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ACCUMULATION AND METABOLISM OF DDT IN THE CRAYFISH ORCONECTES RUSTICUS (GIRARD).

The Ohio State University, Ph.D., 1966
Zoology

ACCUMULATION AND METABOLISM OF DDT IN THE CRAYFISH

ORCONECTES RUSTICUS (GIRARD)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree Doctor of Philosophy in the Graduate School of
The Ohio State University

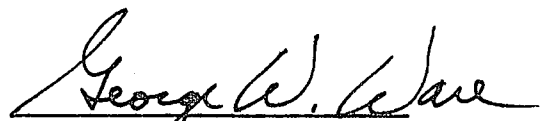
By

Ronald Allan Engel, B. S., M.S.

* * * * *

The Ohio State University

Approved by



Adviser
Department of Zoology and
Entomology

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VITA

July 27, 1936	Born - Ashtabula, Ohio
1958	B.S., Baldwin-Wallace College, Berea, Ohio
1961-1963	Teaching Assistant, Department of Zoology and Entomology, The Ohio State University, Columbus, Ohio
1962	M.S., The Ohio State University, Columbus, Ohio
1962-1964	Research Associate, Natural Resources Institute, The Ohio State University, Columbus, Ohio

PUBLICATIONS

1. Eurytemora affinis, a calanoid copepod new to Lake Erie. Ohio J. Sci. 62(5):252. 1962.

2. Toxicity of rubber stoppers to Daphnia magna Straus. Nature 205(4975):1029. 1965. (with J. H. Hubschman)

FIELDS OF STUDY

Major Field: Zoology

Studies in Aquatic Biology. Professor N. W. Britt
Studies in Invertebrate Zoology. Professor A. C. Broad
Studies in Toxicity. Professor G. W. Ware

CONTENTS

TABLES

FIGURES

INTRODUCTION

DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane was first manufactured in this country in 1943 for use by the armed forces. By 1945, 5% of the 33 million pounds produced was released for civilian purposes (Zimmerman and Lavine, 1946). This seems a very small amount by comparison with the 178.9 million pounds produced in 1963 (Shepard and Mahan, 1965). With the wide-spread agricultural, forestry, medical and domestic use of DDT, early concern was given to the effects of the introduction of this compound into the waters near or included in treated areas.

Results of early studies on aquatic organisms provided evidence of great susceptibility of aquatic invertebrates to the wide-spectrum insecticide DDT. Many of these field investigations were carried on in the late 1940's: Bishop, 1949; Hoffmann and Surber, 1949; Springer and Webster, 1949; Tarzwell, 1950, to name a few. Later investigations such as those by Crouter and Vernon (1959), Bridges and Andrews (1961), Cope (1961), Frey (1961) and Gorham (1961) took advantage of nearby DDT applications to show the reduction of stream bottom-dwelling invertebrates during the post-spraying period.

Field studies on the effects of DDT on aquatic life in ponds and lakes were also conducted during this interim. These investigations included bodies of water purposely treated for insect control (Hunt and Bischoff, 1960; Bridges, Kallman and Andrews, 1963; Jones and Moyle, 1963).

Laboratory investigations of pesticide effects on aquatic arthropods have dealt mainly with bioassay. Because of the important use of DDT for control of mosquitoes and other insect pests, the bulk of the literature is concerned with obtaining acute toxicity levels for various insect species. However, the crustaceans Daphnia, Artemia, and Gammarus were also extensively used in studies involving various pesticides, including DDT (Anderson, 1945 and 1960; Tarpley, 1958; and McDonald, 1962).

Literature concerning the effect of DDT on decapod crustaceans is increasing rapidly because of the concern shown by the shellfish industry (Loosanoff, 1960). The appearance of pesticides in some of the large rivers monitored by the U. S. Public Health Service (Breidenbach and Lichtenberg, 1963) has also aroused public interest. Because of this public awareness and concern, the federal agencies are increasing the number of investigations on biological aspects of pesticide pollution of this country's major water systems. Due to the diverse and numerous studies referring to DDT and Crustacea, some of the findings are reported in tabular form (Table 1).

The tolerance of two populations of the fresh-water shrimp Palaemonetes kadiakensis to DDT was reported by Ferguson, Culley and Cotton (1966). The 36-hour total lethal median (TL_m) values for the two populations were 4.5 and 10.0 parts per billion (ppb). However, in the field the more resistant population survived a contamination of DDT which was lethal to highly resistant fish. This fact throws some doubt upon the reliability of these values.

TABLE 1

DDT effects on crustaceans

Crustacean	DDT Concentration	Results	Remarks	References
Penaeus aztecus and *P. duorarum*	1-6 ppb	immobilized 50% adults in 48 hours	lab, running sea water	Butler and Springer, 1964
Copepods	1 ppm	100% mortality 46 hours.	algae cultures	Loosanoff, Hanks and Ganaros, 1957
Artemia salina 3rd instar	0.105 ppm	LD_{50} 5 hours	static tests phototaxic responses	Tarpley, 1958
Daphnia magna	1 ppb	50% immobilization 32 hours	laboratory	Anderson, 1945
Cambarus virilis	0.5 mg/kg	LD_{50} 48 hours	injection	Welsh and Gordon, 1947
Zooplankton	1 lb/A	decreased population	pond	Jones and Moyle, 1963
Procambarus clarkii	0.6 ppb	TL_m 24,48 and 72 hr.	lab, static	Muncy and Oliver, 1963
Daphnia magna	1.4 ppb	immobilization 50 hr.	laboratory	Anderson, 1960
Callinectes sapidus	0.8 lb/A	66% mortality 24 hr.		Springer and Webster, 1951
" "	0.4 lb/A	90% mortality 2 weeks		" "
" "	0.2 lb/A	60% mortality 1 week		" "
Uca pugnax	0.5 lb/A	42% mortality 1 week		" "
Palaemonetes pugie	0.5 lb/A	severe mortality 12 hrs.		" "

