CHRONIC AND ACUTE EFFECTS OF THE INSECTICIDE TOXAPHENE ON *DAPHNIA PULEX*

BY

JAMES DAVID CHILES

CHRONIC AND ACUTE EFFECTS OF THE INSECTICIDE TOXAPHENE ON *DAPHNIA PULEX*

An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

James David Chiles

August, 1978

ABSTRACT

Daphnia pulex was exposed to concentrations of the insecticide, toxaphene, in acute and chronic toxicity bioassays. In the acute tests, daphnids were placed in concentrations of toxaphene to determine 24, 48, 72, and 96 hour IC_{50} values. The chronic tests involved exposing daphnids to toxaphene over a 28 day period. Numbers of immobilized animals and the number of young produced were recorded daily. Body lengths of adults surviving the 28 day tests were measured.

The concentrations tested in the acute tests ranged from 18 to 180 µg/l and the 28 day concentrations tested ranged from 0.56 to 18.0 µg/l. All tests and controls contained 25 animals. There were three replicates in the acute tests and two replicates in the chronic tests.

The IC_{50} values for the acute tests were 137, 126, and 90 µg/l for the 48, 72, and 96 hour tests, respectively. The concentrations tested were not high enough to observe an IC_{50} value at the 24 hour time period.

The chronic bioassay showed little effect on body length with the only significant observed value being a 4.3 percent reduction of body length in the 18.0 µg/l concentration of the second replicate. Significant reduction of survival was observed, during the second

replicate, for the 18.0 µg/l concentration. There was a 44 percent reduction of survival at that treatment.

Significant reduction in mean number of young per surviving adult was observed in both replicates at all concentrations above 1.6 µg/l. Reductions in mean number of young per surviving adult, of 17.7 and 71.7 percent were observed for the 3.2 and 18.0 µg/l concentrations, respectively.

IC_{50} values reported in this research are higher than those found by investigators working with a soft, synthetic water media. The hardness of the spring water was suspected as the cause for the discrepancy.

The observations show that reproduction is drastically affected at levels of 18.0 µg/l with detectable reproductive impairment occurring at a level of 3.2 µg/l. The 17.7 percent reproductive impairment at the 3.2 µg/l concentration occurred at a level 28 times lower than the 96 hour IC_{50} value and 438 times lower than the 48 hour IC_{50} value.

During this study, reproductive impairment was observed to be a more sensitive measure of toxicity than IC_{50} values. The low dosage effects, as compared to effects observed in the acute tests, indicate the need for more chronic bioassay research. Test conditions such as food source, rate of feeding, and test container size need to be standardized.

CHRONIC AND ACUTE EFFECTS OF THE INSECTICIDE TOXAPHENE ON *DAPHNIA PULEX*

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August, 1978

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To the Graduate Council:

I am submitting herewith a Research Paper written by James David Chiles entitled "Chronic and Acute Effects of the Insecticide Toxaphene on Daphnia pulex." I recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Biology.

Major Professor

We have read this research paper
and recommend its acceptance:

Second Committee Member

Third Committee Member

Accepted for the Council:

Dean of the Graduate School

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

The public awareness of pressing environmental problems has subsided somewhat since the early 1970's. The need for extensive research in the area continues. The development of new, more toxic pesticides and chemical compounds will undoubtedly continue. Aquatic pollutants, both new and old, will occur in the aquatic ecosystem in the future.

It is becoming increasingly apparent that low levels of many of these substances are causing subtle, often unnoticed damage to aquatic organisms.

The variety of aquatic organisms that have been used for bioassay purposes is large, but fish have been the most frequently tested organism. Most of these tests have been short term tests of one to four days. Many investigators have avoided the use of chronic (long-term) toxicity tests because such tests are difficult, expensive and time consuming (Mount and Stephan 1967). Chronic toxicity may ultimately cause death or may result in the elimination of species or individuals over a long period through induced sterility or other factors (Johnson 1968).

Cladocerans are an important part of the aquatic ecosystem.

As small primary consumers, they are important, as food, to a large number of organisms. Various studies of the stomach contents of young fish show from 1 to 95 percent cladocerans by volume and very few studies show less than 10 percent (Pennak 1953). Siefert (1973) and Pflieger (1975) have reported that cladocerans serve as first food for a large variety of freshwater, fry fishes. Because of this, daphnids have been used as bioassay organisms by a large number of investigators, however, most of these studies have been of short duration. Low levels of potentially toxic substances in the environment need to be investigated.

Daphnia pulex as a Bioassay Animal

The advantages of D. pulex are much the same as those of Daphnia magna. Anderson (1944) was probably the first to expound upon the advantages of D. magna. He listed small size, short life span and ease of culture as advantages of primary importance. D. pulex has three or four juvenile instars and 18 to 25 adult instars (Pennak 1953). They have an average life span of 38 days at 20°C (Robertson 1971). They also begin producing young at an age of approximately seven days (Anderson et al. 1937) and grow to a maximum length of 3.8 mm (Pennak 1953).

Daphnids reproduce parthogenetically under favorable environmental conditions in natural waters during most of the year with new broods being produced every few days throughout their lives. During

unfavorable conditions, females produce both males and females which mate and produce ephippial eggs which are resistant to environmental factors such as drying. These eggs hatch under favorable conditions with the new individuals resuming parthogenetic reproduction.

In the laboratory, a continuous supply of young daphnids can be produced parthogenetically if favorable conditions are maintained. Genetic variability may be reduced by using a clone originating from one parthogenetic female.

CHAPTER II

LITERATURE REVIEW

There is extensive literature pertaining to the effect of pollutants on D. pulex and other aquatic organisms. For the purpose of this paper the author will review pertinent research on toxaphene and the effects of pesticides and inorganic substances on fish and aquatic invertebrates.

Characteristics, Uses and Action of Toxaphene

The insecticide investigated consisted of mixed isomers of chlorinated camphene. Von Rumpker et al. (1974) gives the following trade names for the substance: Chlor Chem T-590, Phenacide, Strobane-T and Toxakil. Toxaphene is classed as a broad spectrum insecticide with the main targets being cotton, livestock, small grains, soybeans, corn, and many other crops. Curcubits are the only crop listed by the basic manufacturer, Hercules, Incorporated, to be unsuitable for insect control with toxaphene. When applied to crops, the number of applications is from 1-12 times per season. Livestock is usually treated 1-4 times per season, year around, depending on the pest problems present. It has been used also as a piscicide (fish poison), but is no longer recommended for this purpose due to persistence which has often kept fish from inhabiting waters for years (Hughes and Lee 1973).

According to Guyer (1971), the commercial production of toxaphene consists of the reaction between camphene and chlorine activated by ultraviolet irradiation. Certain catalysts are used to yield a final product of 67-69 percent and an empirical formula of $C_{10}H_{10}Cl_8$. The chlorine content in this product is limited to 67-69 percent since insecticidal activity peaks in that band (Guyer 1971). Gas chromatography suggests that 30 or 40 principle constituents may exist (Guyer 1971). Turner et al. (1975) have shown that two separate fractions of toxaphene are six times more toxic to mice and two fold more toxic to houseflies.

