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EFFECTS OF ORGANIC COMPOUNDS ON AMPHIBIAN REPRODUCTION

By

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ABSTRACT

Aquatic toxicity tests were conducted with atrazine, carbon tetrachloride, chloroform, methylene chloride, trisodium nitrilotriacetic acid (NTA), and phenol. Each compound was administered to developmental stages of three to five amphibian species. Exposure was initiated at fertilization and maintained through 4 days posthatching. Test responses included lethality and teratogenesis. Different amphibian species exhibited varying degrees of tolerance to the selected compounds. Greatest tolerance usually was observed for the more broadly adapted semi-aquatic and terrestrial species (e.g., *Bufo americanus*, *Bufo fowleri*). The more sensitive amphibians usually included those species which normally are restricted to aquatic or moist habitats (e.g., *Rana catesbeiana*, *Rana pipiens*).

Median lethal concentrations (mg/l) determined at 4 days posthatching ranged from 0.41 to >48 for atrazine, 0.90 to 2.83 for carbon tetrachloride, 0.27 to 35.14 for chloroform, 17.78 to >32 for methylene chloride, 39.3 to 252.3 for NTA, and 0.04 to >0.89 for phenol. The most toxic compounds always included phenol, carbon tetrachloride, and atrazine, and the least toxic consistently were NTA and methylene chloride. For three chlorinated alkanes, including methylene chloride (CH_2Cl_2), chloroform ($CHCl_3$), and carbon tetrachloride (CCl_4), toxicity increased with chlorination. Toxicity of the different compounds was further characterized by calculating concentrations which produced embryo-larval lethality or teratogenesis at frequencies of 10% (LC_{10}) and 1% (LC_1). On the basis of LC_1 values, *Hyla crucifer*, *Rana catesbeiana*, and *Rana pipiens* generally exhibited sensitivity equal to or slightly greater than that observed for embryo-larval stages of the rainbow trout.

Descriptors:	Identifiers:
Embryos	Atrazine
Larvae	Carbon Tetrachloride
Terata	Chloroform
Organic Compounds	Methylene Chloride
Phenol	NTA
Water Quality	Phenol
	Amphibians*
	Aquatic Toxicity Tests
	Embryo-Larval Tests

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CHAPTER I

INTRODUCTION

In recent years, it has become obvious that organic trace contaminants pose a formidable hazard to aquatic ecosystems. This is particularly true for aromatic and chlorinated hydrocarbons, many of which are characterized by long environmental half-life, high tendencies for bioaccumulation in animal tissues, appreciable levels of biomagnification through aquatic food chains, and a propensity for extreme toxicity to embryos and other reproductive stages (Jensen, et al., 1970; Hansen, et al., 1971; NAS-NAE Committee, 1973; Nelson, 1972; Bowes, et al., 1973; Nebeker, et al., 1974; Hansen, et al., 1975; Stephenson, 1975). However, due to wide variations in volatility and solubility, chlorinated hydrocarbons and many other organic toxicants are extremely difficult to stabilize and test accurately in conventional bioassay systems (Schoor, 1975; Veith and Comstock, 1975). For example, in the open fish tank cultures most commonly used in generating data for environmental standards, volatile compounds are lost from test waters, and compounds of low solubility usually cannot be maintained in uniform suspension or at constant levels without using emulsifying or carrier solvents which introduce undesirable test variables. Such technical problems have led to serious delays in identifying, detecting, and adequately quantifying the toxicity of important categories of organic pollutants, including aliphatic and aromatic compounds (e.g., benzene, phenol), chlorinated hydrocarbons (e.g., PCB, PVC, DDT, chloroform, 2,4-D), phthalates and other plasticizers, and organophosphates.

In view of these problems, a continuous flow system was developed for evaluating effects of insoluble and volatile organics on developmental stages of fish (Birge, et al., 1979b). A closed exposure chamber, devoid of an air-water interface, was used to minimize evaporative loss of water and toxicant. Insoluble compounds were suspended in influent water by mechanical homogenization and maintained in suspension by continuous agita-

tion supplied to the exposure chamber and by regulation of detention time. This flow-through system proved highly effective in toxicity tests with embryo-larval stages of fish (Birge, et al., 1979a, b). The principal objectives of the present study were to (1) adapt this new bioassay system for use with amphibian eggs and larvae, (2) evaluate the toxic effects of organic pollutants on amphibian developmental stages (e.g., lethality, teratogenesis), and (3) compare the sensitivity of a number of representative amphibian species. Concerning the latter, attempts were made to discern adaptive traits which correlate with increased sensitivity or tolerance to pollution stress and to identify amphibian species considered opportune for inclusion in toxicity testing programs. In pursuing this investigation, toxicity tests were performed with six organic compounds, including atrazine, carbon tetrachloride, chloroform, methylene chloride, NTA, and phenol. Most of these compounds appear on EPA's list of priority toxicants (U.S. EPA, 1978), and all have been identified as important, widely distributed aquatic contaminants which are in need of further bioassay characterization (Stephenson, 1975; Shackelford and Keith, 1976). Each compound was tested using embryo-larval stages of three to five amphibian species.

It is particularly germane at this time to evaluate the effects of organic pollutants on amphibian reproduction. As recently noted by various investigators (Gibbs, et al., 1971; Anonymous, 1973), amphibian populations in numerous geographical regions of the U.S. have suffered substantial reductions. Though contributing causes have not been adequately analyzed, it is one consensus that reproductive stages of amphibians may be extremely sensitive to certain organic and inorganic toxicants (Gibbs, et al., 1971; Birge, et al., 1978; Birge and Black, 1979; Birge, et al., 1979d). In a previous investigation (Birge, et al., 1979c), embryo-larval stages of the narrow-mouthed toad were found to be more sensitive than trout embryos and alevins to 18 of 22 inorganic toxicants, based on median lethal concentrations (LC_{50}). The ability of different amphibian species to withstand the effects of certain metals appeared to increase with tolerance to natural environmental stresses. Toxicity data also showed amphibian embryos to be at least 1,000 times more sensitive than adult

frogs to mercury (Birge, et al., 1979d). As amphibian species are of considerable economic value and are essential in the structure and balance of many aquatic ecosystems, it is important to assess their susceptibility to organic contaminants, and to establish environmental safeguards which are adequate to protect amphibian reproduction.

CHAPTER II

TEST SYSTEM AND EXPERIMENTAL PROCEDURES FOR EMBRYO-LARVAL BIOASSAYS WITH AMPHIBIAN SPECIES

Selection of animal species. Amphibians used in this study included *Bufo americanus* (American toad), *Bufo fowleri* (Fowler's toad), *Bufo quercicus* (oak toad), *Hyla crucifer* (spring peeper), *Rana catesbeiana* (bullfrog), *Rana palustris* (pickerel frog), and *Rana pipiens* (leopard frog). These anuran species were selected to represent differences in patterns of reproduction, ecological habitat, and geographical variation, to determine whether such factors correlated with susceptibility to environmental toxicants.

Leopard frogs were procured from Mogul-Ed, Oshkosh, Wisconsin, and oak toads were supplied by Charles Sullivan, Nashville, Tennessee. Eggs were obtained by inducing ovulation with pituitary extract, following the procedure of Rugh (1962). Fertilization was accomplished by mixing eggs with a sperm suspension for 30 minutes. Freshly fertilized eggs from all other species were collected locally from the Frankfort National Fish Hatchery, Frankfort, Kentucky.

Selection of organic toxicants. Toxicity tests were conducted with atrazine, carbon tetrachloride, chloroform, methylene chloride, trisodium nitrilotriacetic acid (NTA), and phenol. All analytical and toxicity data were expressed as concentrations (mg/l) of analytical or spectrophotometric grade compounds, except for atrazine which was reported as the wettable powder (80% pure). These compounds are known to affect important waterways in the eastern U.S. (Stephenson, 1975; Shackelford and Keith, 1976; U.S. EPA, 1976), and all except atrazine and NTA appear on the initial list of 65 priority toxicants identified by EPA (U.S. EPA, 1978).

Test conditions and expression of data. Each organic compound was tested at five or more concentrations, and all bioassays were conducted using medium hard water (100 mg/l $CaCO_3$). Exposure was initiated within

30 minutes of fertilization in *R. pipiens* and *B. quercicus*, and 2 to 6 hours postspawning for the other species. Average hatching times were 5, 4, 4, 3, 3, 3, and 2 days for *R. pipiens*, *R. palustris*, *R. catesbeiana*, *H. crucifer*, *B. americanus*, *B. fowleri*, and *B. quercicus*, respectively. Toxicity tests were performed in temperature-regulated environmental rooms. Test water was monitored at regular intervals for temperature, dissolved oxygen, water hardness, pH, and specific conductivity, using a YSI tele-thermometer with thermocouple (model 42SC), YSI oxygen meter (model 51A), Orion divalent cation electrode (model 93-92), Corning digital pH meter (model 110), and a Radiometer conductivity meter (model DCM 2e). Monitoring data for these parameters were given in Table 1.

