SOME EFFECTS OF 2, 4 - D AMINE SALT ON DAPHNIA MAGNA

STEVEN HENRY REZBA

SOME EFFECTS OF 2,4-D AMINE SALT ON *DAPHNIA MAGNA*

An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Steven Henry Rezba

December 1972

ABSTRACT

Daphnia magna were exposed to dimethylamine salt of 2,4-dichlorophenoxyacetic acid (2,4-D) in two types of tests. The first was a test with unfed animals to determine the IC_{50} values (concentrations at which fifty per cent of the population was immobilized) for 24, 48, 72, and 96 hours. The second type of test was with fed animals in which IC_{50} values, average instar for primiparous adults, and rate of ecdysis were determined. A total of 2623 young were tested.

Daphnids were raised in Frear and Boyd's (1967) medium and fed yeast and Scenedesmus obliquus. The test temperature was 21°C ± 1°. The light period was sixteen hours. The concentrations used of 2,4-D were 1, 2, 3, 5, 10, 20, 30, 40, 50 75, 100, 150, 200, and 300 mg/liter.

The IC_{50} values for unfed animals for 24 hours were greater than 300 ppm; for 48 hours, 192 ppm; for 72 hours, 156 ppm; and for 96 hours, 144 ppm. In comparison the fed animals' IC_{50} values up to 144 hours were greater than 300 ppm. At 144 hours the IC_{50} value was 138 ppm. There was comparison of the rates of ecdysis of 28 runs of the various test concentrations and seven control tests. There were too many conflicting significant and non-significant values to evaluate the data. There was little change in the average

instar for primiparous adults, but 2,4-D did appear to affect the surface tension and decrease the amount of daphnids entrapped in it. It appears that the feeding of test animals does produce different effects. The typical unfed test has its merit, but one should not expect the same results in nature. Further testing is needed in the comparison of unfed to fed daphnids in pesticide studies.

SOME EFFECTS OF 2,4-D AMINE SALT ON *DAPHNIA MAGNA*

A Thesis

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Steven Henry Rezba

December 1972

To the Graduate Council:

I am submitting herewith a Thesis written by Steven Henry Rezba entitled "Some Effects of 2,4-D Amine Salt on *Daphnia magna*." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in biology.

Diane I. Findley
Major Professor

We have read this thesis and recommend its acceptance:

Floyd M. Ford
Minor Professor

or

Second Committee Member

Davis T. Findley
Third Committee Member

Accepted for the Council:

Wayne E. Stamps
Dean of the Graduate School

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Dr. Diane Findley, Assistant Professor of Biology, Austin Peay State University, who aided and counseled him; to Dr. Floyd M. Ford, Professor of Biology, and to Dr. Davis L. Findley for their guidance and constructive criticism.

Appreciation is extended to all the faculty and graduate students of the Biology Department who have aided me in my work, and to Cliff Mitchell of Amchem Products, Inc. for supplying the technical grade 2,4-D.

The author wishes to express appreciation to his wife, Sherry, for her assistance.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE

LIST OF TABLES

CHAPTER I

INTRODUCTION

There has been little research of the effects of herbicides upon the fresh-water invertebrate fauna. Herbicides enter the aquatic environment by run-off, aerial spraying, or direct application. Toxic materials attack any trophic level within the ecosystem. If a herbicide has reduced a population of zooplankton, other members higher on the food chain may starve even though they were tolerant of the herbicide. Direct kills of fish are usually noticed, but there are some alterations to the environment that remain unknown. Pressures are exerted on populations directly or indirectly that would not have taken place otherwise.

Bioassay Animal

The bioassay animal used in these studies was *Daphnia magna* Straus. These arthropods belong to the Class Crustacea, the Order Cladocera, and the Family Daphnidae (Pennak 1953). Cladocerans, commonly known as water fleas, are an important part of the food chain. They filter feed mainly on algae, bacteria, and organic debris (Pennak 1953), and they in turn are eaten by secondary consumers such as fish.

The great majority of cladocerans live in fresh water.

They are widespread and live in rivers, ponds, and lakes. Some species are littoral while others live in the limnetic zone or in the benthic zone.

Daphnia magna reaches a maximum length of five millimeters and has a single carapace that has a bivalve appearance but is actually folded over the dorsal surface. A single large compound eye and a single ocellus are present. All members of the Daphnidae have five pairs of lobed, leaf-like thoracic legs bearing numerous hairs and setae. The legs are enclosed by the carapace and the setae are used to filter food out of the water. There are two pair of antennae; the first pair are small and sometimes chemoreceptive. These biramous antennae are large and muscular and pull the daphnid through the water in a jerky manner.

There are four stages in the development of a daphnid: egg, juvenile, adolescent, and adult. The eggs are released from the ovary into the brood chamber. They develop and the young are released to the outside in the first juvenile instar. The adolescent period is the single instar when the animal is developing eggs in the ovary. After the next molt, the cladoceran is an adult (Pennak 1953).

Reproduction is parthenogenetic during the majority of the year. The parthenogenetic development is from diploid eggs. Under the proper conditions a completely female population can be maintained indefinitely. Banta raised several common cladocera for twenty-seven years of successive parthenogenetic generations (Pennak 1953). Male production occurs

when there is overcrowding and subsequent accumulation of excretory products and a decrease in food. A decrease in temperature may also be a factor in the development of the parthenogenetic males. The changes in the daphnids' environment that produce the male eggs also influence some of the females so that they produce one or several sexual "resting" eggs. These females are also capable of copulation with males. The walls of the fertilized egg become thicker and darker to form an ephippium. The ephippium is able to withstand freezing and drying and is able to survive over the winter and start a parthenogenetically reproducing population in the spring.

Advantages and Disadvantages of Daphnia magna

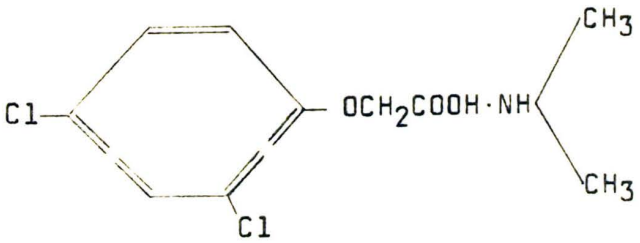
Daphnia magna is an important fresh-water invertebrate; therefore, there is much published information available on culturing. Daphnia magna have a short life span of four to six weeks (MacArthur and Baillie 1929) and are large enough to been seen without a microscope but small enough to raise in large numbers in a limited space. Under favorable environmental conditions, their reproduction is parthenogenetic so that a clone can be developed such as Anderson (1932) did for his studies.

