Bull. Environm. Contam. Toxicol. 29, 392–399 (1982)

Use of *Xenopus laevis* Larvae in 96-Hour, Flow-through Toxicity Tests with Naphthalene

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Increased industrialization has produced many environmental problems. Approximately 90% of man-made chemicals may be hazardous to plants and animals in the environment (WILSON & FRASER 1977). Rapid, reliable, and inexpensive bioassays are essential for detecting the hazards of these chemicals in the environment.

Our laboratory has developed an acute toxicity bioassay using Xenopus laevis larvae (South African clawed frog) and a flow-through diluter system. Xenopus embyryos have been used successfully as sensitive indicator organisms in toxicity tests for heavy metals, pesticides, herbicides, and other chemicals (BIRGE & JUST 1973; COOKE 1972; PRAVDA 1973; DAVIS et al. 1981, respectively). Considerable information is available on Xenopus (DEUCHAR 1975). They are easy to maintain and when induced to breed, as many as 2000 eggs may be produced at a time.

The flow-through bioassay system used for testing toxicity is similar in design to that of BIRGE et al. (1979; Figure 1). Stable temperature, oxygen, and toxicant concentrations are easily maintained. Volatilization and photolysis of naphthalene are also minimized as is the accumulation of feces and bacteria.

The toxicant used in tests was naphthalene ($C_{10}H_8$), a bicyclic aromatic hydrocarbon. Naphthalene is one of the most toxic components of crude oil (SOTO et al. 1975), and the 96 h LC50 of naphthalene has been published using many species. Test results with Xenopus may be compared with the reported LC50 values of other organisms.

The sensitivity of the bioassay system was tested by investigating the effects of naphthalene on the 96 h survival of three week old Xenopus larvae. The EC50 of behavioral and physiological changes (loss of swimming ability and depigmentation of tissues) at 6 h served as early indicators of toxicity.

METHODS AND MATERIALS

Animal Care

The water for holding tanks of Xenopus adults and larvae was filtered through an activated carbon filter (Barnstead™) and aerated for 48 h prior to use, to oxygenate and dechlorinate the water. Adults were fed raw pet-grade liver biweekly, and larvae were fed strained baby peas (Gerber) daily. The food contained

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no artificial additives or preservatives.

The techniques of ETHERIDGE and RICHTER (1975) for breeding and animal care were followed. Prior to breeding, the mating pairs were "conditioned" to ensure maximal response to hormones. Both males and females were injected with diluted Human Chorionic Gonadotropin (Sigma® Chemical Co.) to induce breeding.

Diluter System

Tap water was passed through a chlorine removal column (I.W.T.® Adsorber Model 1), and an activted carbon filter to remove organics (Barnstead™). The water was aerated in a glass reservior for 48 h and was then used for dilution of toxicant and preparation of stock solutions and absorbance standards. The water was tested for total organic carbon (T.O.C.) and heavy metal content. The test results indicted that the water was non-toxic (E.P.A., 1976).

A stainless steel piston pump (F.M.I.® Model G-20) was used to regulate the flow rate of the concentrated toxicant solution. This solution was pumped to a mixing chamber consisting of a 250 ml suction flask with a clamped silicone stopper and a teflon stirbar (Figure 1). Dilution water was delivered to the same mixing chamber at a defined rate by a peristaltic pump (Masterflex® Model 7567). The two solutions were stirred until homogeneous and delivered to the exposure chamber continuously. Exposure chambers consisted of a 2.5 l dessicator (Curtin Matheson Scientific®) with a top opening containing a #8 silicone stopper with inlet and outlet ports. The stopper was tightly sealed with a stainless steel hose clamp around the neck of the chamber. A stainless steel screen (#30 mesh) divided the chamber into a 0.5 l bottom compartment containing a teflon stirbar (to provide continuous stirring) and a 2 l top compartment containing the free-swimming larvae. A glass inlet tube delivered homogeneous, diluted toxicant from the mixing chamber to the bottom area of the dessicator where it was stirred once again. An outlet tube was positioned in the top of the chamber to permit a constant flow through the system. Test chambers were immersed in a Plexiglas water bath held at 28°C by a Brownwill® constant temperature stirrer. The temperature and pH of exposure chambers were continuously recorded. A 12 h diurnal photoperiod was controlled by an automatic timer and a fluorescent light. The entire system was enclosed in a fume hood, and all parameters were monitored from a master control box outside of the hood. The system consisted of glass, teflon, stainless steel and silicone only, as recommended by PELTIER (1978). The five test chambers were placed in series; four chambers each contained a different, but constant toxicant concentration, and the fifth chamber served as a control. All glassware and tubing were acid-base washed and rinsed, and the system was tested for toxicity for at least 24 h prior to each experiment. Data was obtained using lethality, (96 h LC50) depigmentation of skin tissue, (6 h EC50) and the absence of swimming (6 h EC50) as endpoints.

