AN EVALUATION OF BIOASSAY METHODS FOR DETERMINING ACUTE PHENOL TOXICITY TO CHANNEL CATFISH, *ICTALURUS PUNCTATUS* (RAFINESQUE)

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ICTALURUS PUNCTATUS (RAFINESQUE)

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CHAPTER I

INTRODUCTION

Fish bioassays of aquatic pollutants are used to establish permissible levels of industrial pollution in aquatic systems. Standard bioassay methods for determining toxicity values (A.P.H.A. 1971) are designed to gain the greatest information with minimum time. Present recommendations call for introducing test fish into tanks containing the test concentrations of toxin (Sprague 1969; A.P.H.A. 1971). Handling the test fish and suddenly placing them into foreign chemical solutions disturbs the normal metabolic and physiological functions (Fry 1971) and precludes possible acclimation of the fish to the chemical.

It is difficult to maintain stable concentrations of many aquatic pollutants during the bioassay period. Methods for maintaining stable concentrations include periodic replacement of the toxin, transfer of the test fish into fresh solutions, and continuous flow techniques. The most dependable continuous flow systems are similar in design to the automatic dilution apparatus of Mount and Warner (1965).

Phenol is a common industrial pollutant and is difficult to maintain at desired test concentrations because the compound is frequently degraded by microorganisms. The results of several investigations have shown wide differences in the lethal concentrations

of phenol (Table I). Burks (personal communication) found that the 48 hr phenol TLm (median lethal concentration) for fingerling channel catfish, Ictalurus punctatus (Rafinesque), determined by using a continuous flow system, was almost twice the 48 hr static TLm reported by Clemens and Sneed (1959) (Table I). A pilot study was conducted in our laboratory using a continuous flow system to introduce phenol into test aquariums containing fingerling channel catfish. This preliminary study revealed that all fish survived a 48 hr exposure at concentrations higher than the 25 mg/l TLm determined by Burks (personal communication). A TLm determined using the gradual introduction method would be considerably higher than either of the other methods.

The obvious differences in the TLm's obtained by different methods suggested that the standard method may involve unrecognized variables that tend to alter the susceptability of the fish to some toxins. Thus, the present study was planned to evaluate the bioassay methods and hopefully to reveal factors that produce variations in bioassay results. Discrepancies in the TLm's of fingerling channel catfish suggest that one of the variables may be acclimation to phenol when the toxin is gradually introduced to the test fish. Evidence exists that fish accumulate phenol in body tissues (Kariva et al. 1968) and that some fish have a limited ability to detoxify phenol in the liver (Boni 1965; Dutton 1961; Maickel et al. 1958). Activation of a detoxification mechanism is a possible means by which fish could tolerate phenol if they were gradually introduced to the chemical.

Fish are usually subjected to a gradual increase in the concentration of a pollutant in the natural environment and would have some

opportunity to acclimate. For the present study a continuous flow system was developed to determine phenol TLm's and an incipient lethal threshold (LC50) for fingerling channel catfish gradually exposed to phenol solutions, hereafter referred to as slow introduction bioassays. These were compared to a phenol TLm and LC50 for the catfish obtained by standard bioassay methods.

The results of the slow introduction bioassays in the present study suggested that channel catfish may survive higher than expected phenol concentrations in their natural environment. Additional investigations were conducted to determine if the sub-lethal phenol concentrations in the slow introduction bioassays have chronic effects. In addition, a study was designed to determine if a short-term phenol exposure influences the ability of Pimephales promelas Rafinesque to reproduce. Low levels of aquatic pollutants which have no visible acute or chronic effects are known to inhibit or interfere with reproduction (Crandall and Goodnight 1962; Mount 1968; Sprague 1971; Stepanov and Flerov 1969).

As a means of detecting possible physiological effects of phenol concentrations that were sub-lethal in the slow introduction bioassays, experiments were performed to measure the breathing rates during exposure to phenol. Automatic monitoring of fish breathing rates (gill beats/min) is a sensitive and rapid method of detecting pollutants in the aquatic environment. Changes in respiratory activity of fish have been successfully used to determine chronic effects of several pollutants (Heath 1972; Hughes and Saunders 1970; Marvin and Heath 1968; Morgan and Kuhn 1974). In the present study the external electrode method (Spoor et al. 1971) was used to measure the effects

of sub-lethal phenol concentrations on the breathing rates of bluegill sunfish, *Lepomis macrochirus* Rafinesque. Bluegill sunfish were selected after repeated efforts to use fingerling channel catfish failed because they did not produce countable opercle movements even in the absence of phenol.

CHAPTER II

LITERATURE REVIEW

Considerable literature exists concerning various bioassay methods and their use in determining toxicity values for pollutants in the aquatic environment. Sprague (1969, 1970, 1971) summarized the existing literature on bioassay procedures and critically evaluated the commonly used methods. Recommended standard bioassay methods were described by Sprague (1969) and A.P.H.A. (1971).

Phenol toxicity studies have been conducted on many aquatic organisms. Reviews of phenol toxicity to aquatic organisms have been made by McKee and Wolf (1963) and by the British inland fisheries advisory commission (F.A.O. 1973). Phenol toxicity studies have been conducted on a number of fresh water fishes (Belding 1927; Boni 1965; Brown and Dalton 1970; Brown et al. 1967, 1969; Clemens and Sneed 1959; Flerov 1965; Havelka and Effenberger 1957; Herbert 1962; Herbert and Vandyke 1964; Herbert, et al. 1965; Jones 1951; Klinke 1965; Lammering and Burbank 1960; Luk'yanenko and Flervo 1965, 1966a, 1966b; Mitrovic et al, 1968; Trama 1955; Vishnevetskii 1962). Most of these studies were acute, short-term bioassays yielding 24, 48, or 96 hr TLm's for the species tested. Reported concentrations of phenol that are harmful to fish range from 0.08 to 1900 mg/l, with the most reliable TLm values ranging from 4 to 56 mg/l for periods between 6 and 96 hr (F.A.O. 1973). The most frequent TLm values for adult fish in well

aerated fresh water are 9 to 24 mg/l phenol. The reported 24, 48, and 96 hr TLm's of some fresh water fishes (Table I) indicate that if the fish survive the initial 24 hr of phenol exposure they survive for 96 hr.

Changes in any of several physical parameters such as pH, water hardness, temperature, and dissolved oxygen concentrations during the course of a fish bioassay are known to alter the relative toxicity of certain chemicals but the effect of pH on phenol toxicity was found to be negligible from: pH 7.3 to 8.4 for bluegill sunfish (Lammering and Burbank 1960); pH 6.5 to 8.5 for rainbow trout, *Salmo gairdnerii*, (Herbert 1962) and, pH 4 to 11 for crucian carp, *Carassius carassius*, (Luk'yanenko (1967). Studies of monohydric phenols using rainbow trout at water hardnesses of 50 mg/l and 310 mg/l as calcium carbonate also showed no significant difference in the lethal threshold concentration but water hardnesses below 50 mg/l resulted in increased toxicity (Herbert 1962). Similar results were found for phenol alone and for mixtures of phenol with other toxicants (Pickering and Henderson 1966; Luk'yanenko 1967; F.A.O. 1973). Generally, increased temperatures shortened the time of reaction and period of survival of fish exposed to phenol (Burksteeg et al. 1955; Herbert 1962; Gersdorff 1943). However, Brown, et al. (1967) showed that acute toxicity of phenol to rainbow trout decreased with increasing temperature. The 48 hr TLm increased from 5 mg/l at 6 C to 9.8 mg/l at 18 C. A similar but less pronounced reversal was found in juvenile rainbow trout exposed to phenol at temperatures of 4 and 12 C (F.A.O. 1973). Flerov and Luk'yanenko (1966) found no significant change in the minimum lethal concentration for crucian carp over a temperature range

from 5 to 25 C but resistance to high concentrations (100 mg/l phenol) decreased with increasing temperature. Herbert (1962) found that survival of rainbow trout in high concentrations of phenol was reduced by increased temperature. Low dissolved oxygen concentrations shortened the time of response of fish to monohydric phenols and reduced the concentrations that were lethal (F.A.O. 1973). Tests with rainbow trout in mixtures of phenols (Herbert 1962) demonstrated that a reduction in dissolved oxygen from 100% to 50% of the air-saturation value reduced the estimated LC50 by about 20%. A similar reduction was found for mixtures of zinc, copper, lead (Lloyd 1961).

Fishes exposed to toxic concentrations of phenol exhibit avoidance reactions including pronounced locomotive excitability followed by loss of balance reflex, repulsive jerky motions in a lateral position, and eventually total loss of locomotion (Luk'yanenko 1965). Death may follow almost immediately or after a stage of depressed activity interrupted by occasional convulsions (Luk'yanenko 1967). During the course of phenol poisoning there may be color changes (F.A.O. 1973), increased respiration (Morgan and Kuhn 1974), and increased secretion of mucus (F.A.O. 1973). Pathological effects include inflammation and necrosis of the gill filaments, haemorrhage, and degenerative changes in skin, muscle, liver, spleen, and kidney (Waluga 1966a, 1966b). Halsband and Halsband (1963) found a reduction in number and in increase in surface area of erythrocytes of rainbow trout exposed to 1.5 mg/l phenol for 24 hr.

The reproductive period is a critical time in the life cycle and aquatic pollutants which have no visible toxic effects can inhibit reproduction. Mount (1968) found that copper concentrations which had

no significant effect on survival of adult fathead minnows in 11 months produced a pronounced inhibition of spawning. One concentration caused no significant mortality but totally stopped reproduction. Phenol concentrations of 12.5 mg/l significantly reduced the number of live births of guppies, Lebistes reticulatus, (Stepanov and Flerov 1969). Lead nitrate (Crandall and Goodnight 1962) and cadmium (Tafanelli 1972) also reduced fertility in certain species of fish. Since the detoxification process in fishes appears to be poorly developed (Boni 1965; Maickel et al. 1958) and fish exposed to phenol to accumulate phenol in body tissues (Kariya et al. 1968) one might expect that the survivors of an acute phenol exposure might have reduced fertility.

Respiratory activity changes of fish have been suggested as a rapid bioassay method for the detective of toxicants. Several different methods have been employed to study respiratory activity of different fish species under a variety of experimental conditions (Belding 1927; Cairns et al. 1970; Davis and Watters 1970; Hughes and Saunders 1970; Jones 1947; O'Hara 1970, 1971; Schaumberg et al. 1967; Skidmore 1970). The respiratory pressure method and the external electrode method have been successfully used to show the effects of several pollutants on fish respiration. Spoor et al. (1971) found a change in opercle rate of three times the normal rate when bluegill sunfish were exposed to 0.5 mg/l copper. Large mouth bass, Microptrus salmoides, at 1, 5, and 10 mg/l phenol concentrations all showed a 50% or greater change in opercle rate within 4 days (Morgan and Kuhn 1974). Breathing rates of fishes (gill beats/min) have been positively related to ventilation volume and oxygen consumption (Fry 1957;

Heath 1972). These relations indicated that breathing rate could be used as an indirect meature of ventilation or oxygen consumption, or both, in some species. Heath (1972) suggested using a species of test fish such as the bluegill sunfish since it responds to environmental stress with a notable change in breathing frequency. It has been shown that trout respond to environmental stress such as hypoxia by a change in depth of breathing with frequency remaining fairly constant until severe levels of hypoxia are encountered (Hughes and Saunders 1970; Marvin and Heath 1968).

CHAPTER III

MATERIALS AND METHODS

The Test Water and Phenol Determinations

Water for all experiments was well aerated, dechlorinated tap water free of any known pollutants. The test water passed through copper pipes; however, copper levels as determined with an atomic absorption spectrophotometer were below 0.006 mg/l for all experiments. Hardness (as $CaCO_3$) and total carbonate alkalinity were determined for each experiment using standard methods (A.P.H.A. 1971) and are shown along with pH and temperature in Table II. A 90 x 50 x 40 cm water holding reservoir served as a partial temperature buffer chamber for continuous flow experiments. In all experiments the oxygen concentrations were maintained near saturation levels using a forced air supply.

Reagent grade Mallinkrodt phenol crystals were used for all experiments. A Beckman DB-G Grating Spectrophotometer and the spectrophotometric method of Martin et al. (1967) were used to determine phenol concentrations. Water samples were acidified with HCl, centrifuged at 3000 rpm and run in duplicate in early experiments. Since duplicate samples showed consistent readings the duplicate sample was omitted in later experiments unless there was a marked change in phenol concentration. Preliminary studies confirmed that the presence

of fish, their wastes, and secretions did not decrease the accuracy of acidified, centrifuged samples. All phenol samples were analyzed within 3 hr after collection to avoid possible loss of phenol. Standard phenol solutions for standard curves were prepared each day. Stock phenol solutions for continuous flow bioassays were mixed immediately prior to use.

Bioassays

Experimental Animals

Fingerling channel catfish, Ictalurus punctatus, for phenol bioassays were obtained from the Oklahoma State Fish Hatchery, Holdenville, Oklahoma, on the dates indicated in Table III. Test fish were transported to the laboratory in sealed plastic bags with oxygen enriched air over water. The fish were held in 1 kl polyethylene-lined holding tanks for a minimum of 4 days prior to being used for continuous flow bioassays. Fish for static bioassays were held a minimum of 3 days. The fish were fed Purina Complete Ration Trout Chow (No. 6 pellet) once daily until 48 hr before the start of each experiment. Observations of health and behavior of the fish were made twice daily. The fish were in apparent good health and no fish group used in bioassays approached the maximum mortality of 5% cited by Sprague (1969) as the maximum permissible mortality during acclimation to the laboratory. The fish had no known past history of exposure to phenol or other chemical pollutants. Fish wastes and unconsumed food were siphoned off the bottom of the holding tanks once daily and the water was replaced once daily by partial drain and refill operations.

The test fish ranged from 7.0 to 17.8 cm standard length. No attempt was made to select a more uniform size for the bioassays because of the possible effects of additional handling.

Static Bioassays

Two 48 hr static bioassays using standard methods (A.P.H.A. 1971) were conducted using fingerling channel catfish to determine the TLm. Ten, 30 l capacity, all glass aquariums were used with 10 fish per tank and two tanks at each test phenol concentration (Table II). Freshly mixed phenol solutions were added to each tank immediately prior to the addition of fish. Test fish were captured from the holding reservoir with a dip net and transferred to the test tanks according to a random number system (Finney 1964). Phenol solutions were renewed at 24 hr by draw-down and refilling with freshly mixed solutions. Water samples for alkalinity, hardness, and pH were taken at the start and after the 24 hr solution renewal. Observations on fish survival were made at 6 hr intervals for the duration of the experiments. Fish were recorded as dead and removed from the test tanks when there was no apparent opercle movement and no response to mechanical tapping on the body.

Regression analysis was performed on the pooled data from the static bioassays. The two experiments were pooled because the individual experiments did not provide sufficient information to establish separate 48 hr TLm's. An IBM 360/65 computer and the SAS program (Barr and Goodnight 1972) were used to calculate the regression. The 48 hr TLm and error bounds were determined from the regression equation.