TABLE 1
(continued)

Crustacean	DDT Concentration	Results	Remarks	References
Crayfish	0.5 lb/A	near total mortality		Cottam and Higgins,1946
Apus oryzaphagus	0.5-1 lb/A	100% mortality 20 hours		Portman and Williams,1952
Crayfish	1 ppm	100% mortality	field	Lackey and Steinle, 1945
Crayfish (large)	1-20 ppm	100% mortality	laboratory	" "
Palaemonetes sp.	0.1 lb/A	100% mortality		Hess and Kenner, 1947
Crayfish	1-2 lb/A	100% killed	field	Tarzwell, 1947
Callinectes sapidus	0.8 lb/A	100% killed		Springer and Webster, 1951
" "	0.25-0.5 lb/A	20-80% killed		" "
Fiddler Crab	0.25 lb/A	10-20% killed		" "
" "	0.5 lb/A	20-40% killed		" "
Crayfish	0.1-0.2 lb/A	100% killed		Tarzwell, 1950
Callinectes sapidus	3 lb/A	survived	2 different	Tiller and Cory, 1947
	3 lb/A	70% killed	experiments	" "
Uca pugnax	0.2 lb/A	60% killed, 7 wks.		George, Darsie and Springer, 1957
Palaemonetes kadiakensis				
	4.5 ppb	36 hr. TL_m	2 different	Ferguson, Culley and
	10. ppb	36 hr. TL_m	populations	Cotton, 1966

TABLE 1
(continued)

Crustacean	DDT Concentration	Results	Remarks	References	
Artemia salina	0.01 ppm	killed (2-6 da)	sea water 15°C	Richards & Cutkomp, 1946	
Gammarus sp.	0.01 ppm	killed (1-3 da)	fresh-water 15° C	"	"
"	0.01 ppm	killed (1-3 da)	fresh-water 28° C	"	"
Daphnia sp.	0.1	killed (1-5 da)	fresh-water 15° C	"	"
Cyclops sp.	1.0 ppm	killed (3 da)	fresh-water	"	"
Pagurus sp.	0.1 ppm	killed (1-3 da)	sea water	"	"
Emerita talpoida	1.0 ppm	killed (1-2 da)	sea water 15°C	"	"
Daphnia pulex	0.36 ppb	48 hr. EC_{50}	60°F	Sanders and Cope, 1966	
Simocephalus	2.5 ppb	48 hr. EC_{50}	60°F	"	"
serrulatus	2.8 ppb	48 hr. EC_{50}	70°F	"	"
Daphnia magna	1.4 ppb	50 hr. EC_{50}	68°F	"	" 1
" "	4.4 ppb	24 hr. EC_{50}	68°F	"	" 2
Daphnia carinata	2.2 ppb	32 hr. EC_{50}	78°F	"	" 3

[1]values reported by Boyd (unpublished)
[2]values reported by Kawar (unpublished)
[3]values reported by Matida and Kawasaki (1958)

Most toxicity work with crayfish has been limited to observations of mortality after spraying known amounts of pesticides over an area (Cottam and Higgins, 1946, and Bridges, Kallman and Andrews, 1963). A lethal concentration of 0.23-1.0 ppm of DDT in water is suggested by Lackey and Steinle (1945). More recently, Muncy and Oliver (1963) studied the toxicity of 10 insecticides to Procambarus clarkii (the Louisianna red crawfish). They subjected 56 crayfish to aqueous suspensions of DDT and determined the 24, 48 and 72 hour TL_m values in parts per million (ppm). The values given for DDT were 0.6 ppm for all time periods. They also noted that cannibalism was prevalent when more than one animal was tested in each container. It was suggested that the crayfish might be an excellent test animal for sublethal pesticide concentration studies which might be indicated by growth changes.

Because of its economic importance, the effects of twelve pesticides on P. clarkii have been investigated, including laboratory and field studies (Hendrick and Everett, 1965; and Hendrick, Everett, and Caffey, 1966). In the cases reported no significant changes in growth or reproduction were noted.

Tarzwell (1963) recognized the need for sublethal and long term studies of toxicants on aquatic organisms. Several federal laboratories have studied the long term effect of sublethal quantities of pesticides on fish (Lowe, 1964; Butler, 1965;and Mount et al., 1966). However, only a few studies have been conducted with decapods or aquatic insects under similar conditions stated above.

Jensen and Gaufin (1964) subjected stone fly naiads, Acroneuria pacifica and Pteronarcys californica, to DDT and four other organic insecticides. They used concentrations equal to or less than the static four-day TL_m (0.32 and 1.8 ppm respectively) values for a period of 30 days. Under constant-flow bioassay conditions with DDT, the naiads showed only tremors in the concentrations that originally gave 50% mortality with static tests. Reduced molting rates and failure to emerge were also noted.

In another study, Lowe (1965) maintained a continuous flow of 0.5 and 0.25 ppb DDT in sea water for 9 months to determine sublethal effects on juvenile blue crabs, Callinectes sapidus. Only four of the 18 survived the test and they molted, fed and grew similarly to the controls. In preliminary tests, survival was for only a few days in water containing more than 0.5 ppb DDT.

