THE EFFECTS OF FENITROTHION, MATACIL® AND ORTHENE® ON FROG LARVAE

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RESUME

La toxicité du fénitrothion, du Matacil et de l'Orthène pour les larves de Rana clamitans a été étudiée en laboratoire. Pour une exposition de 24 heures, des DL50 de 9,9 247 et 6433 p.p.m, respectivement, ont été obtenues par des tests biologiques statiques réalisés avec environ 2,9ℓ de solution par gramme de tissu de têtard; ce rapport était satisfaisant pour ces tests. Toutefois, la mortalité chez des témoins plus gros et celle qui s'est manifestée en deux temps chez des animaux plus gros exposés au Matacil ont semblé indiquer que le rapport de 1,7ℓ par gramme n'était pas suffisant.

En raison de sa forte solubilité dans l'eau, l'Orthène n'a pas été rapidement absorbé ni par la suite été absorbé dans les tissus de têtards gardés dans un aquarium de laboratoire et dans une mare ayant subi une pulvérisation aérienne. Cela a eu pour effet que les concentrations résiduelles d'Orthène y ont rarement différé de celles du milieu aquatique ambiant. En revanche, le fénitrothion est relativement lipophile et a été facilement absorbé par les larves. Les solubilités de ces insecticides peuvent donc influer sur leur toxicité relative pour les organismes aquatiques.

Alors que le fénitrothion disparaît rapidement des eaux naturelles, à une vitesse dont la courbe est à peu près celle d'une exponentielle négative, l'Orthène s'est révélé relativement rémanent. Ce dernier s'est déposé dans la mare temporaire en quantités beaucoup plus grandes que le fénitrothion (21 et 15%, respectivement) après leur épandage par avion. La concentration d'Orthène dans la mare indique que la quantité déposée est encore plus grande que la quantité recueillie dans les récipients d'échantillonnage des produits pulvérisés.

Il est traité de l'ensemble de l'éthologie des têtards de Rana clamitans dans le laboratoire. Les produits chimiques ont influé sur leur activité, leur comportement natatoire, leur production de bulles, leur position au repos et leur couleur. Il est aussi question des effets que ces produits peuvent avoir sur les prédateurs de têtards, les insectes et le canard sauvage ordinaire, notamment.

L'analyse des teneurs résiduelles de ces produits dans l'eau de la mare a montré que jamais on n'en recontrerait en concentrations nuisibles aux larves d'anoures à la suite de leur application pour la lutte contre les insectes.

ABSTRACT

The toxicity of fenitrothion, Matacil, and Orthene to *Rana clamitans* larvae was investigated in the laboratory. The 24-h LC50 values obtained were 9.9, 247, and 6433 ppm, respectively, for these three chemicals in static bioassays with approximately 2.9 ℓ of test solution per gram of tadpole tissue; this ratio was satisfactory for static bioassays. Mortality of larger control animals and biphasic mortality of larger Matacil-treated test animals, however, suggested that 1.7 ℓ/g was not a sufficient volume of test medium.

In both a laboratory aquarium and an aerially sprayed pond, Orthene, because of its high water solubility, was not readily adsorbed and subsequently absorbed in tadpole tissue. The result was that residual levels of Orthene in tadpole tissue seldom differed from ambient levels present in the water medium. Conversely fenitrothion is relatively lipophilic and was readily taken up by the larvae. The solubilities of these insecticides may thus affect their relative toxicity to aquatic organisms.

While fenitrothion rapidly disappeared from natural waters, at a rate approximating a negative exponential, Orthene was relatively persistent. Of the insecticides emitted from the aircraft, Orthene reached the temporary pond in a greater proportional deposit than did fenitrothion (21% and 15%, respectively). The Orthene concentration in the pond water suggested an even higher deposit than recorded from spray deposit sampling pans.

The behavioral repertoire of *Rana clamitans* tadpoles in the laboratory is discussed. The chemicals affected activity, swimming behavior, bubble-making, resting position, and color in these larvae. The possible effects of these chemicals on tadpole predators are discussed with special reference to predatory insects and Mallards.

Analysis of residual levels in pond water suggested that concentrations detrimental to anuran larvae would never be encountered under normal operational applications of these chemicals.

I Introduction

A. Amphibian - pesticide Interactions

There is a growing concern about the depletion of populations of amphibians in areas inhabited by man. Explanations of declines in populations include destruction of habitat, human predation, and the indiscriminate use of chemicals to control agricultural and forestal pests (Porter, 1972; Cooke, 1974a).

Amphibians come into contact with pesticides sprayed onto their habitats in several ways: direct contact with the spray, inhalation of spray vapors, and by their movements into and out of contaminated waters (Sanders, 1970). They are also exposed to pesticides by ingestion of contaminated foods.

The majority of research on adult amphibian-insecticide interactions has centered around the organochlorines. Direct mortality of adult anurans as a result of DDT-spraying is well known (Logier, 1949; Herald, 1949; Fashingbauer, 1957). Herald (1949) observed the effects of DDT on several anuran species and concluded that it took a heavy dose of direct spray upon an adult amphibian to cause toxicity symptoms. Laboratory toxicity and poisoning symptoms of organochlorines have been determined for a variety of frog species (Kaplan and Overpeck, 1964; Ferguson and Gilbert, 1968; Cooke, 1974b). Prior exposure to these chemicals has resulted in the possible development of DDT- and aldrin-resistance in populations of *Acris* spp. (Hylidae) (Boyd et al., 1963; Vinson et al., 1963). The resistance to aldrin may have resulted from prior exposure to other chemicals in the group, as the populations had not received an aldrin treatment specifically (Vinson et al., 1963).

The organophosphate insecticides have received less attention in terms of anuran biology than the organochlorines. Kaplan and Glaczenski (1965) studied the hematological effects of six organophosphates on adult Northern Leopard Frogs, *Rana pipiens*. With increasing concentrations of each of these

chemicals, they found a progressive anemia and leucopenia until mortality occurred.

Anuran larvae, unlike the adults, are subjected only to contaminants that accumulate in their aquatic habitats. Tadpoles have been used to study the toxicity of copper sulfate (Landé and Guttman, 1973) and as 'fetal' development indicators of methylmercury pollution (Chang et al., 1974). Since *R. pipiens* tadpoles accumulated high residue levels of DDT, Meeks (1968) suggested that they may be useful as indicators of environmental levels of insecticides. Cooke (1970, 1971, 1973a, 1973b) has studied in depth the effects of pp'-DDT on tadpoles of the British Common Frog, *Rana temporaria*, as well as the effects of dieldrin and the herbicide 2,4-D (1973b). Mulla (1963) simulated natural insecticide-interactions by using field ponds to study the lethal dose of 11 organochlorine insecticides to Bullfrog tadpoles *(Rana catesbeiana)*. He found that doses of some of the chemicals used in mosquito larvicide programs were in fact hazardous to the tadpoles. Application of two organophosphates, Phosphamidon and Bidrin, at 1.12 and 0.23 kg/ha, respectively, appeared harmless to the larvae of *R. pipiens* and *R. catesbeiana* (Oliver, 1964). Extensive toxicity-testing of pesticides on anuran larvae was performed by Sanders (1970). He determined the acute toxicity of 16 and 18 pesticides (insecticides, herbicides, defoliants, and synergists) to tadpoles of *Pseudacris triseriata* and *Bufo woodhousei fowleri*, respectively.

Rana clamitans Latreille and *R. sylvatica* LeConte, whose larvae were studied in this investigation, are both common inhabitants of temporary ponds in forest ecosystems. In eastern Canada, where the majority of Canadian forestry spray operations occur, *R. sylvatica* breeds in early spring and *R. clamitans* breeds from May to July (Logier, 1952). Both are present as adults and/or larvae in large numbers when spray operations are occurring

and they are thus exposed to the insecticides. This investigation was designed to study several aspects of tadpole interaction with three insecticides in common use in forest insect control programs.

B. The Insecticides

The effects of two organophosphorus insecticides, fenitrothion and Orthene®, and one carbamate insecticide, Matacil®, on ranid larvae were selected for study in the present investigation. Information concerning these three chemicals is provided in Table 1. Their structures and some reactions discussed in the text are illustrated in Fig. 1.

Fenitrothion has been applied operationally in Canadian forests since 1969, primarily for control of spruce budworm, *Choristoneura fumiferana*. It is registered for restricted use in forested areas at dosage rates of 140-290 g/ha (Taylor, 1975). Matacil has had temporary registration status since 1973 at restricted application rates of 52-86 g/ha for spruce budworm control. Although not registered in Canada, Orthene is registered in the United States for use against a wide variety of pest species. In Canada, experimental applications of this chemical have been made over limited areas to determine its efficacy against spruce budworms and possible wildlife hazards.

The biochemical mode of action of the three chemicals involves the inhibition of cholinesterase (Corbett, 1974). This enzyme is responsible for the hydrolysis of acetylcholine in both insect and vertebrate nervous systems. Both types of compounds react with the enzyme in a manner precisely analogous to the normal substrate (Corbett, 1974). By not allowing the enzyme to hydrolyse the acetylcholine, these chemicals alters the subsequent normal transmission of impulses across synapses.

There is little information on the effects of Matacil and Orthene on aquatic animals except that the toxicity of Orthene to a variety of fish species is documented. The 96-h LC50 values have been found to range from 1000 ppm for rainbow trout (*Salmo gairdneri*) to 9550 ppm for goldfish

TABLE 1

Description of insecticides

Insecticide	Chemical name	Manufacturer	Insecticide class	Active ingredient (%)	
				From label	Calculated*
fenitrothion (Accothion®, Folithion®, Sumithion®)	0,0-dimethyl 0-(4-nitro-*m*-tolyl) phosphorothioate	Sumitomo Chemical Company Osaka, Japan	Organo-phosphate	97	93
Orthene® (acephate, Ortran®)	0,S-dimethyl acetylphosphor-amidothioate	Chevron Chemical Company Richmond, California	Organo-phosphate	90	88
Matacil® (aminocarb)	4-dimethylamino -*m*-tolyl methylcarbamate	Chemagro Corporation Kansas City, Missouri	Carbamate	75	-

* from gas-liquid chromatographic analysis

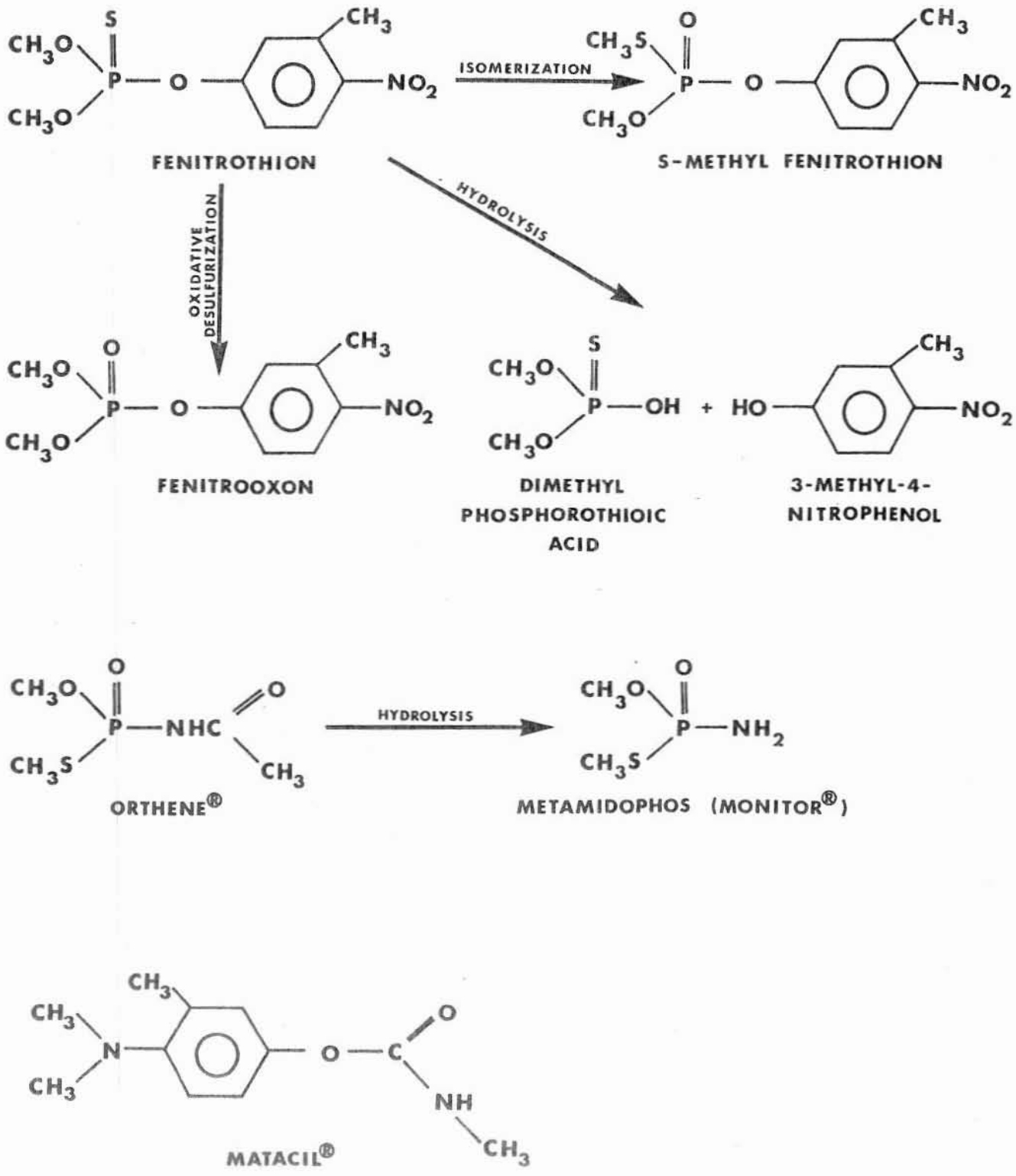

Fig. 1. Chemical structures and reactions discussed in the text.

(Carassius auratus) (Anonymous, 1973).

The 48-h LC50 of Matacil to Atlantic salmon *(Salmo salar)* is much lower at 1.3 ppm than the 1000 ppm value for Orthene to the same species (Nigam, 1975).

The effects of fenitrothion on aquatic animals are much better known. The toxicity of fenitrothion to Atlantic salmon (Wildish et al., 1971), aquatic insects (Wildish and Phillips, 1972), American lobster, *Homarus americanus*, (McLeese, 1974), as well as the toxicity of one of its metabolites, S-methyl fenitrothion, to Atlantic salmon (Zitko and Cunningham, 1975) have been elucidated. Laboratory investigations have determined the effects of fenitrothion on fish behavior (Hatfield and Johansen, 1972; Symons, 1973; Bull and McInerney, 1974; Scherer, 1975), salmonid locomotion (Symons, 1973; Peterson, 1974), fish brain acetylcholinesterase inhibition (Zitko et al., 1970; Wildish et al., 1971), salmonid predator-prey relationships (Hatfield and Anderson, 1972), and the diet of brook trout, *Salvelinus fontinalis*, (Wildish and Lister, 1973). Field studies have also provided in situ information on residues (Hatfield and Riche, 1970; Sundaram, 1974; Eidt and Sundaram, 1975), acetylcholinesterase inhibition (Zitko et al., 1970), and biomass changes in stream fishes (Symons and Harding, 1974). The in vitro inhibition of fish brain acetylcholinesterase by fenitrothion and Orthene is similar, yet there exists a large disparity in their toxicities to trout (Klaverkamp et al., 1975).

Little is known concerning the toxicity or effects of these three insecticides on anuran larvae. Observations have been made on natural and caged populations of *R. sylvatica* and *R. clamitans* under operational and experimental spray applications of fenitrothion (Rick and Gruchy, 1970) and Matacil (Rick and Gruchy, 1971) applied at 140 g/ha and 105 g/ha, respectively.

Even though the deposit was not measured, the authors concluded that these sprays had little direct effect on the tadpoles.

The present study investigates the mortality and behavior of anuran larvae subjected to these insecticides and the degradation dynamics of fenitrothion and Orthene in tadpoles and associated water samples.

II Materials and Methods

A. Toxicity of Fenitrothion, Matacil, and Orthene to Larvae of the Green Frog, *Rana clamitans*.

Green Frog tadpoles *(Rana clamitans)* were collected as required from two ponds in the Larose Forest, Clarence and Cambridge Twps., Russell County, Ontario (Fig. 2) and were transported to the Environmental Impact Laboratory at the Chemical Control Research Institute (C.C.R.I.), Ottawa. The tadpoles were maintained in a mixture of their own pond water and aged tap water in 57-ℓ glass aquaria with aeration. Pond vegetation and organic debris, supplemented with Tetra Min Staple Food for Tropical Fish (Tetra Werke, Melle, W. Germany), were added as food. The aquaria were housed in a controlled environment room at 21 ± 1 °C, a temperature close to the ambient temperatures of their natural ponds. The tadpoles were allowed to acclimate for 48 h prior to toxicity-testing.

Preliminary testing to determine the toxicity range of the three insecticides was carried out with three tadpoles per 250 ml of test solution in a 500-ml Erlenmeyer flask. Technical grade Matacil® (wettable powder) and Orthene® (water-soluble solid) were weighed, with correction for label percent active ingredient, and added directly to the test solution at the desired concentrations. Fenitrothion, a liquid, is relatively insoluble in water and requires a solvent and emulsifier to make it miscible with water. Xylene and Tween®80 (J.T. Baker Chemical Co., Phillipsburg, New Jersey), respectively, were initially tried, but resulted in high mortality of the tadpoles at low concentrations. Corexit-7664®, a nonionic polyethylene glycol dissolved in isopropanol (Enjoy Chemical Co., New York, New York) was then used because of its low toxicity to Atlantic salmon parr (Zitko, 1970). The desired volume of technical fenitrothion was determined from

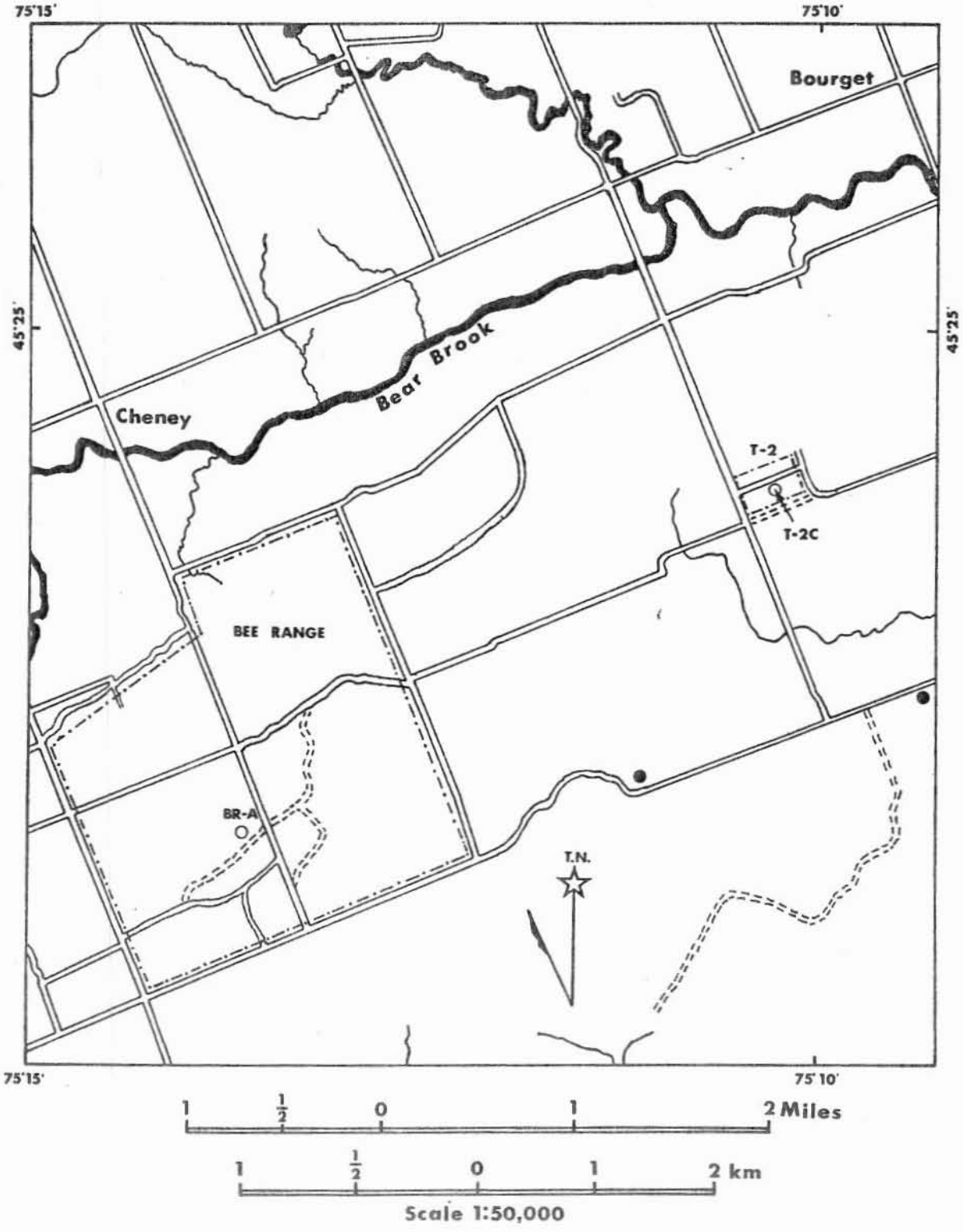

Fig. 2. Map of the study area in Larose Forest showing spray plots ——, test ponds o , and larvae collection locales ● .

the specific gravity (d_4^{25} = 1.3227 (Krehm, 1973)) and dissolved in the Corexit-7664 (volume/volume in a ratio of 1:10) to form a 1000-ppm stock solution. The various test solutions were made from dilutions of this solution. Tap water used in the tests was charcoal-dechlorinated.

