NEGATIVE PHOTOTAXIS OF MOSQUITO LARVAE AS A POTENTIAL TOOL IN THE RAPID BIOLOGICAL MONITORING OF AQUATIC WASTES (DIPTERA : CULICIDAE)

by

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INTRODUCTION

> "Toxicity testing is an exploratory study and must be as versatile and unlimited as the conditions and situations to which the study is to apply." V. M. Brown (1973:93)

Biological assays provide an important tool to evaluate the effects of toxicants on aquatic life under controlled conditions. In most fields, "bioassay" refers to tests which emphasize the comparison of a given response to that evoked by a standard stimulus (Hoskins and Craig, 1962). But in pollution biology, the term is used to describe toxicity tests on effluents. Of primary interest is not the exact nature and magnitude of the toxic conditions; rather, it is an assessment of potential hazards to aquatic life, and an indication of when toxic conditions are developing or have passed (Brown, 1973).

Most recent approaches have emphasized a continuous and/or rapid monitoring capability, and typically the systems have involved fish (Sprague, 1969, 1971, 1973; Heath, 1972; Cairns, et al., 1973a, 1973b). Increasingly, organisms at other levels of organization are being investigated for their potential as monitors, including bacteria (Boudre and Krieg, 1974), algae (Patrick, 1973; Weber, 1973b), and rotifers (Buikema, et al., 1974). In fact, as several authors have pointed out, it is likely that an entire family of biological monitoring methods will be developed to permit versatility in the assessment of water quality under diverse conditions.

Aquatic insects have generally been eschewed in monitoring work because of methodological difficulties. For example, (a) aquatic insects are often awkward to handle, (b) it is necessary to have a continuous supply of test insects, and (c) there is a paucity of suitably quantifiable responses. Careful selection of the test insect, however, can minimize such problems, particularly if a quantifiable response to the stimulus (i.e., pollutant) of interest can be found.

Negative phototactic behavior, exhibited by many insects, is probably one of the more easily investigated quantifiable responses. *Aedes aegypti* L. larvae (Diptera: Culicidae) were selected to investigate the potential of this type of response. They are favorable insects for this work because:

(a) they react vigorously to directional light stimuli;

(b) they are comparatively easy to rear in the laboratory;

(c) handling is relatively simple as the eggs can be dried, stored for several months, and readily shipped;

(d) there is a potential of obtaining eggs from many laboratories throughout the country and world (cf. Gerberg, 1970); and

(e) a broad range of information is available on the structure, function and physiology of *A. aegypti* larvae and adults.

Objectives: The intent of this work was: (a) to develop an exploratory experimental design permitting studies of negative phototaxis by larval *Aedes aegypti* in selected toxicants; (b) to develop a straightforward technique for analyzing the data obtained; (c) to

assess the potential of the photomigratory response as the basis for a rapid biological monitoring system; (d) prior to the photomigration trials *per se*, to assess the acute toxicity of the test solutions, and the effect of the solutions on larval development. In this study, the only toxicants considered were solutions of zinc or copper. Those metals were of particular interest because they are widespread in polluted watercourses and are recognized as belonging to an especially dangerous class of environmental contaminants (Hynes, 1960; Skidmore, 1964; Buhler, 1973).

REVIEW OF THE LITERATURE

The majority of the work on pollutant bioassay has been done on fish (Fujiya, 1964; American Public Health Assoc., 1971; Sprague, 1969, 1971, 1973), but many of the methods described can be applied to aquatic insects as well (Weber, 1973a). Reports on the use of aquatic insects in pollution bioassay *per se* are scarce, and most of the techniques which have been developed were aimed at elucidating the water quality requirements (Bell, 1971; Gaufin, 1960, 1971, 1973a, 1973b) of certain aquatic insects.

There is a comparatively enormous fund of bioassay literature primarily concerned with mosquitoes and insecticide toxicology (Dolby and Corbeau, 1962; Hoskins and Craig, 1962; American Mosquito Control Assoc., 1968; Busvine, 1971). A computerized mosquito data bank (MODABUND) has been developed at the University of Notre Dame to stay abreast of the literature (Crovello, 1972). Certain species, particularly *Aedes aegypti* L., have been the object of many investigations. The book by Christophers (1960) compiled much of the older information available on the life history, bionomics, physiology and structure of *A. aegypti* in all stages.

Types of Stimulus Responses: In a toxicity test, the response of an organism may be assessed by knock-down or mortality, or by any number of sublethal toxicological effects which result in quantifiable

physiological and/or behavioral changes. Mortality, the end point in acute toxicity tests, often is subject to uncertainty because of: (a) differences between observers as to the criteria of death, (b) recovery after apparent death, and (c) persistence of a moribund condition over a variable time period. Partly as a consequence of these uncertainties, increasing emphasis is being placed on the search for sensitive sublethal responses. Tarzwell and Gaufin (1953) were among the first to point out that temporary survival of aquatic organisms is meaningless if they have aberrant behavior patterns, fail to reproduce, have poor growth rates, become deformed, or are not able to function properly in other respects.

There are surprisingly few studies of performance (e.g. locomotion) as affected by pollutants (Sprague, 1971). The best methods of sublethal bioassay, according to Cairns (1966:563), are: (a) the measurement of the ability to swim a specified distance per minute, and (b) measurement of respiration. Both methods can be quantified objectively and with certainty, thus satisfying an important criterion set forth by Hoskins and Craig (1962:449) for proper bioassay procedure.

Phototactic Swimming Response: Many aquatic insects exhibit oriented swimming movements in response to light. Because the phototactic response is part of an adaptive complex directed at survival of animal populations, it is well-suited for toxicological investigations.

While the precise definition of phototaxis is dependent on the research interests of the investigator, Rockwell and Seiger (1973)

proposed this working definition: "Phototaxis is a complex behavioral response to light that begins with the photoreceptor and proceeds through a chain of events that culminates in the locomotion.. of the organism." Depending on the basic direction of the taxis, it is referred to either as a positive, negative, ventral, dorsal, or lateral light reaction (Jander, 1963). Rockwell and Seiger (1973) provided a useful conceptualization of the sources of variation in the phototactic response. They discussed the effects of experimental design and of environmental factors operating prior to and during a test. In addition, they emphasized the genetic aspect of the variability in phototaxis observed among individuals of a given responding population.

One of the most characteristic behavioral traits of larval _Aedes aegypti_ is their intense negative phototaxis (Christophers, 1960: 237). With an experimental design making use of this trait, Burchfield _et al_. (1952) and Burchfield and Hartzell (1955) showed that measurement of larval migration away from a directed light source provides an objective and sensitive means of insecticide bioassay. The technique is based on the assumption that in the presence of toxic materials the larval response is reduced long before death would occur. This principle has been used in investigations on insecticidal activity (Turnipseed and Reed, 1963; Godwin, _et al_., 1965; Beesley, 1972; Kumar and Burkhard, 1972), but not in the field of water pollution control.

Previous reports on the toxicity of metals to mosquito larvae seem to have been limited to screening tests for larvicides. Suzuki

(1959, *in* Novak and Bouda, 1968) found that copper was more toxic than zinc to larvae of Culex pipiens ssp.; $CuSO_4$ was sixth, and $ZnSO_4$ was eleventh in order of toxicity of eleven metals texted.[1/] Novak and Bouda found $CuSO_4$ to be fourth in toxicity of nine metals screened.[2/]

[1/] $AgNO_3 > HgCl_2 > CdCl_2 > CoCl_2 > CuCl_2 > CuSO_4 > SrCl_2 > BaCl_2 > NiCl_2 > MnCl_2 > ZnSO_4$.

[2/] $HgI_2 > AgNO_3 > Cu(NO_3)_2 > CuSO_4 > CdSO_4 > CdCl_2 > CoCl_2 > Co(NO_3)_2 > (NH_4)_2MoO_4$.

PROCEDURES AND MATERIALS

Rearing:--The mosquito eggs used to start a laboratory colony were originally obtained from the laboratory of J. C. Jones, at the University of Maryland, College Park, Maryland. Information on various aspects of mosquito rearing were obtained from Barbosa and Peters (1969), Christophers (1960), Gerberg (1970), Greenough *et al*. 1971), and Peters *et al*. (1969).

About 300 adult mosquitoes in a small cage (35x25x25 cm) provided an adequate continuous supply of eggs for maintaining the colony and for performing various experiments with the larvae. A rabbit was placed on top of the colony cage (Fig. 1) to feed the adults on a regular basis (usually once a day for about 15 minutes). A 10% sucrose Solution was provided continuously by inserting a vial of the solution with a cotton wick through a hole in the side of the cage. The colony cage was kept in a rearing room held at 27° ± 2° C and 80% ± 5% RH.

Oviposition occurred on 9 x 11 cm strips of brown paper toweling, which lined the inside of a 25-dram plastic vial.[1/] The vial, containing 40 ml water, was inserted at a slant, from the outside, into a hole in the side of the cage. In this way, 17 cm of air-water interface was available along the toweling for egg-laying. The vial

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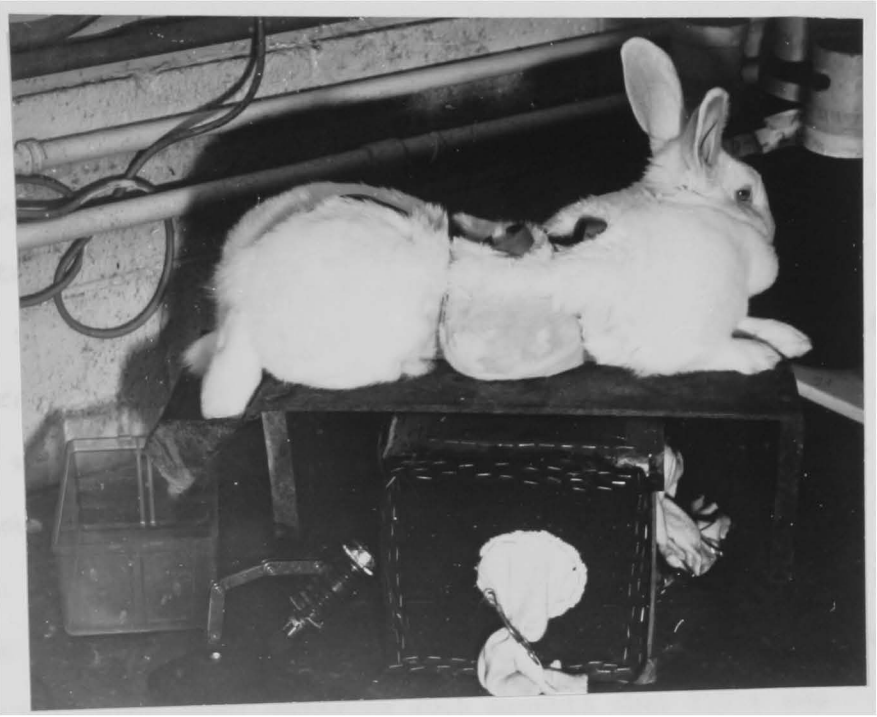
[1/] Thornton Plastic Co., Salt Lake City, Utah.

Fig. 1. Restrained rabbit in position to feed the adult mosquitoes.



remained in the cage for no more than 48 hours at a time.

The eggs were then handled and stored as outlined by Gerberg (1970). Each egg paper was dated to ensure that eggs used for tests were not over eight weeks old. Before the eggs were hatched, they were examined under a dissecting scope to ensure that most were fully embryonated, thus assuring reasonable hatching success. Unsoftened tap water was aged for one day and CO_2 was bubbled through the water for 10-15 minutes to reduce the dissolved oxygen in the water, which encouraged prompt hatching (Barbosa and Peters, 1969; Christophers, 1960; Gerberg, 1970). The eggs were allowed to hatch in this water for 60 to 90 minutes. Usually 80-90% of the larvae hatched in that time. The larval rearing containers used were white enameled trays (29x16x5 cm), to which 1000 ml of water was added. An attempt was made to keep the density of the larvae in the solution at about 2 larvae/cm., a density recommended by Greenaugh _et al_. (1971).

Those larvae intended for colony maintenance were fed Fleischmann's(R) dry activated yeast daily in such amounts that there was always a slight excess of yeast but a clear medium. The larvae destined for the photomigration tests were fed according to a schedule given in Gerberg (1970:54) using a small spatula delivering about 50 mg of yeast per scoop. The rearing trays were kept in chambers held at 27° $\pm$ 3° C, 80% $\pm$ 5% RH, and with a 12 hour photoperiod. Total hardness of the water used for rearing varied between 247 and 290 mg/l as $CaCO_3$; total alkalinity was approximately 120-138 mg/l as $CaCO_3$; dissolved oxygen (D.O.) varied from 5.8 to

8.4 mg/l; free CO_2 from 0 to 20.0, and pH from 7.5 to 8.2

This procedure brought the larvae to the last instar on the fourth day following the hatch, and by the sixth or seventh day, 85 to 95 per cent of the larvae had pupated. Thus, the larvae were fairly uniform in size throughout the growing period, and were used for experiments whenever needed.

For replenishment of the adult colony, fourth instar larvae about to pupate were transferred to an emergence container. This container consisted of a black-painted rigid plastic box[1/] (14x18.3x 8.3 cm) and a pint food carton[2/] modified with a screen top which was inserted into the lid to trap the emerging adult mosquitoes (Fig. 2).

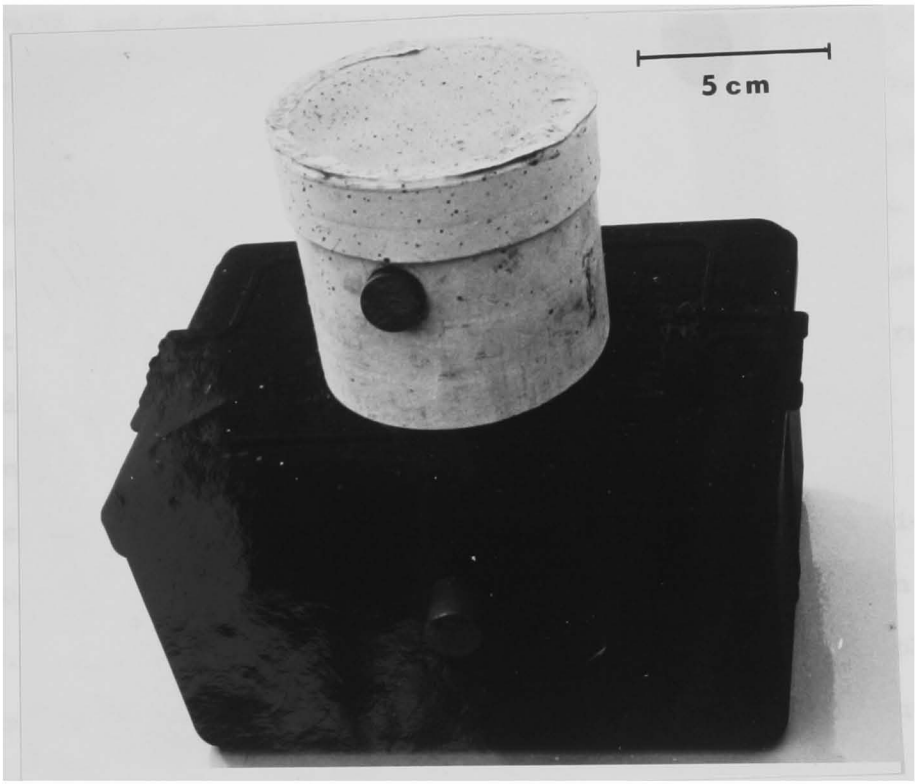
To expedite larval counts in the experiments which follow, a pipette transfer method was developed: the larvae were concentrated in a shaded corner of the rearing tray, an aliquot was removed with a 25 or 50 ml pipette (aperture cut to 4 mm), and a count of the actual number of larvae per aliquot was made. The precision of this technique was established by computing the coefficient of variation in the number of larvae transferred in eight successive trials. (cf. Table 5).