As long as DDT has been used no one has fully elucidated its mode of action, but several hypotheses were suggested, the latest by O'Brien and Matsumura (1964). These workers have continued to study the mechanism of DDT-response which is symptomological of the central nervous system (Matsumura and O'Brien, 1966 a,b). They propose that DDT forms a charge-transfer complex with a component of the axon, thus disrupting transmittance of the impulse. So far the problem has not been reported as solved.

After the publication of "Silent Spring" (Carson, 1962), a program of research was undertaken that was directed toward the long term

residual effects of pesticides on the biosphere. Because of this, DDT has not lacked attention as an often and highly residual chlorinated hydrocarbon (Lichtenstein et al., 1960). Metabolism of DDT and its analogues have been reported by numerous authors.

In mammals, primarily rats, the isolation of DDT metabolites has been done by Jensen et al., (1957); Peterson and Robison (1964); and Pinto, Camien and Dunn (1965). They have suggested that there are at least seven metabolic steps with conjugates and intermediates formed. There is also some evidence for isomeric changes. The metabolic change of DDT to DDD was reported for several animal tissues, including several aquatic vertebrates (Finley and Pillmore, 1963; and Datta, Laug and Klein, 1964).

There have also been extensive studies to gain information on the fate of DDT in insects. The interest in this problem stems from the appearance of DDT resistance in the housefly and other insects. Various investigators have confirmed the conversion of DDT to DDE. This is accomplished in the presence of DDT-dehydrochlorinase and glutathione (Sternburg, Kearns and Moorefield, 1954; and Sternburg, Vinson and Kearns, 1953). Not all the species of insects studied made this conversion to DDE and with some of those that did, it has been impossible to determine the mechanism (Perry, 1960a). In insects, as in mammals, many of the polar metabolites are isolated as conjugates (Perry, 1960b). An excellent account of the known metabolites and probable pathways is given by Perry (1965). In addition to the conversion to DDE, several other insect species oxidize DDT

to the corresponding ethanol derivitive 1,1-bis-(p-chlorophenyl)-2,2,2-trichloroethanol (Menzel et al., 1961). Further evidence of an oxidative conversion pathway is suggested by Plapp et al. (1965). In their study of the DDT resistant Culex tarsalis, the DDT analog DDD (TDE) was converted to the ethanol derivitive [1,1-bis(p-chlorophenyl)-2,2-dichloroethanol] in addition to being dehydrochlorinated to the ethylene compound TDEE. Although insects have not been shown to metabolize DDT to DDD, Perry (1965) suggests this in the light of other known metabolites.

Some studies have been done in vitro in addition to those mentioned in vivo. In comparing in vivo and in vitro metabolism by DDT resistant and susceptible lice strains, Perry, Miller and Buckner (1963) found that where the resistant strain could metabolize the DDT in vivo, both strains could do so in vitro, suggesting that some other factor was responsible for the susceptibility to DDT.

The metabolism of DDT in Crustacea is relatively unknown. In Lowe's 1965 study with blue crabs, one of the test animals that died after four months exposure to 0.5 ppb DDT had a total body residue of 5.36 ppm of DDT and its metabolites (Butler, 1965). Crayfish (Procambarus simulans) from a pond that had been treated with DDT were analysed for DDT, DDD and DDE (Bridges, Kallman and Andrews, 1963). Three weeks after treatment equal to 0.02 ppm DDT, collected crayfish yielded 0.81, 0.46, and 0.55 ppm DDT, DDD,and DDE respectively. Crayfish collected over the next 16 months contained decreasing amounts of DDT, but DDE continued to be prevalent.

From the few studies available, it appears that most aquatic animals tend to concentrate the available DDT and store it or pass it on to the next trophic level in the food web. Because of its role as an omnivore, the crayfish can obtain DDT from many sources. In turn, the crayfish is food for many other animals.

The purposes of this study were to show the effects of various sublethal concentrations of DDT on the crayfish when it was in a continuously renewed medium, the effects of DDT on various stages in the life cycle, the area of concentration of DDT within the crayfish and to determine the quantities of common DDT metabolites that were present after DDT exposures of various periods up to a month.

MATERIALS AND METHODS

Crayfish collections and maintenance

All crayfish used in this study were Orconectes r. rusticus collected from the Big Darby Creek, Union County, Ohio. The majority of these crayfish were collected near the junction of Union County roads #163 and #164. A 6-foot long, quarter-inch mesh seine was used for all collections in the warmer months. Early spring collections of ovigerous females were made with a dip net or by hand, the latter being the most successful way of collecting crayfish from the rocky bottom.

The crayfish were slowly acclimated to laboratory conditions during the first 24 hours after collection. This was especially necessary in the colder months when the stream temperature was 6° to 14°C. There was little mortality during this acclimation period.

After the first 24 hours the crayfish were placed in plastic refrigerator crispers (10 x 12 x 4 inches) with about 1-1/2 inches of charcoal-filtered tap water. They were kept in constant temperature chambers at 20°C with a set 12 hours light, 12 hours darkness in each 24-hour photoperiod. Holding containers were cleaned every two days.

Fresh fish, frozen fish, worms, laboratory mouse food and a commercial pond chow were used to feed the animals. At any one time all the animals in cultures were fed the same food. Animals were kept for several weeks before testing, except in the experiment using ovigerous females.

Bioassay and sublethal exposures

All static bioassay experiments were carried out in 1500 ml glass chambers containing 500 ml charcoal filtered tap water. These chambers readily accommodated one medium-sized crayfish which was fully covered by water. The exposure chambers were held at 20°C under a controlled 12-hour light, 12-hour dark photoperiod. Various DDT concentrates were prepared in acetone or in acetone and emulsifier (Triton X-100[1]) and delivered beneath the water surface by a 1 ml pipette.

