THE USE OF SOUTH AFRICAN CLAWED FROG LARVAE (*XENOPUS LAEVIS*) IN FLOW-THROUGH TOXICITY TESTS FOR NAPHTHALENE

By

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PREFACE

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CHAPTER I

INTRODUCTION

The advent of increased industrialization in past years has subsequently produced a multitude of environmental problems, including hazardous waste dumps, contamination of surface and ground water, and human health hazards. It has been estimated that 90% of man-made chemicals in the environment may be hazardous to man (Wilson and Fraser, 1977). The need for additional knowledge on the mechanisms of chemical toxicity is clearly evident. According to the recent Toxic Substances Control Act (TOSCA), the hazards of approximately 2,000 new compounds which enter the market each year must be evaluated. The chemicals already on the market and in the environment must also be tested. With hundreds of individual laboratories and research facilities in pursuit of this goal, the methods of determining toxicities must be standardized; a rigid mode of investigation must be adopted by all agencies in order to obtain reliable, universal, cost effective results.

The LC50 measurement is widely used as an indication of relative chemical toxicity (Sprague, 1969), and denotes the concentration of toxicant necessary to kill one-half the test organisms within a specified time of exposure.

Flow-through tests to determine the LC50 such as that used by Brungs and Mount (1978); Birge and Black (1977); Birge et al. (1979); and Brenniman et al. (1976) expose the organisms to different concentrations of a toxicant for the desired interval of time. The toxicant is pre-mixed with water (or carrier solvent) to achieve the desired concentration, and this solution is continually pumped through the test chambers. Establishment of a flow-through method of exposure is advantageous in that it is fairly universally accepted, can screen a wide variety of chemicals for toxicity, avoids problems associated with volatilization and degradation of toxicant, may be used with several different organisms, and maintains a stable concentration of toxicant. All of these conditions may be met, continuously monitored, and easily replicated. The system could then serve as one component of a multifaceted study to test teratogenicity, mutagenicity, and carcinogenicity of chemicals. Results obtained from tests using this system may allow extrapolation of data on toxicity of naphthalene and other compounds to other aquatic species and, perhaps, even to humans. Ultimately, it will allow an educated decision on maximum allowable concentrations of these compounds in the environment.

Recent interest has centered around the volatile aromatic hydrocarbons, since much of the toxicity associated with crude oil seems to be associated with this group of compounds (Baker, 1970; Boylan and Tripp, 1971; Soto et al. 1975a, 1975b). The volatile aromatic hydrocarbons are found

to be more toxic than the persistent aromatics (Lee and Nicol, 1978; Moore and Dwyer, 1975; Donahue et al. 1977; Lee et al. 1978).

Naphthalene is a volatile bicyclic aromatic hydrocarbon common in coal tar pitch, and is one of the most toxic components of crude oil (Lee and Anderson, 1977). It is estimated that thousands of kilograms of this compound enter the seas each year (Lee et al., 1972).

One objective of this proposal is to establish a flow-through system for testing the toxicity of polycyclic aromatic hydrocarbons (PAH) and other compounds. The system will be used to determine the 96 h LC50 of naphthalene to 1 wk (stage 22) and 3 wk old (stage 22; Rugh, 1941) Xenopus laevis (Daudin) larvae (South African clawed frog).

Larvae of Xenopus laevis were used as the test organism. They are readily available, totally aquatic, easy to maintain and breed, and have been shown to be sensitive to environmental chemicals. Xenopus embryos have been widely used in toxicity bioassays with heavy metals (Birge and Just, 1973), pesticides (Cabejszek and Wojcik, 1968; Cooke, 1972), herbicides (Pravda, 1973), and other chemicals (Birge et al., 1980; Davis et al., 1981; Greenhouse and Hamburgh, 1968; Greenhouse, 1975, 1976a, 1976b, 1977), and have proven to be a reliable bioassay system. Results from studies with Xenopus laevis larvae correlate well with data from other organisms. No studies have been performed using Xenopus laevis with PAH.

CHAPTER II

REVIEW OF THE LITERATURE

Xenopus laevis

As a Test Organism

Xenopus belongs to the order Anura, and the family Pipidae. Adults were obtained from NASCO (Fort Atkinson, Wisc.). *Xenopus* was selected as a test species for several reasons. They can serve as an ideal assay system to test the effects of aquatic toxins on growth, development, and metabolism. An abundance of information is available on the frog's anatomy, physiology, development, and growth (Deuchar, 1975). *Xenopus* has been used successfully for assessing toxic effects on embryonic and larval stages (Greenhouse, 1976a, 1976b, 1976c,) and thus establishing an LC50.

Genetic variation may be curtailed by selective inbreeding of *Xenopus*, thus augmenting consistent data collection. Commercially available Human Chorionic Gonadotropin (HCG) will readily stimulate mating behavior and ovulation in *Xenopus*. They may be bred year-round, and can produce up to 2000 eggs from one breeding pair, thus facilitating statistical analysis. *Xenopus* are totally

aquatic, and easily maintained. Disease is uncommon, yet they are sensitive to environmental conditions, making them a valuable indicator organism.

Lethal Effects

Xenopus develops from a fertilized egg to a hatching larva in 50 h at 20°C (Rugh, 1941). Xenopus has been primarily used in teratogenicity studies where early embryonic stages (8 cell stage) are exposed to a suspected teratogen for a period of time and embryonic death and abnormality recorded. Xenopus larvae are more sensitive to toxicants than embryos (Greenhouse, 1975), probably due to the differences in membrane permeability. The vitelline membranes of embryos are relatively impermeable to toxicants, while water-borne contaminants may enter through the skin or gills of larvae by passing directly across the cellular membrane. The common sites of tissue damage are the skin, gills, and intestine (Hawkes, 1977). From these areas, contaminants may enter the circulatory system, affecting other organs such as the liver, gall bladder, kidney, muscle, and brain (Anderson, 1977).

Sublethal Effects

Sublethal effects that may occur in fishes, and possibly Xenopus, include a sloughing of the epithelial gill tissue, which allows increased susceptibility to bacterial and fungal contamination. Excessive mucus production has

been shown to cause respiratory deficiency, and even suffocation (Hawkes, 1977). Liver cells have been observed to undergo a massive increase in rough endoplasmic reticulum, a change which may be related to the synthesis of detoxification enzymes. Liver glycogen may be depleted, resulting in reduced liver function and lowered energy reserves (Hawkes, 1977). Vision troubles occur due to lens enlargement in the eye (Baldwin, 1962). Naphthalene metabolites have been observed to stimulate cataract production (Booth and Boyland, 1949). Changes in tissue composition may occur, such as fibrosis (an alteration in which cells die and are replaced with connective tissue instead of new cells). Teratogenic studies with Xenopus in particular have shown production of edematous gut and pericardial regions (Greenhouse, 1976b). Malformations of the axial skeleton, tail and trunk, spinal cord, head, and eyes have also been detected (Greenhouse, 1976a).

Naphthalene

Physical Properties

Naphthalene is an acutely toxic polycyclic aromatic hydrocarbon (Wolfe, 1977). It is a highly volatile compound, which is important in removing this chemical from aquatic environments. Studies indicate that it has a half-life of less than 100 h in streams tested under several conditions of wind and current velocity (Southworth, 1979). Naphthalene is a bicyclic aromatic hydrocarbon, and in its

purified form it is a white crystalline solid at room temperature ~~(Windholz, 1976)~~.

The molecular weight of the compound is 128.18, and it has a chemical formula of $C_{10}H_8$ ~~(Weast, 1979)~~, boiling point of 217.9°C and melting point of 80.6°C ~~(Donaldson, 1958)~~. The solubility of naphthalene has been reported to be 30 mg/l in distilled water at 25°C ~~(Bohon, 1951)~~, and it is highly soluble in ethanol ~~(Weast, 1974)~~. The specific gravity at 20°C is 1.145 ~~(Donaldson, 1958)~~. The octanol/water partition coefficient of naphthalene has been found to be 3.8 (Kudchadker et al., 1978).

Although naphthalene is a colorless compound in water and in ethanol, it absorbs U.V. light at 290 nm and may be detected spectrophotometrically or fluorometrically. In water, it may be detected as low as 0.03 mg/l by fluorometry. In ethanol, as in water, the compound exists as dispersed molecules in solution, not as dimers or crystalline aggregates ~~(Schwarz and Wasik, 1976)~~.

Uses and Sources

Naphthalene is commonly used as an antiseptic dusting powder, insecticide (as in moth balls), as a dyeing agent, and a wood preservative. When the compound is chlorinated, it can be used as a flame retardant, pesticide, electrical insulator, and oil additive ~~(Winter, 1979)~~. In nature, it is often found in coal tar, crude oil, and refined oil (Laughlin and Neff, 1979).

Naphthalene can enter the aquatic environment via oil spills, may be distributed in the air by volatilization ~~(Southworth, 1979)~~, coal power plants ~~(Kingberry et al.,~~ ~~1979)~~, as well as cigarettes, charcoaled meat, and automotive exhausts ~~(Manahan, 1975)~~. Air-borne naphthalene may absorbed to solid particles in air and precipitate to the earth during rain (Corner et al., 1976). Precipitated naphthalene was found to be adsorbed onto organic matter and sediments in the water column (Lee, 1977).

Many derivatives of naphthalene may be formed from chemical breakdown, with or without bacteria and other organisms. Most of these products are more water soluble than naphthalene and according to Wolfe (1977) may be more toxic than the parent compound. Some marine invertebrates may naturally contain derivatives of PAH, including quinone compounds of naphthalene, anthracene, and benzo-a-pyrene (Scheur, 1973).

Metabolism and Elimination

Naphthalene may be adsorbed through the gills, dermally, via inhalation, and by adsorption to food particles. One study indicates that it is not toxic when inhaled, (Clar, 1964), while another believes it is highly toxic (Winters, 1979). It has been shown to produce toxic effects when administered in all other modes.

The accumulation of PAH in aquatic organisms is primarily dependent on the partitioning of the hydrocarbon

between the exposure water and the tissue lipids. This partitioning favors the rapid transfer from the aqueous phase to areas such as biological membranes, macromolecules, and lipid depots in the organism (Leo et al., 1975; Neely et al., 1974). The mechanisms of binding to tissue lipids is by hydrophobic interactions, in contrast to the covalent bonding of some compounds (Neff et al., 1976). Soto et al. (1975b), argue that although there is a high affinity for lipids, partitioning favors a rapid release to the water when the naphthalene concentration in the water is reduced. In this manner, the lipid acts as a source of PAH, however this source is rather insignificant.

It is generally accepted that aromatic hydrocarbons are metabolized by the "mixed function oxidases" which are present in the endoplasmic reticulum of animal cells. The oxygenases are often called the aryl hydrocarbon hydroxylases (AHH) or drug-metabolizing enzymes. They are NADPH-dependent, and catalyze oxidation of the aromatic nuclei in the following manner (Varanasi and Malins, 1977):

$$RH + NADPH + O_2 + H^+ \text{----> } NADP^+ + H_2O + ROH$$

Sims and Grover (1974) support the view that the metabolic conversion of aromatic hydrocarbons to oxygen-containing products is a prerequisite for carcinogenicity. They have shown that aromatic hydrocarbons covalently bind to DNA in rat liver only when microsomal oxygenases are present, presumably to activate the conversion to oxygenated derivatives.

Hepatic monooxygenase enzymes have been shown to catalyze the formation of arene oxides (epoxides) from aromatic hydrocarbons. These enzymes are found in plants, animals, and microorganisms (Jerina and Daly, 1974). The epoxide of naphthalene is naphthalene-1,2-oxide, and is catalyzed primarily by the cytochrome P-450 monooxygenases in mammals. This epoxide may spontaneously isomerize (to 1- or 2-naphthol), be enzymatically hydrated (to trans -1, 2-dihydroxy-1,2-dihydronaphthalene) (Malins et al., 1979) or be conjugated with glutathione (Figure 1). The conjugating enzymes are responsible for converting the potentially toxic metabolic products into less toxic, more water soluble compounds, such as glycosides, glucuronides, sulfates, and mercapturic acids. These compounds are hydrophilic acidic substances which may be excreted rapidly (Dewaide, 1971).

Once conjugated, biliary excretion is the most important mechanism of discharge of organic xenobiotics in marine organisms (Varanasi and Malins, 1977). Biliary excretion in mammals and fishes has been shown to be mediated by glutathione-S-transferase which is in the highest concentration in the liver and the kidney.

Arene oxides are significant as they have been found to bind covalently to DNA, RNA and proteins as demonstrated in cultured cells (Jerina and Daly, 1974). Tests performed indicated guanine as the base most susceptible to attack. This macromolecular binding may be a primary basis of deleterous effects of aromatic hydrocarbons (Gibson, 1977). In

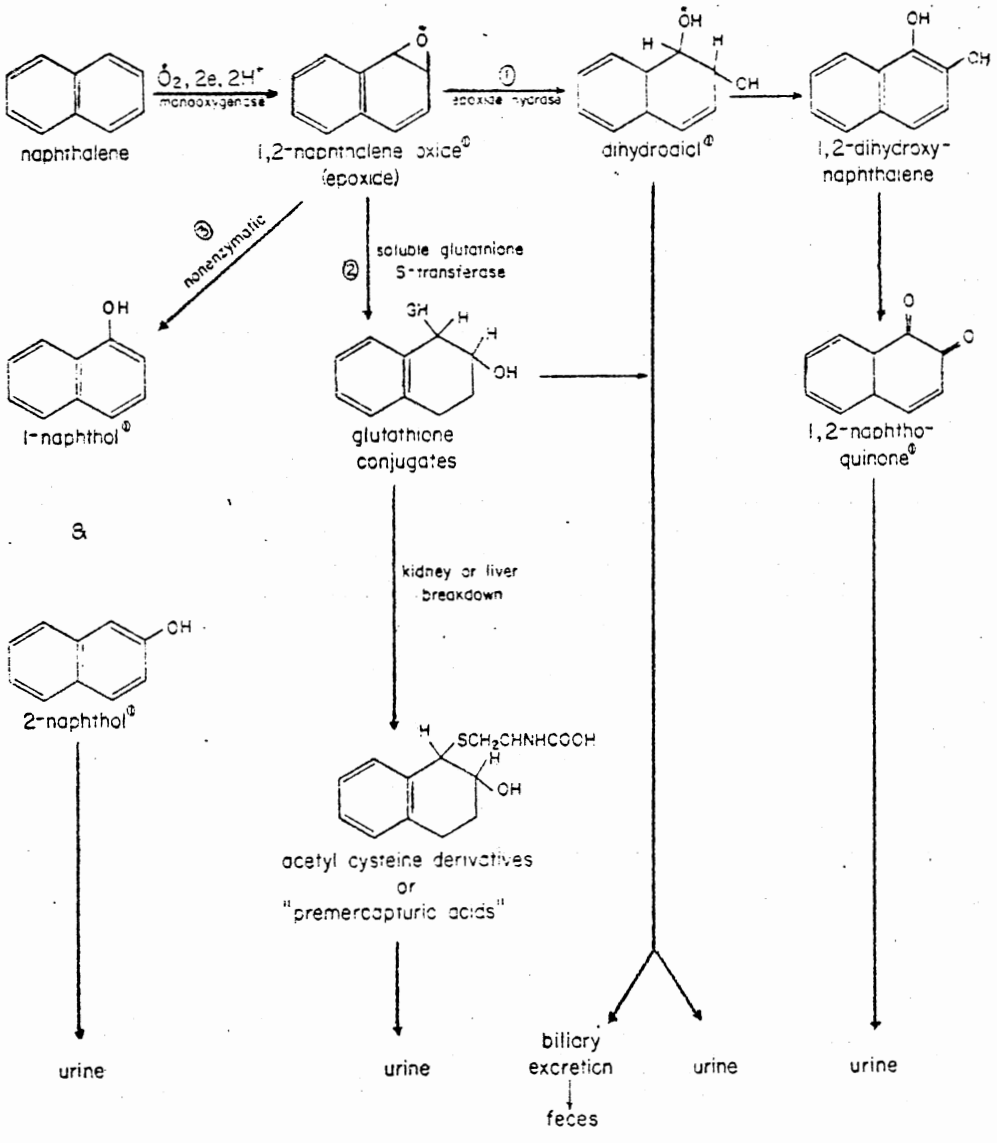


Figure 1. Summary of In Vitro Studies of Naphthalene Metabolism in Aquatic Organisms.

marine organisms and mammals, the enzymes glutathione-S-transferase and epoxide hydrase provide a protective mechanism in which epoxides are converted to non-carcinogenic dihydrodiols and glutathione conjugates (Corner, 1975).

Naphthalene has been shown to bioconcentrate in organisms such as the water flea (Daphnia pulex) (Southworth et al., 1978; Leo et al., 1975; Chiou and Freed, 1977), grass shrimp, (Palaemonetes pugio), clam (Rangia cuneata) (Varanasi and Malins, 1977), killifish, (Fundulus similis) (Anderson, 1975), sheepshead minnow, (Cyprinodon variegatus) (Anderson et al., 1974), and rainbow trout fingerlings (Salmo gairdneri) (Melancon and Lech, 1979).

Naphthalene and its metabolites were found in many organs, primarily the gall bladder, liver, kidney, brain, fat, muscle, and skin. (In fishes, naphthalene enters through the gills, and is metabolized in the liver. The hydrocarbon and metabolites are transferred to the bile, and subsequently excreted in the urine. The gall bladder was found to be a major storage site for these compounds in many organisms (Lee et al., 1972). In a long-term test with rainbow trout fingerlings, bile studies performed with ^{14}C-naphthalene indicated a maximium biliary content of ^{14}C-naphthalene 13,000 times the level in the water (Melancon and Lech, 1978). Over 98% of this ^{14}C-naphthalene was in the form of metabolites, with over 90% conjugated (Melancon and Lech, 1979). In contrast, another study with rainbow trout showed an accumulation of 34-84% of the naphthalene in the skin.

Tests performed indicated that the epidermal mucus was very important in removal of the hydrocarbons and their metabolites (Varanasi et al., 1978).

Klaasen (1975) found that the kidneys were the most crucial organ in excretion of xenobiotics, and subsequent studies found urine to be the most important avenue for discharge from the body in the mudsucker (Gillichthys mirabilis), sculpin (Oligocottus maculosus) and sand-dab (Citharichthys stigmaeus) (Lee et al., 1972).

