ± 0.04					98.3	25.0[e]	2.2	9.2 ± 0.1[e]
3.80 ± 0.05					0	100.0[e]		

[a] Percent exposure hatch relative to percent carrier control hatch
[b] Percent survivors deformed
[c] CF = carrier-free control
[d] CC = carrier control
[e] Significantly ($p < 0.05$) different from carrier control

Guthion 2S test, 0% hatched at the top concentration of 3.8 mg/L; the rate of hatch ranged from 98.3% to 100% for the remainder of the concentrations. The high rate of hatching success in embryos exposed in the Guthion tests was probably due to a lack of effect of the pesticide on nerve transmission at this early stage of the developing nervous system. The lack of hatching at the highest concentration in the Guthion 2S test was probably due to "inert" ingredients (e.g., surfactants) that can penetrate the egg and cause mortality by different modes of action. Control mortality in the carrier-free controls was never more than 5% and averaged 4.3%; mortality in the carrier-controls was never more than 10% and averaged 6.3%.

There were no significant ($p < 0.05$) effects on mortality, length or deformity in Guthion Test 1 (highest test concentration of 0.89 mg/L Guthion) (Table 1). In Guthion Test 2, with a top concentration of 7.62 mg/L, there was also no significant ($p < 0.05$) effect on mortality; however, there was over 90% deformity at 7.62 mg/L Guthion. There was also a significant

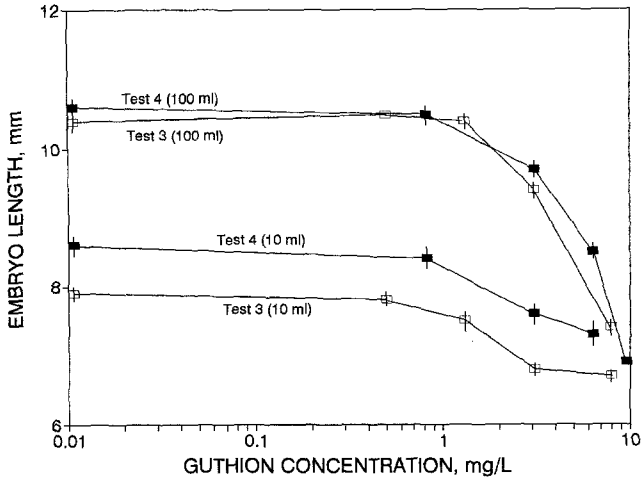

Fig. 1. Total body length (± SE) of *X. laevis* embryos exposed to Guthion for 96 h in 10 and 100 ml test solution (Guthion Tests 3 and 4)

Table 2. Ninety-six hour LC50 and EC50 values (active ingredient) for *Xenopus laevis* embryos exposed to Guthion® and Guthion® 2S

Test no.	Test volume (ml)	LC50 (mg/L) (95% CI)	EC50[a] (mg/L) (95% CI)	TI[b]
Guthion 1	100	>0.89	>0.89	—
Guthion 2	100	>7.62	4.95 (4.73–5.18)	>1.54
Guthion 3	10	6.10 (5.33–6.99)	2.01 (1.67–2.42)	3.03
	100	11.89 ([c])	4.91 (4.62–5.22)	2.42
Guthion 4	10	6.28 (5.69–6.93)	1.66 (1.51–1.84)	3.78
	100	10.63 (10.21–11.07)	7.63 (7.25–8.03)	1.39
Guthion 2S	100	1.60 (1.32–1.95)	>1.30	<1.23

[a] EC50 based on malformed survivors
[b] TI = teratogenic index (LC50/EC50)
[c] CI not calculable

decrease ($p < 0.05$) in embryo length at concentrations of Guthion of 3 mg/L and higher (Table 1).

Embryos exposed to 10-ml volumes of Guthion exhibited greater mortality, increased deformities and decreased size as compared to those exposed to 100 ml at the same concentrations (Table 1). Percent mortality increased dramatically at concentrations greater than 3 mg/L in 10-ml exposure volumes in Guthion Tests 3 and 4 (Table 1). In Guthion Test 3, over 70% were dead at 7.96 mg/L in 10 ml as compared to only 3% dead in 100 ml. In Guthion Test 4, 100% of the embryos were dead at 9.6 mg/L in 10 ml as compared to only 18% mortality in 100 ml.

Deformities in Guthion Tests 3 and 4 occurred in 10-ml exposure volumes at lower Guthion concentrations than in 100 ml (Table 1). In Guthion Test 3, 73% of the survivors were deformed at 3 mg/L in 10 ml as compared to less than 2% in 100 ml. Similarly, in Guthion Test 4 at the same concentration, 89% of the embryos were deformed in 10 ml, while in 100 ml there was less than 2% deformity.

The embryos were smaller in 10-ml exposure volumes than in 100-ml in Guthion Tests 3 and 4 (Table 1, Figure 1). Mean embryo length in the carrier controls in Guthion Test 3 were 7.9 and 10.4 mm, respectively, for 10 and 100 ml. In Guthion Test 4, the carrier control embryo lengths were 8.6 and 10.6 mm, respectively for 10 and 100 ml. Significant decreases ($p < 0.05$) in length as compared to the carrier controls occurred at 1.3 mg/L and greater Guthion in 10 ml and at 3.2 mg/L and greater in 100 ml in Test 3. Similar decreases occurred at 3.08 mg/L Guthion and higher for both exposure volumes in Guthion Test 4.