Toxaphene is an amber-colored insecticide, is relatively stable and has a mild terpene odor. Hercules, Incorporated lists the solubility in water at 3 ppm, however, several investigators have found it to be much lower. Cohen et al. (1960) lists its solubility at 0.4 ppm and Guyer (1971) lists its solubility at 0.5 ppm.

Toxaphene was introduced around 1959 and since that time over a billion pounds have been applied to crops and livestock for pest control. Its use is continuing at the rate of 40 million pounds per year (Casida et al. 1974). From 1964-1974, it was used in greater quantities than any other insecticide (von Rumpker et al. 1974). In 1972, 58 million pounds was the estimated usage in the United States with 51.5 million pounds of this usage being used in the south-central and southeastern United States (von Rumpker et al. 1974). Presently, the most important use is in the control of cotton insects, usually in combination

with methyl parathion. Its level of use is increasing, mainly because of the phasing out of DDT and increased use on cotton, soybean and other field crops. The non-farm uses are small (von Rumpker et al. 1974).

Toxaphene is a broad-spectrum insecticide and is both a contact and a stomach poison. It interacts with the central nervous system and is moderately toxic with low persistence compared to DDT (von Rumpker et al. 1974). Casida et al. (1974) describe it as a complex, biodegradable mixture which can be detoxified to a certain extent by mammals. With the exception of endrin, toxaphene is one of the most lethal of the chlorinated hydrocarbon pesticides to fish on an acute basis (Henderson et al. 1958).

Toxaphene Toxicity Tests

Guyer (1971), in an EPA status report on toxaphene, lists numerous 48 hour LC_{50} values (the concentration lethal to 50 percent of the animals tested) for fish. For 13 species of fish, the LC_{50} values range from 0.0051 ppm for largemouth bass, Micropterus salmoides, to 0.018 ppm for yellow perch, Perca flarescens and the sunfish Lepomis cyanellus. The authors mention that these values are variable depending upon pH, oxygen concentration and water hardness. Henderson et al. (1958) list TL_m values (the value at which half of the test animals survive) for bluegill, Lepomis machrochirus as 0.0075 ppm, 0.0038 ppm and 0.0035 ppm for 24, 48 and 96 hour values, respectively.

Cairns et al. (1975) found that toxaphene was more poisonous to fishes at higher temperature.

Mehrle and Mayer (1975), in a study of the effects of toxaphene on growth and development of brook trout, Salvelinus fontinalis, found that the hatchability of trout was not affected by concentrations of 520 ng/l of toxaphene but young died 30-60 days after hatching. They found that levels from 139-288 ng/l significantly depressed growth of the trout.

Johnson (1968), in a review article on pesticides and fishes, lists 96 hour LC_{50} values of 0.0035 ppm for bluegill and 0.0056 ppm for goldfish. A 24 hour value for rainbow trout is given also as 0.050 ppm.

Skidmore (1964) reviewed the literature on toxicity of zinc to aquatic organisms with special reference to fish. He reported that pH was the most important single factor in determining zinc toxicity to fish. He also found a ten fold decrease in toxicity of zinc to fish with an increase from 12-320 ppm of total water hardness at 2.5 days of exposure. Oxygen concentration had a smaller effect with a 1.4 times greater mortality resulting from a decrease of 8.9 to 3.8 ppm of oxygen. Skidmore also mentioned the importance of minimizing the ratio of biomass to the mass of available poison.

Burdick (1967), in another review article, discussed the use of bioassays in determining levels of toxic wastes harmful to aquatic organisms. He recommended that long term bioassays should be

conducted only in a natural water known to be able to support fish and other aquatic biota over extended periods of time.

Johnson (1968) selectively reviewed the literature concerning fishes and pesticides. He suggested research programs necessary for the detection of sublethal damage to a population. He stressed the need for chronic studies.

Lee (1973) reviewed the chemical aspects of bioassay techniques for establishing water quality criteria. He discussed examples in which changes in the chemical environment of the bioassay test may affect the results and recommended procedures for minimizing problems of this type.

The influence of temperature on chemical toxicity to aquatic organisms was reviewed by Cairns et al. (1975). They stressed the fact that, although investigators often generalize that temperature rises always increase toxicity, other factors may make this assumption false. They list changes in detoxification and increased excretory rates as factors which may reduce the temperature effects on toxin uptake in aquatic organisms.

Factors Affecting Toxicity of Substances to Fish

The most often mentioned factors affecting toxicity in the literature are pH and water hardness. Solbe (1974) found that survival time of trout in 7 mg/l of zinc decreased three times with a decreased water hardness from 504 to 12 ppm. Mount (1966) found

that in a continuous flow toxicity study with fish that zinc was most toxic at a pH of eight and a hardness of 50 ppm and least toxic at a pH of six and a hardness of 200 ppm.

Johnson (1968) found that a pH change from 6.9-8.0 increased the LC_{50} value for rainbow trout five fold when working with the herbicide 2-secondary-butyl-4, 5-dinitrophenol.

One other factor concerning bioassays in the laboratory as opposed to actual aquatic environments was discussed by Lee (1973). He mentions that the lab bioassay may have little significance in relation to the "in situ" situation. Bridges (1961) supports this with the finding that levels of 40 ppb endrin in natural waters did not result in fish kills. Lab bioassays have shown that endrin kills fish at much lower levels (Johnson 1968).

Reviews of Studies Using Daphnids

Blaska (1941) was one of the early investigators of the effects of toxicants on daphnids. He found D. magna and D. pulex more sensitive to $CuSO_4$, $FeSO_4$, and $ZnCl_2$ than were two species of copepods and one species of dipteran larvae.

Anderson (1944) worked with the effects of various industrial wastes on D. magna. He was the first to propose the cessation of swimming (immobilization) as an indicator of the toxic effects of substances. Frear and Boyd (1967) used 12 insecticides in bioassays with D. magna and found that the concentration of the auxilary solvent,

acetone, had a pronounced effect on the response of daphnids.

Zeiss (1963) in a study of the effects of crowding on the copepod Calanus and D. magna found that there was a three fold increase in respiratory rates of D. magna when exposed to a 12 ml per animal concentration as compared to a 0.12 ml per animal concentration. Zeiss used sheep manure as the food source and pond water as the culture medium.

Extensive research on the effects of 86 chemical compounds to nine species of aquatic animals, including D. magna was reported by Dowden and Bennet (1965). Using three different media, their work clearly showed that chemical toxicity to D. magna has high variability in different media. Their study employed one natural water source and two synthetic waters. The amount of the chemical substances required to show a TL_m effect was generally less in the synthetic waters.

Sanders and Cope (1966) worked with numerous insecticides and herbicides and their effects on Simocephalus serrulatus and D. pulex. They found 48 hour EC_{50} values (concentration needed to immobilize 50 percent of the test animals) for S. serrulatus in toxaphene to be 19 ppb and 10 ppb at 60° and $70^{\circ}F$ respectively. A value of 15 ppb was reported for D. pulex at $60^{\circ}F$. Sanders and Cope (1966) used reconstituted water that consisted of 30 mg $MgSO_4$, 48 mg $NaHCO_3$, and 2 mg KCl per liter of deionized water. The pH ranged from 7.4-7.8 in their test media. They found that the elimination of any of these

chemical substances made the media more toxic. A 24 hour median dose value for toxaphene to D. magna was reported by Crosby and Tucker (1966) as 260 µg/l.