Static bioassays, with the insecticide added at the beginning of the test, were chosen for economic reasons and because they best approximate pesticide addition to natural ponds. Bioassays were conducted in 4 ℓ of test solution with 10 tadpoles placed in each test container. The test containers were disposable polystyrene Econo-Cages® (Maryland Plastics Incorporated, New York, New York, E-0210), which when filled with test solution had a depth of 8.8 cm and a surface area of approximately 490 cm^2. Each container held a different concentration of one of the three pesticides. For each insecticide, concentrations were chosen within the toxicity range, as estimated from the pilot experiments. The tadpoles and treatments were assigned to the containers at random. One water control and one Corexit 7664 - water control (concentration equal to the amount in the highest fenitrothion concentration) were also established for each set of experiments. The tadpoles were checked six times daily at the beginning of each test and less frequently as the tests progressed. During each observation, the frequency of poisoning symptoms was observed, and dead animals were removed. If a tadpole failed to respond to a tactile stimulus, it was removed from the test container, placed in a petri dish with test solution, and viewed under a dissecting microscope. If there was no evidence of heart action or blood flow in the cutaneous capillaries, the tadpole was considered dead. Microscopic examination also aided in the diagnosis of post-mortem poisoning symptoms. Live animals were returned to the test container. Total length and snout-vent length of the dead tadpoles were recorded and the carcasses were preserved in 10% formalin. To assess the effects of size on toxicity,

testing was repeated at a later date, using Matacil on tadpoles larger than the ones previously used.

Strictly speaking this conventional method does not, in fact, determine the toxicity of a compound because test animals remain in toxic solution until dead. But death may be irrevocably determined within some shorter period of exposure. To determine this period exactly, it would be necessary to remove groups of test animals from the toxic medium at varying periods of time and assess mortality after some post-exposure period in a non-toxic medium. Therefore, actual toxicities may be higher than those indicated by the method used in this work.

B. Insecticide Degradation Dynamics

1. Laboratory Determination

Rana clamitans tadpoles, obtained and housed in the same manner as described in the previous section, were placed in two 57-ℓ glass aquaria. Each aquarium contained 45 ℓ of test solution, with 1.0 ppm fenitrothion in one, and 1.0 ppm Orthene in the other. The test media were prepared as previously described.

Water and tadpole samples were removed from the aquaria as indicated in Table 2, Columns 1 and 3. Both fenitrothion- and Orthene-treated tadpoles were preserved in 50 ml ethyl acetate and stored at -12 ^{o}C in brown glass bottles, with screw caps and aluminum foil covers, until they were analysed for insecticide residues. Since Orthene is more soluble in water than in other organic solvents (Anonymous, 1973), Orthene water samples were stored immediately at -12 ^{o}C until they too were analysed. Samples (750 ml) of the water containing fenitrothion were extracted into 100 ml of toluene in a 100-ml separatory funnel immediately after collection. The toluene phase was then passed through a plug of anhydrous sodium sulfate to remove any remaining water and collected in a brown glass bottle. The separatory funnel and sulfate were washed several times with an additional 50 ml of toluene which was added to the sample bottle. These samples were also stored at -12 ^{o}C.

2. Field Determination

Two ponds containing natural populations of Wood Frog tadpoles (*Rana sylvatica)* were selected in the Larose Forest. One pond (T-2C) was located within a 16.2-ha plot (T-2) to be treated with an experimental application of fenitrothion; the other (BR-A) was located within a 518-ha plot (Bee Range) to be treated with Orthene (Fig. 2).

TABLE 2

Time relative to insecticide addition when water (W) and larvae (L) samples were collected in the laboratory and field

Time relative to insecticide addition, d	Fenitrothion				Orthene			
	Laboratory		Field		Laboratory		Field	
	W	L	W	L	W	L	W	L
	(1)		(2)		(3)		(4)	
-1			X				X	
0	X	X	X	X	X	X	X	X
1	X	X	X		X	X	X	X
2			X	X			X	X
3	X	X	X		X	X	X	X
5		X	X		X	X	X	X
7	X	X	X				X	X
10			X					
15	X	X						

Tadpole samples were collected at 4-d intervals prior to and following spray application to estimate the developmental stage and the size of the tadpoles with respect to insecticide dynamics. These tadpoles were preserved in 10% formalin, weighed, staged (Gosner, 1960), and measured at a later date. Maximum-minimum thermometers and graduated wooden posts were placed in the ponds, preceding sampling, to record temperature ranges and water depth changes, respectively. On the day prior to spray application, the surface area and depth of each pond were estimated and selected chemical properties (viz. pH, dissolved oxygen, dissolved carbon dioxide, hardness, acidity, and alkalinity) were measured using a Hach water analysis kit. Aluminum pans (17.8 x 14.0 x 2.0 cm deep) were placed at intervals around the ponds, as indicated in Fig. 3, to estimate the amount of spray deposited.

The fenitrothion pond was aerially sprayed at 0645 h on 10 June 1975 and the Orthene pond at 0630 h on 19 June 1975. Insecticides were emitted at the rates of 280 and 560 g active ingredient per hectare, respectively, from two Cessna aircraft, both fitted with Micronair nozzles. Fenitrothion was sprayed in an oil (Arotex) solution and Orthene was sprayed in a water solution. Even though this was an experimental spray, it was applied under the same conditions that would prevail during an operational control program.

Water and tadpole samples for residue analysis were collected on days indicated in Table 2, columns 2 and 4. Day 0 represents one hour after spray application. The remaining post-spray samples were collected at approximately the same time each day. Water samples were collected in 900-ml glass Mason jars approximately 15 cm below the surface of the pond at the same location each time. Tadpole samples were collected from various locations in the pond, at random, with a dip net, 30 cm in diameter, and preserved in 50 ml ethyl acetate. All samples were transported immediately in an ice

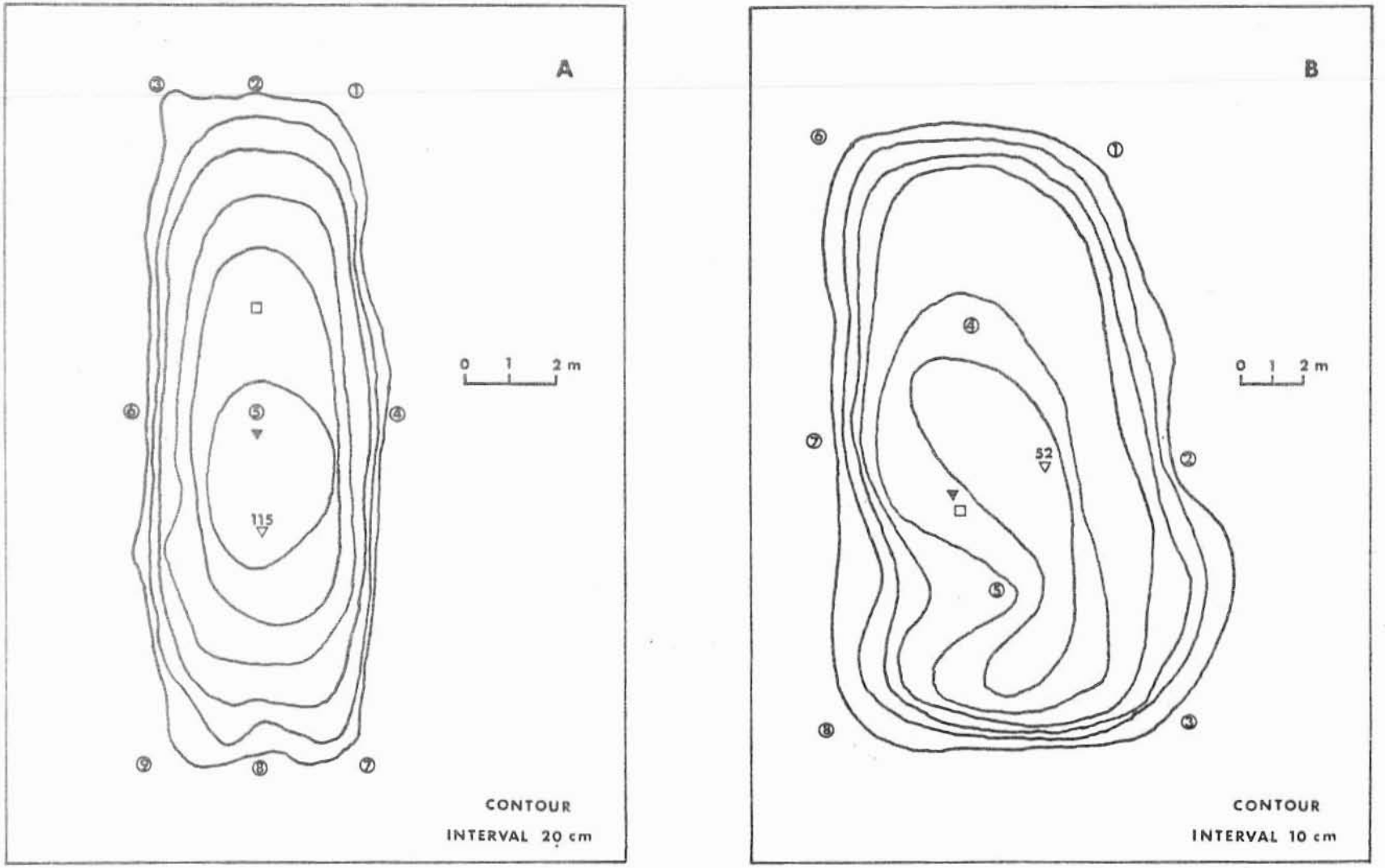

Fig. 3. A, Fenitrothion experimental pond (T-2C) and B, Orthene experimental pond (BR-A). Depths recorded on 9 and 18 June 1975 respectively. Spray deposit pan o ; depth post □ ; maximum depth in cm ▽ ; water collection site ▼ .

chest to the laboratory and all but the fenitrothion water samples were stored at -12 °C. The fenitrothion in the water samples was extracted with toluene as previously described and then stored at -12 °C.

Rainfall data were obtained from the Larose Forest Forestry Station, Ontario Ministry of Natural Resources. Spray pans were analysed by the Spray Physics Laboratory, Chemical Control Research Institute, Environment Canada, Ottawa.

3. Extraction and Analysis of Fenitrothion (Sundaram, 1974).

Tadpole samples were weighed and the fenitrothion and metabolites extracted in the original 50 ml of ethyl acetate plus an additional 50 ml in a Sorvall-Omni-Mixer (5 min, speed 6). The extract was filtered through a sharkskin filter paper and washed with an additional 10 ml of ethyl acetate. The extract was then passed through a plug (5 g) of anhydrous sodium sulfate into a 500-ml round-bottom flask and flash-evaporated to approximately 5 ml. The residue was dissolved in 25 ml of acetonitrile and partitioned twice with 25 ml of hexane. The hexane phase was discarded and the acetonitrile phase was flash-evaporated to approximately 2 ml. The residue was transferred quantitatively to a column containing 2.5 g of an activated charcoal- Celite 545 mixture (6:4 w/w ratio) between two 5-g layers of anhydrous sodium sulfate and eluted with 100 ml of 25% benzene in ethyl acetate. The eluate was then flash-evaporated to 2 ml for gas-liquid chromatographic (GLC) analysis.

The toluene extract partitioned from the water samples was measured and flash-evaporated to a small volume. The extract was transferred to a graduated centrifuge tube and the volume was adjusted to 10 ml for GLC analysis without further clean-up.

GLC analysis was carried out using a Hewlett-Packard model 7610A gas chromatograph fitted with a flame photometric detector. The operating parameters are outlined in Table 3. This method allows for the identification of the parent compound, fenitrothion, and two of its metabolites, fenitrooxon and S-methyl fenitrothion. Chromatographs were standardized, using standard solutions, prepared from analytical grade samples obtained from the Sumitomo Chemical Company, Japan.

A sample of the technical fenitrothion used in the toxicity and behavioral studies was also analysed using the GLC.

During the toluene-partitioning of fenitrothion from the laboratory water sample, difficulties were experienced in obtaining complete separation of the two immiscible phases. The formation of a cuff probably resulted from the use of the Corexit-7664 which functions as a surfactant. Subsequent passing of the toluene phase through the sodium sulfate plug resulted in the loss of measurable amounts of the solvent and presumably fenitrothion residues. Since partition coefficients are not dependent on volume, it was assumed that the proportion of solvent lost was equal to the proportion of fenitrothion lost. The data were corrected accordingly.

4. Extraction and Analysis of Orthene (C.C.R.I., unpublished data)

Orthene and its metabolite, Monitor®, were extracted from the tadpoles in a manner similar to the method described for fenitrothion-treated tadpoles. The technique was identical until the solvent-partitioning stage. Here 50 ml of acetonitrile and 20 ml of hexane were used instead of the volumes already noted. The acetonitrile phase was then flash-evaporated to dryness. A column containing a 5-g layer of SiO_2 between two 5-g layers of sodium sulfate was utilized for the clean-up procedure. The column was initially rinsed with 20 ml of ether. The residue was dissolved in 40 ml of ether and transferred quantitatively to the column. The column was then rinsed with 25 ml of a 5% methanol in ether solution. All these rinsings were discarded. The insecticide residue was eluted with 150 ml of a 10%

TABLE 3

Gas-liquid chromatograph operating parameters

Parameter	Gas-liquid chromatograph	
	Hewlett-Packard 7610A	Hewlett-Packard 810
Detector	FPD (P-mode)	FPD (P-mode)
Column:		
Length	1.83 m	0.46 m
Inside diameter	4 mm	6.4 mm
Support	Chromosorb W A.W. DMCS	1% Reoplex-400 on Gaschrom Q
Mesh	80/100	100/200
Temperature:		
Injection port	240 °C	200 °C
Oven	195 °C	130-180 °C
Change rate	-	0.25 °C/s
Detector	175 °C	200 °C
Gas flow:		
Nitrogen (carrier)	1.30 ml/s	0.33 ml/s
Air	2.50 ml/s	2.50 ml/s
Oxygen	0.83 ml/s	1.25 ml/s
Hydrogen	0.33 ml/s	0.20 ml/s
Attenuation	32	32
Range	10^3	10^3
Chart speed	0.21 mm/s	0.11 mm/s
Retention time (min)	4.4 (fenitrothion)	4.28 (Monitor) 5.91 (Orthene)

methanol in ether solution. The resulting eluate was then flash-evaporated to dryness and then dissolved in ethyl acetate for GLC analysis.

A 50-ml aliquot from each of the Orthene water samples was mixed, in a Sorvall container, with approximately 150 g of anhydrous sodium sulfate. A slurry resulted from continuous stirring of the mixture while the container was immersed in a cold water bath. The insecticide was extracted from the slurry by adding 150 ml of ethyl acetate to the container and mixing in a Sorvall-Omni-Mixer for 5 min at speed five. The resulting solution was then passed through two 50-g plugs of Na_2SO_4. The plugs were each rinsed with an additional 25 ml of ethyl acetate. The ethyl acetate was flash-evaporated to dryness. The residue was then dissolved in 10 ml of methyl isobutyl ketone for GLC analysis. A Hewlett-Packard model 810 gas-liquid chromatograph with a Tracor flame photometric detector was used for the insecticide analysis. The GLC operating parameters are listed in Table 3. Both Orthene and its hydrolysis product methamidophos (Monitor) can be detected using this method.

Technical Orthene used in the lethality and behavior experiments was also analysed.

5. Matacil

Analytical techniques to assess aquatic levels of Matacil are currently under development by the Pesticide Chemistry Section of the Chemical Control Research Institute, Environment Canada, Ottawa, and were thus unavailable for this investigation.

C. Behavior of Insecticide-treated *Rana clamitans* Larvae

Preliminary observations on individual tadpoles in 4 ℓ of water resulted in the definition of a five-category behavioral repertoire (Table 4). As the movements of one tadpole affect the movements of another, larvae were studied singly. Individual larvae (mean (±SE) body length, 11.2 ± 0.3 mm; range of developmental stages (after Gosner, 1960), 27-30), randomly selected, were subjected to sublethal concentrations of one of the three insecticides formulated and applied as described in Section A. Five concentrations, all lower than the concentrations used in the toxicity tests, were selected for each insecticide (fenitrothion, 0.25 - 3.00 ppm; Matacil, 1.0 - 50.0 ppm; Orthene, 100 - 2000 ppm). Test concentrations were assigned at random with 5 - 15 larvae tested daily. Tadpoles were allowed to acclimate for 24 h in the insecticide medium in a controlled environment room at 21 ± 1°C with a photoperiod of 12 h light and 12 h dark. After 24 h the room was entered and a 15-min period was allotted to allow the tadpoles to resume their normal activity. This precaution was taken to lessen the effect of the initial disturbance. Each larva was observed for 10 min from a distance of 1 m. Individual behavioral events were counted using a multi-event mechanical counter and total activity time (s) was determined using a cumulative-time stop-watch. The amount of time each tadpole spent resting on the surface was recorded to the nearest minute and the color of each animal during the observation period was noted. Seven tadpoles were observed in each insecticide concentration and in a Corexit-7664 control, the concentration being equal to the amount used to emulsify the highest fenitrothion concentration. Ten tadpoles were observed in water controls. Opaque cardboard partitions were placed between adjacent test tanks so the movements of one tadpole would not affect the movements of the next.

TABLE 4

Behaviors of *Rana clamitans* larvae used in the behavioral study

Behavior	Definition
A. Bubble-making	Movement from resting position to air-water interface with the formation of a small bubble from the mouth
B. Unidirectional swimming	Movement in one direction (may be interrupted by the end of container resulting in a new direction)
C. Multidirectional swimming	Movement in more than one direction (includes erratic movements)
D. Feeding movements	Slow undulating movement along the sides or bottom of the container, with accompanying jaw movement
E. Single tailbeat	Movement of tail in one full oscillation or less (may not result in net movement of the animal)

III Results

A. Toxicity-testing

1. Log-probit Transformed Distributions of Time to Death

To obtain linear plots from the time-response data, cumulative percent mortalities were converted to probits, standard deviations above and below the mean response with 5.0 added to eliminate negative numbers (Sprague, 1969), and times were converted to logarithms (Figs. 4-7). This method assessed whether or not the log times approximated a normal distribution.

Complete kills were recorded at all concentrations of fenitrothion (Fig. 4) indicating that the incipient LC50 value, lethal concentration for 50 percent of the individuals on long exposure (Sprague, 1969), was exceeded. Total mortality occurred within 160 h, a comparatively short exposure interval.

Approximate straight-line distributions of time to death were obtained for small larvae treated with Matacil (Fig. 5), except at the lowest concentration, 75 ppm (Fig. 5A). The two lowest concentrations, 75 and 100 ppm (Fig. 5A, B), resulted in partial mortalities of 70 and 80% after 365 and 332 h, respectively.

Reductions in the mortality rate and incomplete mortality of the tadpole sample occurred in groups treated with Orthene at 6000 - 7000 ppm (Fig. 6A-C). These events coincided with the appearance, at approximately 48 h, of a precipitate at the air-liquid interface. The amount of precipitate appeared to be positively correlated with the concentration of Orthene in solution. This film was observed on the surface of all Orthene test containers. The remainder of the Orthene concentrations resulted in normally distributed mortality, in less than 48 h.