Naphthalene may be broken down by microorganisms. In degradation studies with an initial naphthalene concentration of 30 mg/l, various species of Pseudomonas and Acinetobacter were isolated from estuarine, coastal, and gulf stream study sites (Neff, 1979). In a ground water study, naphthalene was completely degraded in 216 h by four genera of Pseudomonas (Kappeler and Wuhrmann, 1978a). Lee and Anderson (1977) determined that microbes can degrade naphthalene to 1,2-dihydroxy-1,2-dihydronaphthalene, and ultimately to CO_2 and H_2O. Fungi, like mammals, (but unlike bacteria) possess a cytochrome P-450 enzyme system, and degrade naphthalene in the same manner as mammals (Table I) (Neff, 1979). Figure 2 depicts the differences between the reactions used by eukaryotes and prokaryotes to initiate the oxidation of aromatic hydrocarbons.

The elimination of naphthalene was found to be biphasic in nature in rainbow trout using thin layer chromatographic techniques. Tests were performed on tissues of the liver,

TABLE I

COMPARISON OF ISOLATED METABOLITES OF NAPHTHALENE FROM VARIOUS ORGANISMS

Compound Isolated	Organism	Reference
	rat	Booth and Boyland, 1949
	rabbit	Booth and Boyland, 1949; Young, 1947
	mice	Corner and Young, 1954
	guinea pig	Corner and Young, 1954
	coho salmon (*Oncorhynchus kisutch*)	Varanasi and Malins, 1977
	mudsucker (*Gillichthys mirabilus*)	Lee et al., 1972
	sanddab (*Citharichthys stigmaeus*)	Lee et al., 1972
1,2-dihydroxy-1,2-dihydro-naphthalene	sculpin (*Oligocottus maculosus*)	Lee et al., 1972
	rainbow trout (*Salmo gairdneri*)	Malins et al., 1979
	crab (*Maia squinado*)Herbst	Corner et al., 1973
	fungus (*Cunninghamella elegans*)	Ferris et al., 1973

TABLE I (continued)

Compound Isolated	Organism	Reference
	Bacillus naphthalinicum non-liquefaciens	Jeffrey et al., 1975
	Pseudomonas 53/1	Jeffrey et al., 1975
	P. 53/2	Jeffrey et al., 1975
	P. desmolyticum	Jeffrey et al., 1975
	P. putida	Jeffrey et al., 1975
	P. NClB9816	Jeffrey et al., 1975
	P. fluorescens	Jeffrey et al., 1975
	Nocardia NRRL 3385	Jeffrey et al., 1975
	N. Strain R	Jeffrey et al., 1975
1,2 naphthalene oxide	rat	Jerina and Daly, 1974; Grover and Sims, 1974
	Chinese Hamster Ovary cultured cells	Jerina and Daly, 1974
	"Fishes"	Lee et al., 1972
	"Plants and Animals"	Jerina and Daly, 1974
1-naphthol	rat	Badger, 1962
	coho salmon	Varanasi and Malins, 1974

TABLE I (continued)

Compound Isolated	Organism	Reference
	rainbow trout	Malins et al., 1979
	sheepshead minnow (*Cyprinodon variegatus*)	Melancon and Lech, 1979
1-naphthyl-sulfate	crab	Corner et al., 1973
	coho salmon	Varanasi and Malins, 1977
	rainbow trout	Melancon and Lech, 1979
1-naphthyl-glucoside	crab	Corner et al., 1973
	coho salmon	Varanasi and Malins, 1977
	rainbow trout	Malins et al., 1977
1-naphthyl-mercapturic acid	crab	Corner et al., 1973
	coho salmon	Varanasi and Malins, 1977
2-naphthol	rat	Badger, 1962
	fungus	Ferris et al., 1973
1-naphthyl-glucopyranoside	rainbow trout	Melancon and Lech, 1979
	sheepshead minnow	Melancon and Lech, 1979
2-methyl-1-naphthol	rainbow trout	Melancon and Lech, 1979
	sheepshead minnow	Melancon and Lech, 1979

TABLE I (continued)

Compound Isolated	Organism	Reference
dimethyl naphthalene	oysters (*Crassostrea virginica*)	Neff et al., 1976a
1-methyl glucuronic acid	coho salmon	Varanasi and Malins, 1977
1,2-naphthoquinone	fungus	Ferris et al., 1973
1,4-naphthoquinone	fungus	Ferris et al., 1973
4-hydroxy-1-tetralone	fungus	Ferris et al., 1973
salicylic acid	*Pseudomonas* spp.	Strawinski and Stone, 1954; Walker and Wiltshire, 1953

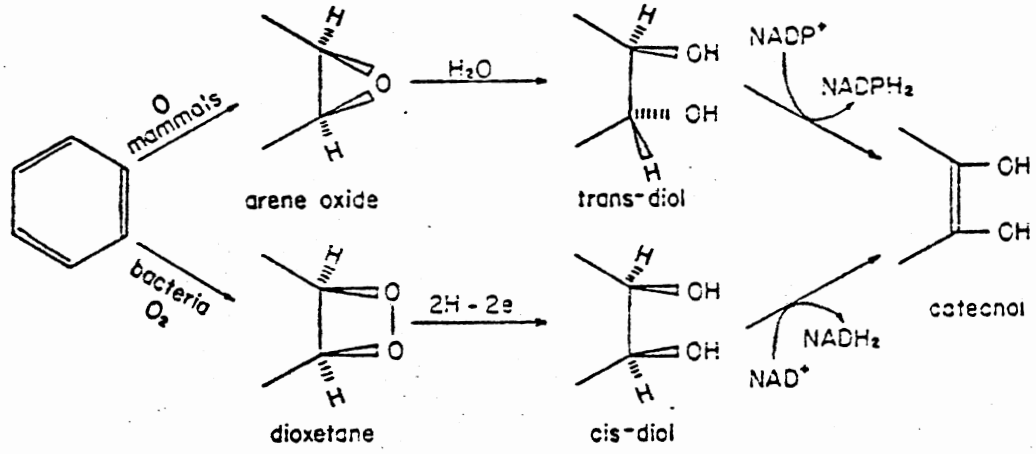


Figure 2. Comparative Oxidation Of Aromatic Hydrocarbons (From Gibson, 1977).

blood, gills, fat and whole fish. The half-life of depuration was hundreds of hours in a long-term test, however, after an 8 h exposure to naphthalene, most elimination half-lives were less than 24 h. Fat depuration took a longer period of time, with a half-life of 62.2 h (Melancon and Lech, 1979). Anderson et al. (1974) found a biphasic depuration in sheepshead minnow (Cyprinodon variegatus) as well. At first, elimination was very rapid, then slowly declined over time.

Studies with a clam (Rangia cuneata) showed that after an 8 h exposure to an oil-water dispersion (OWD), 79% of the naphthalene and metabolites were released from the tissues after 24 h (Varanasi and Malins, 1977), while killifish (Fundulus similus) that were exposed to 2 mg/l of the water-soluble fraction (WSF) of #2 fuel oil took 366 h to eliminate all of the naphthalene. ^{14}C-naphthalene was eliminated faster than labeled Benzo-a-pyrene (Lee et al., 1972) and ^{14}C-2-methylnaphthalene (Melancon and Lech, 1979). In mammals, elimination may also be via body secretions such as tears, sweat, and milk (Klaasen, 1975). Corner et al. (1976) found that in several marine organisms, the route of administration of naphthalene determined the elimination time. When uptake was directly from seawater, depuration was rapid, with less than 5% left in the tissues after 10 d. However, if uptake was via food, one-third of the naphthalene remained in the tissues after 10 d.

Toxic Effects

The LC50 for naphthalene has been determined for several organisms, using various testing procedures (Table II). For static tests, LC50 concentrations ranged from 0.45 mg/l (*Pimephates prometas* larvae) to 8.6 mg/l (*Daphnia magna*), with a mean value of 3.1 mg/l. Using sublethal exposures, naphthalene has been shown to cause diverse deleterious effects. Several dye workers developed bladder cancer after working with naphthalene derivatives (Windholz, 1976), and the compound itself caused tumors in rats at 3500 mg/kg doses when injected intraperitoneally (Toxic Substances List, 1974). It may cause cataracts due to a degeneration of the lens proteins in the eye (Bourne and Young, 1934). The epoxides have been associated with cancer (Swaisland et al., 1974), and have actually been shown to bind to macromolecules in the cell (Melancon and Lech, 1979). Growth may be altered, as demonstrated by a study with the larvae of mud crabs, which developed faster than the controls (Laughlin and Neff, 1979). In algae (*Chlamydomonas angulosa*), naphthalene killed numerous cells, and the surviving cells underwent a long lag phase prior to resuming normal growth (Soto et al., 1975a). There was a total loss of their photosynthetic ability in media containing naphthalene. When transferred to fresh media, the cells quickly metabolized the naphthalene and began to function normally (Soto et al., 1975b).

TABLE II

LC50 VALUES FOR NAPHTHALENE IN VARIOUS ORGANISMS

Organism	LC50 Value (mg/l)	Time of Exposure	Type of Test *	Reference
polychaete (Neanthes arenaceodentata)	3.8	96 h	S	Neff et al., 1976
grass shrimp (Palaemonetes pugio)	2.4	96 h	S	Neff et al., 1976
grass shrimp (Palaemonetes pugio)	2.6	24 h	S	Anderson et al., 1974
Crab zoea Stage 1	> 2.4	96 h	S	Caldwell et al., 1977
barnacle larvae	2.9	1 h	S	Donahue et al., 1977
amphipod (Elasmopus pectenicrus)	2.7	96 h	S	Lee and Nicol., 1978
sheepshead minnow (Cyprinodon variegatus)	2.4	24 h	S	Anderson et al., 1974
brown shrimp (Penaeus aztecus)	2.5	24 h	S	Anderson et al., 1974
cladoceran (Daphnia magna)	8.6	96 h	S	E.P.A., 1980
rainbow trout (Salmo gairdneri)	2.3	96 h	F-T	E.P.A., 1980
Fathead minnow (Pimephales promelas)	4.9	96 h	F-T	E.P.A., 1980
Fathead minnow larvae (P. promelas)	.45-.86	96 h	S	E.P.A., 1980

* S = Static test; F-T = Flow-through test.

Degradation of Naphthalene by Non-Biological Mechanisms

(Naphthalene has been shown to oxidize in the presence of light and air (Ludzack and Ettinger, 1963), when in the presence of high temperatures (Josephy and Radt, 1948). According to Kirk and Othmer (1967), the process involves an initial conversion to naphthoquinone, followed by lysis of one of the aromatic rings, and liberation of CO_2.) Because naphthalene is capable of photodecomposition, studies on naphthalene photodegradation were performed to determine if this process was occurring during a 4 d toxicity test.

CHAPTER III

METHODS AND MATERIALS

Test System

Dilution water was passed through a chlorine removal column (I.W.T. adsorber Model 1), and an activated carbon filter to remove organics (Barnstead). The dilution water was then held in a glass reservior, and aerated until needed. This water was used for dilution of the toxicant, and the preparation of solutions and standards. It has been tested for heavy metal content including K, Zn, Fe, Na, Ca, Mn, Cu, and Pb (Water Quality Research Lab, O.S.U.). The concentration of all metals were well within E.P.A. limits.

A stainless steel piston-flow pump (FMI Model G-20) was used to regulate the flow rate of the concentrated toxicant (Figure 3). This solution was pumped to a mixing chamber consisting of a 250 ml suction flask with a silicone stopper and Teflon stirbar. Dilution water was delivered at a defined rate to the mixing chamber by a peristaltic pump (Masterflex, Model 7567). Both solutions were mixed until homogeneous in the mixing chamber, and then delivered to the exposure chamber.

Nominal concentrations were estimated in the following manner. Ex: if concentrated toxicant was pumped at 200 ml/h

and dilution water was pumped at 400 ml/h, the total flow rate was 600 ml/h. To obtain a dilution factor:

$$[1] \qquad \frac{\text{total rate (ml/h)}}{\text{toxicant rate (ml/h)}} = \text{dilution factor}$$

When the dilution factor is multiplied by the exposure chamber concentration, the product is the concentration of the stock solution in the brown bottles.

One organic compound, naphthalene, was tested at four different concentrations, and results compared to a control. Twenty one-week Xenopus larvae or twelve three-week larvae were used in each 2.5 l chamber. Four concentrated solutions of naphthalene in water were prepared from a stock solution of 2000 mg/l naphthalene in 50% ethanol, and stored in silicone- stoppered, photoresistant brown bottles to discourage photodecomposition and volatilization. The concentrated solutions varied, depending on the final desired concentration, and on the pump rates for delivery of toxicant and dilution water to the mixing chamber (Figure 3).

Because naphthalene is a volatile organic, the test chambers were totally sealed, and filled entirely with solution to alleviate an air-water interface. The flow-through diluter was specifically designed to accomodate the most volatile of organic compounds. All vessels and tubing that came in contact with the toxicant were sealed from the air. No headspace was allowed in either the sample mixing flasks or exposure chambers. The only volatilization possible was

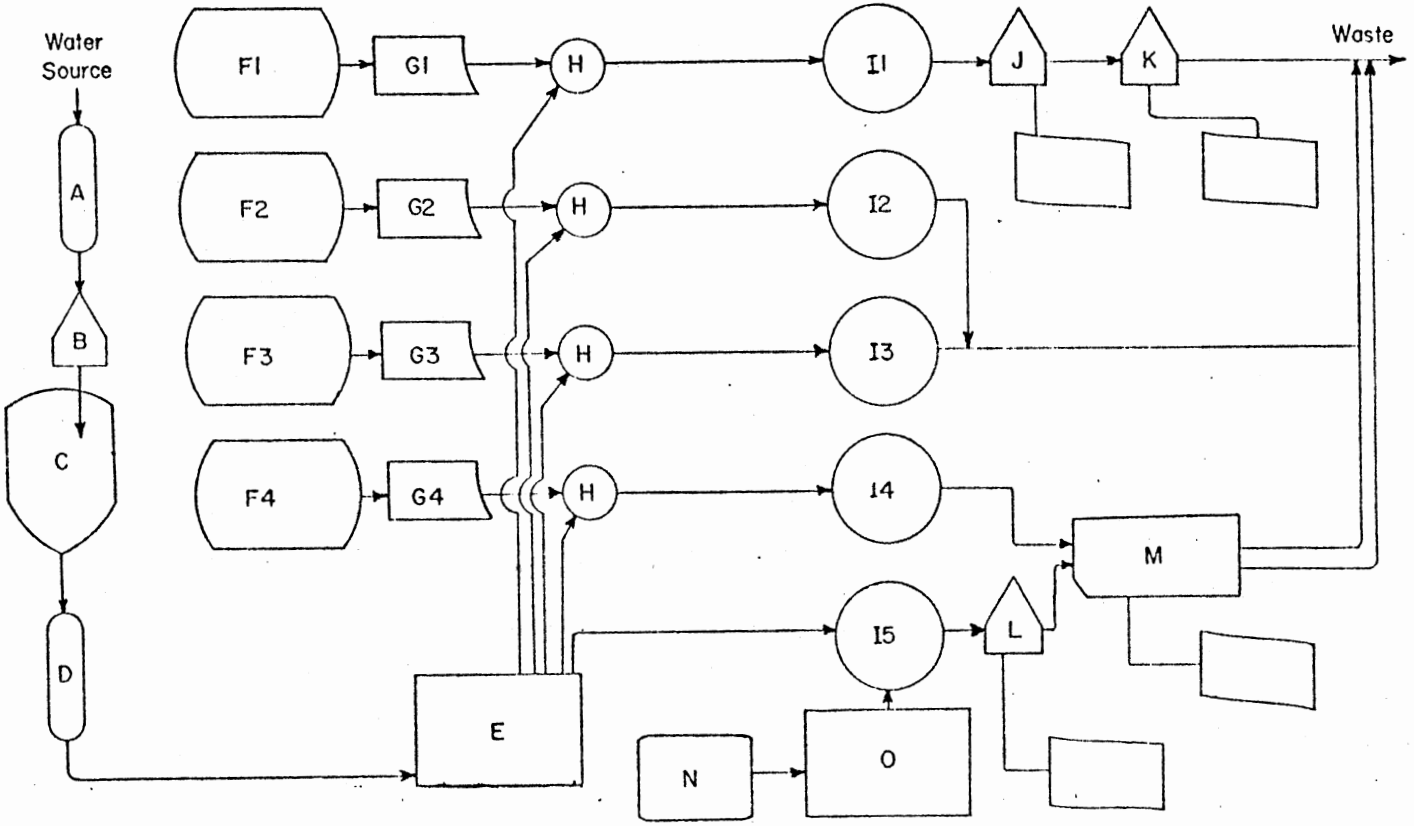


Figure 3. Diluter System Design and Layout. A. Chlorine filter. B. Automatic filler. C. Reservoir. D. Organic removal filter. E. Masterflex Pump. F. Toxicant bottles. G. FMI pumps. H. Mixing Chambers. I. Exposure Chambers. J. Dissolved Oxygen Monitor. K. pH Monitor. L. Temperature Monitor. M. ISCO Concentration Monitor (Spectrophotometer). N. Ethanol Source. O. Ethanol pump. Exposure Chamber Number 5 is used as the control. Arrows denote fluid flow through system.

in the brown toxicant storage bottles. In these bottles, the access to air was restricted by corking the bottle. This was effective in minimizing volatilization. For other volatile organics, the container can be pressurized with an inert gas such as nitrogen in order to minimize the loss of toxicant from the water.

The chambers consisted of a 2.5 l dessicator (Curtis Matheson Scientific) with an opening in the top for a #8 silicone stopper and the inlet and outlet ports. The stopper was clamped down by a stainless steel O-ring around the neck of the chamber. A stainless steel mesh (#30 mesh-Small Parts Inc.), approximately 7 cm from the bottom of the dessicator, served to divide the chamber into two compartments - 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 permitted delivery of diluted toxicant to the bottom chamber where it was stirred once again, and an outlet glass tube was positioned at the top of the chamber to remove the "old" solution. To avoid temperature fluctuations, the test chambers were immersed in a plexiglas water bath held at 28^{o}C by a Haake FK constant temperature bath. The temperature was continuously monitored (thermistor and Sargent model SR recorder) as was the pH of the highest concentration (Model 10 Corning pH meter and probe, Honeywell recorder). A 12 h day-night photoperiod was controlled by an automatic timer and a fluorescent

light mounted on the hood. Glass tubing was used for all connections, and fitted together with small pieces of silicone tubing. The system consisted of glass, teflon, stainless steel, and silicone only, as recommended by E.P.A. (Peltier, 1978).