Dilution Water and Stock Solutions: In all toxicity tests performed in this study, a "synthetic"hard water (SHW) proposed by Cairns (1969)

[1/] Tri-State Plastic Molding Co., Henderson, Ky.

[2/] Neptune Paper Products, Inc., Jamaica, Long Island, N.Y.

Fig. 2. Rearing chamber into which 4th instar *A. aegypti* larvae and pupae were placed for adult emergence.


5 cm

was used. The composition of the water (Table 1) was modified by the omission of silicic acid (H_2SiO_3), which was originally included primarily for the benefit of diatoms (Cairns, personal communication). A concentrated (100x) stock solution was prepared by dissolving the reagent grade chemicals in 20 liters of deionized and distilled water in a 5 gal (ca. 20 l) glass carboy. The low solubility of the carbonates, $MgCO_3$ and $CaCO_3$, required that they be dissolved separately from the other chemicals by bubbling carbon dioxide gas through the water. The carbonate stock was stored in quart (0.9 l) glass jars.

Concentrated stock solutions of zinc were prepared by thoroughly dissolving reagent grade $ZnSO_4 \cdot 7H_2O$ in 900 ml of deionized and distilled water held in quart (0.9 l) glass jars. Stocks were prepared at concentrations of 100, 1000, and 10,000 mg/l. Stock solutions of copper ($CuSO_4 \cdot 5H_2$) were prepared similarly. The constancy of the zinc stock concentrations was checked by periodically analyzing samples by atomic absorption spectrophotometry in the V.P.I. & S.U. Center for Environmental Studies. Zinc was emphasized in this study, though some "exploratory" bioassays were conducted with copper.

The chemical parameters of the test solutions, once diluted with the synthetic hard water (SHW), were:

total hardness	140 to 147 mg/l
total alkalinity	95 to 103 mg/l as $CaCO_3$
dissolved oxygen	7.0 to 8.4 mg/l
free CO_2	0 to 10 mg/l
pH	6.7 to 7.8

TABLE 1. Chemical Composition of the Synthetic Hard Water (SHW)[1/]

Chemical	Solubility (20° C) g/l	Stock Concentration g/20 l	Concentration When Diluted g/l
KCl	347	20	0.01
$NaHCO_3$	69	40	0.02
$MgSO_4$ $7H_2O$	710	200	0.10
$MgCO_3$	0.1	40	0.02
$CaCO_3$	0.015	220	0.11
NH_4NO_3	192	0.74	0.0037
$FeC_6H_5O_7$ (ferric citrate)	freely soluble	0.08	0.0004

[1/] Modified from Cairns (1969)

Acute Toxicity: To obtain an estimate of the acute toxicity of zinc and of copper against immature *Aedes aegypti*, survival of second, third, and fourth instar larvae was evaluated in a series of metal solutions. No food was added to these solutions during the periods of observation. The larval density for these tests was 100 larvae/200 ml. The extent of the larval mortality and morbidity was judged for each instar by tapping and/or slightly tipping the test containers. This caused a characteristic and vigorous reaction among unaffected larvae. Larvae which failed to react in any way were considered dead. Those with clearly sluggish and uncoordinated swimming movements were recorded as being moribund.

Development: In addition to evaluations for 24 and 48 hour periods, the effects of prolonged exposure were assessed by rearing newly hatched (less than 3 hours old) larvae in various concentrations of zinc to determine the time to initiation of pupation, the number of adults that successfully emerged, or, the instar at which development ceased. The larval density was 25 larvae/100 ml, and the larvae were fed at the rates suggested by Gerberg (1970:54). These tests were replicated twice in time using larvae from different hatches. All tests were conducted in rearing chambers maintained at about 27° C and 85% RH.

Photomigration apparatus: The trough used in this work conformed to "design I" in the classification system of Rockwell and Seiger (1973: 342). They grouped the experimental designs which can be used in the

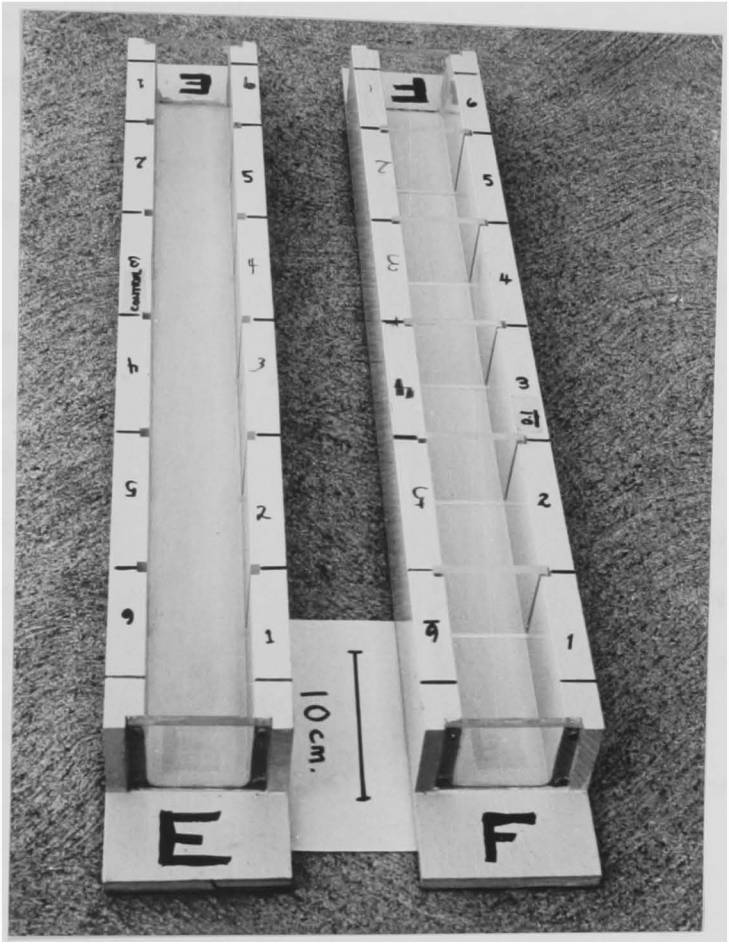
study of phototaxis into three general classes; design I involves a directed light source parallel to the plane of movement, and is thought to be particularly suitable for the study of the locomotory ability of animals responding to light.

The chambers used for the photomigration bioassays were wooden troughs coated with cream-colored epoxy paint (Fig. 3). The inside dimensions were 60x5x5 cm, and the ends were made of clear plexiglass. The troughs were divided into six equal sections by grooves spaced at 10 cm intervals; clear plexiglas barriers could be dropped into these grooves when required. A movable screen barrier could be placed 3 cm from the end. In the experiments, the troughs held 800 ml of a given test solution; the depth of the solution was 2.1 cm.

The lights inducing photomigration were six-watt fluorescent tubes, one at each end of the troughs. The lights were positioned 1 cm below the water line, and centered so that they illuminated two side-by-side troughs equally (cf. Fig. 4). In each photomigration experiment, two sets of larvae were tested simultaneously in the juxtaposed troughs. One trough served as a control chamber, the other contained the test solution. Using six troughs, it was possible to test three concentrations in succession.

Prior to an experiment, the larvae were washed with distilled water, transferred in aliquots (by pipette) to petri dishes, and kept in the dark until used (within eight hours). The overhead lights in the room were off during the tests, and the room temperature was $26° \pm 2°$ C.

Fig. 3. Two of the six troughs used in the negative photomigration trials.


E
F
10 cm.

The aliquot containing the larvae was added to the solution between the 3 cm barrier and the end of the trough. After the larvae had acclimatized for about 10 minutes, the light was turned on, and the barriers in the two troughs were removed. Larval movement along the trough was recorded by flash photography.[1/] Four photographs were taken during each photomigration "run," 15, 30, 60 and 90 seconds after the light was switched on (Fig. 4 a, b, c, d). After two minutes (30 sec after the last photograph), the light was turned off and the barriers at 40 and 50 cm were dropped simultaneously. The number of larvae in the last two sections was recorded.

The runs were repeated with the same group of larvae at intervals of 15 to 30 minutes until 50% or more of the test larvae were unable to migrate at least 50 cm in 120 sec. This cut-off was arbitrary. The object was to find an empirically justifiable criterion by analyzing the photographs, a criterion which would allow inactivation to be followed to at least the 70% point.

A replicate consisted of several successive migration runs performed on one test concentration and its respective control. For the first run of each experiment, the light nearest the end where the larvae had been introduced was turned on. For each following run, the light at the end towards which the larvae had last migrated was used. This eliminated the necessity of repeatedly returning the larvae to

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[1/] Two types of fine-grain films were used: Kodak Panatomic-X and an exceptionally fine-grain, thin emulsion film, H & W Control VTE Pan (H & W Co., St. Johnsbury, Vt. 05819, U.S.A.).

a single point of release, and thus lessened handling-induced trauma among the larvae.

Analysis of data and presentation of results: Usually one roll of 36-exposure film was sufficient to record each replicate. The photographs were placed in a film-strip viewer and projected onto a screen to facilitate counting the larvae. The number of larvae in each 10 cm section, as well as the total number in both troughs was determined, and the numbers in each section were converted to percentages of the respective totals. The results of replicates were averaged for each section and time interval. Histogram interpretations of the larval distribution, as recorded from photographs of the troughs, were based on the percentage distribution of larvae among the 10-cm sections. The histograms in Fig. 5 were derived from Fig. 4. The distribution was totaled over distance to construct cumulative distribution curves (cf. Fig. 10). From each photograph, one cumulative distribution (CD) curve could be constructed. Approximately 100 such curves were constructed during the course of the migration analyses.

From these curves, the position of any desired proportion of the migrating larvae could be estimated by interpolation, and the relative migration rate of the control and of the test larvae could be directly compared. This information was used to establish an objective criterion of toxicity as a prelude to the construction of regression lines and response surves.

Basic statistics, including standard deviations and regression equations, were run on the pooled data for all replicates at each

concentration with the use of a statistical package program, STIL, at the Virginia Tech Computing Center. The text by Sokal and Rohlf (1969) was consulted in the analysis of results.

RESULTS AND DISCUSSION

Acute toxicity: The present acute toxicity tests provided a basis for the photomigration trials in that they gave an indication of how susceptible the *Aedes aegypti* larvae are to zinc and copper. The 24- and 48-hour readings on the lethality of zinc and copper solutions (ranging in concentration from 0.001 to 1000 ppm) are summarized in Table 2. The concentration required to produce a given response increased by approximately one order of magnitude with each larger instar. After 24 hours, the concentration of Cu^{++} required to kill 50% of the 2nd:3rd: 4th instar larvae was 1-10 : 10-100 : 100-1000 ppm Cu^{++} respectively. The larvae were clearly more sensitive to copper than to zinc in all instars. Levels of zinc at least one power of ten greater than those of copper were required to induce equivalent degrees of mortality.

The percentage of all larvae affected by 0.001 and 0.01 ppm Cu^{++}, and 0.001, 0.01 and 0.1 ppm Zn^{++} did not differ appreciably from the controls after 48 hours, but all second and third instar larvae were dead or morbid after 24 hours in 1000 ppm of both metals. Fourth instar larvae, 5 and 20% of which were still alive after 48 hours in 1000 ppm Cu^{++} and Zn^{++} respectively, were clearly the most tolerant of all.

Growth and development: Zinc interferes with growth and survival at levels far below those which are acutely toxic (Table 3). The larval development time increased, and the percent adult emergence decreased,

Table 2. Percent mortality and moribundity[1/] in second, third and fourth instar *Aedes aegypti* with 24- and 48-hour exposures to various zinc ($ZnSO_4 \cdot 7H_2O$) and copper ($CuSO_4 \cdot 5H_2O$) solutions.

TOXICANT	CONC. (ppm)	INSTAR Second 24h	Second 48h	Third 24h	Third 48h	Fourth 24h	Fourth 48h
COPPER [2/]	0.0	0	3	1	1	1	1
	0.001	1	3	0	2	0	1
	0.01	5	12(5)	2	5	0	0
	0.1	19(8)	3(30)	6	9	1	1
	1.0	31(20)	69(25)	11(10)	18(14)	2	3
	10.0	84(15)	98(2)	26(20)	35(28)	16(12)	22(19)
	100.0	98(2)	100	58(35)	90(10)	39(30)	61(24)
	1000.0	100	100	100	100	74(16)	95(5)
ZINC[3/]	0.0	0	5	1	3	0	0
	0.001	0	6	0	4	0	0
	0.01	1	6	1	5	1	1
	0.1	4	11	2	5	0	3
	1.0	9	16	5	13	1	6(2)
	10.0	20(8)	31(20)	6(4)	18(25)	3(6)	10(10)
	100.0	66(25)	100	34(35)	79(12)	20(29)	51(30)
	1000.0	100	100	100	100	60(21)	80(10)

[1/] Numbers in parentheses indicate moribund larvae

[2/] Based on 100 larvae per concentration per instar (single replicate)

[3/] Based on 200 larvae per concentration per instar (two replicates)

Table 3. Effects of continuous exposure to zinc solutions ($ZnSO_4 \cdot 7\ H_2O$) on growth and survival of *Aedes aegypti* larvae. Each value is the mean of four replicates with 25 larvae per replicate.[1/]

Conc. (ppm)	Percent adult emergence	Time to first larval-pupal ecdysis (hrs)
0.0	86.7 [a]	116.8 [d]
0.01	82.5 [a b]	121.5 [d]
0.1	77.5 [b c]	138.8 [e]
1.0	67.5 [c]	145.5 [e]
10.0	34.5 [d]	206.0 [f]
100.0	0.0 [2/]	

[1/] Means for each column followed by the same letter were not significantly different at the 5% level of significance (t-test)

[2/] Only 11 percent reached the third instar; none completed development

with increasing concentration. The test periods ranged up to ten days. There was no significant difference, at the 5% probability level, between the controls and 0.01 ppm Zn^{++}, nor between 0.1 ppm and 1.0 ppm Zn^{++}, but in 0.1 ppm Zn^{++}, both the development time (ca. 139 hrs.) and successful adult emergence (77.5%) differed significantly (5% level) from the corresponding values for the controls (ca. 117 and 86.7% resp.). By contrast, the mortality during the acute toxicity tests in 0.1 ppm Zn^{++} was not significantly different (5% level) from the mortality in the respective controls (Table 2).

Although no larvae pupated in the 100 ppm solutions, 11 percent reached the third instar, which is surprising in view of the 100% mortality observed during the acute toxicity tests with second instar larvae. The discrepancy may be due to adsorption of the comparatively highly concentrated metal (100 ppm) onto the food particles which had been added to the larval medium. The metal concentrations in all test dilutions probably decreased considerably (cf. Warnick and Bell, 1969: 281) because the solutions were not renewed.