<u>Continuous Flow Apparatus</u>

Four automatic dilution units similar to those described by Mount and Warner (1965) were constructed. Each unit delivered at regular intervals a given volume of diluted chemical to each of four experimental 30 l glass aquariums equipped with surface drains. The dilution units permitted a controlled rate of flow and the use of large volumes of chemical solution without manual mixing. Each unit was adjusted to a flow rate of 20 l/hr which exchanged the volume of each test tank in 6 hr. This flow rate insured complete exchange in less than 24 hr (Sprague 1971). Each dilution unit was designed to maintain a given concentration of chemical in each of four experimental tanks. Figure 1 shows a view of one dilution unit and four experimental aquariums. Water was held in a large 90 x 50 x 40 cm, epoxy-lined plywood reservoir. A plastic float valve at the inlet maintained a constant water level in the reservoir. The reservoir water was aerated with forced air. Tygon tubing (9.5 mm inside diam) served as a siphon for the flow of water from the holding reservoir through a teflon-lined solenoid valve into the mixing bucket. The mercury switch activated the solenoid valve to interrupt the siphon flow. The solenoid valve was closed when no power was supplied to it. When the mixing bucket was tilted upward the solenoid valve opened and the bucket filled with water from the holding reservoir. As the mixing bucket filled it tilted downward and the toxin dipper delivered a measured volume of concentrated chemical into the mixing bucket. The level of concentrated chemical solution in the toxin delivery reservoir was maintained by atmospheric pressure through an

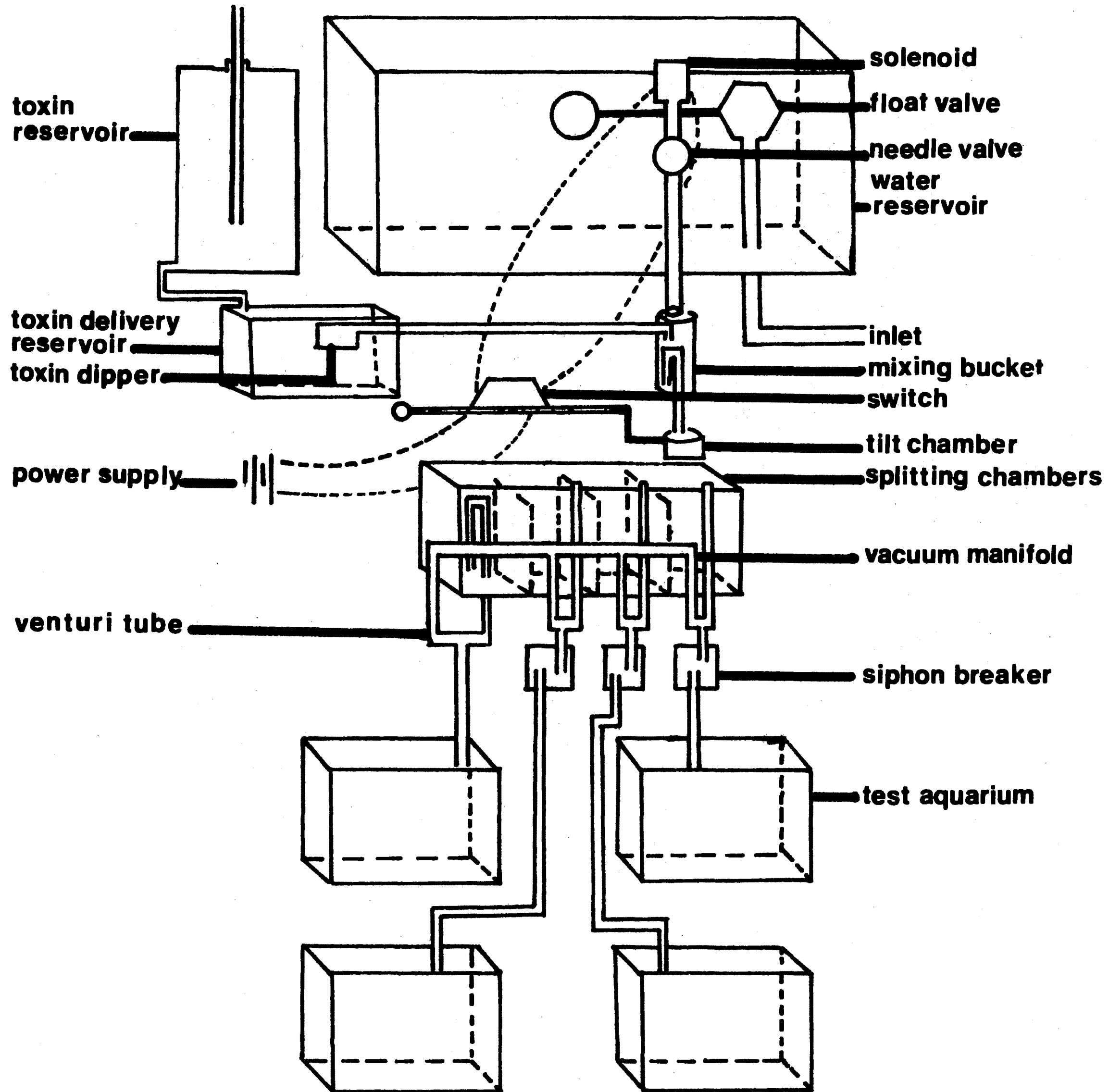

Figure 1. The Dilution Apparatus

open glass tube inserted into the toxin reservoir. As the mixing bucket filled, the water level in the bucket covered the automatic siphon tube and siphoned the mixture into the first splitting chamber. The small perforated tilt chamber filled and pivoted downward cutting off water flow to permit recycling of the unit. The volume of each splitting chamber was equal to the volume of one dump from the mixing bucket. After four successive dumps of the mixing bucket the automatic siphon in the last splitting chamber began siphons of all four splitting chambers dumping the diluted chemical into each of four experimental aquariums. A special venturi tube connected to a vacuum manifold started the splitting tank siphons during each cycle. Siphon breaker chambers prevented continuous siphoning of successive dumps from the mixing bucket. Control of the final concentration of chemical in the test aquariums was made by adjusting the concentration of chemical in the toxin reservoir and by changing the size of the toxin dipper. Control of flow rate was made by adjusting the needle valve. Final volume adjustments of the splitting chambers were made by adding large glass beads to these chambers. Although the apparatus required considerable time for volume adjustments and flow rate regulation, the units gave relatively trouble-free operation once these initial adjustments were made.

Continuous Flow Bioassays

Fish were introduced to phenol concentrations in two ways. Slow introduction involved placing test fish in the experimental tanks and during the next 24 hr interval water only flowed through the dilution apparatus and the test tanks to permit the fish to recover from the

stress of handling and adjust to the aquariums. Phenol was added after 24 hr and the concentrations in the test tanks gradually increased to test levels. Rapid introduction involved placing fish in the test tanks 24 hr after phenol was started through the dilution apparatus. The 24 hr period insured that test concentrations were reached before fish were placed in the experimental tanks. In both types of introduction the test fish were selected and distributed to test tanks according to the recommendations of Finney (1964).

Four continuous flow phenol bioassays were conducted to determine the LC50 and the 48 hr TLm for the two types of fish introduction. Since all four experiments were below the lethal concentrations, a fifth phenol bioassay using only slow introduction was conducted to include the slow introduction 48 hr TLm and to determine 64 and 72 hr slow introduction TLm's. Experiments I and II were designed to test three experimental concentrations of phenol and one control (zero) level (Table II). Experiments III, IV, and V were designed to test four experimental phenol concentrations and one control. With the exception of Experiment II each experimental phenol concentration was replicated in each of four test tanks. Two test tanks at each test phenol concentration were used for slow introduction and the other two tanks at each test phenol concentration were used for rapid introduction. Only two test tanks at each experimental phenol concentration were used in Experiment II because the flow rate was doubled to determine if the rate of phenol increase was a factor on survival. Doubling the flow rate brought phenol concentrations up to test levels in 6 hr instead of 12 hr. One tank at each test phenol level in Experiment II was used for slow introduction and the other

tank used for rapid introduction.

Water samples from the continuous flow system were taken daily during the bioassays to determine pH, water hardness, and alkalinity. Temperature in the first two continuous flow experiments was monitored using Ryan continuous recording thermometers. Since little temperature change occurred during these experiments, a maximum-minimum thermometer in the holding reservoir and standard laboratory thermometers in each experimental tank were used to monitor temperature in later experiments. Water temperatures were recorded from each experimental tank each time phenol samples were collected (Table II). Photoperiod was not controlled.

The results from the five continuous flow bioassays were pooled to determine a slow and rapid introduction 48 hr TLm because the results of individual experiments were not sufficient to determine individual TLm's. The concentrations used for determining the 48 hr TLm were the average concentrations of the 24, 36, and 48 hr phenol measurements. It has been shown that the average of fluctuating concentrations of ammonia, phenol, and zinc (Brown et al. 1969) and of pesticides (Abram 1967) predicted survival time of fish adequately. The TLm's and error bounds were determined using methods already described. The median lethal times (TL50's) from the five continuous flow bioassays were pooled with the TL50's of the static bioassays to determine LC50's for the two types of fish introduction using the graphic method recommended by Sprague (1969).

Reproduction of Fathead Minnows

Adult fathead minnows for reproduction experiments were obtained from a stock population reared and maintained in 30 l aquariums by the Oklahoma Reservoir Research Center Bioassay Laboratory and had no prior exposure to foreign chemicals. The fish were fed twice daily with Tetra Minn Tropical Fish flakes before and after phenol exposures, but were not fed for 48 hr before phenol exposure or during the exposure period.

Two continuous flow phenol experiments were conducted to determine if a 24 or 48 hr phenol exposure, near the reported TLm for the species, would interrupt or delay spawning. Each experiment consisted of a control and three different phenol concentrations in duplicate 30 l aquariums. Eight female and four male minnows were placed in each aquarium and phenol was introduced 24 hr later through the continuous flow diluters. The phenol concentrations and experimental conditions are shown in Table IV. Aquariums were illuminated with fluorescent lights programmed for a 16 hr photoperiod. Approximate light intensity was 18.3 lux at the water surface. Black plastic sheeting draped over the aquarium rack eliminated extraneous light. Each aquarium was equipped with a surface drain, forced air supply, and two 10 cm spawning tiles. A continuous flow of fresh water, started at the end of the exposure period, gradually removed phenol from the tanks. Observations on survival were made every 6 hr during the phenol exposures and twice daily after the removal of phenol. Reproductive behavior and spawning activities were monitored twice daily during the 30 day observation period following exposure. Spawning tiles

were replaced after each spawn. The eggs in each spawn were counted and placed in wide mouth jars for incubation and hatching. The number of eggs fertilized was determined by counting the embryos present on the third day following each spawn. Eggs were held until hatching was completed but fungal growth on most egg masses prevented the hatching data from being meaningful.

Water samples of the dilution water for pH, alkalinity and hardness were collected at the beginning of each experiment. Temperature was monitored daily during the experiments.

Opercle Rates of Bluegill Sunfish

Adult bluegill sunfish were collected from a local farm pond using hook and line and small-mesh minnow seines. Fish from 8.5 to 12 cm standard length were selected and only individuals superficially hooked were kept. The sunfish were transported to the laboratory and held a minimum of 48 hr in 85 l holding tanks.

Six glass electrode chambers, 30 x 6 x 10.5 cm, equipped with surface drains, baffle chambers, and stainless steel grid electrodes were constructed according to the specifications of Spoor et al. (1971) (Fig. 2). The size of each electrode chamber, excluding the baffled ends, was 20 x 6 x 10.5 cm. Each electrode chamber was equipped with an air stone at the inlet connected to an aquarium pump to maintain oxygen levels near saturation at 24 C. The grid electrodes of each test chamber were connected by Belden No. 301 shielded cables to a small preamplifier located behind each electrode chamber. These preamplifiers were connected by shielded cables to 3-M physiograph preamplifiers located outside the test room (Fig. 3). A remote power

switch, located outside the test room, supplied power to the chamber preamplifiers from six, 1.5 volt dry cell batteries wired in series. The six electrode chambers were placed on 8 cm foam rubber pads and separated from each other by cardboard partitions. Each electrode chamber received a continuous flow of premixed phenol solution from a gravity flow stock reservoir. Flow rates were adjusted to exchange the volume of each electrode chamber in 6 hr and stock reservoirs held a 24 hr supply of phenol solution. Drains from the electrode chambers emptied into an 85 l polyethelene barrel equipped with a submerged pump to remove waste solutions from the test room. The electrode chambers, stock reservoirs, chamber preamplifiers, and drain barrel were located inside an electrically shielded 3 x 3 x 2.5 m test room to eliminate electrical "noise." An electrically shielded 500 watt surgical light provided continuous illumination in the test room during the experiments. The apparatus was designed to permit periodic monitoring of opercle movements without entering the test room or disturbing the test fish. It was necessary to enter the test room to start the chemical flow and to measure dissolved oxygen and phenol concentrations of each test chamber once during each phenol exposure.

Preliminary studies using fingerling channel catfish failed to provide consistent opercle breathing rates even after 64 hr acclimation to the electrode chambers. Variation in opercle rates among different individuals and variation in individual rates at different monitoring times failed to show a predictable pattern. After repeated trials this species was abandoned as a test animal.

Bluegill sunfish were chosen as the test animal because they are

known to respond to environmental stress with a notable change in breathing frequency (Spoor et al. 1971; Heath 1972). Preliminary trials using bluegill sunfish produced fairly uniform recordings of opercle beats which could be distinguished from other types of muscular movements such as swimming. Differences existed in the amplitude of opercle beats among individuals; therefore, amplitude was not considered in the analysis of data.

Two 12 hr experiments of the effect of phenol on opercle rates of bluegill sunfish were conducted using one fish in each experimental chamber. The fish were placed in the experimental chambers 24 hr prior to the introduction of phenol. Exposure concentrations were 0, 1, 5, and 0, 10, and 20 mg/l phenol. Opercle rates were monitored continuously for 2 hr beginning 1 hr before phenol introduction. A 15 min recording was made 2 hr after phenol introduction and at 2 hr intervals during the remainder of the experiments. Opercle rates were determined by taking ten, 1 min counts at each time interval and averaging the counts to obtain opercle beats/min. Change in opercle rate was determined by subtraction of the rate before phenol was added from the rate after phenol introduction. A completely randomized design was used to measure changes in opercle rates. Comparisons of opercle rate for the lower phenol concentrations (0, 1, and 5 mg/l) with the higher concentrations (10 and 20 mg/l) were made using orthogonal comparisons (Snedecor and Cochran 1967).

Phenol and dissolved oxygen determinations were made at the beginning of each experiment and once during each exposure period. Measurements of dissolved oxygen were made using a dissolved oxygen meter.

CHAPTER IV

RESULTS

Maintaining Treatment Levels of Phenol

Several factors resulted in fluctuations in phenol concentrations in the open, aerated solutions of both static and continuous flow bioassays. Precise control of delivery volumes to individual test tanks was not possible and measured delivery volumes varied by as much as 200 ml/hr within one dilution unit, i.e. four test tanks at one treatment concentration. Flow into the splitting tanks, i.e. total flow to four experimental aquariums, varied by a maximum total of apprixímately 600 ml/hr among the four dilution units. This variation contributed to the differences between test tanks during the period necessary to achieve desired test concentrations of phenol but did not effect the final concentrations within each unit once they were reached.

The number of fish in the aquariums and artificial aeration of the phenol solutions did not alter the concentrations. Pilot studies using different rates of aeration in the continuous flow system failed to show significant change in phenol concentration in 48 hr. Bucksteeg et al. (1955) also found that evaporation of phenol from aerated 30 l test tanks was negligible. Preliminary studies using different numbers of test fish in aquariums of the continuous flow system failed

to show a correlation between the number of fish per tank and change in phenol concentration in 48 hr. Phenol concentrations decreased in tanks with low mortality in Experiment II. However, the continued presence of fish in the aquariums and the resulting build-up of their excrement may have provided a more suitable environment for increased microbial degradation of phenol.