The test chambers were set up in series with each chamber containing a different, but constant concentration of the toxicant. Four toxicant concentrations and one control were used. All glassware and tubing were acid-base washed and rinsed prior to hook-up, and preliminary system toxicity tests were performed for at least 24 h prior to an actual test, to ensure that these components were not the cause of lethal effects. Prior to each test, the pumps were recalibrated, and nominal bottle and chamber concentrations were estimated from these values. The bottle (4000 ml), and initial mixer and chamber solutions (3000 ml) were prepared, and after actual concentrations were checked by fluorometry, the solutions were added to the proper vessels, and stirred. Twelve to twenty tadpoles at the proper stage were then randomly distributed to each chamber. The animals were fed one day prior to the test, but were not fed for the duration of the test. Trial runs showed that larvae exhibited no visible signs of stress when food was withheld for 10 d. Feeding may present problems, as organics can preferentially adsorb to the food particles and may be ingested in this manner, thereby altering test results. Feeding also increases the fecal material in the chambers, thus allowing

enhanced growth of microorganisms. Tests were performed until an LC50 value was repeated with stage 22 (1 wk and 3 wk old) larvae.

Decontamination

The test chambers, components of the holding tanks, the flow-through system and glassware were washed in the following manner to remove surface contaminants:

1. All surfaces rinsed with 95% ethanol.
2. Glassware soaked and washed with Micro^{R} cleaning solution in hot water.
3. Rinsed three times with hot water.
4. Rinsed thoroughly with a fresh solution of acetic acid (5%).
5. Rinsed with hot water.
6. Rinsed with a fresh solution of sodium hydroxide (5%).
7. Rinsed three times with distilled water and allowed to air dry.

Care of Organisms

Larvae.

Each holding tank was acid-base washed as previously described. For maximium growth, the larvae were fed daily, and the fecal material and excess food were siphoned out every other day. At the same time, approximately half of the water was drained, and fresh water was added to the

tanks. The culture water was filtered through an activated carbon filter (Barnstead) to remove any organic compounds present. The water was held in detoxified polyethylene carboys, and bubbled at least 48 h prior to usage, to oxygenate and to remove any residual chlorine. Each individual holding tank was filled with water, and gently aerated using air stones and pumps (Whisper 800). Food consisted of strained baby peas (Gerber) with no artificial additives or preservatives.

Adults

The adult frogs were fed and their water changed twice weekly. The frogs were kept in 10-20 gallon aquaria with a minimum of two 3-5 inch frogs per gallon of water. The water depth was maintained at approximately three inches. Each frog was fed 1-2 g of fresh raw liver (pet grade) at each feeding.

Breeding

Xenopus reach breeding age at about 3 years old. The female Xenopus is usually larger, and has protruding cloacal lips, while the male is characterized by "nuptial pads" or dark areas on the inside of the forearm. These pads darken, and become more obvious when the male is ready to breed.

Breeding was facilitated using techniques described by Etheridge and Richter (1975). Prior to breeding, the frogs

were "conditioned" to ensure maximal response to hormones. The males and females were separated, fed fresh meat, and the aquaria cleaned daily for seven days. Human Chorionic Gonadotropin (HCG; Sigma, St. Louis, Mo.), was diluted with 0.9% sterile NaCl solution (Abbott Labs., Chicago, Ill.) to a final concentration of 500 I.U./ml (International Units). Solution was refrigerated if not used on day of preparation. Using a 1 cc syringe with a 26 gauge needle, the male was injected in the dorsal lymph sac with 150 I.U. HCG 30-36 h before the female was added to the tank. Injections penetrated approximately 0.4 cm at about 0.6 cm above the frogs cloaca. Both frogs were injected with 500 I.U. HCG and after 8-12 h the pair were placed together in a breeding tank for amplexis. The breeding tank was a 10 gallon aquarium, with a plastic mesh bottom, which allowed the eggs to fall to the bottom away from the frogs. The tank was filled about a third of the way full with filtered water, and was aerated. The top and sides were covered with aluminum foil to create a darkened environment and the frogs were left undisturbed. After 16-20 h, the breeders were returned to their respective holding tanks. Fecal material was siphoned from around the eggs, and the water was replaced with fresh water of the same temperature. The water was changed every other day for 4 days. On the fifth day, the tadpoles were fed, and to ensure proper growth, thinned to a density of approximately 10 tadpoles/gallon to ensure proper growth conditions. Up to 95% fertilization may occur using this

breeding method.

Analytical Procedures

The concentrated toxicant was prepared fresh daily. The concentration of the stock and diluted solution were checked daily on an Aminco-Bowman spectrophotofluorometer. The mean value for concentrations in each chamber was recorded after 96 h. This value was calculated for each chamber by adding together each concentration obtained and dividing it by the total number of samples collected from the exposure chamber (N). The test solution was continuously monitored for temperature using a thermistor-recorder combination, and for pH using a Corning model 10 pH meter connected to a Honeywell recorder. Daily samples were obtained for analysis of dissolved oxygen (D.O.; YSI model 54A Oxygen meter). The total number of survivors was determined at 0, 4, 8, 16, 24, 48, 72, 96 h. Death was determined using heartbeat and circulation as the criteria.

The 2000 mg/l stock solutions were prepared from naphthalene (Baker Reagent grade, Lot #415952) and 50% ethanol (U. S. Industrial Chemical Co., Inc.). This solution was used to prepare the concentrated stock solutions and the standards for fluorometry.

Naphthalene standards of 0, 1, 10, 15, 20 mg/l were prepared to establish a linear relationship between concentration and fluorescence. A correlation coefficient, y intercept, and unknown concentration values were determined

from the fluorescence values obtained. Concentrated toxicants were diluted with distilled-deionized water using a 50 ml volumetric cylinder and a Pipetman pipet. Wavelength emission spectra were done periodically to ensure that the naphthalene breakdown was not occurring during the 96 h of testing. Spectra were compared to previously made standard spectra. An excitation wavelength of 290 nm, and an emission wavelength of 200 to 750 nm was routinely used. Detection levels of 8µg/l range have been tested on this instrument accurately (Elaine Stebler, 1981, personal communication).

Data Evaluation

Data was evaluated using Litchfield and Wilcoxon's (1949) method and the 2-point analysis. The Litchfield-Wilcoxon procedure involves plotting the data on logrithmic-probability paper, using a modified Chi-square test, and fitting a straight line through the points. From this line, an LC50 was established. The slope of the line, a calculated value from the data, and a provided nomograph allowed calculation of confidence limits. Once calculated by hand, the test results were confirmed by computer analysis, and thereafter, all data analysis was performed by computer (Radio Shack Model II).

CHAPTER IV

RESULTS

Degradation of Naphthalene by Non-Biological Organisms

Volatilization

It is difficult to assess the toxicity of naphthalene because of its volatility. Because of this property, glass bottles with ground glass stoppers were used for containing all samples. Preliminary tests were performed to determine percent loss of sample over time.

Three types of containers were used: 1.)open jars, non-aerated and aerated; 2.)jars with aluminum foil and screw lids; and 3.)jars with ground glass stoppers. Concentrations of samples were determined by fluorescence spectrometry before and after a certain time period. Values were compared to a regression curve using 0, 1, 5, 10, 15 mg/l standards run with each set of samples.

In the four open containers with no aeration, the 5 mg/l samples lost 96% of the naphthalene, and the 10 mg/l samples lost 84% after 12 h. In the aerated open containers, 99% volatilized from the containers after 3 hours.

The samples with aluminum foil and screw lids were

monitored three times over 24 h for volatilization. At 1 h, there was less than 10% loss in the 8 samples tested (4 samples of 5 mg/l, and 4 samples of 10 mg/l naphthalene). After 9 h, there was 10-20% loss, and after 24h, the loss averaged of 34.7% with a range from 16-55%.

Several tests were conducted to determine percent volatilization of samples from bottles with ground glass stoppers, since this method was used for static tests in this study. In four out of five studies conducted, the loss of naphthalene from the three bottles averaged 4% after 24 h (range 1-8%). In the fifth test the three bottles lost 11-19%, with an average of 16%. These values are quite different and as much as 5% of this difference may be attributed to instrument error. The stoppers may not have been as tightly closed on these samples or, perhaps, were off the jars for a longer period of time when collecting or sampling the solutions. Naphthalene may also have come out of solution in the water (solvent) and thus was not detected by the analysis. These data clearly illustrate the need for tightly closed vessels when using naphthalene and other volatile compounds not only within the bioassay system, but also for collection and concentration analysis.

Photodegradation

The study performed was designed to determine the toxicity of naphthalene, not the toxicity of the resultant products. Compound degradation was therefore studied to

define breakdown that might have been occurred over a 4 d bioassay test, and if breakdown was taking place, to determine methods to alleviate the problem.

Fluorometry was used to determine the change in emission spectra of naphthalene and compounds suspected as oxidation products. Sodium borohydride was used to reduce compounds, and wavelength spectra were performed on solutions both in the reduced and oxidized state. Changes in spectra implied that some breakdown had in fact occurred. In samples kept in the light for 14 d, there was an obvious color change from clear to yellow. Compounds tested included duroquinone (tetramethylbenzoquinone), juglone (5-hydroxy-1,4-naphthoquinone), toluene (1 methylbenzene), 2-hydroxy-1,4-naphthoquinone (2-OH, 1,4-NQ) and solution "X". Solution X was a naphthalene solution made 8/8/80 and kept in the light for 24 h. After this time, the solution was stored in a ground glass stoppered bottle in a dark closet for 47 d. Emission spectra were checked at this time and also after the solution was again exposed to light for 3 d and 4 d respectively. Although there were no significant changes in spectra of solution X during this time period, the 96 h solution contained an extra peak at 432 nm. This peak could have been present before this time, and remained undetected. It is also possible that some of the compound was in the process of decomposing in the presence of light over 96 h into a new compound, and it is feasible that this new compound would not be detected until after 72 h in the

light.

Also tested were three types of solutions of 1,2-naphthoquinone (1,2-NQ). Spectra were performed on fresh solution (Od), after 3 d, (3d-1,2-NQ) and after 5 d (5d-1,2-NQ) in the light in ground glass stoppered bottles. A naphthalene solution named solution "Z" was prepared 9/10/80, kept in the light in ground glass stoppered bottles until 9/30/80, and checked for changes in emission spectra. Figure 4 lists the concentrations used, wavelengths, colors, and major peaks of each compound tested.

The "control" spectrum of sodium borohydride in water resulted in a small peak at approximately 572 nm. The peak also occurred in two oxidized forms of 1,2-naphthalene, as well as in all reduced compound spectra but naphthalene and toluene. Toluene did not reduce using sodium borohydride, and it is possible that inadequate resolution in the naphthalene spectrum precluded the appearance of this peak.

Emission peaks found in spectra of fresh naphthalene were at 325 nm and 641 nm, both in oxidized and reduced states. The 325 nm peak ($\pm$ 10 nm) was found in the spectra of reduced duroquinone in both reduced and oxidized solution X, and in the reduced form of solution Z.

Another fairly common peak occurred at 403 nm, and was found in oxidized and reduced states of solution X, reduced forms of 3 d and 5 d-1,2-naphthoquinone, and the oxidized form of solution Z. There were four spectra which contained peaks at approximately 432 nm--the reduced forms of solution

Compound and State*	Emission Peak Wavelengths (300 350 400 450 500 550 600 650 700 750 800)	Color of sample	Concentration (mg/l)
Naphthalene-ox	-325 ——— 641 ———	C	1.75
Naphthalene-rd	-325 ——— 641 ———	C	1.75
Duroquinone-ox	—341 ——— 641 ———	Y	0.06
Duroquinone-rd	—330 ——— 578 — 651 ———	DY	0.55
Juglone-ox	———	C	0.04
Juglone-rd	312 —— 378 ——— 569 ———	C	0.07
Toluene-ox	———	C	0.01
Toluene-rd	———	Y	- -
2-OH-1,4-NQ-ox	———	C	0.01
2-OH-1,4-NQ-rd	-325 —— 403 ——— 641 ———	C	0.10
Solution X-OH-ox	-325 —— 403 ——— 641 ———	C	0.51
Solution X-OH-rd	—328 —400 ——— 576 641 ———	C	0.44
Solution X-72h-ox	-325 — 400 ——— 641 ———	C	0.34

Figure 4. Summary of major peaks, colors, and concentrations of compounds fluorometrically scanned at 290 nm excitation from 300-750 nm emission.

Compound and State*	Emission Peak Wavelengths	Color of sample	Concentration (mg/l)
	300 350 400 450 500 550 600 650 700 750 800		
Solution X-72h-rd	328 — 400 — 576 — 641	C	0.44
Solution X-96h-ox	325 — 400 — 641	C	0.42
Solution X-96h-rd	325 — 400 — 432 — 641	C	0.43
Od-1,2-NQ-ox	573	Y	0.02
Od-1,2-NQ-rd	438 — 453 — 571	C	0.04
3d-1,2-NQ-ox	571	Y	0.02
3d-1,2-NQ-rd	300 — 413 — 434 — 578	C	0.04
5d-1,2-NQ-ox		Y	0.02
5d-1,2-NQ-rd	397 — 421 — 432 — 571	C	0.05
Solution Z-ox	403	Y	0.05
Solution Z-rd	328 — 571 — 633	Y	0.05
Sodium Borohydride	573	C	0.01

*State denotes oxidized or reduced; ox= oxidized, rd= reduced, C= colorless, Y= Yellow, DY= Dark Yellow.

Figure 4 (continued)

X and the fresh, 3 d, and 5 d-1,2-naphthoquinone solutions. In fresh 1,2-naphthoquinone, 5d-1,2-naphthoquinone, oxidized duroquinone, and reduced juglone, there were several peaks which did not match those spectra of other tested compounds.

The dilutor and exposure chambers were designed to minimize photodecomposition. With a turnover time of once every 5 h (5 times in 24 h), fresh naphthalene was constantly replacing metabolic decomposition products (theoretically). It is doubtful that photodecomposition occurred during this time period, since studies indicate that naphthalene breakdown products did not appear in fluorescence spectra until after 5 d in light.

Figure 5 outlines the summary of the breakdown of naphthalene to other products, while Figure 6 graphically illustrates the peaks of naphthalene and each breakdown product. The fluorescence data allowed the conclusion that naphthalene did not photodecompose in the exposure chambers over 4 d. However, the analysis of breakdown products are not complete, as all compounds do not fluoresce at 290 nm or at other wavelengths. Compounds implied as breakdown products are merely speculation and further studies using an ultraviolet scanner may provide more concrete results.

Water Quality

The quality of the water used in all tests was checked by the OSU Water Quality Research Lab to insure that the water used in the toxicity tests was acceptable. Table III

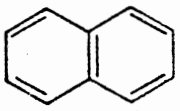
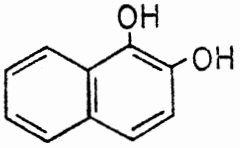
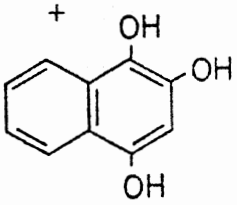
	Structure of Compound	Name of Compound	Wavelength Peaks of Emission Scans*(nm)
1		naphthalene	325, 641
2		1,2-naphthohydroquinone	432
3	UNKNOWN + 	UNKNOWN + 2-hydroxy-1,4 hydroquinone	403 + 572

Figure 5. Summary of Degradation of Naphthalene in Light. *Spectrum performed on an Aminco-Bowman spectrophotofluorometer, at 290 nm (excitation).

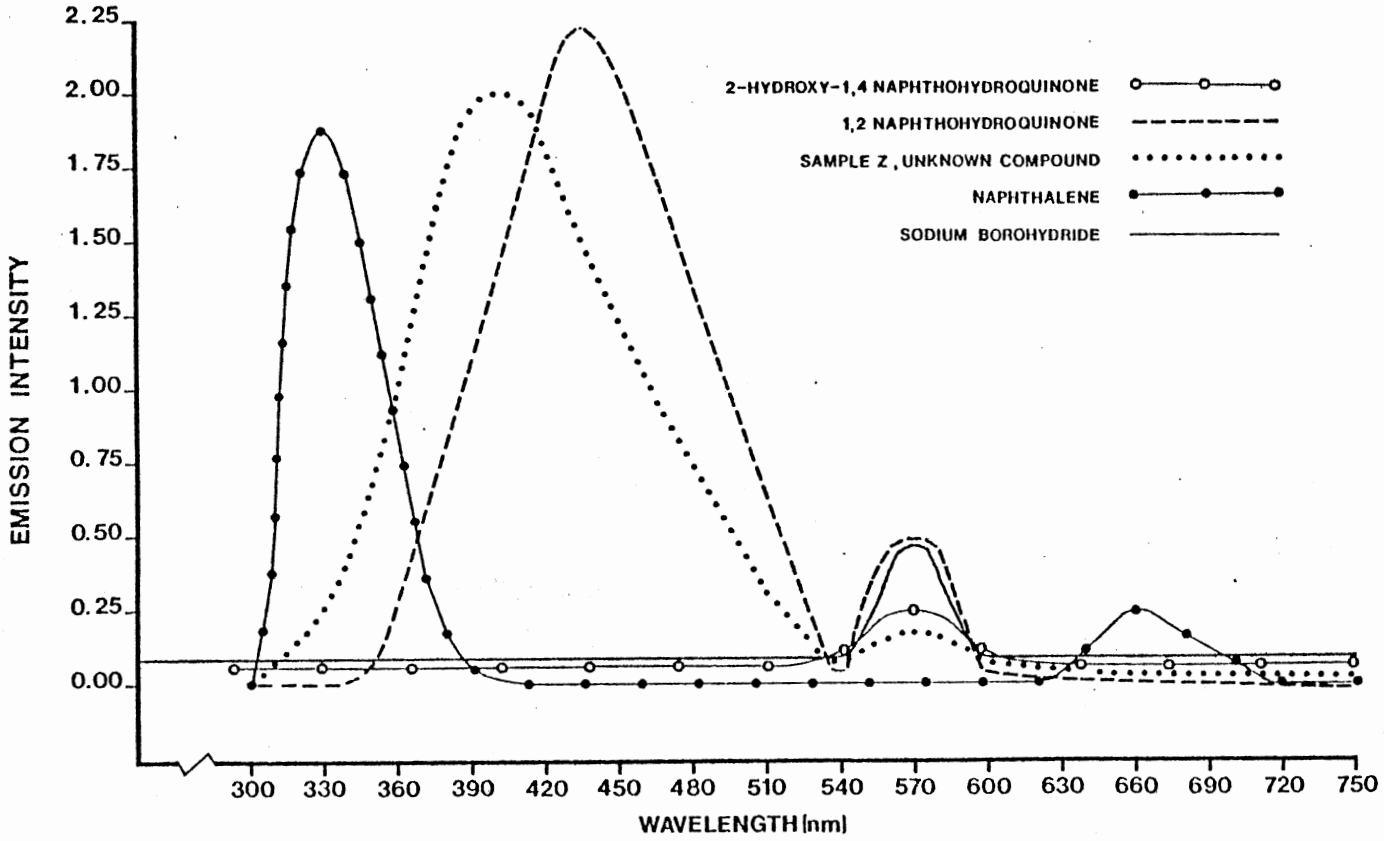


Figure 6. Fluorescence Emission Wavelengths of Naphthalene and Breakdown Products When Excited at 290 nm. Emission intensity is in arbitrary units.