Experiments on the development of newly-hatched larvae in toxic solutions may well give a more valid estimate of larval sensitivity than estimates provided by acute toxicity tests. As pointed out by Dolby and Corbeau (1962), the latter type of test does not account for larvae which would eventually either recover or succumb. It was clearly indicated from the two preceding tests that, in static-system bioassays, *A. aegypti* larvae were comparatively tolerant of zinc and, to a lesser degree, of copper.

Larval photomigration: Several factors affecting the photomigratory response of untreated larvae were investigated to set standards for the tests prior to measuring the toxic action of the metals by photomigration. These were (a) the type and intensity of light used to induce migration and (b) the migration rate of untreated free-swimming larvae.

The intensity gradient of the light produced by the 6-watt cool-white fluorescent light ranced from ca. 7500 lux at the point of release (3 cm) to 81 lux at the far end of the trough (Table 4). The difference in the intensity gradients between two juxtaposed troughs illuminated by the same light was considered inconsequential, as was the variation between different lights. Because of the low thermal output of the lights used, the temperature of the solutions did not increase appreciably over ambient room temperature, even after prolonged testing.

First and second instar larvae were not adequately resolved due to limitations of the camera lens used in making the photographic record. Crowding of larvae which had reached the end of the chamber made it necessary that the number of larvae in the end section be determined indirectly, using the counts made from previous frames. The reproducibility of the counts varied slightly from frame to frame, but was usually within 5% of the true value. Inadvertent reflection of the flash from the water's surface occasionally caused difficulties in counting all the larvae in the affected region of the trough. The grooves intended to accommodate the removable plexiglass barriers provided a refuge for a small number of larvae, which were not included

Table 4. Intensity of the light gradient parallel to the longitudinal axis of the photomigration trough (light source: one 6 watt fluorescent tube)

Distance from source (cm)	Luminous intensity	
	lux	foot-candles
0.5	19,250	1,750
3.0 (origin)	7,500	682
10.0	2,170	201
20.0	700	64
30.0	337	31
40.0	162	15
50.0	119	11
60.0 (far end)	81	7.2

in the total counts. The seal of some plexiglass barriers was not always satisfactory as a few of the smaller larvae were able to pass.

The pipette technique delivered larvae for the tests in adequately reproducible numbers (Table 5). The coefficient of variation tended to decrease in a regular fashion from the smallest to the largest stage, except that the third instar larvae were delivered in more variable numbers than the second instar larvae. For the third instar larvae, primarily employed in the photomigration trials, the mean number was approximately 112, with a range from 96 to 127. Within this range the differences in migration rate due to differing larval densities would not appreciably affect the photomigration test results.

Locomotory Ability, Activity and Reactivity[1/] of the Control Larvae: The innate activity level of the larvae was high, even in the absence of any apparent stimuli. This was probably a result of their high food-energy requirements (Christophers, 1960). In low-level, non-directional light, the larvae foraged, often by gliding along the bottom head-first, using their mouth brushes as impellers. They lingered only briefly at the surface to obtain air.

When a point source of light was turned on, a vigorous "escape response" ensued immediately, regardless of the strength of the source The larvae whipped themselves along tail-first with side-to-side lashing movements. It appeared that the speed of locomotion increased

---

[1/] Reactivity is differentiated from activity in that reactivity is a function of response to various features of the environment (Connolly, 1965, in Rockwell and Seiger, 1973).

Table 5. Precision of the pipette technique in delivering immature mosquitoes for the toxicity tests.

Instar	Mean number delivered	C. V. [1/] %
First	141.0(±21.5)	22.1
Second	127.2(±14)	13.4
Third	111.6(±15.5)	19.6
Fourth	98.0(±11)	13.1
Pupae	95.2(±10)	12.8

[1/] Coefficient of variation

with an increase in light intensity until the maximum swimming rate was reached. The extent of this increase was not quantified

The difference in locomotory ability between second, third and fourth instar larvae migrating away from the 6-watt fluorescent light is shown in Table 6. In 120 second, 98.1, 97.0 and 87.4 per cent of the fourth, third and second instar larvae, respectively, had passed 30 cm; 96.3, 90.6, and 78.6 per cent had reached 40 cm; and 93.8, 84.0 and 58.5 per cent had passed 50 cm. The distribution of the larvae became less skewed with greater instar, that is, the older the larvae, the more vigorous and the less variable the response among the population of migrating larvae. Omardeen (1957) demonstrated that the negative phototactic response in A. aegypti larvae increased in the later instars and was strongest in the pupal stage; he thought this to be associated with the development of the imaginal eye. Seldin et al. (1972) showed that the general sensitivity of the lateral ocelli of fourth instar larvae was greater than that of second instar larvae, but that the spectral sensitivity was qualitatively equivalent.

The extent of the difference in photoresponse is depicted in Fig. 6 with the regression lines of the distribution of the second, third and fourth instar larvae after 15 seconds' exposure to the photomigration light. From the curves, it was possible to interpolate the distance attained by 50 per cent of the larvae: (2nd instar larvae, 8.5 cm; 3rd, 16 cm; 4th, 21.5 cm). The distance values were used to derive a "migration rate factor" (MRF) which quantified the

Table 6. Distance migrated by control larvae when exposed to light for different periods of time (mean of six successive runs with ca. 100 larvae each).

Length of Exposure	Percent of larvae in interval (cm)					
(sec)	0-10	10-20	20-30	30-40	40-50	50-60
2nd Instar						
15	29.1	30.4	26.0	8.8	4.7	0.6
30	13.0	29.2	31.5	16.0	7.1	4.2
60	7.8	11.7	15.6	14.1	38.1	12.1
90	2.4	8.2	12.8	19.6	21.3	35.5
120	0.8	3.9	8.4	8.8	20.1	58.5
3rd Instar						
15	23.1	22.3	30.7	18.0	4.5	1.6
30	9.3	21.1	23.1	22.4	12.5	12.4
60	3.0	5.8	16.1	14.8	20.1	40.8
90	1.0	1.8	5.8	8.1	20.4	63.1
120	0.3	0.3	2.0	4.4	8.6	84.0
4th Instar						
15	8.3	27.1	30.0	19.1	13.0	2.8
30	2.0	7.7	9.2	11.2	31.3	38.9
60	1.0	5.0	3.1	9.6	19.9	61.2
90	0.3	1.8	2.0	2.5	14.6	78.2
120	0.2	0.7	1.1	1.8	2.5	93.8

Fig. 4. One series of four photographs recording a single run of a given experiment in 10 ppm Cu^{++}, initiated at 51 minutes. Frame (a) was taken 15 sec., (b) 30 sec., (c) 56 sec. and (d) 86 sec. after the light was switched on.

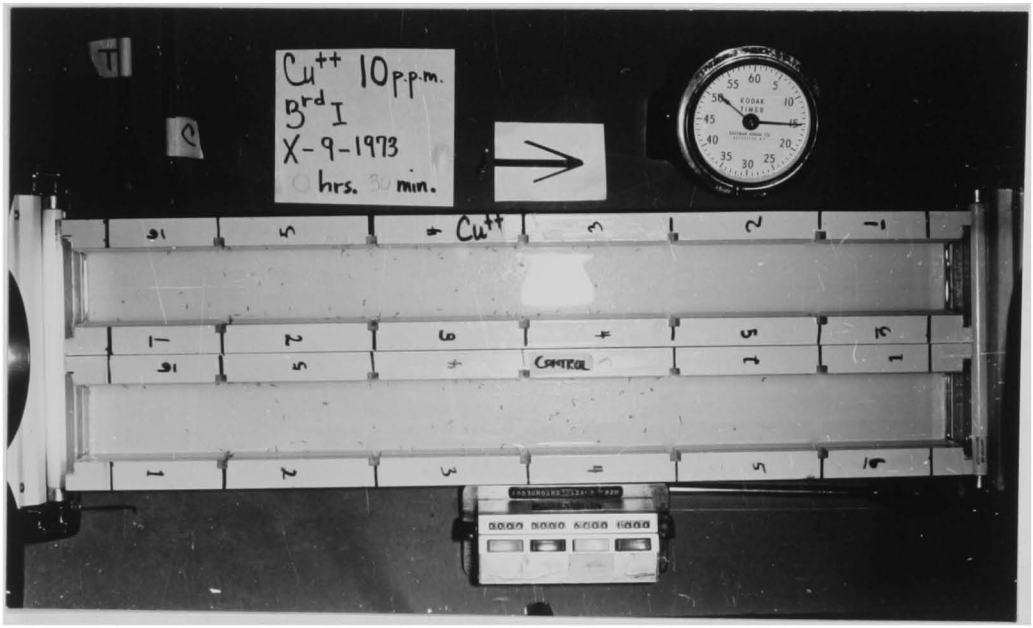

Cu++ 10 p.p.m.
3rd I
X-9-1973
hrs. 30 min.
Cu++
CONTROL

Fig. 4 a

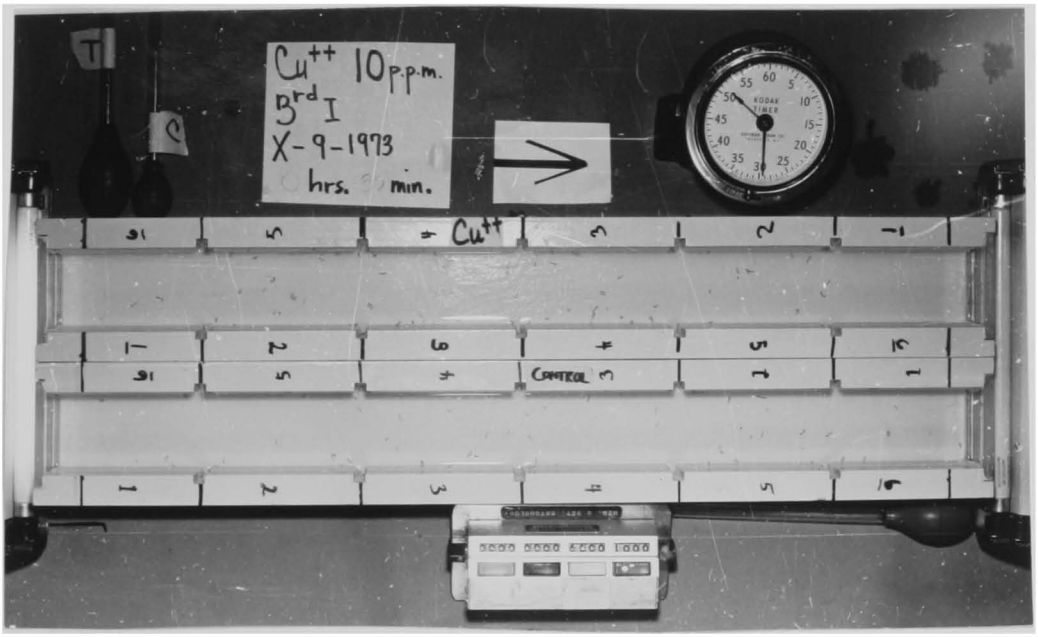

Cu++ 10 p.p.m.
3rd I
X-9-1973
hrs. min.
Cu++
CONTROL

Fig. 4 b

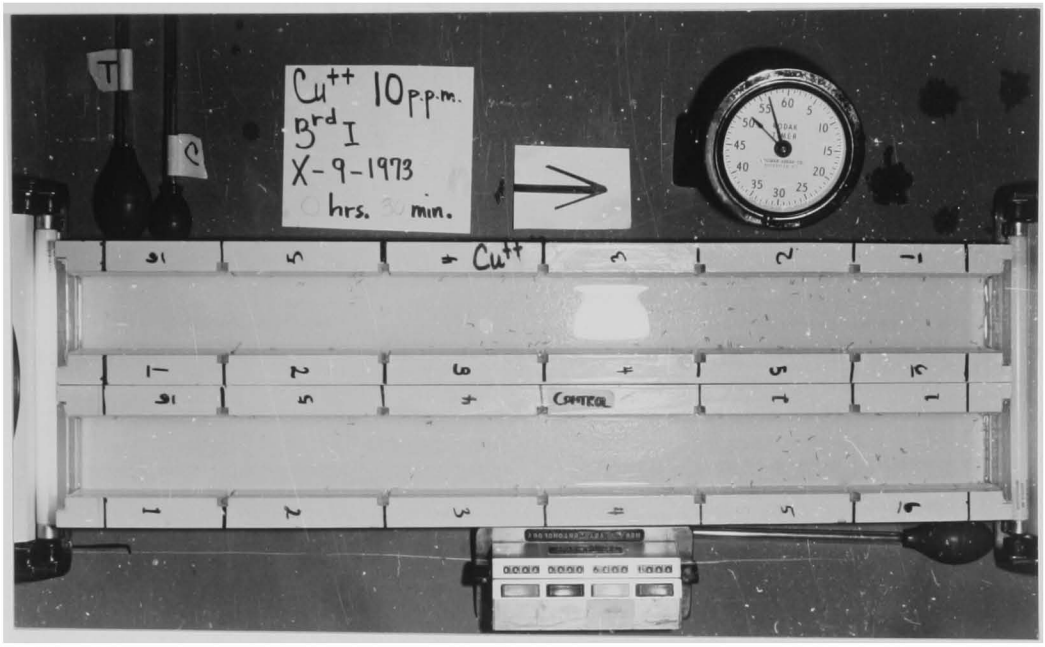

Cu++ 10 p.p.m.
3rd I
X-9-1973
hrs. min.
Cu++
CONTROL

Fig. 4c

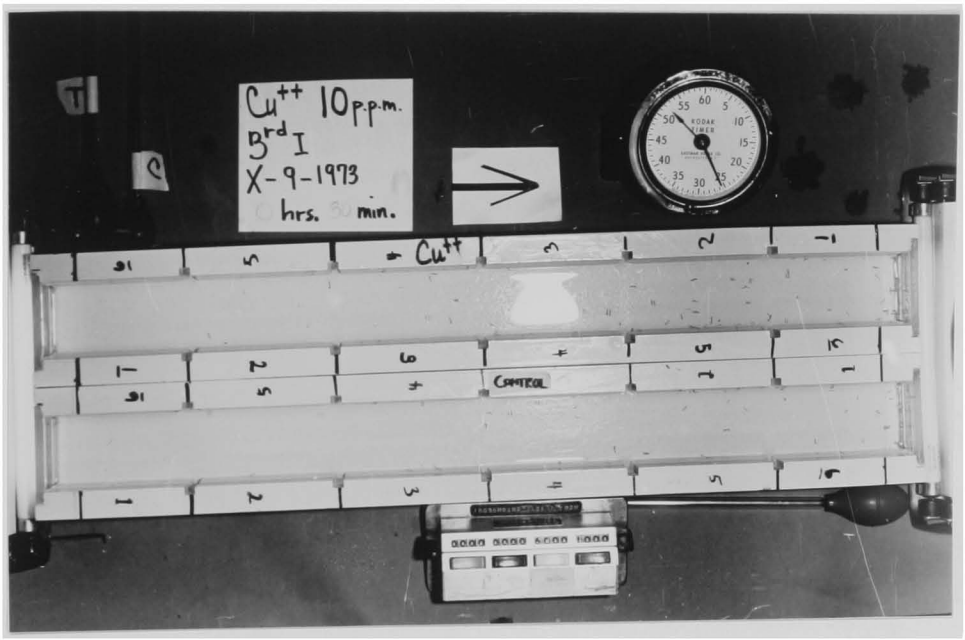

Cu++ 10 p.p.m.
3rd I
X-9-1973
hrs. min.
Cu++
CONTROL

Fig. 4d

Fig. 5. Histograms of the Relative Migratory Rates of the Larvae Shown in the Troughs in Fig. 4.