Frear and Boyd (1967) reported a 24 hour LD_{50} value (the dose lethal to 50 percent of the test animals) of 94 ppb for D. magna in a toxaphene bioassay. They used a standard test medium consisting of 200 mg $NaHCO_3$, 224 mg $CaCl_2$, and 26 mg K_2SO_4 per liter of distilled water. Their tests were conducted at 19°C and the dissolving solvent was ethanol.

Findley (1969) extensively investigated the effects of the herbicide amino triazole on selected aspects of the life history of D. magna. She found IC_{50} values for adults at 120 hours and 16 days, of 40.65 ppm and 2.20 ppm respectively. In contrast to this, values of 53.50 and 1.12 were found at 24 hours and 5 days respectively for animals that were 2 to 26 hours old at the initiation of the test. A total inhibition of reproduction was recorded at 3.5 ppm. At 2 ppm, 33.3 percent of the adult daphnids lost their eggs.

Biesinger and Christensen (1972) conducted a variety of tests on the effects of various metals on D. pulex. They found that fed animals in 48 hour LC_{50} tests, with 18 metals, had higher LC_{50} values, in all cases, than did unfed animals. They performed a 3 week chronic test on D. pulex and observed that a 16 percent, 3 week reproductive impairment level for cadmium was only 3.4 percent of that required for a 48 hour LC_{50} value. In the 3 week studies, they observed

reproductive impairment to be a more sensitive measure of toxicity than survival.

Rezba (1972) attempted reproduction studies with D. magna and 2,4-D herbicide in Frear and Boyd's medium. The data from this study could not be evaluated due to too many conflicting significant and non-significant values.

Biesinger et al. (1973) in a 3 week study of the effects of nitriloacetate (NTA) on D. magna found a ten fold decrease in toxicity in hard water. They also reported a strong negative correlation between LC_{50} values and water hardness. Tests were run with Lake Superior water and two concentrations of artificially hardened Lake Superior water.

Sherr and Armitage (1973) worked with D. pulex and the toxicity of dichromate ions. They found a zero percent survival at 1.0 mg/l in 24 hours. They used Chlamydomonas moewusii as the food source for the daphnids and found difficulty in culturing enough daphnids for the tests.

Nebeker and Puglisi (1974) used a food source of wheat leaves and trout chow in a bioassay with D. magna and polychlorinated biphenols. They found a 3 week LC_{50} value of 25 µg/l and a 50 percent reproductive impairment at 24 µg/l.

Goss and Bunting (1976) used trout chow and alfalfa leaves as food in a study of the thermal effects on D. magna and D. pulex. They found that D. pulex was much less succeptible to changes of water

temperature than was D. magna.

Objectives of the Study

Since the ban of the agricultural use of DDT in 1973, toxaphene use has increased (Casida et al. 1974), with an estimated 40 million pounds being used yearly. Due to widespread use, the insecticide frequently occurs in the aquatic ecosystem. Drift from aerial applications and other uses is so widespread that the insecticide has been recovered from the atmosphere near Bermuda (Bidleman and Olney 1975). Fifty six samples averaged 0.63 ng/cubic meter. Nicholson et al. (1964) have listed toxaphene as the most common water contaminant of northern Alabama. Nicholson et al. (1964) also observed many fish kills that were directly related to toxaphene toxicity. Due to the relatively low bio-magnification and low cost, the use of toxaphene will probably continue to increase (von Rumpker et al. 1974).

Since these conditions exist, there is a great need for chronic toxicity studies. According to Brooks (1957) and Pennak (1953), D. pulex ranges over most of the United States. Due to the widespread application of toxaphene, the potential for damage is great. Since chronic and sublethal toxicity of many elements and compounds occurs at levels which may sometimes be more than 100 times less than the concentration needed for acute lethal effects of the same organism (Lee 1973), it is desirable to determine the chronic level at which toxaphene significantly affects reproduction, growth and life span.

For the purpose of this investigation, the following terms will be used as defined by Sprague (1969).

acute--coming speedily to a crisis

chronic--continuing for a long time, lingering (may be lethal or non lethal)

sublethal--below the level which directly causes death

cummulative--brought about, or increased in strength by successive additions

This investigation will attempt to show the effects of technical toxaphene on growth, longivity and reproduction in D. pulex. Since static bioassays of 24-96 hour duration are routinely done with all potential pollution causing pesticides, acute tests of 24 to 96 hour duration will also be conducted for comparison purposes with the 28 day bioassays. This will allow the comparison of this research with other reports in the literature.

CHAPTER III

METHODS AND MATERIALS

General

The organism used for the bioassay was Daphnia pulex. The original culture was purchased from the MacMillan Science Company and was maintained for approximately two months before any were used for testing. The stock cultures were maintained in 1-gallon, wide-mouth jars at $20^{\circ}C \pm 1^{\circ}$ in a Percival Incubator with an automatically controlled, 12 hour photoperiod. Light was supplied by a single 40 watt fluorescent bulb over the test animals. Additional light was received from lights over the other shelves in the incubator. The daphnids were fed a suspension consisting of Purina Trout Chow and Fleischmann's activated, dried yeast. The spring water in these cultures was changed weekly. The jars were covered with small plates of Lexan transparent material to reduce evaporation and to keep out extraneous material.

Test Water

The water used in all tests and for growing the test animals was filtered spring water. The spring is located in Christian County, Kentucky at an altitude between 570-580 feet according to the Casky quadrangle U. S. topographic map. The spring has approximately 1/2

acre of land surrounding it from which precipitation runoff can occur. The spring forms a pool approximately 30 feet in diameter and occurs in a limestone formation. The flow from this spring is great enough to provide a continuous flow from the pool year around.

The spring water was collected in a 3 gallon polyethylene bucket and poured into a 6 gallon polyethylene storage jar. Water in the jar was continuously aerated with compressed air and stored in the laboratory until needed.

Chemical analysis of aerated spring water was performed three times at one week intervals using procedures outlined in the 8th edition of the Hach Chemical Company manual for use with a Hach DR-EL direct-reading portable kit. Total alkalinity, calcium hardness, total hardness, nitrate nitrogen, phosphate (meta), and phosphate (para) were measured. The pH of the spring water was monitored with a Leeds Northrup pH meter. The results of these tests are listed in Table I of the results.

Before use, the spring water was filtered through cotton cloth containing 10,816 squares per square inch to remove any pieces of debris which might have been introduced during the collection of the water at the spring.

Insecticide

A sample of purified, technical toxaphene was purchased from Chem Service, Inc., P. O. Box 194, West Chester, Pa. 19380. It

TABLE I

Chemical Analysis of Aerated Spring Water

Analysis	Method	Mean (ppm)	Range (ppm)
Alkalinity (total)	Methyl orange	162.2	143.3-190
Calcium hardness	EDTA-complex	185	163.3-210
Hardness (total)	EDTA-complex	194.5	175.0-221.7
Nitrate nitrogen	Cadmium reduction	0.034	0.022-0.05
Phosphate (meta)	Stannaver with solutions	2.36	2.33-2.42
Phosphate (ortho)	Stannaver with solutions	0.172	0.037-0.40
pH [a]			

[a] The pH of the spring water was monitored with a Leeds Northrup pH meter and was found to range from 7.3-8.2. All other analyses were performed with a DR-EL Hach Kit.

consisted of four 1-gram samples of the waxy solid sealed in individual bottles. One gram was sufficient for running all of the tests reported in this paper. A stock solution was prepared approximately one hour before use. Ten milligrams were dissolved in 3 ml of 95% ethanol Three tenths ml of the stock solution were added to one liter of spring water to make a stock solution of 1 mg per liter. From this stock solution, the other concentrations were prepared.