Toxicant Preparation and Fluorescence Analyses

Stock naphthalene was prepared fresh daily. Four concentrated solutions (20-30 mg/l) of naphthalene in water were prepared from a stock solution of 2000 mg/l naphthalene (Baker® Reagent

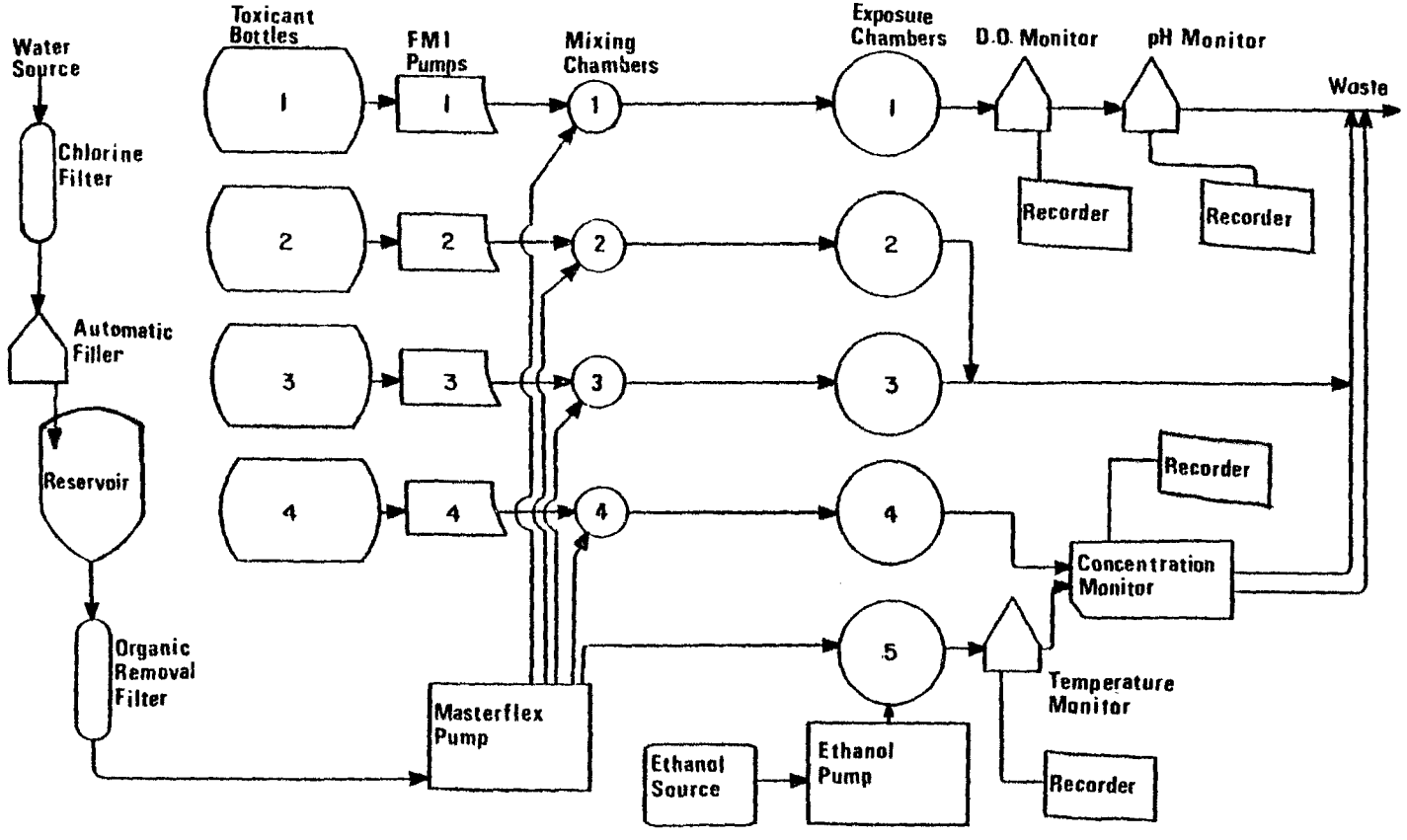

Figure 1. Diluter system design and layout.

grade) in 50% ethanol, and stored in silicone-stoppered brown bottles to minimize photodecomposition and volatilization. Photodegradation of naphthalene was monitored over time to ensure that the toxicity observed was attributed to naphthalene and not its metabolites. The control chamber contained a 1% ethanol solution in filtered water.

An Aminco-Bowman® Spectrophotofluorometer was used to analyze concentrations of stock solutions (in brown bottles) and diluted solutions (in mixing and exposure chambers) daily. Naphthalene standards of 0, 1, 5, 10 and 15 mg/l were prepared daily to establish a linear relationship between concentration and fluorescence, and the concentrations of solutions from all chambers and bottles were determined using linear regression analysis. A value for the mean concentration of each solution over 96 h was calculated. Wavelength emission spectra were also performed daily from 300-750 nm to ensure that naphthalene did not undergo photolysis within the 5 h turnover time in the exposure chamber. Data was analyzed using dose-response statistics (LITCHFIELD and WILCOXON 1949) with a Radio Shack® Model II Computer.

Data Collection

When toxified with naphthalene above a certain concentration, tissues of Xenopus larvae change color first from grey to whitish, then to very white. Generally, once the larvae turn white, death occurs in 1-6 h, depending on concentration of toxicant. The 6 h EC50 for depigmentation of larvae was calculated.

As the concentration of toxicant increases, larvae exhibit the inability to swim. This behavior was used as an indication of toxicity, and the 6 h EC50 for absence of swimming was calculated.

Every 8 h the number of dead larvae was tabulated and then removed from the exposure chambers. The 6 h EC50 for depigmentation, absence of swimming and the 96 h LC50 was calculated using the probit analysis method of LITCHFIELD and WILCOXON (1949).

RESULTS

Diluter System

The rate of volatilization from the diluter system stock vessels was found to be very low (4% over 24 h). Changes in fluorescence emission spectra indicted that no measureable photolysis of naphthalene occurred within 96 h.

In Tests 1 and 2, mean temperatures were 28°C while pH was 7.0 and 7.1 respectively.

Depigmentaion

The EC50 of depigmentation for Test A was 3.7 mg/l(Table 1). The EC50 for Test 2 was not determined due to insufficient data.

Absence of Swimming

The EC50 for absence of swimming (6 h EC50) served as a good indication of the 96 h LC50 with values of 2.3 mg/l and 1.7 mg/l in Tests A and B. Table 2. outlines Xenopus toxicity behavior.

Lethality

The 95 h LC50 for the 3-week larvae was 2.1 mg/l in both Test 1 and 2 respectively (Table 1, Figure 2).

TABLE 1
Effect of naphthalene on percent loss of pigment, absence of swimming, and lethality in two tests with three week Xenopus laevis larvae.