TABLE III

HEAVY METAL ANALYSIS OF TEST WATER*

Metal	Dilution Water Metal Concentration (mg/l)	Tap Water Metal Concentration (mg/l)
K	4.57	5.08
Zn	0.02	0.04
Pb	<0.005	0.02
Cd	<0.005	<0.005
Cr	<0.001	0.04
Cu	0.03	0.05
Fe	0.05	0.07
Ca	41.48	52.70
Na	12.62	26.41
Mn	0.1	<0.10

*Analyzed by O.S.U. Water Quality Research Laboratory.

illustrates results of the heavy metal analyses on the water.

Total organic carbon (TOC) analyses were performed, and the values obtained were well within normal limits. The water samples, and a sample with a freshly prepared 1 mg/l naphthalene spike were assayed with a mass spectrometer. No abnormal spectra were detected, and all spectra were determined free of contamination by other chemicals.

Static Tests

In order to determine the relative toxicity of naphthalene, static tests were performed. The results were used to estimate concentrations for the flow-through bioassay system. Tests were performed using 500 ml glass jars with ground glass stoppers (Pyrex) which were acid-base washed and rinsed prior to each use. Five concentrations of naphthalene and a control were used. The jars were entirely covered with foil to discourage compound photodegradation, and emission wavelengths were recorded each 24 h to determine whether decomposition had occurred. No measurable degradation was detected. Larvae of stage 23 (6 wk old) were tested in this system, however they died from lack of oxygen probably due to the development of lungs. This stage was therefore eliminated from subsequent studies. One wk and 4 d old embryos were tested and results indicated a 24 h LC50 of approximately 11 mg/l at 22^{0}C (Table IV). Several static tests were also performed at 25^{o}C. The 24 h

TABLE IV

IN VIVO STATIC NAPHTHALENE LC50 TESTS IN SEVERAL STAGES OF *XENOPUS LAEVIS* LARVAE AT DIFFERENT TEMPERATURES

Animal Age weeks (stage)	Temp(°C) Test Duration	Number Animals	Naphthalene Concentration (mg/l)	Percent dead at end of test (%)	Approximate LC50 (mg/l)
1 (22)	22	10	control	0	
		10	7.6	0	~ 10
	24 h	10	13.9	100	
		10	17.9	100	
1 (22)	22	10	control	0	
		10	4.4	10	~ 11
	24 h	10	6.7	0	
		10	9.1	30	
2 (22)	22	10	control	0	
		10	5.8	0	~ 12
	24 h	10	10.6	0	
		10	14.0	100	
6 (23)	22	10	control	0	
		10	6.9	20	~ 11
	24 h	10	12.8	80	
		10	16.7	100	
7 (23)	22	10	control	0	
		10	5.9	30	~ 11
	24 h	10	10.6	40	
		10	14.1	100	

TABLE IV (continued)

Animal Age weeks (stage)	Temp(°C) Test Duration	Number Animals	Naphthalene Concentration (mg/l)	Percent dead at end of test (%)	Approximate LC50 (mg/l)
1 (22)	25	15	control	0	
		13	3.6	0	~ 8.8
	24 h	15	5.6	7	
		18	8.0	17	
		16	9.3	100	
1 (22)	25	20	control	0	
		20	3.1	20	~ 6.5
	96 h	20	5.4	40	
		20	7.1	60	
		20	8.5	100	
3 (22)	25	20	control	0	
		20	5.0	2	~ 6.8
	96 h	20	6.3	25	
		20	7.0	40	
		20	7.6	90	
0 (cleaving eggs)	28	40	control	28	
		41	2.6	53	~ 2.5
	24 h	40	5.1	65	
		40	9.9	73	
0 (17)	28	10	control	0	
		10	1.1	10	~ 4.3
	24 h	10	3.2	10	
		10	5.4	100	

naphthalene LC50 for 1 week larvae was 8.8 mg/l, while the 96 h LC50 was 6.5 mg/l. With 3 week larvae, the 96 h LC50 was 6.8 mg/l (Table IV). A test at 28°C with cleaving eggs resulted in a 24 h LC50 of 2.5 mg/l, while a test with "oral sucker" stage larvae (Stage 17) resulted in a 24 h LC50 of 4.3 mg/l. The concentration of naphthalene in the bottles was determined fluorometrically and concentration values were estimated by reference to a standard curve prepared on the day of the experiment. Standard curves were prepared using 0, 1, 5, 10, 15, 20 mg/l naphthalene and were linear over this range. Twenty-four h static tests gave an indication of relative toxicity and initial flow-through tests incorporated a value of 11 mg/l as a median concentration.

Flow-Through Tests

Lethality

One purpose of the flow-through diluter system was to determine the 96 LC50 of naphthalene. This value may be compared to similar tests using other compounds and may serve as a system to assign values of relative toxicity to compounds.

The 96 h LC50 for one week tadpoles in Test A (the first successful test with one week larvae) was 4.3 mg/l as compared to 3.3 mg/l in Test B (the second successful test with one week larvae) (Table V). Test A values were 33% higher than Test B values (Tables VI-IX).

TABLE V

SUMMARY AND COMPARISON OF NAPHTHALENE EC50 FOR DEPIGMENTATION AND ABSENCE OF SWIMMING, AND LC50 FOR LETHALITY FOR ONE WEEK AND THREE WEEK LARVAE

Age of Larvae	Date	EC50 Depigmentation (mg/l)	EC50 Absence of swimming (mg/l)	96 h LC50 Lethality (mg/l)
1 week	8/20-8/24	5.3 (6 h)	1.9	4.3
	9/16-9/20	2.6 (0.8 h)	0.4	3.2
	9/16-9/20	3.0 (4 h)	3.0	3.2
3 week	8/30-9/3	3.7	2.1	2.05
	10/13-10/22	3.4	1.7	2.1
	8/29-8/30	2.3	2.3	---
	9/27-10/3	3.0	2.1	---
	9/29-10/3	2.1	3.0	---

TABLE VI

EXPOSURE CHAMBER NAPHTHALENE AND DISSOLVED OXYGEN CONCENTRATIONS OVER 96 HR FOR ONE WEEK LARVAE TEST; 20 AUG.-24 AUG.

Naphthalene concentration* (mg/l)	95% Confidence interval of the mean naphthalene concentration	Dissolved Oxygen* (mg/l)	95% Confidence interval of the mean dissolved O_2 concentration
0	—	5.4 ± 0.2 (6)	± 0.3
1.0 ± 0.1 (7)	± 0.1	5.7 ± 0.4 (3)	± 1.0
2.8 ± 0.2 (7)	± 0.1	4.9 ± 0.2 (2)	± 1.9
4.9 ± 0.4 (7)	± 0.4	5.3 ± 0.3 (2)	± 2.5
5.0 ± 0.7 (7)	± 0.7	5.3 ± - - (1)	- -

* Values are means ± SD (n).

TABLE VII

EFFECT OF NAPHTHALENE ON PERCENT LOSS OF PIGMENT, ABSENCE OF SWIMMING AND LETHALITY FOR ONE WEEK LARVAE; 20 AUG.- 24 AUG.*

Naphthalene Concentration (mg/l)	95% Confidence interval (mg/l)	Mean Dissolved Oxygen (mg/l)	95% Confidence interval (mg/l)	Loss of pigment at 6 hr (%)	Absence of swimming at 6 hr (%)	Dead at 96 hr (# dead/# total) (%)
0	-	5.4	5.1-5.7	0	0	0 (0/20)
1.0	0.9-1.1	5.7	4.7-6.7	0	35.0	0 (0/20)
2.8	2.7-2.9	4.9	2.9-6.8	10.0	45.0	10.0 (2/20)
4.9	4.5-5.3	5.3	2.8-7.8	30.0	70.0	45.0 (9/20)
5.0	4.3-5.7	5.3	-.- -.-	40.0	100.0	80.0 (16/20)
			EC_{50}	5.3	1.94	- -
			EC_{16}	3.8	0.6	- -
			EC_{84}	7.5	6.2	- -

* Temperature mean was 28.5°C, range 27.5-29.5°C; pH mean was 7.04, range 6.5-7.3.

TABLE VIII

EXPOSURE CHAMBER NAPHTHALENE AND DISSOLVED OXYGEN CONCENTRATIONS OVER 96 HR FOR ONE WEEK LARVAE TEST; 16 SEPT.-20 SEPT.

Naphthalene concentration* (mg/l)	95% Confidence interval of the mean naphthalene concentration	Dissolved Oxygen* (mg/l)	95% Confidence interval of the mean dissolved O_2 concentration
0	—	5.5 ± 0.2 (6)	± 0.3
1.4 ± 0.2 (7)	± 0.2	5.5 ± 0.1 (3)	± 0.1
2.6 ± 0.1 (7)	± 0.1	5.4 ± 0.3 (3)	± 0.5
3.1 ± 0.1 (7)	± 0.1	5.2 ± 0.1 (3)	± 0.2
4.8 ± 0.2 (7)	± 0.2	5.3 ± 0.2 (3)	± 0.3

* Values are means ± SD (n).

TABLE IX

EFFECT OF NAPHTHALENE ON PERCENT LOSS OF PIGMENT, ABSENCE OF SWIMMING AND LETHALITY FOR ONE WEEK LARVAE; 16 SEPT.- 20 SEPT.*

Naphthalene Concentration (mg/l)	95% Confidence interval (mg/l)	Mean Dissolved Oxygen (mg/l)	95% Confidence interval (mg/l)	Loss of pigment at 6 hr (%)	Absence of swimming at 6 hr (%)	Dead at 96 hr (# dead/# total) (%)
0	-	5.5	5.3-5.8	0	0	0 (0/20)
1.4	1.2-1.6	5.5	5.4-5.6	25.0	70.0	6.3 (1/16)
2.6	2.5-2.6	5.4	4.9-5.8	40.0	75.0	15.0 (3/20)
3.1	3.0-3.2	5.2	5.0-5.4	80.0	80.0	30.0 (6/20)
4.8	4.7-5.0	5.3	5.0-5.6	60.0	85.0	100.0 (20/20)
			EC_{50}	2.6	0.4	3.3
			EC_{16}	0.9	0.1	2.1
			EC_{84}	7.7	4.6	5.0

* Temperature mean was 28.0°C; pH mean was 7.1, range 6.9-7.1.

naphthalene lethality dose-response curves for Test A and B with 1 week larvae are shown in Figures 7-8. The log concentration (mg/l) was plotted against the probability (probit) of response.

The 96 h LC50 for 3 week tadpoles in Test A (the first successful test) was 2.1 mg/l and 2.1 mg/l in Test B (the second successful test) with 100% correlation between test results (Tables X-XIII). Figures 9-10 represent the dose-response curve for these tests.

Depigmentation

When toxified with naphthalene above a certain concentration, Xenopus larvae may change the color of their pigmentation from grey to whitish, and then to very white. Generally, once they turn white, they will die within 6-8 h. This change in pigmentation occurs at a high naphthalene concentration and is easy to differentiate from normal pigmentation. The lack of swimming, however, may occur at a concentration as low as 1 mg/l. The $EC50_{Depig}$, or the concentration of naphthalene it takes to change 50% of the larvae to a white color, may be used as an indicator of toxicity, and perhaps may allow prediction of the 96 h LC50. A 6 h time interval was used for the tests to correlate the data with the lack of swimming data. Both sets of data together may enhance prediction of the LC50 value.

Using 1 wk organisms, 6 h $EC50_{Depig}$ values were calculated based on percent of animals that appeared white in

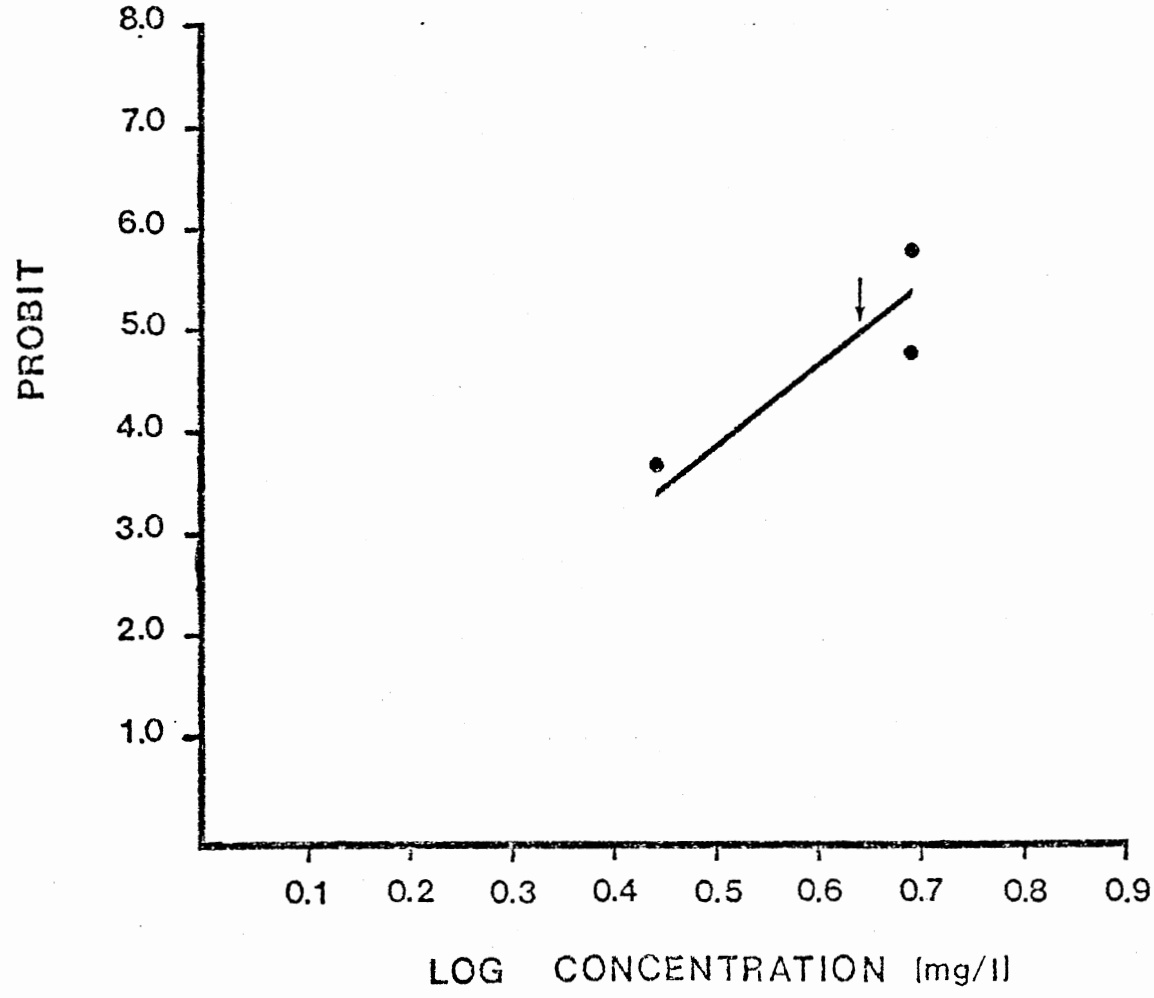


Figure 7. Naphthalene Dose-Response Lethality Curve For 96 Hour Exposure Test With 1 Week Old Larvae; 20 Aug.-24 Aug. Arrow denotes LC50.

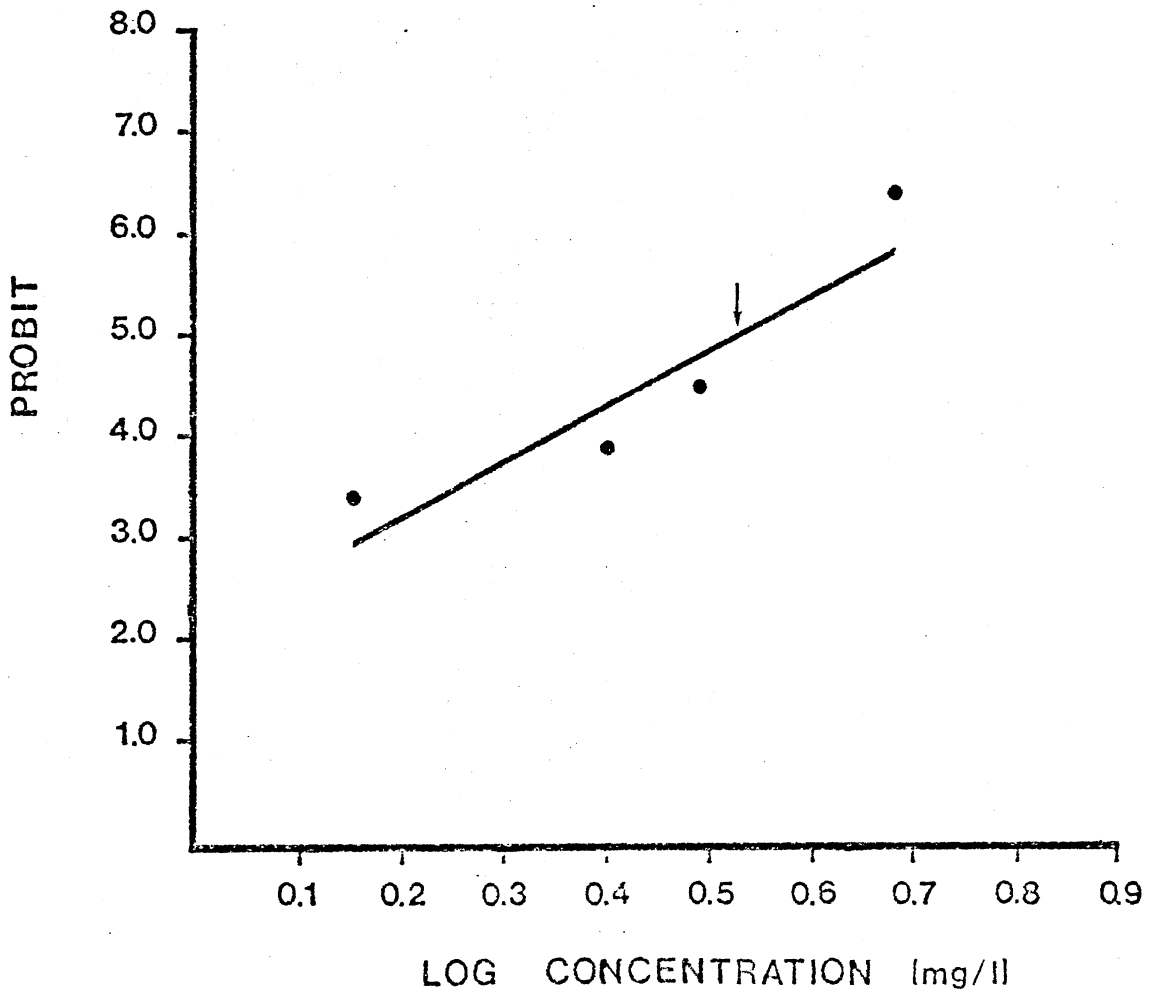


Figure 8. Naphthalene Dose-Response Lethality Curve For 96 Hour Exposure Test With 1 Week Old Larvae; 16 Sept.-20 Sept. Arrow denotes LC50. Values from Table IX.