Due to using the solvent, ethanol, a second control was used in all tests. This control contained the maximum amount of ethanol contained in the highest toxaphene concentration. The usual control differed from the test solutions only by the lack of toxaphene and ethanol. Earlier attempts by this investigator, using acetone, showed it to be more toxic than the toxaphene concentrations that it contained. Dowden and Bennett (1965), in a study with Daphnia magna found it to be toxic to 50 percent of the animals in 24 hours at a concentration of 10 mg/l. Several investigators have used acetone in insecticide bioassays and failed to report its effects.

Food

Each gallon jar of daphnids was fed at three day intervals between 1600 and 1900 hours. The food source was a modification of one used by Biesinger and Christensen (1972). In this study, food was prepared in the same manner, except with the deletion of the dried grass and the use of filtered spring water. Ten grams of

Purina Trout Chow were mixed at high speed for 5 minutes with 250 ml of filtered spring water with a blender. An additional 50 ml of water were used for rinsing the blender and were added to the suspension which was then filtered through number 20 bolting cloth and stored in the refrigerator at 4°C.

An additional solution of activated, dried yeast was prepared on feeding days by suspending 0.1 g of yeast in 100 ml of filtered spring water. This was fed to the stock cultures at the rate of 3 ml per gallon jar. The trout chow suspension was fed to the daphnids at the same time and at the same rate. The use of the dried yeast eliminated some of the variability of using grass of unspecified nature.

The toxicity tests required a different food dilution. One drop of the trout chow suspension proved to reduce longevity of the daphnids in the test containers, probably by depleting the oxygen supply. A dilution of 5 ml of trout chow suspension and 0.1 g of dried yeast to 100 ml of spring water was found to be satisfactory. This was fed to the test animals at 48 hour intervals, at the time the animals were counted, at a rate of one drop per shell vial. The shell vials contained 25 ml of solution. In addition to the food mixture, the daphnids may have obtained some nutrients from bacteria, algae and detritus in the spring water.

Glassware and Equipment

All glassware used for cultures and preparation of test solutions

was scrubbed in detergent solutions and rinsed a minimum of three times in tap water. The glassware was then soaked in 10 percent hydrochloric acid for a minimum of 5 minutes and rinsed five times with distilled water. All polyethylene material and cloth were washed in detergent, rinsed in tap water three times, and rinsed five times with distilled water. Materials other than glassware were not soaked in acid.

Collection of the Test Animals

Twenty four hours before young were needed for testing they were separated from the adults. A device for separation had been prepared by removing the bottom of a 1/2 gallon polyethylene jar and covering the mouth with number 30 nylon sieve material having openings of 0.595 mm. The material was held in place with a metal screw top lid that had the center removed to within 1 cm of the edge. The sieve material was fixed to the inside of this lid with silicone glue.

With the lid in place, the cultures were filtered to separate the young from the adults. The young passed through the sieve material with the spring water. The daphnids remaining on the sieve at the conclusion of filtering were gently rinsed with spring water to remove any young that might have been entangled with the adults. The sieve was then inverted over a gallon jar of fresh water and rinsed again to remove the adults. The whole process took less than 1 minute, with the daphnids showing little adverse effects from remaining on the sieve.

The young daphnids, in old spring water, were returned to a gallon jar and fed. Debris at the bottom of the jar was removed with a large pipette after feeding. The adults were also fed, cleaned and returned to the incubator.

At the end of the 24 hours, the same procedure was followed to separate the young and the adults. The spring water containing the 0-24 hour young was then filtered through the filtration device which had a piece of cotton cloth containing 10,816 squares per square inch over the sieve. The level of the spring water was kept above the cloth by keeping the separation device in a polyethylene bucket. The young in the device were removed with a rubber-bulbed pipette and placed in a 1 liter beaker for randomization. Using this method, the young were never exposed to the air. The young from all the cultures were allowed to randomize for 30 minutes before use. They were then placed in 400 ml beakers containing 100 ml of the appropriate test solutions for washing before placing in the appropriate test vials with pasteur pipettes with the tips broken off.

Immobilization Tests

Ninety, six hour immobilization tests were performed on young which were 1-25 hours old. The concentrations investigated ranged from 18 to 180 micrograms per liter. The tests were conducted in 8 dram, flat bottom, 25 by 95 mm shell vials containing 25 ml of the test solution. The test vials were held in wooden racks with holes drilled

1.5 inches into the block. Each rack was used to hold 25 shell vials.

Immobilization was used, rather than death, to eliminate the need for microscopic examination of the animals. If an individual daphnid was lying at the bottom of the vial and could stroke her antenna a few times, she was considered alive.

The daphnids were fed when they were placed in the vials and at the time of counting on the second day of testing. Sheets of 1/8 inch Lexan transparent material were used to cover the shell vials.

Growth, Reproduction and Survival Tests

D. pulex was exposed to concentrations to toxaphene ranging from 0.56 to 18.0 µg/l for 28 days. Test solutions were replaced at four day intervals at which time all young were discarded and living adults were transferred by pasteur pipette to the fresh solutions. The test daphnids were fed one drop of the combination food mixture at 2 day intervals at the conclusion of daily observation of the animals.

On the 28th day, the animals were counted in the usual manner and the surviving adults were transferred to vials containing 95 percent ethanol for subsequent measurement. All animals were measured within 1 week using a Carl Zeiss Standard W L Research microscope with image splitting eyepiece and ocular micrometer. The length was measured according to Anderson et al. (1937) and was the longest dimension exclusive of the spine.

CHAPTER IV

RESULTS

Twenty Four to Ninety Six Hour IC_{50} Tests

There were 25 daphnids in each concentration in each replicate during the IC_{50} tests. The concentrations tested were 18, 32, 56, 100 and 180 µg/l. Immobilizations were recorded at 24 hour intervals for 96 hours. The results of those tests are shown in Figure 1. The data were adjusted so that natural immobilizations are considered in the values. The data for each similar concentration were combined for the three replicates. The combined data were tested for significance against the control by the chi-square method with values shown in Table II. All values which determined the slope of the lines in Figure 1 were significant at the 5 percent level. The data were plotted on semi-logarithmic paper with the dosages plotted logarithmically on the abscissa and the survival percentages on the ordinate scale arithmetically. This method is known as straight-line graphical interpolation (Doudoroff 1951). This method is recommended in Standard Methods for the Examination of Water and Waste Water (1971) and has been used by Frear and Boyd (1967), Sprague (1964), and Rezba (1972). The IC_{50} values were determined from the intersection of the percent immobilization line with the time line by interpolating to the concentration axis.