Naphthalene Concentration (mg/l)	Dissolved Oxygen (mg/l)	Loss of Pigment at 6 h (%)	Absence of Swimming at 6 h (%)	Lethality at 96 h (%)
Test 1				
0.0	5.5 (± 0.4)	0.0	0.0	0.0
2.0 (± 0.2)	5.8 (± 0.4)	16.7	50.0	50.0
3.3 (± 0.2)	5.7 (± 0.1)	33.4	58.0	83.3
4.1 (± 0.1)	4.7 (± 0.5)	50.0	41.6	91.6
4.9 (± 0.6)	6.0 (± 0.4)	67.0	67.0	100.0
	EC50	3.7(3.1-4.5)*	2.3(0.8-5.3)*	2.1(1.3-3.8)*
Test 2				
0.0	5.5 (± 0.1)	0.0	0.0	0.0
1.3 (± 0.1)	5.2 (± 1.2)	0.0	33.0	16.7
2.4 (± 0.2)	5.3 (± 0.1)	0.0	75.0	41.7
3.1 (± 0.2)	5.4 (± 0.3)	8.0	75.0	100.0
3.8 (± 0.2)	5.5 (± 1.2)	100.0	100.0	100.0
	EC50	— **	1.7(1.2-2.3)*	2.1(1.4-2.9)*

*EC50 or LC50 mean value (95% confidence interval)
**Insufficient data to calculate EC50.