Sporadic mortality occurred in several of the large tadpole samples (mean (± SE) body length, 10.9 ± 0.1 mm; mean (± SE) total length, 31.1 ± 3.2 mm) treated with Matacil (Fig. 7A-C, F). Complete mortality occurred at all

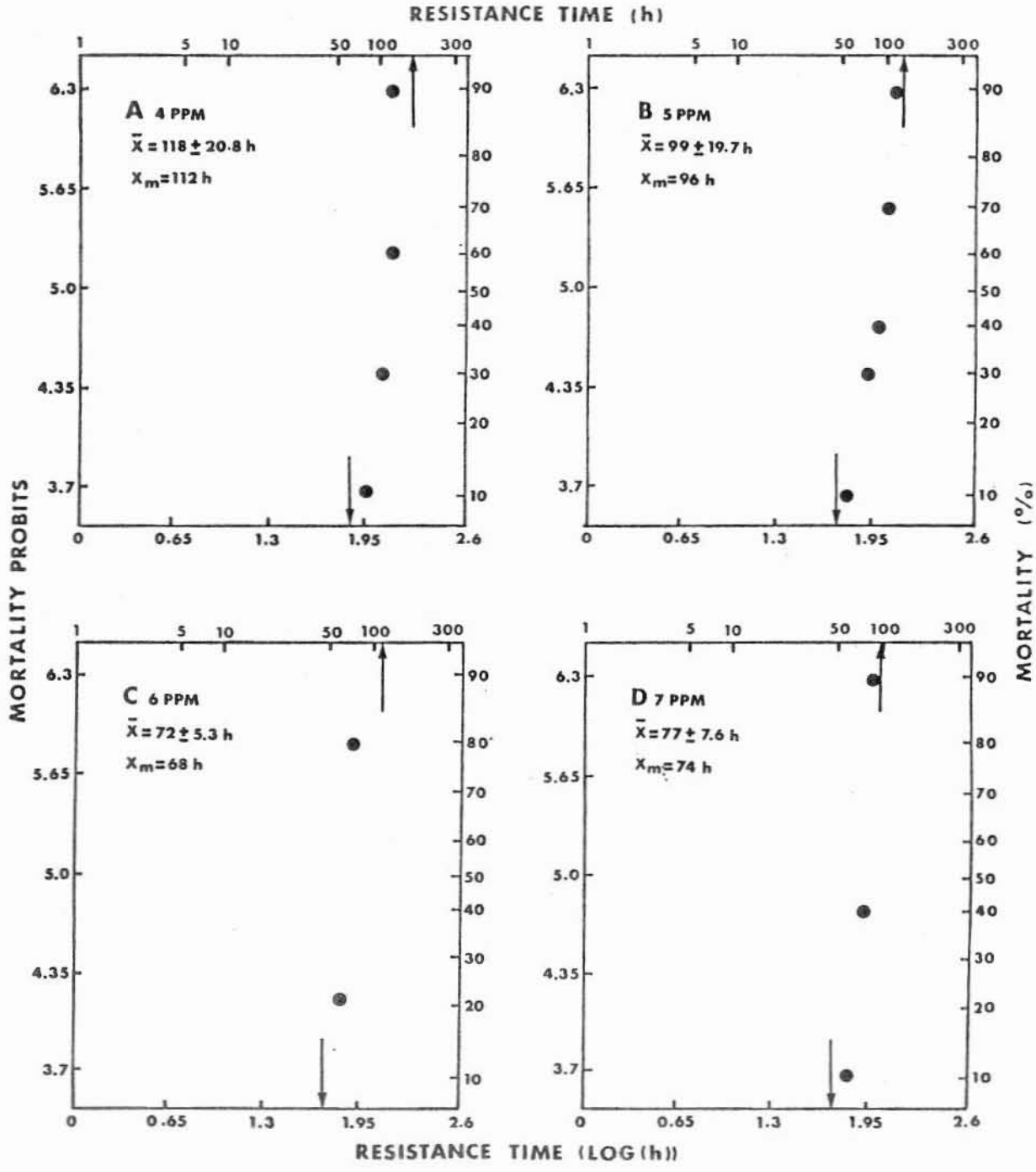

Fig. 4A-D. Log-probit transformed distributions of time to death for fenitrothion-treated *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

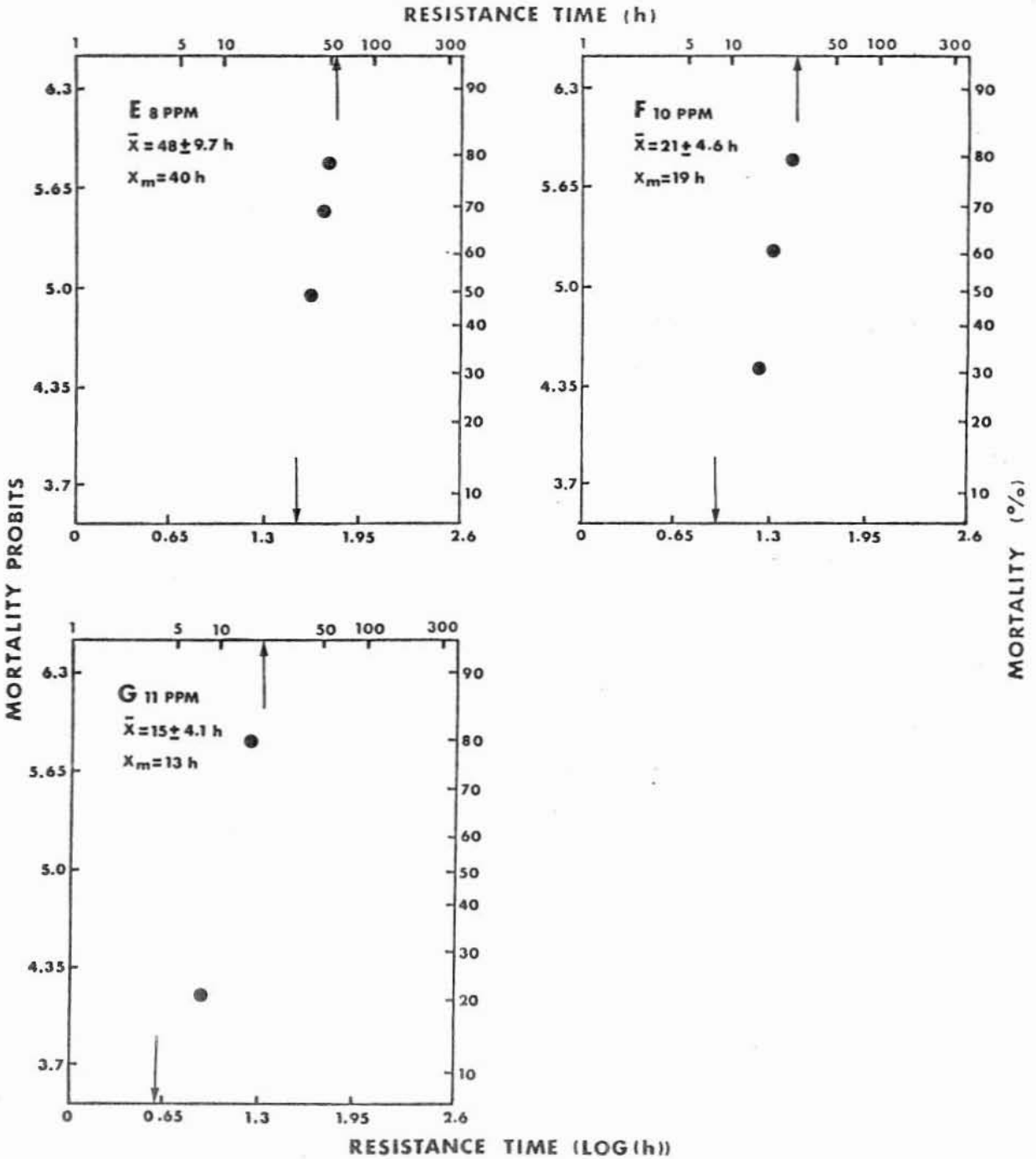

Fig. 4E-G. Log-probit transformed distributions of time to death for fenitrothion-treated *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

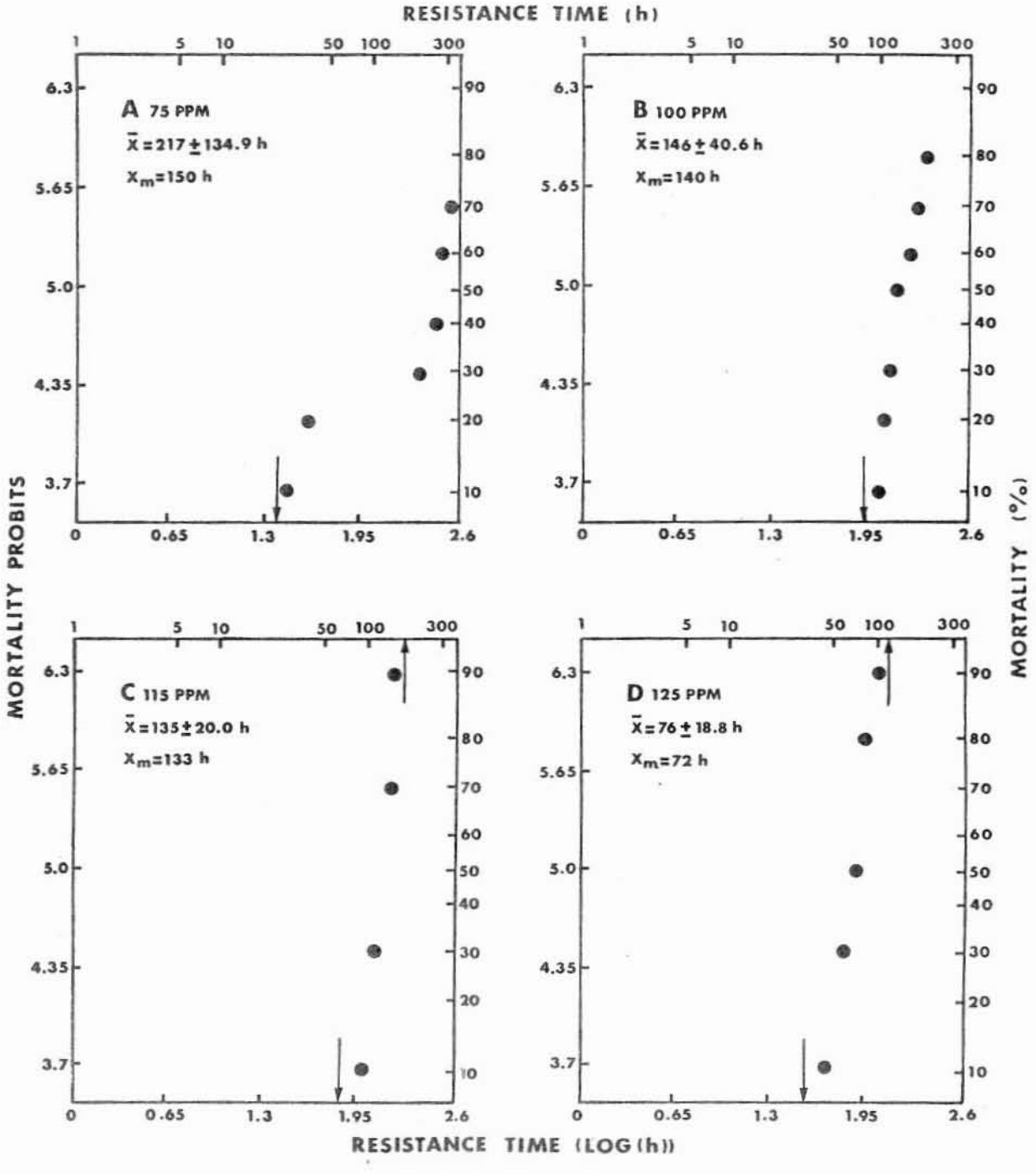

Fig. 5A-D. Log-probit transformed distributions of time to death for Matacil-treated small *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

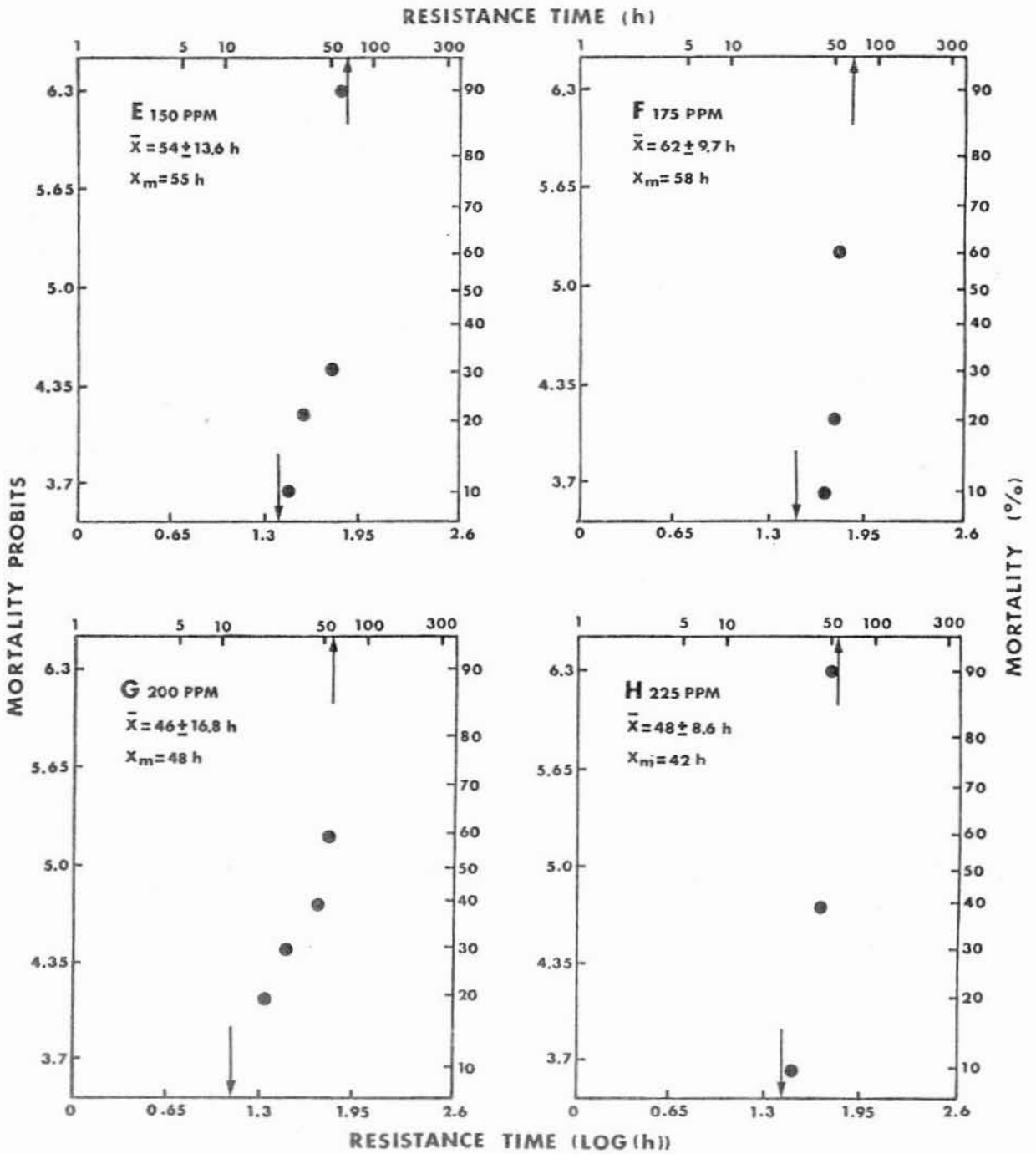

Fig. 5E-H. Log-probit transformed distributions of time to death for Matacil-treated small *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

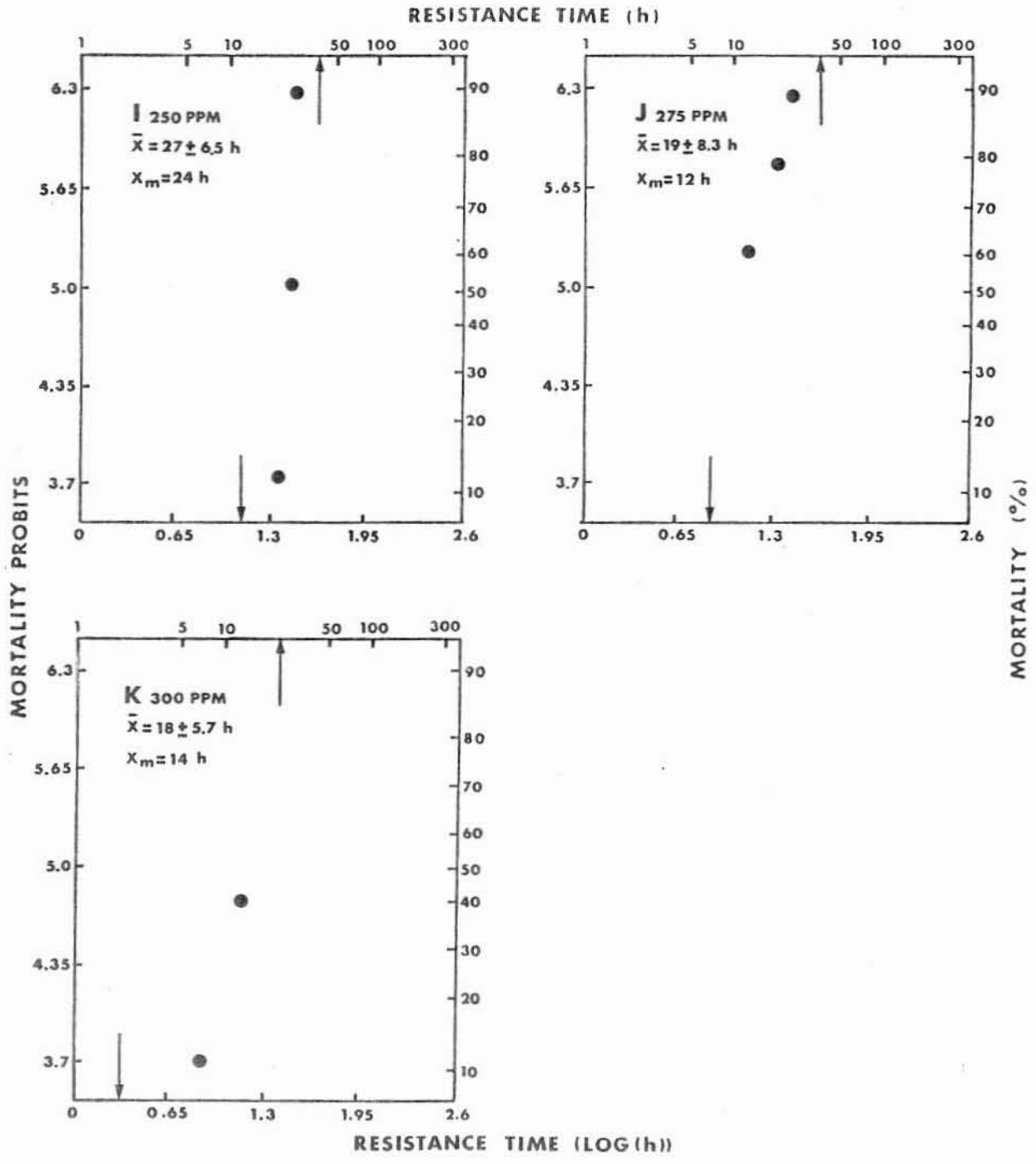

Fig. 5I-K. Log-probit transformed distributions of time to death for Matacil-treated small *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