Bioassay and chronic exposure experiments in flowing water with a constant amount of DDT added were also conducted. For these tests a serial-dilution apparatus for continuous delivery of various concentrations of materials in water was used. This particular apparatus was one of the early models made by Dr. D. I. Mount of the Federal Water Pollution Control Administration, Public Health Service, Department of Health, Education and Welfare, Newtown, Ohio. A later improved model was extensively described by Mount and Warner (1965). The apparatus worked by water flowing from a constant head box, overflow siphons and venturi siphons, and is shown in Figures 1, 2 and 3.

This model provided four different pesticide dilutions at a flow rate of 30 ml/min. Tap water was moved to a 20-gallon aquarium head box by city water pressure. The water was thoroughly aerated upon entering the aquarium. Because of fluctuations in water pressure, a larger volume of water was delivered than was used. To compensate for this, a constant level siphon was fixed to the side of the aquarium

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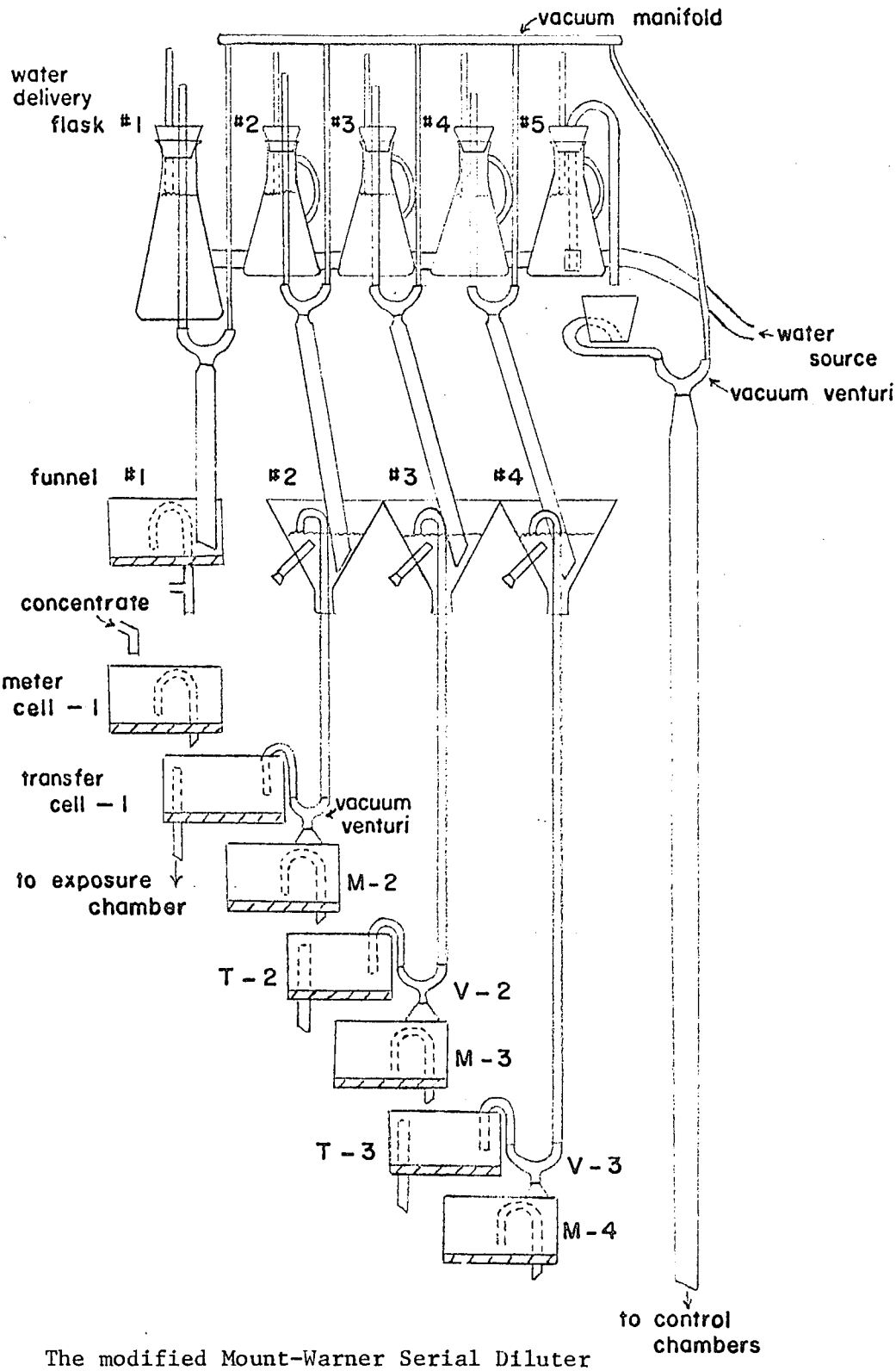
[1]Alkyl phenoxy polyethoxy ethanol, Rohm and Hass Co., Philadelphia