There are disadvantages to using D. magna as a bioassay animal. Anderson and Jenkins (1942) pointed to the need for a more controllable culture medium that could be easily reproduced. Freeman (1953) had developed a Standard

Reference Water. This medium was simplified by Frear and Boyd (1967). However, there are still many researchers that use different media. Thus, there are obvious problems in comparing data that was acquired from animals raised in water of different pH and dissolved compounds and minerals.

Herbicide Being Investigated

The herbicide investigated was the dimethylamine salt of 2,4-dichlorophenoxyacetic acid. The trade name is Weedar 64 and is produced by Amchem Products, Inc. Common names are 2,4-D or 2,4-D amine salt. According to the Herbicide Handbook of the Weed Society of America (1970), it is widely used for control of broadleaf weeds in cereal crops, turf, pastures, and non-crop lands. Its application is usually postemergence with rates that vary from one quarter pound to four pounds per acre. The structural formula is shown below:



The molecular formula is $C_{10}H_{13}Cl_2NO_3$ with a molecular weight of 266.1. It is a white crystalline, odorless salt with a melting point of 85 to 87°C. It also decomposes at this point. The solubility at 20°C is 300 g of 2,4-D to 100 g of distilled water. The acute oral IC_{50} of different for-

mulations are from 300 to 1000 mg/kg for rats, guinea pigs, and rabbits. Sanders (1970) found the forty-eight hour TL_{50} value of 4 mg/liter at 21°C for Daphnia magna with 2,4-dimethylamine salt (technical grade) supplied by Amchem Products Inc.

Objectives of the Investigation

The total production of herbicides in the United States declined between 1968 and 1969. The total amount of herbicides produced in the United States in 1968 was 402,199,000 pounds, and in 1969 was 371,230,000 pounds. The decline was in the production of 2,4-D and 2,4-5-T that were used as defoliants in Vietnam (Frear 1972). The total amount of 2,4-D acid esters and salts produced in 1968 was 94,116,000 pounds and 46,998,000 pounds in 1969. Thus in 1969 2,4-D was roughly twelve per cent of the total United States production. It seems likely that the production of herbicides for domestic use will increase because of the demand for increased yields.

Salts of 2,4-D are readily leached in sandy soils and undergo a low rate of bacterial breakdown in warm, moist soils and minor loss from photodecomposition (Herbicide Handbook, 1970). Besides run-off, there is also direct application of 2,4-D to the aquatic environment to remove nuisance macrophytes. There were 170,000 gallons of 2,4-D, at four pounds per gallon, added to to two TVA reservoirs for this specific purpose (Wojtalik et al, 1971).

According to Brooks (1957) D. magna range over a large area of northern and western North America. This range does coincide with application, so there are many possibilities of exposure. Since different studies of the stomach contents of young fish show concentrations from one to ninety-five per cent cladocerans by volume (Pennak, 1953), any toxin that affects D. magna could drastically affect all higher trophic levels in certain aquatic ecosystems. Therefore, the purpose of this study was to determine if sublethal concentrations would affect growth, reproduction, and ecdysis of D. magna. Also it was to determine the IC_{50} values (those values at which fifty per cent of the population were immobilized) on young at 24, 48, 72, and 96 hour time periods and also to determine if there was a difference in IC_{50} values between fed and unfed animals.

CHAPTER II

METHODS AND MATERIALS

Reproducing Population

The bioassay animal was *Daphnia magna* Straus. A culture was obtained from Ward's Natural Science Establishment. The reproducing population was maintained in one gallon wide-mouth jars. Fifty to seventy-five female *D. magna* were in each one gallon jar with a total of about eight jars. The young were filtered off to be used as experimental animals and for restocking the cultures. For restocking these young were kept in separate jars until they became primiparous. Then they were returned to the reproducing population. Every seven days the adults were filtered out of their jars and fresh medium was added. Debris was removed then the adults were returned and fed. The jars were maintained in Percival Incubators at 21°C ± 1°. The light cycle was from 0800 to 2300 hours. One twenty-watt white fluorescent light per shelf was reflected up through the bottom of the containers to attract the daphnids to reduce the number of floaters.

Culture and Test Medium

The test medium used was developed by Frear and Boyd (1967). This was a simplified version of Freeman and Fowler's

(1953) Standard Reference Water. The composition of the medium is found in Table I. The chemicals were added from stock solutions to deionized glass-distilled water. Compressed air was bubbled through the medium for twenty-four hours. A twenty-four hour period was allowed for equilibrium, and the pH was adjusted to 7.5 with hydrochloric acid or sodium hydroxide. Frear's medium was made and stored in a 75-liter polyethylene container.

The glassware for cultures was cleaned with a cleanser then soaked in fifteen to twenty per cent hydrochloric acid. None of the test vials were scrubbed with the cleanser. Instead, they were scrubbed with a brush and soaked in fifteen to twenty per cent hydrochloric acid and rinsed in tap water.

Feeding

Each one gallon jar of the reproducing population of daphnids was fed daily between the hours of 0700 to 2100 hours. Dewey and Parker (1964) found a combination of Scenedesmus obliquus and fresh yeast suspension to be an excellent food for maintaining population growth. A fresh suspension of Fleischmann's dry yeast was made daily at the ratio of 50 mg of yeast to 50 ml of distilled water. One milliliter of yeast suspension and 2 ml of Scenedesmus obliquus were added daily to each jar.

The Scenedesmus obliquus culture was originally obtained from the Indiana University Culture Collection of Algae at Bloomington (Starr, 1964). It was cultured on slants of

TABLE I

COMPOSITION AND CONCENTRATION OF STANDARD TEST MEDIUM

Salt	Concentration in Stock Solution (mg/ml)	Final Concentration in Medium (mg/ml)
$NaHCO_3$	100	200
$CaCl_2$	112	224
K_2SO_4	13	26

Beijerinck's medium (contents listed in Table II). The Scenedesmus was again transferred to a dozen or more agar slants. These were maintained at room temperature at approximately 22-25°C in continuous fluorescent lighting of cool white fluorescent lights until there were thick, dark green growths of algae. From these agar slants, the algae were suspended and transferred to sterile Beijerinck's medium in 250 and 300 ml Pyrex Erlenmeyer flasks that were aerated with compressed air and maintained under the same conditions as the agar slants. Scenedesmus obliquus was grown until there was a thick layer on the bottom of the flasks.

In the growth and reproduction tests the animals were fed a standardized suspension of algae. A suspension of Scenedesmus obliquus that gave a Spectronic 20 reading between .60 to .70 absorbance at 680 mu was used. If culture dilution was necessary, Beijerinck's medium was used.