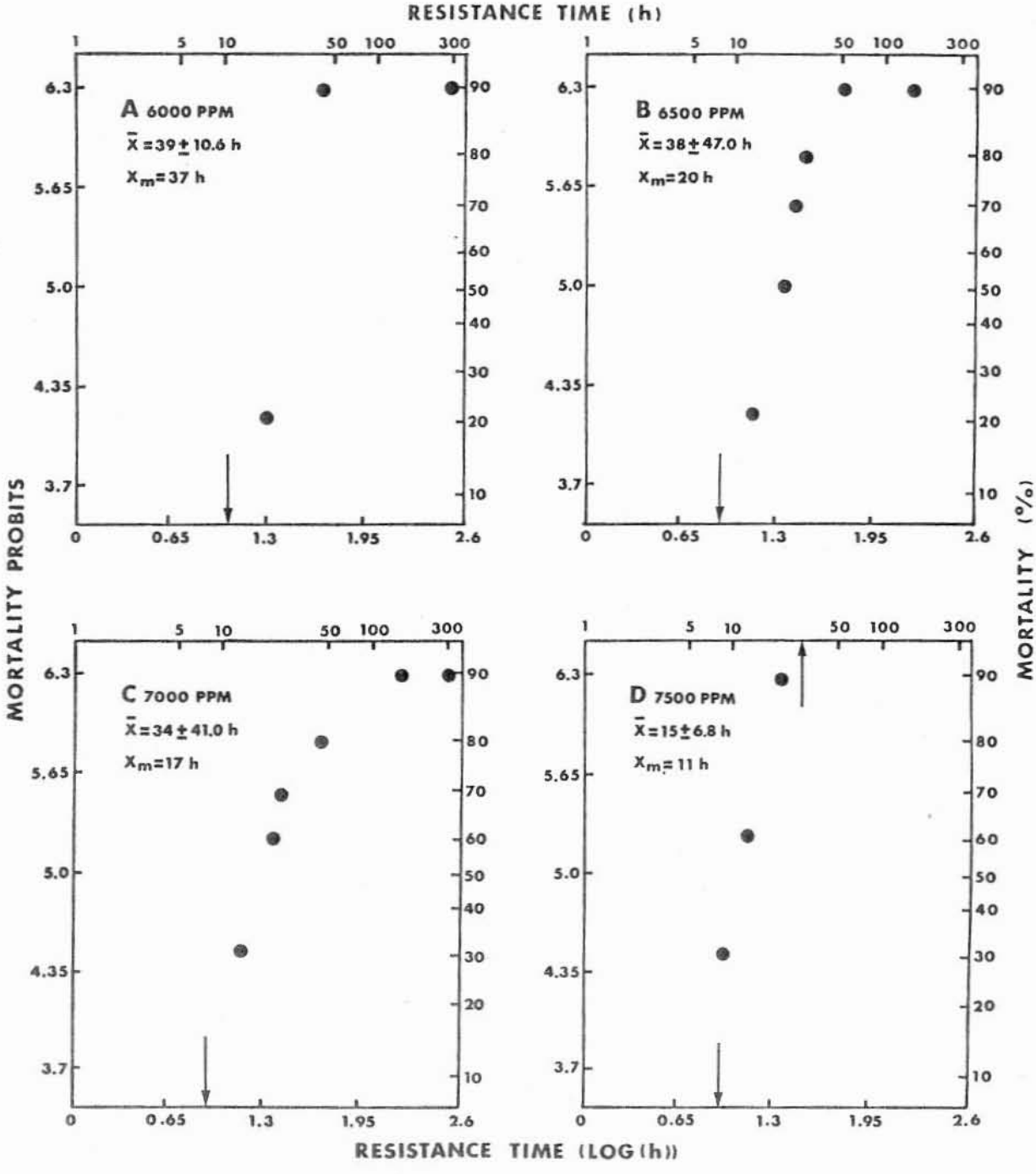

Fig. 6A-D. Log-probit transformed distributions of time to death for Orthene-treated *Rana clamitans* larvae. $\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

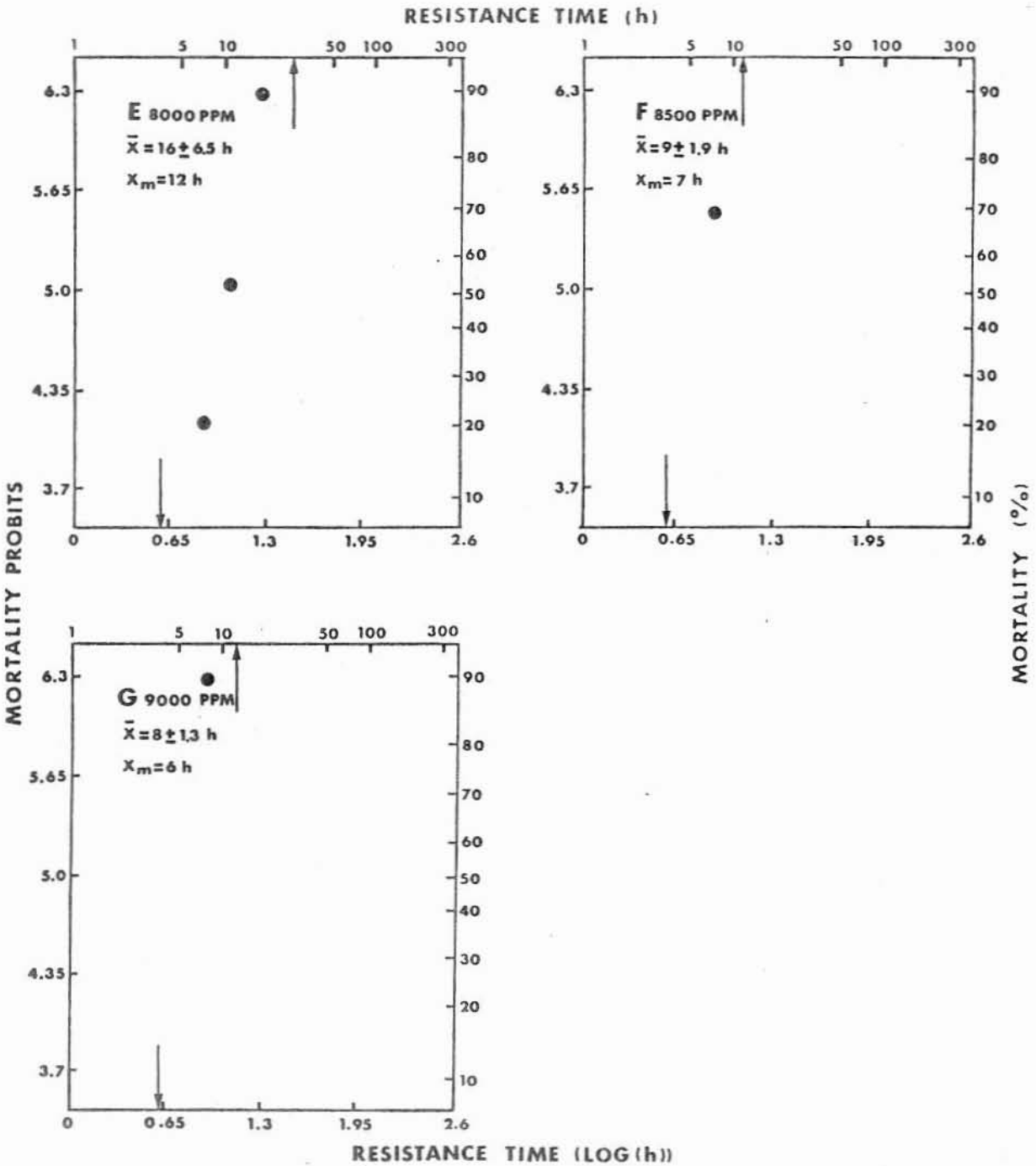

Fig. 6E-G. Log-probit transformed distributions of time to death for Orthene-treated *Rana clamitans* larvae. $\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

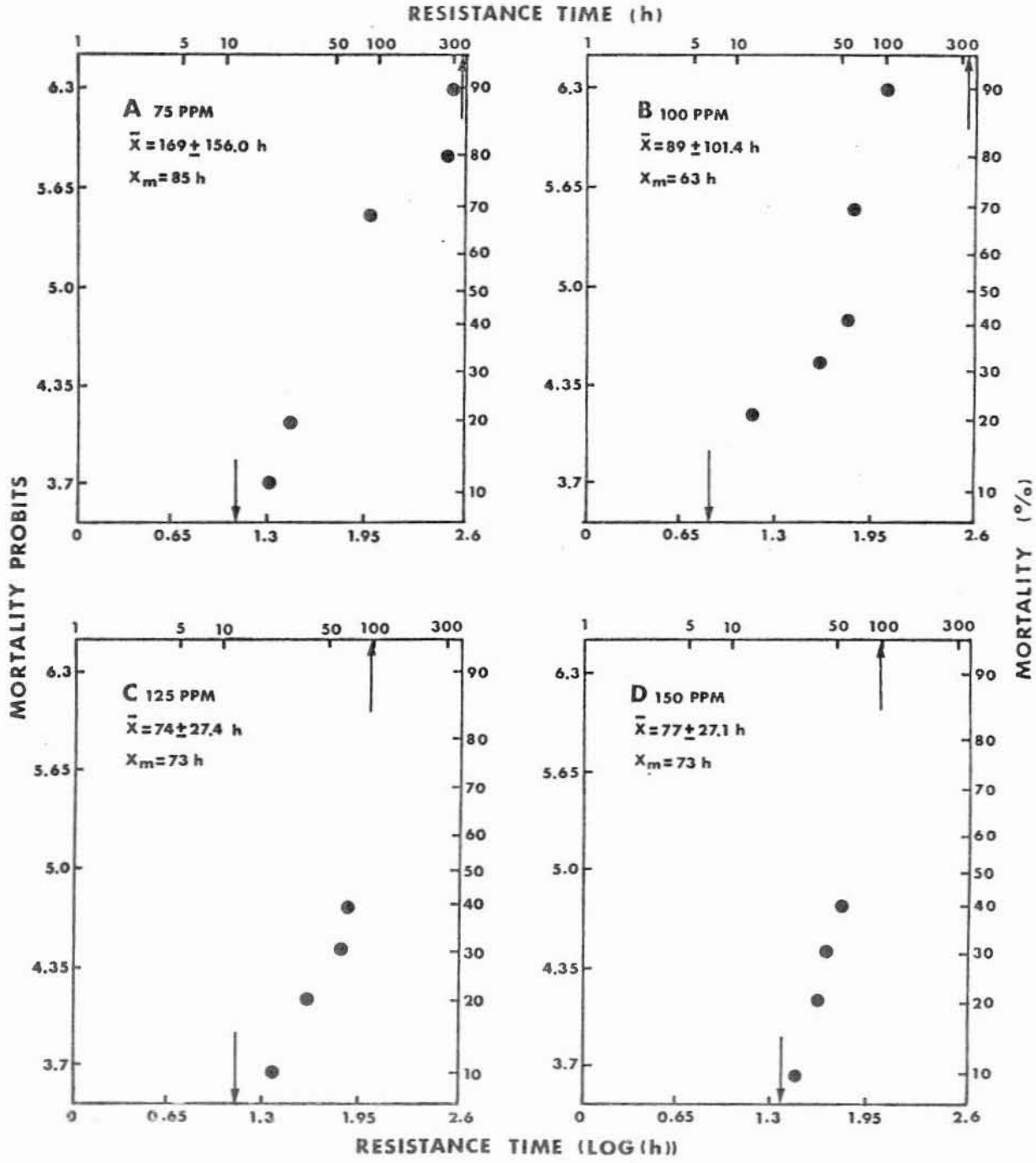

Fig. 7A-D. Log-probit transformed distributions of time to death for Matacil-treated large *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

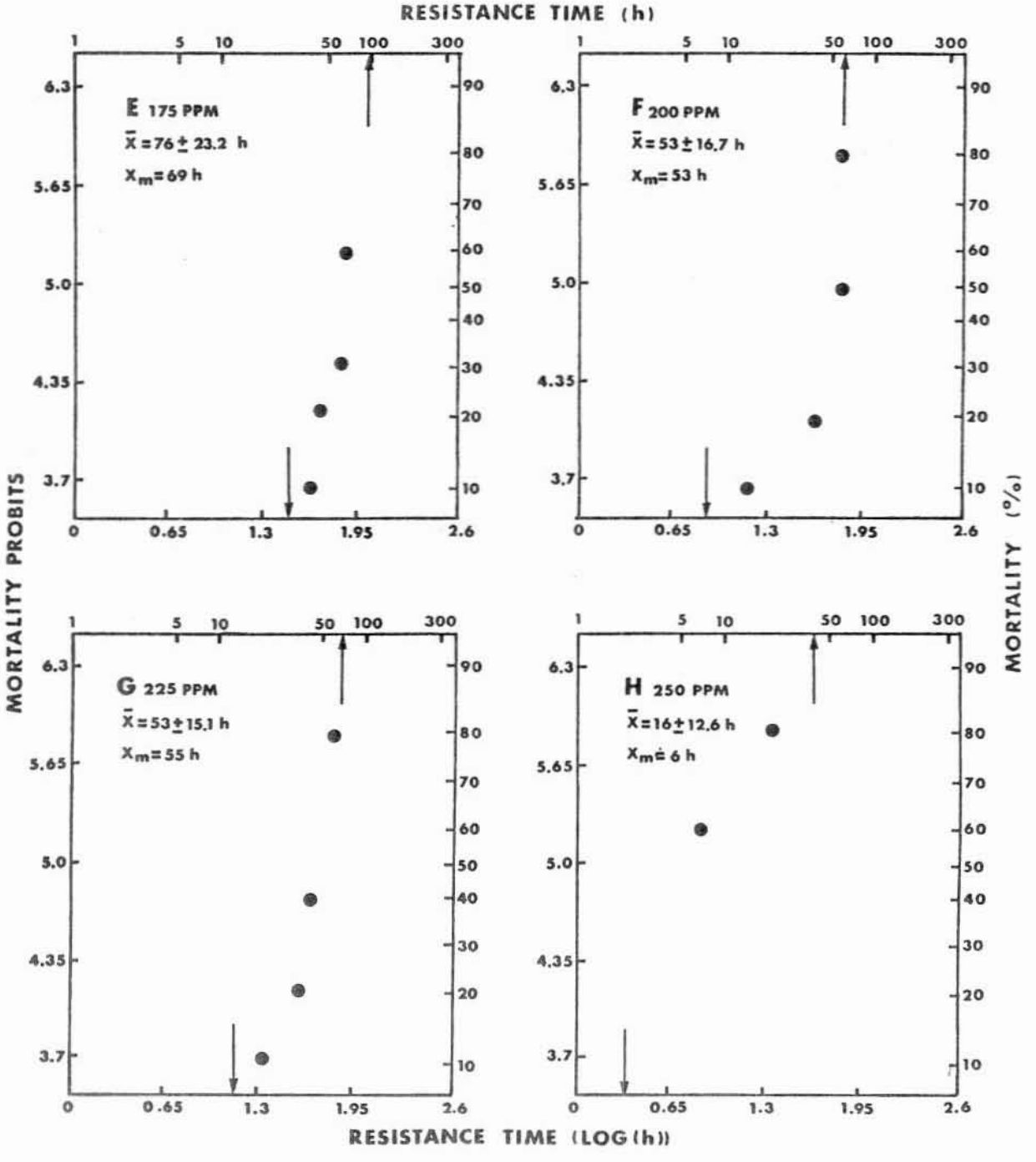

Fig. 7E-H. Log-probit transformed distributions of time to death for Matacil-treated large *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

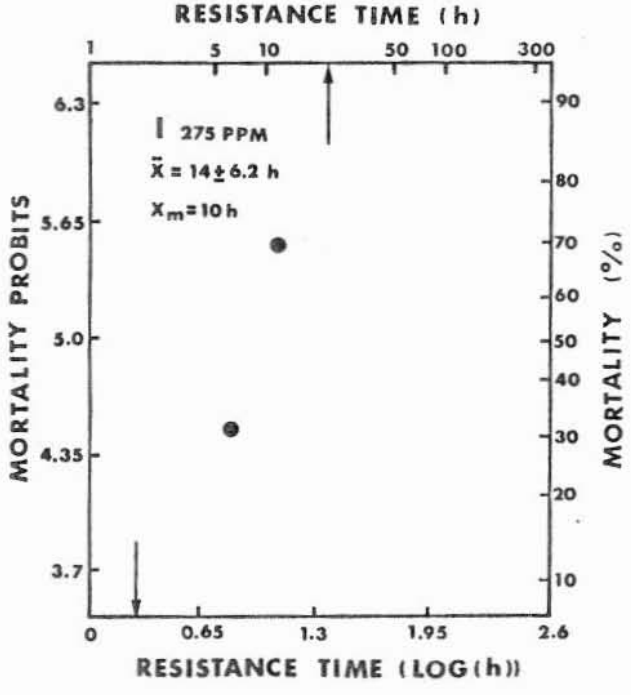

Fig. 7I. Log-probit transformed distributions of time to death for Matacil-treated large *Rana clamitans* larvae.

$\bar{X}$ = mean ± standard deviation; X_m = median; ↓ = 0 % mortality; ↑ = 100 % mortality.

concentrations. The irregular nature of the tadpole mortality seems to be related to the high mortality (30%) in the corresponding control group for this series of tests. No mortality occurred in either the Corexit-7664 or water controls for the insecticide treatments of the small larvae (mean (± SE) body length, 8.2 ± 1.1 mm; mean (± SE) total length 22.4 ± 2.3 mm).

Volumes of test solution per mass of tadpole tissue values were approximately 2.9 and 1.7 ℓ/g for the small and large tadpoles, respectively.

2. Estimation of LC50 Values

The LC50 values(concentration required to kill 50% of the individuals in a given time) marked by an asterisk in Table 5 were estimated using maximum likelihood probit regression analysis with fiducial limits (Finney, 1952) calculated accordingly. This method compares the likelihood of mortality in the control groups with mortality in the treated groups.

Because of the complex distribution of the response times at certain dose levels, LC50 values could not be calculated for all the biased times (24, 48, 72, and 96 h). For this reason a model that would combine the relationship between mortality, time, and dose was sought. A low parametric model was used, estimating median lethal times directly and seeking a relationship with dose. Approximate linear regressions were obtained using logarithmic transformations of dose for the fenitrothion - (Fig. 8) and Matacil-treated (Fig. 9) small larvae. Transforming both median lethal times and dose to logarithms resulted in an approximate linear regression for the Orthene-treated larvae (Fig. 10). Departures from these regressions (Figs. 8-10) provided estimates of error variance and approximate 95% fiducial curves. These limits agreed well with the limits obtained by maximum likelihood regression analysis.

TABLE 5

LC50 (± 95 % fiducial limits) values for insecticide-treated *Rana clamitans* larvae

	Insecticide treatment			
LC50	Fenitrothion, ppm	Matacil, small larvae, ppm	Matacil, large larvae, ppm	Orthene, ppm
24 h	9.9(8.9-?)	247(232-262)*	234(223-246)*	6433(5857-6775)*
48 h	7.8(7.1-8.5)	206(191-220)*	-	-
72 h	6.2(5.6-6.8)*	161(145-180)	-	-
96 h	4.9(4.2-5.3)	118(112-125)*	-	-

* from maximum likelihood regression analysis

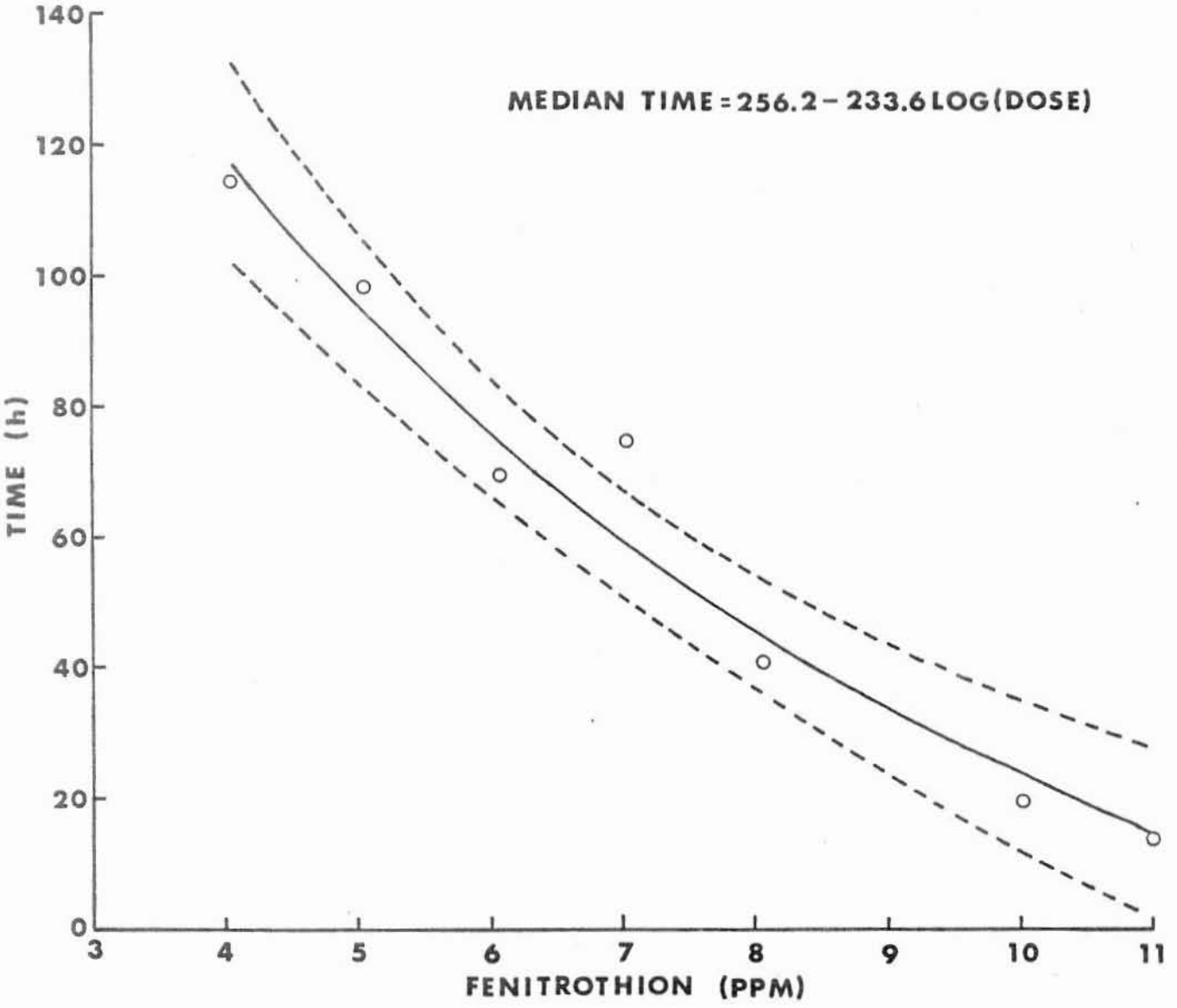

Fig. 8. Toxicity curve of fenitrothion to *Rana clamitans* larvae. Solid line indicates regression curve; dashed lines represent 95 % fiducial limits; circles denote median mortality responses.