Microorganisms became established in the test aquariums containing phenol. The time when significant populations would develop was unpredictable but there was visual evidence of white floculant colonies of microorganisms in most test phenol concentrations by 48 hr. A marked drop in phenol concentration occurred in static aquariums shortly after microbial growth became visible (Fig. 4). The rate of microbial growth appeared to be more rapid in phenol concentrations below 30 mg/l and phenol concentrations dropped in these tanks during the 24 to 48 hr period. The progressive microbial degradation of phenol resulted in lower phenol concentrations in some continuous flow aquariums during the second and third days of the 48 and 72 hr experiments. The microorganisms were not identified but it was assumed that they used phenol as a carbon source because control aquariums, with fish and no phenol, never developed visible microbial growth.

Static Experiments

In Experiments I and II the rate of phenol loss from static test tanks was not reproduceable from experiment to experiment (Fig. 5 and 6). Phenol concentrations in Experiment I dropped below detection limits by 48 hr. Since no phenol measurements were taken between 36

and 48 hr the graphs fail to show the time at which phenol became undetectable. Phenol concentrations in Experiment II dropped in the 18 to 24 hr interval. No measurements were taken of phenol concentrations at 24 hr before solution renewal, but they probably would have shown even lower concentrations than those recorded for 18 hr.

<u>Continuous Flow Experiments</u>

Desired treatment concentrations of phenol were reached in 6 to 18 hr after initial introduction into test tanks (Fig. 6-10). The phenol concentrations above 50 mg/l were more stable with less fluctuation among test tanks at each treatment concentration. Treatment concentrations below 50 mg/l had less stability and wider phenol fluctuation among tanks. In some test tanks phenol concentrations dropped during the final 12 hr of the exposure period. Continuous renewal of phenol using the automatic dilution units permitted the maintenance of more uniform concentrations of phenol than was attained in the static bioassays.

Fish Survival

<u>Analysis of Data</u>

Date derived from the static and slow introduction bioassays were subjected to simple linear regression analysis. Regression analysis was chosen because it provides reasonably detailed analysis of the linear relationship between two variables. Scatter plots of datum points for the 48 hr static and 48 hr slow introduction experiments indicated a linear relationship. Preliminary plots of datum points

for the 64 and 72 hr slow introduction experiments indicated a sigmoid type curve. Several transformations were tried and a probit transformation of concentrations was chosen as the best transformation of the 64 and 72 hr data.

Static Bioassays

The channel catfish exhibited typical rapid swimming movements and apparent avoidance reactions immediately after being placed in phenol solutions. Within minutes after introduction the fish in the higher concentrations of phenol (30, 35, and 40 mg/l) began shallow, rapid movements of the opercles and erratic jerky swimming movements. Within 1 to 2 hr at the higher phenol concentrations the fish turned on their sides on the bottom of the test tanks and ceased all swimming movements except for an occasional spasmodic contraction of the body. Most fish that died did so in the initial 6 hr of exposure (Fig. 5 and 6). In phenol concentrations above 20 mg/l only one fish died during the final 12 hr of each of the 48 hr experiments. Although considerable reduction of phenol concentrations occurred during the static bioassays it was apparent that concentrations of phenol above 30 mg/l were extremely toxic and concentrations below 25 mg/l had little effect on the ability of fingerling channel catfish to survive a 48 hr exposure. The 48 hr static TLm was calculated from the fitted regression line (Table V and Fig. 11). A static 48 hr TLm of 27.75 mg/l phenol with error bounds of 25.42 and 30.35 mg/l phenol was determined from the pooled data of the two static bioassays.

Slow Introduction Bioassays

The 48, 64, and 72 hr slow introduction TLm concentrations were determined in the same manner as described for static experiments. The progressive decrease in the TLm with time (48 hr, 67.01; 64 hr, 41.74; 72 hr, 38.24 mg/l) indicated that exposure time influenced survival. Only a few fish died in the first 36 hr of exposure (Fig. 6-10). In Experiment II only four fish died during the first 24 hr of exposure even when desired test concentrations were reached in 6 hr instead of the 12 hr required in the other slow introduction experiments (Fig. 7). In a preliminary experiment, raising the phenol concentrations from zero to 28.5 mg/l in 18 min failed to produce mortality of fingerling channel catfish in 48 hr. Within minutes the fish showed typical symptoms of phenol poisoning described for static bioassays above, but by 12 hr the symptoms had disappeared.

Avoidance was the initial reaction of the fish exposed to a slow introduction of phenol. The fish remained at the outlet end of the aquariums except for an occasional brief dash to the inlet. After the initial 10 to 20 min of exposure the fish began random movements within the tanks. Within 1 to 2 hr following phenol introduction the fish at high concentrations began erratic, jerky swimming movements symptomatic of phenol intoxication. Swimming movements diminished after 2 or 3 hr exposure to high phenol concentrations and the fish became quiescent on the bottom unless disturbed. Rapid opercle movements were characteristic of fish exposed for more than 3 hr to phenol concentrations above 30 mg/l. After remaining in a quiescent state for approximately 12 hr, catfish in concentrations below 50 mg/l appeared to recover

somewhat and assumed sluggish, jerky swimming movements. Some individuals in concentrations above 40 mg/l developed distended abdomens after about 24 hr continuous exposure. Autopsy revealed an accumulation of excess abdominal fluid but no visible swelling of internal organs. The individuals that developed distended abdomens retained the condition for the duration of the experiments.

Rapid Introduction Bioassays

Dumping fish into phenol solutions produced results similar to the static bioassays. The test fish reacted immediately to the phenol and most fish died in the initial 6 hr. The rapid introduction 48 hr phenol TLm was below 30 mg/l which was similar to the static 48 hr TLm of 27.75 mg/l. Phenol concentrations were held more constant in the continuous flow bioassays. This was not the case for the static bioassays but since the catfish died in the initial few hours of exposure when rapidly introduced into phenol solutions the maintenance of test levels beyond the initial 6 hr did not appreciably change the 48 hr TLm.

The Incipient Lethal Threshold

The LC50's were determined using the graphic method described by Sprague (1969). Although the data were insufficient to determine incipient LC50's quantitatively, the approximate values of 31 mg/l for the static and 82 mg/l for the slow introduction bioassays show that a marked difference occurred in threshold concentrations of catfish exposed to phenol. The LC50 values differ by more than the 48 hr TLm's for the two types of introduction (Table V). Although separate

LC50 concentrations for the static and rapid introduction continuous flow bioassays were not determined, the two bioassay methods produced similar results (Fig. 15). In all experiments rapid introduction produced high mortality in the first 24 hr at concentrations above the static 48 hr TLm. This was expected since both methods involved a rapid transfer of fish into the phenol solutions.

The minimum time for lethal action in the static and rapid introduction bioassays was less than 6 hr. The graph fails to show the actual minimum time for lethal action because the first record of mortality was taken at 6 hr. However, many fish died during the initial 6 hr of both static and rapid introduction experiments.

Reproduction of Fathead Minnows

Phenol concentrations were maintained within 8 mg/l at each test concentration using the continuous flow system. Test concentrations were reached by 12 hr at all test concentrations except the 41 mg/l in the 48 hr experiment. The 41 mg/l concentration was established by 24 hr. Test concentrations remained relatively constant throughout the two experiments and microbial growth was not apparent in any of the test aquariums during the phenol exposure period. The 28 mg/l concentrations did show a slight drop in concentration between 12 and 36 hr.

Effects of 24 hr Phenol Exposure on Reproduction

Surviving fathead minnows spawned following a 24 hr exposure to concentrations of 21, 40, and 52 mg/l phenol (Table VI). These phenol concentrations included the reported 24 hr TLm of 40.6 mg/l phenol

(Pickering and Henderson 1966). Mortality was 50% in the 52 mg/l concentrations but the survivors produced viable eggs from two successful spawns on the 28th and 30th day following phenol exposure. The survivors of 52 mg/l phenol also produced the largest number of eggs of any group, except the controls, during the 32 day experiment.

The time to the first successful spawn was not delayed by 24 hr exposure to phenol. Control fish spawned on the 28th day and fish exposed to 21 mg/l phenol spawned 11 days following exposure. Spawns were observed at 27 and 30 days after phenol exposures of 40 and 52 mg/l, respectively. During the 32 day test, fish in both control tanks produced repeated spawns but only fish in one tank at each phenol concentration spawned. Spawns in some tanks could have been overlooked because the parent fish occasionally consume the eggs. Known spawns were lost from predation by parent minnows in the 48 hr experiment but none were known to be lost in the 24 hr experiment. One experimental aquarium at 21 mg/l, one aquarium at 52 mg/l, and one control aquarium were treated for suspected disease on day 17 of the experiment. Malachite Green (5 mg/l) and formalin (1 mg/l) were used for 20 min followed by a rapid flush with fresh water. Only the fish in one 52 mg/l aquarium failed to spawn following treatment.

Effects of 48 hr Phenol Exposure on Reproduction

No obvious impairment of spawning activity occurred following 48 hr exposure to phenol concentrations of 21, 28, and 41 mg/l. Only minnows in one test tank (28 mg/l) failed to spawn during the 32 day test. Fish exposed to the highest test concentration (41 mg/l) produced the greatest number of eggs during the observation period.

Control fish spawned earlier but produced fewer eggs per spawn than fish exposed to phenol. Survival was 58% at the end of the 32 day test for fish exposed to 41 mg/l phenol.

Some spawns were lost because the eggs were consumed by the fish before the spawning cups were removed to count the number of eggs. No spawns in control tanks were known to be lost.

No apparent relationship existed between the percent of eggs fertilized and phenol exposure. The percent fertilization ranged from 39% in the 28 mg/l phenol to 93% in the 41 mg/l. Breeding behavior was normal in all aquariums. No behavioral differences were observed among control or experimental minnows during the 30 day observation period.

Effects of Phenol on Respiratory Rate of Bluegill Sunfish

The breathing rate of bluegill sunfish, measured by opercle beats per minute, was increased by phenol concentrations of 10 mg/l and above (Fig. 17). Both phenol concentration and time were judged to have a significant effect on the opercle rate ($P<0.05$). The 0, 1, and 5 mg/l concentrations did not show a significant difference among concentrations ($P<0.05$) but the 5 mg/l concentration was approaching significance at the 0.05 level. A significant difference ($P<0.001$) was found when group I (0, 1, and 5 mg/l phenol) was compared with group II (10 and 20 mg/l phenol) (Fig. 17). This change in opercle rate indicated that the threshold concentration of phenol necessary to cause a rate change in 12 hr was between 5 and 10 mg/l, probably closer to 5 mg/l phenol. With respect to the 0, 1, and 5 mg/l levels, time was not judged to be significant ($P>0.1$), and the phenol-time interaction

was also nonsignificant ($P>0.1$). With respect to the 10 and 20 mg/l phenol levels, both phenol and time were significant at the 0.001 level but the phenol-time interaction was nonsignificant ($P>0.025$). Phenol-time interaction was significant when all four levels of phenol were considered together. Since no significant phenol-time interaction occurred in either of the two groups when considered separately, the interaction obtained when all four phenol levels were considered together was attributed to experimental error.

A split-plot analysis with a completely randomized design was performed on the data. One assumption in the use of this design is the independence of measurements. In the opercle study the fish served as their own control and repeated measurements were made on each fish. The repeated measure violated the independence of measurements and the tests of significance may show significance when none should exist. Winer (1971) states that the degrees of freedom can be determined using the formula:

$$df = \phi\ (K-1)$$

The value of ϕ is somewhere between unity and 0.33 and the value of K is the observed degrees of freedom. When ϕ is one, the degrees of freedom are the actual observed degrees of freedom and a test of significance may show significance when none should exist. This is the liberal test used to state levels of significance in the present study. The conservative test uses 0.33 as ϕ and may not show significance when significance exists. When this conservative test was used only the phenol-time interaction changed from significant to nonsignificant.

Observations on opercle movements of individual fish revealed considerable variation in the pattern of breathing. Some individuals

produced even, regular movements while others showed a series of usually two or three rapid opercle movements followed by a short pause (Fig. 16). In the 10 and 20 mg/l phenol concentrations no pauses were observed, presumably because the respiration demand was too great to permit even temporary interruptions of water flow over the gill surfaces. The opercle beat tracings did not show a flat baseline because of the high sensitivity of the recording apparatus. Only the sharply defined spikes were counted as opercle beats because the smaller, more irratic spikes were not associated with visible opercle movements. Coughing was observed frequently during the experiments. Coughing was detected by a reversal in the spike during regular opercle movement tracings. No apparent relationship existed between frequency of coughs and phenol concentration.

CHAPTER V

DISCUSSION

Phenol Concentrations

Phenol concentrations were more constant in continuous flow bioassays than in the static bioassays but the build-up of microorganisms within test tanks produced some unpredictable variation (Davis 1956; Evans 1963; Mickerson 1956). Brown et al. (1969) found that fluctuating levels of phenol between a maximum of 1.5 and a minimum of 0.5 times the 48 hr TLm had no significant effect on median survival times of rainbow trout. Since the variation in my study was well within these limits in all test tanks, it was assumed that the effects of phenol fluctuations on survival of channel catfish were minimal.

Bioassays

Analysis of the slow introduction bioassays resulted in significantly higher TLm and LC50 values than those obtained using standard bioassay methods. Standard phenol bioassays conducted on several different species have shown rapid mortality during the initial hours of exposure and fish that survive the initial few hours tend to survive indefinitely at the test concentration (Brown et al. 1967; Burks personal communication; Clemens and Sneed 1959; Lammering and Burbank 1960; Pickering and Henderson 1966; Trama 1955). Perch,

Perca flavescens, (Bucksteeg et al. 1955) and bitterling, Rhodeus sericeus, (Malacea 1968) maintained for several days in low concentrations of phenol took twice as long to react to higher concentrations of phenol when compared with previously unexposed fish. Similar results have been reported for crucian carp (Luk'yanenko 1967) and blue bream, Abramis ballerus, (Volodin et al. 1966). My results suggest a similar development of resistance in fingerling channel catfish exposed to phenol. Bioassays that involved the transfer of fingerling channel catfish into phenol solutions produced rapid mortality during the initial hours of exposure, but in the slow introduction bioassays mortality started slowly and increased with increasing exposure time. Only concentrations considerably above the slow introduction 72 hr TLm produced any significant mortality in the initial hours of exposure in the slow introduction bioassays.

Fish collected in different seasons showed variation in their sensitivity to phenol. Late February specimens used in continuous flow bioassay I (Fig. 6) were more susceptible to phenol poisoning than fish taken in May or July (Fig. 7-9). The apparent seasonal differences may have involved other factors. Brown et al. (1967) reported a complicated temperature reversal for phenol toxicity using rainbow trout. The greater susceptibility of chennel catfish collected in February may have been influenced by the temperature reversal phenomenon.

Handling stress and the shock of sudden immersion into a foreign chemical solution would not be a normal occurrence in nature. Fish exposed to a foreign chemical in their natural environment would have a period of a few minutes to several hours to acclimate in gradually changing concentrations of the chemical. Although it is difficult to

extrapolate laboratory results to actual field conditions, the slow introduction bioassay may yield more useful information concerning the survival potential of a fish population in a given concentration of a pollutant in their natural environment because the effects of handling and sudden immersion are virtually eliminated. Measurement of the toxicity of a chemical by using both rapid and slow introduction bioassays and determining the two LC50's provides a method for measuring the effects of handling stress and possible acclimation to a particular chemical.

A direct comparison of the TLm's derived from the different bioassay methods used in this study may not be entirely valid because of the time necessary to bring the concentrations of phenol from a zero level up to the desired test level in the slow introduction bioassays. TLm values are expressed with respect to a specified exposure period, i.e., a 48 hr TLm is determined from continuous exposure of fish to the test concentrations of a toxin for a full 48 hr (Sprague 1969). A slow introduction TLm, as used in the present study, expresses the total time the fish are exposed to phenol, beginning with the introduction of phenol into the test tanks, i.e., a shorter exposure to the desired test concentrations than would be the case in a standard bioassay.