TABLE X

EXPOSURE CHAMBER NAPHTHALENE AND DISSOLVED OXYGEN CONCENTRATIONS OVER 96 HR FOR ONE WEEK LARVAE TEST; 30 AUG.-3 SEPT.

Naphthalene concentration* (mg/l)	95% Confidence interval of the mean naphthalene concentration	Dissolved Oxygen* (mg/l)	95% Confidence interval of the mean dissolved O_2 concentration
0	—	5.5 ± - - (1)	± 0.4
2.0 ± 0.1 (5)	± 0.2	5.8 ± 0.4 (3)	± 1.0
3.3 ± 0.2 (5)	± 0.2	5.7 ± 0.1 (2)	± 1.3
4.1 ± 0.1 (5)	± 0.1	6.0 ± 0.5 (2)	± 4.5
4.9 ± 0.4 (3)	± 0.9	4.7 ± 0.4 (4)	± 0.6

* Values are means ± SD (n).

TABLE XI

EFFECT OF NAPHTHALENE ON PERCENT LOSS OF PIGMENT, ABSENCE OF SWIMMING AND LETHALITY FOR THREE WEEK LARVAE; 30 AUG.- 3 SEPT.*

Naphthalene Concentration (mg/l)	95% Confidence interval (mg/l)	Mean Dissolved Oxygen (mg/l)	95% Confidence interval (mg/l)	Loss of pigment at 6 hr (%)	Absence of swimming at 6 hr (%)	Dead at 96 hr (# dead/# total) (%)
0	-	5.5	5.2-5.9	0.0	0.0	0.0 (0/12)
2.0	1.8-2.2	5.8	4.8-6.8	16.7	50.0	50.0 (6/12)
3.3	3.2-3.5	5.7	4.4-7.0	33.4	58.0	83.3 (10/12)
4.1	4.0-4.2	4.7	4.2-5.3	50.0	41.6	91.6 (11/12)
4.87	4.2-5.5	5.95	1.5-10.4	67.0	67.0	100.0 (12/12)
			EC_{50}	3.7	2.3	2.1
			EC_{16}	2.1	0.1	1.3
			EC_{84}	6.5	90.61	3.2

* Temperature mean was 28.3°C, range 27-29°C; pH mean was 7.09, range 7.0-7.2.

TABLE XII

EXPOSURE CHAMBER NAPHTHALENE AND DISSOLVED OXYGEN CONCENTRATIONS OVER 96 HR FOR ONE WEEK LARVAE TEST; 18 OCT.-21 OCT.

Naphthalene concentration* (mg/l)	95% Confidence interval of the mean naphthalene concentration	Dissolved Oxygen* (mg/l)	95% Confidence interval of the mean dissolved O_2 concentration
0	—	5.5 ± 0.1 (3)	± 0.1
1.3 ± 0.1 (6)	± 0.1	5.2 ± 0.2 (2)	± 1.9
2.4 ± 0.1 (6)	± 0.1	5.3 ± 0.1 (2)	± 0.6
3.1 ± 0.1 (6)	± 0.1	5.4 ± 0.3 (2)	± 0.7
3.8 ± 0.2 (6)	± 0.2	5.5 ± 0.2 (2)	± 2.0

* Values are means ± SD (n).

TABLE XIII

EFFECT OF NAPHTHALENE ON PERCENT LOSS OF PIGMENT, ABSENCE OF SWIMMING AND LETHALITY FOR THREE WEEK LARVAE; 18 OCT.- 21 OCT.*

Naphthalene Concentration (mg/l)	95% Confidence interval (mg/l)	Mean Dissolved Oxygen (mg/l)	95% Confidence interval (mg/l)	Loss of pigment at 6 hr (%)	Absence of swimming at 6 hr (%)	Dead at 96 hr (# dead/# total) (%)
0	-	5.5	5.3-5.6	0	0	0 (0/12)
1.3	1.1-1.4	5.2	3.2-7.1	0	33.0	16.7 (2/12)
2.4	2.2-2.5	5.3	4.6-5.9	0	75.0	41.7 (5/12)
3.1	2.9-3.3	5.4	4.7-6.1	8.0	75.0	100.0 (12/12)
3.8	3.5-4.0	5.5	3.5-7.4	100.0	100.0	100.0 (12/12)
			EC_{50}	- -	1.7	2.1
			EC_{16}	- -	1.0	1.3
			EC_{84}	- -	3.0	3.2

* Temperature mean was 28.0°C; pH mean was 7.00, range 6.95-7.05.

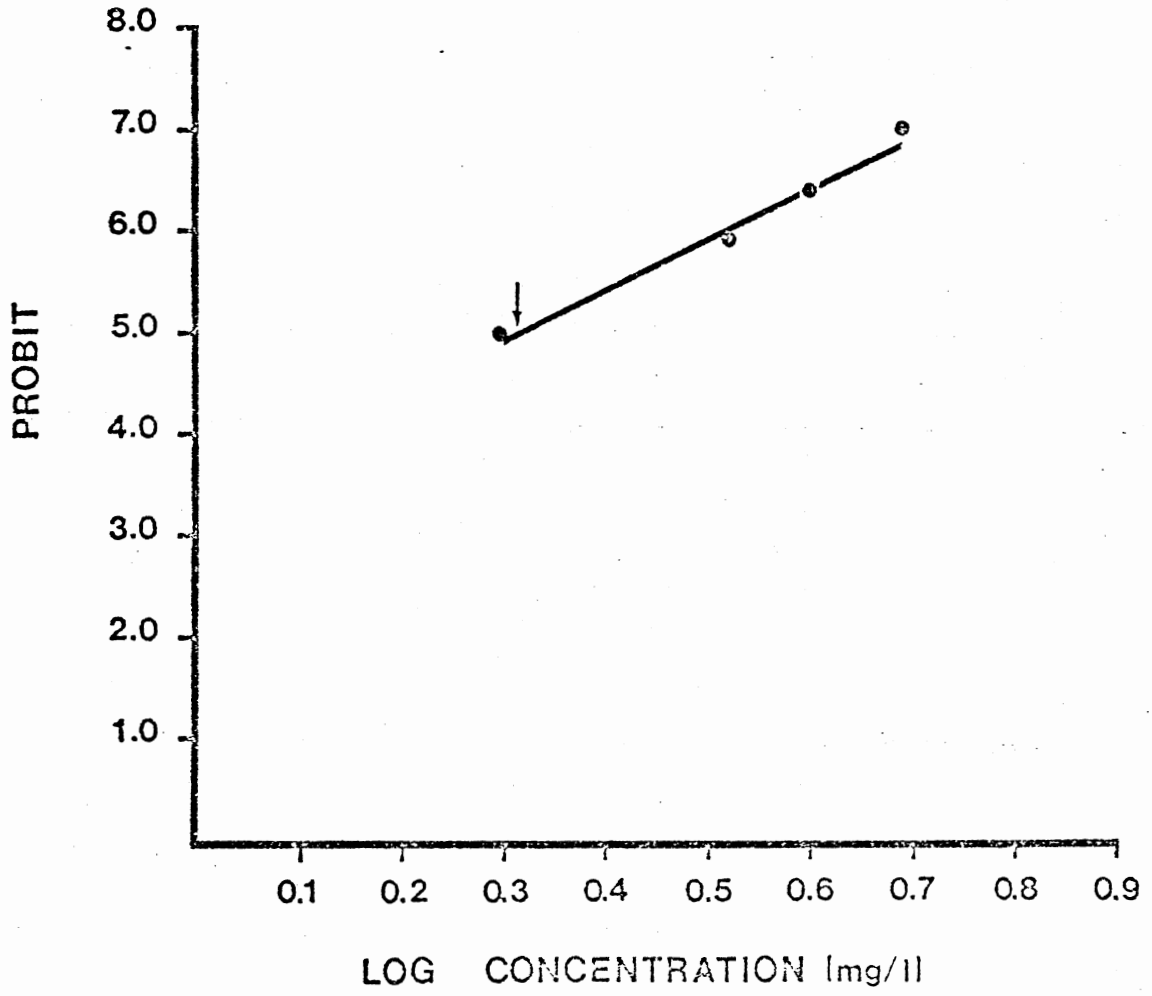


Figure 9. Naphthalene Dose-Response Lethality Curve For 96 Hour Exposure Test With 3 Week Old Larvae; 30 Aug.-3 Sept. Arrow denotes LC50. Values from Table XI.

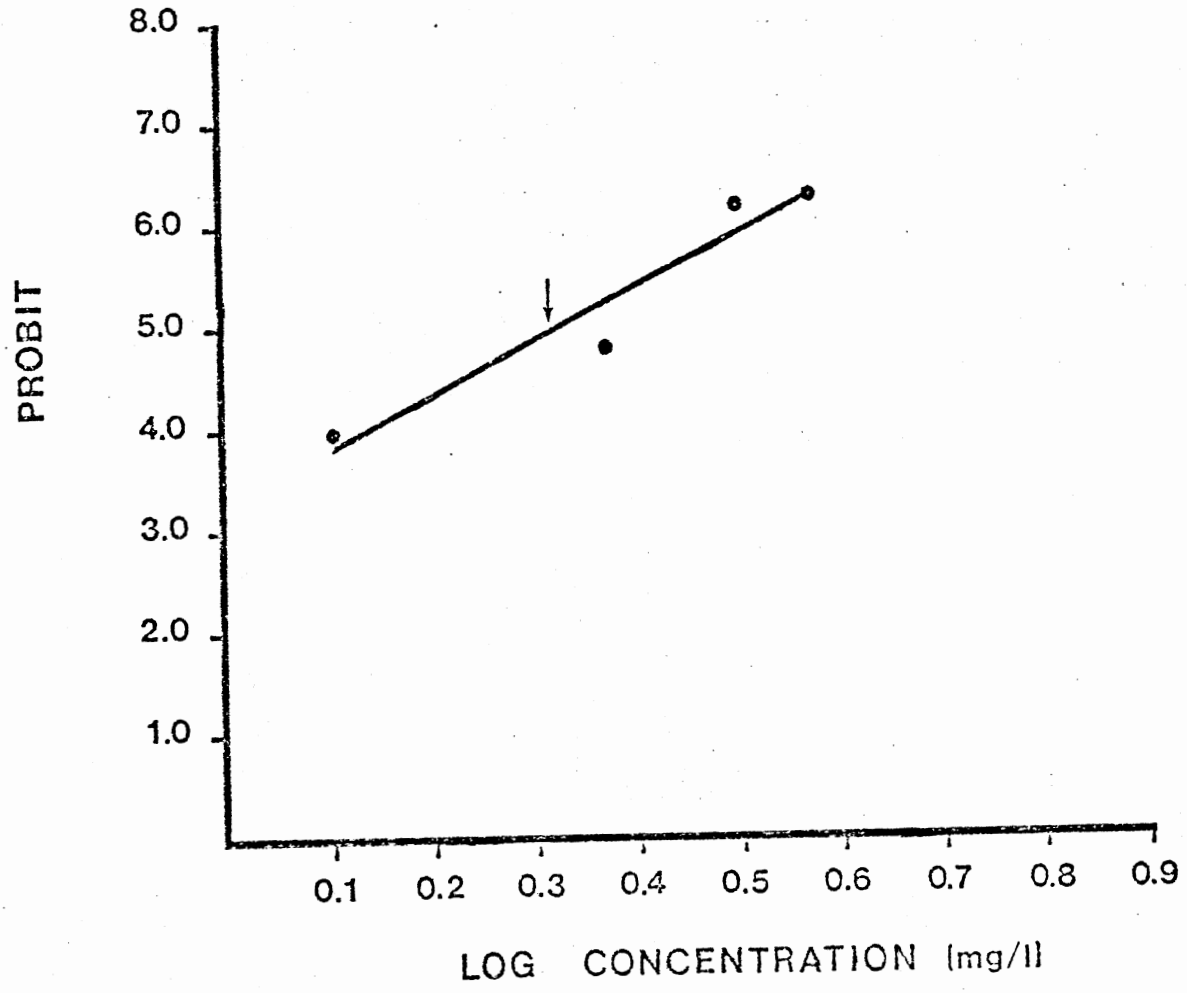


Figure 10. Naphthalene Dose-Response Lethality Curve For 96 Hour Exposure Test With 3 Week Old Larvae; 18 Oct.-22 Oct. Arrow denotes LC50. Values from Table XIII.

each concentration. In Test A, the $EC50_{Depig}$ was 5.3 mg/l, and the 96 h LC50 was 4.3 mg/l. In Test B, the $EC50_{Depig}$ data was 2.6 mg/l, compared to a 96 h LC50 of 3.3 mg/l (Tables VI and VIII; Fig. 11). These values were within 18% and 21% of the true LC50 values. Using data of Depigmentation at 4 h with Test B larvae, the correlation is very good with an $EC50_{Depig}$ of 3.0 mg/l and 96 h LC50 of 3.3 mg/l. Using 3 week old organisms, in Test A 6 h $EC50_{Depig}$ was approximately 2.1 mg/l and the 96 h LC50 was 2.1 mg/l. Test B had a 6 h $EC50_{Depig}$ of approximately 3.4 mg/l, while the 96 h LC50 was 2.1 mg/l (Tables X and XII). In test A the $EC50_{Depig}$ was the same as the LC50 value, and in Test B, the $EC50_{Depig}$ was 64% greater than the 96 h LC50. Test B values did not correlate well, or serve as a good indication of the LC50. Figures 12-15 represent data of 6 h depigmentaion from four tests--Test A and Test B, as well as two tests which did not last over 24 h.

Absence of Swimming

One behavorial trait which may indicate toxicity is the lack of swimming by the larvae. The 6 h EC50, using lack of swimming as criteria, ($EC50_{swim}$) was calculated for each 96 h toxicity test, and also for several tests which did not last for the entire 96 h (Tables VI, VIII, X, and XII). The 6 h time interval was chosen because the animals were just beginning to acclimate to the naphthalene. At this time, at lower concentrations, the larvae can begin to swim

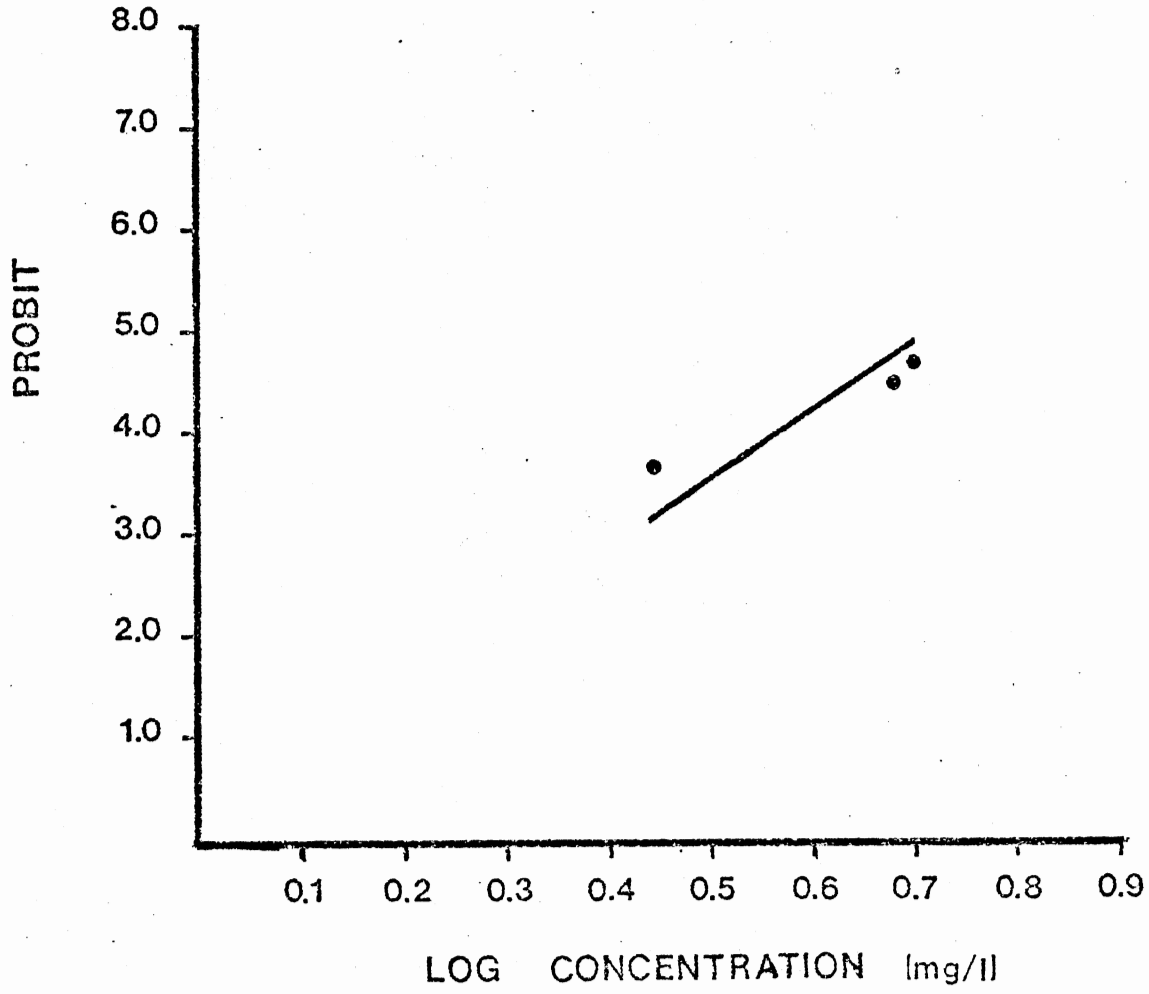


Figure 11. Naphthalene Dose-Response Curve For Depigmentation At 6 Hr With 1 Week Old Larvae; 20 Aug.-24 Aug. Values from Table VII.