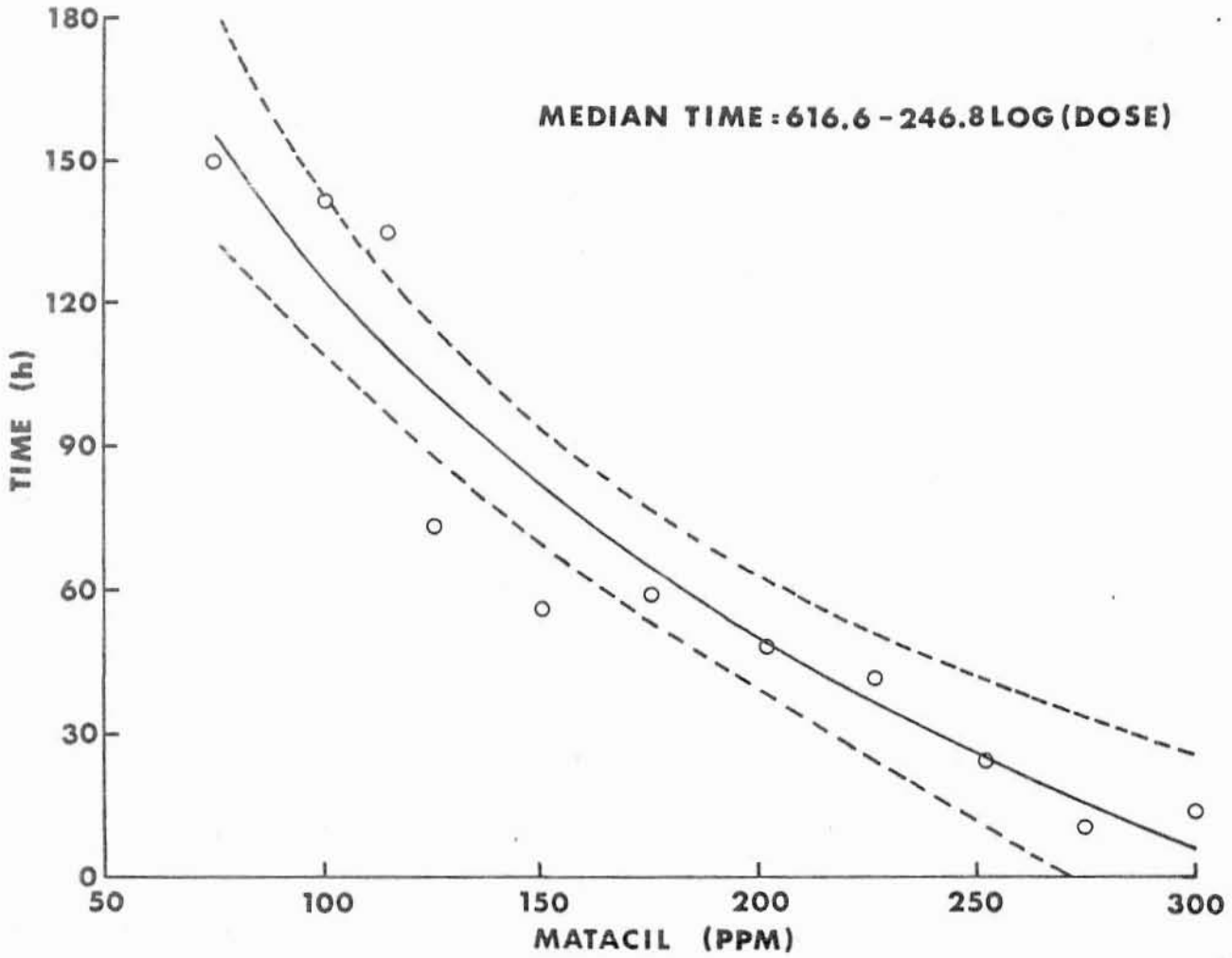

Fig. 9 Toxicity curve of Matacil to small *Rana clamitans* larvae. Solid line indicates regression curve; dashed lines represent 95 % fiducial limits; circles denote median mortality responses.

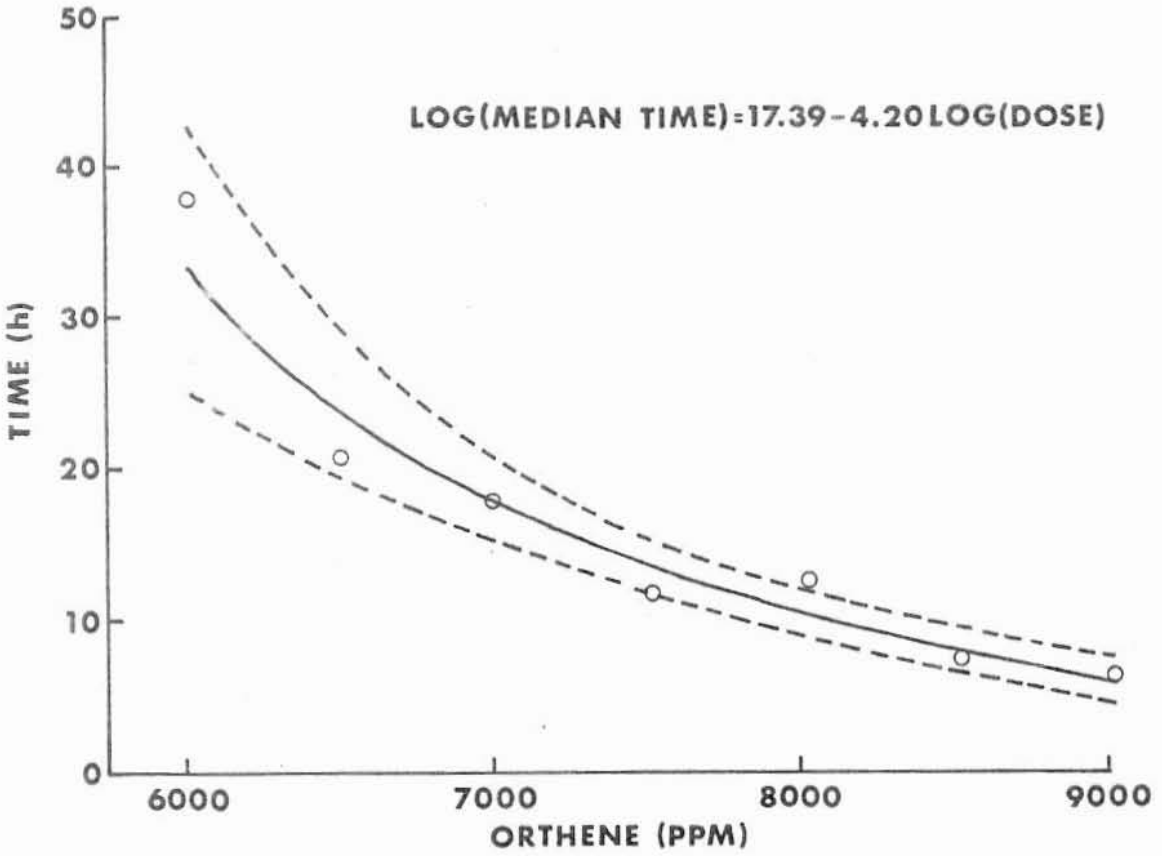

Fig. 10. Toxicity curve of Orthene to *Rana clamitans* larvae. Solid line indicates regression curve; dashed lines represent 95 % fiducial limits; circles denote median mortality responses.

As a result of the mortality pattern exhibited by the Matacil-treated large larvae (Fig. 11), an approximate model could not be determined.

LC50 values at 48, 72, and 96 h could not be estimated for Orthene-treated larvae because the curve does not intercept these values, (Fig. 10).

At the 50% response level, fenitrothion was found to be 25 times more toxic to small larvae than Matacil which in turn was 26 times more toxic than Orthene to the larvae at a 24-h exposure interval. The 24-h LC50 estimates for Matacil-treated small and large tadpoles were in close agreement (247 and 234 ppm, respectively).

3. Toxicity Symptoms

Table 6 lists the toxicity symptoms exhibited by the tadpoles treated with each of the three insecticides. Post-mortem examination revealed extensive tissue necrosis in almost all the Orthene-treated tadpoles. Some of the living larvae also had necrotic tissue on the distal portion of their tails. Fenitrothion- and Matacil-treated animals showed physical symptoms involving extensive hemorrhagic regions, jaw-twitching, and ecdysis. For all three chemicals, exposed tadpoles experienced some degree of swimming difficulty, buoyancy problems, and color change. Symptoms related only to Matacil treatments included deformation of the mouth, minimal necrosis, edema, and bent tails. Live trematodes were observed under the integument in dead tadpoles. These were most frequently located at the base of the tail and less frequently posterior to the lower jaw.

Tadpoles typically became moribund (failure to respond to a tactile stimulus) and died shortly thereafter. Some tadpoles, however, became moribund soon after they were placed in the test medium but recovered within the first 24 h only to die later.

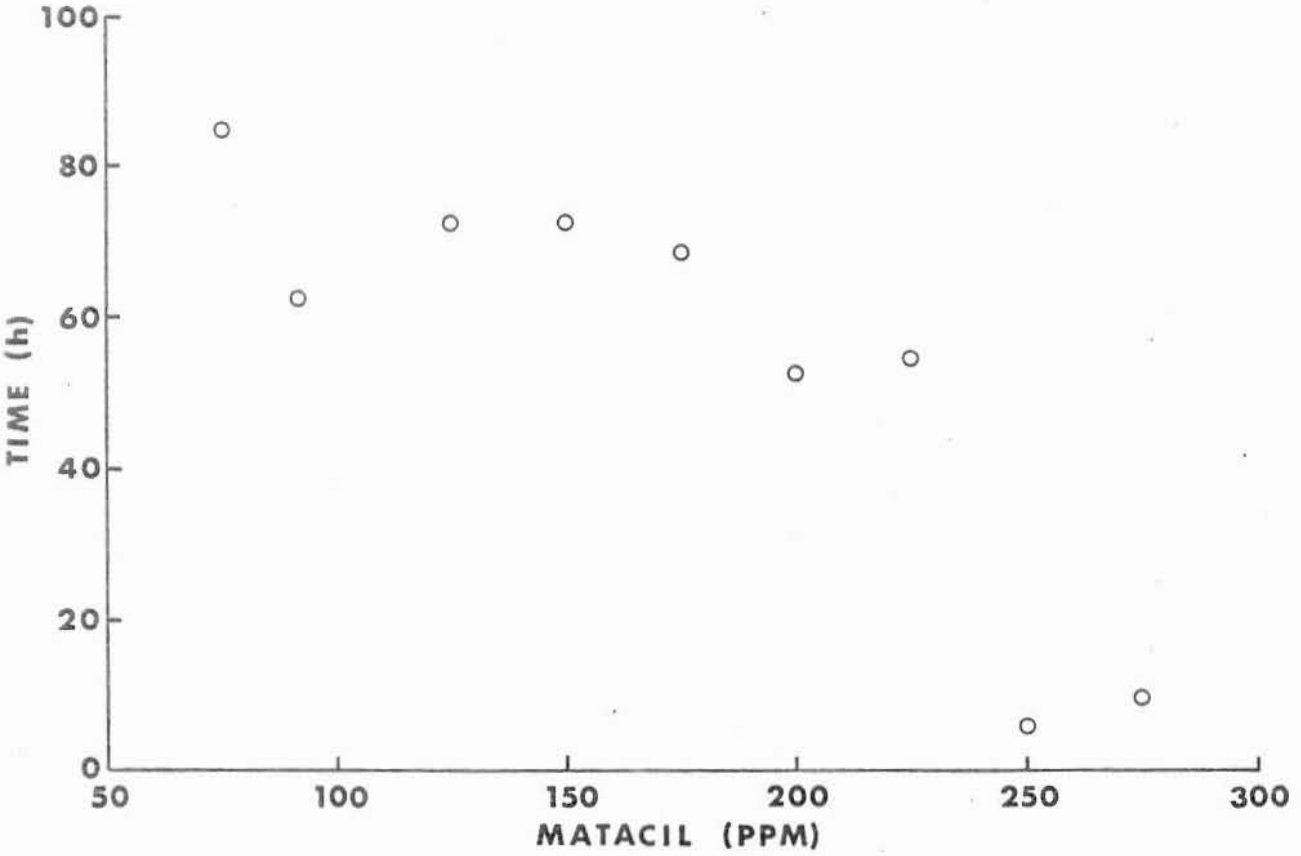

Fig. 11. Median mortality responses for Matacil-treated large *Rana clamitans* larvae.

TABLE 6

Frequency of pre- and post-mortem observations made during toxicity-testing on *Rana clamitans* larvae. 1, <10%; 2, 10-49%; 3, 50-90%; 4, >90%; -, not observed; A, all concentrations; H, higher concentrations; L, lower concentrations

Observation	Insecticide treatment		
	Fenitrothion	Matacil	Orthene
Swimming difficulty	3A	3A	2A
Hemorrhagic areas:			
Heart	2H	2A	-
Base of tail	2H	2A	-
Elsewhere	1L	1H	-
Trematodes alive:			
Base of tail	2A	2A	-
Elsewhere	1A	-	-
Mouth swollen	-	2A	-
Edema	-	1L	-
Necrosis:			
Tail while alive	-	-	3A
Body after death	-	-	4A
Elsewhere	-	1H	-
Dark color	4A	4A	3A
Bent tail	-	2A	-
Jaw-twitching, gulping	1H	1H	-
Ecdysis	1H	-	-
Trouble staying on bottom	3A	3A	2A

B. Degradation Dynamics of the Insecticides

The + 1-h concentration (0.523 ppm) of fenitrothion in the water sample from the laboratory (Fig. 12A) does not correspond with the 1 ppm test medium formulated. The low value suggests that the fenitrothion had not mixed thoroughly when the + 1-h sample was collected. The higher Day 1 value (0.899 ppm) suggests that subsequent aeration and tadpole activity probably resulted in the dispersal of the insecticide. The decline in residual insecticide levels in the aquarium water then followed an exponential decline over time with an approximate half-life of 3 d (Fig. 12A). The fenitrothion residues in the *Rana clamitans* larvae were 5.30 ppm after 1 h and peaked at 11.50 ppm at Day 1. The insecticide level in the tadpoles was relatively constant up to and including the 5-d sample and then exhibited a gradual decline (Fig. 12B) with an approximate half-life of 7 to 8 d.

The highest fenitrothion concentration in the water from the field (0.003 ppm) occurred 1 h after spray application. The disappearance of the residues roughly approximated a negative exponential (Fig. 13D) with a small increase in concentration at Day 2. This increase in concentration corresponded to the only rainfall recorded over the 10-d sample period (Fig. 13A). Only traces of residues were encountered after Day 5 and by Day 10, residues were not detected. The field half-life of 2 to 3 d is consistent with the laboratory observations. The + 1-h sample of tadpoles (Fig. 14A) contained 0.61 ppm in whole body residues. This is greater than 185 times the concentration in the water sample collected at the same time. By Day 2 the residue level in the larvae had diminished to 0.04 ppm, which was still 18 times the concentration in the Day 2 water sample. The pH of the water samples ranged from 7.8 to 8.0 and the water temperature (Fig. 13C) of the pond gradually increased over the 10-d period. These conditions of alkalinity and temperature are both beneficial for organo-

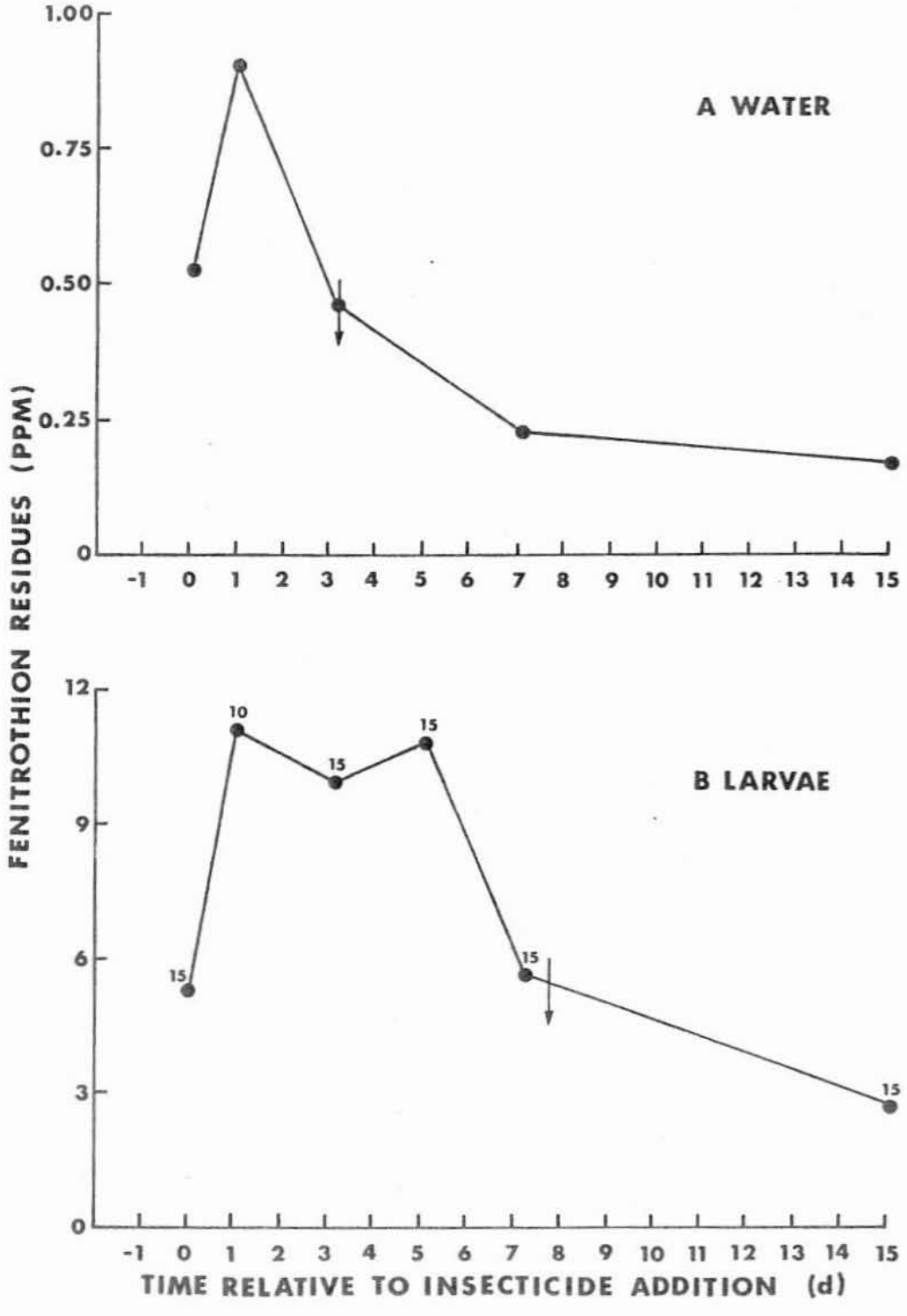

Fig. 12. Fenitrothion residues in A, water and B, *Rana clamitans* larvae from the laboratory. Numbers refer to bulked larvae sample size; arrow indicates approximate half-life.