Control eggs were cultured simultaneously with experimentals and under identical conditions, except for omission of the toxicants. Eggs were examined daily to gauge extent of development and to remove dead specimens. Sample size ranged from 50 to 130 eggs per exposure concentration. Percent survival of normal organisms was expressed as the frequency in experimental populations/controls and was determined at hatching and 4 days after hatching. Normal organisms were defined as those animals free of gross teratic defects. In all instances, survival frequencies were based on accumulative test responses incurred from onset of treatment. Percent egg hatchability included all embryos, normal or aberrant, which completed the hatching process. Teratogenesis was determined at hatching and expressed as the percent of survivors affected by gross, debilitating abnormalities likely to result in eventual lethality (Birge and Black, 1977). Terata were infrequent in control populations and seldom exceeded 1%. Counting teratic larvae as lethals, log probit analysis (Finney, 1971) was used to compute control-adjusted LC_{50}, LC_{10}, and LC_1 values with 95% confidence limits. The LC_1, taken as the concentration which produced 1% control-adjusted impairment in test populations, was used to estimate the toxicity threshold for each compound.

Test water. Reconstituted test water was prepared by the addition of reagent-grade calcium, magnesium, sodium, and potassium salts to distilled, double deionized water. Physicochemical characteristics were summarized in Table 2. Concentrations of cations and anions were within

Table 1. General water characteristics observed during toxicity tests with amphibian embryo-larval stages.

Embryo-Larval Bioassays		Observed Test Parameters (Mean ± Standard Error)				
Compound	Test Species	Temperature (°C)	Dissolved Oxygen (mg/l)	Water Hardness (mg/l $CaCO_3$)	pH	Conductivity (µmhos/cm)
Atrazine	*R. catesbeiana*	20.0 ± 0.10	8.9 ± 0.1	113.3 ± 2.2	7.4 ± 0.04	174.3 ± 6.6
	R. pipiens	19.9 ± 0.11	8.7 ± 0.2	114.6 ± 2.0	7.6 ± 0.01	153.9 ± 1.3
	R. palustris	20.5 ± 0.10	9.0 ± 0.1	103.4 ± 1.5	7.5 ± 0.02	159.3 ± 2.9
	B. americanus	19.0 ± 0.00	8.5 ± 0.1	102.9 ± 1.7	7.4 ± 0.05	170.1 ± 2.6
Carbon tetrachloride	*R. catesbeiana*	20.7 ± 0.57	8.8 ± 0.1	107.9 ± 2.1	8.0 ± 0.02	172.1 ± 2.6
	R. palustris	21.5 ± 0.01	8.8 ± 0.1	103.8 ± 1.0	7.7 ± 0.01	152.7 ± 0.8
	B. fowleri	21.5 ± 0.01	8.8 ± 0.1	103.8 ± 1.0	7.7 ± 0.01	152.7 ± 0.8
Chloroform	*H. crucifer*	20.5 ± 0.10	9.0 ± 0.1	107.5 ± 2.6	7.6 ± 0.03	158.3 ± 0.9
	R. pipiens	20.4 ± 0.11	8.1 ± 0.1	107.9 ± 1.6	7.5 ± 0.01	161.8 ± 1.0
	R. palustris	21.5 ± 0.01	8.7 ± 0.2	104.2 ± 1.0	7.6 ± 0.03	154.2 ± 1.2
	B. fowleri	21.5 ± 0.01	8.7 ± 0.2	104.2 ± 1.0	7.6 ± 0.03	154.2 ± 1.2
Methylene chloride	*R. catesbeiana*	20.7 ± 0.57	8.8 ± 0.1	106.8 ± 1.3	7.9 ± 0.02	171.3 ± 2.1
	B. fowleri	21.5 ± 0.01	8.8 ± 0.1	106.8 ± 0.9	7.6 ± 0.03	158.6 ± 1.1
	R. palustris	21.5 ± 0.01	8.8 ± 0.1	106.8 ± 0.9	7.6 ± 0.03	158.6 ± 1.1

Table 1 - continued.

Embryo-Larval Bioassays		Observed Test Parameters (Mean ± Standard Error)				
Compound	Test Species	Temperature (°C)	Dissolved Oxygen (mg/l)	Water Hardness (mg/l $CaCO_3$)	pH	Conductivity (μmhos/cm)
NTA	*R. pipiens*	19.9 ± 0.11	8.4 ± 0.2	115.0 ± 2.1	7.7 ± 0.05	155.8 ± 2.1
	R. catesbeiana	20.0 ± 0.10	9.0 ± 0.0	114.8 ± 3.4	7.8 ± 0.07	196.7 ± 7.8
	R. palustris	20.5 ± 0.10	8.9 ± 0.0	110.4 ± 4.4	7.9 ± 0.09	168.7 ± 2.4
	B. fowleri	20.0 ± 0.10	9.0 ± 0.0	114.8 ± 3.4	7.8 ± 0.07	196.7 ± 7.8
	B. quercicus	23.7 ± 0.33	8.2 ± 0.1	96.0 ± 3.7	7.8 ± 0.02	196.0 ± 3.4
Phenol	*R. pipiens*	19.0 ± 0.00	9.1 ± 0.1	113.8 ± 1.4	7.6 ± 0.02	147.1 ± 0.9
	R. catesbeiana	20.0 ± 0.10	9.0 ± 0.1	113.1 ± 2.1	7.5 ± 0.04	178.1 ± 4.1
	B. fowleri	20.0 ± 0.10	9.0 ± 0.1	113.1 ± 2.1	7.5 ± 0.04	178.1 ± 4.1
	R. palustris	20.5 ± 0.10	8.9 ± 0.1	106.4 ± 0.9	7.7 ± 0.01	167.0 ± 0.7
	B. americanus	19.0 ± 0.00	8.5 ± 0.1	105.1 ± 1.1	7.4 ± 0.02	165.1 ± 1.8

Table 2. Reconstituted test water.

Hardness as $CaCO_3$:	50 mg/l	100 mg/l	200 mg/l
DISSOLVED SALTS[1], mg/l			
$CaCl_2$	37.5	75.0	150
$MgSO_4 \cdot 7H_2O$	37.5	75.0	150
$NaHCO_3$	100	100	100
KCl	5	5	5
CHEMICAL COMPOSITION, mg/l			
Ca	13.6	27.1	54.2
Mg	3.7	7.4	14.8
Na	27.4	27.4	27.4
K	2.6	2.6	2.6
Cl	26.3	52.3	98.2
HCO_3	72.6	72.6	72.6
SO_4	14.6	29.2	58.5
PHYSICOCHEMICAL CHARACTERISTICS[2]			
Hardness, as mg/l $CaCO_3$	53.3 ± 1.3	101.6 ± 4.4	197.5 ± 5.8
pH	7.84 ± 0.02	7.70 ± 0.01	7.78 ± 0.02
Total alkalinity, as mg/l $CaCO_3$	66.7 ± 0.4	65.0 ± 0.4	65.3 ± 0.6
Conductivity, µmhos/cm	133.6 ± 1.4	176.0 ± 1.0	282.0 ± 1.9
Osmolarity, mOsm/Kg H_2O	8.9 ± 0.2	10.8 ± 0.3	12.7 ± 0.4
Total dissolved solids, mg/l	121.4 ± 4.4	171.8 ± 2.0	336.7 ± 7.8
Dissolved oxygen, mg/l at 13.5°C	9.9 ± 0.2	10.1 ± 0.2	10.1 ± 0.2

[1]Prepared in distilled, deionized water with a specific conductivity of 0.25 µmhos or less.

[2]Measurements made at 25°C except where noted. Mean with standard error determined for 10 replicates.

ranges published for freshwater resources in Arizona (Dutt and McCreary, 1970), Kentucky (U.S. Geological Survey, 1970), and other areas of the U.S. (McKee and Wolf, 1963; Mount, 1968). Total chloride content, total dissolved solids, and the concentration of sodium plus potassium were under maximum levels of 170 mg/l, 400 mg/l, and 85 mg/l observed for 95% of U.S. waters found to support a good, mixed aquatic fauna (Hart, et al., 1945). Specific conductivity compared favorably with values of 150 to 500 µmhos/cm recommended for fish propagation (McKee and Wolf, 1963), and osmolarity was well under the maximum limit of 50 mOsm/kg water suggested for U.S. freshwaters (National Technical Advisory Committee, 1968). Total alkalinity and pH also were within optimum ranges for aquatic habitat (Baas Becking, et al., 1960; McKee and Wolf, 1963; NTAC, 1968). As maintained in the test system described below, dissolved oxygen ranged from 8.4 to 9.1 mg/l at temperatures of 19.0 to 23.7°C.

This reconstituted water was used previously in embryo-larval tests with a broad array of inorganic and organic toxicants, and results compared closely with those obtained when toxicants were administered in natural waters of similar composition (Birge, et al., 1979a, e). Reconstituted water was used in this study to provide reproducible test conditions required in evaluating comparative sensitivity of different animal species. Natural waters often are subject to substantial seasonal fluctuations in composition (e.g., dissolved solids, hardness, pH), and they frequently contain background contaminants.