This temporal discrepancy is also a factor when a comparison is made of the two LC50's determined from the two different bioassays. However, the incipient LC50's are determined from median lethal times, i.e., the time at which the mortality in each test container reaches 50%. The only values that are needed to determine the LC50 are those in which mortality reaches or exceeds 50% regardless of the time

required to reach the 50% mortality level (Sprague 1969). Therefore, a comparison of the LC50's would seem to be a valid comparison of the two methods even though the temporal difference exists.

Reproduction of Fathead Minnows

The results of the reproduction experiments failed to show an interruption or prolonged delay in spawning after phenol exposure. The extreme variability in the number of eggs spawned by different females and the variation in the time necessary for individuals to reach spawning condition made the analysis of subtle effects of phenol on reproduction impossible.

There appeared to be positive effects on the total number of eggs spawned and the number of eggs per spawn after phenol exposure, but the variation in number of eggs per spawn among different fish could account for the differences observed. The eggs/spawn of fathead minnows in the laboratory range from fewer than 50 to over 1200 (Burks, personal communication). Considering the huge variation in number of eggs/spawn among females the values of 90 and 195 eggs/spawn for controls in the present study compared well with control averages of 165 eggs/spawn (Eaton 1973) and 164 eggs/spawn (Pickering Gast 1972) for fathead minnows.

No apparent relationship was found between phenol concentrations and the percent fertilization. The high fertilization percentage in the 41 mg/l concentrations may have resulted partially from the bacteriosidal effect of phenol and/or later spawning may have resulted in a greater percentage of mature eggs being spawned.

Some spawns were lost by predation by the parent fish, but no

known spawns were lost in control tanks. Predation of eggs by fathead minnows was observed by Eaton (1973) in chronic bioassays of mixtures of copper, cadmium, and zinc, and no spawns were lost through predation in his control tanks. Further testing is needed to determine the effect of pollutants on this behavioral phenomenon.

No numerical data were obtained on the hatchability of eggs and survival of larval minnows. Some eggs hatched in 4-6 days and the larval fishes appeared to be normal during a two-week observation period.

Although the experiments exceeded the recommendations of Mount (1968) that 10 fish per container and 20 fish per concentration is a sufficiently large sample size to measure the effect of pollutants on fish reproduction, it is obvious that much larger numbers of fish and test containers at each concentration would be needed to obtain reliable reliable estimates of the number of eggs per spawn.

Effects of Phenol on Respiratory Rate of Bluegill Sunfish

Although no statistically significant change in opercle rate was observed in the 1 and 5 mg/l phenol concentrations (P 0.05), fish in the 5 mg/l concentrations showed a change in opercle rate greater than that of the controls. Recently Morgan and Kuhn (1974) reported that concentrations of phenol of 0.5, 1, and 5 mg/l all produced an increase in opercle rates of largemouth bass. They found that the bass took 23 hr at 5 mg/l, 53 hr at 1 mg/l, and 63 hr at 0.5 mg/l to reach an opercle rate greater than the initial rate when the fish were first placed in the test chambers. If bluegill sunfish had been exposed for longer periods at the 1 and 5 mg/l phenol concentrations in the

present study perhaps one or both levels would have produced significant changes in opercle rate. In the present study the opercle rate of fish exposed to 10 and 20 mg/l phenol increased to three times the pre-test rate. It was suspected that the opercle rates of these fish were probably near maximum.

Considerable variation occurred in both the rhythm and rate of opercular movements among individuals. Initially, most fish placed in the electrode chambers gave uniform, evenly spaced tracings but after several hours in experimental chambers the breathing rhythm became more irregular. Some individuals produced a fairly even rhythm of breathing which persisted for the 24 hr pre-test period while others produced a rhythm consisting of a series of usually three rapid opercle movements followed by a brief pause.

The amplitude of opercle movement tracings changed during the experiments. Heath (1972) found that fish close to the electrodes gave a greater response than fish a few centimeters away. Removing the air supply and cutting off water flow resulted in an increased amplitude and a more regular breathing pattern for bluegill sunfish in my experimental chambers. Therefore, amplitude changes were not used even though the higher concentrations of phenol appeared to produce more forceful opercle movements.

The size of the test chambers and the isolation of previously unisolated bluegill sunfish may have contributed to changes in opercle rates. The fish were able to turn around in the test chambers but not without touching the sides of the chamber and this confinement may have caused some stress.

The acclimation time before the beginning of phenol exposure may have been too short. The increased opercle rates of control fish during the course of the experiments suggested that the fish were stressed. However, Sparks et al. (1972) found that bluegill sunfish acclimated to a laboratory for 2 months and acclimated in isolated 20 l aquariums for an additional 2 weeks showed variation in opercle rates comparable to those observed in this study.

The use of the external electrode method to monitor breathing as a means of detecting chronic concentrations of chemicals needs further study. Selection and pretreatment of test fish needs to be standardized before the method can be used for predictive determination.

Investigations correlating gill beats/min with changes in respiratory pressures of different fish species is needed to understand the complexity of respiratory changes associated with a changing environment. Channel catfish, for example, proved to be totally unsuitable as test animals in this study. Spoor et al. (1971) reported that fathead minnows failed as test animals for the external electrode method but guppies and largemouth bass gave reliable respiratory tracings. Rainbow trout responded to environmental stress by changing the depth of breathing with breathing frequency remaining relatively constant (Heath 1972).

The increase in opercle rate of bluegill sunfish at phenol concentrations of 10 mg/l and the evidence that 5 mg/l phenol was almost significant at the 0.05 level shows that there was a significant physiological effect of phenol on bluegill sunfish at concentrations well below the reported TLm for this species. Since opercle-rate can be used as an indirect measurement of oxygen consumption (Fry

1957; Heath 1972), it is probable that a continuous exposure of bluegill to phenol concentrations above 5 mg/l would be detrimental to the health of this species in the natural environment.

CHAPTER VI

SUMMARY

Static and continuous flow 48 hr phenol bioassays with immersion of fish into phenol solutions and continuous flow 48, 64, and 72 hr phenol bioassays with slow introduction of phenol into tanks containing fish were made using fingerling channel catfish. Comparison of the TLm and LC50 values obtained from the different bioassay procedures indicated that handling the catfish and the chemical shock caused by sudden immersion into phenol solutions significantly influenced the results. Sudden immersion of fish into phenol solutions resulted in greater susceptibility and mortality during the initial hours of exposure, whereas slow addition of phenol produced lower susceptibility and mortality increased as exposure time increased. Although the TLm values decreased with increased exposure time, the 72 hr slow introduction TLm was still above the 48 hr static TLm.

The slow introduction bioassays with renewal of phenol by continuous flow provides a more meaningful estimate of phenol toxicity because it more closely simulates natural conditions. This method eliminates stress caused by handling the fish immediately prior to the experiment and prevents the accumulation of excretory wastes and other by-products. By gradually increasing the concentration of the test chemical, some time is allowed for acclimation as would occur usually under natural conditions.

Comparison of the LC50's determined from sudden immersion and by slowly adding phenol solutions into tanks containing fish can provide a useful measure of the combined effects of stress produced by handling, chemical shock, and possible acclimation of the fish. TLm comparisons would not be as useful because they are based on specific time periods.

The reproductive potential of fish exposed to phenol was evaluated by two experiments. Adult fathead minnows that survived 24 and 48 hr slow introduction phenol exposures at concentrations above the reported TLm reproduced within 30 days. Time to first spawn, number of eggs spawned and the percentage of eggs fertilized were used as criteria to measure reproductive success. No measurable differences were found.

Breathing rates of bluegill sunfish exposed to phenol were investigated to evaluate chronic effects of sub-lethal concentrations. Significant increases in opercle rates occurred within 6 hr following the slow introduction of phenol at concentrations of 10 and 20 mg/l. Opercle rates of sunfish exposed to lower concentrations for 12 hr were not significantly different from those of unexposed fish. Although 10 mg/l phenol may not be lethal in a standard bioassay the increased opercle rates suggest that longer exposure times could produce significant mortality.

The magnitude of the difference in phenol toxicity values determined by standard bioassay methods and those determined by slow introduction bioassays illustrates the need for additional research using other pollutants to determine if the observed difference is unique for phenol. The amount of variation in reproduction of fathead minnows found in this study demonstrates that extensive replication

would be needed before meaningful information concerning the effects of a pollutant on reproduction in this species can be ascertained. Examination of changes in opercle rates of fish exposed to low concentrations of pollutants shows considerable potential in determining the subtle effects of aquatic pollutants on fish.

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APPENDIXES

TABLE I

TOXICITY OF PHENOL TO FISH

Species	24 hr TLm (mg/l)	48 hr TLm (mg/l)	96 hr TLm (mg/l)	Reference
Fathead Minnows (soft water)	40.6	40.6	34.3	Pickering and Henderson (1966)
Fathead Minnows (hard water)	38.6	38.6	32	Pickering and Henderson (1966)
Bluegill Sunfish (soft water)	25.8	23.8	23.9	Pickering and Henderson (1966)
Bluegill Sunfish	22.7*	22.2		Lammering and Burbank (1960)
Bluegill Sunfish			13.5	Patrick et al. (1968)
Bluegill Sunfish	20.5		19.3	Trama (1955)
Channel Catfish fingerlings		16.7	16.7**	Clemens and Sneed (1959)
Channel Catfish fingerlings	25	25		Burks (personal communication)
Goldfish (soft water)	49.9	49.1	44.5	Pickering and Henderson (1966)
Guppies (soft water)	49.9	49.8	39.2	Pickering and Henderson (1966)

*28 hr

**72 hr

TABLE II

SUMMARY OF EXPERIMENTAL BIOASSAY CONDITIONS

Experiment	Phenol mg/l	No. Test Tanks Rapid	No. Test Tanks Slow	No. Fish Rapid	No. Fish Slow	Alkalinity (mg/l)	pH	Hardness (mg/l)	Temp. (C) (Range)	Phenol Sampling Times (hr) 0	6	12	18	24	36	48	64	72
	0	2		20						+		+		+	+	+		
STATIC	15	2		20						+		+		+	+	+		
I	20	2		20		94	7.7	171	25	+		+		+	+	+		
	25	2		20					(24-25)	+		+		+	+	+		
	30	2		20						+		+		+	+	+		
	0	2		20						+	+	+	+	+	+	+		
STATIC	25	2		20						+	+	+	+	+	+	+		
II	30	2		20		95	7.6	172	25	+	+	+	+	+	+	+		
	35	2		20					(24-25)	+	+	+	+	+	+	+		
	40	2		20						+	+	+	+	+	+	+		
CONTINUOUS	0	2	2	20	20	112	7.7	170	20	+		+		+	+	+		
FLOW	25	2	2	20	20				(19-21)	+		+		+	+	+		
I	30	2	2	20	20					+		+		+	+	+		
	40	2	2	20	20					+		+		+	+	+		
CONTINUOUS	0	1	1	10	10					+	+	+		+	+	+		
FLOW	25	1	1	10	10					+	+	+		+	+	+		
II	55	1	1	10	10	130	7.8	174	20	+	+	+		+	+	+		
	65	1	1	10	10				(20-21)	+	+	+		+	+	+		
CONTINUOUS	0	1	1	10	10					+		+		+	+	+		
FLOW	20	2	2	20	20					+		+		+	+	+		
III	30	2	2	20	20	134	7.8	181	25	+		+		+	+	+		
	40	2	2	20	20				(24-25)	+		+		+	+	+		
	50	2	2	20	20					+		+		+	+	+		
CONTINUOUS	0	1	1	10	10					+	+	+	+	+	+	+		
FLOW	40	2	2	20	20					+	+	+	+	+	+	+		
IV	50	2	2	20	20	94	7.6	169	25	+	+	+	+	+	+	+		
	50	2	2	20	20				(25-26)	+	+	+	+	+	+	+		
	50	2	2	20	20					+	+	+	+	+	+	+		
CONTINUOUS	0		2		20					+	+	+	+	+	+	+	+	+
FLOW	30		4		40					+	+	+	+	+	+	+	+	+
V	40		4		40	94	7.6	181	26	+	+	+	+	+	+	+	+	+
	70		4		40				(25-27)	+	+	+	+	+	+	+	+	+
	80		4		40					+	+	+	+	+	+	+	+	+

TABLE III

TIME SCHEDULE FOR CHANNEL CATFISH

Experiment	Arrival Date	Experiment Date
Continuous Flow I	February 24, 1972	February 28, 1972
Continuous Flow II	May 3, 1972	May 9, 1972
Continuous Flow III	July 5, 1972	July 11, 1972
Continuous Flow IV	June 28, 1973	July 9, 1973
Continuous Flow V	July 17, 1973	July 23, 1973
Static I	June 28, 1973	July 7, 1973
Static II	July 17, 1973	July 20, 1973

TABLE IV

SUMMARY OF EXPERIMENTAL CONDITIONS FOR REPRODUCTIVE STUDIES

Experiment	Exposure (hr)	Phenol (mg/l)	Alkalinity (mg/l)	pH	Hardness (mg/l)	Temp. C. (range)	Phenol Sample Times (hrs) 0	6	12	24	36	48
		0					+	+		+		
I	24	21	134	7.7	170	25	+	+		+		
		40				(24-26)	+	+		+		
		52					+	+		+		
		0					+		+	+	+	+
		21					+		+	+	+	+
II	48	28	130	7.8	172	25	+		+	+	+	+
		41				(24-26)	+		+	+	+	+

TABLE V

SUMMARY OF COMPUTED TLm'S FROM REGRESSION ANALYSIS OF STATIC AND CONTINUOUS FLOW BIOASSAYS

Type of Introduction	Exposure (hr)	No. Reps.	Degrees Freedom	TLm (mg/l)	High Bound (mg/l)	Low Bound (mg/l)	Data Transformation
Static	48	16	15	27.75	30.35	25.42	None
Slow	48	40	39	67.01	74.87	61.5	None
Slow	64	18	17	41.74	49.22	32.92	Probit
Slow	72	18	17	38.24	44.4	30.84	Probit

TABLE VI

SUMMARY OF REPRODUCTION FOLLOWING 24 HR AND 48 HR PHENOL EXPOSURE

Exposure (hr)	Phenol (mg/l)	No. Males*	No. Females*	No. Spawns	Total No. Eggs Produced	Average No. Eggs/Spawn	% Fertilization***
	0	7	15	6	1172	195	
24	21	6	14	1	79	79	
	40	6	14	1	157	157	
	52	2	10	2	415	208	
	0	6	16	9	813	90	48
48	21	8	16	10**	873	146	75
	28	6	15	5**	360	120	39
	41	3	11	7**	1372	229	93

*Number of survivors after 32 days.