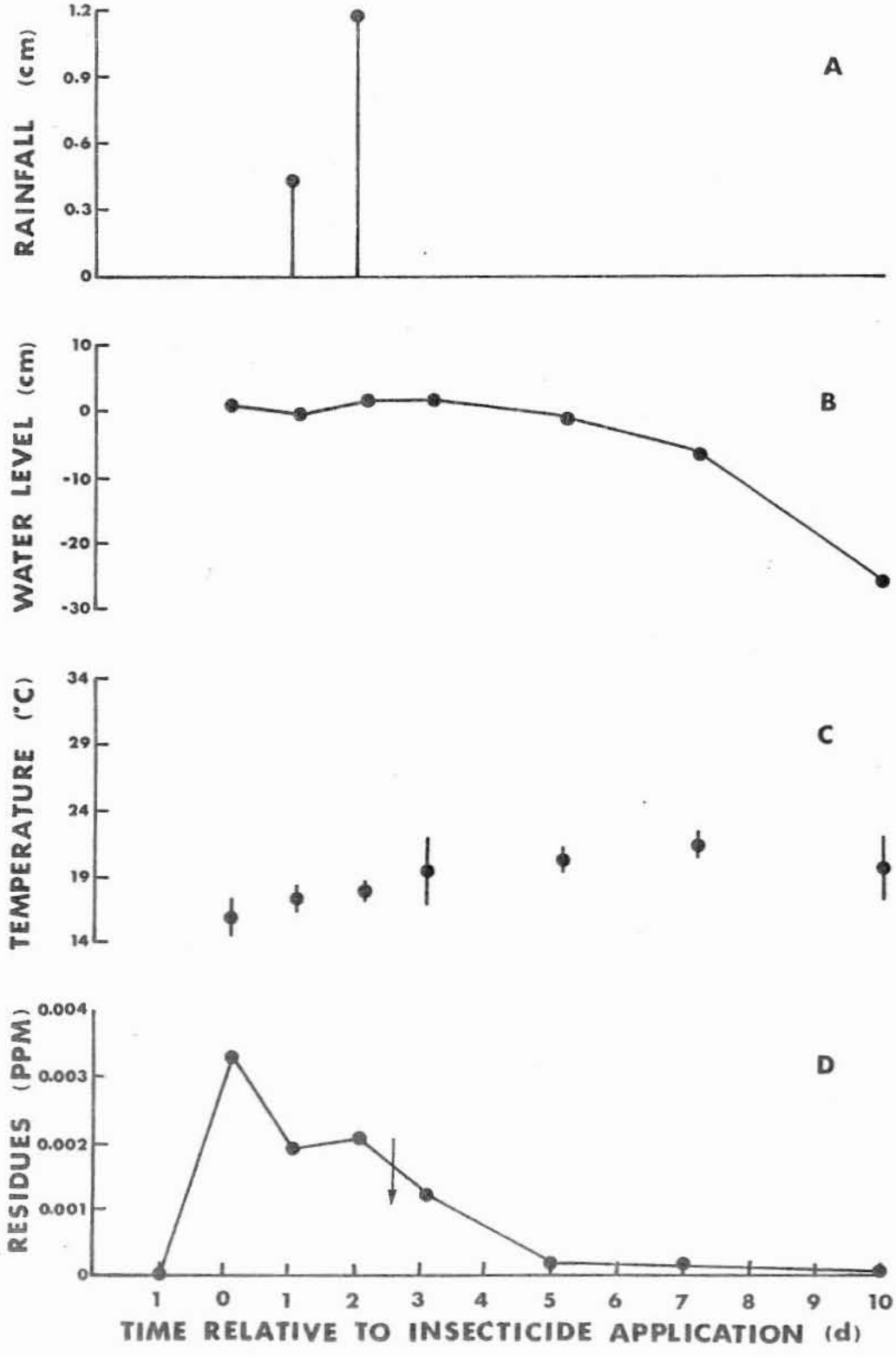

Fig. 13. A, Rainfall; B, water level; C, water temperature midrange; D, fenitrothion residue levels in pond water. For C, vertical bars denote temperature range; in D, arrow indicates approximate half-life.

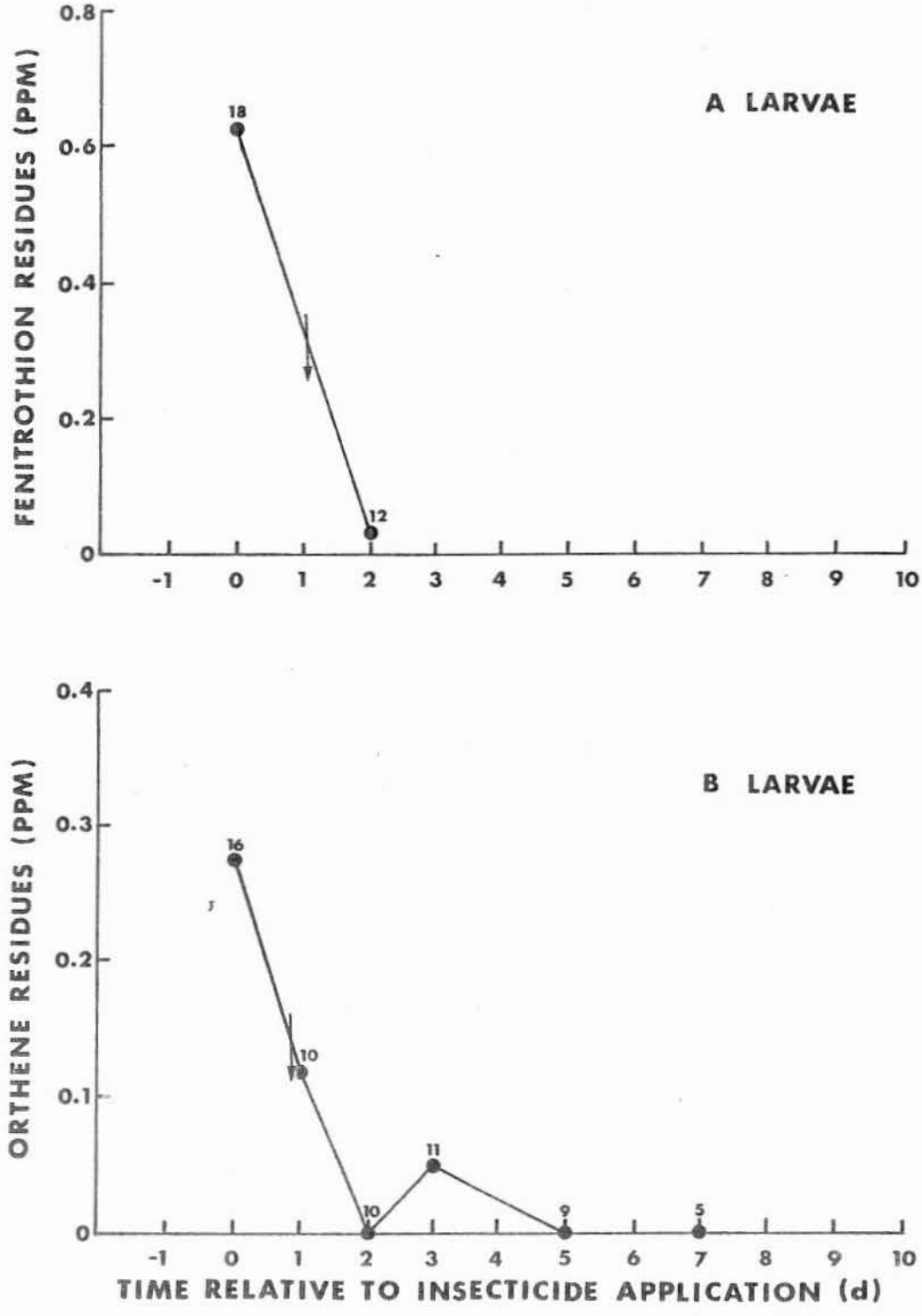

Fig. 14. A, Fenitrothion and B, Orthene residues in *Rana sylvatica* larvae from the field. Numbers refer to bulked sample size; arrow indicates approximate half-life.

phosphate hydrolysis (Wildish et al., 1971). The water level of the pond (Fig. 13B) changed little until towards the end of the sample period, when residue levels were low and therefore water level had little effect on insecticide concentration.

Neither of the derivatives fenitrooxon and S-methyl fenitrothion were ever detected in either water samples or tadpole tissue from the field or from the laboratory.

The Orthene concentration in the aquarium after 1 h was consistent with the 1 ppm concentration initially set up (Fig. 15A). After 24 h the concentration had declined; however, by Day 3 the concentration had risen six-fold (Fig. 15A). This sharp increase in concentration may have resulted from contamination of the sample by a surface precipitate like the ones noted in the Orthene toxicity tests. The + 1-h concentration in the tadpoles (Fig. 15B) was approximately one-fifth the concentration in the associated water sample. At Day 1 the concentration in the tadpoles was identical to the concentration in the water sample and decreased thereafter (Fig. 15B). By Day 5 the residual level in the larval sample was 34 times that in the corresponding water sample.

There was evidently an absence of Orthene in the + 1-h water sample from the field (Fig. 16D). The corresponding *Rana sylvatica* tadpole sample, however, contained 0.27 ppm (Fig. 14B). A sample of newly metamorphosed *Rana sylvatica* occupying both the water and the shoreline contained a similar residue level (0.25 ppm). By Day 1 the concentration in the water was 0.130 ppm and the level in the tadpoles had fallen to 0.12 ppm, a very similar concentration. This is consistent with finding similar concentrations by Day 1 in the laboratory tadpole and water samples. After Day 2 the concentration in the water sample was 0.046 ppm and fluctuated little for the next three samples (Fig. 16D). Residues were not detected in the Day 2 larval sample (Fig. 14B) but were again detected

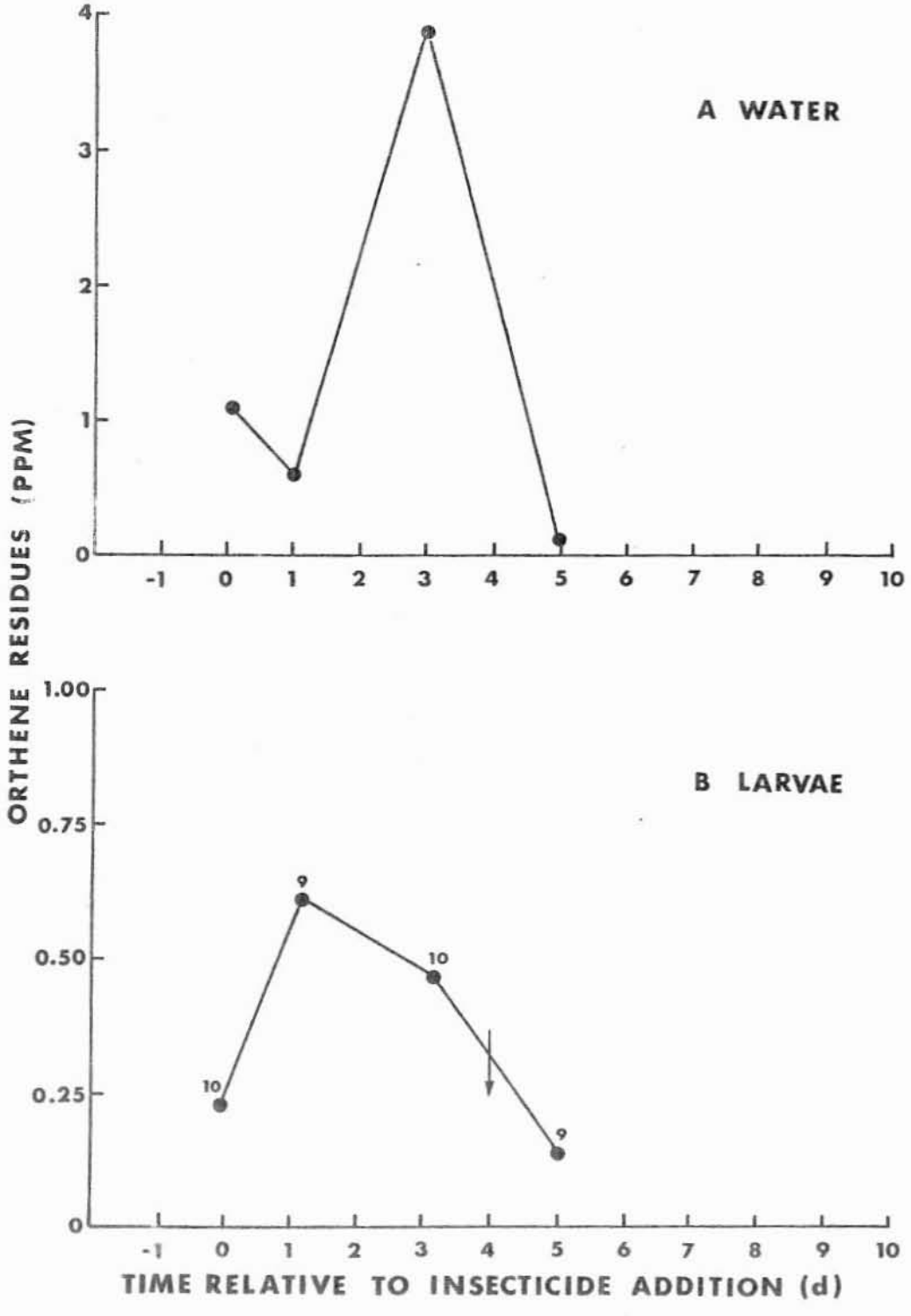

Fig. 15. Orthene residues in A, water and B, *Rana clamitans* larvae from the laboratory. Numbers refer to bulked larvae sample size; arrow indicates approximate half-life.

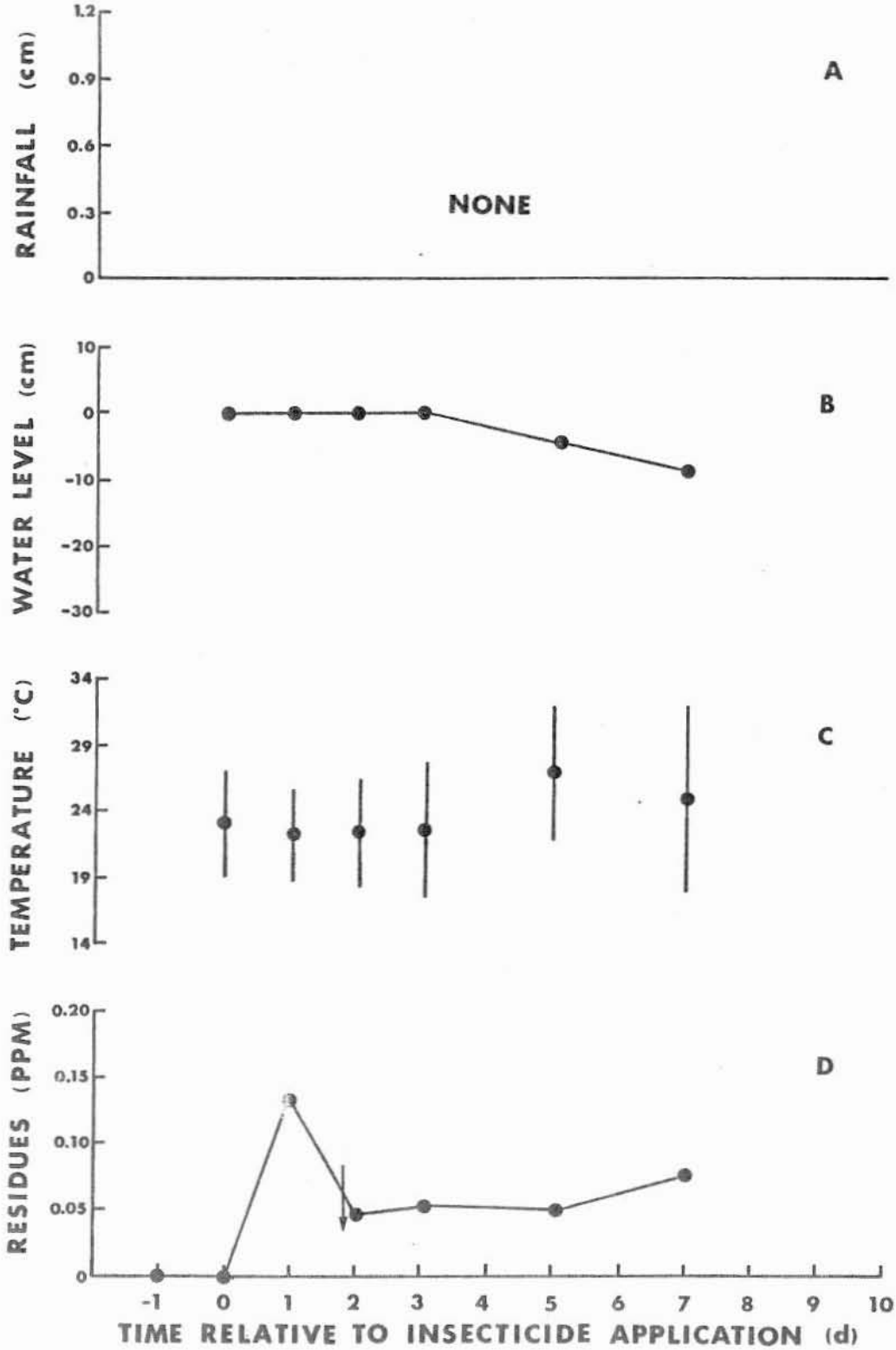

Fig. 16. A, Rainfall; B, water level; C, water temperature midrange; D, Orthene residue levels in pond water. For C, vertical bars denote temperature range; in D, arrow indicates approximate half-life.

in the Day 3 sample. Orthene was not detected in either of the remaining samples. Since metamorphosis was actively occurring in the population, these samples contained a mixture of morphologically different larval stages (Fig. 17B); this heterogeneity might explain these ambiguities. No rainfall occurred during the sample period, but a reduction in water level (Fig. 16B) and an increase in water temperature (Fig. 16C) were recorded.

The metabolite Monitor was detected (0.005 ppm) only in the Day 5 water sample from the field.

The pH of the Orthene pond (BR-A) ranged from 6.8 to 7.1 during the sample period. Laboratory water samples were slightly alkaline (7.4 - 7.7). The chemical properties of the water for both ponds are shown in Table 7.

The approximate volume of each pond was determined (Table 8) and the average amount of active ingredient incident on the ponds was calculated (Table 9). Extrapolating from the above and assuming total miscibility of the insecticides in the pond water, the expected concentrations of fenitrothion and Orthene in the ponds were estimated. The expected concentration of fenitrothion (0.007 ppm) is approximately twice the actual level found in the pond at + 1 h. Less than 15% of the insecticide sprayed from the aircraft actually reached the pond, as estimated from spray deposit. For the Orthene pond, the actual concentration in the pond water at Day 1 was 3.5 times the expected concentration as determined from the spray deposit. From the spray pan estimate, the amount of Orthene incident on the pond was about 21% of the aircraft emission.

Metamorphosis of the *Rana sylvatica* population was actively occurring in the Orthene pond during the 7-d sample period as indicated by the presence of newly metamorphosed frogs at + 1 h. The development

TABLE 7

Physical characteristics of pond water on the days prior to insecticide applications

Pond	pH	Dissolved oxygen, ppm	Dissolved carbon dioxide, ppm	Total hardness, ppm	Total acidity, ppm	Total alkalinity, ppm
T-2C (fenitrothion)	8.0	9	10	5.6	0.2	5.6
BR-A (Orthene)	7.0	8	20	2.8	0.7	2.1

TABLE 8

Determination of residue levels in ponds from spray deposit

Pond	Surface area, m^2	Mean (± SD) depth, m	Volume, ℓ	Mean (± SD) spray deposit g/ha	Expected conc., mg/ℓ	Actual conc., mg/ℓ
T-2C (Fenitrothion)	68	0.56 (±0.35)	38080	40.4 (±16.5)	0.007	0.003*
BR-A (Orthene)	215	0.31 (±0.16)	66650	118.3 (±62.5)	0.038	0.130**

* 1 h after spray deposit

** 24 h after spray deposit

TABLE 9

Spray deposit on each experimental pond

Spray card location (Fig. 3)	Volume of insecticide formulation deposited, ℓ/ha		Amount of active ingredient deposited, g/ha	
	Fenitrothion*	Orthene**	Fenitrothion	Orthene
1	0.095	0.643	60.7	246.4
2	0.080	0.292	51.3	112.0
3	0.058	0.380	37.3	145.6
4	0.015	0.175	9.3	67.2
5	0.095	0.285	60.7	109.2
6	0.073	0.358	28.0	137.2
7	0.044	0.102	46.6	39.2
8	0.058	0.234	37.3	89.6
9	0.051	-	32.6	-
Mean ± SE	0.063 ±0.053	0.309 ±0.143	40.4 ± 1.4	118.3 ± 2.8

* emission rate - 966 g active ingredient per ℓ of solution

** emission rate - 384 g active ingredient per ℓ of solution

curve (Fig. 17B) resulted from difficulties in collecting random samples from both aquatic and terrestrial habitats. The result was a series of tadpole samples with morphologically different individuals. The comparatively smooth development curve (Fig. 17A) for the tadpoles in the T-2C pond indicates a lack of metamorphosis in the population and relatively uniform tadpole samples.