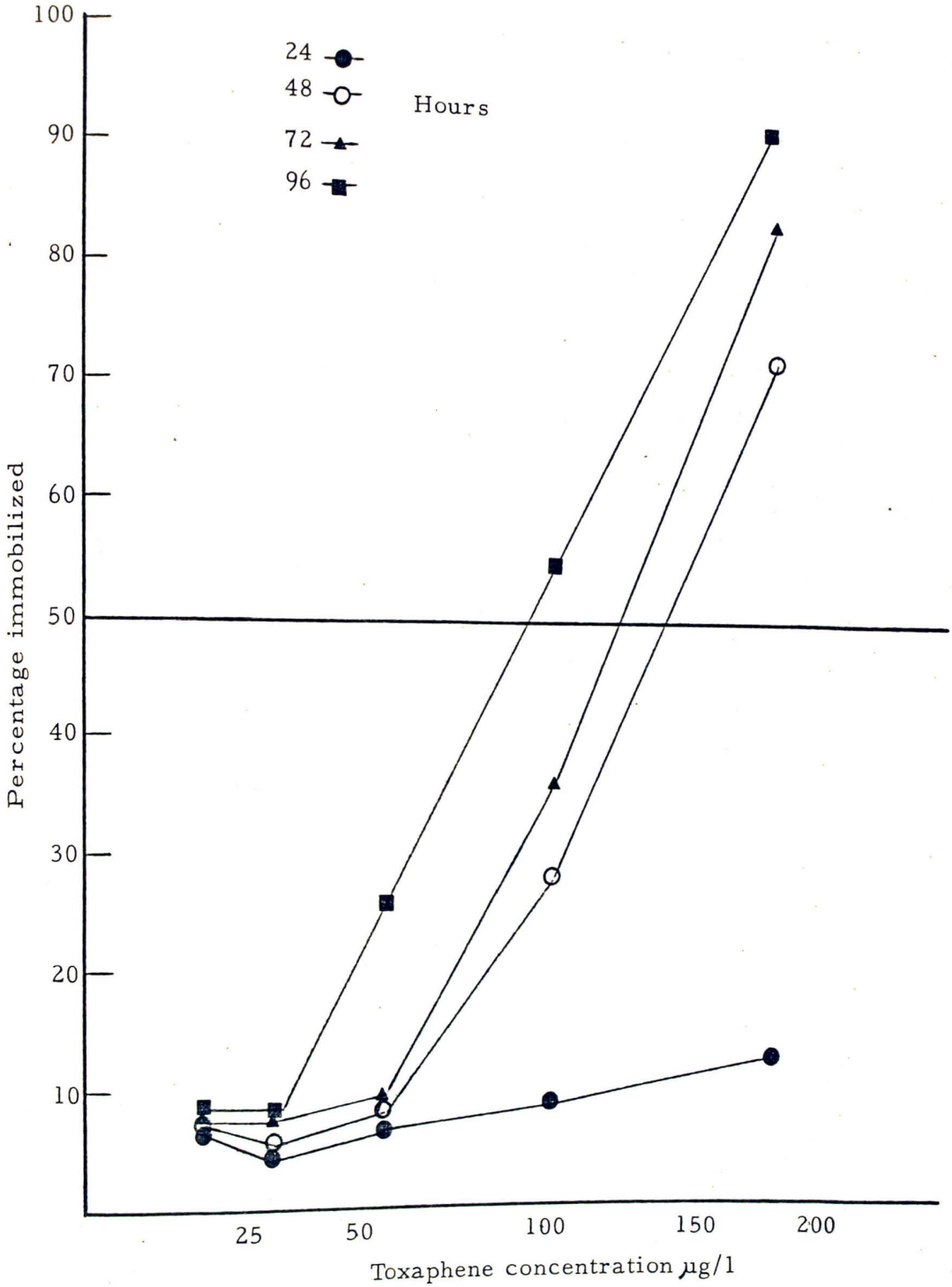

Figure 1. Combined replicate immobilization rates for D. pulex. There were three replicates with 25 animals at each concentration.

TABLE II

Chi-Square Values for the Immobilization Rates Found in Figure 1

Micrograms/ Liter Toxaphene	Time in Hours			
	24	48	72	96
18	1.349	2.453	2.453	4.133[a]
32	1.325	1.714	2.452	3.260
56	2.955	3.261	4.127[a]	18.610[b]
100	3.857[a]	19.919[b]	28.322[b]	52.086[b]
180	6.805[a]	81.000[b]	110.425[b]	130.989[b]

[a]Significant at the .05 level from the control.

[b]Significant at the .01 level from the control.

There were 75 animals at all concentrations.

The IC_{50} values observed during the 24 hour intervals were 137, 126, and 90 µg/l for the 48, 72, and 96 hour times respectively. The concentrations tested were not high enough to observe an IC_{50} value at the 24 hour time period and should be over 180 µg/l.

General: Growth, Reproduction, and Survival Tests

Daphnids, 1 to 25 hours old, were introduced to 8-dram shell vials containing the toxaphene solutions. The solutions were changed at four day intervals for 28 days. The concentrations tested were 0.56, 1.8, 3.2, 5.6, 10.0 and 18.0 µg/l with 25 daphnids in each concentration. There were two replicates. Immobilized individuals and young produced were recorded daily. Surviving adults at each concentration were measured according to Anderson et al. (1937). The summarized results of the 28 day tests are shown in Tables III and IV.

Growth Tests

Body length measurements for daphnids in concentrations of toxaphene were tested for significance using Duncan's Multiple Range. Tables III and IV show the mean body lengths and standard deviations for the first and second 28 day replicates. The mean body lengths of the first replicate test animals were not significantly different at the 5 percent level from the control. In the second replicate the mean body lengths ranged from 2.04 ± 0.04 mm for the 18.0 µg/l concentration to 2.13 ± 0.04 mm for the 0.56 µg/l concentration. The mean body length for the 18.0 µg/l concentration represented a 4.3 percent decrease in

TABLE III

Summarized Results of 1st Replicate, 28 Day
Toxaphene Toxicity to Daphnia pulex

Characteristics	Concentration in µg/l 0.00	0.56	1.8	3.2	5.6	10.0	18.0
Surviving Adult Animals	17	16	16	16	14	16	15
Percent Immobilized	32	36	36	36	44	36	40
Number of Young	1289	1181	1159	1007	703	754	356
Number of Young per Surviving Adult	75.6	73.8	72.4	60.5[b]	49.2[ab]	45.1[b]	22.5[b]
Mean Body Lengths and SD (mm)	2.12 ±0.08	2.12 ±0.06	2.11 ±0.08	2.10 ±0.05	2.10 ±0.06	2.11 ±0.05	2.09 ±0.04

Underlined results are not significantly different at the .05 level.

[a]Not significant at the .05 level from the results of the replicates.

[b]Significant at the .05 level from the control of this replicate.

TABLE IV

Summarized Results of the 2nd Replicate, 28 Day
Toxaphene Toxicity to Daphnia pulex

Characteristics	Concentration in µg/l 0.00	0.56	1.8	3.2	5.6	10.0	18.0
Surviving Adult Animals	24	21	22	24	22	20	13
Percent Immobilized	4	16	12	4	12	20	48
Number of Young	1398	1459	1243	1175	1055	593	293
Number of Young per Surviving Adult	56.8	60.2	55.5	48.1[b]	46.4[ab]	25.9[b]	15.2[b]
Mean Body Lengths and SD (mm)	2.13 ±0.04	2.13 ±0.04	2.10[b] ±0.04	2.10[b] ±0.04	2.08[b] ±0.04	2.09[b] ±0.06	2.04[b] ±0.04

Underlined results are not significantly different at the .05 level.