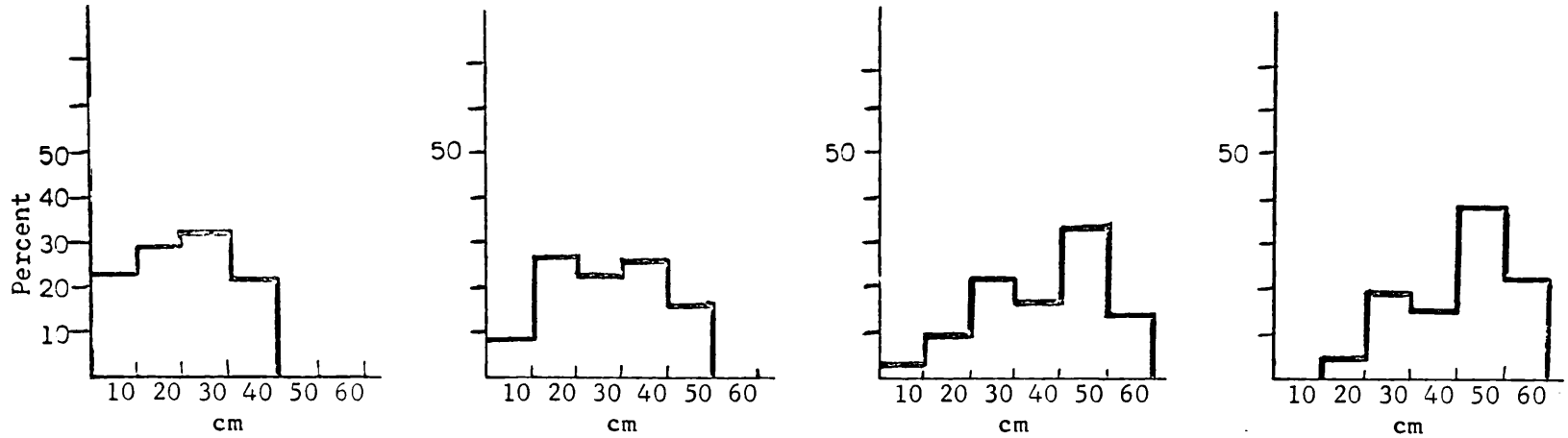
NOTE: The abscissa denotes the incremental (10 cm) distances from section to section in the troughs; the ordinate represents the percent distribution of larvae in the various increments at the times indicated.

Test solution: 10 ppm Cu as $CuSO_4 \cdot 5H_2O$; Run #4; Total time in trough at start of run: 51 minutes.

Time of exposure to light since start of run:

15 sec. 30 sec. 56 sec. 86 sec.

TEST LARVAE



CONTROL LARVAE

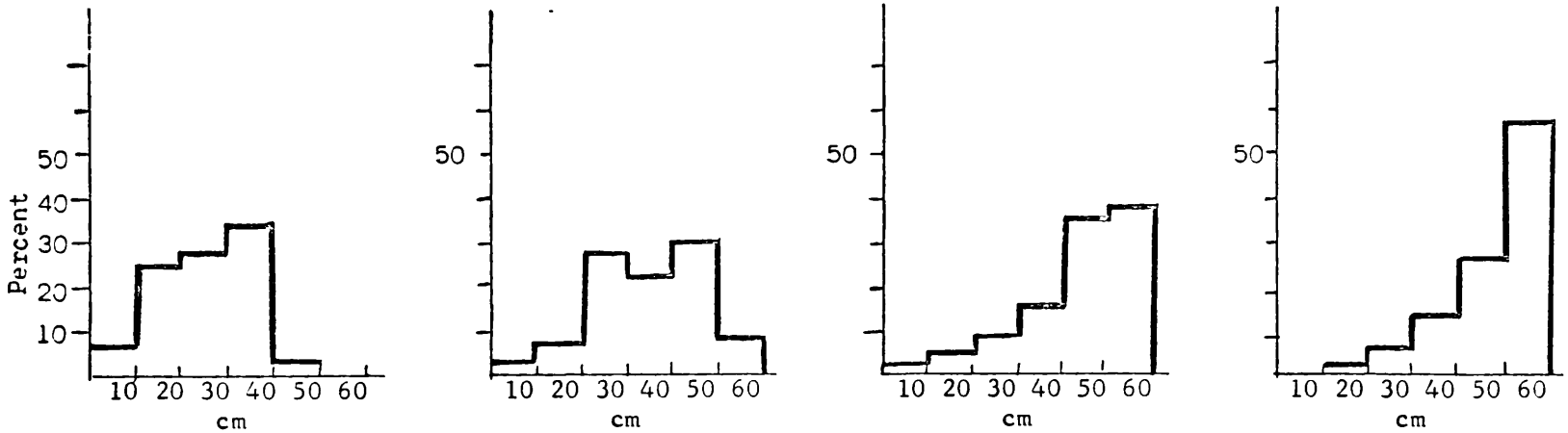
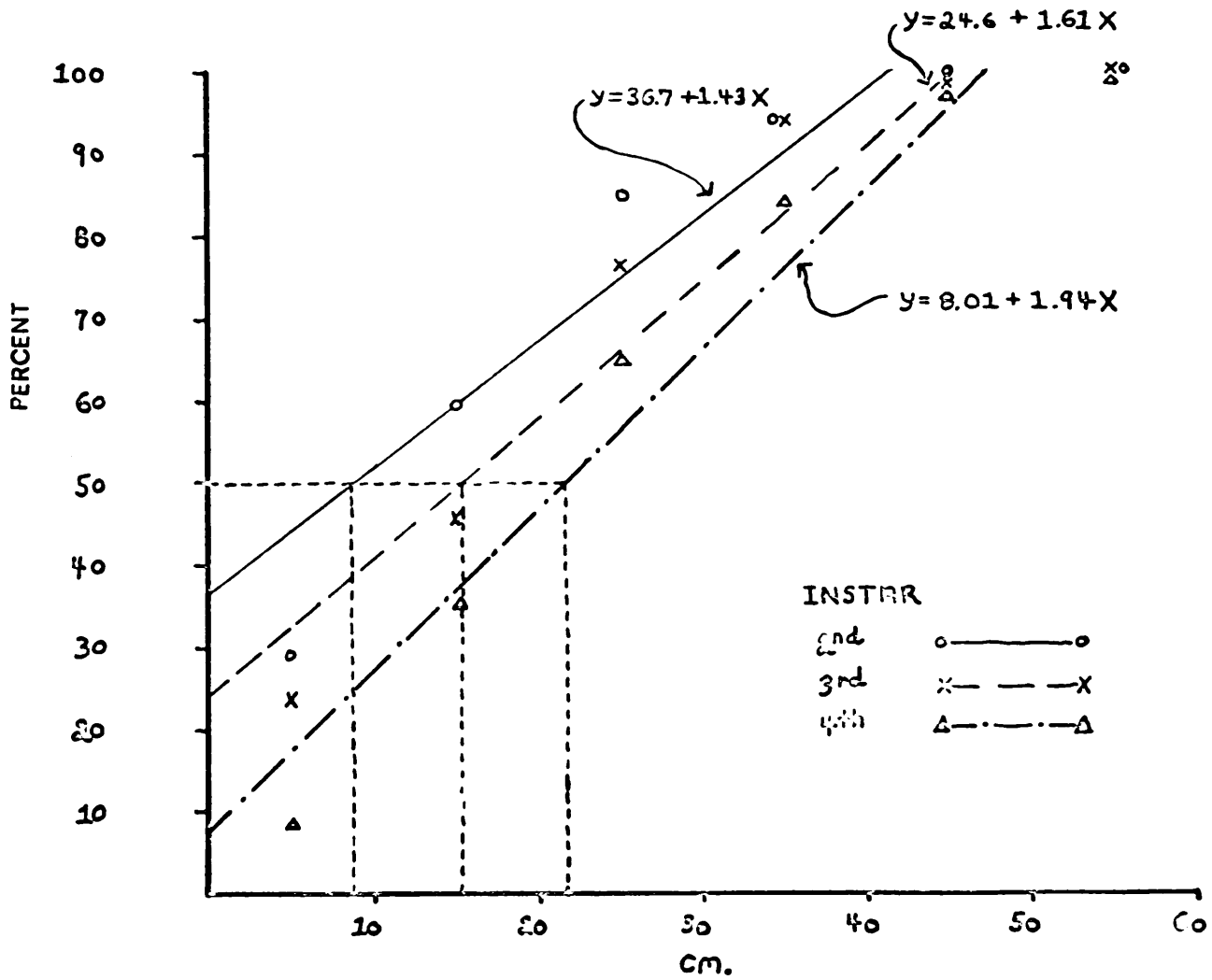


Fig. 6. Regression lines comparing the relative photomigratory ability of second, third and fourth instar larvae, recorded 15 seconds after the light was switched on.


y=24.6 + 1.61 X
y=36.7 +1.43 X
y= 8.01 + 1.94X
PERCENT
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
cm.
INSTAR
2nd
3rd
4th

relative migratory rate of the various instar larvae (Table 7). The units of MRF are cm/sec.

Burchfield et al. (1952) reported that a 100-watt incandescent bulb was not intense enough to induce satisfactory migration among 20 to 22 hour-old larvae (early second instar); a 500-watt lamp proved satisfactory for their purposes. Hassett et al. (1960) utilized a 40-watt incandescent bulb with satisfactory results. The preliminary results of the present study appeared to indicate that an adequate migratory response was induced by the 6-watt fluorescent light (cool-white).

It may be significant to note that over 60% of the total radiant energy of a cool-white fluorescent light is found in the wavelength band from 460 to 600 nm (Klein, 1973); this band corresponds to the region of maximal spectral sensitivity in A. aegypti lateral ocelli (Seldin, et al. 1972). By contrast, less than 20% of the total radiant energy of a 40-W incandescent lamp is found in the 460-600 nm band. Thus, it might be said that the overall effective intensity of a 40 watt incandescent bulb, instead of being seven times as strong as a 6-watt fluorescent tube, is just over twice as strong in terms of its migration-inducing capacity.

Choice of test instar: Fourth instar larvae were the first to be ruled out because as indicated earlier in this work, they are less susceptible to copper and zinc than younger instar larvae. Fay (1959) pointed out that fourth instar A. aegypti are the most tolerant of all instars to toxicants in general.

Table 7. Migratory rate factor (MRF) of untreated *Aedes aegypti* larvae under the influence of a 6-watt fluorescent light for 15 seconds.

Instar	MRF	Range in MRF
Second	0.61	0.50 - 0.82
Third	1.02	0.92 - 1.18
Fourth	1.43	1.24 - 1.46

The incremental distribution of second and third instar larvae after 60 and 90 seconds' exposure to light is illustrated in Fig. 7. The variability of the response of the second instar larvae was generally greater than that of the third instars. Segregation of the third instar larvae therefore was the more satisfactory. For this reason, and because the third instar larvae showed up more clearly on film than the second instar larvae, the third instars were chosen as the primary test organism in the photomigration trials.

Possible Criteria of Poisoning: Having established the photomigration test conditions, the next objective was to select an impartial criterion to make judgments on the degree of poisoning. The effort focused on quantifying enfeeblement of the negativephototactic response. However, a temporarily increased migration rate was also observed which was briefly analyzed for its potential as a quantifiable response. The latter response is discussed first.

Zinc and copper in some cases were found to have an initial excitatory effect on the larval migration rate; the effect was most distinct during the first minutes of exposure to the toxicants. It was best demonstrated in the tests involving 100 ppm copper and 10 ppm zinc, respectively, 15 seconds after the photomigration light was turned on. The effect apparently involved only a brief burst in the migration rate after the light was turned on; after 30 seconds, the difference was less apparent. Figures 8 and 9 show that after exposure for 10 minutes to solutions of copper and zinc respectively, the larvae in the test solutions had migrated farther (in 15 seconds) than the control

Fig. 7. Mean percent distribution, per 10 cm interval in the photomigration trough, of untreated second- and third-instar larvae after 60 and 90 sec exposure to the directed light source. The vertical bars denote one S. E. of the mean (n = 6).

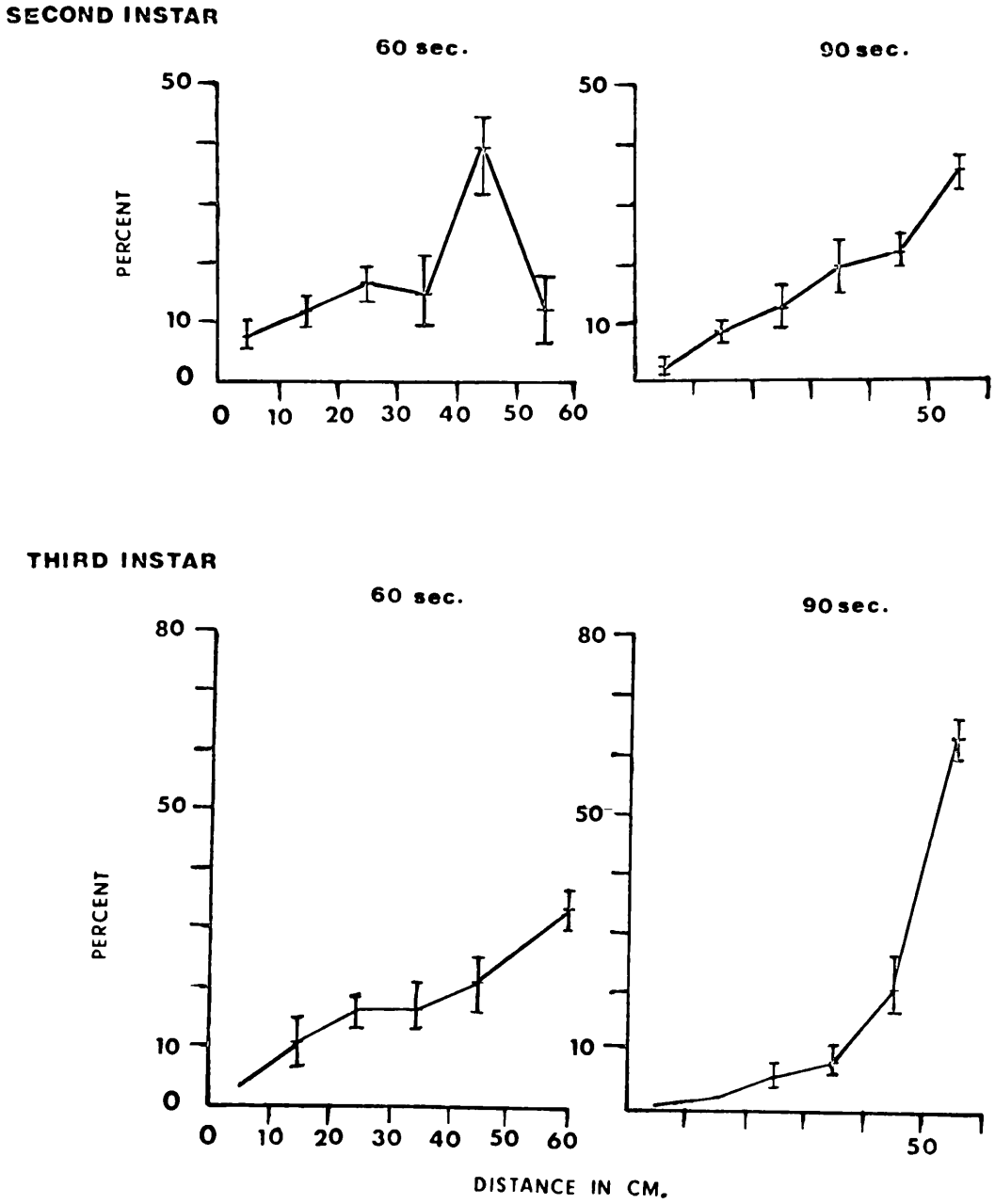

SECOND INSTAR
60 sec.
90 sec.
PERCENT
50
10
0
0 10 20 30 40 50 60
50
THIRD INSTAR
60 sec.
90 sec.
PERCENT
80
50
10
0
0 10 20 30 40 50 60
DISTANCE IN CM.