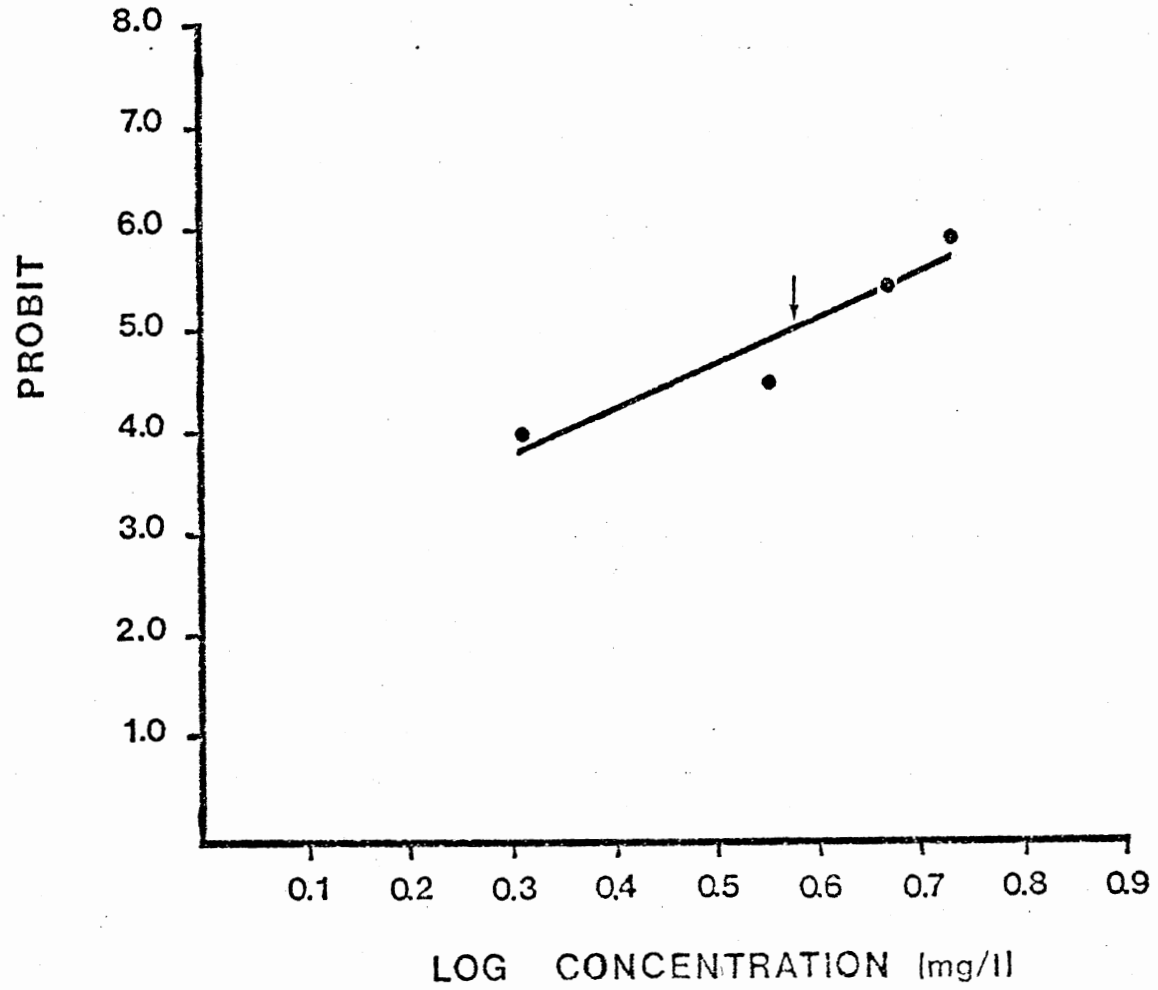


Figure 12. Naphthalene Dose-Response Curve For Depigmentation At 6 Hr With 3 Week Old Larvae; 30 Aug.-3 Sept. (First Organisms). Arrow denotes EC50. Values from Table XI.

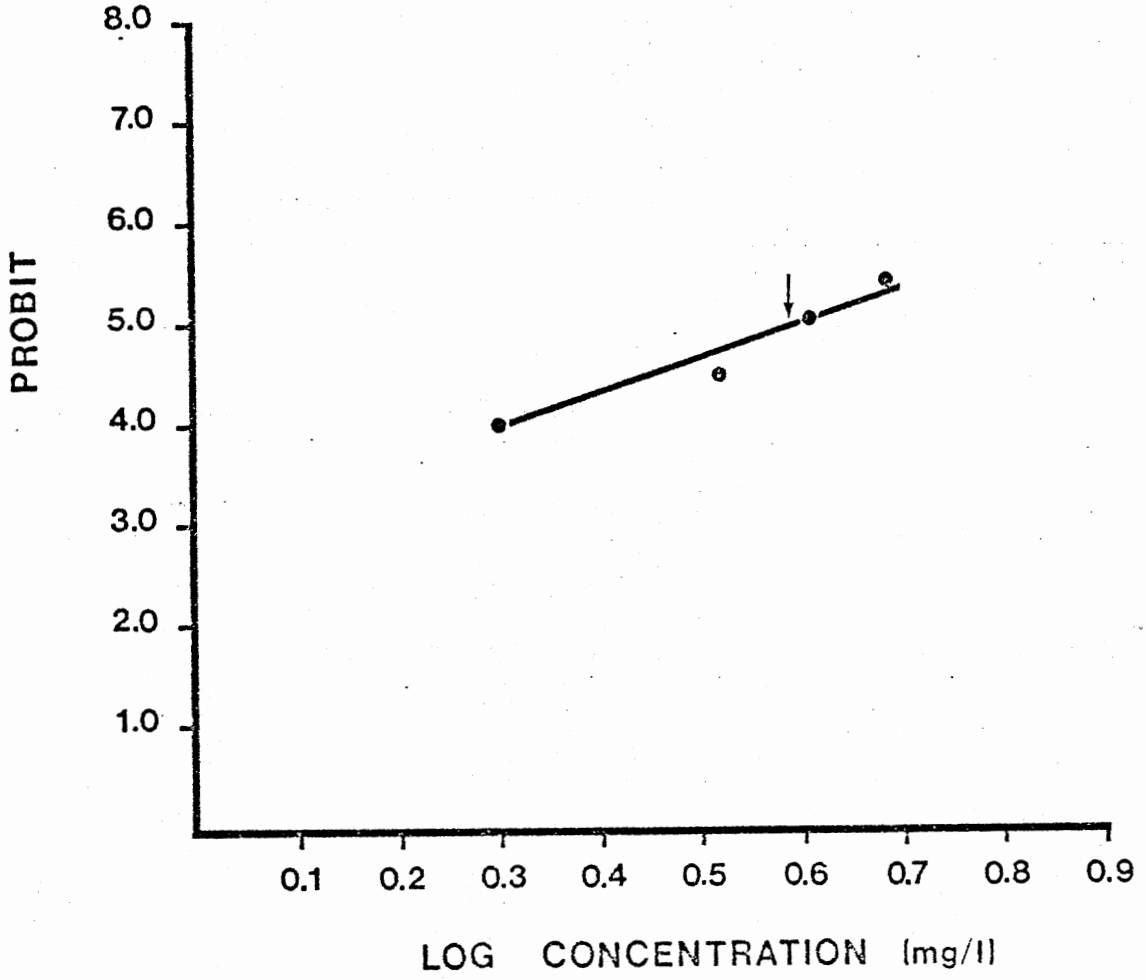


Figure 13. Naphthalene Dose-Response Curve For Depigmentation At 6 Hr With 3 Week Old Larvae; 30 Aug.-3 Sept. (Second Organisms). Arrow denotes EC50. Values from Table XI.

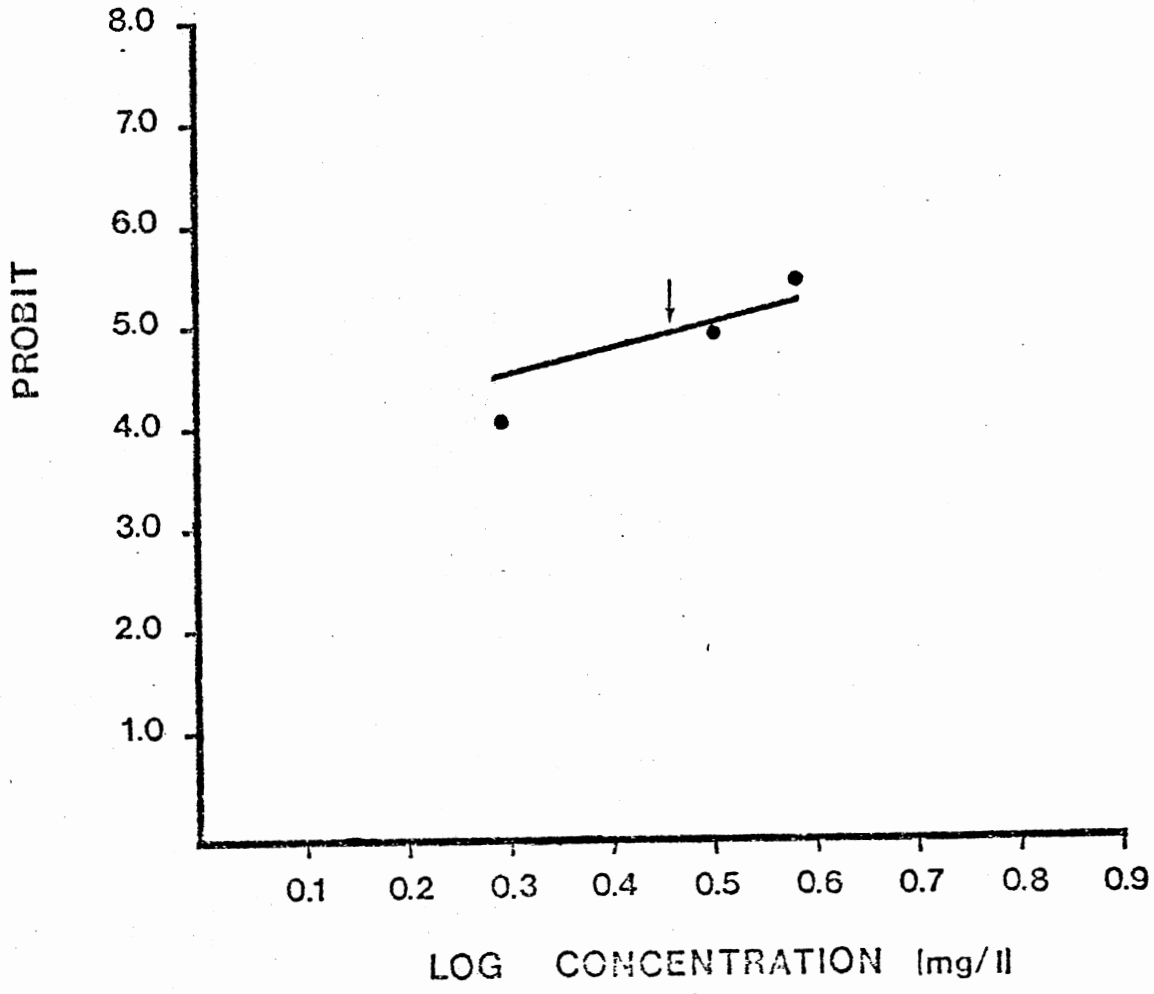


Figure 14. Naphthalene Dose-Response Curve For Depigmentation At 6 Hr With 3 Week Old Larvae; 27 Sept.-28 Sept. Arrow denotes EC50.

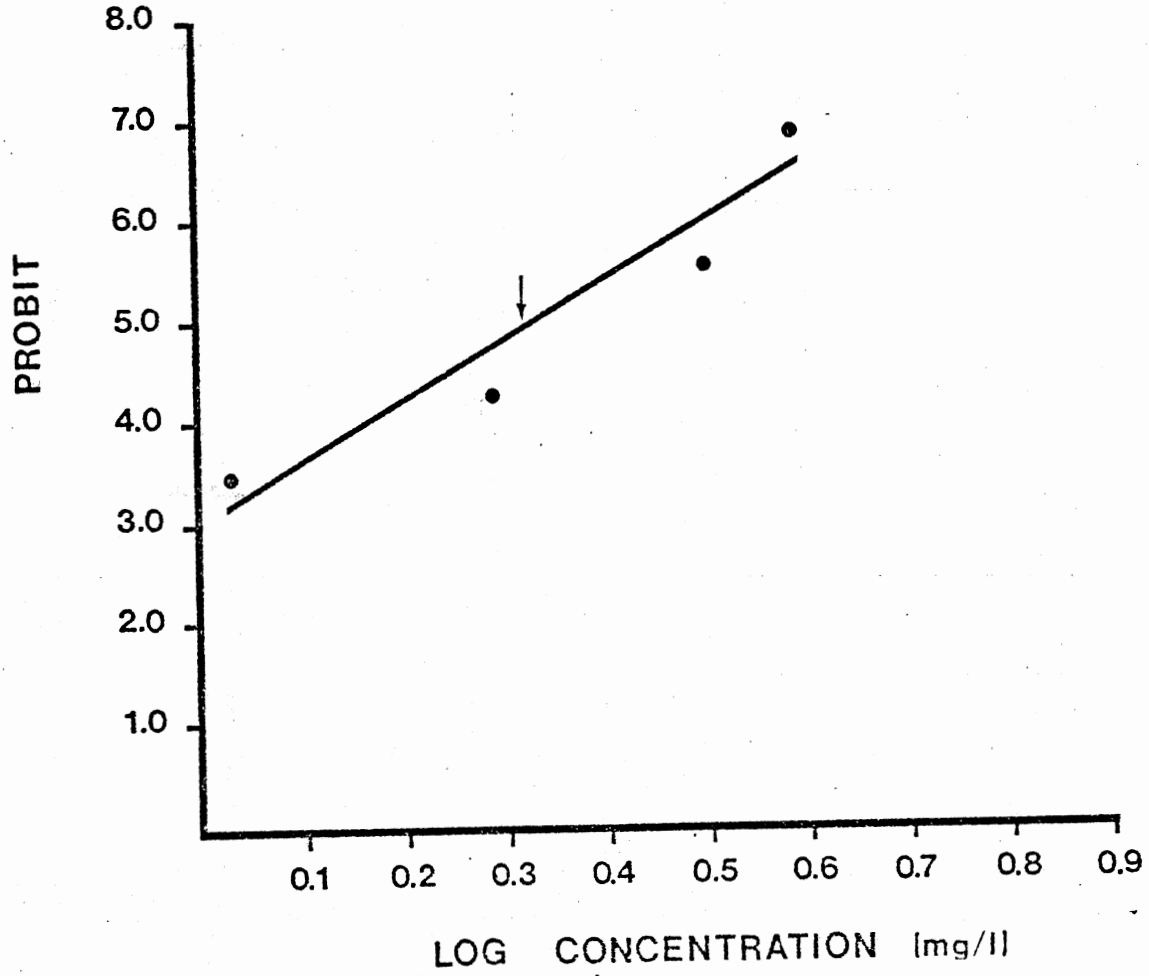


Figure 15. Naphthalene Dose-Response Curve For Depigmentation At 6 Hr With 3 Week Old Larvae; 29 Sept.-3 Oct. Arrow denotes EC50.

somewhat normally.

The $EC50_{swim}$ was calculated to determine if this behavior could serve to predict the 50% lethality at 96 h for each concentration. The $EC50_{swim}$ for 1 week Test A organisms was 1.9 mg/l (Table V). The 96 h LC50 for the same organisms was 4.3 mg/l, or 2.2 times the $EC50_{swim}$ value. In Test B the $EC50_{swim}$ value was 0.4 mg/l with the 96h LC50 value at 3.3 mg/l, a 7.4 fold difference between values.

The 6 h $EC50_{swim}$ values for 3 week larvae correlated very well with the 96 h LC50 values, This proved to be a much better predictor of the 96 h LC50. In Test A the 6 h $EC50_{swim}$ was 2.3 mg/l, and the corresponding 96 h LC50 value was 2.1 mg/l. In Test B the values also were of predictive value, with a $EC50_{swim}$ of 1.7 mg/l, and a 96 h LC50 of 2.1 mg/l. In Test A, the $EC50_{swim}$ values were within 10% of the correct LC50, and in Test B, they were within 18% (Table V). Several other depigmentation responses were recorded, but the 96 h LC50 were not obtained with these tests. Dose-response curves for absence of swimming with 1 week and 3 week larvae are shown in Figures 16-21.

In many cases, naphthalene has been found to have a "biphasic" effect on organisms. In Xenopus, the effect is represented by graphs of toxicity behavior over time (Figure 22). The criteria used was the number of animals not swimming (i.e. those organisms that have fallen to the screen). Most of the animals fell to the screen within the first few

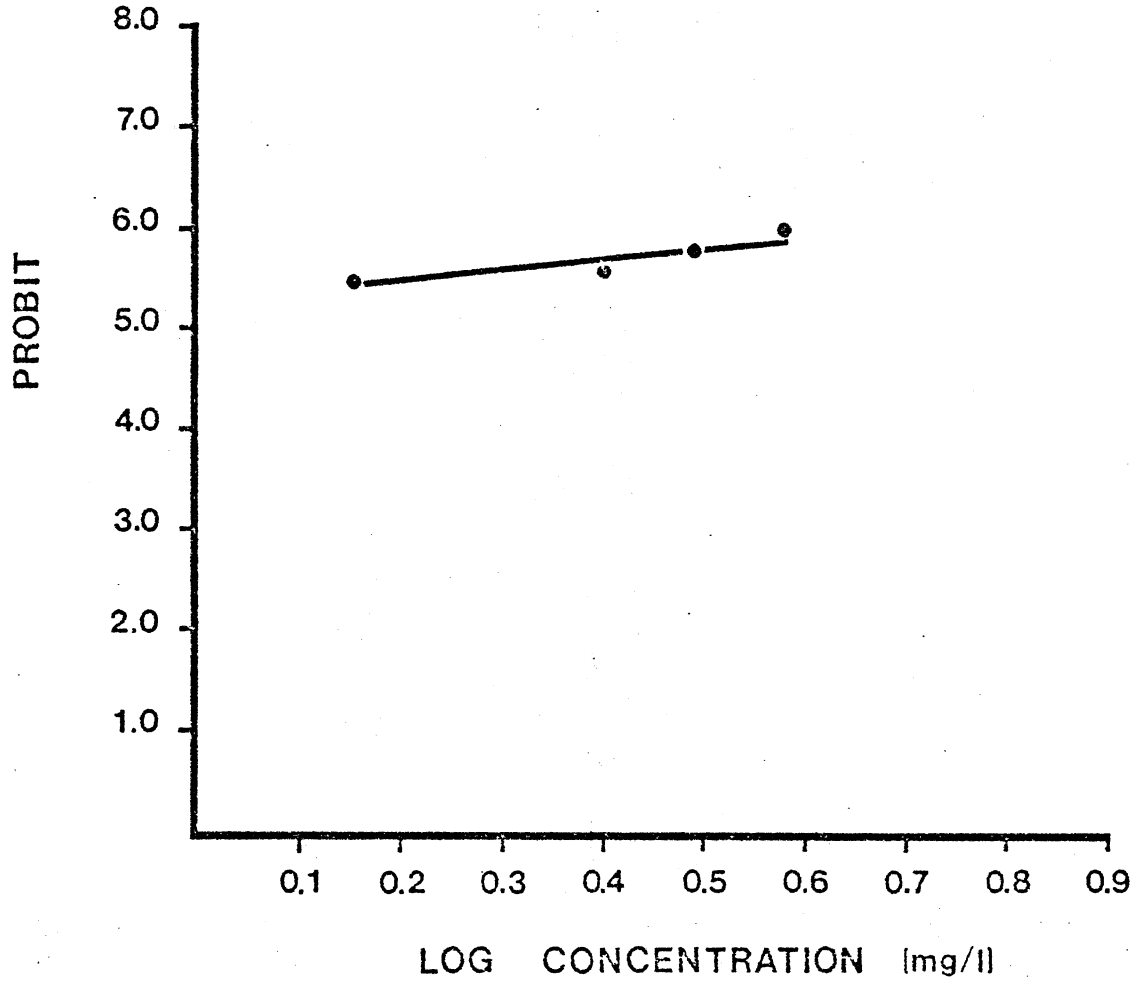


Figure 16. Naphthalene Dose-Response Curve For Absence of Swimming At 6 Hr With 1 Week Old Larvae; 16 Sept.-20 Sept. Values from Table IX.