**Includes spawns in which counts were lost.

***Using only spawns that were not lost.

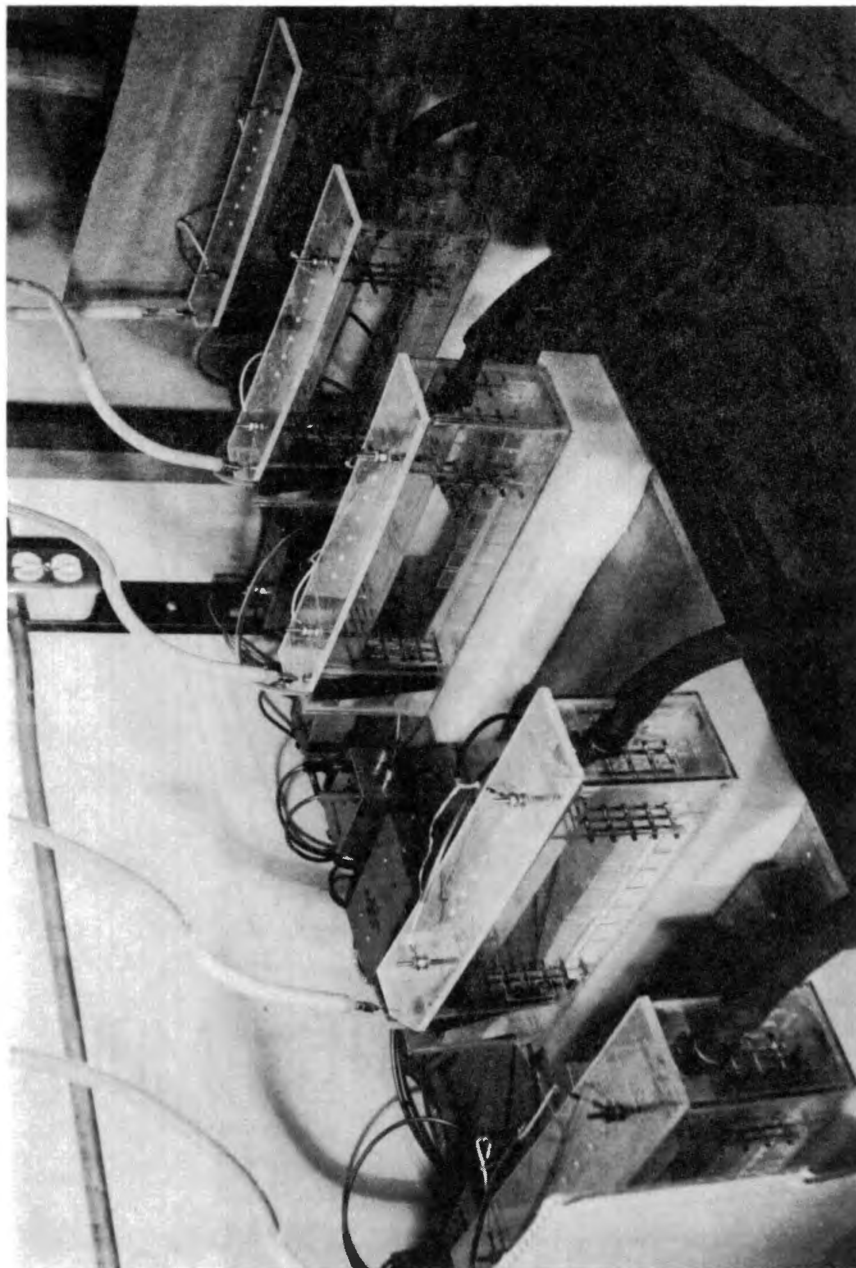

Figure 2. The Electrode Chambers

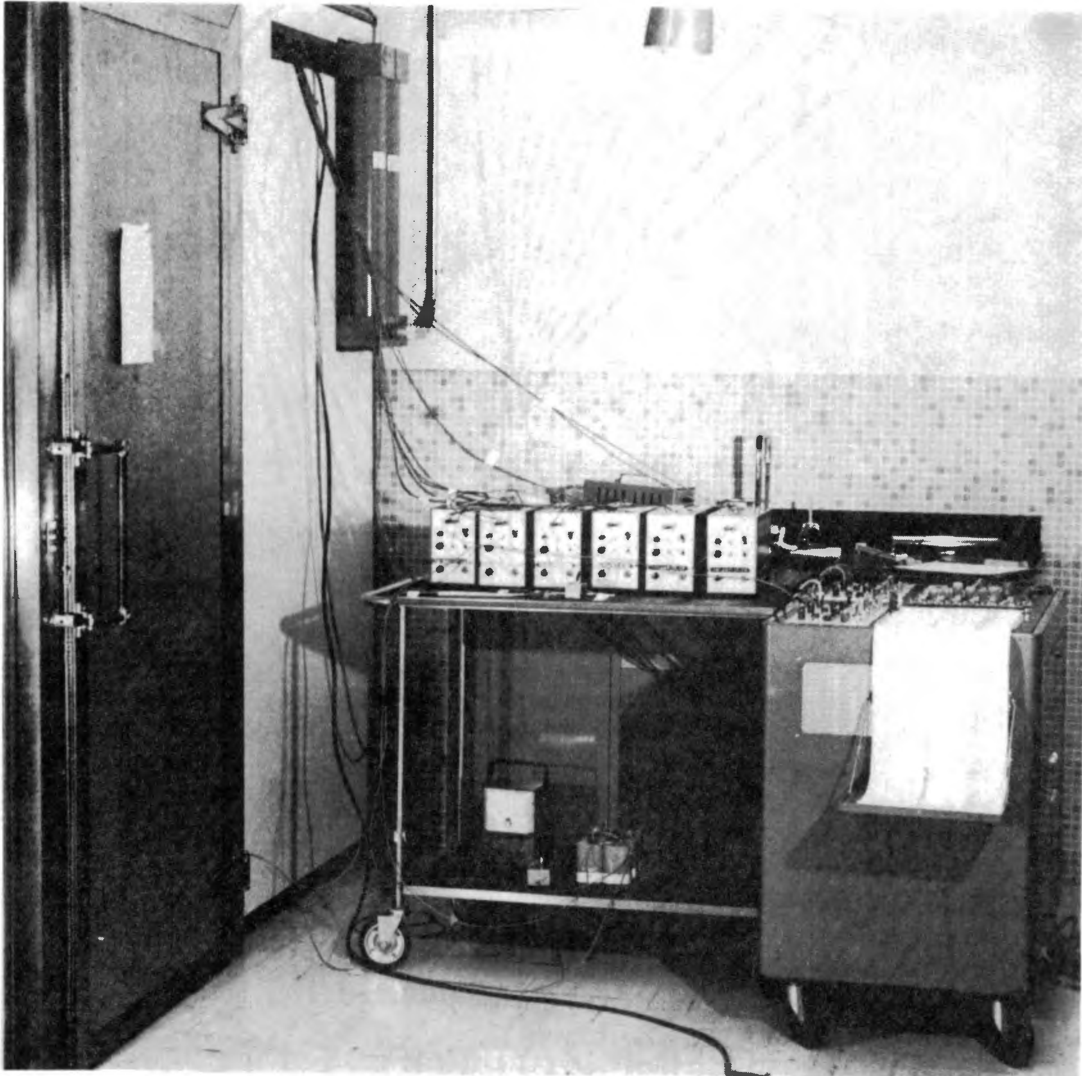

Figure 3. The Test Room and Recording Equipment

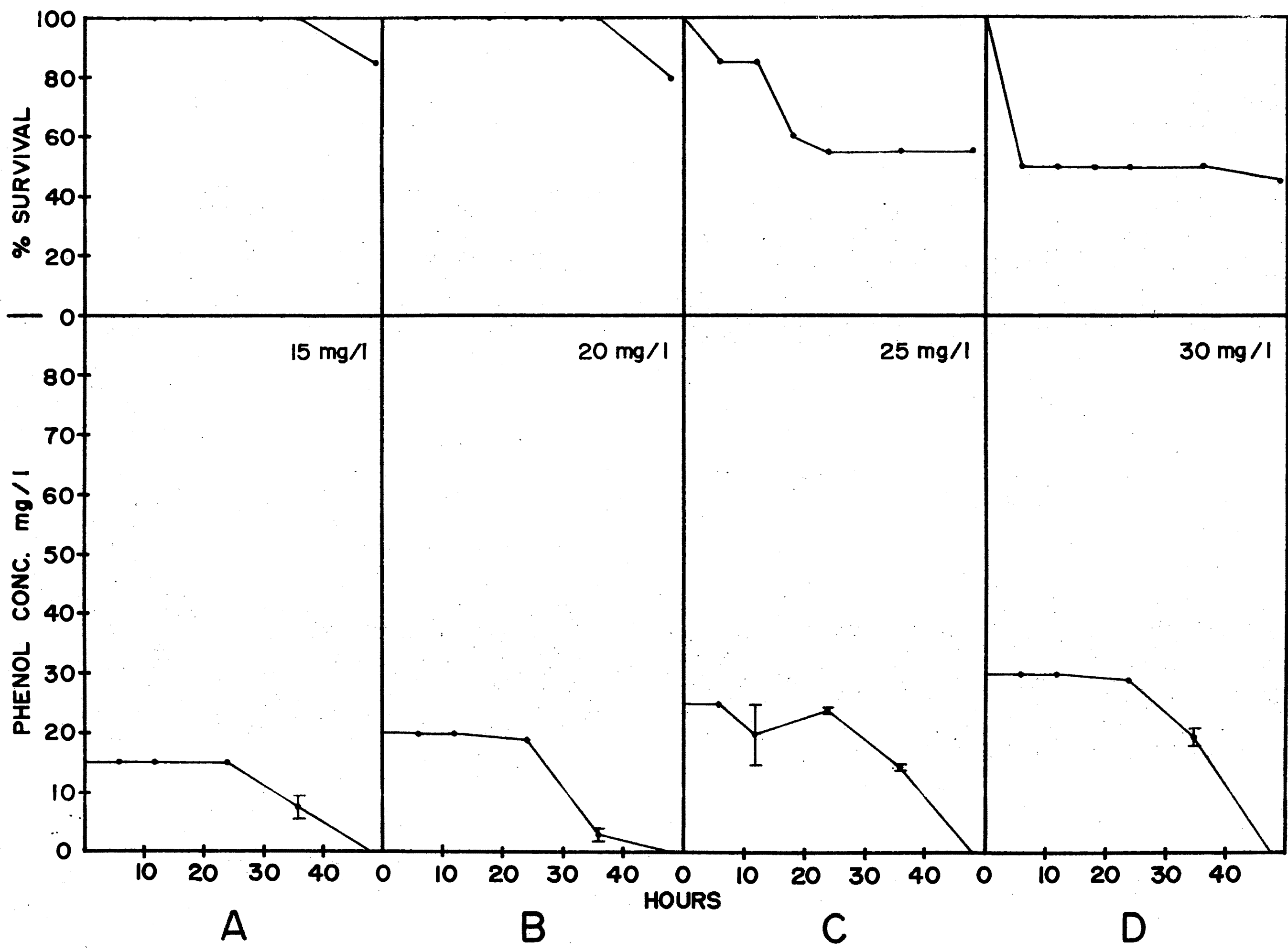

Figure 4. Static Bioassay I - Phenol Concentrations and Survival

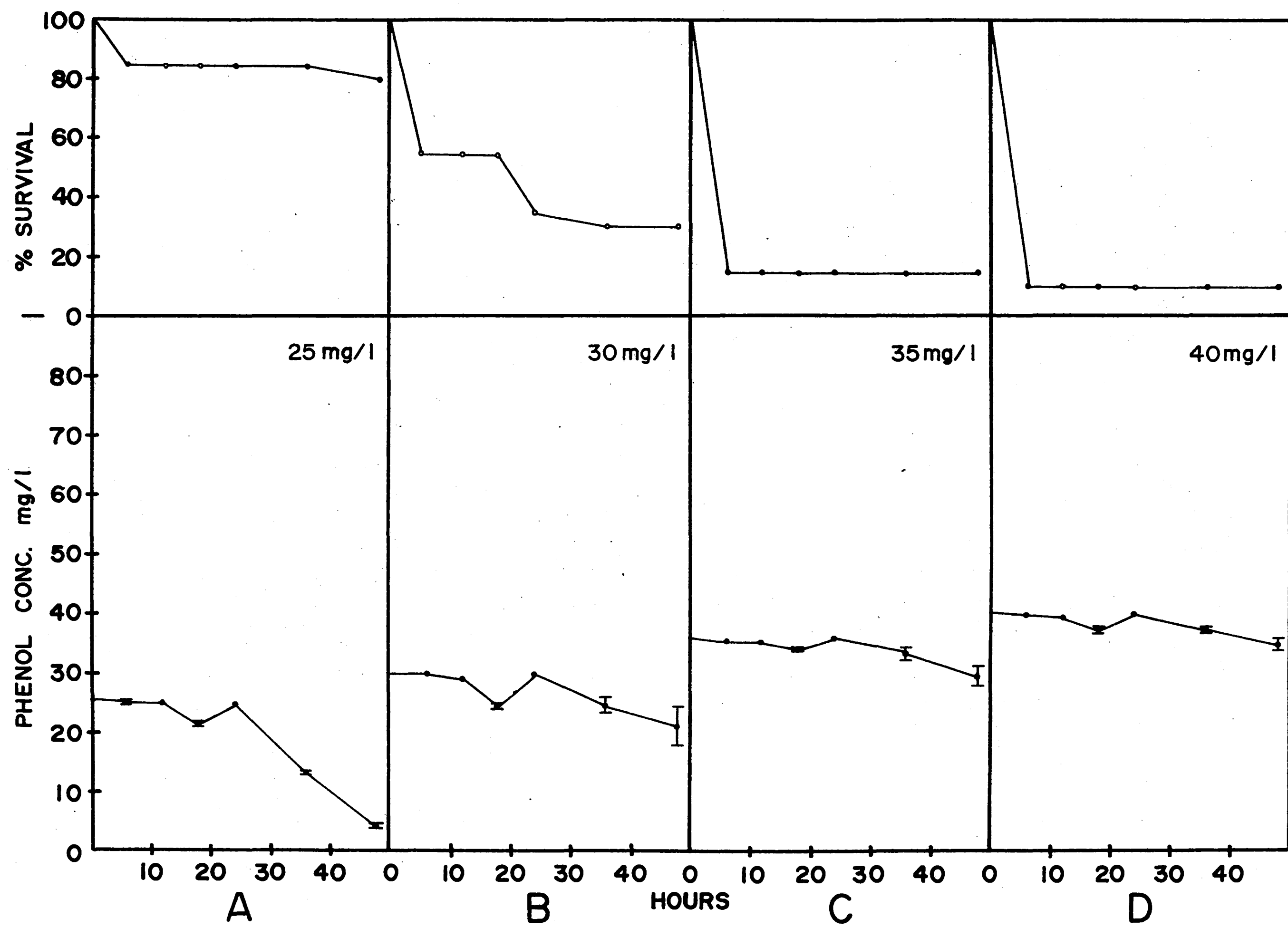

Figure 5. Static Bioassay II - Phenol Concentrations and Survival

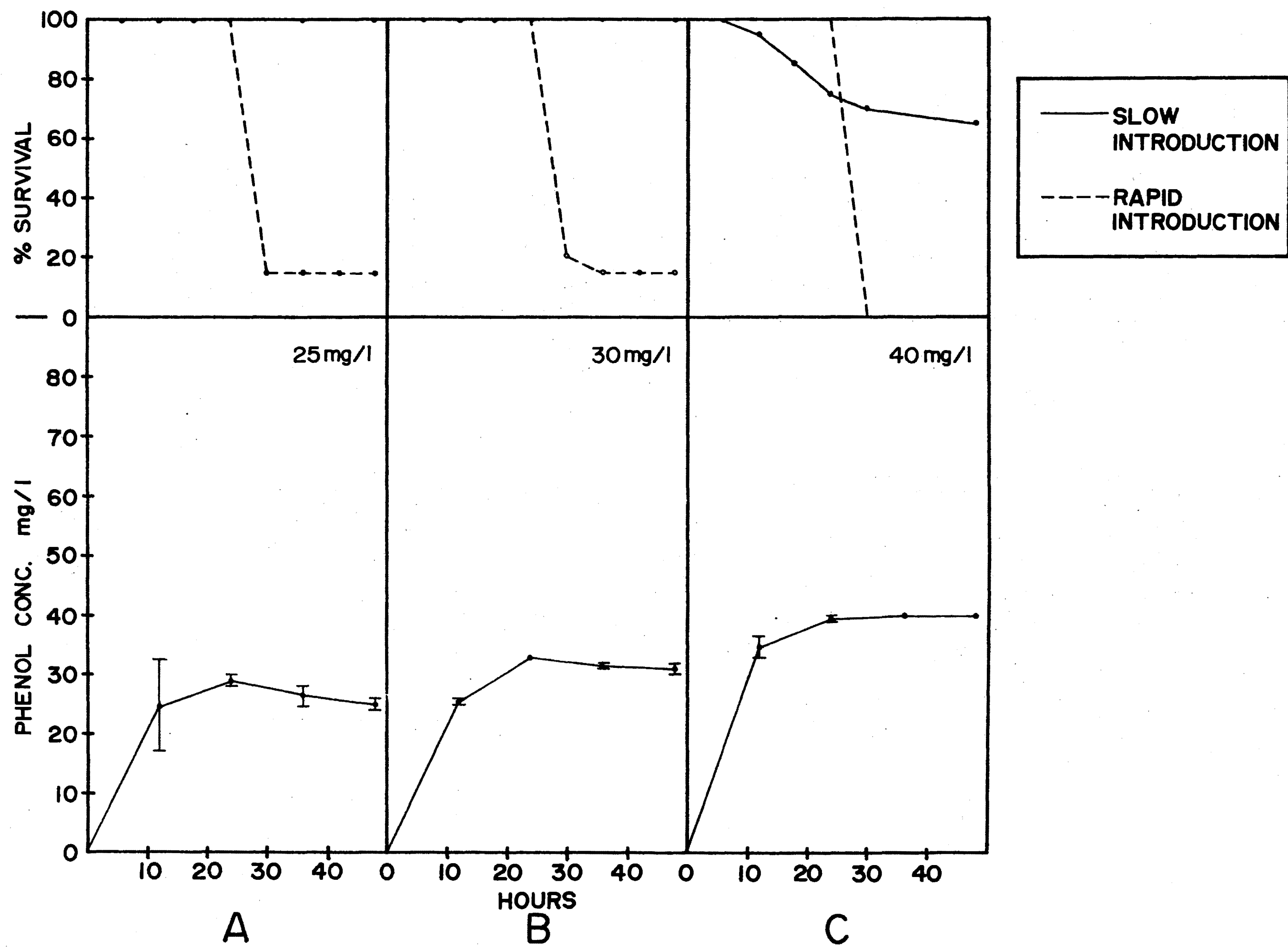