Fig. 8. Regression curves for cumulative migration, 15 sec. after light turned on, showing the relative positions of the control larvae and those exposed to 10 ppm Cu^{++} for 10 and 120 min.

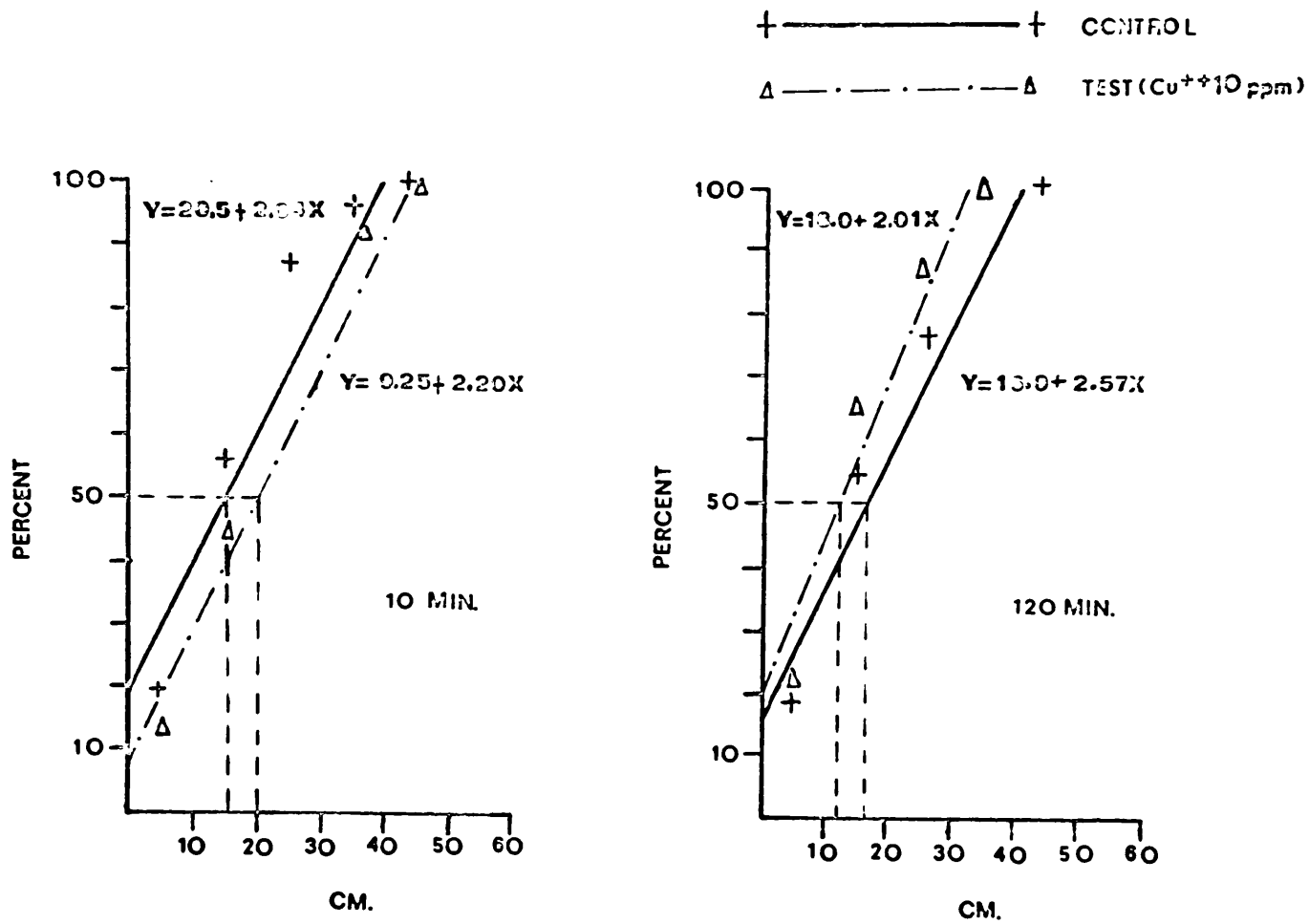
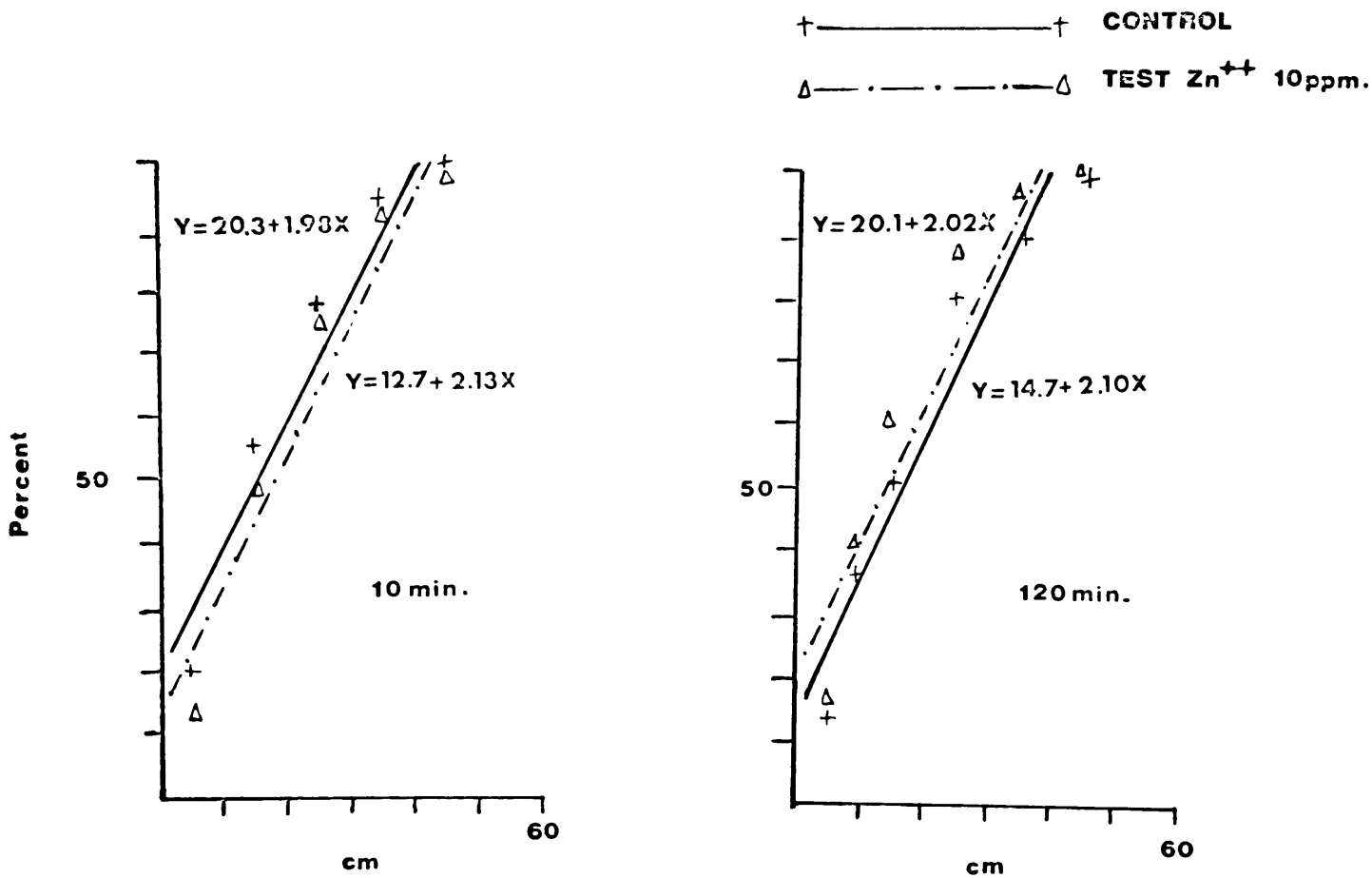

CONTROL
TEST (Cu++10 ppm)
Y=20.5+2.00X
Y= 0.25+2.20X
10 MIN.
PERCENT
CM.
Y=13.0+2.01X
Y=13.0+2.57X
120 MIN.
PERCENT
CM.

Fig. 9. Regression curves of cumulative migration, 15 sec. after light turned on, showing the relative positions of the control larvae and those exposed to 10 ppm Zn^{++} for 10 and 120 min.


CONTROL
TEST Zn++ 10ppm.
Percent
50
Y=20.3+1.98X
Y=12.7+2.13X
10 min.
60
cm
Y=20.1+2.02X
Y=14.7+2.10X
120 min.

larvae, but after 120 minutes' exposure the situation was clearly reversed. Arthur and Leonard (1970) noted a similar response in a species of Gammarus (Crustacea), which exhibited an immediate increase in locomotor activity when introduced to solutions of copper sulfate; the organisms' powers of motility were lost prior to death. In the present experiments, the excitatory effect was less noticeable at the lower concentrations.

The regression lines (Figs. 8 and 9) were used to derive MRF values (Table 8), which indicate the magnitude of the difference in migration rates. The excitatory effect was more pronounced in copper than in zinc. For copper, the increase in the MRF between the control and test larvae was 65% higher than the increase in MRF for zinc. This could perhaps be interpreted as a manifestation of the fact that Aedes aegypti larvae are more sensitive to copper than to zinc.

When the standard error for estimated values along the regression lines in Figs. 8 and 9 were taken into account, it was apparent that the true MRF values (Table 8) were not significantly different. The uncertainty about the slope of the regression lines (due to the low number of points) made it unlikely that the excitatory effect per se could be used as a criterion of toxicity.

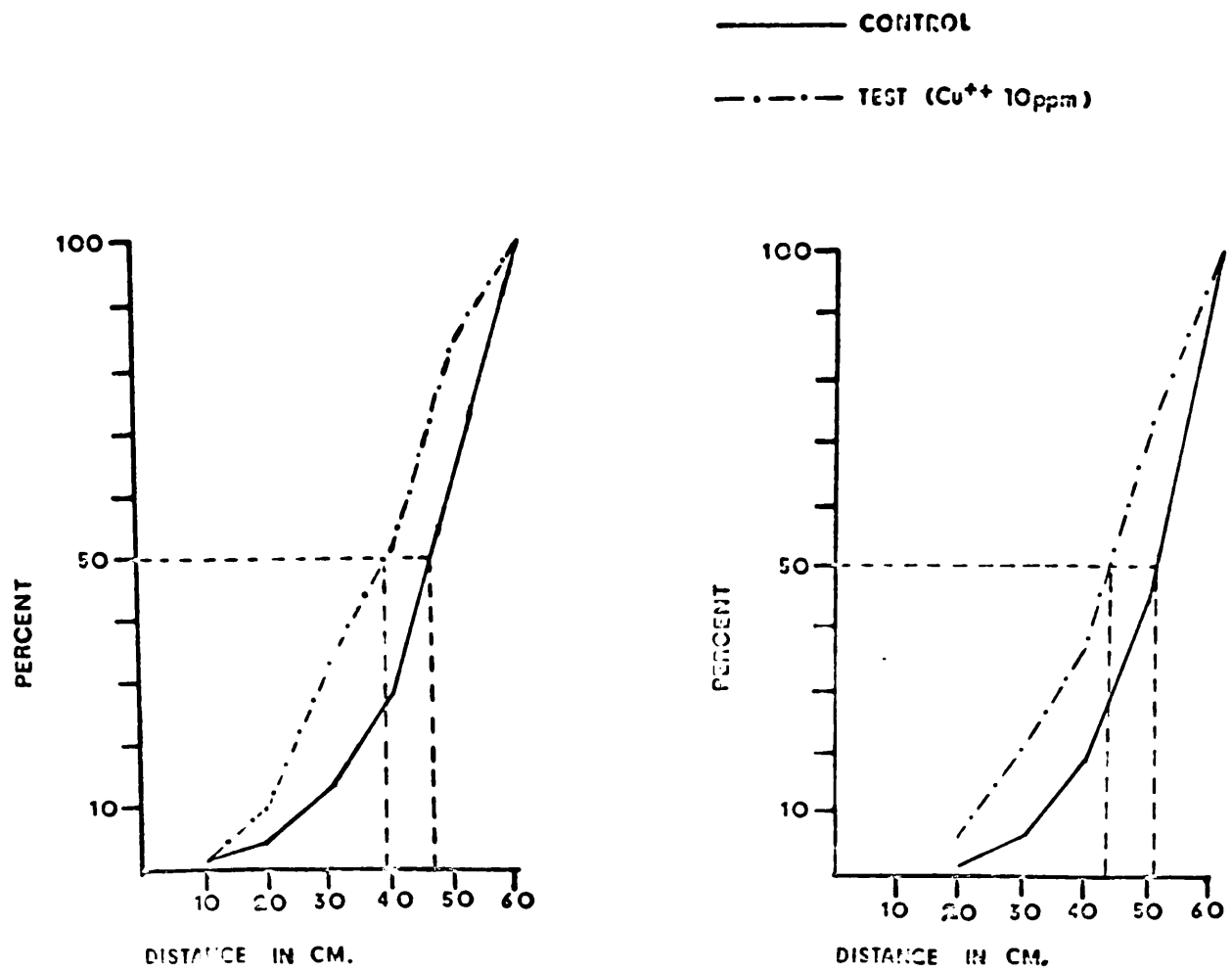
Inhibition of photomigration: Figures 4 and 5 showed the characteristic reduction in the migratory rate of the test larvae when compared with the control larvae (in this case after exposure for 51 minutes to 10 ppm Cu^{++}). Cumulative distribution (CD) curves allowed the reduction to be better visualized. By interpolating from Fig. 10 we see that

Table 8. MRF (migration rate factor) analysis of the temporarily increased migration rate (measured 15 sec after "lights-on") for larvae exposed to 10 ppm Cu^{++} and 10 ppm Zn^{++} respectively.

Treatment	Time Exposed to Toxicant (min)	MRF
10 ppm Cu	10	1.30
Control	10	0.97
		+0.33
10 ppm Cu	120	0.87
Control	120	1.07
		-0.20
10 ppm Zn	10	1.20
Control	10	1.00
		+0.20
10 ppm Zn	120	0.97
Control	120	1.06
		-0.09

Fig. 10. Cumulative percentage distribution of third instar larvae in the photomigration trough, comparing the control larvae with the larvae in 10 ppm Cu^{++}, 56 sec. and 86 sec. after the light was switched on (cf. Figs. 4 and 5).