Embryo-larval test system. Toxicity tests were conducted using the flow-through system illustrated in Figures 1 and 2. Using graduated flow from a syringe pump, toxicant was administered to a mixing chamber which was situated ahead of each egg exposure chamber. Test water was delivered to the mixing chamber by regulated flow from a peristaltic pump. Continuous aeration was supplied to the peristaltic pump reservoirs. Solutions from the two pump channels were mixed by mechanical stirring or homogenization, and delivered from the mixing unit to the test chamber under positive pressure. Toxicant exposure level was regulated by adjusting the mixing ratio between pumping units and/or by varying the concentration of toxicant delivered from the syringe pump. Flow rates from syringe

and peristaltic pumps were monitored using Gilmont micro and no. 12 liquid flow meters, respectively. Flow rate was set at 200 ml/hr for 500-ml test chambers, giving a detention time of 2.5 hr. The flow-through system was operated using Brinkmann (model 131900) and Gilson (model HP8) multichannel peristaltic pumps and Sage syringe pumps (model 355). Sage pumps were fitted with modified syringe holders, as noted previously by Birge, et al. (1979b), and each unit was operated using up to six double-ground glass syringes. Syringe capacity varied from 1 ml to 100 ml, depending upon the toxicant.

To preclude loss of organic toxicants of high volatility (e.g., methylene chloride), a closed exposure chamber devoid of an air-water interface was used to house test animals. These test chambers were constructed from 3" Pyrex pipe joints, provided with clamp-locking O-ring seals. Using standard glass-blowing techniques, the pipe was cut and sealed to give a capacity of 0.5 liter (Figure 3). An outlet tube was annealed to the cover, with an inlet positioned near the bottom of the chamber. A stainless steel inlet screen was positioned 3 cm above the bottom of the dish, dividing the chamber into an upper egg compartment and a lower stirring compartment. Amphibian eggs were supported on the inlet screen, and a Teflon-coated magnetic stirring bar was used in the lower compartment to provide moderate, continuous agitation of test water. An upper outlet screen was used to retain test organisms. The outlet screen was held in place by a Pyrex pedestal, and the inlet screen was supported on the constricted upper wall of the stirring compartment (Figure 3). Access to test organisms was obtained by opening the water-tight joint and removing the chamber cover. Prior to opening the chamber, a rapid-disconnect was used to remove the inlet line and drain the fluid level down to the O-ring seal. When perfused with a continuous flow of oxygen-saturated water, the sealed chamber was essentially free of standing air space.

As noted above, toxicant and test water were blended by either mechanical mixing or homogenization, using mixing chambers. A stoppered 250-ml side-arm flask, operated with a magnetic stirrer (Magnestir, model S8290), was adequate for maintaining stable concentrations of water-

soluble organic compounds such as NTA (Figure 2). However, high speed homogenization was required to suspend less soluble organics (i.e., carbon tetrachloride) in test water. This was accomplished with an Oster homogenizer, equipped with a 400-ml glass container. The latter was provided with terminal inlets for syringe and peristaltic pump lines and a side outlet for supply of water-toxicant homogenate to the test chamber (Figures 3.1, 3.2). Pyrex tubing (3 mm O.D.) was used to extend pump inlet lines to a depth of 3 cm above the stirring blades. Though homogenization initially was maintained continuously, intermittent operation generally proved adequate. Blending time was regulated with an electronic timer and varied for different organic compounds, depending on the stability of their aqueous suspensions. In addition, moderate agitation supplied to the exposure chamber and regulation of flow rate were used to prevent immiscible organics from partitioning out of test water.

Analytical procedures. Exposure concentrations for all organic toxicants were confirmed by daily analyses of test water, using either gas chromatography (GLC) or spectrophotometric methods. The GLC determinations (i.e., carbon tetrachloride, chloroform, methylene chloride) were performed on a Hewlett Packard gas chromatograph (model 5838A), equipped with a Purge and Trap System (model 7675A) and a flame ionization detector. Spectrophotometric analyses (i.e., atrazine, NTA, phenol) were conducted using a Varian-Techtron spectrophotometer (model 635).

Carbon tetrachloride, chloroform, and methylene chloride were analyzed directly from 2 to 15 ml aliquots of test water, using the Purge and Trap System described above. Each sample was purged with dry, pre-purified nitrogen at 10 ml/min for 10 minutes. Each compound was adsorbed on a Tenex GC trap at ambient temperature, desorbed at 200°C, and analyzed at programmed temperatures of 70 to 105°C on a 2 m X 2 mm I.D. glass column. The stationary phase was 10% Carbowax 20 M on 80/100 Anakrom U, and the detector temperature was 250°C. Nitrogen was used as the carrier gas, with a flow rate of 19 ml/min. Detection limits were 5 µg/l, 1 µg/l, and 0.5 µg/l for carbon tetrachloride, chloroform, and methylene chloride, respectively.

Atrazine was determined employing a modification of a previously reported procedure (White, et al., 1967). A 100-ml test water sample was

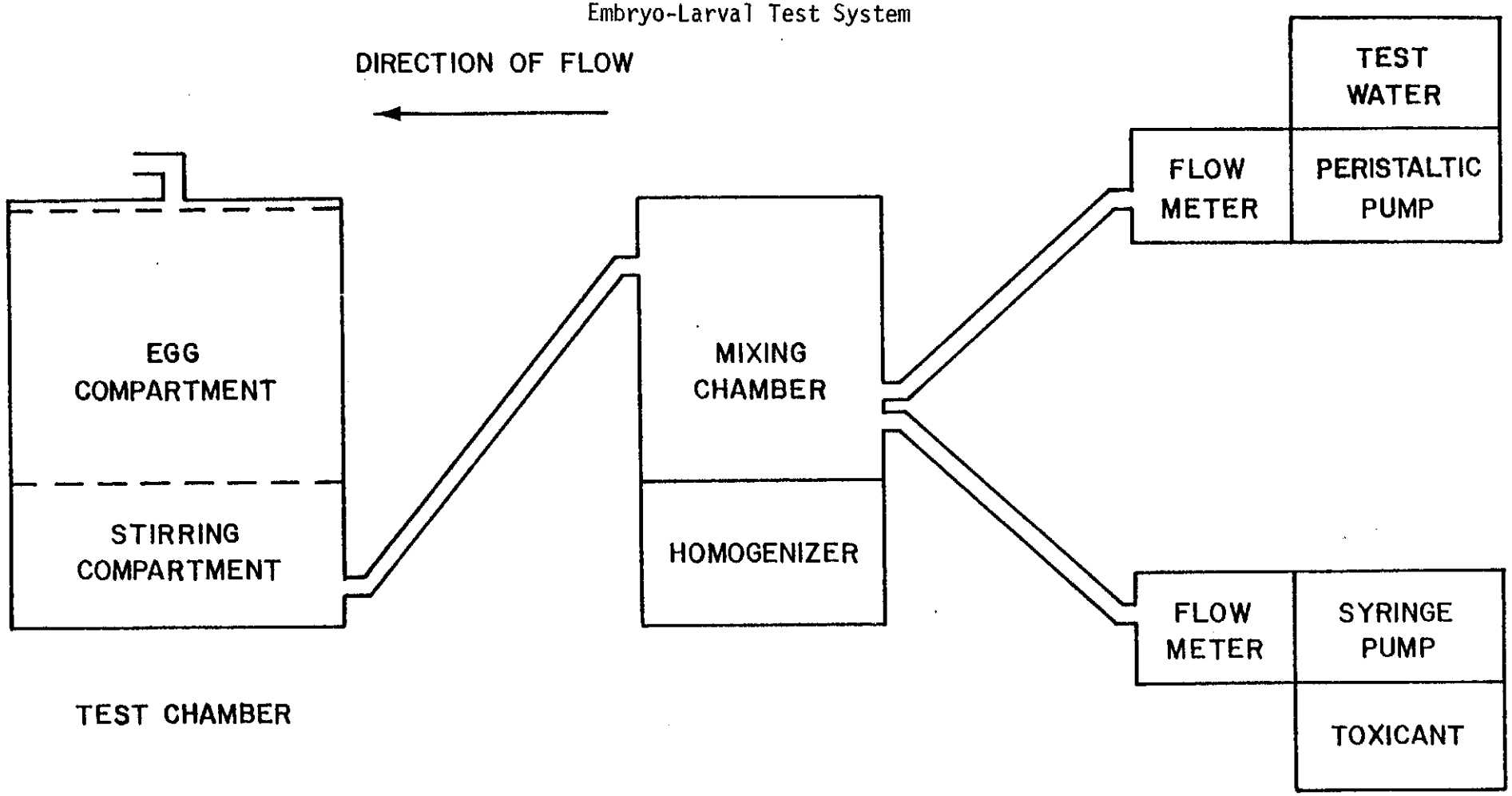
extracted with chloroform. Carbon tetrachloride (5 ml) and 50% sulfuric acid (2 ml) were added to the chloroform layer, and this mixture was shaken for 30 seconds at 15-minute intervals over a 2-hour period. The solution was transferred to a 125-ml erlenmeyer flask, mechanically mixed for 15 minutes with 20 ml of water, and allowed to stand for 2 hours. Atrazine in the water layer was analyzed spectrophotometrically at 225, 240, and 255 nm, and the detection limit was 10 µg/l.

Trisodium nitrilotriacetic acid (NTA) was analyzed by the zinc-zincon method (U.S. EPA, 1974). To prevent interference with calcium and magnesium ions, NTA samples were batch-treated with ion exchange resin (Dowex 50W-X8, 50-100 mesh). Prepared samples were quantified spectrophotometrically at 620 nm, and the detection limit was 0.5 mg/l.

Phenol concentrations were determined using the 4-aminoantipyrine procedure with chloroform extraction as described in Standard Methods (American Public Health Association, 1975). Samples were quantified spectrophotometrically at 460 nm, and the detection limit was 1.0 µg/l.