Separation of Young

Isolation of young daphnids was obtained by pouring the contents of each gallon population jar through two Dacron nets in a polyethylene funnel. The first net, made out of white Dacron curtain material with 672 squares per square inch, filtered out the adults. The second net, which would filter out the smallest young, was made out of Dacron fabric with 9216 squares per square inch. After the contents of the population jars were poured through the nets, the nets were turned upside down and rinsed off with Frear and Boyd's

TABLE II

BEIJERINCK'S ALGAE MEDIUM

	Chemical	Conc. of Stock Sol.	Vol of Stock/ Liter of D-H_2O
I.	NH_4NO_3	14 g/liter	10ml
II.	$MgSO_4$	20 g/liter	1ml
III.	$CaCl_2$ $2H_2O$	10 g/liter	1ml
IV.	KH_2PO_4	40.28 g/liter	10ml
V.	K_2HPO_4	69.66 g/liter	10ml
VI.	Trace Elements		1ml

Composition of 100ml Trace Element Stock (Hutner 1950)

EDTA	5.00 g
$ZnSO_4$ $7H_2O$	2.20 g
H_3BO_3	1.00 g
$CaCl_2$	0.50 g
$MnCl_2$ $4H_2O$	0.50 g
$FeSO_4$ $7H_2O$	0.50 g
$CoCl_2$ $6H_2O$	0.15 g
$CuSO_4$ $5H_2O$	0.15 g
$(NH_4)_6Mo_7O_{24}$ $4H_2O$	0.10 g

medium. Agitation and short periods of time on the wet nets did not appear to affect the daphnids. The maximum time that the daphnids were left on the nets was approximately sixty seconds.

Before a test was to be started, the young were filtered off and the adults were returned to their jars. Twenty-four hours later the stock population was filtered again. The young would then range from zero to twenty-four hours old. The test animals were then placed in a randomizing beaker without food for one hour. The young, now one to twenty-five hours old, were ready for testing. They were quickly filtered through a small net made of Dacron with 9216 squares per square inch. The net was dipped in a beaker containing the lowest concentration of herbicide to be used. The young were released directly into the solution. The control groups were filtered through a net used to transfer them to the control medium. Using this method, all the test animals could be in the proper test solution in a matter of minutes. Then the young were individually transferred to the test vials. The vials were 25 mm by 95 mm, eight dram, flat bottom, holding approximately 25 ml to 30 ml of medium. Pasteur pipettes were used for transferring. The tips were broken off to accommodate the larger molts taken out of the vials.

Herbicide

The herbicide tested was dimethylamine salt of 2,4-dichlorophenoxyacetic acid (2,4-D). A stock solution was obtained from Amchem Products, Inc. It consisted of one

quart of .78 grams/ml of 2,4-dichlorophenoxyacetic acid mixed in distilled water. Three and one-half milliliters of this stock were added to 3.5 liters of Frear and Boyd's medium for a 1:1000 dilution. This was a more workable stock of 480 mg/liter. From this stock the other dilutions were made. All the dilutions were with Frear and Boyd's medium so that the only difference between the control medium and the test medium was the herbicide.

Immobilization Tests

Two types of tests were run. One was the IC_{50} test which was to determine the dose of a toxin at a given time that would immobilize fifty per cent of the individuals tested. Immobilization rather than death was used as an index because the actual time of death in daphnids was difficult to determine. If an individual daphnid was lying at the bottom of the jar and could stroke her antenna a few times, she was considered alive. The concentrations of 2,4-D used were 20, 30, 40, 50, 75, 100, 150, 200, 250, and 300 milligrams per liter. The tests were started when the young were one to twenty-five hours old. They were rechecked at 24, 48, 72, and 96 hour exposures. The IC_{50} test animals were not fed.

Growth and Reproduction Tests

The growth and reproduction tests were followed through the entire life of the individuals. Reproduction, number of young, survival, ecdysis, and immobilizations were recorded daily. The concentrations used were 1, 2, 4, 5, 10,

20, 30, 40, 50, 75, 100, 150, 200, 250, and 300 milligrams per liter. The growth and reproduction test animals were fed one drop of yeast suspension and two drops of Scenedesmus obliquus suspension from Pasteur pipettes. The test animals were fed after they were transferred to their vials and after their progress was recorded every twenty-four hours until all the animals were immobilized.

CHAPTER III

RESULTS

IC_{50} Tests Using Unfed Animals

All tests were maintained at 21°C ± 1°. Daphnids were one to twenty-five hours old at the beginning of the tests. A total of 1233 unfed animals were tested to determine the IC_{50} values. There were 197 controls and 1036 test animals. The results of the mortality rates of the unfed daphnids are recorded in Figure I. The data are adjusted with the controls, so the natural mortality is considered in the IC_{50} values. The tests were replicated three times. The data were plotted on semi-logarithmic paper. The dosages were plotted on the logarithmic scale and the survival percentages on the arithmatic scale. This method is known as straight-line graphical interpolation (Doudoroff 1951). The IC_{50} values were determined from the intersection of the per cent immobilization line with the time line by interpolating to the concentration axis.