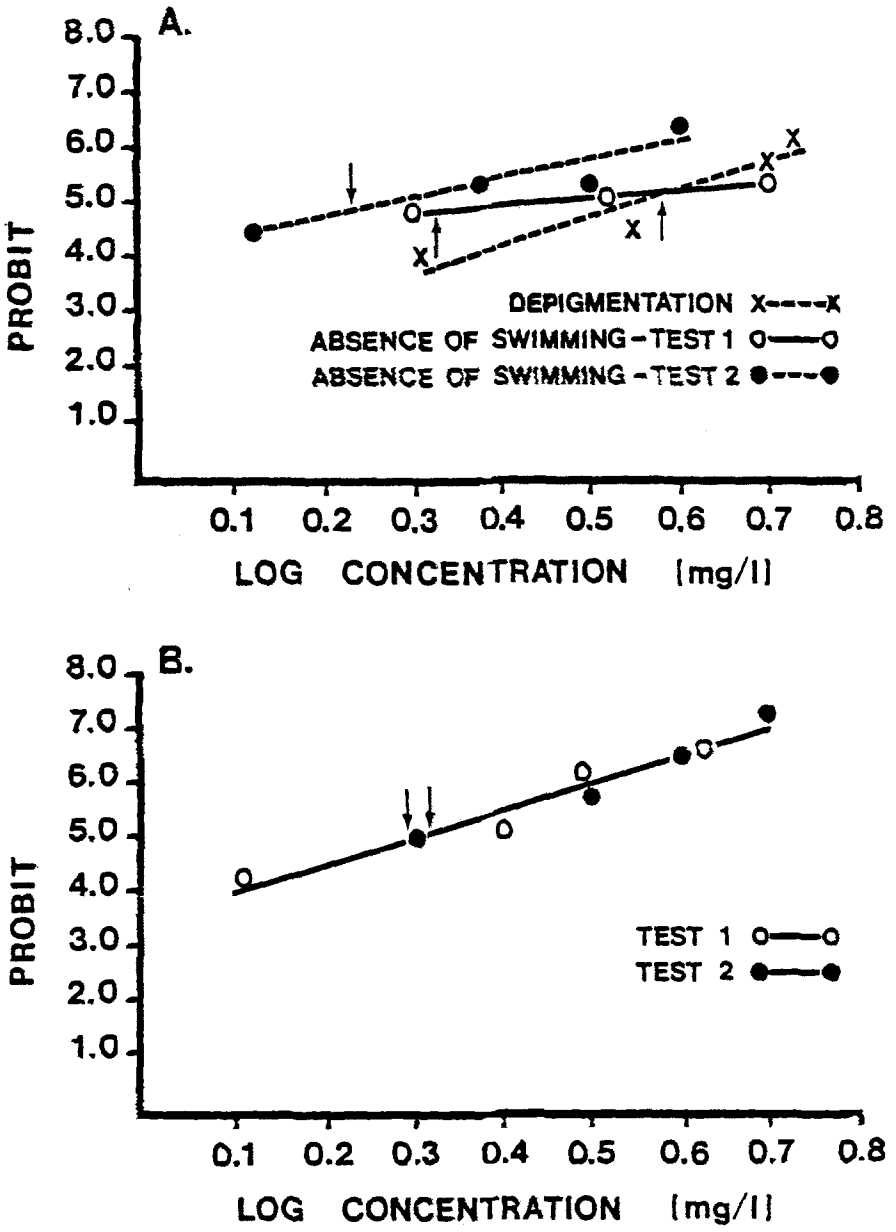

Figure 2. Dose-response curves for two 96 h naphthalene toxicity tests. A. Depigmentation-Test 1 only; Absence of swimming-Test 1 and Test 2. B. Lethality-Test 1 and Test 2.

TABLE 2
Naphthalene toxicity behavior phases and traits

Phase 1.	[Slightly Toxic]
	a). Swim to top of chamber for oxygen.
	b). Lie on screen.
	c). Breathe rapidly in gulps.
	d). Heartbeat very fast.
Phase 2.	[Moderately Toxic]
	a). Loss of bouyancy-"float" down to screen.
	b). Spiral while swimming.
	c). Nervous "flitters" when disturbed.
	d). Breathe moderately-lie on screen.
	e). Heartbeat fast.
Phase 3.	[Very Toxic]
	a). Lie on the screen breathing normally (slowly, easily).
	b). Heartbeat slow.
	c). Nervous "flitters".
	d). Whitish appearance.
Phase 4.	[Extremely Toxic]
	a). Lack of apparent breathing.
	b). White appearance.
	c). Lie on side or back.
	d). Do not move when disturbed.
Phase 5.	[Lethal]
	a). No heartbeat.
	b). Very white pigment.

DISCUSSION

Xenopus laevis served as an excellent toxicity indicator organism. Death was easily detected in Xenopus as the heartbeat may be viewed through the transparent tissue on the ventral side of the organism. Control larvae survived well in the flow-through diluter apparatus, and no deaths occurred in the tests.

The flow-through bioassay system provided stable experimental conditions. The concentration of naphthalene, temperature and pH were controlled throughout the experimental period and the volatilization and photodecomposition was minimal over the 96 h.

One objective of the study was to determine which mode of testing is most accurate for assessing toxicity, and if the 6 h EC50 values could serve to provide toxicity information quickly and reliably. The 96 h LC50 proved to be the most sensitive indicator of toxicity, and replication of data and the narrow confidence intervals in the two tests indicated that values were reliable.