Figure 1

Embryo-Larval Test System



Test water and toxicant were supplied to the mixing chamber using peristaltic and syringe pumps. After blending, the water-toxicant mixture was perfused through the test chamber (egg compartment) under positive pressure. A magnetic stirrer was used to insure homogeneous distribution of toxicant.

Figure 2

Flow-through Bioassay System for Amphibian Embryo-Larval Stages

Peristaltic pumps (A) and syringe pumps (B) were used to supply diluent water and toxicant to mixing chambers (C and D). Water and toxicant were blended with homogenizers (C) or magnetic stirrers (D) and delivered under positive pressure to test chambers (E). The multichannel system was maintained in an environmental room and syringe pumps were mounted on the outside wall to avoid effects of high humidity on operation.

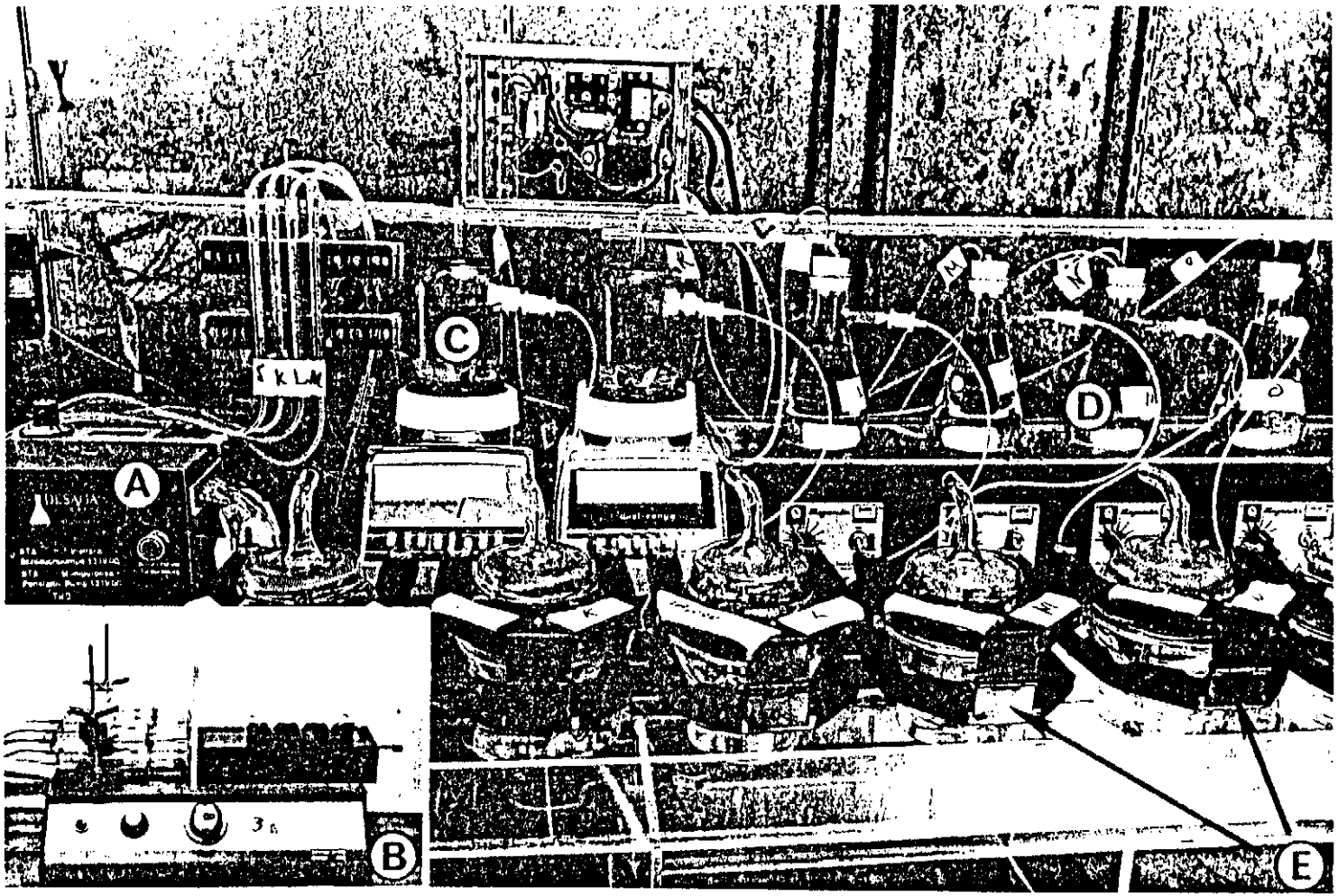
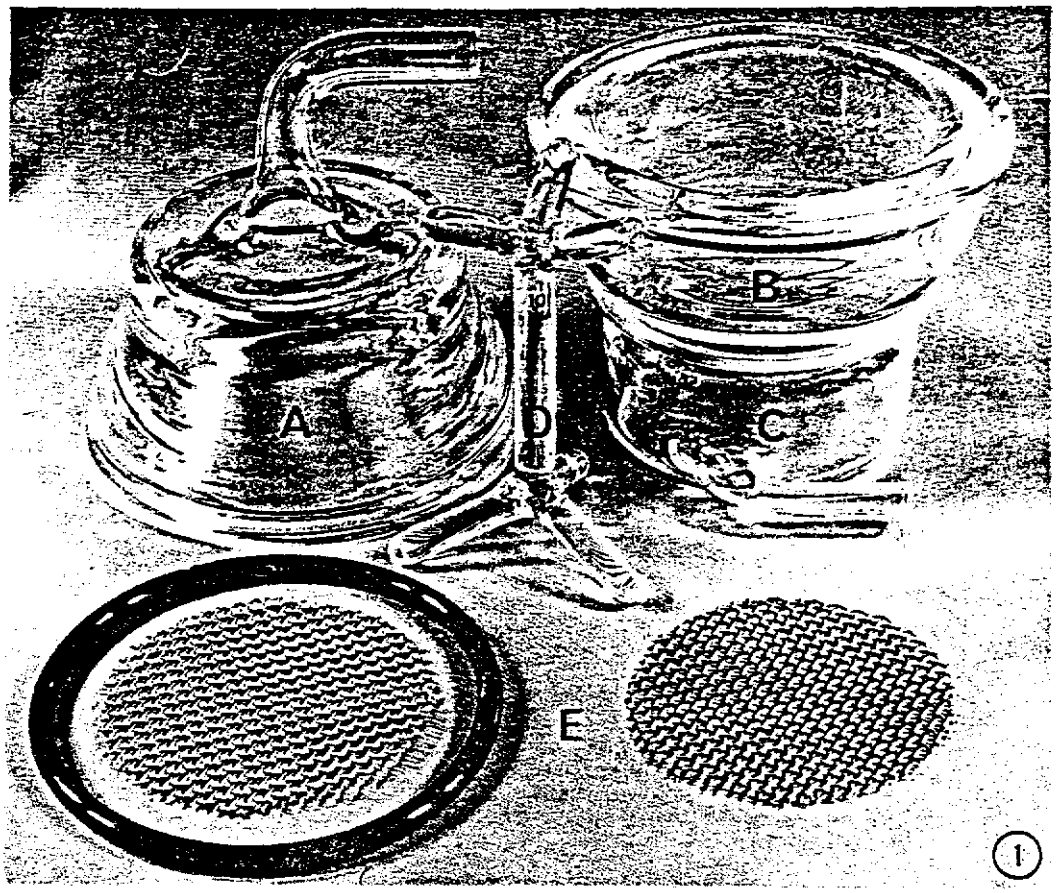

A
B
C
D
E

Figure 3

Exposure Chamber

3.1 Disassembled chamber, including cover (A), egg compartment (B), stirring compartment (C), screen support (D), and O-ring with inlet and outlet screens (E).

3.2 Assembled test chamber, showing outlet from egg compartment (A), locking clamp (B), and stirring compartment inlet (C).