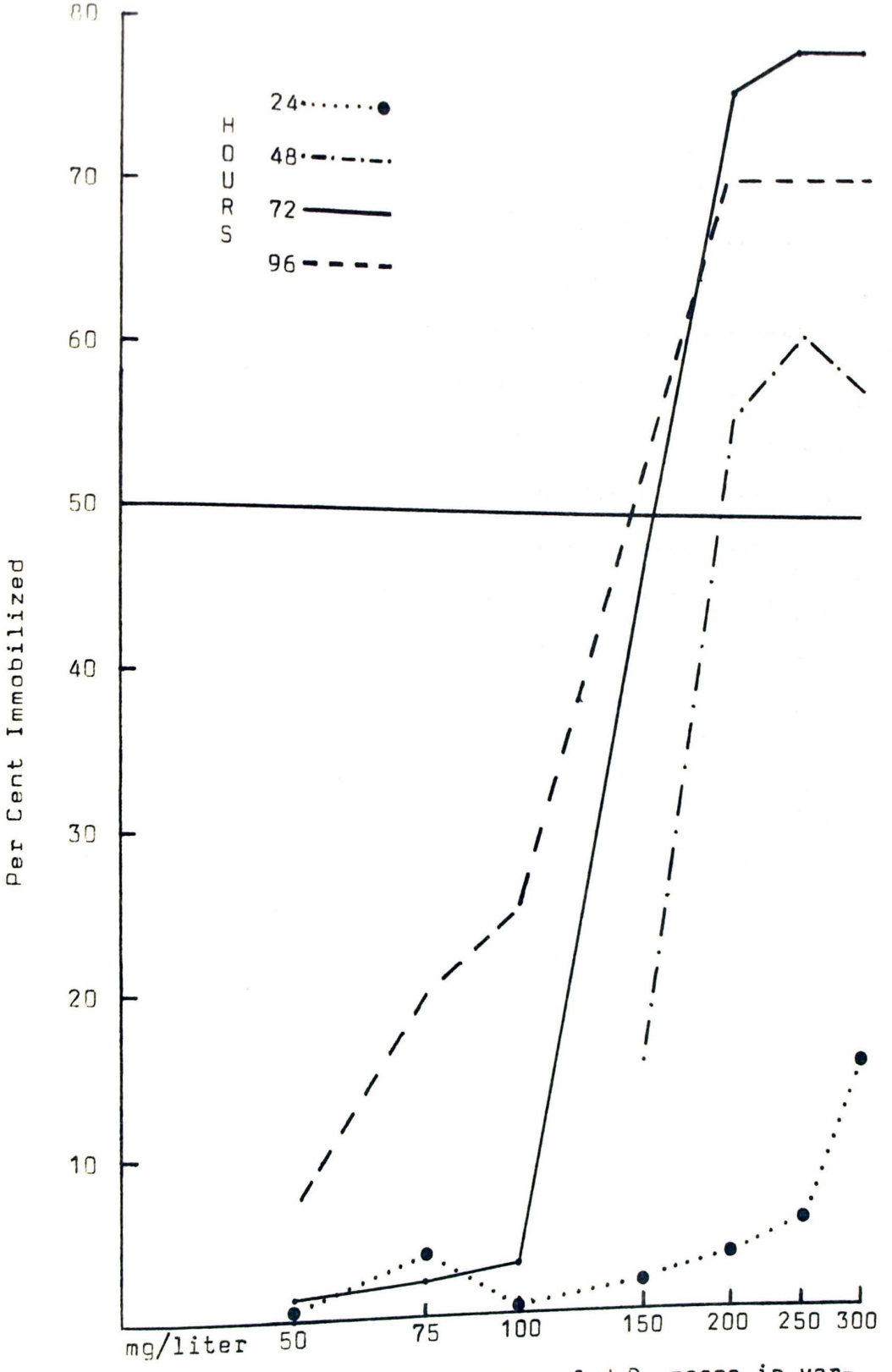


Figure 1: Immobilization rates for unfed D. magna in various concentrations of 2,4-D

The IC_{50} values found by interpolation for the various time periods are the following:

24 hours	over 300 mg/liter
48 hours	192 mg/liter
72 hours	156 mg/liter
96 hours	144 mg/liter

The 48 and 72-hour lines are not plotted in the lower concentrations because the values are negative. After the per cent immobilizations of the controls were subtracted from test immobilization values, some values were found to be negative. The significance of these negative and positive immobilization values is found in Table III. This table presents the chi-square values for Figure 1. The values are used for determining the significance of the immobilization of D. magna in different concentrations of 2,4-D. Chi-square values were obtained for each concentration by combining data of equal concentrations. Typical tests had forty animals per concentration. Each concentration was then tested for significance against its corresponding combined controls.

Growth and Reproduction Tests

All tests were maintained at $21^{o}C \pm 1^{o}$, and all daphnids were one to twenty-five hours old at the beginning of the tests. A total of 1380 animals were tested to determine IC_{50} values for fed animals, average number of instars for primiparous adults, and any change in ecdysis and reproduction. Two hundred seventy-eight control animals and 1112

TABLE III

CHI-SQUARE VALUES FOR THE IMMOBILIZATION RATES FOUND IN FIGURE 1

TOTAL NO. OF ANIMALS	MG/LITER 2,4-D	TIME IN HOURS			
		24	48	72	96
80	20	.213	.485	.012	.021
78	30	.659	.548	.066	4.490+
80	40	.010	.579	7.080*	16.319*
79	50	.008	8.295*	3.539	5.367+
119	75	.606	2.334	1.352	.502
120	100	0.0	5.069+	5.069*	3.573
120	150	.504	12.001*	42.161*	40.142*
120	200	3.268	77.380*	105.217*	65.065*
120	250	3.232	89.617*	142.198*	27.875*
120	300	14.632*	99.918*	173.093*	106.201*

*Significant at 5% level

+Significant at 1% level

test animals were used in this part of the study. The tests were duplicated at each concentration. The results of the immobilization rates of the unfed daphnids are recorded in Figures 2 and 3 and the data are plotted on a semi-logarithmic scale. IC_{50} values were determined in the same manner as for Figure 1. The only IC_{50} immobilization concentration determined for Figure 2 is 138 mg/liter for 144 hours. For Figure 3 no IC_{50} value could be determined because immobilization did not exceed fifty per cent natural mortality. The data are adjusted with the control so that the natural mortality is considered in the IC_{50} values.

Concentrations of 50 to 300 mg/liter of 2,4-D are plotted on Figure 2, and concentrations of one to fifty mg/liter on Figure 3. On Figures 2 and 3 some values are not plotted because the values are negative after the per cent immobilization of the controls were subtracted from test immobilization values to determine natural mortality.

Table IV presents the chi-square values for determining the significance of the immobilization of *D. magna* in test concentrations of 2,4-D. Table IV presents the values from 1 to 300 mg/liter.

Figure 4 presents the mean instar and standard deviations for primiparous *D. magna* for the controls and each test concentration. The mean instar was determined by recording each molt of each individual until each daphnid reached adulthood. The instar of each primiparous adult was listed according to concentration. The mean and standard