[a]Not significant at the .05 level from the results of the replicates.

[b]Significant at the .05 level from the control of this replicate.

body length when compared to the control of that replicate.

Duncan's Multiple Range was also used to test for significance between replicate treatments. No significant differences for mean body lengths for animals exposed to experimental treatments were observed for the first and second replicate series. Therefore, the mean body length for the animals exposed to various concentrations of toxaphene was fairly consistent between replicates.

Reproduction Tests

The total number of young produced per treatment is listed in Tables III and IV. There were 1289 young produced in the control as compared to a range from 1181 to 356 for the 0.56 to 18.0 µg/l toxaphene concentrations. The second replicate showed a wider range in number of young between the control and the highest concentration. The control daphnids produced 1398 young with 293 and 1459 young being produced by the adults in the 18.0 and 0.56 µg/l concentrations, respectively. There was a regressive value at 10.0 µg/l in the first replicate, possibly due to higher adult survival rates at that treatment.

The combined replicate values are shown in Figure 2. This clearly shows the progressive reproductive impairment of the insecticide to *D. pulex*.

The adult daphnids in the second replicate, 0.56 µg/l concentration, produced more young than the control, however the difference was not significant. Table V shows the percentage impairment of the

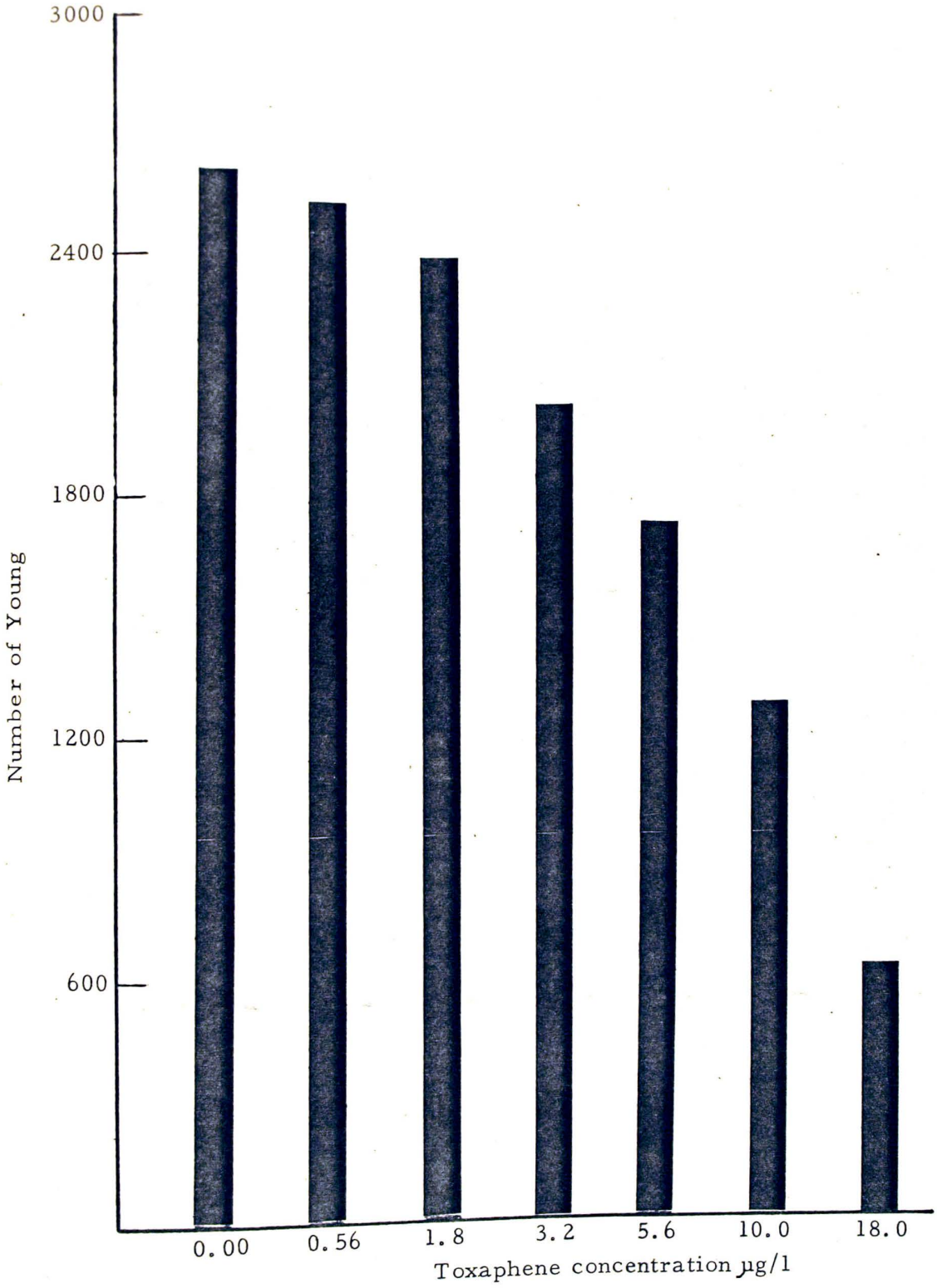

Figure 2. Combined number of young produced for two 28 day chronic toxicity tests. Young were discarded at 4 day intervals.

TABLE V

Percentage Reproductive Impairment
of D. pulex in Toxaphene

	Treatment in µg/l					
Replicate	0.56	1.8	3.2	5.6	10.0	18.0
1st	8.4	10.0	21.9	45.5	41.5	72.4
2nd	4.0	11.1	16.0	24.5	57.6	79.1
Replicates Combined	6.2	10.6	19.0	35.0	49.6	75.8

Each replicate control and all test treatments began with 25 daphnia.

Numbers of young, per treatment, used in calculating the reproductive impairment are shown in Tables III and IV.

total number of young for the separate and combined replicates. The first replicate percentage reproductive impairment ranged from 8.4 to 72.4 percent for the 0.56 and 18.0 µg/l concentrations respectively. The second replicate ranged from 4.0 to 79.1 percent for comparable tests in the second replicate. A 49.6 percent reproductive impairment value was observed for the 10.0 µg/l concentration of the combined replicates.

The mean number of young per surviving adult and significance for the 28 day tests are given in Tables III and IV. The mean number of young in the first replicate was significantly different at the 5 percent level from comparable tests of the second replicate except for the 5.6 µg/l concentration. This indicates varability in reproductive rates of animals from one experimental time period to another. Mean number of young produced in the first replicate ranged from 75.6 in the control to 22.5 in the 18.0 µg/l concentration. The range in the second replicate was from 56.8 young in the control to 15.2 young in the 18.0 µg/l concentration. Although the mean number of young differed considerably, the percentage impairment did not. At the 18.0 µg/l concentration, there were 70.2 and 73.2 percent reproductive impairments for the two replicates. The close agreement between the two replicates indicates the regular nature of the toxicant to reproduction.