NOTE: The dotted lines denote the distances attained by 50% of the larvae.



(a.) 50 sec.

(b.) 60 sec.

50% of the control larvae had passed 46 cm after migrating 56 seconds, whereas 50% of the test larvae had reached only 39 cm (Fig. 10). At 86 seconds, 50% of the control and test larvae had reached 51 cm and 43 cm respectively (Fig. 10).

It soon became apparent in plotting CD curves that after 15 and 30 sec the difference in the relative migration rate of the test and control larvae was usually not sufficiently distinct to obtain adequate separation. Therefore, the 15 and 30 sec data was omitted from consideration in the inactivation analysis. Likewise, the 90 second ($\pm$ 5 seconds) data was omitted because of difficulties in counting the larvae which had reached the end plates and became clumped. At 60 seconds ($\pm$ 5 seconds), the distribution of the two simultaneously migrating populations was clearly separable and there was a range of distances which could be used as cut-off points. So, the concentration- and time-response curves were constructed using data interpolated from the 60-second CD curves.

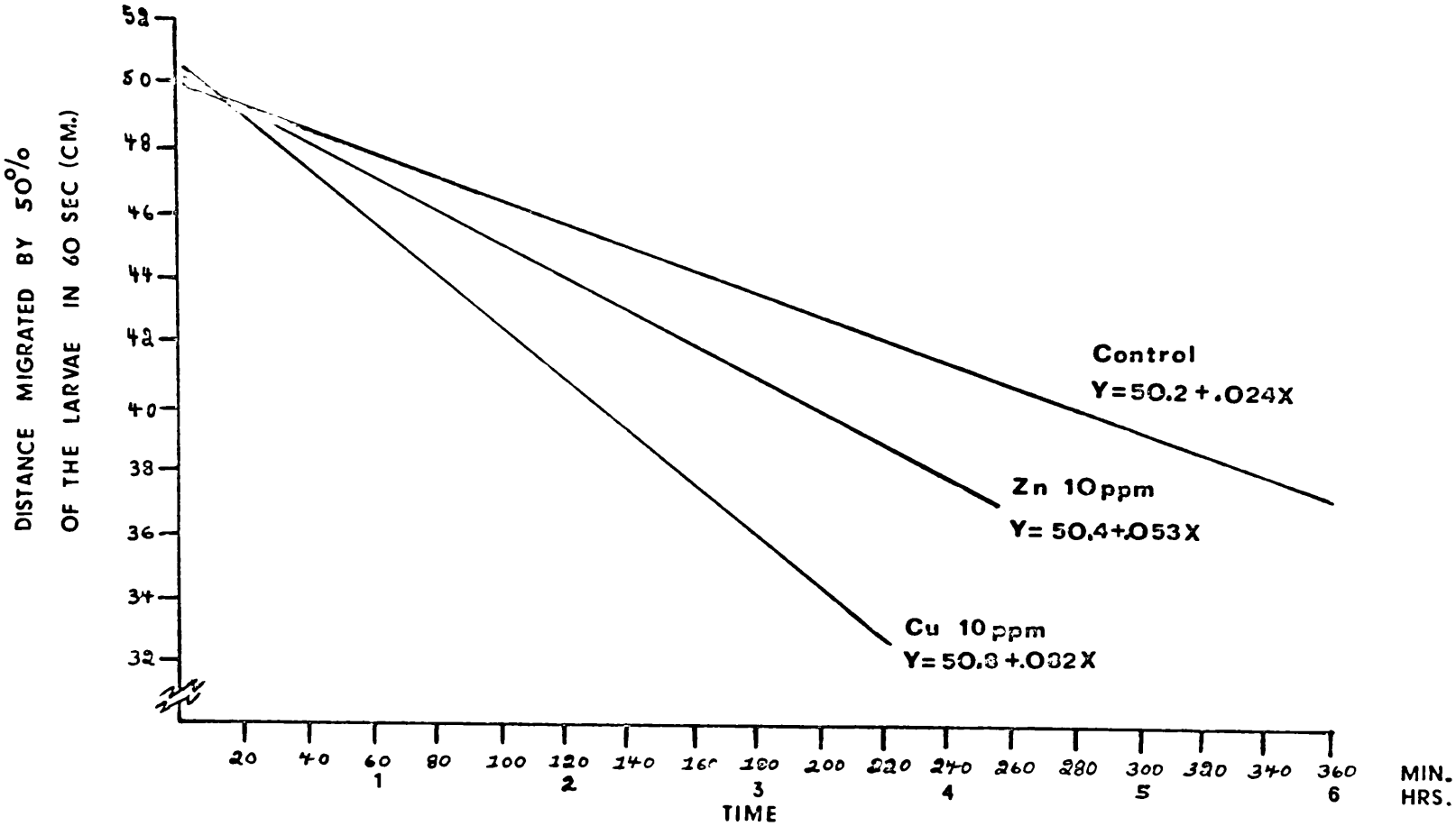
The methods of interpolating the median distances from the CD curves was chosen in preference to plotting regression lines for the interpolations (as was done for the MRF analyses in Figs. 6, 8, 9) because, in view of the comparatively large number of graphs that was required, the CD curve method was most expedient. A comparison of the two methods showed that regression lines yielded somewhat lower estimates of the median distances than did the cumulative curves, but the differences between the control and test curves obtained by the two methods were proportionally equivalent.

To estimate the relationship between the median distances migrated and the time the larvae were exposed to toxicant, distance-on-time regression lines were fitted (Fig. 11) using data obtained from the 60-sec CD curves. Part of the rationale behind fitting these distance-time regression lines was to provide some empirical basis for the selection of a "cut-off distance," i.e., the threshold distance which 50% of the larvae had to pass. In addition, the lines helped provide insight into the phenomena involved. For example, the upper curve in Fig. 11, representing the pooled data of 10 groups of control larvae, depicts a declining response pattern. It was clear that any test-inactivation response, to be distinguished from the control response, had to manifest itself quicker than the natural course of inactivation of untreated larvae. This "natural" inactivation probably was due to a lack of food during the test period.

During the course of an experiment, the distance reached by at least half of the larvae in 60 sec typically decreased from nearly 50 cm to less than 35 cm, with a mean near 40. Distances attained by 50% of the control larvae decreased from about 50 cm to 40 cm in a period of five hours (300 min.). By comparison, the mean distance migrated by larvae in the 10 ppm Zn^{++} and 10 ppm Cu^{++} solutions (two replicates each) decreased by the same amount in 200 and 130 min. respectively (cf. Fig. 11). A 40-cm cut-off point was adopted for the subsequent analyses.

The quantitative definition of the critical response used as the criterion of toxicity was: the ability of the larvae to swim 40 cm

Fig. 11. Regression on time of the distances migrated by 50% of the larvae in 60 seconds. Compares the relative rates of larval inactivation in 10 ppm Cu^{++}, 10 ppm Zn^{++} and in no toxicant.


DISTANCE MIGRATED BY 50% OF THE LARVAE IN 60 SEC (CM.)
52
50
48
46
44
42
40
38
36
34
32
Control
Y=50.2 +.024X
Zn 10ppm
Y= 50.4+.053X
Cu 10 ppm
Y=50.3 +.082X
20
40
60
1
80
100
120
2
140
160
180
3
200
220
240
4
260
280
300
5
320
340
360
6
TIME
MIN.
HRS.

away from the light within 60 seconds. When applied to 50% of the population, the response was referred to as the ET_{50}, or more precisely, the "40-cm, 60-sec ET_{50}." The term ET (for "effective time") is applied to the time required for a particular effect to influence a given proportion of the responding population.

Time-inactivation curves: The percentage of control larvae inactivated as a function of time is shown in Fig. 12. This is the pooled result of several replicates for each time interval, and was used as the standard curve against which the test curves were compared (Figs. 13 and 14). The spread of the values--characteristic of larval response in low concentrations--shows that the rate of inactivation increased appreciably after five hours. Sokal and Rohlf (1969) cautioned about extrapolating from a regression line if one has any doubts about the linearity of the relationship. In this case, the curvilinearity (skewness) manifested itself primarily after the practical upper limit of the test (the time for 50% of the control larvae to be inactivated), so it would appear that ET_{50} interpolations done in the 60 to 300 minute range were justified. In the early stages of all tests the response was relatively uniform before the toxicant caused inactivation. The curves were most linear at the higher concentrations.

The time-inactivation curves in Figs. 13 and 14 illustrate the degree to which the metal salts were capable of inducing larval inactivation. When the concentration of metal was increased, the ET_{50} decreased. The useful range of inactivation was from 30 to 70 per cent.

Fig. 12. Regression curve of control larval migration, where the ordinate (Y) equals percentage of larvae unable to migrate 40 cm in 60 sec., and the abscissa (X) is the time exposed to the test solution.

NOTE: The vertical dots indicate the range of Y variates per value of X variate. The " x " signs denote the means of the ranges.

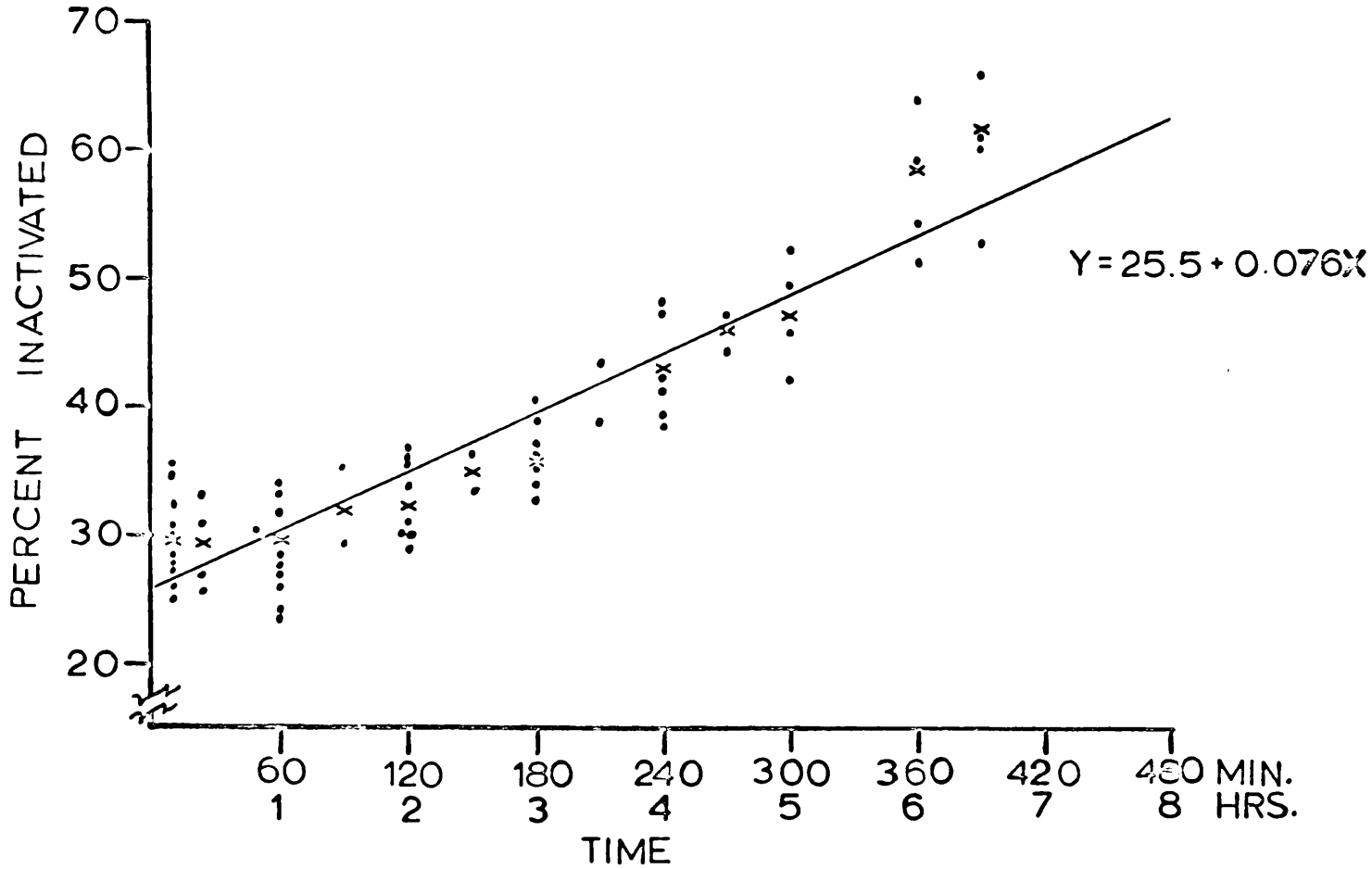

PERCENT INACTIVATED
70
60
50
40
30
20
Y=25.5+0.076X
60
120
180
240
300
360
420
480 MIN.
1
2
3
4
5
6
7
8 HRS.
TIME

Fig. 13. Time-inactivation curves for various concentrations of zinc.

NOTE: Individual data points were omitted. The dashed lines indicate the 40-cm, 60-sec. ET_{50} values.