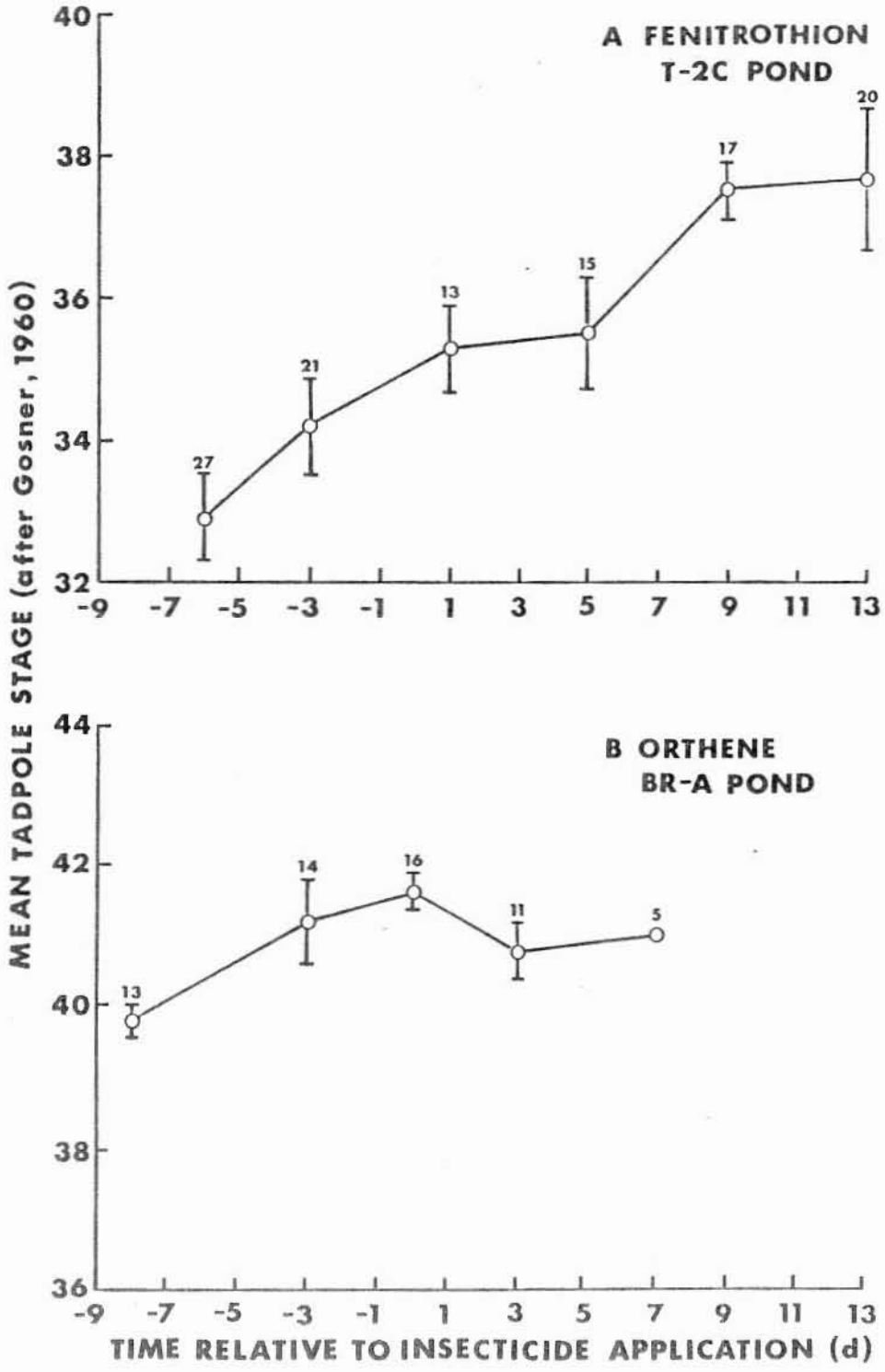

Fig. 17. Development of *Rana sylvatica* larvae from both experimental ponds. Numbers refer to sample size; vertical lines denote one standard error each side of the mean.

C. Behavior of Insecticide-treated *Rana clamitans* Larvae

Since the variances for the behavioral event means were found to be different, weighted *t*-tests ($p < 0.1$) (Cochran and Cox, 1957) were performed to compare mean values for each event for treated and untreated tadpole groups. The mean values for each behavioral event and the significance of their differences from the corresponding control group are shown in Fig. 18.

Significant differences were obtained for bubble-making and unidirectional swimming in Matacil- and Orthene-treated tadpoles and for multidirectional swimming in tadpoles treated with all three chemicals. Tadpole bubble-making showed a significant increase ($p < 0.05$) at 1000 ppm Orthene and then a significant decrease at 2000 ppm ($p < 0.1$). Matacil-treated tadpoles showed a significant reduction ($p < 0.1$) in this behavior only at 25.0 ppm and not at the higher concentration of 50.0 ppm. The unidirectional swimming behavior exhibited a significant reduction at 10.0 ppm Matacil ($p < 0.1$) and again at 50.0 ppm Matacil and at 2000 ppm Orthene ($p < 0.05$). Multidirectional swimming was reduced in the two highest concentrations of fenitrothion and Matacil and in the highest concentration of Orthene. The highest concentrations where no significant behavioral changes from the control group occurred in fenitrothion, Matacil, and Orthene were 1.00, 5.0, and 500 ppm, respectively.

Least-squares regression equations with log-transformed dose and mean total activity time of the larvae in each of the three chemicals are illustrated in Fig. 19. Concentrations in which the mean activity times were significantly different (*t*-test, $p < 0.05$) from the control mean (79.2 ± 24.3 (SE)s) are also indicated. Significant decreases were recorded at the two highest concentrations of fenitrothion and Orthene, and at the three highest concentrations of Matacil.

Feeding behavior was recorded only in the control group and in

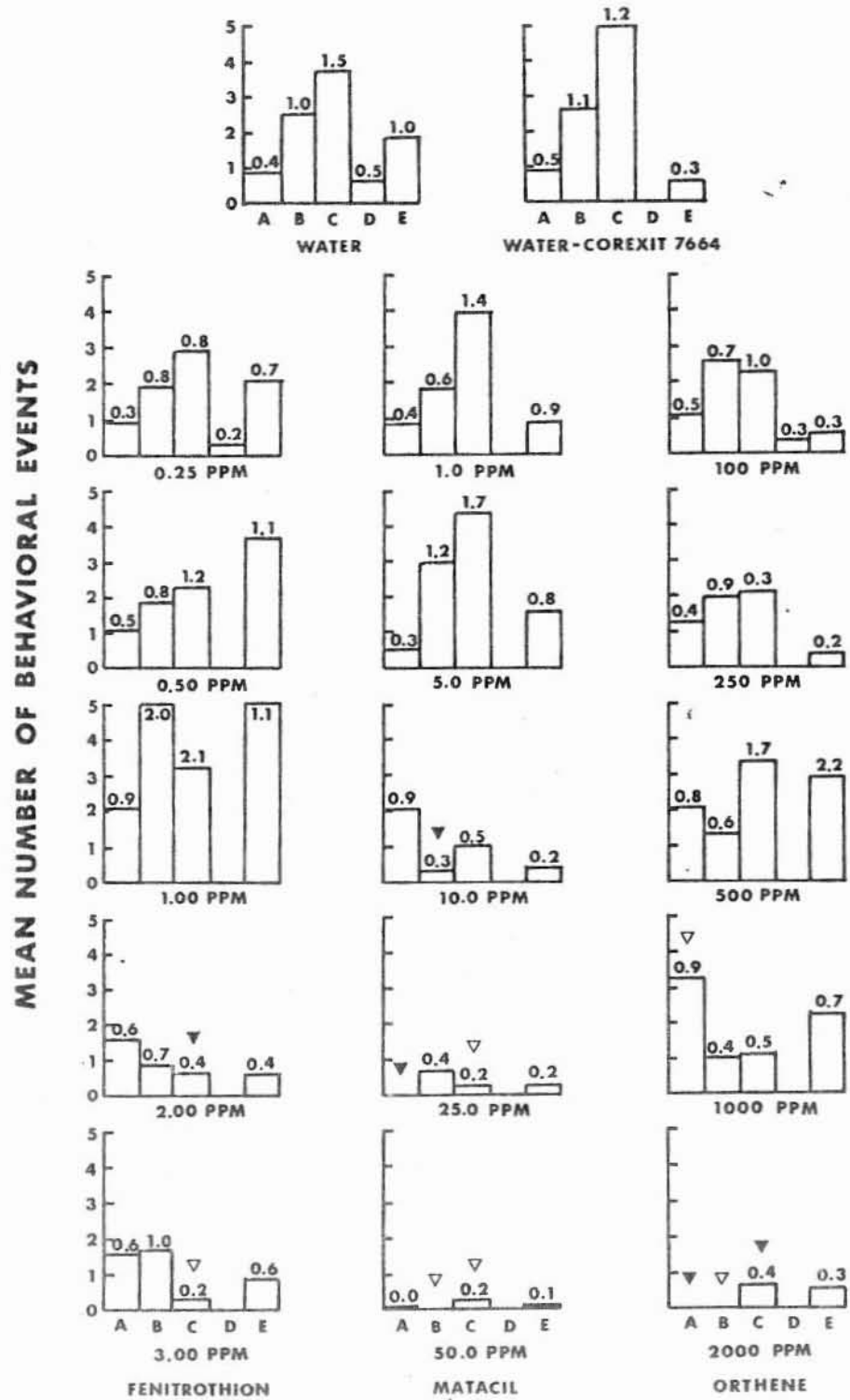

Fig. 18. Mean number of behavioral events for each behavioral category for *Rana clamitans* larvae in controls and various concentrations of the three insecticides. Numbers refer to standard errors; ▽ = significantly different from water control ($p < 0.05$; *t*-test); ▼ = significantly different from water control ($p < 0.1$; *t*-test). Concentrations refer to the initial concentrations formulated.

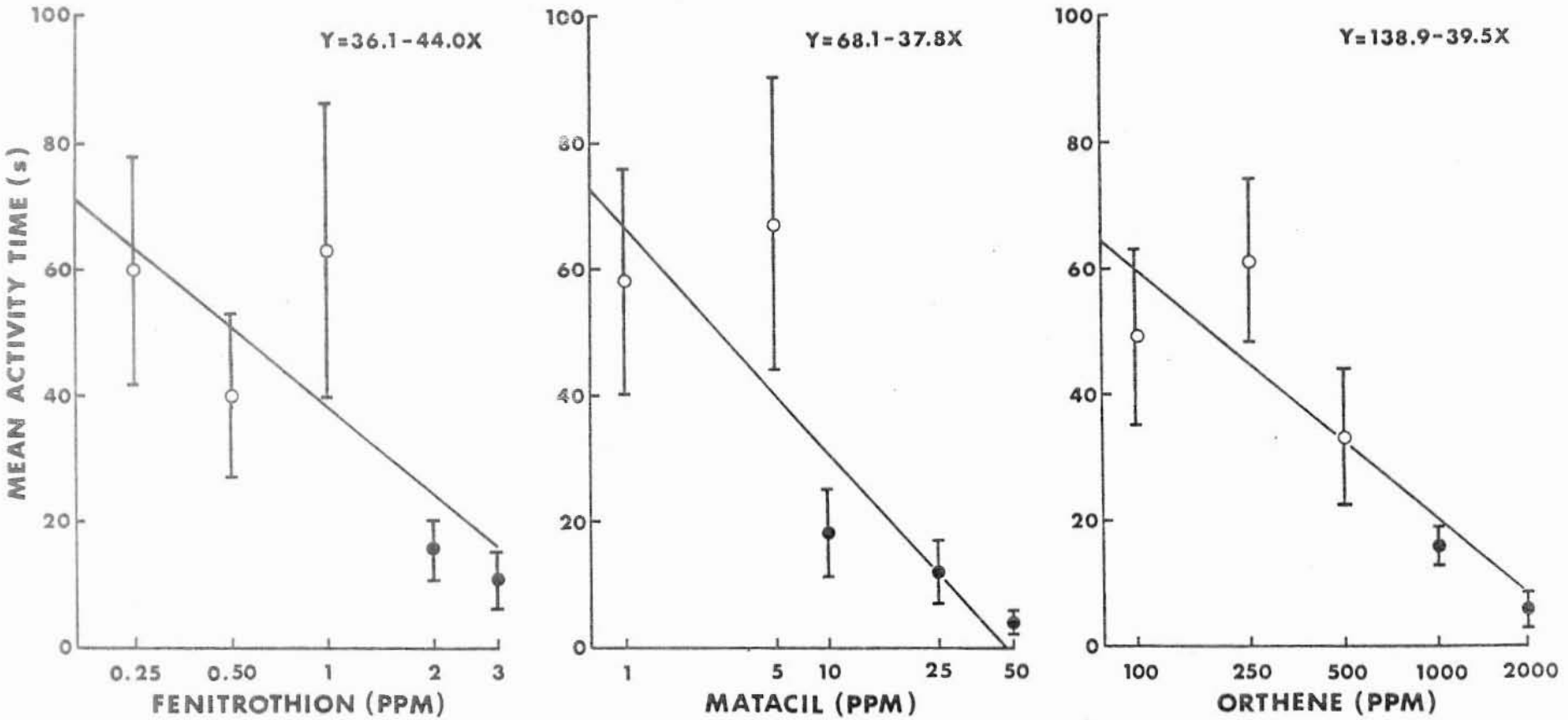

Fig. 19. Mean activity times for *Rana clamitans* larvae in various concentrations of the three insecticides. Closed circles represent significant differences from control ($p < 0.05$; t-test); vertical lines denote one standard error each side of the mean.

the lowest concentrations of fenitrothion and Orthene. The low incidence of feeding in these groups is not significantly different ($p > 0.05$) from the absence of this behavior in the other test groups.

Color changes and resting position of the larvae are recorded in Table 10. *Rana clamitans* tadpoles are typically light green in color. Tadpoles in the higher insecticide concentrations, however, turned dark green to brown. Inactive larvae observed in holding tanks, control tanks, and in natural ponds normally rest on the substrate. Resting on the surface by treated tadpoles may reflect insecticide-induced buoyancy or respiratory problems.

Treated tadpoles were maintained up to 192 h in their respective test media to determine if these concentrations were indeed sublethal. The only mortality observed occurred after 144 h in the 2.00 and 3.00 ppm fenitrothion tests. After each series of 10-min observation periods, the tadpoles were given a tactile stimulus as a check for moribundity. This condition was never encountered.

TABLE 10

Effects of sublethal concentrations of the three insecticides on color and resting position in *Rana clamitans* larvae

Chemical	Conc., ppm	Color, % dark	Resting position, % of time on the surface
Water	-	0	0
Corexit-7664	-	0	0
Fenitrothion	0.25	0	0
"	0,50	0	14
"	1.00	100	57
"	2.00	100	29
"	3.00	100	57
Matacil	1.0	0	0
"	5.0	0	0
"	10.0	0	0
"	25.0	100	29
"	50.0	100	43
Orthene	100	0	0
"	250	0	0
"	500	14	57
"	1000	43	66
"	2000	43	60

IV Discussion

A. Comparative Toxicity and Poisoning Symptoms of the Three Insecticides in Frog Larvae.

The response curve for each concentration shows whether one or more modes of toxic action occurred. Split probits are indicative of resistance developing in the population (Sprague, 1969). The response curves obtained for fenitrothion-, Orthene-, and Matacil-treated small larvae indicate only one mode of toxic action. Biphasic response curves obtained for Matacil-treated large larvae possibly resulted from the gradual buildup of toxic metabolic wastes of the tadpoles.

Fenitrothion was found to be many times more toxic to *Rana clamitans* tadpoles than Matacil or Orthene. The described symptoms of fenitrothion- and Matacil-poisoning suggest a neurotoxological mode of action. Swimming difficulties and bent tails are probably indicative of primary effects of acetylcholinesterase inhibition at nerve-muscle synapses. Jaw-twitching, ecdysis, and color change in anurans are also under neurohormonal control (Porter, 1972) and would be affected by a neurological disorder.

O'Brien (1967) stated that organophosphate-induced mortality results from the interaction of the pesticide with the respiratory centre or as a direct effect on the respiratory muscles causing asphyxiation. Gulping observed in the fenitrothion-treated larvae would support this view. Buoyancy problems may be an indirect effect of asphyxiation. The buildup of gases, probably in the viscera, would buoy the tadpoles to the surface.

The other toxicity symptoms may have been secondary responses to the presence of foreign material in the body or a response to physiological stress. Chang et al. (1974) stated that edema in methylmercury chloride-treated *Rana pipiens* larvae was probably the result of an osmotic disturbance.

Because of the design of the experiment, these symptoms could be observed only in moribund or dead animals and may be typical of the normal post-mortem condition.

The presence of live trematodes in the dead insecticide-treated tadpoles may be an example of toxicant specificity. The cholinesterases present in platyhelminths are generally not inhibited by organophosphates or carbamates (Silver, 1974). Alternative hypotheses are that the insecticide did not penetrate into the areas where the parasites were observed, or the lethal concentration (LC50) for the trematodes may have differed from that of the tadpole host.

The extensive necrosis observed in the Orthene-treated larvae suggests direct damage to the integument cells. This may have been the poisoning mode of action. Some of the observations (i.e. hemorrhagic areas, trematodes, dark color) made on larvae treated with fenitrothion and Matacil did not appear in the Orthene-treated tadpoles. The extensive tissue necrosis in the latter may, however, have masked these observations.

The deformation of the snouts observed in the Matacil-treated tadpoles probably resulted from the movement of the tadpoles along the surface of the container. Pesticide adsorbed on the container could adsorb on their snouts causing tissue necrosis. Cooke (1970) observed a similar condition in *Rana temporaria* tadpoles exposed to DDT but gave no explanation for it.

B. The Effect of Insecticide Formulations on Relative Toxicity

The water solubility properties of the three chemicals will regulate their degree of assimilation in an aquatic organism and hence their relative toxicity. Orthene, which is 65% water soluble, readily goes into solution. The high water solubility of this insecticide decreases the rate with which it will adsorb on the surface of an aquatic organism and subsequently be absorbed into it. Conversely, fenitrothion and Matacil are highly hydrophobic as pure formulations. The technical Matacil used in this investigation was a wettable powder. As such it contains the insoluble crystalline insecticide and various inert hydrophilic and lipophilic chemicals. This system allows the pesticide to be miscible with water and disperse throughout it in particles of approximately the same size. Insecticide-emulsion systems, such as the one utilized for fenitrothion, function in a manner similar to that of the wettable powder complex, but droplets of varied size result. The stability of emulsions depends on the amount of emulsifier used. Since fenitrothion and Matacil are dispersed in the test media they are still available for adsorption by the tadpoles.

Orthene, which is more toxic to Mallards (*Anas platyrhynchos*) than are fenitrothion and Matacil and which is as toxic to spruce budworms as is fenitrothion (Nigam, 1975), is relatively innocuous to aquatic organisms. Tarzwell (1950) found that DDT emulsions were more toxic to amphibians than DDT solutions or dusts. The availability of the three insecticides to an aquatic organism becomes instrumental in their relative toxicity. Matacil, which is more toxic than fenitrothion to Atlantic salmon (Nigam, 1975), is much less toxic to the tadpoles. This may also be the result of the type of formulations used.

Upon standing, films of material formed on the air-liquid interfaces of the Orthene test media. Since volatilization and codistillation of a pesticide are inversely proportional to solubility (Kenaga, 1974), evaporation from the containers would leave the water-soluble Orthene behind as a surface film. This material would become unavailable to the tadpoles and result in the observed reduction in mortality rate after approximately 48 h.

The effects of fenitrothion noted here could have been potentiated by the emulsifier. Detergents are known to solubilize and activate acetylcholinesterase (Wildish et al., 1971).

C. Other Factors Influencing Toxicity

Toxicity of insecticides also depends on the degree of penetration into an organism, translocation in the organism, and the organism's ability to eliminate and detoxify them. Detoxification of organic pesticides in fishes involves enzymatic hydrolysis throughout the body, but primarily in the liver (Wildish et al., 1971). Brodie and Maikel (1962), however, reported a low oxidative ability in livers of *Rana* sp., but suggested that amphibians can remove toxicants into the surrounding water via their gills.

The use of static bioassays may have compounded the toxic effects of the chemicals. Lincer et al. (1970) found that the toxicity of DDT to fathead minnows, *Pimephales promelas*, was greater using static as opposed to dynamic bioassays. Similarly, Wildish and Phillips (1972) considered control mortality in fenitrothion-treated insect tests related to the stagnant conditions in static bioassay vessels. Their rationale was that static bioassays allow for the buildup of metabolic products and thus enhance toxicity. Anuran larvae excrete ammonia and urea, both of which are highly toxic (Porter, 1972). The 30% mortality of the large tadpoles in the control group and the apparent biphasic mortality in the Matacil-treated large tadpoles support this contention. Sprague (1969) suggested the use of 2-3 ℓ of test solution per gram of fish, changed daily, for static bioassays. Changing test media daily would, however, result in fluctuations in pesticide concentration and not the exponential decline that occurs in natural waters. Using a volume of 2.9 ℓ/g for the small tadpoles appeared to be satisfactory for long exposure intervals. The lower value of 1.7 ℓ/g for the large tadpoles, however, resulted in complicating factors.