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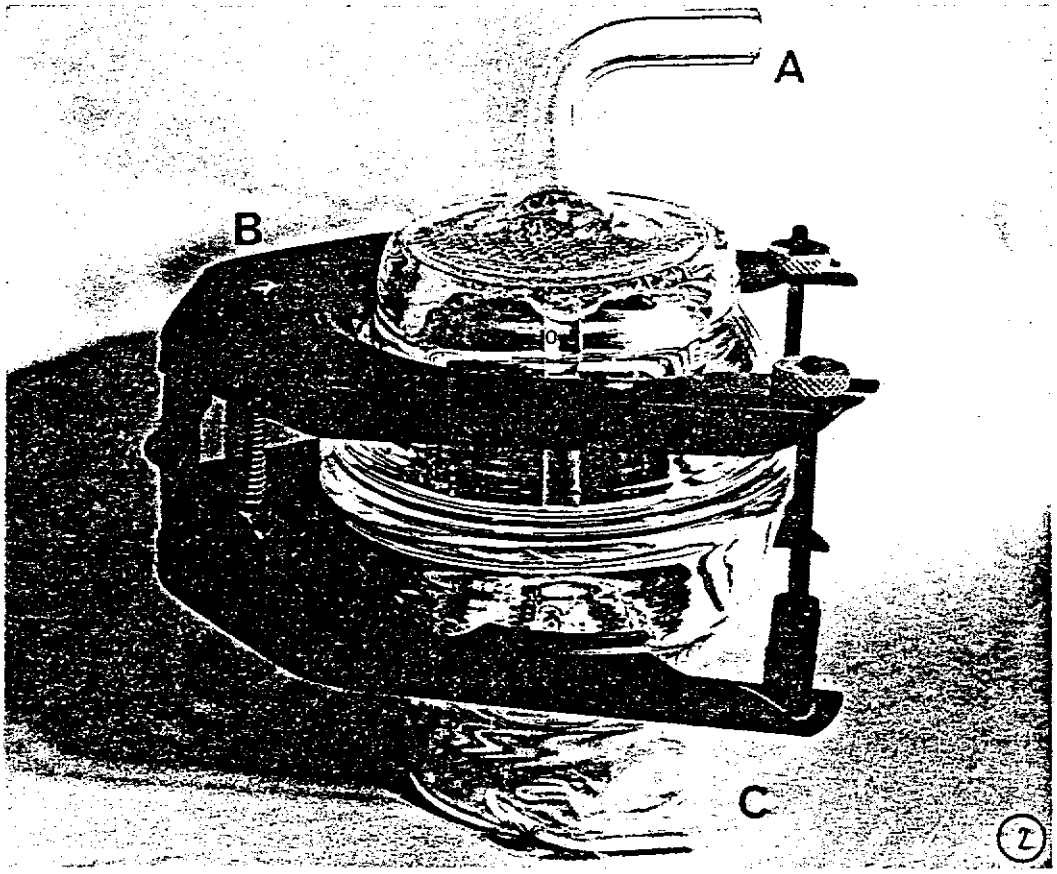

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CHAPTER III

RESULTS AND DISCUSSION

Toxicity tests on amphibian embryo-larval stages were performed with six organic compounds, using medium hard water (100 mg/l $CaCO_3$). As noted above, survival data were control-adjusted. Control survival ranged from 82% to 98%. Toxicant monitoring data indicated good reproducibility of exposure concentrations for the selected test compounds (Table 3).

Toxicity tests with atrazine were performed on embryo-larval stages of four amphibian species, including *B. americanus*, *R. catesbeiana*, *R. palustris*, and *R. pipiens* (Table 3). *Rana catesbeiana* was the most sensitive species, suffering complete lethality at atrazine concentrations as low as 14.8 mg/l. Embryopathic effects (e.g., lethality, teratogenesis) were detectable at all exposure concentrations, which ranged down to 0.051 mg/l. Frequencies of teratogenesis were 7%, 47%, and 100% at atrazine exposure levels of 6.33, 26.4, and 45.8 mg/l. Survival of normal larvae at 4 days posthatching decreased from 86% at 0.051 mg/l to 10% and 0% at 6.33 and 14.8 mg/l, respectively. Concerning all species tested, the order of increasing tolerance was *R. catesbeiana*, *R. pipiens*, *R. palustris*, and *B. americanus*, for which the LC_{50}'s at 4 days posthatching were 0.41, 7.68, 17.96, and >48 mg/l, respectively (Table 4). Embryo-larval mortality was the major test response. Though substantial frequencies of terata were observed in tests with *R. catesbeiana* and *R. pipiens*, teratogenesis was not an appreciable factor when atrazine was administered to more tolerant amphibian species (Table 3).

Carbon tetrachloride was administered to embryo-larval stages of *B. fowleri*, *R. catesbeiana*, and *R. palustris* (Table 3). *Rana catesbeiana* was the most sensitive species, and complete lethality was observed at 7.81 mg/l. Terata occurred at frequencies of 1% to 17% over a concentration range of 0.060 to 7.81 mg/l. Embryo-larval survival of normal organisms was 99%, 89%, and 63% at exposure levels of 0.026, 0.060, and 1.18 mg/l,

respectively. The order of increasing tolerance for the three test species was *R. catesbeiana*, *R. palustris*, and *B. fowleri*, based on LC_{50} values for carbon tetrachloride of 0.90, 2.37, and 2.83 mg/l taken at 4 days post-hatching (Table 4). Carbon tetrachloride was less teratogenic than atrazine to amphibian embryos. In tests with *R. palustris* and *B. fowleri*, appreciable frequencies of anomalous larvae occurred, but only at the highest exposure concentrations (Table 3).

Chloroform was tested on developmental stages of *B. fowleri*, *H. crucifer*, *R. palustris*, and *R. pipiens* (Table 3). The least tolerant species was *H. crucifer*, which exhibited complete mortality at a chloroform concentration of 7.34 mg/l. Both lethality and teratogenesis were detected at exposure levels as low as 0.0087 mg/l. Teratic larvae were observed at frequencies of 4% and 10% at chloroform concentrations of 0.073 and 0.69 mg/l. Survival of normal larvae decreased from 88% at 0.0087 mg/l to 46% and 0% at 0.69 and 7.34 mg/l. Other amphibian species were less affected by exposure to chloroform. The order of increasing tolerance was *H. crucifer*, *R. pipiens*, *R. palustris*, and *B. fowleri*, for which LC_{50}'s at 4 days post-hatching were 0.27, 4.16, 20.55, and 35.14 mg/l, respectively (Table 4). Teratogenesis was not observed to be a significant test response for the two more tolerant animal species (i.e., *R. palustris*, *B. fowleri*).

Toxicity tests with methylene chloride were conducted on eggs and larvae of *B. fowleri*, *R. catesbeiana*, and *R. palustris* (Table 3). Comparing the three amphibian species, *R. catesbeiana* was the most sensitive to this compound. Control-adjusted embryo-larval survival varied from 74% to 101% over a concentration range of 6.73 to 0.017 mg/l and decreased to 28% at 46.8 mg/l. Anomalous larvae were observed at frequencies of 1%, 6%, and 20% at toxicant exposure levels of 0.66, 6.73, and 46.8 mg/l, respectively. *Bufo fowleri* and *R. palustris* were relatively tolerant to methylene chloride, as survival of normal embryo-larval stages for both species did not drop below 65% at the highest concentration of methylene chloride administered (32.1 mg/l). The order of increasing species tolerance was *R. catesbeiana*, *R. palustris*, and *B. fowleri*, for which LC_{50} values at 4 days posthatching were 17.78, >32, and >32 mg/l, respectively (Table 4). Though responses were quite similar for the last two species,

survival of normal larvae at 4 days posthatching was somewhat higher for *B. fowleri* than for *R. palustris* (Table 3). Frequencies of teratogenesis induced by treatment with methylene chloride were generally low, but the trend observed for the three species was consistent with that given for LC_{50} values.

Trisodium nitrilotriacetic acid (NTA) was administered to *B. fowleri*, *B. quercicus*, *R. catesbeiana*, *R. palustris*, and *R. pipiens* (Table 3). Compared to the other compounds, NTA was substantially less toxic. Complete mortality was not observed except when NTA was administered at concentrations of 222 to 479 mg/l. *Rana pipiens* was the most sensitive species. Embryo-larval lethality and teratogenesis were observed at all exposure levels, which ranged down to 0.97 mg/l. Frequencies of teratogenesis were 3%, 11%, and 63% at NTA concentrations of 0.97, 10.9, and 97.5 mg/l, respectively. Survival of normal larvae at 4 days posthatching decreased from 87% at 0.97 mg/l to 57% and 0% at 48.8 and 479 mg/l. Concerning all species tested, the order of increasing tolerance was *R. pipiens*, *R. catesbeiana*, *R. palustris*, *B. fowleri*, and *B. quercicus*. Given in the same order, LC_{50}'s at 4 days posthatching were 39.3, 113.4, 134.6, 175.5, and 252.3 mg/l (Table 4). Embryo-larval mortality was the predominant test response. Although teratogenesis was observed at all exposure concentrations in tests conducted with *R. pipiens*, appreciable frequencies of terata occurred only at the higher NTA concentrations in tests with *R. catesbeiana*, *R. palustris*, and *B. fowleri*. Furthermore, no teratogenesis was observed when NTA was administered to *B. quercicus*.

Aquatic toxicity tests with phenol were performed using embryo-larval stages of five amphibian species, including *B. americanus*, *B. fowleri*, *R. catesbeiana*, *R. palustris*, and *R. pipiens* (Table 3). Phenol was decidedly more toxic to *R. pipiens* than to other species. A concentration of 1.09 mg/l produced complete mortality at 4 days, and phenol at 0.0047, 0.0073, and 0.074 mg/l reduced survival of normal larvae to 91%, 83%, and 36%, respectively. Anomalous *R. pipiens* larvae were detected at frequencies of 2% to 9% for this exposure range. Terata averaged 9% when phenol was administered at 0.89 mg/l to *B. americanus*, but appreciable levels of teratogenesis were not observed for the remaining species (i.e., *B. fowleri*,

R. catesbeiana, *R. palustris*), except when phenol was administered at high concentrations (≥ 10 mg/l). Increasing tolerance for the five species was in the order of *R. pipiens*, *R. catesbeiana*, *B. fowleri*, *R. palustris*, and *B. americanus*, based on LC_{50} values of 0.04, 0.23, 2.45, 9.87, and >0.89 mg/l, respectively (Table 4).

In most tests, amphibian embryos proved to be considerably more sensitive than larvae to the selected organic compounds. Survival usually did not decrease substantially during the posthatched period, and, except in several instances, LC_{50} values calculated at 4 days posthatching were not decidedly lower than those recorded at hatching (Table 4). Examples of high larval mortality included those tests in which atrazine was administered to *R. catesbeiana* and carbon tetrachloride, NTA, and phenol were administered to *B. fowleri* (Table 3).