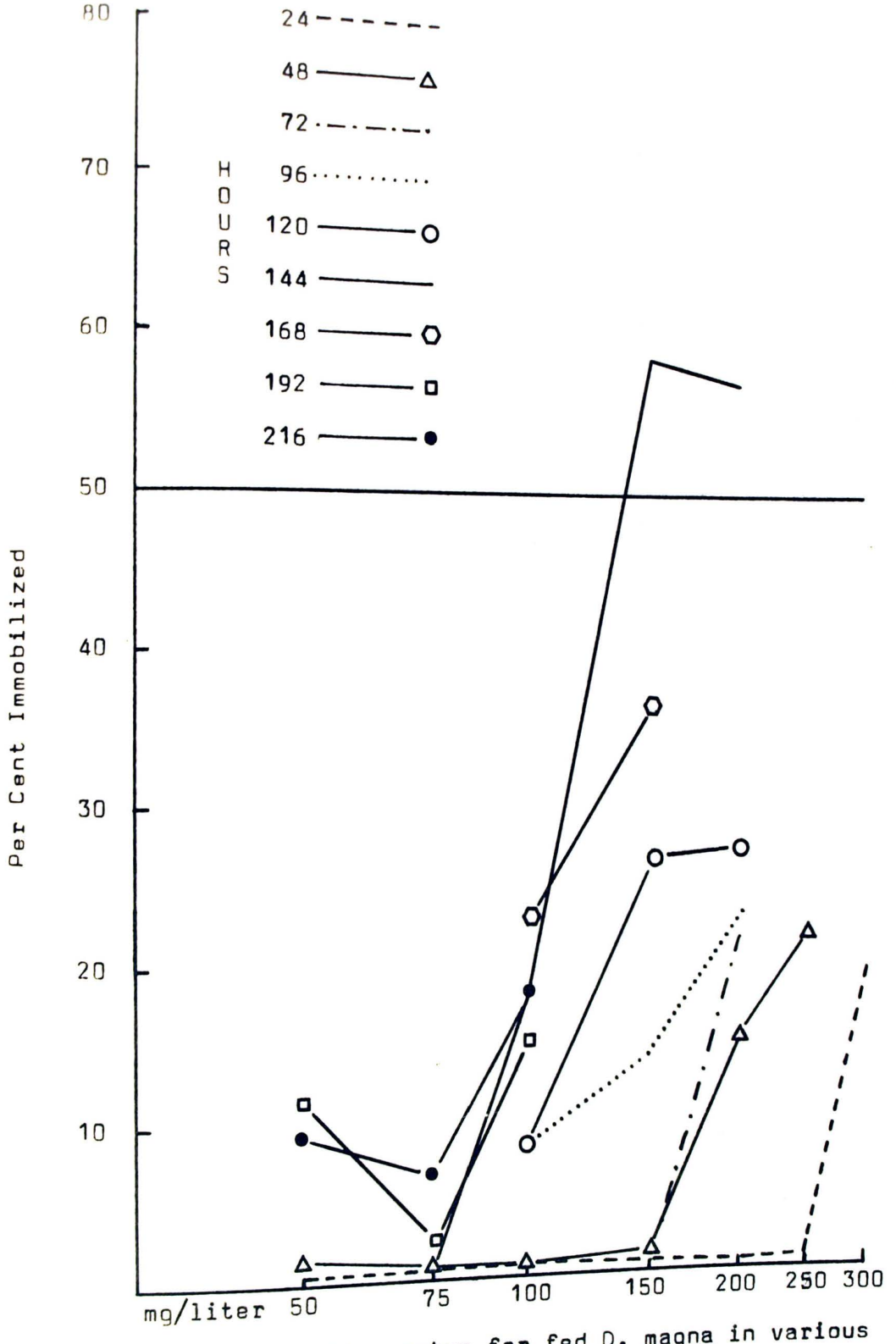


Figure 2: Immobilization rates for fed D. magna in various concentrations of 2,4-D

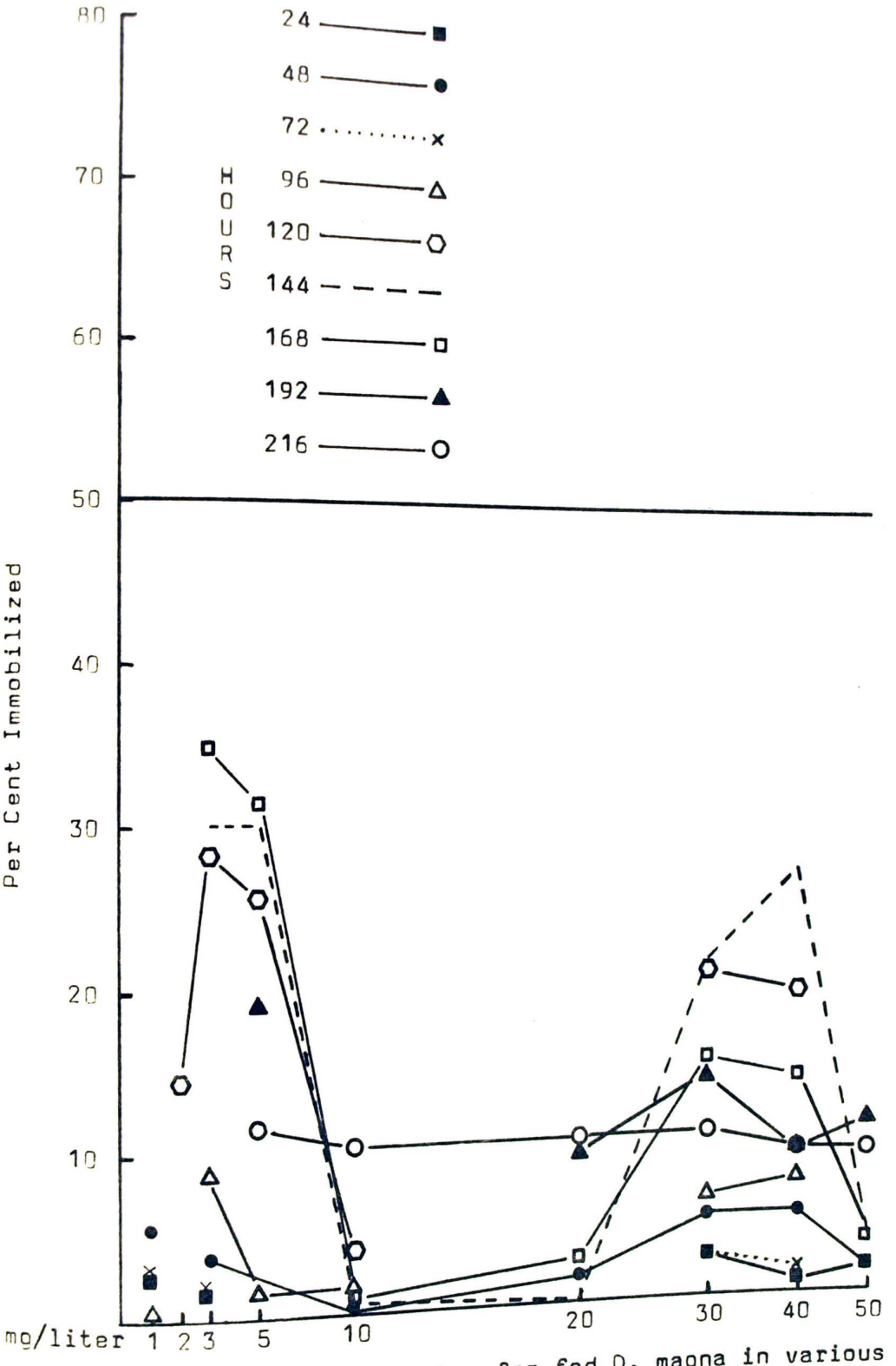


Figure 3: Immobilization rates for fed D. magna in various concentrations of 2,4-D