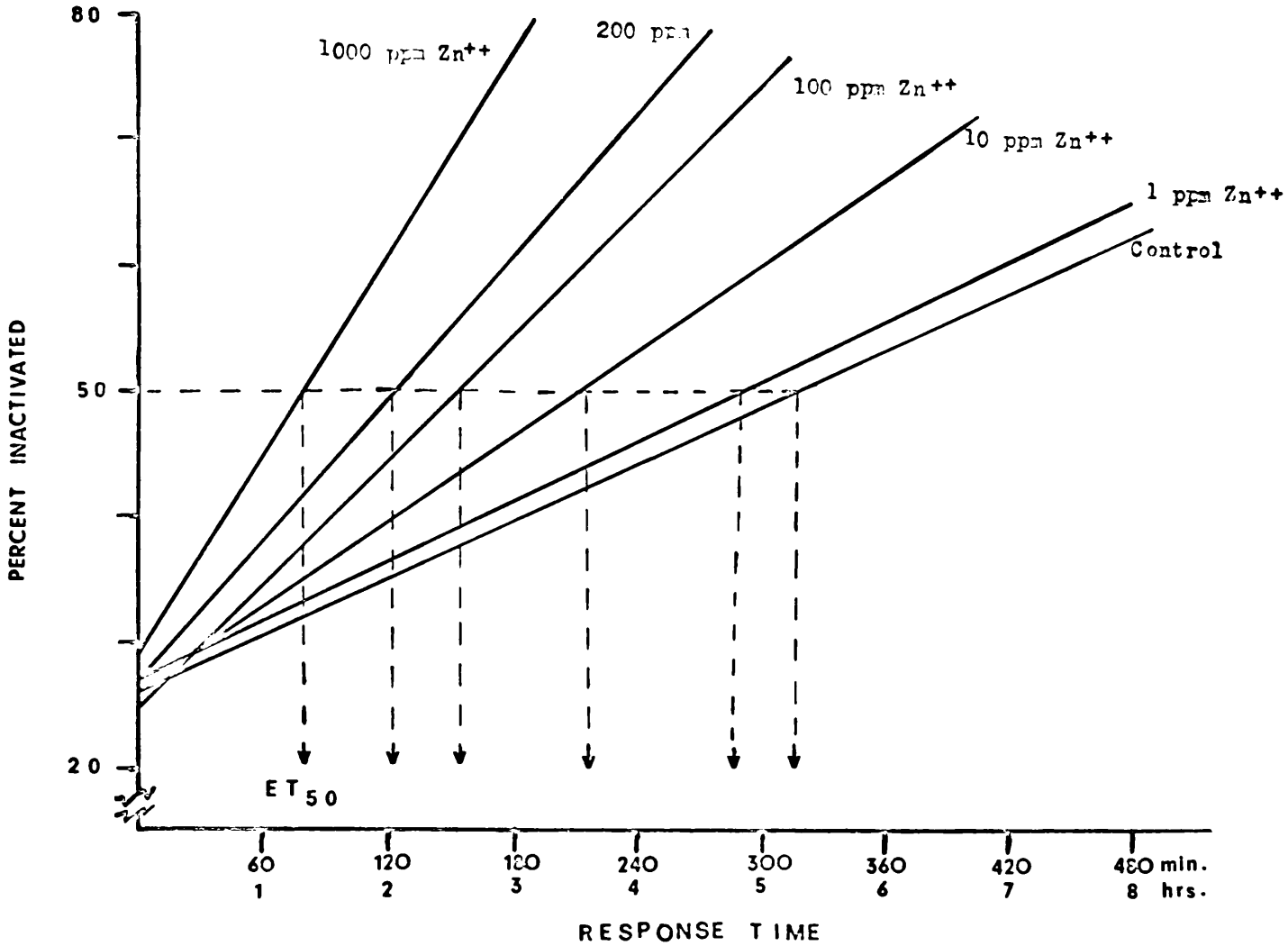
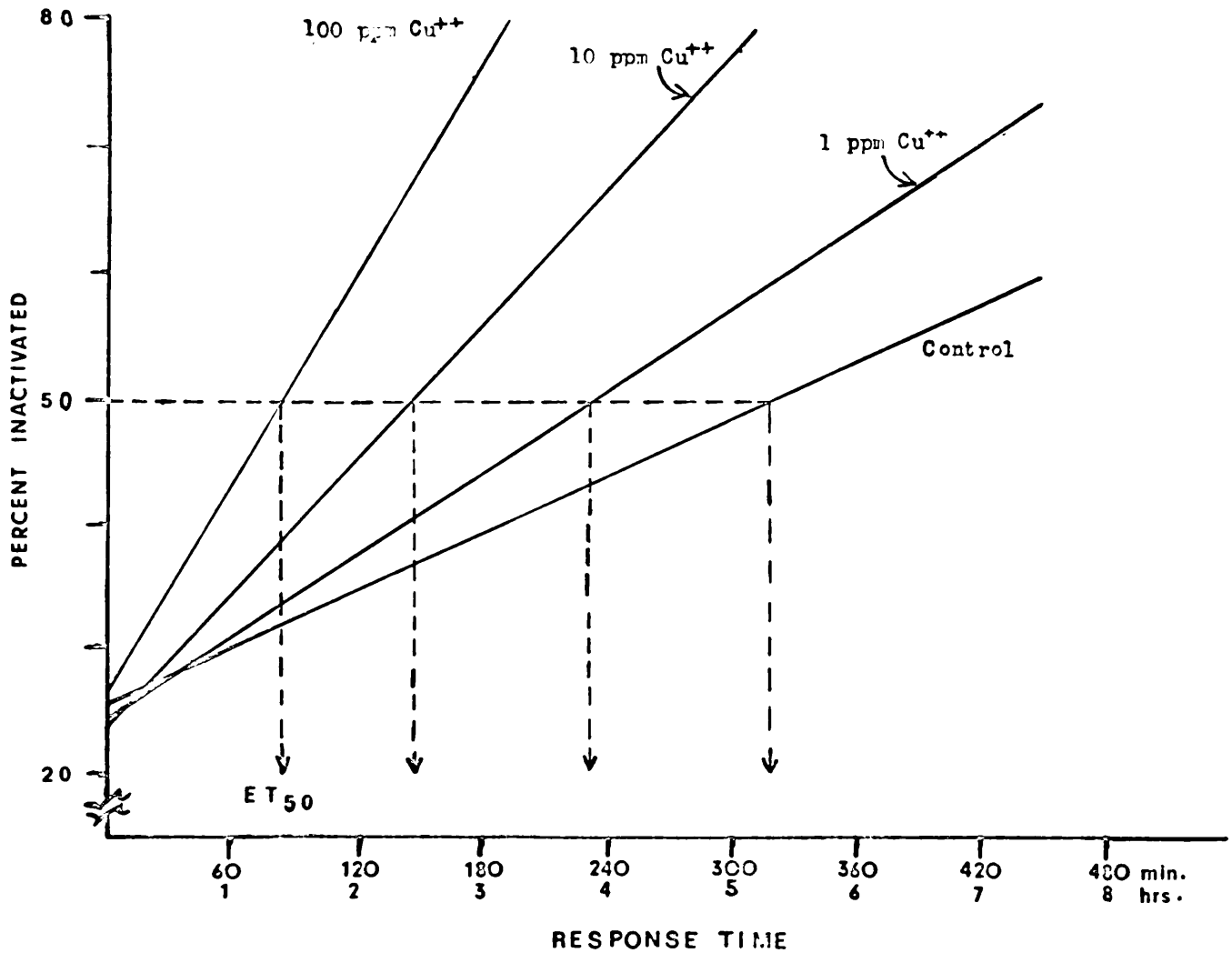

80
50
20
PERCENT INACTIVATED
1000 ppm Zn++
200 ppm
100 ppm Zn++
10 ppm Zn++
1 ppm Zn++
Control
ET50
60
1
120
2
180
3
240
4
300
5
360
6
420
7
480 min.
8 hrs.
RESPONSE TIME

Fig. 14. Time-inactivation curves for various concentrations of copper.

NOTE: Individual data points were omitted. The dashed lines indicate the 40-cm, 60-sec ET_{50} values.


80
50
20
PERCENT INACTIVATED
100 ppm Cu++
10 ppm Cu++
1 ppm Cu++
Control
ET50
60
1
120
2
180
3
240
4
300
5
360
6
420
7
480 min.
8 hrs.
RESPONSE TIME

For 0.1 ppm Zn^{++}, the inactivation response was not distinguishable from the control ; 1 ppm Zn^{++} induced only a slightly greater rate of inactivation (Fig. 13). Copper at the same concentration (1 ppm) caused a distinctly quicker inactivation (Fig. 14). The ET_{50} values derived from the time-response curves are listed with the regression equations and standard errors of estimation (S.E.) in Table 9. The standard errors are sums of squares of the deviations of experimentally derived percentage values from values on the lines, and indicate the spread of points around the fitted regression lines.

Initial analyses of the time-response regression lines indicated that the use of logarithmic values for time would have imparted no analytical advantage. Likewise, little was improved by plotting the percentage inactivation in probability units (probits): the lower "lag" in the data would have straightened, but the upper skewness would have increased.

Dosage-response curves: For further interpretation, various types of dosage-response curves might be constructed by interpolation from the time-response regression lines. Examples are the toxicity curves in Figs. 15 and 16, which are plots of the $\log_{10}$ of ET_{50} *versus* the $\log_{10}$ of concentration. Log-dosage probit lines could have been constructed for any selected period of exposure (e.g., 120 and 300 min.). However, a rigorous interpretation of the quantitative relation between dosage and response by log-probit regression lay beyond the scope of this study.

The shape of the toxicity curves (Figs. 15 and 16) defines the inactivation characteristics of the metals as determined by the

Table 9. ET_{50} values, regression equations, and standard errors of estimate (S.E.) for the time-inactivation curves in Figs. 13 and 14.

Metal	Conc. (ppm)	ET_{50} (min)	Regression Equation	S.E.[1/]
Zinc	0.0	315	Y = 25.5 + 0.076X	3.03
	0.1	319	26.6 + 0.073X	6.72
	1.0	289	27.2 + 0.079X	6.04
	10.0	209	25.9 + 0.21X	5.66
	100.0	151	25.0 + 0.166X	4.82
	1000.0	78	29.5 + 0.267X	3.22
Copper	0.0	315	Y = 25.5 + 0.076X	3.03
	1.0	228	24.8 + 0.107X	6.21
	10.0	147	25.3 + 0.171X	4.12
	100.0	86	26.1 + 0.279X	3.53

[1/] Units are the same as those of the ordinate in Figs. 13 and 14, i.e., per cent.

Fig. 15. Toxicity curves for third instar larvae in Zn^{++}, using the data on the 40-cm, 60-sec. ET_{50} inactivation times derived from the response curves in Fig. 13 and plotted against concentration.

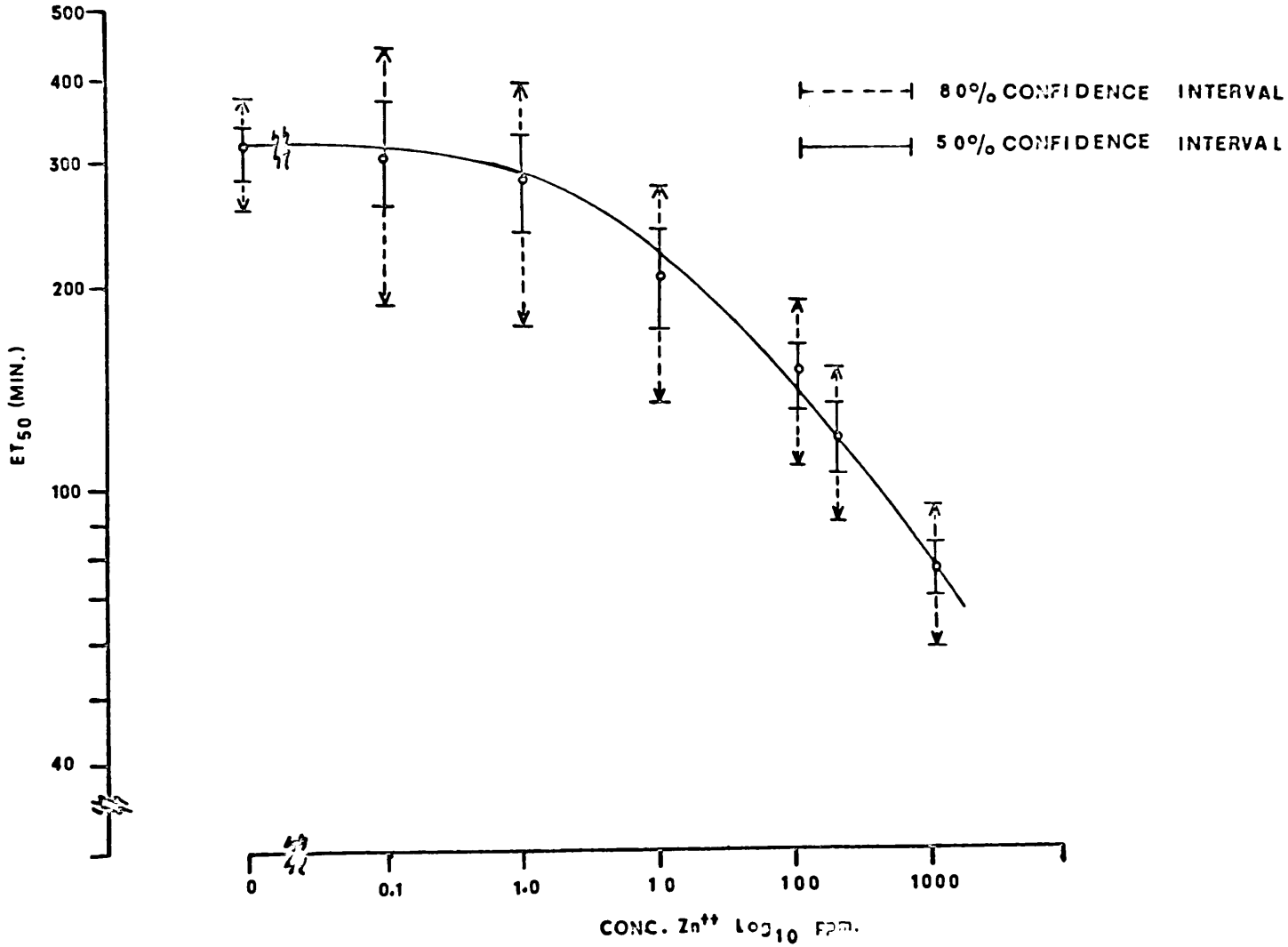
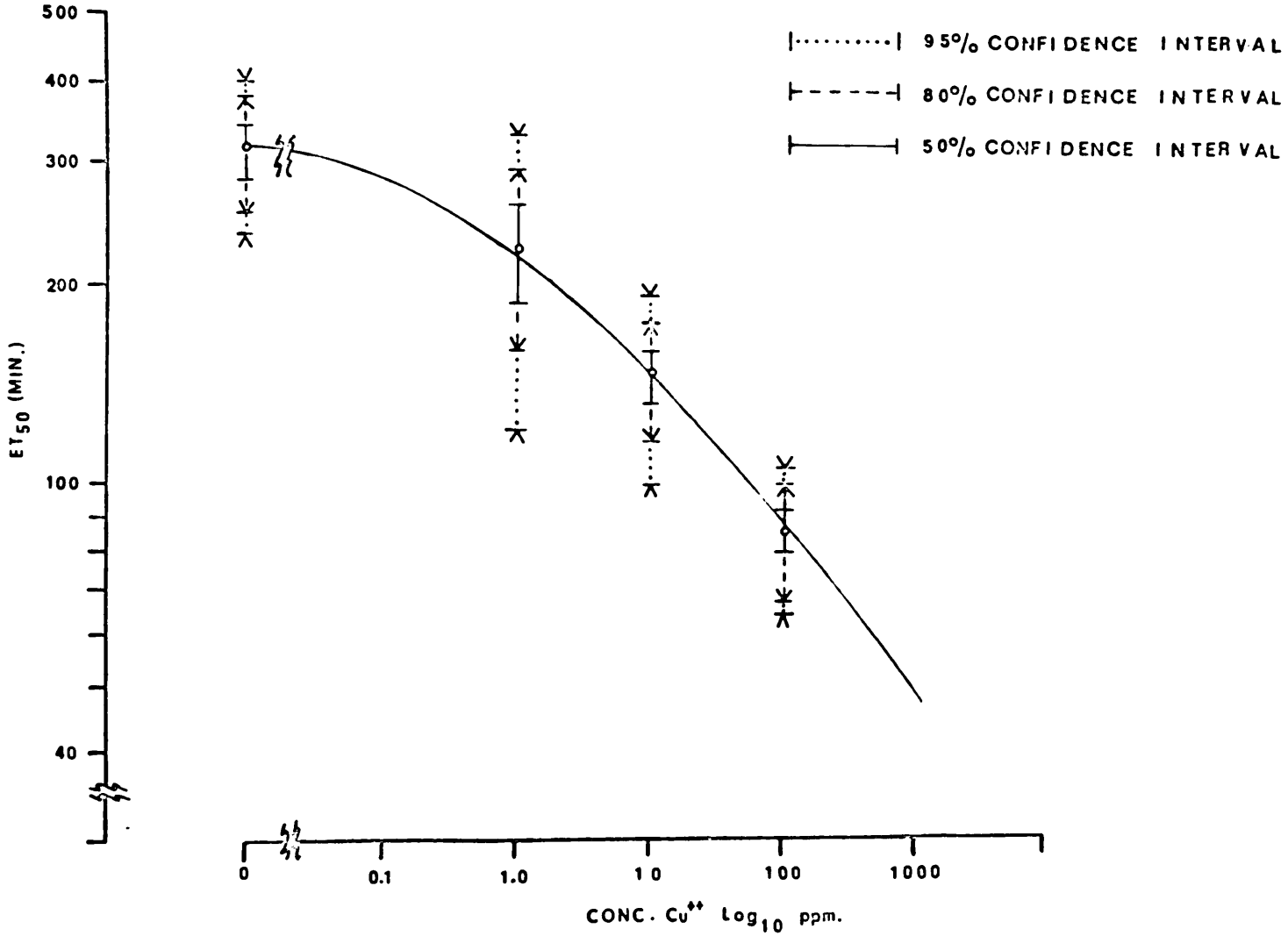

80% CONFIDENCE INTERVAL
50% CONFIDENCE INTERVAL
ET50 (MIN.)
500
400
300
200
100
40
0
0.1
1.0
10
100
1000
CONC. Zn++ Log10 ppm.