To compare the six organic compounds for toxicity to amphibian embryo-larval stages, all were used in tests with *R. palustris*, and five toxicants were tested against each of two additional species (i.e., *B. fowleri*, *R. catesbeiana*). Based on median lethal concentrations determined at 4 days posthatching in bioassays with *R. palustris*, the order of decreasing toxicity was as follows: carbon tetrachloride (2.37 mg/l), phenol (9.87 mg/l), atrazine (17.96 mg/l), chloroform (20.55 mg/l), methylene chloride (>32 mg/l), and NTA (134.6 mg/l). The toxicological ranking was phenol (0.23 mg/l), atrazine (0.41 mg/l), carbon tetrachloride (0.90 mg/l), methylene chloride (17.78 mg/l), and NTA (113.4 mg/l) in tests with *R. catesbeiana*, and phenol (2.45 mg/l), carbon tetrachloride (2.83 mg/l), chloroform (35.14 mg/l), methylene chloride (>32 mg/l), and NTA (175.5 mg/l) in tests with *B. fowleri* (Table 4). Though the order of toxicity varied somewhat for the different amphibian species, several trends were evident. The most toxic compounds included phenol, carbon tetrachloride, and atrazine, and the least toxic compounds were always methylene chloride and NTA. A particularly interesting relationship was observed for the three chlorinated alkanes (i.e., carbon tetrachloride, chloroform, methylene chloride). Toxicity to embryo-larval stages increased with chlorination. For example, in tests with *R. palustris*, LC_{50}'s at 4 days posthatching were 2.37, 20.55, and >32 mg/l for carbon tetrachloride

(CCl_4), chloroform ($CHCl_3$), and methylene chloride (CH_2Cl_2), respectively (Table 4). This is consistent with results obtained in earlier investigations with polychlorinated biphenyls, in which toxicity to fish and amphibian embryo-larval stages increased with percent chlorination (Birge, et al., 1978).

In earlier investigations in which developmental stages of several species of amphibians were treated with metallic toxicants, a correlation was observed between species sensitivity and particular ecological and reproductive adaptations. Results indicated that anuran species which were more narrowly adapted for ecological requirements and/or mode of reproduction generally were more susceptible to pollution stress. Conversely, greater tolerance was observed for more broadly adapted anurans, particularly those capable of withstanding greater latitudes of natural environmental stress. Initial support for this concept was presented in studies with copper (Birge and Black, 1979), and embryo-larval bioassay data for mercury provided an even closer relationship between tolerance and adaptability (Birge, et al., 1979d).

Results of the present study were used to compare the relative sensitivities of the various amphibian species to organic compounds. In studies with chloroform, the order of decreasing species sensitivity was *H. crucifer*, *R. pipiens*, *R. palustris*, and *B. fowleri*, based on LC_{50} values at 4 days posthatching of 0.27, 4.16, 20.55, and 35.14 mg/l (Table 4). In tests conducted with phenol, based on median lethal concentrations (LC_{50}), species sensitivity decreased in the order *R. pipiens* (0.04 mg/l), *R. catesbeiana* (0.23 mg/l), *B. fowleri* (2.45 mg/l), *R. palustris* (9.87 mg/l), and *B. americanus* (>0.89 mg/l). The most sensitive amphibian species included *H. crucifer*, *R. catesbeiana*, and *R. pipiens*, while *R. palustris*, *B. americanus*, *B. fowleri*, and *B. quercicus* consistently were the most tolerant species. Similar results were obtained for atrazine, methylene chloride, carbon tetrachloride, and NTA. Ecological requirements and reproductive characteristics of the above species have been reviewed by Birge and Black (1979), Birge, et al. (1979d), Conant (1975), Vial (1973), and Wright and Wright (1949). Those species observed to be more sensitive to organic pollutants are known to be restricted largely to

aquatic or moist terrestrial habitats. Those species found to be most tolerant generally are more broadly adapted ecologically. They include semi-aquatic and terrestrial species which, for the most part, can frequent a greater variety of habitats. These and other considerations further support the hypothesis that more broadly adapted amphibian species usually exhibit greater tolerance to pollution stress.

In order to estimate toxicity thresholds for embryopathic effects, log probit analyses were used to calculate concentrations of the organic compounds which produced 10% (LC_{10}) and 1% (LC_1) impairment of test populations. These determinations were based on combined frequencies for lethality and teratogenesis observed for embryo-larval stages and were calculated using dose-response data taken at 4 days posthatching for the most sensitive amphibian species (Table 5). The selection of sensitive species was based on comparisons of median lethal concentrations (LC_{50}) determined for the six different organic toxicants.

In fish embryo-larval tests with both inorganic and organic aquatic contaminants (Birge, et al., 1979 a, e), it was established that probit LC_1 values taken at 4 days posthatching generally compared closely with maximum acceptable toxicant concentrations (MATC) determined in chronic life-cycle studies. McKim (1977) has provided additional support for using embryo-larval tests to estimate MATC's for freshwater aquatic life. As few chronic life-cycle studies have been conducted with amphibians (Birge, et al., 1979d), LC_1 values determined in embryo-larval tests provide a basis for estimating the tolerance of amphibian species to aquatic toxicants (Birge, et al., 1978, 1979d). In addition, LC_{10} values may prove useful in delineating the concentration at which toxicant exposure begins to produce appreciable reproductive impairment.

In bioassays performed with atrazine, the two most sensitive amphibian species tested were *R. catesbeiana* and *R. pipiens*. Probit LC_1 and LC_{10} values ranged from 7.4 to 32.6 µg/l and 44.9 to 378.9 µg/l, respectively (Table 5). The former did not differ substantially from LC_1's of 29.0 and 77.2 µg/l determined when atrazine was administered in soft water (i.e., 50 mg/l $CaCO_3$) and hard water (i.e., 200 mg/l $CaCO_3$) to embryo-larval stages of the rainbow trout (Birge, et al., 1979a).

A maximum acceptable toxicant concentration (MATC) for atrazine was reported to fall between 65 and 120 µg/l, when partial life-cycle studies were conducted with the brook trout (Macek, et al., 1976). These data indicate that *R. catesbeiana* and *R. pipiens* are slightly more sensitive to atrazine than are trout.

Carbon tetrachloride was administered to three amphibian species, and *R. catesbeiana* and *R. palustris* exhibited the highest sensitivity. Probit LC_1 and LC_{10} values calculated at 4 days posthatching were 23.6 and 113.0 µg/l in tests with *R. catesbeiana* and 109.6 and 435.7 µg/l in bioassays with *R. palustris* (Table 5). No other chronic data have been reported for freshwater organisms treated with this compound. However, based on the above data, it would appear that reproduction of more sensitive amphibian species could be impaired appreciably by concentrations of carbon tetrachloride exceeding 0.1 mg/l.

Embryo-larval stages of four amphibian species were exposed to chloroform. The most sensitive species were *H. crucifer* and *R. pipiens*, and the LC_1 values were 1.9 and 54.9 µg/l, respectively. The corresponding LC_{10}'s for chloroform were 17.7 and 383.4 µg/l (Table 5). The LC_1 determined with *H. crucifer* was close to values of 4.9 and 6.2 µg/l obtained in studies with the rainbow trout (Birge, et al., 1979a). The chronic value recently cited for freshwater invertebrates was 500 µg/l (U.S. EPA, 1979). This was based on results obtained for *Daphnia magna* but no details were given concerning test conditions. If conventional bioassay procedures were used, it is likely that substantial quantities of chloroform may have been lost from test water due to volatilization. It should be noted that data reported for amphibian species in Table 5 were determined using a new procedure designed to preclude volatility as a test variable (Birge, et al., 1979b). As seen in Table 3, this method provided precise regulation of exposure concentrations down to 7.5 µg/l. Brenniman, et al. (1976) also have called attention to problems involved in evaluating the toxicity of volatile organics. In view of the foregoing considerations, the suggested criterion of 500 µg/l would not appear to afford adequate protection for more sensitive fish and amphibian species. Critical life-cycle stages may be affected by concentrations as low as

2 to 5 µg/l, and embryopathic effects may become appreciable in the range of 18 to 400 µg/l, depending upon animal species and test conditions.

Methylene chloride was administered to embryo-larval stages of three amphibian species, and *R. catesbeiana* proved to be the most sensitive. Probit LC_1 and LC_{10} values were 92.5 and 981.0 µg/l, respectively (Table 5). Other species (i.e., *B. fowleri*, *R. palustris*) were considerably more tolerant to methylene chloride, and reliable LC_1 and LC_{10} values could not be calculated. No chronic studies with methylene chloride have been reported for freshwater organisms. However, it appears from the above data that developmental stages of *R. catesbeiana* are affected by concentrations as low as 0.1 mg/l and that concentrations in excess of 1 mg/l could prove hazardous to reproduction in sensitive amphibian species.