TABLE IV

CHI-SQUARE VALUES FOR IMMOBILIZATION OF FED D. MAGNA IN IC_{50} TESTS (FIGURES 2 AND 3)

mg/liter of 2,4-D \ hrs.	24	48	72	96	120	144	168	192	216	240	264	288	312	336	360 *
1	.28	.15	0.0	0.0	0.0	.01	0.0	.02	.08	.01	0.0	0.0	0.0	.48	.48
2	0.0	1.38	1.59	.49	6.67	2.39	7.29	0.0	1.49	2.49	4.49	4.1	7.0	4.16	.49
3	0.0	0.0	0.0	3.82	12.56	15.63	24.72	9.76	1.35	1.35	.50				
5	.50	.17	0.0	.33	10.59	15.63	18.25	5.35	.25	.50	0.0				
10	.49	1.44	.16	0.0	.36	.18	.05	0.0	8.07	1.33	.49				
20	.49	0.0	0.0	0.0	1.22	.02	.10	0.0	0.0	1.35	.50				
30	0.0	.13	.10	2.36	6.86	8.13	3.51	1.73	0.0	1.35	.50				
40	.50	.25	.10	.04	.02	.02	.03	.05	.17	0.0					
50	.51	.51	.26	0.0	0.0	3.02	.34	1.74	0.0	0.0					
75	.51	.26	0.0	.08	1.99	0.0	.02	0.0	0.0	0.0					
100	0.0	.50	2.39	3.25	4.47	1.62	6.45	3.28	.17	0.0					
150	1.33	.80	0.0	4.92	10.75	23.45	12.58	1.38	0.0	0.0					
200	25.13	32.41	28.93	43.44	44.48	14.89	12.90	1.30	0.0	0.0					
250	0.0	17.21	57.71		*All values after 360 hrs. are 0.0										
300	14.24	71.24													

TABLE V

DATA ON PRIMIPAROUS *D. MAGNA* SUBJECTED TO 2,4-D

MG/LITER	n	$\bar{X}$	SD	T-test
0	132	5.52	.7255	
1	39	5.49	.6013	.260
2	44	5.39	.7537	1.000
3	2	5.00	0.0000	8.234
5	14	5.50	.7595	.090
10	13	5.69	.6304	.914
20	26	5.46	.5817	.460
30	19	5.68	.7492	.873
40	14	5.71	.6112	1.084
50	34	5.65	.5970	1.080
75	31	5.74	.5143	1.966
100	28	5.75	.4409	2.199
150	4	4.75	.4082	3.605
200	6	5.50	.7071	.067
250	0			
300	0			

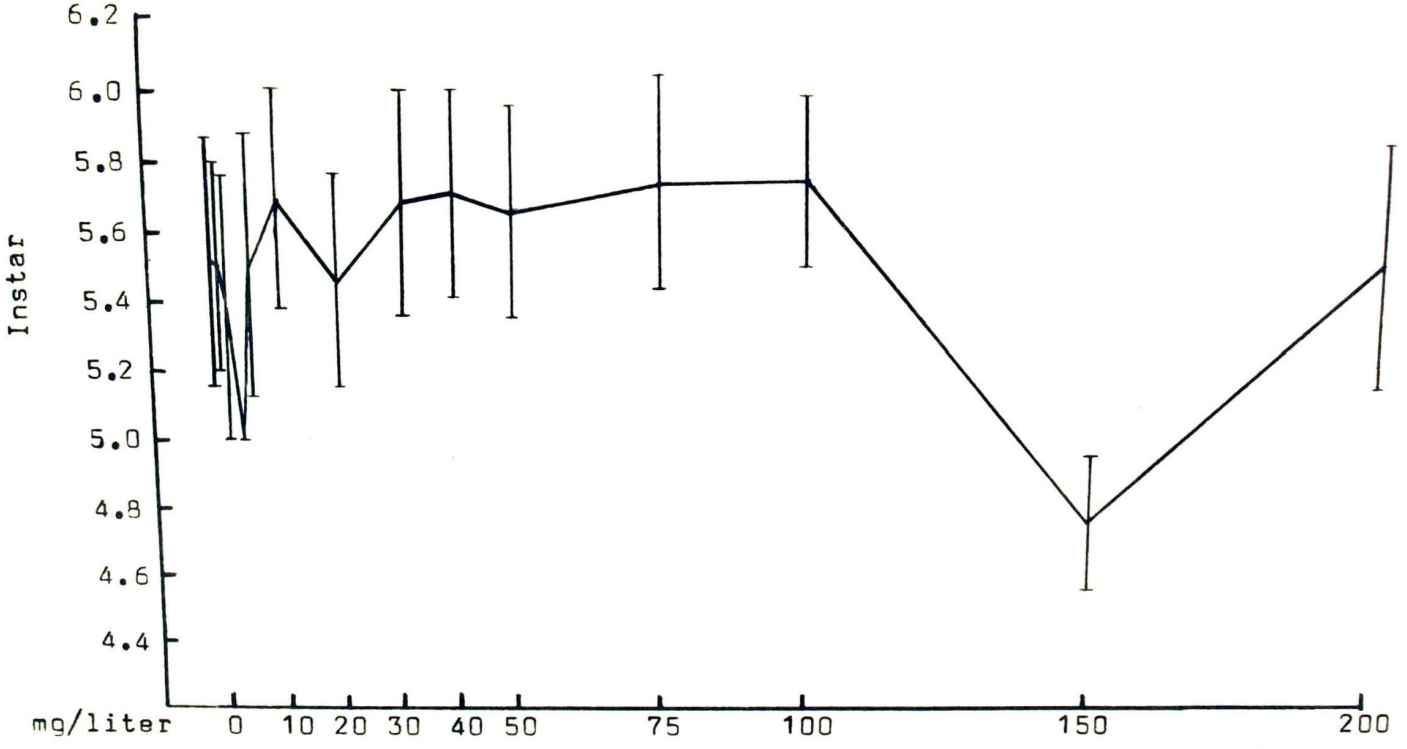


Figure 4: Average instar and standard deviation of primiparous D. magna in various concentrations of 2,4-D

deviation were determined.

The molts of each animal in the growth and reproduction tests were recorded every twenty-four hours. Recorded in Table VI are the chi-square values to determine if there was a significant difference in the rate of ecdysis. The comparison was for a five day period. Each concentration was compared against its control.