Mean numbers of young per treatment and reproductive impairment for the various treatments at 4 day intervals are shown in Tables VI and VII. The mean reproductive impairment for the first three

TABLE VI

Number of Young per Adult per Treatment per 4 Day Interval
with Percentage Impairment or Stimulation
1st Replicate

Treatment	Day of Observation 8		12		16		20		24		28	
	#	%	#	%	#	%	#	%	#	%	#	%
Control	2.17	00	18.44	00	15.88	00	11.06	00	13.47	00	12.94	00
0.56 µg/l	3.00	38	21.13	15	15.44	3	8.75	21	11.81	12	12.38	4
1.8 µg/l	2.55	18	19.31	5	14.19	11	11.50	4	9.94	26	13.38	3
3.2 µg/l	2.55	18	14.28	23	13.59	14	8.18	26	11.19	17	9.13	29
5.6 µg/l	1.09	50	15.67	15	6.80	57	7.36	33	8.14	40	9.57	26
10.0 µg/l	1.09	50	12.00	35	6.94	56	7.25	34	7.44	45	9.63	26
18.0 µg/l	0.62	71	5.71	69	3.41	79	1.76	84	3.59	73	6.47	50

The controls and treatments began with 25 animals.

TABLE VII

Number of Young per Adult per Treatment per 4 Day Interval
with Percentage Impairment or Stimulation
2nd Replicate

Treatment	Day of Observation 8		12		16		20		24		28	
	#	%	#	%	#	%	#	%	#	%	#	%
Control	14.16	00	7.44	00	11.08	00	9.28	00	6.48	00	7.79	00
0.56 µg/l	14.16	00	7.52	1	11.52	4	9.75	5	7.20	11	9.68	24
1.8 µg/l	12.96	8	6.67	10	11.46	3	7.21	22	5.83	10	8.73	12
3.2 µg/l	10.80	24	7.04	5	9.00	19	7.60	18	6.82	5	6.83	12
5.6 µg/l	9.20	35	7.48	1	8.48	23	7.26	22	5.17	20	7.73	1
10.0 µg/l	6.83	52	3.67	51	6.50	41	3.55	62	2.18	66	2.81	64
18.0 µg/l	3.22	77	2.14	71	4.74	43	1.65	82	1.50	77	1.53	80

The controls and treatments began with 25 animals.

observations was compared, using Duncan's Multiple Range, to the mean reproductive impairment for the last three observations. There was little significance between the 12 day periods. The only significant values were for the 10.0 and 18.0 µg/l concentrations of the second replicate.

Twenty Eight Day Immobilizations

The chi-square method was used to test for significance between immobilizations in the test solutions and the control. The same method was used to determine significance between comparable treatments in the replicates. Chi-square values for the immobilizations are shown in Table VIII. The only significant value observed for the comparison of immobilizations in toxaphene treatments to the control, for each concentration replicate, was observed in the 18.0 µg/l treatment of the second replicate. This skewed the data for combined replicate immobilizations. The combined immobilizations for the replicates are shown in Figure 3.

When the replicates were compared, there were significant differences between controls, 18.0, 5.6, and 3.2 µg/l treatments. There were 4 percent and 32 percent immobilization rates for the controls of the two replicates. Some unknown factor caused significant immobilization differences between the two replicates (Table VIII).

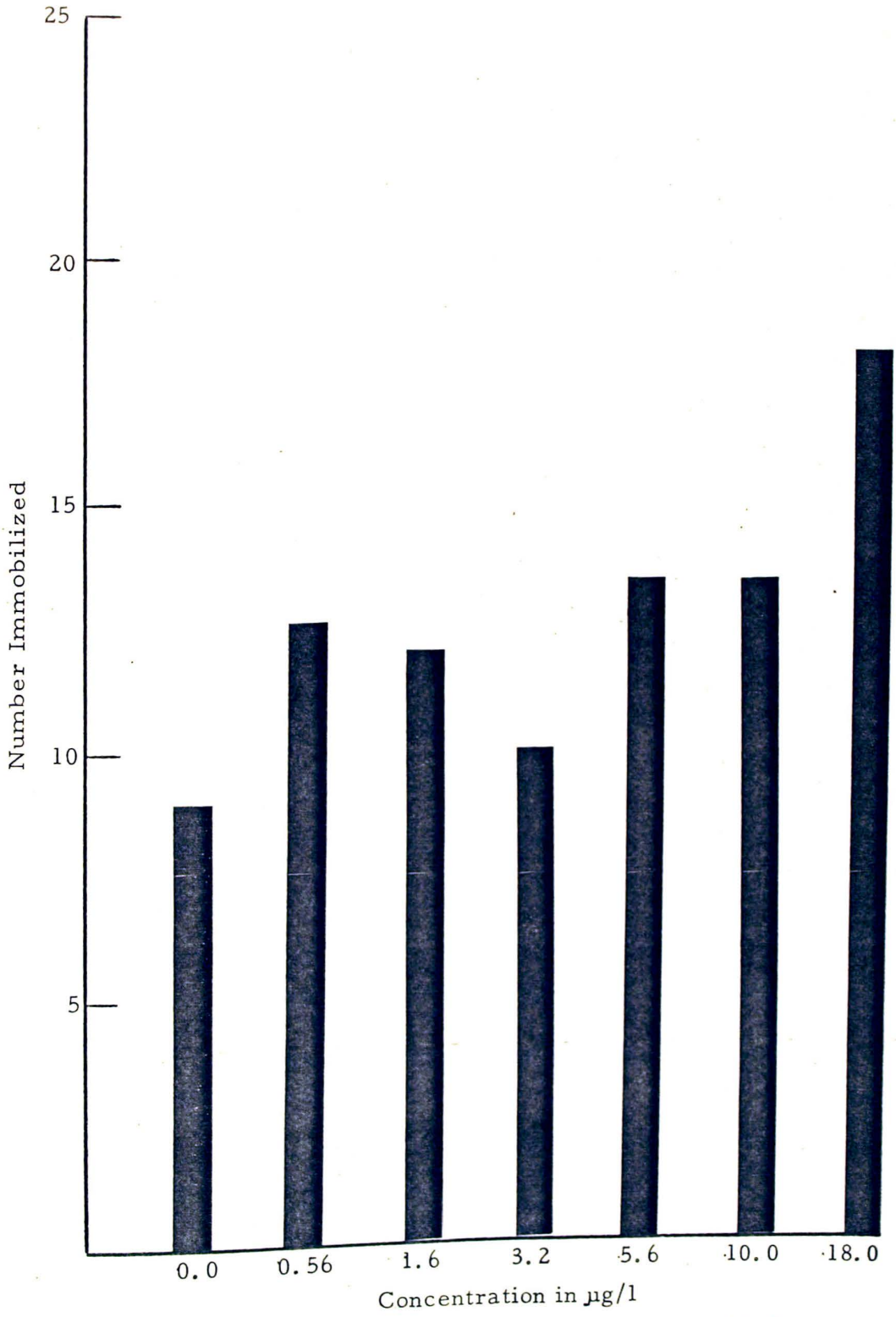

Figure 3. Combined number of immobilized daphnids for two 28 day chronic toxicity tests. Each treatment replicate involved 25 animals with one animal per vial.