Figure 1



The modified Mount-Warner Serial Diluter

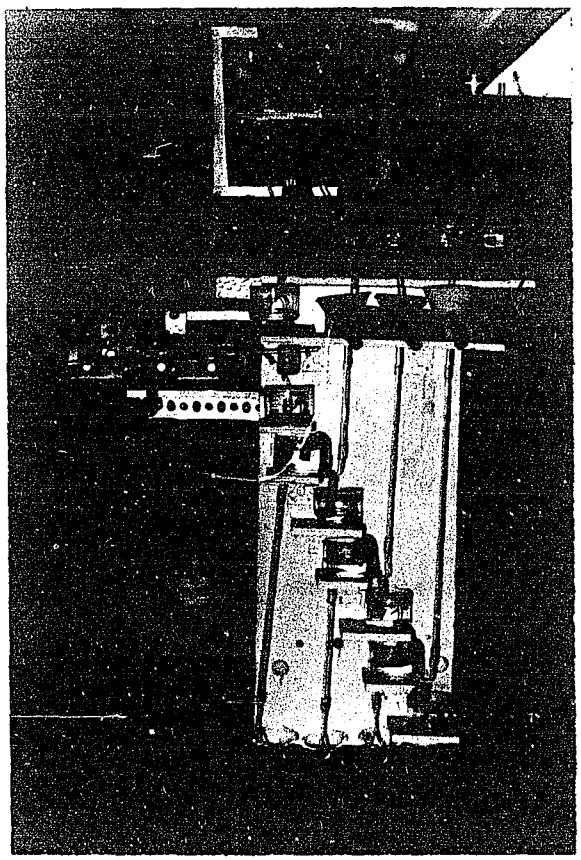


Figure 2. The serial-dilution apparatus in operation.

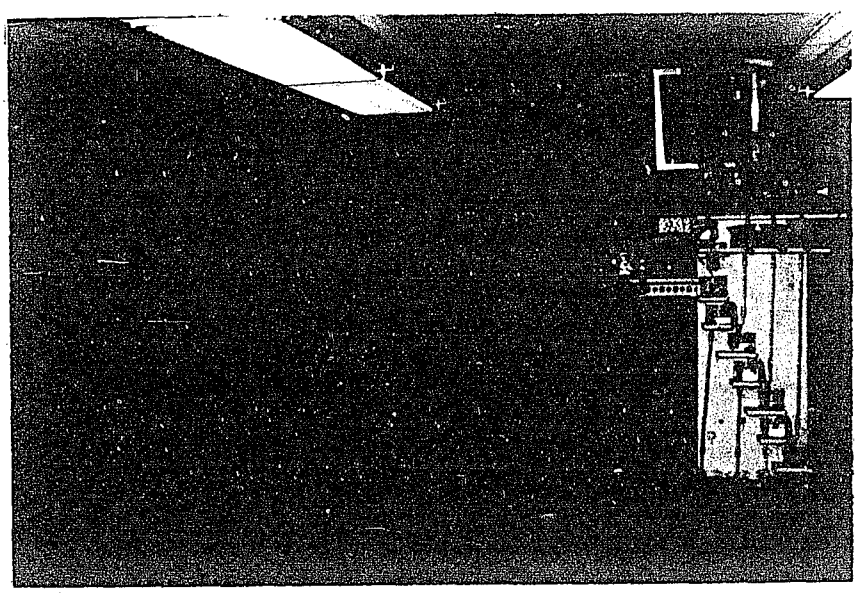


Figure 3. The serial-dilution apparatus and exposure chambers.

through which the excess water was passed to the drain. Later, an automatic starting siphon was installed to prevent accidental overflow.

Water from the aquarium was siphoned into an activated charcoal-filled aquarium filter in order to remove free chlorine and particulate material. A Tygon® tube led from the filter to the manifold which fed the water delivery system for the apparatus. Five 125-ml vacuum filter flasks were used for the water delivery system. Their siphons were calibrated to deliver 60, 30, 30, 30, and 60 ml (#1-5, respectively). These siphons were started with the overflow from flask #5 which ran through a vacuum venturi that in turn placed a vacuum on the ends of the siphons of flasks numbered 1 through 4. The water flowing to the vacuum venturi first spilled into a plastic cup which lowered with the increased weight, thus pinching the rubber delivery tube for the manifold (Figures 4 and 5). This momentary stoppage of water to the flasks was enough to assure clearance of the flask siphons so that they would not deliver more than the calibrated quantity. Water which passed through the vacuum venturi fell into a plastic cup. This trickered a counting device and the water flowed on to the control exposure chambers. The water delivery flasks (#2-4) emptied into funnels (#2-4) fitted with siphons. These chambers provided a closed end so that a vacuum could be applied to start the flask's siphon. A modification (funnel #1) for this particular machine required a larger volume to pass water to the first meter cell. Two 60-ml quantities of water from delivery flask #1 were necessary before water would overflow funnel siphon #1. This provided more stirring action and better mixing of the pesticide concentrate with the water.

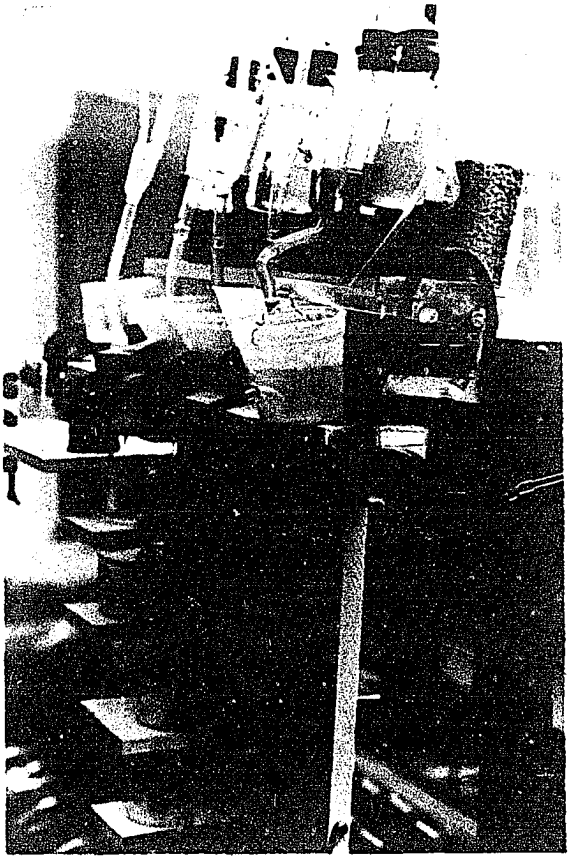


Figure 4. The rubber delivery tube open allowing water to enter the flasks.