D. Deposit and Dynamics of Fenitrothion and Orthene in Temporary Pond Water

The maximum concentration of a pesticide sprayed in aqueous or oil solutions should be apparent in temporary pond water 1 h after spray deposit. This was in fact the case in the fenitrothion pond. Orthene, a material that is highly soluble in water, however, was not detected at this sampling time. The explanation for its apparent absence from the water samples when high concentrations were evident in associated tadpole and frog samples from the same pond may reflect technical errors. Incomplete mixing of the water in the pond by 1 h after spray deposit may have left the Orthene in the upper strata. Tadpoles and young frogs could move in and out of these layers and acquire Orthene residues. Water collected 15 cm below the surface may have been devoid of residual insecticide.

The discrepancy between expected (0.038 ppm) and actual (0.130 ppm) pond concentrations of Orthene may have resulted from the loss of deposited material from the spray pans. Evaporation of the liquid phase from the spray mist would result in the deposit of dry particles. The dry particles of Orthene and dye would not as readily adhere to the spray pan and may have been lost during transport of the pans.

The calculated deposit of Orthene (21%) was greater than the fenitrothion deposit (15%). Pond concentrations of Orthene indicated that the deposit should have been much greater than observed. This suggests that Orthene, a solid, reaches ground level of the target area in a greater proportional deposit than does fenitrothion, a liquid. Fenitrothion is probably lost from the spray mist via volatilization and is deposited in minute quantities outside the target area.

The effect of rainfall on insecticide concentrations in natural waters has been documented previously (Sundaram, 1974). Rain on 11-12 June resulted in an increase in fenitrothion residues in the Day 2 water sample.

The increase in pesticide concentration results from the input of new material washed from nearby foliage and soil and to a lesser extent fallout from the atmosphere.

The rate of disappearance of the fenitrothion from the pond closely approximated first order kinetics. Fenitrothion, like most organophosphates and carbamates undergoes alkaline hydrolysis (Eto, 1974). Since the pond was slightly alkaline, hydrolysis of the fenitrothion would occur fairly rapidly. This hydrolysis is also temperature-and concentration-dependent. An increase in water temperature towards the end of the sample period would favour a hydrolytic degradation pathway. The GLC technique used to analyse the samples is specific for the oxidative desulfurization (fenitrooxon) and isomerization (S-methyl fenitrothion) products of fenitrothion and would not identify the hydrolytic metabolite, 3-methyl-4-nitrophenol (Fig. 1).

Other routes for the disappearance of fenitrothion from temporary ponds include adsorption on the substrate, codistillation, volatilization, biotransformation, and photodecomposition. The movements of insecticides in temporary ponds are summarized in Fig. 20. Zitko and Cunningham (1974) hypothesized that fenitrothion is rapidly adsorbed on suspended solids and removed from the system by sedimentation. They also found that a large percentage of the fenitrothion is also directly adsorbed on the substrate.

The neutral pH and high turbidity of the Orthene pond may have been instrumental in the slow disappearance of this insecticide. Both these factors would reduce the rate of chemical reactions. The water solubility of Orthene would reduce its adsorption on the sediment and prevent it from undergoing codistillation from the surface. Evaporation would then concentrate Orthene and not remove it from the system. The net result is that Orthene is moderately persistent (Eto, 1974).

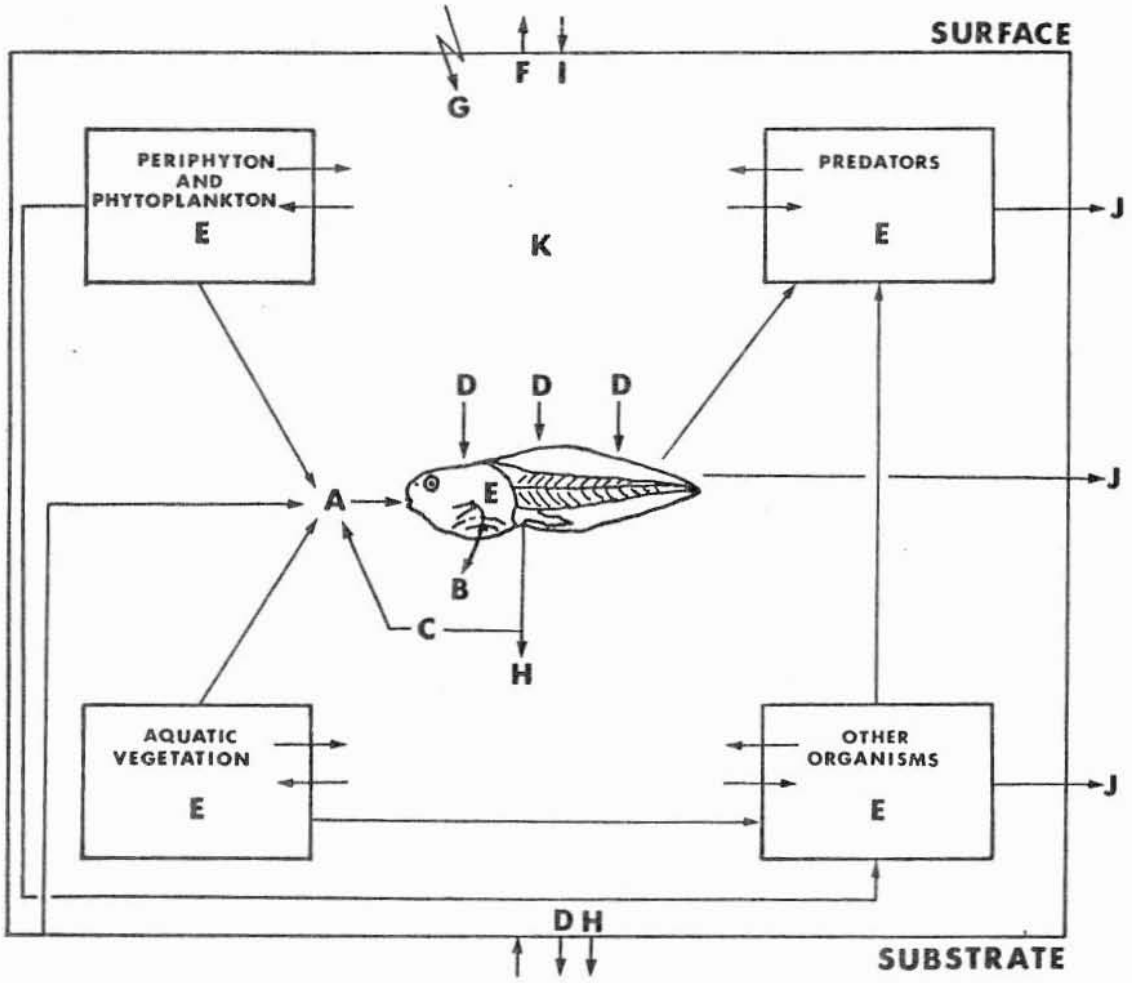

Fig. 20. Model for the movements of pesticides in natural ponds with special reference to anuran larvae populations. A = ingestion and gill ventilation; B = removal of toxicants via gills; C = cannibalism, necrophagy, and coprophagy; D = adsorption; E = biotransformation; F = volatilization, codistillation; G = photodecomposition; H = sedimentation; I = runoff, fallout; J = emigration; K = hydrolysis.

E. Dynamics of Fenitrothion and Orthene in Aquarium Water.

Loss of the pesticides from the aquaria would be governed by the same principles as observed in the field, with additional emphasis on adsorption on the sides of the containers and loss to the tadpoles' bodies. By GLC analysis, Bull and McInerney (1974) found that fenitrothion in a laboratory aquarium and bioassay vessels had decreased 27% after 16 h, but then remained constant up to 111 h. The loss of fenitrothion from the aquarium in this experiment, however, closely followed a negative exponential curve with an approximate half-life of 2-3 d. This is similar to the results of Zitko and Cunningham (1974) for laboratory-treated river water.

Approximately 45% of the Orthene disappeared from the aquarium water after 24 h. The surge of Orthene in the Day 3 water sample probably resulted from the film of material left behind on the surface when the water evaporated, as previously mentioned.

F. Dynamics of Fenitrothion and Orthene in Frog Larvae.

For both insecticides the maximum whole body residues in the larvae were evident within the first 24 h after insecticide application. This holds true for both the field and laboratory situations. Kenaga (1974) stated that, typically, well over 50% of the total adsorptive uptake of pesticides in aquatic organisms occurs in the first few hours after exposure with no significant uptake after the first day.

The highest residue levels of the two insecticides observed in the tadpoles are not necessarily the highest concentrations reached. Maximum concentrations may have been reached between the 1-h sample and the Day 1 sample with a point recorded at Day 1 being on the decline. Residue levels in tadpoles follow a predictable pattern. There is an initial rapid rate of assimilation, probably involving adsorption, with little elimination. Then an equilibrium is reached where the uptake of the insecticide is equal to the amount lost. This is best illustrated with fenitrothion in the laboratory tadpoles, where the equilibrium plateau lasts for approximately 3 d. This peak is followed by a net loss in whole body residues. Half-lives of the toxicants in the tadpoles can be interpolated from the observed maximum concentrations since these are equal to or less than the actual maximum concentration reached.

Adsorption of pesticides on an organism is dependent on four factors: temperature, speed of circulation of the flow system, ratio of biomass to flow system volume, and mode of intake of the pesticide into the organism (Kenaga, 1974). Subsequent absorption of the pesticide is dependent on the impedance of the surface to the chemical (Eto, 1974). Anuran larvae potentially have three surfaces on which pesticides can be adsorbed from the aquatic environment: the integument, the alimentary tract, and the gills and associated structures. The significant respiratory

surface in tadpoles is dependent on the size of the tadpoles, the smaller tadpoles utilizing the skin for gaseous exchange. The larger the tadpole the greater the dependence on gills as the respiratory surface (Savage, 1951). Movements of water over the gills also allow the involuntary extraction of nutrient material by filtration. Thus insecticide adsorbed on suspended organic and inorganic material would pass through the alimentary tract. Factors controlling pesticide uptake by tadpoles include their degrees of movement and gill ventilation which regulate the flow rate over the skin and gills, respectively. Temperature also regulates metabolic rate which in turn regulates gill ventilation. Thus the small size of the tadpoles and higher temperature in the field may account for the higher residues of fenitrothion in the field situation as compared to the laboratory.

Anuran larvae are also coprophagic, necrophagic, and cannibalistic (Beiswenger, 1975). These feeding modes would be particularily significant in pesticide accumulation in laboratory-treated tadpoles where only minimal food was supplied.

Since Orthene is highly water-soluble, concentrations in tadpole bodies are never much different than the concentrations in the associated water, in both laboratory and field situations. The occurrence of similar residue levels in newly metamorphosed frogs and the larvae suggests that the tadpole skin was more important in accumulating Orthene residues than were the gill apparatus and digestive tract. Newly metamorphased frogs would be utilizing pulmonary respiration and terrestrial food sources, if they were feeding, and not the methods available to the larvae. The common denominator for both life stages was their contact with the treated water and substrate. Since metamorphosis was not occurring during the sample period in the fenitrothion pond, a similar comparison was not possible.

The difference in the ratio of tadpole fenitrothion residues to water fenitrothion residues between the field pond and laboratory aquarium may reflect species differences, concentration effects, abiotic effects, or formulation systems. Differences in this ratio were not observed for Orthene-treated tadpoles.

G. Derivative Dynamics

Phosphorothionates like fenitrothion with P = S groups are poor anticholinesterases. Inhibition of the enzyme depends on the desulfurization in the animal to form P = O bonds forming fenitrooxon (Wildish et al., 1971). The apparent absence of the oxygen analogue in the tadpole samples does not, however, mean that the oxidative desulfurization was not taking place. Fenitrooxon is less stable than the parent compound and would be deactivated and eliminated faster. Detection of fenitrooxon by GLC analysis is also less sensitive than for fenitrothion (Zitko and Cunningham, 1974). It was suggested by Zitko and Cunningham (1974) that the oxon may partially decompose on the GLC column.

Fenitrothion can also undergo isomerization to S-methyl fenitrothion. This reaction may be catalysed by sunlight under environmental conditions (Zitko and Cunningham, 1975). However, like fenitrooxon, it is also less stable than the parent compound and was not detected.

Orthene and its metabolite, Monitor, are both relatively poor anticholinesterases and also require metabolic activation (Eto, 1974). Metabolic products of these compounds were not analysed. Monitor is as soluble in water as Orthene and would be easily eliminated from tadpole bodies. The presence of this metabolite in the Day 5 water sample from the field may have resulted from any of several catalysed pathways. Because of the low number of tadpoles in the pond at the time, it is doubtful that its presence is related to tadpole metabolic activity.

H. Behavioral Bioassays

Bioassays using behavioral changes in test organisms reveal lower thresholds than physiological techniques, because the response is derived from an intact integrated system (Scherer, 1975). *Rana clamitans* larvae, unlike those of *Scaphiopus* spp. and *Bufo americanus* (Beiswenger, 1975), lack a well defined social organization. Ranid tadpoles in general do not exhibit territorial behavior nor social hierarchies (DeBenedictus, 1974). Thus behaviors used in pesticide interaction experiments must be defined from the behavioral repertoire of the individual.

Changes in total motor activity in these larvae seem to be relatively sensitive to insecticide treatment. For all three chemicals the revealed reductions in total activity time at increased concentration probably resulted from partial or total paralysis. O'Brien (1967) stated that paralysis is typical of organophosphate poisoning. This contrasts with the hyperactivity for DDT-treated tadpoles (Cooke, 1970). Reductions in activity and locomotion have been previously noted in other fenitrothion-treated aquatic organisms (Hatfield and Riche, 1970; Symons, 1973). This may be typical of anti-cholinesterase pesticides. The high incidence of resting in the control tadpoles (average, 87% of total observation time) suggests that low activity is the norm. Further reduction in activity may be a reaction to stress.

The reduction in the two swimming behaviors are probably related to reduction in total activity time. Matacil- and Orthene-treated tadpoles showed significant declines in both swimming behaviors. Fenitrothion-treated larvae, however, exhibited a reduction in just the multidirectional swimming behavior.

The observed chromatic condition is effectively a quantal response,

light to dark, since objective determination of intermediate color change is difficult to record. Peaslee (1970) found that DDT-treated *Rana clamitans* showed a significant increase in melanocyte-stimulating hormone (MSH). Although amphibian color change is not as closely related to neural control as color change in fishes, a neural imbalance produced by these insecticides could cause the reported melanin response.

As already mentioned, resting of the larvae on the surface may indicate buoyancy problems. Wasserburg and Seibert (1975) suggested that surface swimming in amphibian larvae may, however, be a behavioral response to low dissolved oxygen. This would not appear to be the case in this experiment, since the behavior reported by Wasserburg and Seibert involved continuous swimming and the larvae observed here were stationary. Wildish et al. (1971) reported surface swimming and the formation of visceral gas bubbles in fenitrothion-treated salmon alevins and fry, but concluded that it was not neurotoxic in origin. The occurrence of the typical head up-tail down response in fish has been reported for many toxicants (Warner, 1967), yet the mechanism of the reaction has not been elucidated.

On the other hand the significant increase in the bubble-making behavior in the Orthene-treated tadpoles (1000 ppm) may be in response to low dissolved oxygen. Wasserburg and Siebert (1975) referred to this behavior as 'bobbing' (Swimming to the surface for air) and concluded that it functions as a means of orally taking in air for respiration. In this experiment a significant increase in this behavior occurred only in tadpoles treated with Orthene. The significant reductions in this behavior (Matacil, 25.0 ppm; Orthene, 2000 ppm) would probably result from paralysis due to the interaction of the pesticides with acetylcholinesterase at the nerve-muscle synapses.

Rana clamitans larvae have been described as continuous feeders (Jenssen, 1967), actively filtering material in their branchial apparatus from water flow during gill ventilation. Tadpoles also use their horny teeth to rasp material off surfaces. The feeding behavior described here would correspond to the latter mechanism. This behavior did not occur frequently enough to have been regularly recorded in the 10-min observation periods or to show a significant difference between treated and untreated tadpole groups. It is, however, noteworthy that the behavior was recorded only for untreated larvae and for tadpoles exposed to low pesticide concentrations. Absence of feeding has been described for other fenitrothion-treated aquatic organisms (Bull and McInerney, 1974) and may be occurring here. Feeding reduction could result from damage to peripheral sense organs (Sprague, 1971) or general stress.

With the relatively large number of *t*-tests performed, one might expect a few significant differences to occur by chance alone. However, chance differences would be expected to be randomly distributed throughout all concentrations. The fact that they occurred only at the higher concentrations tends to lend credence to the speculations presented.

I. Possible Insecticide Effects on Predator-prey Relationships

Conspicuous changes in a prey species could conceivably disrupt predator-prey relationships. Cooke (1971) concluded that hyperactivity in DDT-treated *Rana temporaria* larvae made them more susceptible to predation by warty newts, *Triturus cristatus*. Hatfield and Anderson (1972) also noted that fenitrothion-exposed Atlantic salmon parr were more readily preyed upon by brook trout, even when fenitrothion has been shown to reduce activity. A lack of activity, color changes, and surface-resting positions in tadpoles treated with the chemicals used in this investigation could cause changes in predator-prey relationships at the reported concentrations.

Since the tadpoles are relatively resistant to these insecticides and could be rendered predator-susceptible, what would ingesting contaminated tadpoles do to a toxin-sensitive predator? Aquatic insects are particularly sensitive to fenitrothion at residue levels as low as 2 ppb for some species (Wildish and Phillips, 1972). Populations of belostomatids, dytiscids, nepids, notonectids, and Odonata nymphs, all known tadpole-predators (Calef, 1973; DeBenedictis, 1974; Licht, 1974) might be adversely affected. Direct mortality of these insects would also reduce natural mortality in the tadpole population. The oral LD50s of fenitrothion and Orthene to Mallards, avian tadpole predators, have been estimated as 1190 and 350 mg/kg, respectively (Nigam, 1975). Assuming each tadpole weighs 0.5 g, each duck weighs 1.13 kg, and using the maximum whole body residues observed in the field, one Mallard would have to consume approximately 4.7×10^6 or 3.1×10^6 larvae respectively to reach lethal levels. This is a most unlikely occurrence. However, the ingestion of smaller quantities of these insecticides might cause detrimental sublethal effects.

V General Discussion and Conclusions

Normal spray applications using Matacil and Orthene within the recommended dose ranges would have little direct effect on tadpole populations. Concentrations required to cause acute mortality or significant behavioral changes would never be encountered in the field unless accidental spillage of the insecticides occurred. Fenitrothion levels in shallow ponds after insecticide-spraying could more closely approach expected harmful levels. The peak concentration (3 ppb) observed in this investigation and the levels reported by Sundaram (1974) for ponds in the same forest (9-25 ppb), however, suggest that concentrations affecting anuran larvae are not reached. Peak stream values of less than 15 ppb (Eidt and Sundaram, 1975) would also prove innocuous to stream-inhabiting ranid tadpoles.

Suggested 'safe levels' of 0.1 to 0.05 of the incipient LC50 (Sprague, 1971), the concentration where no detrimental effects occur, are substantiated here.

Orthene has been shown to be more effective than fenitrothion in control of spruce budworms (Nigam and Hopewell, 1973). Toxicity values for fenitrothion, Matacil, and Orthene to budworms are 0.31, 0.04, and 0.42 $\mu g/cm^2$ (contact 72 h LD50) (Nigam, 1975). Thus Orthene and Matacil are as effective as fenitrothion in control of the target organism and of lower toxicity to anuran larvae.

To better understand movements of insecticides in temporary ponds, more samples should be collected and analysed. These samples should include sediment samples, biota samples, and water samples from various depths and areas of the pond. This is not feasible with the present technology, for GLC analysis is costly and generally very laborious.

Sprague (1970) suggested the need for an indicator species to evaluate the effect of pollutants on aquatic organisms. Tadpoles, because of their rapid accumulation of organic pesticides as indicated here and by Meeks (1968) are useful in determining levels of toxicants in aquatic habitats, yet their relative resistance to the insecticides compared to other aquatic organisms makes them an insensitive indicator of lethal levels. Anuran larvae undergo complex changes in their rapid development and would be useful organisms to study the effects of these pesticides on 'fetal' growth and development. Species such as *R. sylvatica* have a relatively short breeding season and would be present in large numbers in temporary ponds at a relatively uniform size and developmental stage. *Rana clamitans*, which in some studied populations overwinter as larvae, would also be useful for long-term study of development.

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