TABLE VIII

Chi-Square Values for Replicates of
28 Day Adult Immobilizations with
Comparison between Replicates

	Treatment in µg/l						
Replicate	0.0	0.56	1.8	3.2	5.6	10.0	18.0
1st		0.100	0.100	0.100	0.764	0.100	0.347
2nd		2.000	1.050	0.000	1.050	3.050	12.578[a]
Replicates Compared	6.64[c]	2.599	3.947[b]	8.000[c]	6.349[b]	1.587	0.325

[a]Significant at the .01 level from the replicate control.

[b]Significant difference at the .05 level between comparable replicate treatments.

[c]Significant difference at the .01 level between comparable replicate treatments.

There were 25 animals per replicate.

CHAPTER V

DISCUSSION

Bioassays with aquatic organisms are performed most often over short periods of time. Observations are made usually at 24, 48, 72, and 96 hours, with few tests being run over longer periods of time. IC_{50}, TL_m or LD_{50} values are typically calculated for these test runs.

While bioassays of this type are important for comparison to the toxicity of other substances, they may have little significance to the natural situation. Undetected damage to the aquatic ecosystem may occur over long periods of time at much lower levels. Since toxicants may not enter waters all at once, the effects of continuing application of the toxicant become important.

In this author's investigation, the effects of a common environmental contaminant, toxaphene, were observed in a natural water situation over a major portion of the organisms life span. Significant effects on reproduction were observed at levels below those causing acute toxicity.

IC_{50} values of 137, 126, and 90 µg/l were observed for the 48, 72, and 96 hour tests, respectively. The concentrations investigated were not high enough to produce an IC_{50} value for the 24 hour time. The value should be over 180 µg/l. Several values are present in the

literature for the toxicity of toxaphene to daphnids. Guyer (1971) lists a 48 hour LC_{50} value of 15 µg/l for D. pulex. There is a large discrepancy between this value and the value observed in this study. This may be explained partly by the investigators note that the value was dependent on pH, oxygen concentration and hardness. Sanders and Cope (1966) found an EC_{50} value of 11-20 µg/l at 60°F. Their study was conducted in a reconstituted water medium consisting of 30 mg of $MgSO_4$, 30 mg of $CaSO_4$, 48 mg of $NaHCO_3$ and 2 mg of KCl per liter of distilled water. The pH ranged from 7.4-7.8. Their test water was a much softer medium than water which was used in this study. The spring water had a mean total hardness of 194.5 ppm.

Frear and Boyd (1967) found a 26 hour LD_{50} value in toxaphene of 94.0 µg/l for D. magna in a hard synthetic medium. Crosby and Tucker (1966) found a value of 260 µg/l for a 24 hour median dose for D. magna. While these values are for another closely related species, the conditions of the test median are more comparable to the work of this researcher. A highly oxygenated condition of the test water in this study may have led also to the higher observed values, although water hardness is the more likely factor. While the IC_{50} values determined in this study differ from those in the literature, they do serve as a basis for comparison with longevity and reproduction data. The observed IC_{50} values indicate the need for more standardization of test procedures using daphnids.

There are no studies in the literature for comparison with the

reproductive data found by this researcher. Cladoceran studies of this type have been performed with very few substances during the last 10 years and prior to that time are almost non-existent. The emphasis has been on investigations of fish.

The data on reproduction were both significant and regular in nature as shown by Figure 2 and Tables V, VI, and VII. There were significant effects on reproduction at all levels above 1.8 µg/l. Combined values of 19, 35, 49.6 and 75.8 percent reduction in number of young were recorded for the 3.2, 5.6, 10.0, and 18.0 µg/l concentrations. This represents nearly a 50 percent reduction at the 10.0 µg/l concentration.

The data or impairment of mean number of young per treatment showed impairment levels similar to those of total reproduction impairment values. No significant reproductive impairment was found at concentrations below 3.2 µg/l. A 70.3 and 73.2 percent impairment in mean number of young per surviving adult was found for the replicates at the 18.0 µg/l concentration. There were impairment values of 20.0 and 15.3 percent at the 3.2 µg/l replicate concentrations. This indicates uniformity in the effect of the insecticide on reproduction. A combined impairment level of 46.3 percent was observed at the 10.0 µg/l concentration. This is only a 3.3 percent difference in the value recorded for the total reproduction impairment at the same concentration. Although a 50 percent impairment value was not calculated statistically, it was noted to be slightly above 10.0 µg/l. This value is approximately

14 times lower than the 24 hour IC_{50} value and nine times lower than the level of toxaphene producing a 96 hour IC_{50} effect. When impairment of reproduction for the 8, 12, and 16 day observation times was compared to the values for the 20, 24, and 28 day observation periods of the replicates, there was little significant difference between reproduction impairment for the two twelve day periods. The only significant values were for the 10.0 and 18.0 µg/l concentrations of the second replicate. There were slightly lower mean impairment values for the first 12 days of observation in the second replicate. Observations of this data show that 3-week reproductive studies would give similar percentages to those found at the 28 day time period.

The mean measurements of the adults surviving the 28 day toxicity studies are shown in Tables III and IV. There were no values significantly different from the control in the first replicate, but the 5.6 to 18.0 ug/l mean lengths were significant at the 5 percent level for the second replicate. The mean length was 2.04 ± 0.04 mm in the 18.0 µg/l concentration and 2.13 ± 0.04 mm in the control. There was a 4.3 percent reduction in the total body length at the 18.0 µg/l level of the second replicate. Compared to the reduction in reproduction at the same levels, the effects on growth are not very significant.

Immobilizations for the toxaphene concentrations tested were non-significant when compared to the replicate controls for each replicate. Only one value in the second replicate 18.0 µg/l treatment was significant from the control. Chi-square values for significance between

treatments and controls for the two replicates are shown in Table VII. Chi-square values comparing equal treatments between replicates are also shown. Several significant values indicate that there was significant difference between comparable treatments of the replicates. The large discrepancy between immobilization rates in the replicates cannot be explained.

This author feels that the data presented are more applicable to the natural aquatic environment than would have been if performed with a chemically prepared artificial water source. This researcher also feels that water hardness is of primary importance in determining the toxicity of toxaphene to daphnids. Biesinger et al. (1973) have reported total hardness values and pH values for the Ohio, Mississippi, Minnesota and Missouri rivers which are very close to the means of the spring water used in this study. The values obtained in this study should be comparable to such bodies of water when water hardness is considered. Perry (1969), Filson (1974), and Harned (1976) have reported values of 70-143 ppm, 10-30 ppm, and 30-50 ppm total hardness for Percy Priest Lake, a farm pond and a woodland pond respectively. In situations of this type, levels of toxaphene reported in this paper would probably have a more toxic effect.

This investigation has shown reproductive impairment to be a more sensitive measure of toxaphene toxicity than immobilization rates. Biesinger et al. (1973), in a 3-week reproduction study with D. magna, has also found this to be true.

Bioassay work with cladocerans requires more study. Chronic studies are required for many toxicants. More attention needs to be given to keeping bioassay conditions similar to those in the natural environment. Test animal age, food, container size and sources of animals should be standardized.

The results of this study indicate that relatively low levels, compared to acutely toxic concentrations, may affect the reproductive rate of an important food source organism. If reductions in reproductive rate observed in this study occurred in nature, the entire aquatic ecosystem would be affected. Young fish, requiring food of this type, would be significantly affected.

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