TABLE VI

CHI-SQUARE VALUES FOR NUMBER OF MOLTS IN THE FIRST FIVE DAY PERIOD

Test K		Test M		Test F	
mg/liter	chi^2	mg/liter	chi^2	mg/liter	chi^2
40	.953	40	11.465	1	34.669
30	.926	30	7.558	2	.698
20	.0	20	10.010		
10	.359	10	11.041		
5	.654	5	10.710	Test I	
3	.245	3	6.959	mg/liter	chi^2
		2	6.081	150	.901
		1	7.855	100	.295

Test N		Test H		Test J*	
mg/liter	chi^2	mg/liter	chi^2	mg/liter	chi^2
200	4.146	150	.343	300	87.613
100	.112	75	.369	250	22.945
75	9.072	50	.403	200	49.690
50	1.292				

*figured on three days

CHAPTER IV

DISCUSSION

Toxicity tests with cladocerans typically have been used to determine concentrations that immobilize fifty per cent of the population within twenty-four hours or intervals of twenty-four hours. The test animals were unfed. In Figure 1 the data were derived by this method. The IC_{50} value for 24 hours is in excess of 300 mg/liter. The IC_{50} value for 48 hours is 192 mg/liter. Sanders (1966) using Daphnia magna at 21°C, in untreated well water, determined a 48-hour TL_{50} (medium tolerance limit) value of 4 mg/liter for 2,4-D (technical grade dimethylamine salt). This is a tremendous conflict in the amount of toxin needed to immobilize D. magna. Sanders also reports the 48-hour TL_{50} of seed shrimp to be eight mg/liter for 2,4-D and for all other animals he tested (scud, sowbug, glass shrimp, crayfish, and bluegill). He reported that the TL_{50} values would be greater than 100 mg/liter. He reported using ten early instar animals at four to five concentrations with appropriate controls. There was no mention of duplicate runs. The main differences between the author's test and Sanders' work are the test medium and the number of test subjects. This author believes

that with 1233 test subjects and 197 controls and ten concentrations, the results are valid for this situation.

From Figure 1 two other IC_{50} values were derived. The 72-hour IC_{50} was 156 mg/liter; the 96-hour IC_{50}, 144 mg/liter. The use of unfed animals for testing is supposed to eliminate one of the many variables. Investigators using cladocerans have used a wide choice of media because there is little known about the nutritional requirements of D. magna. Media have contained everything from egg yolk to herring meal. It is obvious that lake, river, or any natural waters are going to present many variables with which to contend. One solution is a synthetic medium made from distilled water and inorganic salts. Freeman (1953) developed a "Standard Reference Water." Frear and Boyd (1967) refined and simplified this medium and called it "Standard Test Medium." It would be ideal if this medium, or another medium were used by all investigators performing cladoceran bioassays.

In Figure 2 the IC_{50} value for fed animals at 144 hours was 138 mg/liter. Any shorter time period was greater than 300 mg/liter or indeterminable at the fifty per cent immobilization level. There were not any IC_{50} values that reached the fifty per cent level at the 168-hour, 192-hour, and 216-hour time periods. The closest IC_{50} value for the unfed animals in Figure 1 was 144 mg/liter at 96 hours. It appears that feeding daphnids during the testing period reduced the immobilization rate. This may be due to the uptake

of 2,4-D by the alga and yeast; but if this were true, one would expect the daphnids to still be exposed to the toxin at even greater concentrations since the daphnids filter feed continuously. Feeding the animals added a new variable to IC_{50} testing, but a variable that can be standardized and one that is necessary for lengthy testing. A standardized alga suspension and a yeast suspension were used. An equal proportion of these suspensions was used to minimize differences in each vial.

Table III presents the chi-square values for the unfed IC_{50} tests shown in Figure 1. The only point on the 24-hour line that is significant is at the one per cent level for 300 mg/liter. For the 48-hour line two values are negative. For 50 mg/liter the chi-square value is negative and significant at the one per cent level. At 100 mg/liter it is a negative value significant at the five per cent level. The other values on the 48-hour line from 150 to 300 mg/liter are significant at the one per cent level. On the 96-hour line the value for the 50 mg/liter concentration was significant at the five per cent level. Five and 100 mg/liter concentrations were not significant and 150 through 300 mg/liter concentrations were significant at the one per cent level. A possible explanation for the non-significant levels was biological variability in the test population. The only significant value in the 24-hour period was at 300 mg/liter. The values through the range of 20 to 250 mg/liter were not significant. Since molting occurred

at 39.5 to 46.3 hours at 20°C (Findley, 1969), it appears that young daphnids were more susceptable at molting rather than in the first instar.

The IC_{50} tests with unfed animals with 2,4-D concentrations of 20, 30, and 40 mg/liter were not plotted on Figure 1. Of the twelve values, six were negative and three of these were significant. These twelve values, if plotted, would not contribute to the main time line from which the IC_{50} values were interpolated. The reason for the negative values is that the death rate of the controls exceeded that of the test subjects. The exact cause of the significant difference in the negative numbers is unknown, but a possible explanation is that 2,4-D may have enhanced the environment of the vial at low concentrations. The reduction of the surface tension appears to be a possibility. There was one test that was not included in any of the previous results and calculations because it had one animal per vial whereas the remainder of the unfed experiments had two animals per vial. Except for this factor, this test was identical to the other IC_{50} tests with unfed animals. Below are the per cent of animals floating at 24 hours in this previously unreported test.

mg/liter	per cent
0	67
20	24
30	0.03
40	0.03
50	17

The floating animals led to a higher mortality rate

because daphnids actually have a water-repelling carapace; however, once caught in the surface tension they are doomed.

The per cent of animals floating in the previously reported tests of unfed animals at 24 hours are as follows:

mg/liter	per cent
0	
20	49
30	26
40	11
50	21
75	7
100	4
150	5
200	2
250	1
300	3
	3

It is obvious that as the concentration increased, daphnids were caught less often in the surface tension. This factor alone could have increased control deaths so that negative values were obtained in many cases.

Frear and Boyd (1967) reported the per cent mortality of daphnids in their medium. The tests were run with unfed animals. These values are recorded below. Frear and Boyd did not report the number of test subjects. Also recorded below are the author's per cent of immobilization of six control tests (IC_{50} tests of unfed animals) with a total of 197 animals.

Time	n	24	48	72	96
Frear & Boyd	?	0	14*	28	--
Author	197	.51	6.6	19.8	27.92

*Recorded at 58 hours

From the chart one can see that in all cases, except at 24 hours, the author's controls survived better than those of Frear and Boyd. Therefore, an unduly large number of deaths could not be contributed directly to the medium.

Table IV presents the chi-square values for Figure 2. None of the negative values is significant. The first value is 100 mg/liter at 168 hours and is significant at the five per cent level. For 150 mg/liter there are values significant at the one per cent level at 120 to 168 hours. At 200 mg/liter, twenty-four through 168 hour values are significant at the one per cent level. For 250 mg/liter there is a one per cent significant value at 72 hours and for 300 mg/liter values for 24 and 48 hours are significant at the one per cent level.

Table IV presents the chi-square values for Figure 3. All the values from 120 to 360 hours are negative but are not significant. All the values for 2 mg/liter are negative with the exception of the 120-hour which is significant at the one per cent level. Four other values from 265 to 336 hours are significant but as negative values. These are the only significant values in the range from one to 300 mg/liter. Only one is at the one per cent level, i.e. at 312 hours. At 3 mg/liter significant values are found at 120 to 192 hours. There are non-significant negative values in 5 mg/liter at 24 and 72 hours. Also in 5 mg/liter there are significant values from 120 to 192 hours. For 10 mg/liter there are non-significant values at 24 and 72 hours,

and significant values at 216 hours. At 20 mg/liter there are negative values at 24, 72, 96, and 120 hours of which none are significant. For 30 mg/liter there are significant values at 96 to 144 hours. No values are significant or negative at 40 mg/liter. In Figures 2 and 3 none of the negative values were significant. Even though there was a large number of test animals, this number is low in comparison to a natural population which occurs in large numbers. Perhaps the test population should be of an infinitely larger number of animals to rule out the possibility of biological variability affecting the results.