Fig. 16. Toxicity curves for third instar larvae in Cu^{++}, using the data on the 40-cm, 60-sec. ET_{50} inactivation times derived from the response curves in Fig. 14 and plotted against concentration.


95% CONFIDENCE INTERVAL
80% CONFIDENCE INTERVAL
50% CONFIDENCE INTERVAL
500
400
300
200
100
40
ET50 (MIN.)
0
0.1
1.0
10
100
1000
CONC. Cu++ Log10 ppm.

photomigration technique: a progressive enfeeblement of larval migration is clearly implicated. In the lower concentration range, the exposure period required to produce the response was relatively constant. At a higher range of concentrations, the larvae were unable to cope with the toxicant. The result was an abrupt downward inflection of the dosage response (toxicity) curve. The toxic action of copper was more rapid than that of zinc at both low and high concentrations. Thus, the ET_{50} for 1 ppm Zn^{++} was 279 ($\pm$87)[1/] min. and for 1 ppm Cu^{++}, 228 ($\pm$21) min.; at 100 ppm zinc and copper, the values were 151 ($\pm$27) and 86 ($\pm$13) min. respectively.

Compared with exponential toxicity curves of a more conventional nature, these curves are "unusual" in that they are downward-directed, and that neither axis is approached asymptotically. The reason for the termination on the time (ET_{50}) axis is the gradually reduced response in the control population (discussed above), which sets a lower limit on the sensitivity of the photomigration technique as presently developed. Although it is not evident from the figures, it is reasonable to assume that there is a minimum time for zinc and copper to inactivate 50% of a given population, at least at very high doses, because a finite amount of time is required for the toxicant to reach its site of action.

The confidence intervals for the values in the toxicity curves were graphically derived from the time-inactivation regression lines

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[1/] One standard deviation

with the S.E. values given in Table 9. The lower the test concentrations and, therefore, the longer the exposure to test conditions, the more variable the response, i.e., the wider the intervals. The confidence limits were lowest for the control values because of the greater number of degrees of freedom (results of the controls of all successful tests were pooled).

The comparatively large confidence intervals in the figures probably do not represent the variability inherent in the negative photomigration response *per se* as much as the variability in conditions subject to errors introduced by equipment, operator and environment. The use of control populations concurrently with each test population at least partially enhanced reproducibility. Equipment and operator error accounted for the low number of successful replicates. Part of the variation in response times no doubt was due to normal genetic variability in phototaxis and in physiological tolerance among individuals of a given responding population (cf. Rockwell and Seiger, 1973:343). One important factor in the variability and magnitude of the response was the physiological state of the test organisms, e.g., age and nutritional conditions. The most likely causes for variation during the tests were: (a) lack of food in, and/or hypotonicity of the test medium, (b) influence of previous migrations and handling on subsequent runs, (c) acclimation to the light stimulus by the larvae once they had moved a distance away from it, and, possibly (d) loss of the test material during the experiment due to adsorption phenomena, etc.

A significant cause for variability may lie in the very fact that

the third instar larvae are relatively tolerant of copper and zinc. These metals are slow-acting, and do not cause as clear a reaction as, for example, materials like organic pesticides. Burchfield et al. (1952:83) stated that for all insecticides they studied with their photomigration technique, "the T_{50} at 0.1 ppm was less than 100 minutes with the exception of ionic materials such as nicotine sulfate..." In the present study, responses to copper and zinc were significantly different from the control beginning only at 10 ppm, with 95% resp. 50% confidence. The ET_{50} values were: 147 minutes for copper and 209 minutes for zinc.

It must be recognized that both metals are known to be essential elements in animal metabolism, and so clearly cannot be toxic at low levels (Table 10). Copper is required in more than a dozen oxidative and other enzymes; zinc is also incorporated into several metal-ion-activated enzymes (Bailar, 1972, and Frieden, 1971). At higher concentrations, zinc has generally non-specific toxic action, but exhibits some nephrotoxic properties (Skidmore, 1964). In the case of copper, Hubschman (1967), who worked on crayfish metabolism, suggested that several toxic mechanisms were operative. At very high concentrations, (1000 ppm +), cellular proteins were coagulated. At concentration above 1 ppm, respiratory enzymes were inhibited (e.g. succinic dehydrogenase). Below 1 ppm, long-term exposure caused degenerative changes in tissues and cells.

Table 10. Major essential metallo-enzymes and -proteins incorporating zinc and copper. Required for the successful functioning of living organisms.

Metal	Enzyme or Protein	Biological Function
Copper	Ceruloplasmin	Iron utilization; copper storage
	Cytochrome oxidase	Principal terminal oxidase
	Lysine oxidase	Elasticity of aortic walls
	Tyrosinase	Pigmentation
	Hemocyanin	Oxygen transport in invertebrates
Zinc	Carbonic anhydrase	CO_2 formation, regulation of acidity
	Carboxypeptidase	Protein digestion

Source: Adapted from Bailar (1972) and Frieden (1971)

CONCLUSIONS

Negative phototaxis by mosquito larvae provides a viable approach to toxicity testing in the field of water quality control. Several features recommend this approach:

1. Larval inactivation can be quantified with certainty and objectivity, once a criterion of toxicity has been established.

2. Its sensitivity is comparable to that of acute toxicity tests, and is potentially more sensitive.

3. Results are available within a few hours, rather than days or weeks as required for conventional tests. For rough approximations of toxic activity, only minutes are necessary.

4. Reliability is enhanced by the use of large populations (50-100+) of larvae; simultaneous controls with each test provide a "baseline" with which to make comparisons.

5. Mosquitoes can be readily mass-reared, and the eggs stored for use as needed, or the eggs can be shipped from a source laboratory.

The photomigration approach could be expanded to include other species of insects. The test is suitable for a wide range of non-turbid toxicants. As presently conceived, it would be particularly useful in assessing the quality of standing waters because mosquitoes naturally inhabit such waters.

The following concluding statements can be made about the methodology developed in this study:

1. Use of a control with each pair of runs was necessary for successful interpretation of test outcomes; in this way, variables which modulated the photomigratory response were compensated for in both chambers.

2. Several changes in the experimental procedure could enhance sensitivity of the photomigration technique: (a) use of younger instar larvae, and (b) increased light intensity: these factors may reduce variability by reducing the time larvae spend under rearing conditions and by augmenting stimulus intensity; (c) pre-exposure of several replicates of larvae to the toxicant, followed by one or only a few successive "runs" each to establish inactivation; this would reduce the possible confounding influence of trauma caused by retesting the same group of larvae repeatedly; and (d) more careful attention to uniformity in the mosquito rearing techniques.

3. The photographic procedure is not essential to small-scale toxicity tests, and makes the process of migration analyses unnecessarily involved. Photographs do, however, provide a convenient record for later reference.

4. The alternative to the above is to select a cut-off point by means more arbitrary than those developed in this study. Thus, a barrier could be strategically placed in the trough based on approximation of the distance which a given proportion of the population swims in a set amount of time. Direct counting of the larvae would then be feasible. Conceivably, in certain larger-scale applications, automated techniques incorporating computer interfacing technology might be employed.

5. The initial excitatory effects caused among the larvae upon introduction to zinc and copper are not amenable to quantification. Nor does the monitoring of intermediate changes in response intensity appear to offer an "experimental handle."

SUMMARY

A little-known approach to toxicity testing--based on negative phototaxis of larval Aedes aegypti--was investigated as a contribution to the search for rapid methods applicable to the field of water pollution control. Zinc and copper were the toxicants tested. All tests were conducted with a standard "synthetic" dilution water.

A mosquito colony was established to provide a uniform supply of test larvae. Preliminary tests were performed on the acute toxicity of zinc and copper against A. aegypti larvae, as well as tests on larval growth and development at various concentrations of the metals. A number of prefatory tests were performed to assess the nature of the photomigration response.

For the photomigration toxicity tests, two juxtaposed troughs were used, one containing the test solution, the other a control. Third instar larvae migrated away from a six-watt fluorescent light for two minutes per run. This was repeated at intervals until 50% were unable to migrate 50 cm in 120 sec. Photographs were taken of the larval migrations. From the pictures an empirical criterion was derived (the 40-cm, 60-sec ET_{50}) through a series of graphical interpolations. All inactivation analyses were based on this criterion. In general, the evaluations were based on multiple observations of from one to ten replicates of up to 125 larvae each.

From time-inactivation regression lines (Figs. 13 and 14),

exponential toxicity curves were obtained by interpolation. The curves were of an unusual shape, depicting the characteristic nature of the dosage-response (Figs. 15 and 16). The confidence limits on the curves indicated the degree of variability in the experimental procedures and migration response. The control larvae in this study imposed a natural limit on the sensitivity of the photomigration test: they themselves were inactivated at a slow but steady rate, probably due to a lack of larval food during the tests, and due to effects of repeated migrations with the same larvae. Several sources of variation were taken into account when interpreting the results.

The sensitivity of the inactivation technique was comparable to that of the acute toxicity tests. However, inactivation was far quicker; depending on concentration, it occurred within one to five hours. The developmental tests showed that larval growth and adult emergence were inhibited at levels far below those acutely toxic, but the tests required up to ten days to perform. By all methods used in this study, zinc and copper were judged to be slow-acting and of low overall toxicity (when compared to insecticides, for example). Copper was, however, consistently more toxic than zinc by at least one order of magnitude.

Some possible improvements in technique were discussed. It was suggested that the photomigration approach to toxicity testing can be of definite practical use to biologists in water pollution control.

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APPENDIX

It seems appropriate to mention my early efforts to locate a candidate bioassay organism among the aquatic Diptera; these efforts were undertaken prior to the development of the photomigration technique. Emphasis was placed on larvae of chironomid midge flies, in connection with concurrent research on the midge fauna of several polluted watercourses in and around Montgomery Co., Virginia.

Chironomid larvae have been reported to develop distinctive deformities in the presence of industrial wastes (Hamilton & Saether, 1971). It appeared that this tendency for the larvae to develop morphological deformities had potential in the detection of sublethal dosages of heavy metals and other toxicants, at least in laboratory bioassays.[1/]

Over ten chironomid species have been successfully reared in the laboratory (Biever, 1965, 1971; Caspary and Downe, 1971; Credlund, 1973; Downe and Caspary, 1973; Fischer, 1969; Hilsenhoff and Narf, 1967). Among these is *Chironomus riparius* Meigen, a locally abundant species. Attempts to colonize *C. riparius* therefore were initiated to provide a continuous supply of laboratory reared larvae for testing. However, the prospects of obtaining an adequate number of larvae from the colony were discouraging due to the difficulties in inducing

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[1/] Personal communication in 1972 with Drs. A. L. Hamilton and O. A. Saether, Fisheries Res. Bd. Canada, and Dr. J. A. Spence, McGill University, Montreal.

mating swarms in the cages used, and because of complications in maintaining the vigor of the larval colony. For this reason, it was decided to focus on *Aedes aegypti* as an aquatic dipteran more amenable to laboratory colonization.

VITA

Walter Ingolf Knausenberger was born in September, 1948 near State College, Pennsylvania. His family moved to West Germany in 1959. He attended the Fuerstenfeldbrucker Oberschule, 1960-61, and then the Munich American High School, graduating in 1966. He attended the University of Maryland (European Division) for the following two years.

In January, 1969, he enrolled at the Pennsylvania State University, University Park campus, where he majored in Biophysics. He received the B.S. degree in June, 1971. That Fall, after ROTC Basic Training Summer Camp, he enrolled as a graduate student in the Department of Entomology at the Virginia Polytechnic Institute and State University, where he has served as graduate research assistant.

He has membership in the American Institute of Biological Sciences, American Nature Study Society, Association of Southeastern Biologists, Entomological Society of America, North American Benthological Society, Student Conservation Association, Inc., Virginia Academy of Sciences and the Wilderness Society. He belongs to Gamma Sigma Delta (Honor Society of Agriculture) and Phi Sigma (Biological Sciences Honor Society). He was the recipient of part of the McCormick scholarship from the College of Agriculture at V.P.I. & S.U., in 1974.

He has participated in a variety of organized extracurricular activities. He was one of the co-founders and the first Vice-President

of the Entomological Society of V.P.I. & S.U. He has twice served as the Departmental Representative to the Graduate Assembly, and was elected as the graduate student representative to the Learning Resources Center Advisory Committee. He is fluent in German and French and has a working knowledge of Spanish.

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NEGATIVE PHOTOTAXIS OF MOSQUITO LARVAE AS A POTENTIAL TOOL IN THE RAPID BIOLOGICAL MONITORING OF AQUATIC WASTES (DIPTERA: CULICIDAE)

by

Walter Ingolf Knausenberger

(ABSTRACT)

A little-known approach to toxicity testing--based on negative phototaxis of larval Aedes aegypti--was investigated as a contribution to the search for rapid methods applicable to the field of water pollution control. Zinc and copper were the toxicants tested. All tests were conducted with a standard "synthetic" dilution water.

A mosquito colony was established to provide a uniform supply of test larvae. Preliminary tests were performed on the acute toxicity of zinc and copper against A. aegypti larvae, as well as tests on larval growth and development at various concentrations of the metals.

For the photomigration toxicity tests, two juxtaposed troughs were used, one containing the test solution, the other a control. Third instar larvae migrated away from a six-watt fluorescent light for two minutes per run. This was repeated at intervals until 50% were unable to migrate 50 cm in 120 sec. Photographs were taken of the larval migrations. From the pictures an empirical criterion was derived (the 40-cm, 60-sec ET_{50}) through a series of graphical

interpolations. All inactivation analyses were based on this criterion.

From time-inactivation regression lines, exponential toxicity curves were obtained by interpolation. The curves were of an unusual shape, depicting the characteristic nature of the dosage-response.

The sensitivity of the inactivation technique was comparable to that of the acute toxicity tests. However, inactivation was far quicker; depending on concentration, it occurred within one to five hours. By all methods used in this study, zinc and copper were judged to be slow-acting and of low overall toxicity . Copper was, however, consistently more toxic than zinc by at least one order of magnitude. The ET_{50} in 10 ppm Cu^{++} was 147 min.; in 10 ppm Zn^{++}, it was 209 min.

Some possible improvements in technique were discussed. It was suggested that the photomigration approach to toxicity testing can be of definite practical use to biologists in water pollution control.