Of the five amphibian species tested with NTA, the two most sensitive were *R. pipiens* and *R. catesbeiana*. The LC_1's for this relatively non-toxic compound were 3.2 mg/l (*R. pipiens*) and 4.8 mg/l (*R. catesbeiana*), and the LC_{10}'s were 9.9 and 19.8 mg/l (Table 5). As noted above for several other compounds, tolerance of developmental stages of these sensitive amphibian species is comparable to or slightly less than that observed for embryo-larval stages of the rainbow trout. In tests with the latter species, LC_1 values for NTA ranged from 16.9 to 20.2 mg/l. By comparison, the MATC for NTA determined in an 8-month life-cycle study with the fathead minnow was set in the range of 54 to 114 mg/l (Arthur, et al., 1974). Viewing these results, it appears that NTA would not exert appreciable effects on most fish and amphibian species at concentrations of 10 mg/l or less.

Toxicity tests with phenol were performed on embryo-larval stages of five amphibian species, and *R. pipiens* and *R. catesbeiana* were the least tolerant. These species exhibited about equal sensitivity to phenol, as LC_1's ranged from only 1.0 to 1.1 µg/l and LC_{10}'s varied from 5.2 to 8.5 µg/l (Table 5). The LC_1's were within the range of 0.3 to 8.6 µg/l obtained in tests with the rainbow trout (Birge, et al., 1979a), and they did not differ significantly from values determined for several other species of freshwater fish (e.g., goldfish, bluegill). Based on the above data and other considerations (U.S. EPA, 1976), it would appear

that the maximum concentration of 0.1 mg/l originally suggested for phenol in 1973 (NAS-NAE Committee, 1973) is inadequate for protection of sensitive life-cycle stages of certain fish and amphibian species. In 1976, the Environmental Protection Agency established a criterion of 1 µg/l phenol for domestic water supplies and for protection against fish flesh tainting (U.S. EPA, 1976). It is of interest that the LC_1 values reviewed above are in good agreement with the organoleptic threshold, and this further supports feasibility of a freshwater criterion for phenol of 1 µg/l. It is obvious that this limiting concentration is essential not only to prevent fish flesh tainting but also to protect reproductive potential of sensitive aquatic species.

Considering the limited data available from chronic life-cycle studies, and the low cost feasibility of such tests, short-term embryo-larval bioassays provide a useful means of quantifying the toxicity of aquatic contaminants. When care is taken to develop an adequate dose-response relationship, log probit analysis can be used to calculate LC_1 values. The latter generally provide a reliable approximation of the threshold for toxic effects (e.g., lethality, teratogenesis), and as LC_1 values generally are in reasonable agreement with MATC's determined in chronic life-cycle tests, such data appear applicable to the promulgation of freshwater criteria. Furthermore, the LC_{10} can be used to provide an additional reference point for assessing toxic effects. Considering the combined effects of long-term pollution stress and natural environmental stresses, it is likely that 10% or greater impairment of reproductive potential would significantly affect population dynamics in natural communities (Gerking, 1978; Birge, et al., 1979a, e). Also, the concentration intervals between LC_1 and LC_{10} values may be useful in the hazard evaluation process. As the difference between the two values decreases, accuracy in defining a regulatory criterion becomes more critical.

CHAPTER IV

CONCLUSIONS

Aquatic toxicity tests were conducted with six organic toxicants, including atrazine, carbon tetrachloride, chloroform, methylene chloride, trisodium nitrilotriacetic acid (NTA), and phenol. Each compound was administered to developmental stages of three to five amphibian species. The different species exhibited varying degrees of tolerance to the selected organic compounds. Combining frequencies for lethality and teratogenesis, LC_{50}'s calculated at 4 days posthatching ranged from 0.41 to >48 mg/l for atrazine, 0.90 to 2.83 mg/l for carbon tetrachloride, 0.27 to 35.14 mg/l for chloroform, 17.78 to >32 mg/l for methylene chloride, 39.3 to 252.3 mg/l for NTA, and 0.04 to >0.89 mg/l for phenol. In most instances, higher LC_{50} values were obtained in tests with *B. americanus*, *B. fowleri*, *B. quercicus*, and *R. palustris*. Those species which exhibited the greatest susceptibility to organic compounds, based on LC_{50} determinations, consistently included *H. crucifer*, *R. catesbeiana*, and *R. pipiens*. The more sensitive amphibians generally included those species which normally are restricted to aquatic or moist terrestrial habitats, whereas the more tolerant amphibians included those semi-aquatic and terrestrial species known to be more broadly adapted ecologically.

Concerning median lethal concentrations (LC_{50}) for the six organic compounds, NTA and methylene chloride always were the least toxic. Though the order varied somewhat in tests with different amphibian species, phenol, carbon tetrachloride, and atrazine were the most toxic compounds. Attention also was given to the comparative toxicology of three chlorinated alkanes, including methylene chloride (CH_2Cl_2), chloroform ($CHCl_3$), and carbon tetrachloride (CCl_4). For these compounds, toxicity consistently increased with the degree of chlorination. This and other correlations between chemical structure and toxicity (Birge, et al., 1978) indicate that predictive toxicology may prove useful in hazard assessment programs for aquatic contaminants.

Sensitivity of amphibians was further characterized by calculating toxicant concentrations which produced embryo-larval lethality or teratogenesis at frequencies of 10% (LC_{10}) and 1% (LC_1). The LC_1 values, which provided a reliable means of estimating toxicity thresholds, varied from 1 µg/l for phenol to about 4.8 mg/l for NTA. On the basis of LC_1 values for the selected organic toxicants, developmental stages of *H. crucifer*, *R. catesbeiana*, and *R. pipiens* exhibited sensitivity equal to or slightly greater than that observed for embryo-larval stages of the rainbow trout. Therefore, the high susceptibility of embryos and larvae of certain amphibians to aquatic contaminants 1) should be considered in addressing the causal analysis of factors underlying the decline of natural amphibian populations and 2) stresses the need to consider amphibian species in promulgating freshwater regulatory criteria.

Table 3. Toxicity of organic compounds to embryo-larval stages of amphibian species.

Compound	Species	Toxicant Concentration Mean ± S.E. (mg/l)	Percent Hatchability[1]	Percent Survival[2] Normal Organisms	
				Hatching	4 Days Posthatching
Atrazine	*Rana catesbeiana*	0.051 ± 0.016	95(1)	94	86
		0.41 ± 0.12	92(3)	90	51
		6.33 ± 0.74	79(7)	73	10
		14.8 ± 3.3	54(22)	42	0
		26.4 ± 2.1	32(47)	17	0
		45.8 ± 1.5	8(100)	0	0
	Rana pipiens	0.11 ± 0.08	100(2)	98	95
		0.21 ± 0.17	97(2)	95	88
		1.13 ± 0.04	90(5)	85	86
		6.54 ± 0.59	92(9)	83	75
		13.2 ± 1.2	84(13)	73	57
		48.7 ± 1.2	40(46)	21	5
	Rana palustris	0.16 ± 0.08	98(0)	98	97
		0.56 ± 0.20	99(0)	99	98
		5.84 ± 0.66	92(0)	92	91
		10.4 ± 1.0	85(2)	84	81
		20.6 ± 1.6	64(5)	61	54
		33.9 ± 0.9	16(18)	13	4
	Bufo americanus	0.058 ± 0.003	101(0)	101	100
		0.49 ± 0.05	102(2)	100	101
		5.56 ± 0.28	99(2)	97	91
		10.8 ± 0.6	96(3)	93	95
		24.8 ± 0.7	90(6)	85	85
		48.2 ± 1.7	80(17)	66	68

Table 3 - continued.

Compound	Species	Toxicant Concentration Mean ± S.E. (mg/l)	Percent Hatchability[1]	Percent Survival[2] Normal Organisms	
				Hatching	4 Days Posthatching
Carbon tetrachloride	*Rana catesbeiana*	0.026 ± 0.004	100(0)	100	99
		0.060 ± 0.006	92(1)	91	89
		1.18 ± 0.10	70(8)	65	63
		7.81 ± 0.98	23(17)	19	0
		65.7 ± 7.1	0	0	0
	Rana palustris	0.020 ± 0.003	96(0)	96	93
		0.032 ± 0.022	100(0)	100	99
		0.69 ± 0.08	86(0)	86	81
		4.98 ± 0.92	44(0)	44	30
		92.5 ± 28.4	5(100)	0	0
	Bufo fowleri	0.020 ± 0.003	100(0)	100	94
		0.032 ± 0.022	100(0)	100	100
		0.69 ± 0.08	98(0)	98	78
		4.98 ± 0.92	77(3)	75	41
		92.5 ± 28.4	66(11)	59	0
Chloroform	*Hyla crucifer*	0.0087 ± 0.0016	97(2)	95	88
		0.073 ± 0.006	90(4)	86	78
		0.69 ± 0.05	66(10)	60	46
		7.34 ± 1.96	4(0)	4	0
		32.9 ± 9.01	0	0	0
	Rana pipiens	0.013 ± 0.001	100(0)	100	93
		0.021 ± 0.002	101(1)	100	99
		0.16 ± 0.01	95(2)	93	92
		0.66 ± 0.02	80(2)	78	77
		11.8 ± 0.5	61(12)	54	50
		26.9 ± 0.2	18(100)	0	0

Table 3 - continued.