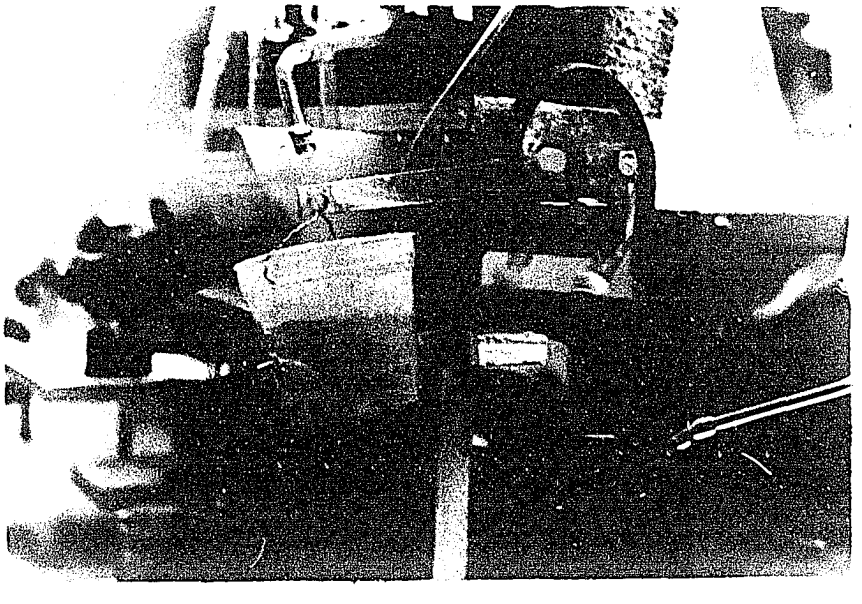


Figure 5. The rubber delivery tube pinched closed allowing the flask siphons to clear.

Water from the funnels fell into meter cells. The meter and transfer cells were 120-ml glass crystalization dishes with a 1/2-inch hole in the bottom fitted with a one-hole rubber stopper. This was the outlet for the automatic siphon tube of the meter cell and was fitted with a standpipe in the transfer cells. The automatic siphon delivered 120 ml of water and toxicant to the transfer cell once each full cycle. Of the 120 ml delivered to the transfer cell, 60 ml overflowed the standpipe and were delivered to the exposure chambers. The other 60 ml were siphoned by venturi to the next meter cell in line. It can be seen that for every 60 ml of water and toxicant, two 30-ml water portions were delivered to the meter cell so that each dilution delivered to the exposure chambers was half the concentration of the previous transfer cell.

The toxicant concentrate was delivered into meter cell #1 by a different mechanism than that described by Mount and Warner (1965). A five-ml syringe was held stationary and the plunger pushed in by a threaded rod. The threaded rod was placed through a threaded gear and held against the syringe plunger with a rubber stopper so that it would not turn. The forward movement was caused by turning the gear while it was stationary to any lateral movement. Volumes of the concentrate from the syringe were determined by the distance the threaded rod travelled with each turn of the gear. Some of the water falling from funnel #1 was by-passed to run by the syringe needle. The remainder fell into a plastic bucket which was balanced at the end of a lever. The upward movement of the other end of the lever rotated the gear 1/21 revolution, thus pushing the threaded rod and syringe

plunger to deliver a calculated amount of DDT concentrate (Figure 6). A small hole in the bottom of the plastic bucket provided drainage, allowing the empty bucket to return to its original position (Figure 7).

The exposure chambers were 500 x 100 mm stainless steel troughs, 127 mm deep, partitioned with stainless steel so that five 100 mm-square chambers were provided (Hubschman, 1966). The water with toxicant came from the diluter by glass tubes and spilled into one end of the trough. Holes in the stainless steel partitions facilitated the flow of the medium through the troughs. The water and toxicant left the trough by over-flowing a 50-mm high standpipe at the opposite end of the chamber and joining the overflow of the others in a common drainpipe.

The apparatus was set to deliver four different concentrations of DDT in water. Each of the three highest concentrations spilled into two exposure troughs. The lowest concentration was split to spill into four troughs. All chambers, therefore, received the same volume. This was possible because the last metering cup had 120 ml of toxicant and no transfer cell.

The first bioassays with high concentrations of DDT in acetone proved that an emulsifier was necessary to keep the DDT from crystallizing and clogging the syringe. Subsequent bioassays were conducted with an emulsifiable concentrate of DDT in 10% Triton X-100 in acetone. Various concentrates were used to obtain the desired DDT level in the water and analyses were made periodically to determine the actual DDT concentration in the water. When a bioassay test or chronic exposure was run, the machine was calibrated to deliver the desired DDT concentrations. Water and toxicant were run through the flow-through chambers