Figure 6. Continuous Flow Bioassay I - Phenol Concentrations and Survival

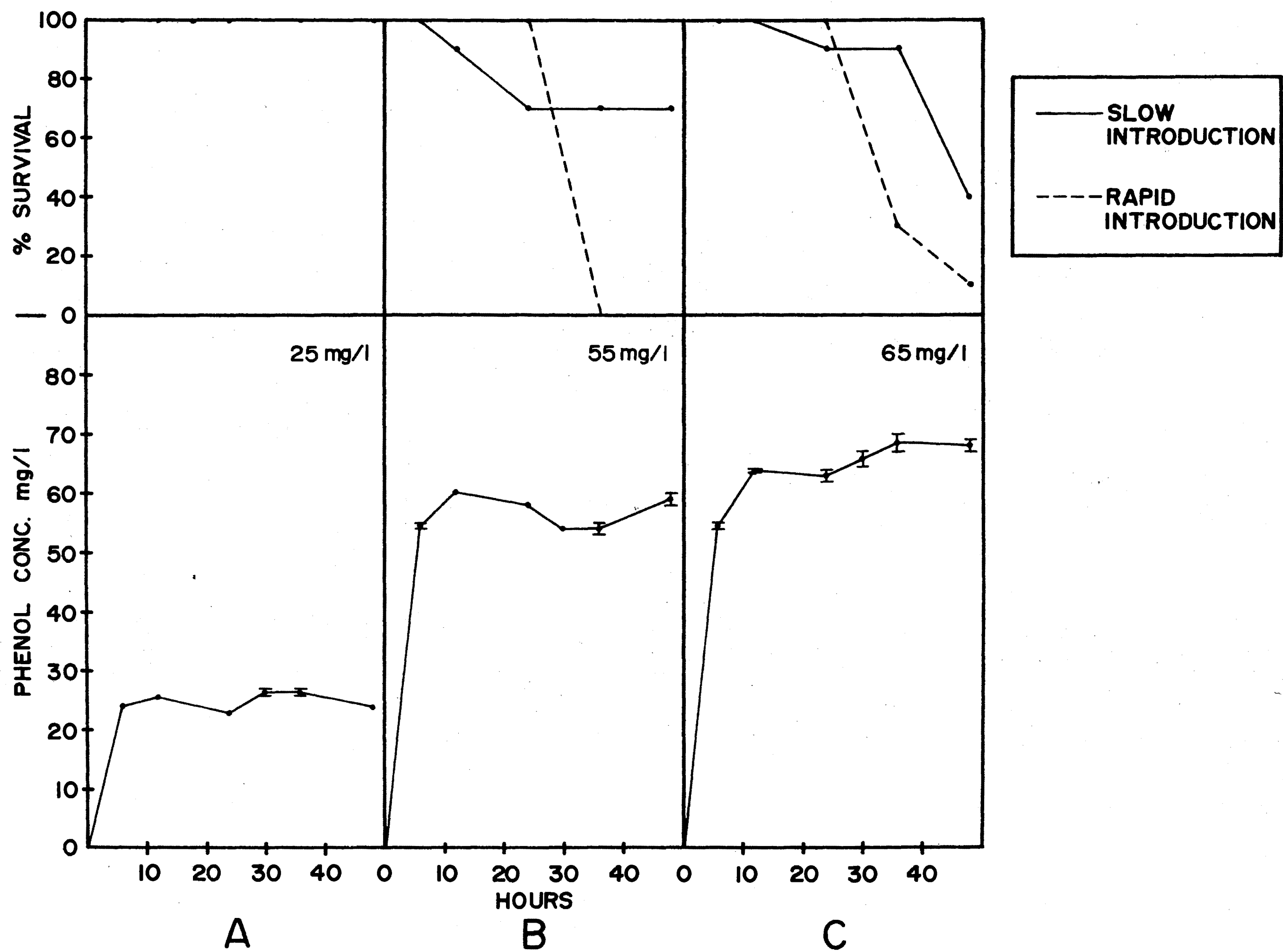

Figure 7. Continuous Flow Bioassay II - Phenol Concentrations and Survival

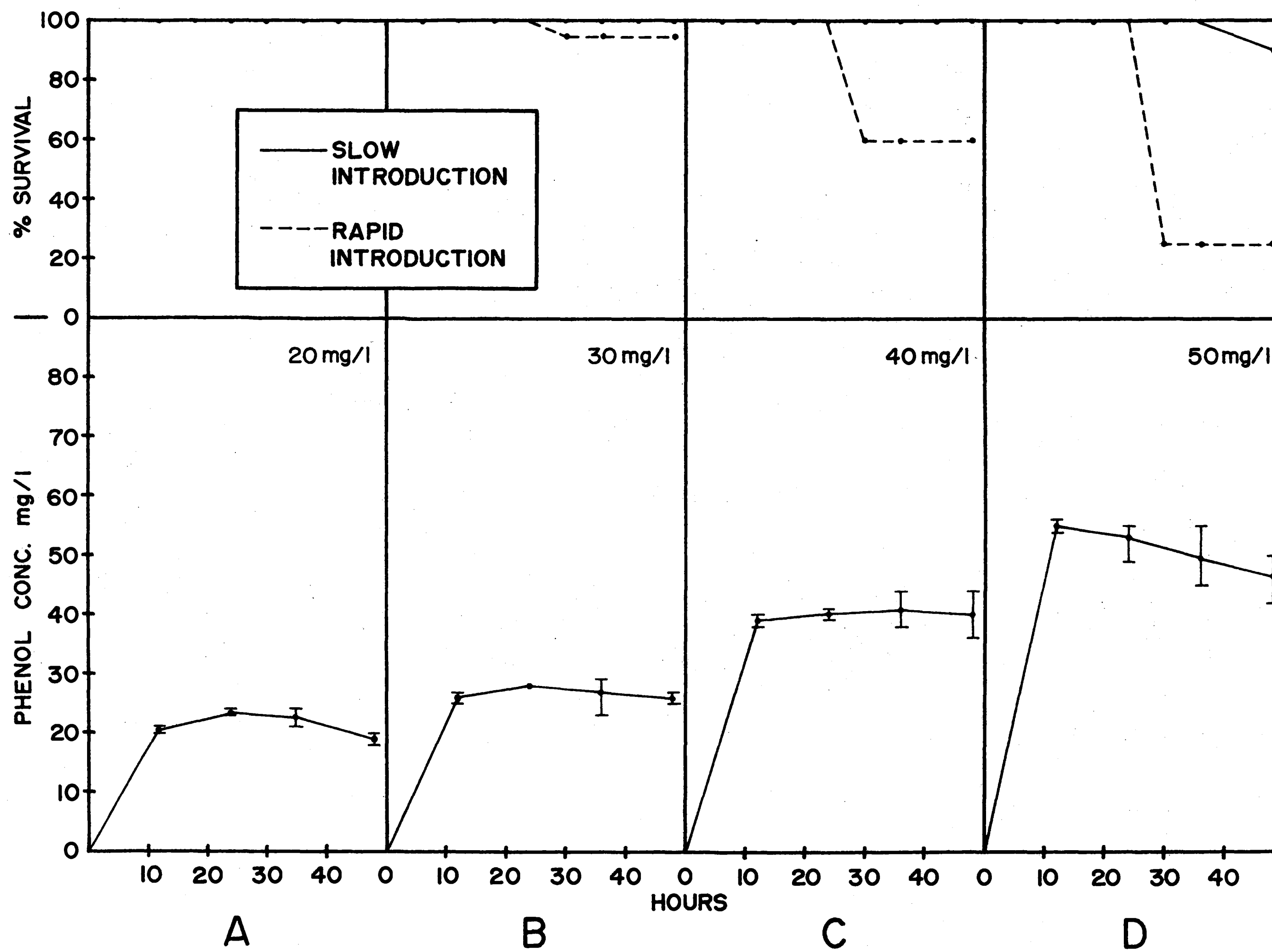

Figure 8. Continuous Flow Bioassay III - Phenol Concentrations and Survival

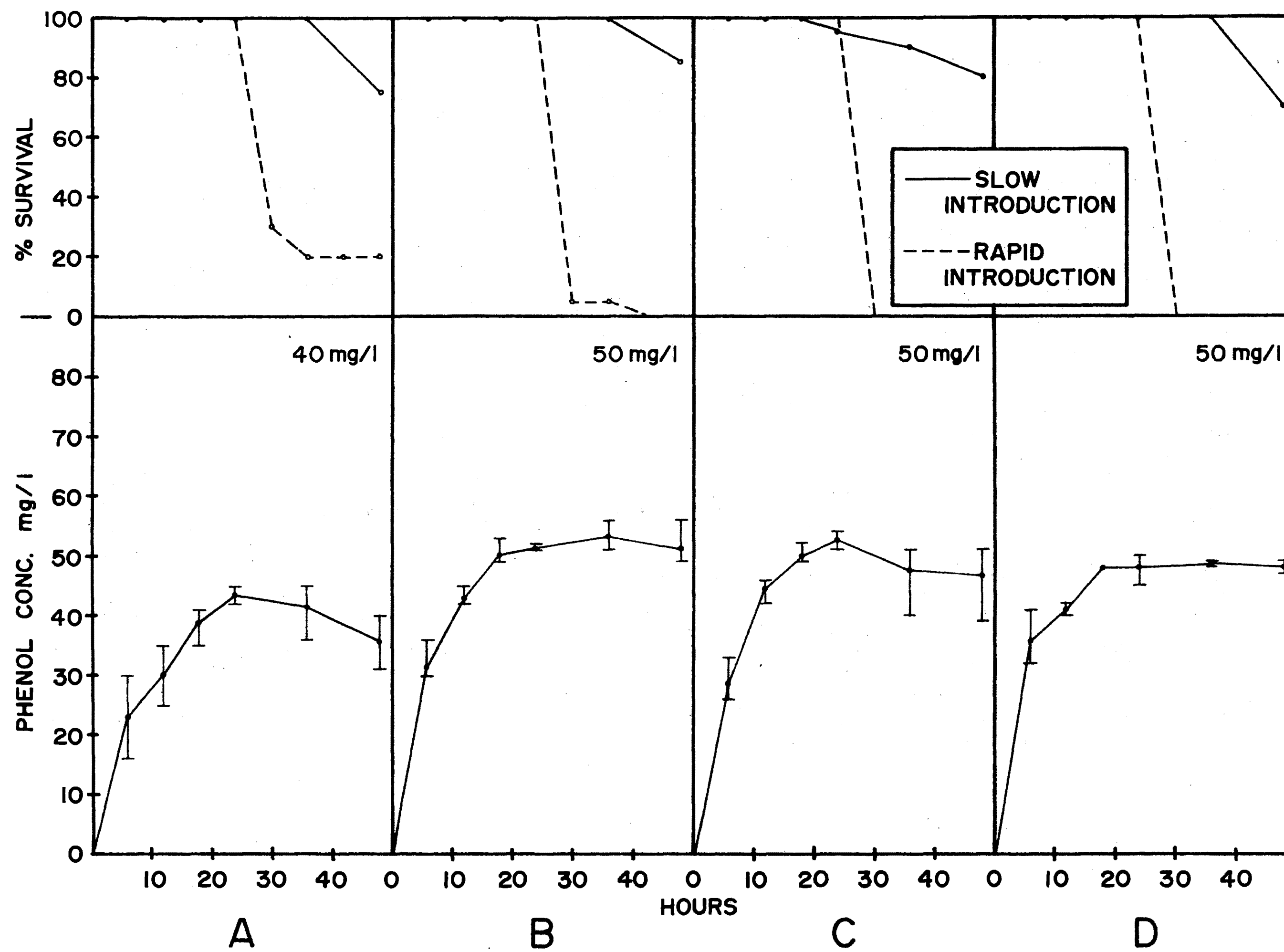

Figure 9. Continuous Flow Bioassay IV - Phenol Concentrations and Survival

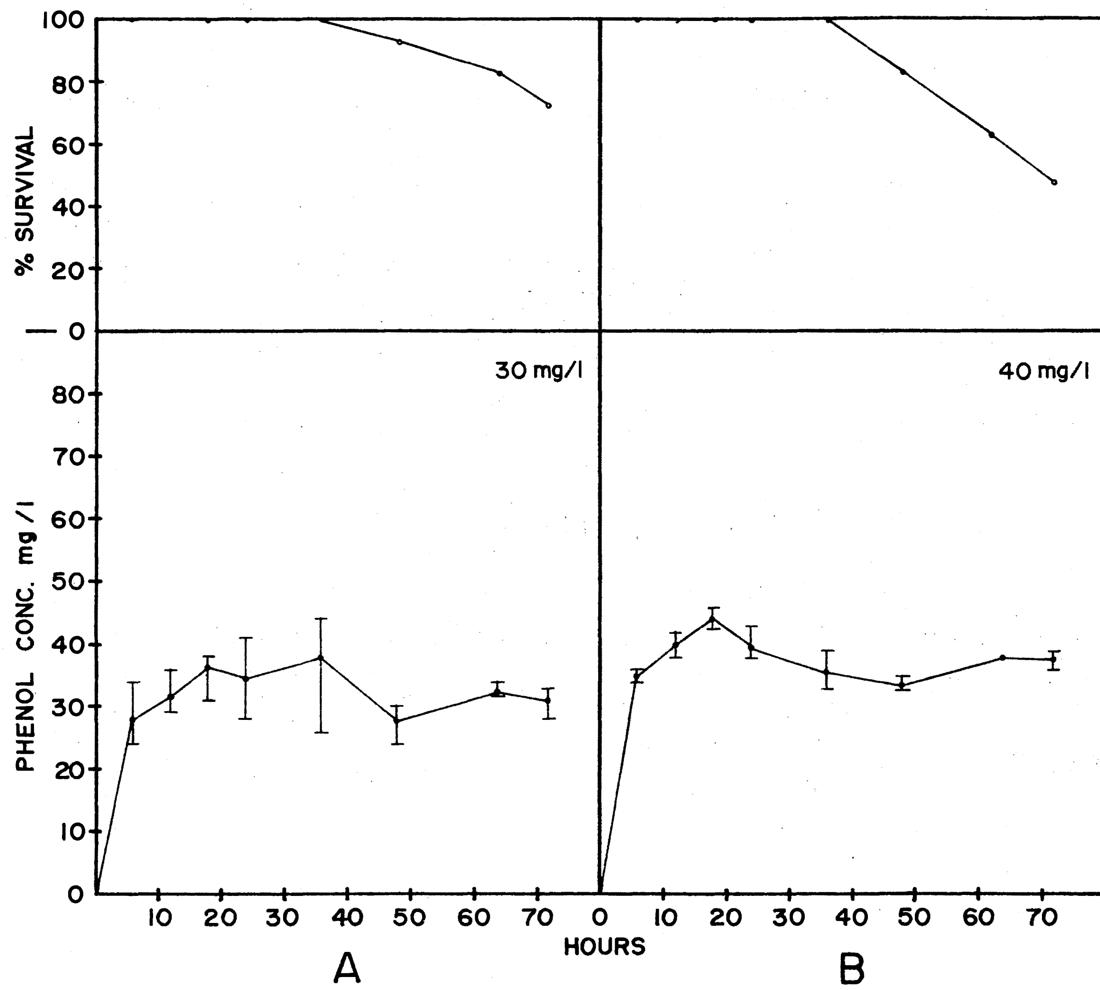

Figure 10. Continuous Flow Bioassay V - Phenol Concentrations and Survival