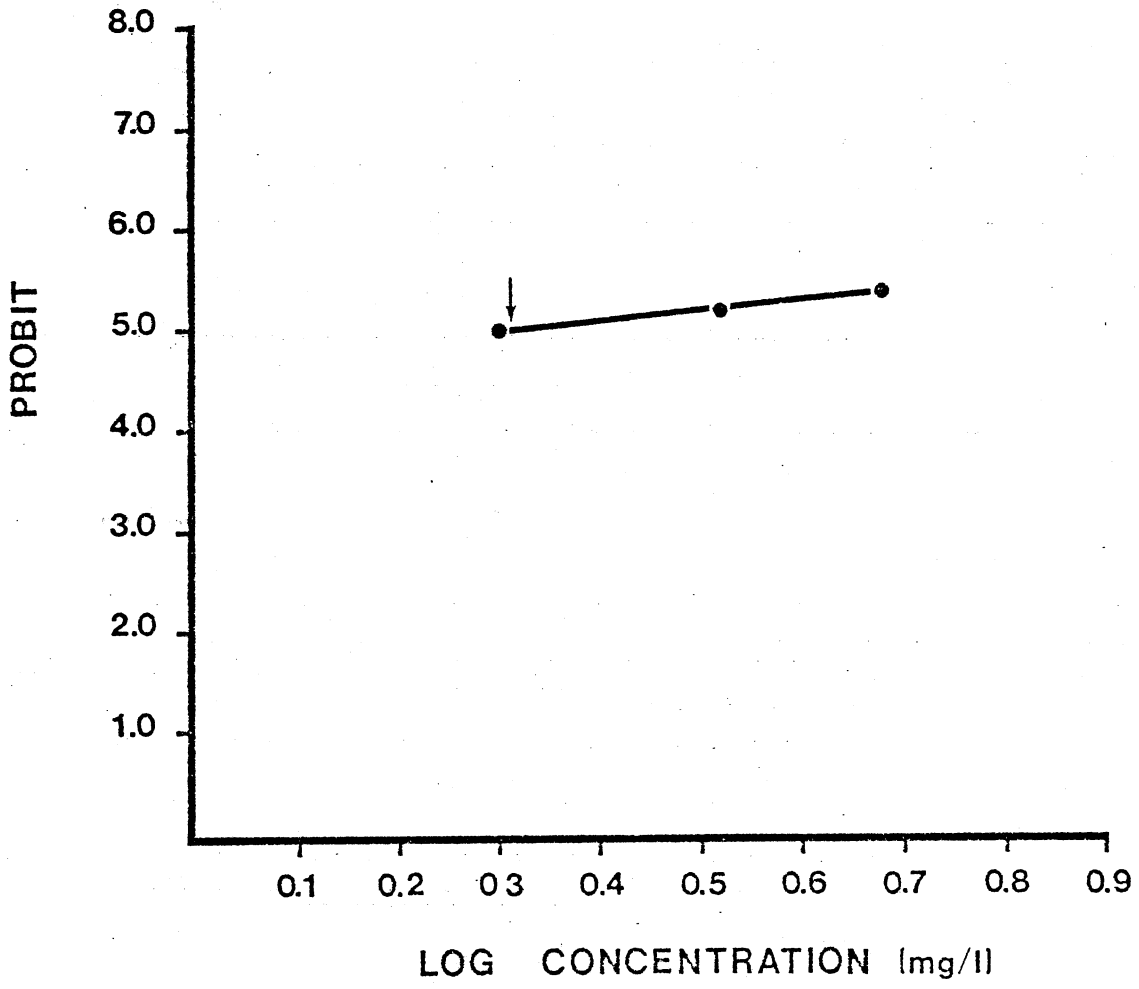


Figure 17. Naphthalene Dose-Response Curve For Absence of Swimming At 6 Hr With 3 Week Old Larvae; 30 Aug.-3 Sept. Arrow denotes EC50. Values from Table VII.

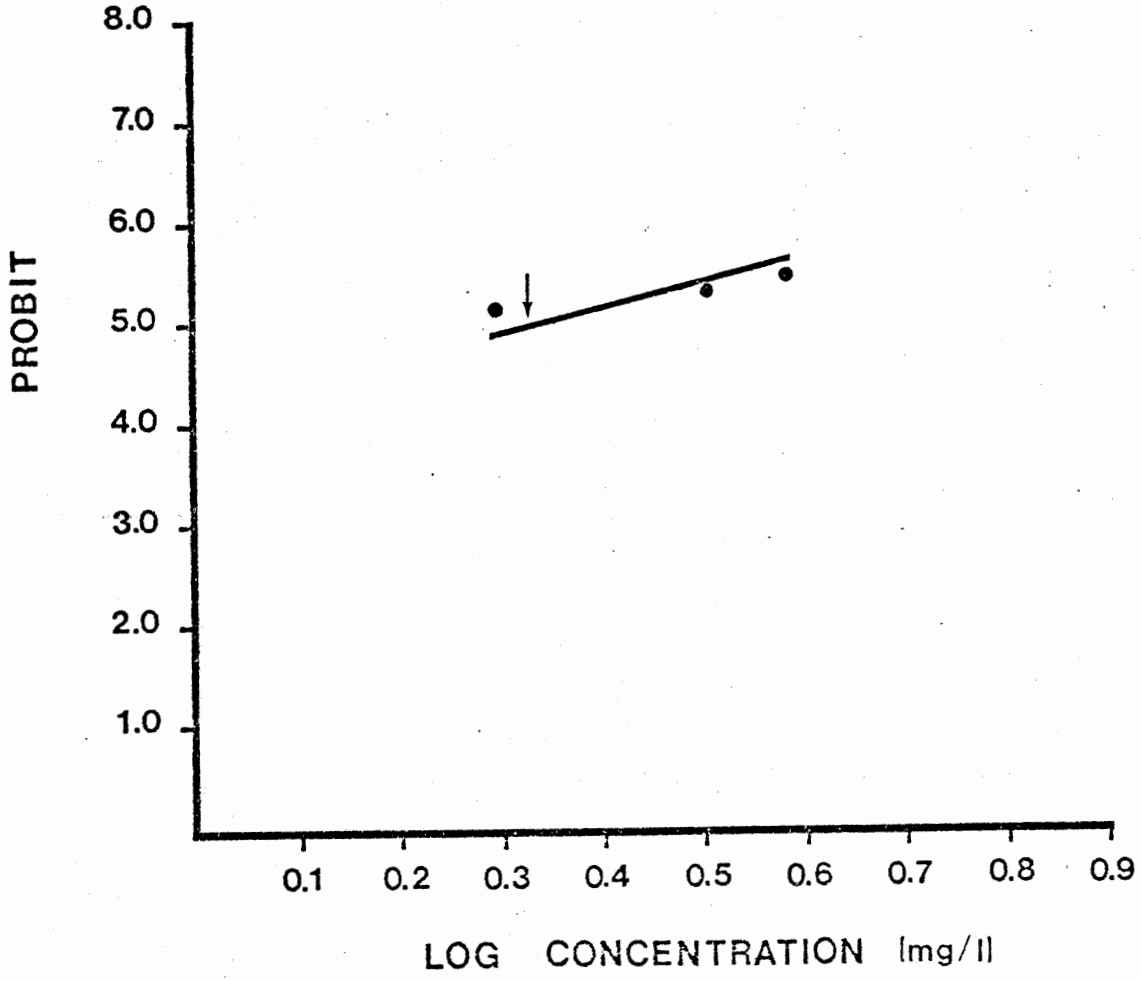


Figure 18. Naphthalene Dose-Response Curve For Absence of Swimming At 6 Hr With 3 Week Old Larvae; 27 Sept.-28 Sept. Arrow denotes EC50.

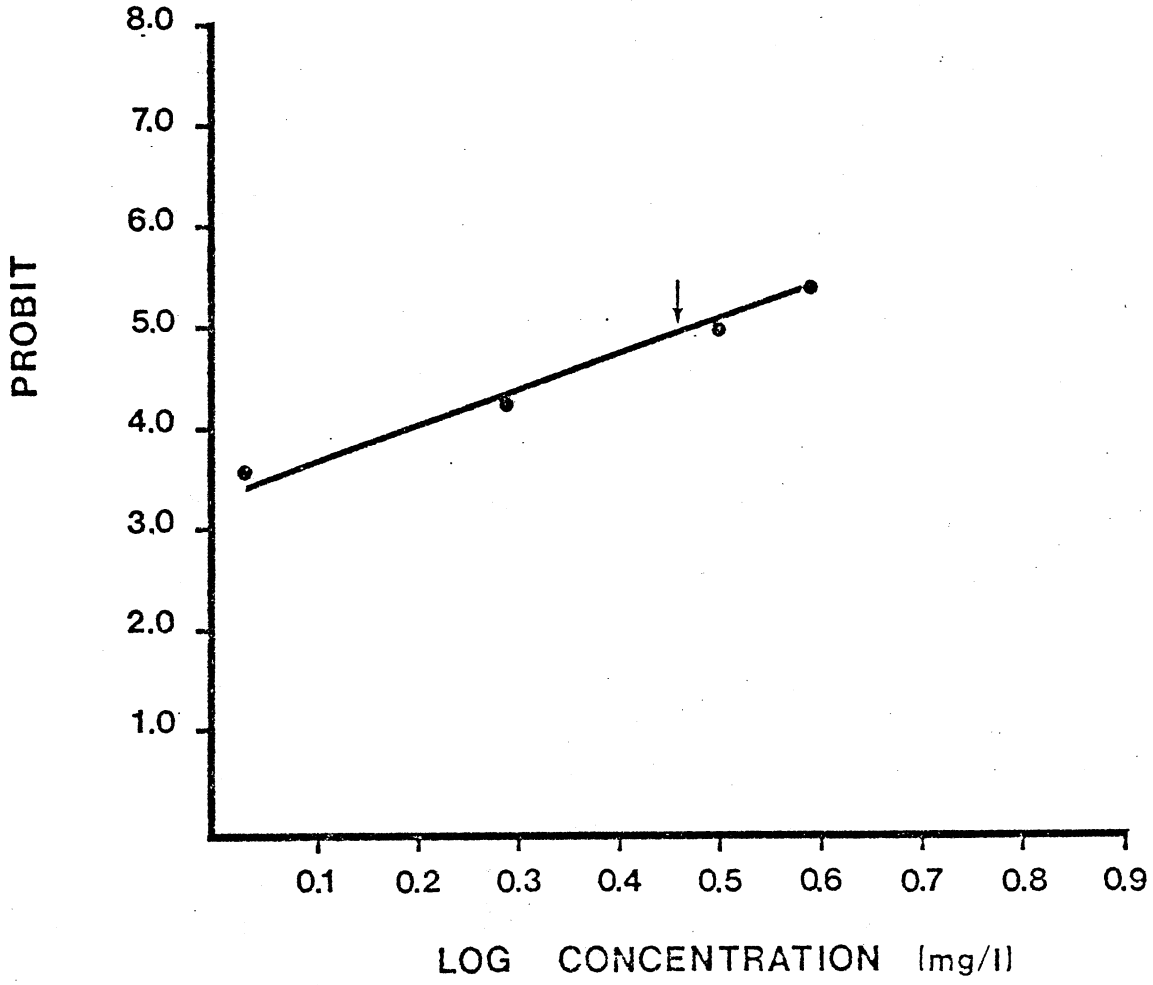


Figure 19. Naphthalene Dose-Response Curve For Absence of Swimming At 6 Hr With 3 Week Old Larvae; 29 Sept.-3 Oct. Values from Table XI.

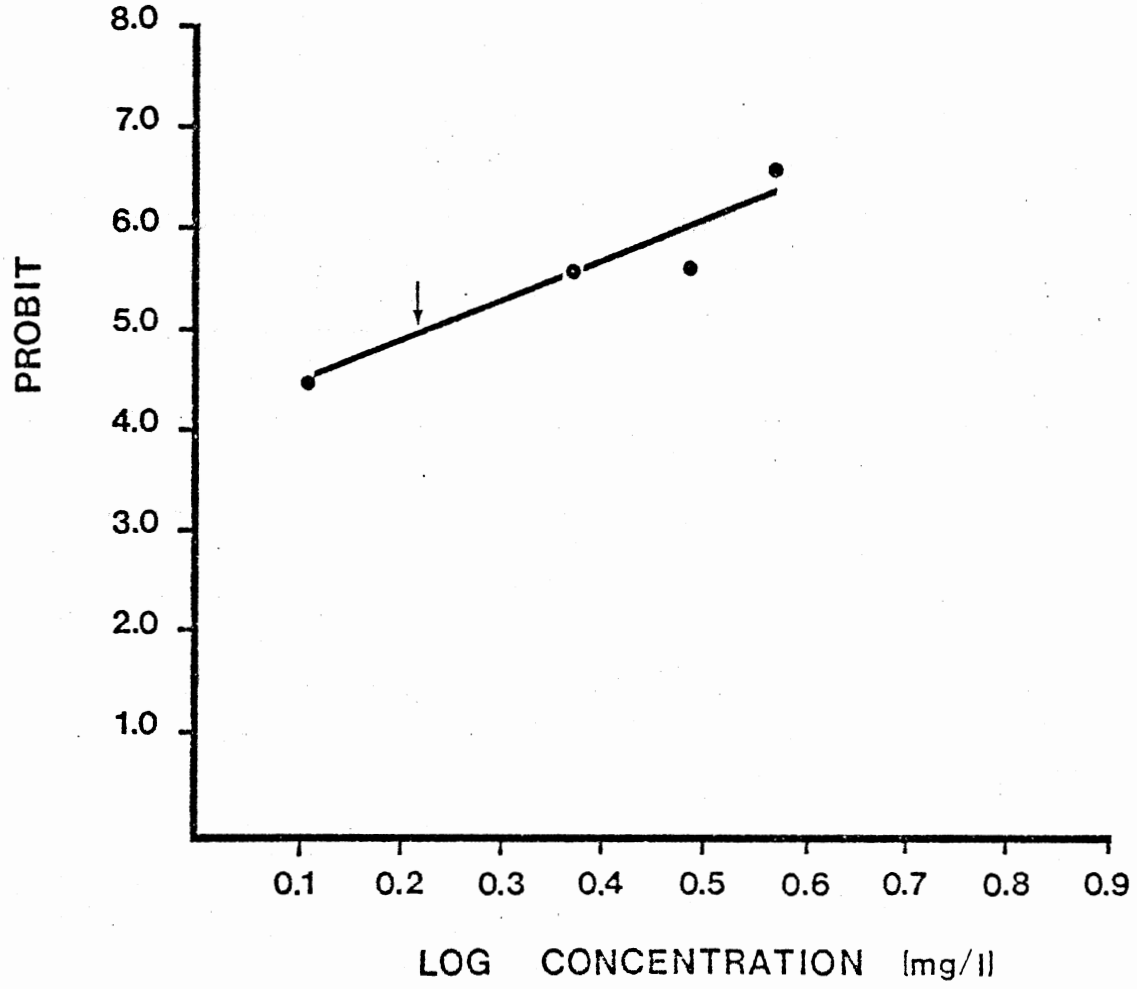


Figure 20. Naphthalene Dose-Response Curve For Absence of Swimming At 6 Hr With 3 Week Old Larvae; 18 Oct.-22 Oct. Arrow denotes EC50. Values from Table XIII.