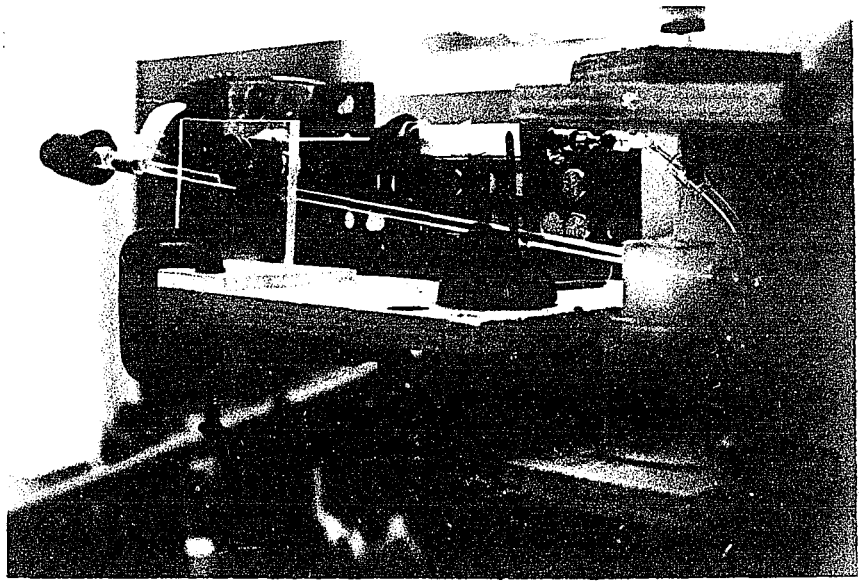


Figure 6. The concentrate injection system showing bucket filled with water and gear rotated.

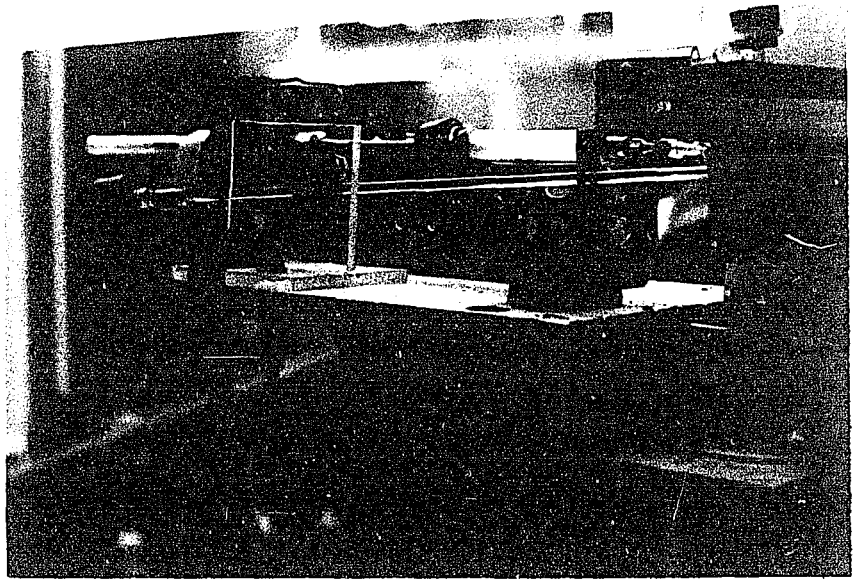


Figure 7. The concentrate injection system showing empty bucket in normal position.

for at least 12 hours before test animals were introduced. Under these conditions the test animal was subjected to one continuous DDT concentration. One animal was placed in each compartment after noting sex, and except for alternating the sexes in adjacent compartments, test animals were chosen at random from a large group of similar sized animals.

The activity and condition of the test animals were noted several times each day and the apparatus tended at least twice a day. All dead animals were removed from the chronic exposure tests and frozen. When the exposures were terminated, all animals were weighed, measured and destroyed or in the case of chronic exposure tests, placed in vials and frozen for future analysis.

Analytical techniques

All chemicals used throughout these experiments were reagent grade unless otherwise noted. All DDT used in experiments came from the same 10-g quantity of 99.9% p,p'-isomer provided by the Geigy Chemical Corporation[1]. Other standards used were reference standards from the Federal Food and Drug Administration. DDMU (1-chloro-2,2-bis(p-chlorophenyl) ethylene) was from the Denver Wildlife Research Center, Denver, Colorado, and the DDA (bis(p-chlorophenyl) acetic acid) was made in this laboratory following the procedure of Grummitt, Buck and Sterns (1945). Acetonitrile, acetone and n-hexane were all practical grade chemicals which were glass distilled in the laboratory. Periodic checks were run on the distilled products to insure analytical reliability.

[1]Ardsley, New York

Hydrogen ion concentration, total hardness, and calcium hardness, as well as DDT analyses of the water in the test chambers were undertaken periodically. The DDT was analyzed by gas chromatography and the other chemical conditions were measured with a Hack chemical kit[1]. DDT was removed from 500 ml of the test water by thorough shaking with three 50-ml portions of n-hexane. The hexane fractions were combined and the water removed with anhydrous sodium sulfate powder. The hexane was then evaporated to dryness with filtered air over a hot plate and the sample transferred to a capped glass vial.

Before analysis, the frozen crayfish was weighed, and the carapace measured. If the individual organs were to be analyzed, the animal was dissected and the hepatopancreas, gonads, antennal gland, alimentary tract, abdominal muscle and gills removed. Each kind of organ was combined with the four others obtained from crayfish in the same trough. The combined weight of each kind of organ was noted and then five times this weight of anhydrous sodium sulfate was used to macerate the organs or the animal with a mortar and pestle. Grinding was continued until a free-flowing powder resulted.