Table V presents the chi-square values for the rate of ecdysis in a five-day period. There are complete tests such at Test M in which all values from one to forty mg/liter are significant, and Test K at three to forty mg/liter with none of the values significant. After looking at the remainder of the data, it seems that no significant conclusions could be made.

Figure 4 presents the average instar and standard deviation of primiparous D. magna. The mean instar at 3, 100, and 150 mg/liter were significantly different than the controls. There were only two primiparous adults at 3 mg/liter, and for 150 mg/liter there were only four. Such low numbers are not desirable to work with because they do not represent the actual population's true response. For 100 mg/liter there were twenty-eight primiparous adults with a mean of 5.75 instars. At 75 mg/liter with thirty-

one animals the mean instar was 5.74. The 5.75 instar mean was significant. It would be difficult to say that 2,4-D had any effect on ecdysis since there seems to be no definite pattern in Figure 4.

Reproduction rates are as important as immobilization rates. This investigation failed to produce the data needed to give adequate analysis of 2,4-D on reproduction. Findley (1969), working with amino triazole, showed that the twenty-six hour IC_{50} value was 26.75 mg/liter for D. magna, but at 3.5 mg/liter, one hundred per cent of the animals lost their eggs. It is obvious that there is a need for further research of the reproduction of fish-food organisms such as D. magna. Researchers should also review the established IC_{50} values which have been determined for pesticides and investigate the effects of these pesticides on reproduction. It is possible that Frear and Boyd's medium is not the proper medium for long range testing. Even with the nutrients contained in the alga, the combination or the proportions may have been lacking in some area that led to shorter lives and to varied rates of reproduction.

The original problem was to include a study of the number of young produced. In this research that number was so varied that the author believes that no valid conclusions could be made without further testing. A look at the n value on Table V will demonstrate that variability of primiparous adults. The low rate of production is puzzling when one considers that the populations in the one gallon jars flour-

ished. From the time the culture arrived and throughout all the research, the population reproduced continuously. During the investigation a small number of subcultures was started from young daphnids not used in experimentation. These were sufficient to restock the reproducing populations. These subcultures were maintained in identical situations and fed the same food. They had little trouble reproducing.

The test animals were maintained in the same incubator as the reproducing population, but the test daphnids were in small shell vials whereas the reproducing population was maintained in gallon jars. Obviously, the amount of surface area between the vials and jars was different, but Findley (1969) did not encounter any problem in raising single D. magna in identical vials. The food and medium came from identical containers, but one possibility is contamination of the vials. The vials were scrubbed then soaked in fifteen to twenty per cent hydrochloric acid, then rinsed and soaked and rinsed again in tap water and allowed to dry. The possibility of 2,4-D remaining is small, but it was possible that some chlorinated compounds would remain, but not chlorine gas. To alleviate this problem, perhaps a distilled water rinse should have been used.

Summary

An investigation of the effects of 2,4-D on D. magna was undertaken. IC_{50} values were determined for unfed D. magna as well as fed D. magna. It appears that D. magna are

less susceptable to 2,4-D when they are fed or at least when they are maintained in the presence of yeast and Scenedesmus obliquus. There was conflicting significance in the data of the rate of ecdysis in a five-day period. No attempt was made to draw any conclusion in the rate of reproduction, for the data were too limited. There was little change in the average instar for primiparous adults although there were some significant values. It appeared that 2,4-D did affect the surface tension and decreased the amount of daphnids entrapped in it.

To enable investigators to compare results, tests should conform to a standard. Temperature ranges, light, media, age ranges, and feeding should be standardized. Tests must be run to approximate conditions found in nature for useful figures to be obtained.

LITERATURE CITED

Anderson, B. G. 1932. The number of pre-adult instars, growth, relative growth, and variation in Daphnia magna. Biol. Bull. 63:81-98.

Anderson, B. G., and J. C. Jenkins. 1942. A time study of events in the life span of Daphnia magna. Biol. Bull. 83:260-272.

Brooks, A. S. 1957. The systematics of North American Daphnia. Mem. Conn. Acad. Arts Sci., Conn. 13:1-180.

Dewey, J. E., and B. L. Parker. Mass rearing of Daphnia magna for insecticide bioassay. J. Econ. Entomol. 57:821-825.

Doudoroff, P. 1951. Bio-assay methods for the evaluation of acute toxicity of industrial wastes to fish. Sewage and Industrial Wastes. 23:1380-1347

Findley, Diane I. 1969. Effects of amino triazole weed-killer on selected aspects of the life history of Daphnia magna. Ph.D. dissertation. University of Tennessee, Knoxville, 51 p.

Frear, D. E. H. 1972. Pesticide handbook-entoma. College Science Publishers, Pennsylvania. 253 p.

Frear, D. E. H. and J. E. Boyd. 1967. Use of Daphnia magna for the microbioassay of pesticides. J. Econ. Entomol. 60:1228-1236.

Freeman, L. 1953. A standardized method for determining the toxicity of pure compounds to fish. Sewage Ind. Wastes. 25:848.

Freeman, L., and I. Fowler. 1953. Toxicity of combinations of certain inorganic compounds to Daphnia magna. Sewage Ind. Wastes 25:1191

Hutner, S. H., L. Procasolig, A. Schatz, and C. P. Hauskin. 1950. Some approaches to the study of the role of metals in the metabolism of microorganisms. Proc. Amer. Philo. Soc. 94:152-170

MacArthur, J. W., and W. H. T. Baillie. 1929. Metabolic activity and duration of life. I. Influence of temperature on longevity in Daphnia magna. Jour. Exper. Zool. 53:221-242.

Pennak, R. W. 1953. Freshwater invertebrates of the United States. Ronald Press Co., New York. 769 p.

Sanders, H. O. 1970. Toxicities of some herbicides to six species of fresh-water crustaceans. Water Poll. Con. Fed. J. 42:1544-1550.

Starr, R. C. 1964. The culture collection of algae at Indiana University. Amer. J. Bot. 51:1013-1044.

Weed Society of America. 1967. Herbicide handbook. W.F. Humphrey Press, Inc., New York, 293 p.

Wojtalik, T. A., T. F. Hall, and L. O. Hill. 1971. Monitoring ecological conditions associated with wide-scale applications of DMA, 2,4-D to aquatic environments. Pest. Mon. J. 4:184-202.