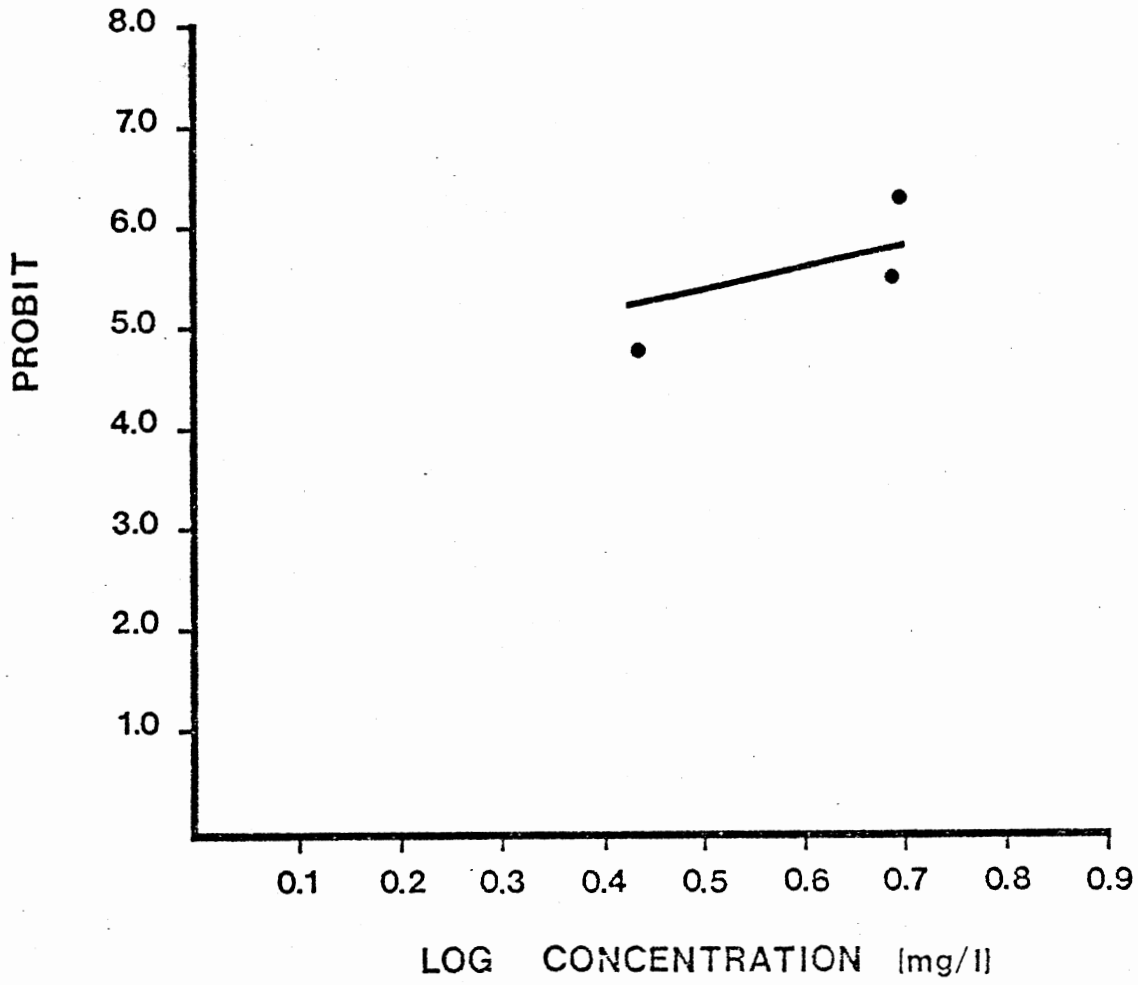


Figure 21. Naphthalene Dose-Response Curve For Absence of Swimming At 6 Hr With 1 Week Old Larvae; 20 Aug.-24 Aug. Arrow denotes EC50. Values from Table VII.

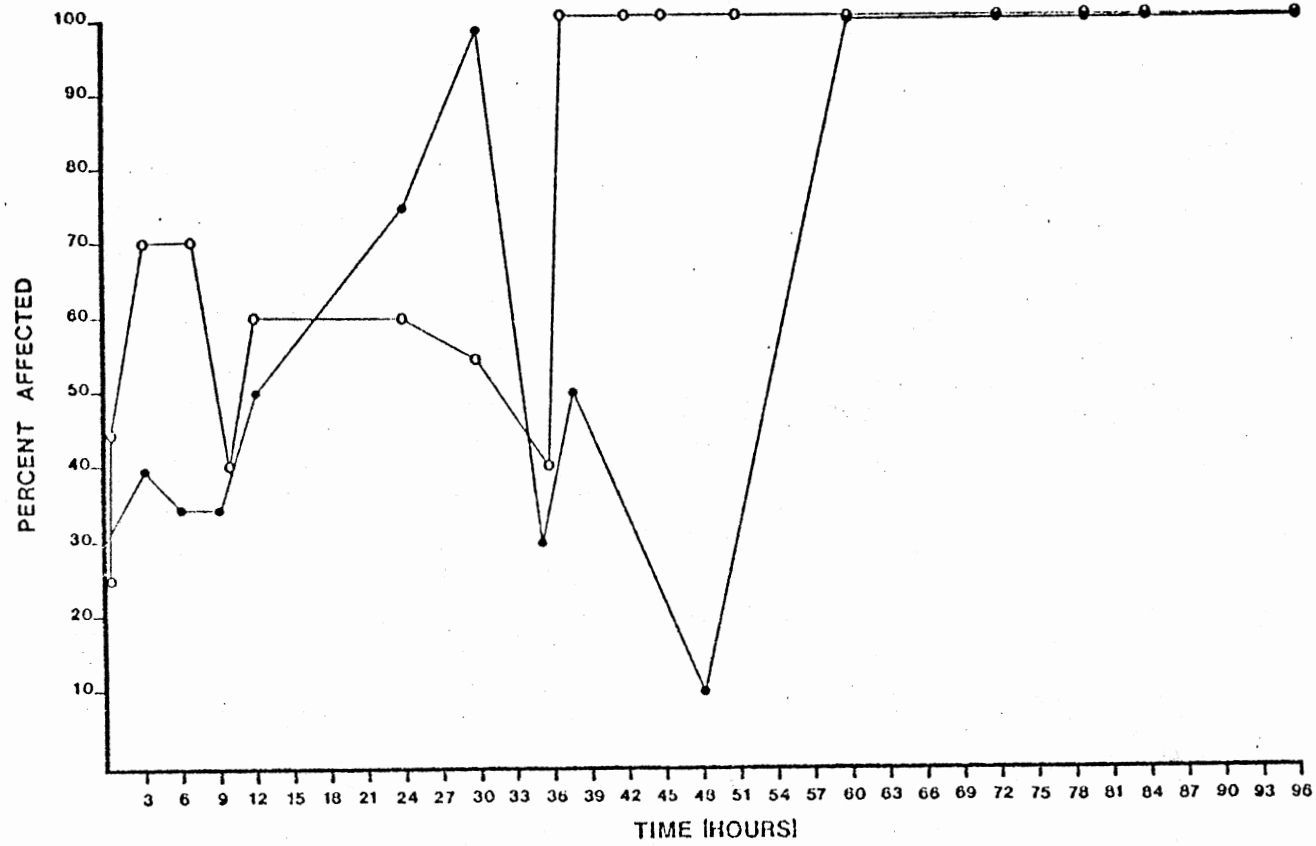


Figure 22. Comparison Of Biphasic Behavior Response To Naphthalene With Xenopus Larvae At 1 mg/l (-O-O-O-) And 4.8 mg/l (-O-O-O-), Using Absence Of Swimming As The Behavior Criteria.

minutes of exposure. At approximately 6-8 h, depending on the concentration, the animals began to acclimate to the naphthalene, and some began to swim once again. The animals continued swimming until the 24 h to 60 h, when they fell back to the screen. At this time, the animals may or may not die, and most usually will remain on the screen for the duration of the experiment.

Behavior Phases and Traits

Xenopus larvae exhibit certain toxicity responses when subjected to naphthalene. These responses are listed as "Phases" in Table XIV. Within a 96 h at 28°C, and approximately 0.5-1.0 mg/l, the 3 week larvae exhibit behavior associated with Phase 1. Phase 2 occurs at 1.0-1.3 mg/l, Phase 3 at 1.3-1.5 mg/l and Phase 4 at 1.5-2.0 mg/l. Phase 5 represents death, and occurs at approximately 2.1 mg/l. All phases, from 2-4, may end in at least partial death of the population within 96 h.

TABLE XIV

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, 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 medium while on screen.
e). Heart beat fast.

Phase 3. [Very Toxic]

a). Lie on the screen breathing normally-slow, easy.
b). Heart beat 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.
e). Heart beat slow.

Phase 5. [Lethal]

a). no heartbeat.

CHAPTER V

DISCUSSION

Diluter System

The flow-through system was ultimately used to determine the acute toxicity of naphthalene to Xenopus larvae. The bioassay is superior to that of a static test in that the concentration of toxicant may be kept stable without significant degradation, volatilization, or photolysis. Oxygen content is constantly replenished, alleviating stress on the organisms. During a static test, the organisms may deplete the toxicant or the oxygen and may foul the water, therefore altering the survival rate. This system tests the effects of a specific concentration of a chemical, maintained over a period of 96 h. It is a relatively short-term test with a high degree of accuracy. The entire system is enclosed in a fume hood, and a master control unit is located outside the hood enabling manipulation of all components without entering the area.

Naphthalene Volatilization and Photodecomposition

The results of the photodecomposition study show that of all solutions tested, only one (toluene) did not

fluoresence when excited at 290 nm, either in oxidized or reduced form. The toluene spectrum illustrated that other chemical changes may have occurred in the samples, but were not detected at this particular excitation wavelength or did not yield a solution that fluoresced.

In the reduced forms of 2-OH-1,4-naphthoquinone, fresh, 3 d and 5 d - 1,2-naphthoquinone, the 572 nm peak had significantly greater area under the curve than could be attributed to the presence of sodium borohydride (SB). This variation could have been caused by the addition of differing amounts of sodium borohydride added to these sample solutions, since no measurements were made when adding the reducing agent. This is doubtful however, since the solutions were thoroughly mixed until no bubbles could be visually detected.

It is interesting that the 572 nm peak occurred also in two oxidized samples of 1,2-naphthoquinone (0 d and 3 d samples), which indicates that part of the 1,2-naphthoquinone molecule fluoresced at this wavelength. This peak representing the presence of the 1,2-naphthoquinone could perhaps explain the increased peak surface area for reduced solutions as well. If this were true, 1,2-naphthoquinone is one of the photodegradation compounds of naphthalene.

When analyzing the peaks in solution X (the naphthalene from 1 d in light and 47 d in dark), the standard 325 $\pm$ 3 nm and 641 $\pm$ 1 nm peaks, representative of naphthalene are observed. The peak at 572 $\pm$ 4 nm is most likely sodium

of the spectrum. All spectra, for all time periods (Solution X fresh, and at 3d and 4d), in oxidized and reduced solutions, resulted in a peak at 400 ± 3 nm. This peak is believed to be a degradation product of naphthalene, and was not repeated on any other spectra but the reduced form of solution Z, and possibly in 3 d-1,2-NHQ. The peak in 3 d-1,2-NQ, however, was shifted 13 nm higher than the average of all other spectra with this peak (from 400 nm to 413 nm). Noting the consistency of all other curves, it is doubtful that the 413 nm curve represents the same solution as the 403 nm curve. In analyzing the peaks in solution Z, the reduced form has both naphthalene peaks and the oxidized form has only the 403 nm peak. Solution Z was left in the light on the shelf for 29 d and then analyzed for photodecomposition. It is highly probable then, that the peaks in solution Z and X represent the same solution. If this were the case, in solution X the peak began to appear at 5 d (total days in light) and at this time naphthalene was still present (as illustrated by the presence of the 325 nm and 641 nm peaks). After 29 d in the light however, all naphthalene was degraded and only the 403 nm peak remained as shown by the peak in the oxidized form of solution Z.

It was determined that these changes did not occur within 4 d at normal temperatures in light and air. Of course the solution may very well be metabolized by the Xenopus larvae and/or bacteria, and be oxidized in this manner.

Static Tests

Static toxicity tests were performed to determine a range of naphthalene toxicity to Xenopus, and to assess the predictive value of 24 h and 96 h static tests to a flow-through system. The 24 h tests were performed at 22^0C, and results were very consistent, with an average of approximately 11 mg/l as shown in Table IV. Several were performed at 25^0C, and the 24 h LC50 lowered to approximately 8.8 mg/l. Further static tests were conducted, and when the temperature was increased to 28^0C, the 24 h LC50 lowered to approximately 4.3 mg/l. Similar results have been published by other authors (Table II). Presumably, as the temperature increases, the metabolism also increases and the inhibition of cell processes by naphthalene becomes more pronounced. Several 96 h static tests were performed, and again results varied due to temperature. At 22^0C, the 96 h LC50 was approximately 12 mg/l, at 25^0C it dropped to 6.8 mg/l, and at 28^0C, was approximately 3.8 mg/l. All of these values correlated well with the values from the 24 h LC50 tests, which indicates that the 24 h tests were fairly adequate indicator tests. At this time, flow-through was begun, and temperatures were maintained at 28°C. The 24 h static values were used as a median concentration for flow-through experiments at a temperature of 28°C.

Flow-Through Tests

Static Versus Flow-through Tests

The 24 h static tests were not as adequate an indication of toxicity as the the 96 h static test. Flow-through tests were much more accurate, because the larvae were exposed to a solution of a constant concentration and oxygen content. The flow-through tests also theoretically flush out the naphthalene metabolites approximately once every 5 h, so that the organisms are exposed to naphthalene itself, and not an array of other derivatives of naphthalene. In static tests, the organisms may foul the water, and this may have an effect on toxicity as naphthalene may be absorbed by the organic waste.

Lethality

One objective of this study was to compare several different types of toxicity data, using *Xenopus laevis* larvae and the described flow-through diluter system, and determine which methods prove to be the most accurate testing method for assessing toxicity.

The 96 h LC50 is a widely accepted measurement, and in this study proved valuable. The response (death) is easily identifiable in *Xenopus*, as the heart and heartbeat may be viewed through the transparent tissue on the ventral side of the organism. Ninety-six hours is a good time interval to

determine the overall toxicity. Frequently, no deaths would occur with Xenopus until after 48 h of exposure. If the test had lasted 24 h or 48 h, one would not determine the actual toxic concentration of the compound. The 96 h test also takes sublethal dosages into consideration, in that, a concentration determined to be sublethal in a 24 h toxicity bioassay may become lethal in a 96 h test. In these cases, the longer the test the better the accuracy in determination of toxicity. Some effects may also be "latent", with a metabolic "chain reaction" occurring before the compound is toxic to the organism, as with Benzo-a-pyrene, and the 96 h test provides an adequate time interval for this to occur.

The 96 h flow-through tests were performed at 22^0C, 25^0 and 28^0C, and as the temperatures increased, the LC50's decreased, with values at 11-12 mg, 7-8 mg/l and 2-3 mg/l, respectively.

There was a difference in 1 week old and 3 week old larvae 96 h LC50 values. One week values were not as consistent, while 3 week tests almost exactly duplicated each other. Different parents were bred for each set of larvae tested, so there could have been genetic variation differences between tests. Growth differences would be more pronounced among the 1 week animals, while the 3 week animals were old enough that some of these differences may not have been as apparent. At one week old, much of organogenesis has just occurred, and the larvae are still rapidly developing. With differential growth rates, the slight variations

in stage may cause quite a difference in LC50 values. The three week old organisms, however, have temporarily stopped organogenesis and are engaged in feeding and growth. Because of this, only slight changes in structure and metabolism are expected at this time.

The three week old larvae were more sensitive to naphthalene, which could be due to greater metabolism rate, or greater gill surface area. It could be that naphthalene is metabolized to its toxic form with inducible enzymes, and with a greater metabolic rate, more naphthalene is degraded to more toxic by-products in the 3 week larvae.

The three week Xenopus larvae proved to be as sensitive, or more sensitive, than most of the species listed in Table II for 96 h exposures, and the LC50 values correlated quite well with those obtained with the grass shrimp, crab zoea, and rainbow trout.

Depigmentation

With 1 week old organisms, one can successfully use the depigmentation data as an indication (within 20%) of the 96 h lethality value. However, with the 3 week old organisms, this value cannot be used to correctly assess the 96 h LC50. Perhaps if calculations were performed using data from a different time period than at 6 h (i.e. 24 h) the data would serve a better indication of 96 h LC50.

Absence of Swimming

With the 1 week larvae, the 6 h $EC50_{swim}$ values were not adequately predictive of 96 h LC50. The difference in $EC50_{swim}$ values for Test B, 1 week larvae, was much greater than the differences in the other tests, which could indicate that the values are erroneous. The values obtained were also quite variable.

The data is much more indicative of the 96 h LC50 with 3 week tadpoles, and in these two tests, predicted values were within 20% of the true LC50 values. With one week larvae however, the values were quite different than the true LC50 values and should not be used as an indication of toxicity. While naphthalene EC50 values for the absence of swimming accurately predicted the 96 h LC50 for 3 week larvae, studies with other compounds should be performed to insure the predictive capabilities of this approach.

The absence of swimming behavior was clearly biphasic in nature as the animals temporarily recovered after 6 h exposure and then ceased swimming again (Figure 18). This biphasic effect observed during naphthalene exposure may be caused by an activation of induction enzymes which degrade naphthalene to a more toxic compound. When first exposed to naphthalene, the animals respond and toxic effects are observed. After a period of several hours, enzymes responsible for metabolizing naphthalene are induced and the level of naphthalene falls. At this time, the animals begin to recover but this recovery period is cut short by the

accumulation of naphthalene metabolites. Toxic effects are again observed followed by lethality.

Behavior

The behavior data as indicated by the "Phases" listed in Table 5, served very well as an indication of toxicity. Of course, no "value" is provided, as is with an LC50, but through interpretation of the data, and dividing behavior into phases, one can determine the relative toxicity of the compound tested. This relative toxicity may be useful for double checking toxicity, or for a quick check of the absence of a toxic compound in a vessel or body of water. By executing a test for 8-12 h, one can detect the presence of toxicant behavior characteristics, and if detected, further studies such as the LC50 may be performed.

It is possible that Xenopus has a particular behavior only with one compound, and the type of behavior changes with each compound, depending upon the mechanism of toxicity. With naphthalene, the larvae exhibit particular responses depending on the concentration of the solutions. A greater percent death (Phase 5) will occur with a greater concentration, and if the concentration is above 3.0 mg/l, the larvae may enter Phase 3 or 4 immediately without ever exhibiting behavior associated with Phases 1 and 2. If the concentration is higher than 4.5 mg/l, Phase 4 will occur within a few hours. On the other hand, if the concentration

is low enough, i.e. approximately 0.5-1.0 mg/l, the larvae may stay in Phase 1 for the entire 96 h.

Summary

In summary, naphthalene is toxic to Xenopus laevis larvae at 2-4 mg/l. One week larvae were not found to be as valuable an indicator organism as the three week larvae. This could be due to the present developmental flux of the one week organisms. The three week larvae were more sensitive to naphthalene than the one week larvae, probably due to a greater metabolic rate and gill surface area in the older organisms. Absence of swimming was a more accurate indication of the 96 h LC50 than was depigmentation. The behavior of both ages of Xenopus was quite reproducible and was an excellent assesment of toxicity. The larvae responded in a particular manner when exposed to a certain concentration of naphthalene. These behavior "phases" were characterized for naphthalene.

Naphthalene photodecomposition and volatilization were studied, and volatility was shown to be an important characteristic in removing naphthalene from solution. It was determined by flourescence spectrometry that naphthalene did not photodecompose during the 4 d bioassay test, but would begin to decompose after this time period. To alleviate problems associated with these parameters, sealed exposure chambers and diluter system were incorporated, and a complete turnover time in exposure chamber was 5 times per 24 h.

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