Compound	Species	Toxicant Concentration Mean ± S.E. (mg/l)	Percent Hatchability[1]	Percent Survival[2] Normal Organisms	
				Hatching	4 Days Posthatching
Chloroform	*Rana palustris*	0.0075 ± 0.0030	100(0)	100	100
		0.034 ± 0.003	97(0)	97	96
		0.33 ± 0.05	96(1)	95	93
		6.04 ± 0.85	72(9)	66	63
		40.0 ± 6.0	58(19)	47	43
	Bufo fowleri	0.0075 ± 0.0030	99(0)	99	102
		0.034 ± 0.003	101(0)	101	105
		0.33 ± 0.05	98(0)	98	99
		6.04 ± 0.85	83(2)	82	69
		40.0 ± 6.0	76(6)	71	54
Methylene chloride	*Rana catesbeiana*	0.017 ± 0.002	101(0)	101	101
		0.071 ± 0.008	98(0)	98	96
		0.66 ± 0.06	96(1)	95	90
		6.73 ± 0.48	84(6)	80	74
		46.8 ± 2.1	52(20)	42	28
	Bufo fowleri	0.022 ± 0.003	100(0)	100	99
		0.13 ± 0.01	100(0)	100	99
		1.42 ± 0.10	98(0)	98	98
		10.1 ± 0.8	91(0)	91	84
		32.1 ± 6.6	80(2)	79	66
	Rana palustris	0.022 ± 0.003	100(0)	100	100
		0.13 ± 0.01	100(0)	100	97
		1.42 ± 0.10	98(0)	98	92
		10.1 ± 0.8	86(3)	84	80
		32.1 ± 6.6	72(5)	69	65

Table 3 - continued.

Compound	Species	Toxicant Concentration Mean ± S.E. (mg/l)	Percent Hatchability[1]	Percent Survival[2] Normal Organisms	
				Hatching	4 Days Posthatching
NTA	*Rana pipiens*	0.97 ± 0.28	95(3)	93	87
		5.26 ± 0.31	93(7)	87	81
		10.9 ± 1.3	92(11)	83	81
		48.8 ± 2.7	78(15)	66	57
		97.5 ± 4.0	37(63)	14	13
		479 ± 14	0	0	0
	Rana catesbeiana	1.19 ± 0.24	105(0)	105	102
		9.60 ± 0.64	99(1)	98	94
		78.8 ± 1.5	85(3)	83	71
		103 ± 2	68(5)	65	45
		206 ± 6	63(4)	60	48
		451 ± 8	40(25)	30	4
	Rana palustris	2.49 ± 0.37	100(0)	100	99
		13.3 ± 0.4	98(6)	92	92
		88.7 ± 2.5	95(11)	85	85
		121 ± 6	92(19)	74	75
		222 ± 12	70(36)	45	0
		520 ± 70	0	0	0
	Bufo fowleri	1.19 ± 0.24	102(0)	102	100
		9.60 ± 0.64	102(0)	102	99
		78.8 ± 1.5	101(1)	100	94
		103 ± 2	99(2)	97	64
		206 ± 6	72(5)	68	40
		451 ± 8	73(7)	68	14

Table 3 - continued.

Compound	Species	Toxicant Concentration Mean ± S.E. (mg/l)	Percent Hatchability[1]	Percent Survival[2] Normal Organisms	
				Hatching	4 Days Posthatching
NTA	*Bufo quercicus*	99.3 ± 7.0	100(0)	100	100
		203 ± 14	68(0)	68	63
		317 ± 13	46(0)	46	43
		400 ± 20	20(0)	20	0
		547 ± 31	0	0	0
Phenol	*Rana pipiens*	0.0047 ± 0.0007	94(5)	90	91
		0.0073 ± 0.0008	88(2)	87	83
		0.074 ± 0.008	51(9)	46	36
		1.09 ± 0.09	0	0	0
		11.5 ± 1.0	0	0	0
	Rana catesbeiana	0.0009 ± 0.0004	100(0)	100	99
		0.0028 ± 0.0012	99(0)	99	95
		0.056 ± 0.010	88(0)	88	79
		0.062 ± 0.008	75(0)	75	61
		0.53 ± 0.13	56(2)	55	40
		10.2 ± 1.5	14(15)	12	6
	Bufo fowleri	0.0009 ± 0.0004	98(0)	98	96
		0.0028 ± 0.0012	97(0)	97	94
		0.056 ± 0.010	91(0)	91	85
		0.062 ± 0.008	89(0)	89	72
		0.53 ± 0.13	84(1)	84	63
		10.2 ± 1.5	68(5)	65	28

Table 3 - continued.

Compound	Species	Toxicant Concentration Mean ± S.E. (mg/l)	Percent Hatchability[1]	Percent Survival[2] Normal Organisms	
				Hatching	4 Days Posthatching
Phenol	*Rana palustris*	0.0007 ± 0.0006	98(0)	98	99
		0.013 ± 0.003	98(0)	98	99
		0.092 ± 0.010	100(0)	100	100
		0.15 ± 0.04	97(0)	97	99
		1.86 ± 0.22	67(2)	66	61
		21.8 ± 0.7	53(15)	45	45
	Bufo americanus	0.010 ± 0.004	102(0)	102	103
		0.022 ± 0.005	103(0)	103	105
		0.036 ± 0.009	99(2)	97	97
		0.063 ± 0.009	94(1)	93	88
		0.22 ± 0.05	82(2)	80	79
		0.89 ± 0.25	76(9)	69	66

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

[2]Normal organisms were defined as those free of gross teratic defects.

Table 4. Log probit LC_{50} values for organic compounds administered to amphibian embryo-larval stages.[1]

Compound	Species	Exposure Days Beyond Hatching	LC_{50} (mg/l)	95% Confidence Limits
Atrazine	*Rana catesbeiana*	0	11.55	9.80 - 13.26
		4	0.41	0.27 - 0.59
	Rana pipiens	0	22.89	17.18 - 30.01
		4	7.68	4.84 - 11.90
	Rana palustris	0	20.20	17.77 - 22.96
		4	17.96	15.86 - 20.11
	Bufo americanus	0	>48	-
		4	>48	-
Carbon tetrachloride	*Rana catesbeiana*	0	1.50	1.11 - 2.09
		4	0.90	0.68 - 1.18
	Rana palustris	0	3.62	2.68 - 4.97
		4	2.37	1.74 - 3.21
	Bufo fowleri	0	>92	-
		4	2.83	1.95 - 4.05
Chloroform	*Hyla crucifer*	0	0.76	0.54 - 1.01
		4	0.27	0.19 - 0.37
	Rana pipiens	0	4.56	2.20 - 7.67
		4	4.16	1.97 - 7.06
	Rana palustris	0	28.17	15.57 - 64.43
		4	20.55	11.53 - 43.83
	Bufo fowleri	0	>40	-
		4	35.14	18.37 - 92.25

Table 4 - continued.

Compound	Species	Exposure Days Beyond Hatching	LC_{50} (mg/l)	95% Confidence Limits
Methylene chloride	*Rana catesbeiana*	0	30.61	21.22 - 64.66
		4	17.78	11.51 - 29.83
	Bufo fowleri	0	>32	-
		4	>32	-
	Rana palustris	0	>32	-
		4	>32	-
NTA	*Rana pipiens*	0	60.4	49.5 - 69.4
		4	39.3	26.3 - 52.5
	Rana catesbeiana	0	237.9	196.2 - 300.6
		4	113.4	95.0 - 134.5
	Rana palustris	0	181.2	163.5 - 200.9
		4	134.6	126.3 - 144.1
	Bufo fowleri	0	>451	-
		4	175.5	157.2 - 196.4
	Bufo quercicus	0	271.8	242.3 - 297.6
		4	252.3	225.4 - 275.3
Phenol	*Rana pipiens*	0	0.05	0.03 - 0.07
		4	0.04	0.03 - 0.05
	Rana catesbeiana	0	0.60	0.44 - 0.85
		4	0.23	0.15 - 0.35
	Bufo fowleri	0	>10	-
		4	2.45	1.26 - 5.61
	Rana palustris	0	11.23	7.11 - 19.86
		4	9.87	5.73 - 19.95
	Bufo americanus	0	>0.89	-
		4	>0.89	-

[1]Grossly teratic larvae were counted as lethals.

Table 5. Log probit LC_{10} and LC_1 values determined at 4 days posthatching for organic compounds administered to amphibian embryo-larval stages.[1]

Compound	Species	LC_{10} (µg/l)	95% Confidence Limits	LC_1 (µg/l)	95% Confidence Limits
Atrazine	*Rana catesbeiana*	44.9	21.8 - 77.7	7.4	2.6 - 15.9
	Rana pipiens	378.9	111.5 - 812.2	32.6	4.2 - 110.9
Carbon tetrachloride	*Rana catesbeiana*	113.0	71.3 - 163.8	23.6	11.7 - 40.4
	Rana palustris	435.7	216.6 - 681.3	109.6	34.8 - 219.7
Chloroform	*Hyla crucifer*	17.7	9.9 - 28.1	1.9	0.8 - 3.9
	Rana pipiens	383.4	60.1 - 985.0	54.9	3.1 - 225.0
Methylene chloride	*Rana catesbeiana*	981.0	327.2 - 1,908	92.5	12.7 - 287.0
NTA	*Rana pipiens*	9,890	3,900 - 16,550	3,210	770 - 6,880
	Rana catesbeiana	19,810	12,784 - 27,239	4,778	2,308 - 8,002
Phenol	*Rana pipiens*	5.2	2.8 - 8.1	1.1	0.4 - 2.1
	Rana catesbeiana	8.5	3.2 - 17.1	1.0	0.1 - 1.7

[1]Grossly teratic larvae were counted as lethals.

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