Depigmentation of tissue was used to indicate relative toxicity; however, this data did not correlate well with the 96 h LC50 values. The EC50 of the absence of swimming was more indicative of the 96 h LC50, and predicted values were within 20% of the true LC50 values. Other substances must be tested to determine if this relationship is valid in the majority of cases.

The absence of swimming is an integral aspect of Xenopus behavior during toxicity. Behavior served as a reliable indicator of toxicity. When the concentration of naphthalene is approximately 0.5-1.0 mg/l, most of the larvae remained in Phase 1 for the entire 96 h (Table 2). If the concentration was above

approximately 3 mg/ml, the larvae entered Phase 3 or 4 immediately without exhibiting Phase 1 and 2 behavior. If the concentration was above 4.5 mg/l, Phase 5 occurred within a few hours.

In summary, Xenopus laevis is a sensitive indicator organism. Death is easily identifiable, and the 96 h LC50 and larval behavior facilitated the assessment of toxicity. The LC50 values for naphthalene obtained with Xenopus correlate well with the LC50 of the grass shrimp (NEFF et al. 1976), crab zoea (CALDWELL et al. 1977) and rainbow trout (E.P.A., 1980). Because of the commercial availability of Xenopus, its ability to provide large numbers of offsrping year round, and its well understood biology, the use of Xenopus is a practical alternative to other currently used species in toxicity testing.

ACKNOWLEDGEMENT

Research was supported by a Presidential Challenge Grant from Oklahoma State University. We would like to thank Mr. K. Shelby, Ms. P. Simpson and Mr. K. Fisher for technical assistance, and Ms. R. Odell for typing the manuscript.

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