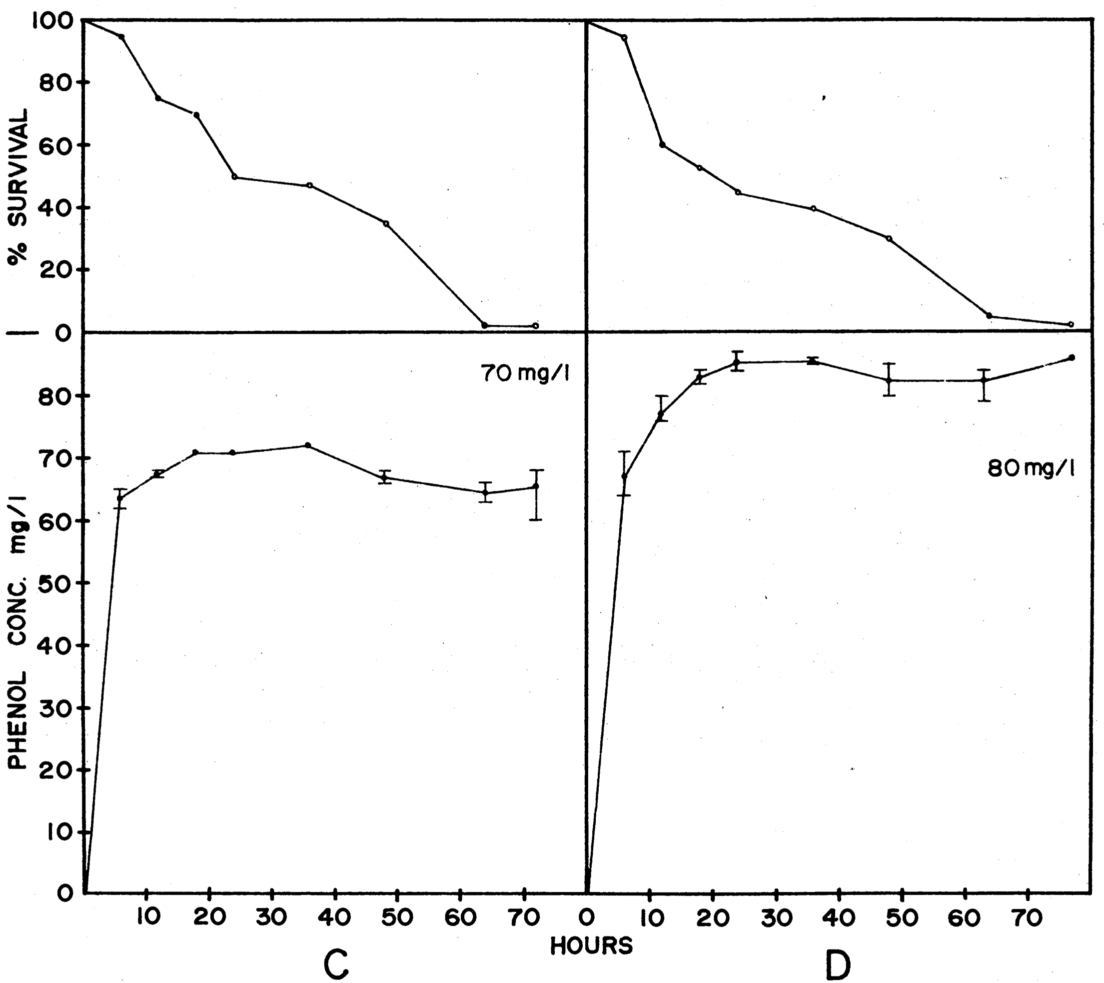

Figure 10. (Continued)

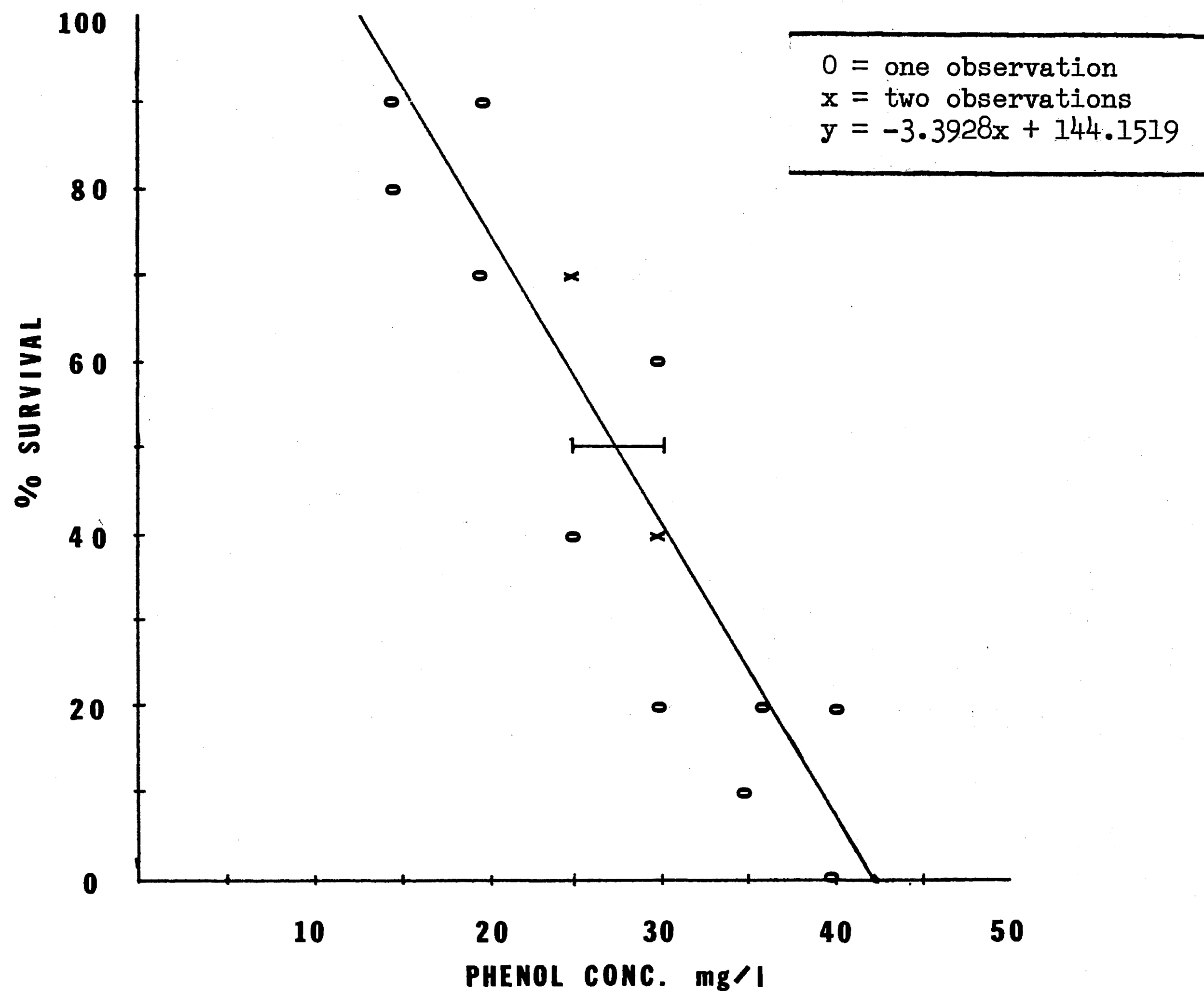

Figure 11. 48 Hr Static Bioassays - The Regression of Channel Catfish Survival on Phenol Concentrations

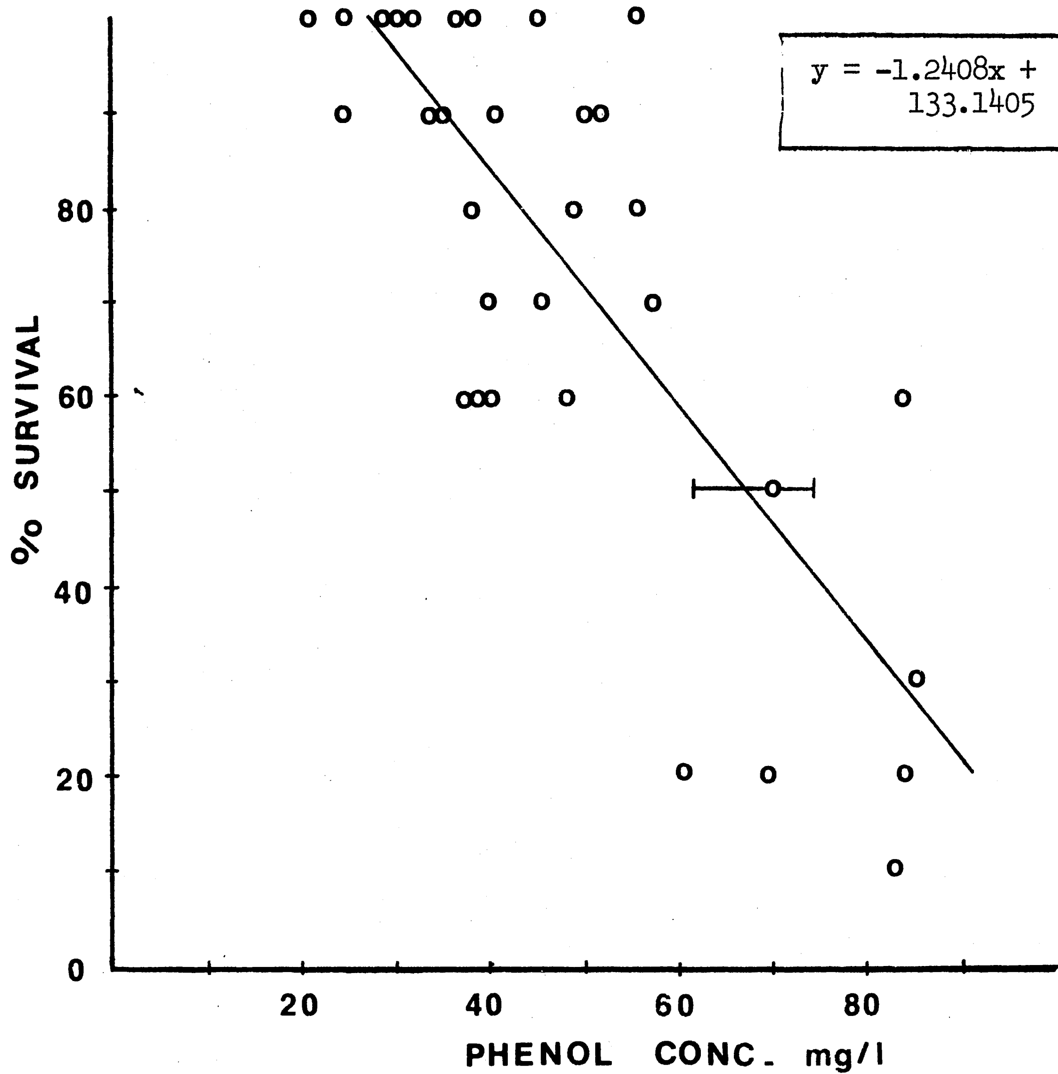

Figure 12. 48 Hr Continuous Flow Bioassays - Regression of Channel Catfish Survival on Phenol Concentrations

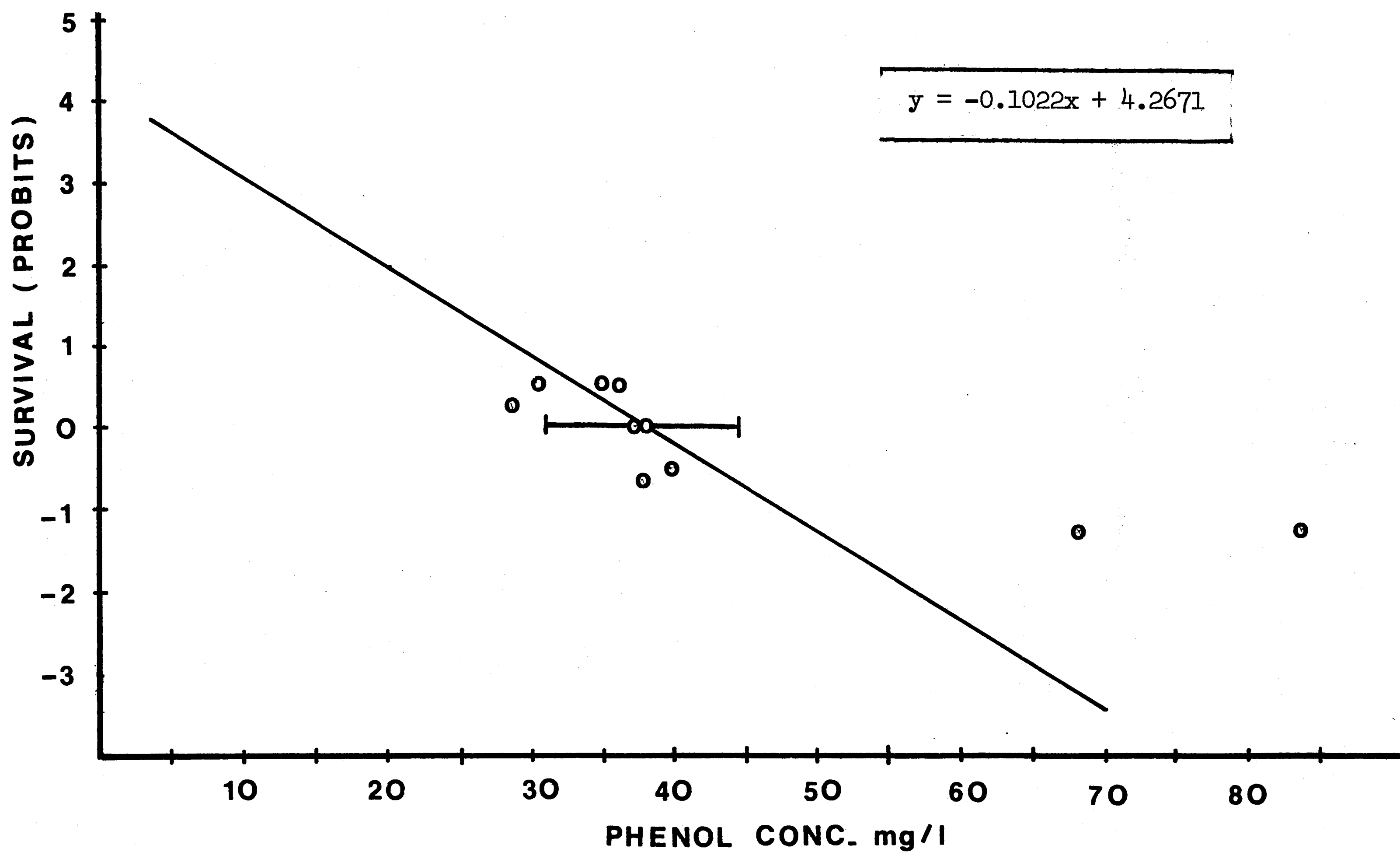

Figure 13. 64 Hr Continuous Flow Bioassay - Regression of Channel Catfish Survival on Phenol Concentrations