Ninety-six hour LC50 values for Guthion in 100-ml exposure volumes ranged from >7.62 mg/L to 11.89 mg/L (Table 2). Higher Guthion toxicity was indicated in 10-ml volumes by 96-h LC50s which ranged from 6.10 to 6.28 mg/L. A similar pattern occurred for the 96-h EC50s where values for 100 ml ranged from 4.95 to 7.63 mg/L and the values for 10 ml were only 1.66 to 2.01 mg/L. The teratogenic index (TI = LC50/EC50) is a measure of teratogenicity (Bantle *et al.* 1989); a value of over 1.5 indicates a significant teratogenic risk. The mean TI obtained from Guthion Tests 3 and 4 in 10 ml was 3.4 (Table 2). The mean value for 100 ml in these tests was 1.9, indicating less teratogenicity when using the larger exposure volumes.

The NOAEL and LOAEL values also indicated greater toxicity in 10-ml exposure volumes as compared to 100-ml volumes of Guthion (Table 3). Mean NOAELs for length, deformity, and mortality for 10-ml volumes were 0.66, 0.51, and 3.14 mg/L Guthion, respectively. Corresponding values for the 100-ml volumes were 1.13, 3.11, and 7.16 mg/L Guthion. Mean corresponding LOAELs were 2.20, 1.31, and 7.16 mg/L Guthion in the 10-ml volumes and 3.11, 7.31, and 14.06 mg/L Guthion in the 100-ml volumes.

The embryos were more sensitive to Guthion 2S (which was tested only in 100 ml) than Guthion. There was 100% mortality in the Guthion 2S test at a concentration of 3.8 mg/L as compared to only 3% or less mortality at a concentration of ≤ 3.2 mg/L Guthion in Guthion Tests 2-4 (Table 1). The LC50 of 1.6 mg/L (AI) obtained in the Guthion 2S test was much lower than the LC50s of 11.89 and 10.63 mg/L obtained in Guthion Tests 3 and 4 (Table 2). Embryo length was significantly less than the controls ($p < 0.05$) at a concentration of 1.3 mg/L Guthion 2S (AI) (Table 1). There was no increase in percent deformity with increasing concentration of Guthion 2S (Table 1). Total mortality in the Guthion 2S test occurred at 3.8 mg/L AI (Table 1). This concentration was less than the 6.36 to 7.96 mg/L, which resulted in significant deformities but very low mortality in the 100 ml Guthion tests (Table 1), apparently resulting from the "inert" ingredients in the Guthion 2S formulation. Linder (1990) observed a threefold increase in acute toxicity to *Rana pipiens* for a commercial formulation of paraquat as compared to the technical grade and emphasized the need to evaluate formulations of pesticides since they are commonly encountered in the environment. Mayer and Ellersieck (1986) noted that the effects of "inert" ingredients of waterborne compounds may be as high as 2.5 orders of magnitude.

Reduced toxicity, decreased deformity, and increased growth all appeared related to the 10-fold increase in test exposure volume in the larger test containers at the same concentrations. This larger volume may have provided a greater supply of dissolved oxygen and assimilative capacity for excreted

Table 3. Ninety-six hour NOAEL and LOAEL values (active ingredient) based on length, deformity and mortality for *Xenopus laevis* embryos exposed to Guthion® and Guthion® 2S

Test no.	Length		Deformity		Mortality	
	NOAEL (mg/L)	LOAEL (mg/L)	NOAEL (mg/L)	LOAEL (mg/L)	NOAEL (mg/L)	LOAEL (mg/L)
			10 ml Exposure Volume			
Guthion 3	0.51	1.31	0.51	1.31	3.20	7.96
Guthion 4	0.82	3.08	<0.99	<0.99	3.08	6.36
Mean	0.66	2.20	0.51	1.31	3.14	7.16
			100 ml Exposure Volume			
Guthion 2	1.25	3.05	3.05	7.62	—	—
Guthion 3	1.31	3.20	3.20	7.96	7.96	18.57
Guthion 4	0.82	3.08	3.08	6.36	6.36	9.56
Mean	1.13	3.11	3.11	7.31	7.16	14.06
Guthion 2S	0.48	1.30	—	—	1.30	3.80

waste products such as ammonia. However, no chemical measurements were performed due to the small volumes in the 10-ml containers. Embryos in the larger containers would be under less crowding stress enabling a greater potential for growth during the test period. Cooke (1979) reported that *Rana temporaria* tadpoles reared at 50 animals/L were half the size of those reared at 10 animals/L and that the smaller tadpoles appeared to be more sensitive to DDT. The presence of breakdown products and infections from dead embryos would have less effect upon the survivors in the larger test containers. In a smaller container, decomposing embryos can cause a cascading death effect among the survivors. The larger container also has the advantage of providing sufficient volumes of test solution from which to measure actual exposure concentrations.

The lowest NOAEL values obtained in this study (Table 3) are many times greater than the USEPA water quality criterion value of 0.01 μg/L (USEPA 1986), suggesting that developmental stages of predominantly aquatic frogs such as *X. laevis* would be protected by existing water quality criteria. Amphibians can be sensitive indicators of environmental contamination especially because of their biphasic life cycles and presence in both aquatic and terrestrial environments. This study adds to the information base needed from which to draw knowledgeable conclusions about water quality criteria that protect wildlife.

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