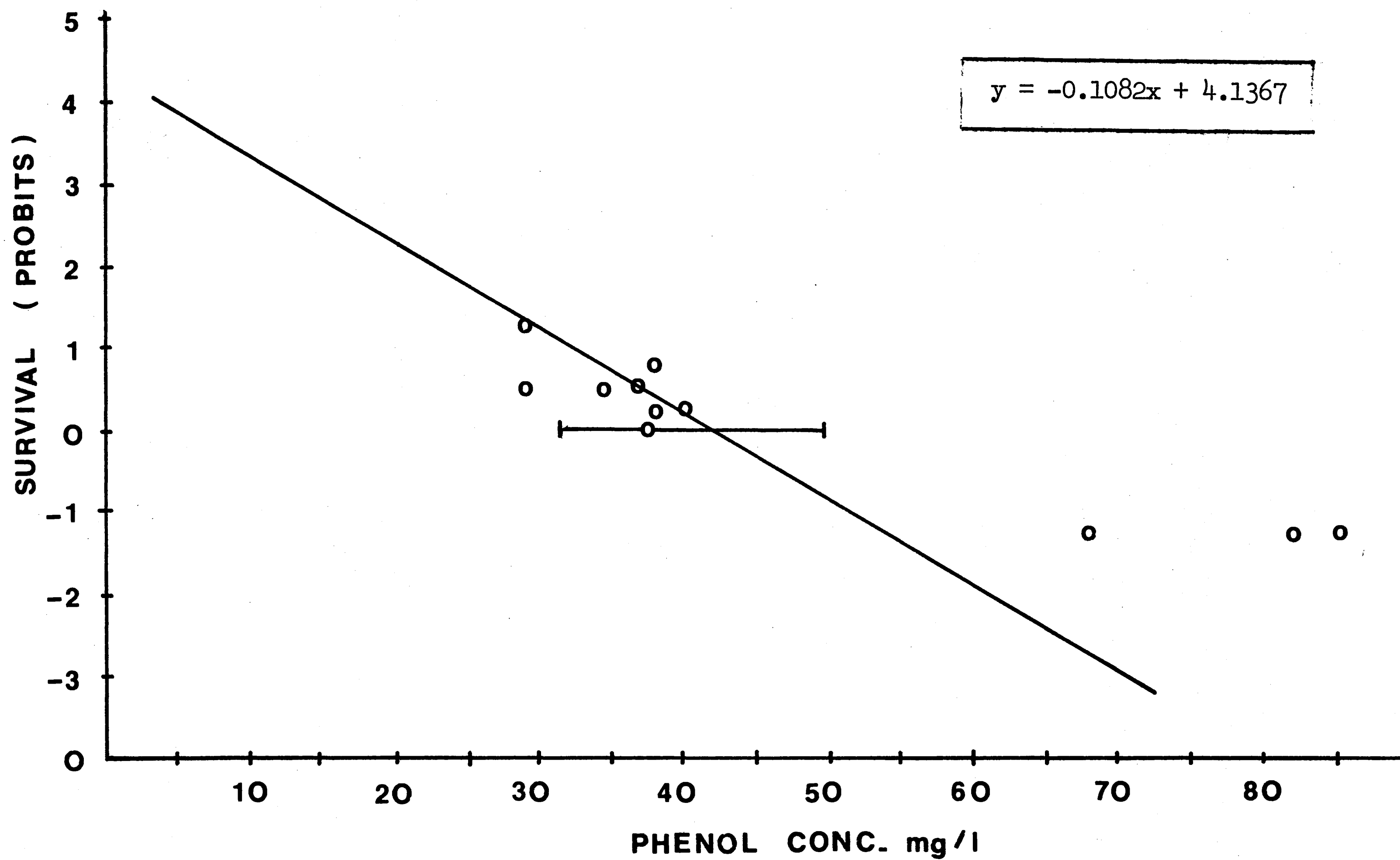

Figure 14. 72 Hr Continuous Flow Bioassay - Regression of Channel Catfish Survival on Phenol Concentrations

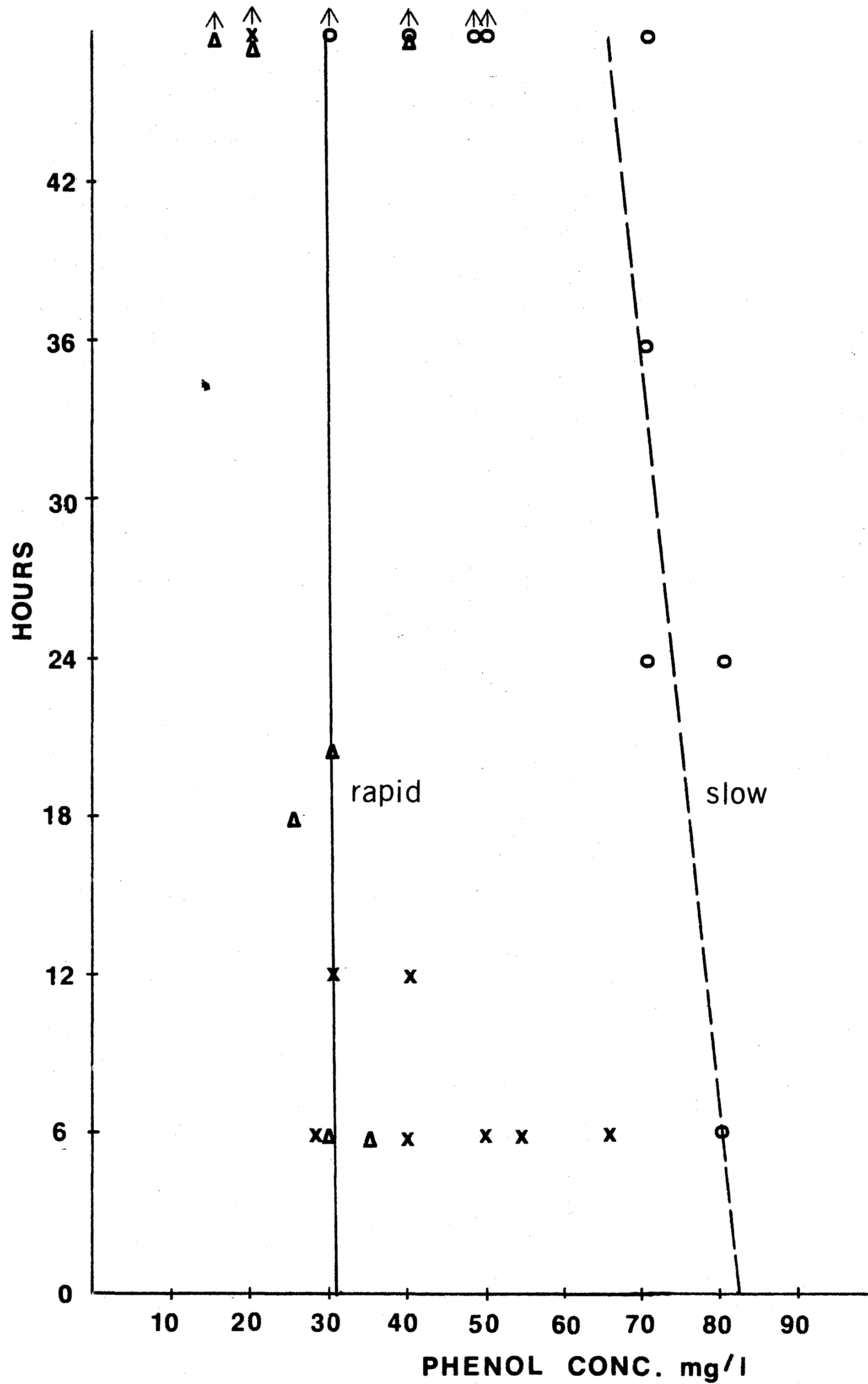

Figure 15. Relationship of Rapid and Slow Introduction on the Toxicity of Phenol to Channel Catfish
Δ = Rapid Introduction (Static Bioassays),
x = Rapid Introduction (Continuous Flow Bioassays),
O = Slow Introduction Bioassays

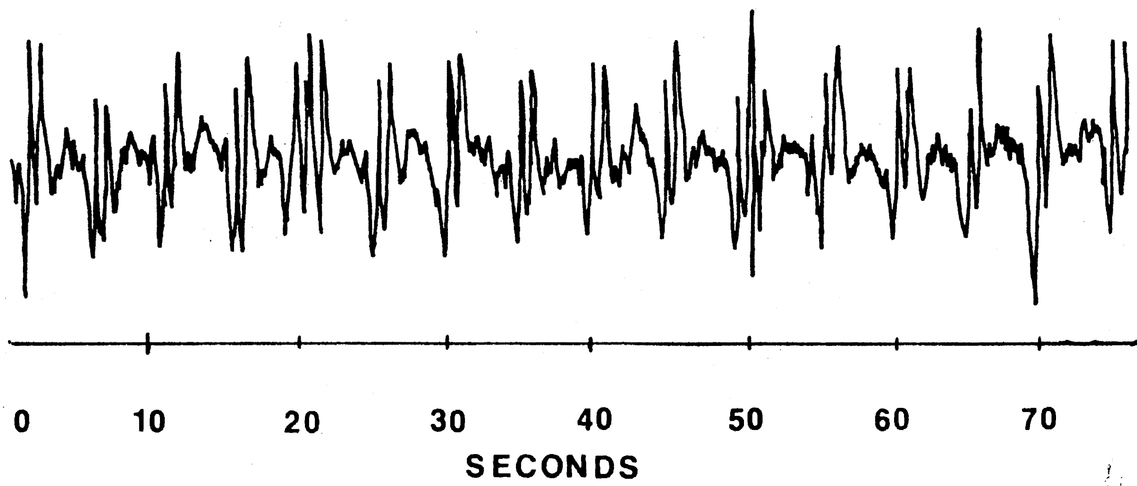

Figure 16. A Typical Tracing of Breathing Pattern in Bluegill Sunfish

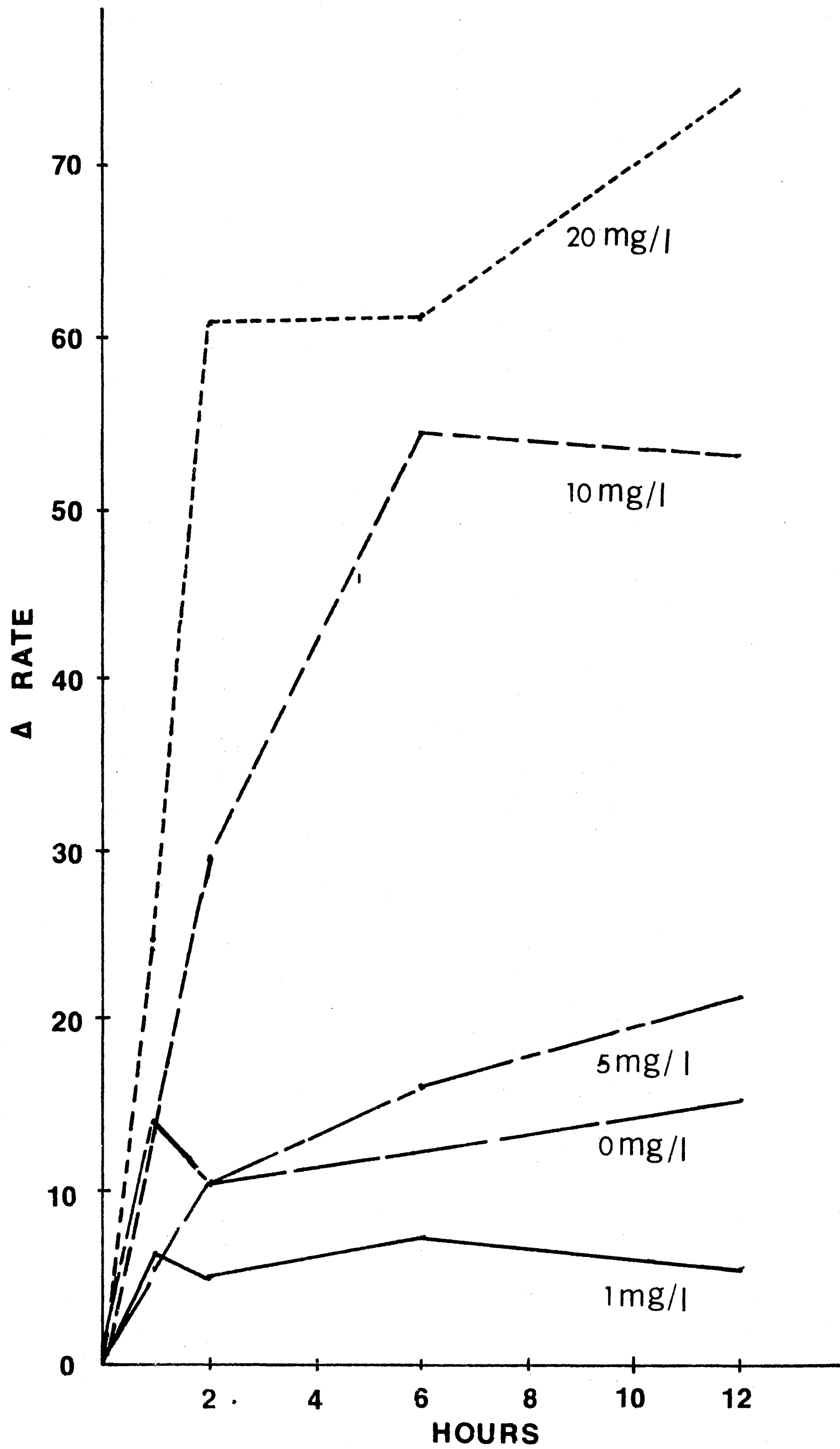

Figure 17. Effect of Phenol Concentrations on Opercle Breathing Rates of Bluegill Sunfish

VITA

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Doctor of Philosophy

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