The tissue-sulfate mixture was ground five times in separate 25-ml volumes of hexane-saturated acetonitrile. The five portions were combined in a beaker, filtered, evaporated to a known volume and extracted with three hexane portions, each equal to the acetonitrile fraction. The acetonitrile was removed from the separatory funnel, evaporated to dryness, dissolved in 10% ethyl acetate in hexane, and stored at 20°C in a screw-capped glass vial for future clean-up. The

[1]Hack Chemical Co., Ames, Iowa

hexane layers were combined and evaporated to a convenient volume for immediate clean-up. Clean-up of the hexane fractions was accomplished by mixing 3 ml of 1:1 mixture of concentrated sulfuric acid; fuming sulfuric acid in a separatory funnel with a 50-ml hexane sample. After agitating vigorously the precipitate was released and the rest was filtered through sodium sulfate to remove any suspendible material. Centrifugation was also used to remove the acid material and resulted in better recovery of the hexane. Apropriate recovery factors were applied to correct for losses in the extraction and clean-up procedures. The acid clean-up procedure was also applied to standards in order to determine any degradation.

The acetonitrile fraction clean-up was done on a sulfuric acid-treated silicic acid-packed column (Peterson and Robison, 1964). The column packing was prepared by treating each gram of $SiO_2 \cdot XH_2O$ with 0.5 ml of 0.5 N aqueous H_2SO_4 in 5 ml of acetone. The resultant slurry was mixed thoroughly and dried to a free-flowing powder. Before the column was packed, the treated silicic acid was mixed with an equal weight of Celite®[1]. Three grams of the packing was placed in a 10 (I.D.) x 450 mm glass tube which had been drawn out at one end to restrict a glass wool plug. The resulting 60-mm column capped with 1/4-inch Celite® was prewetted with n-hexane before the acetonitrile fraction was added to the column and forced just to the top of the Celite®. Thirty ml of 5% ethyl acetate in hexane was then added

[1]Diatomaceous silica products, Johns-Manville's registered trademark

as the eluting solvent. The first 10 ml through the column was discarded and the remainder was used for thin layer chromatography.

Gas liquid chromatography (GLC) was done on a Packard Gas Chromatograph[1], Series 7500 equipped with a dual column oven model 802, power supply model 835, a single electrometer model 840, temperature controller model 871, an electron capture detector with tritium foil, and a Westronics single-pen recorder model S11A/U-1/DV6H[2]. The dual column oven held one 5' and one 3' coiled Pyrex column, 4 mm I.D. The 5' column was packed with 1.5% SE 52 on 70/80 mesh Anakrom ABS. The 3' column was packed with 5% DC 11 on Anakrom A. Other conditions kept constant were injection port temperature, 230°C; column temperature, 205°C; detector temperature, 210°C; voltage, 25; N_2 gas flow rate, 150 ml/min. at 31 psi, and chart speed, 1/5 inch per minute. A sensitivity of 3×10^{-9} or 1×10^{-9} amperes was used throughout the tests depending upon the amount of DDT in the samples and the absence of interfering substances.

All quantitative estimates of residues were based on the chromatograms of GLC. Residues were determined by comparing their peak heights with those of standards injected into the column periodically throughout the day. One standard solution containing 0.1 ng/µl DDMU, 0.2 ng/µl DDT, 0.01 ng/µl DDD and 0.02 ng/µl DDE in hexane was used for quantitative analyses. Replicates of three known quantities of the standard

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[1]Packard Instrument Co., Inc., La Grange, Illinois

[2]Westronics, Inc., Fort Worth, Texas

solution were injected into the chromatograph to provide data for linear standard curves. The amount of a compound in a sample was then determined by plotting its peak height on the standard curve and reading the resultant quantity from the abscissa. Qualitative determinations of the compounds in the sample were done by comparing their retention times with those of known compounds. Additional compounds, or confirmation of those found by GLC, were sought by thin-layer chromatography.

Thin-layer chromatography (TLC) was done on 200 x 200-mm glass plates coated with 0.25 mm layer of Silica Gel G. This was applied with a Desage/Brinkmann[1] adjustable applicator model 250012. The coating was prepared by slowly adding 40 ml distilled water to 30 g Silica Gel in an Erlenmeyer flask. When it was thoroughly wetted and free of air bubbles, an additional 20 ml of distilled water was added and the flask vigorously shaken, a total time of less than two minutes. Five plates were mounted on a board and coated at one time, and allowed to dry in place for 15 minutes before activating for 30 minutes at 75°C. The coated plates were pre-washed with distilled water by placing them in an upright position in a shallow dish with about 1/2 inch distilled water. An even flow was obtained by adhering a narrow piece of filter paper to the bottom edge of the plate. After prewashing, the plates were dried in the hood and activated in an oven at 75°C.

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[1]Brinkmann Instruments, Westbury, New York

Development of plates spotted with apolar compounds was done with 2% acetone in n-hexane (Kovacs, 1963) in a glass tank 10 x 22 x 22 cm with a fitted lid. Thin-layer plates spotted with the treated acetonitrile phase (now in 5% ethyl acetate in hexane) were developed in 2% ammonium hydroxide in absolute ethanol. After the solvent rose 12 cm, the plates were removed from the tank, air-dried in the hood, and then sprayed with chromogenic agent.

The chromogenic agent was made by adding 10 ml of 2-phenoxyethanol to 0.4 g $AgNO_3$ in 1 ml of water. The mixture was diluted to 200 ml with acetone and a drop of 30% H_2O_2 was added (Kovacs, 1963). After the plate was sprayed and air-dried, it was placed in 75° oven for 15 minutes. The spots were produced by exposure to ultra-violet light for approximately 10 minutes.

For identification of unknowns, the resulting spots were compared with standards applied to the same plate or with Rf values reported in the literature (Kovacs, 1963; Onley, 1964, and Morley and Chiba, 1964). In some cases, it was necessary to compare Rf's to standards spotted on other plates in this laboratory. It was not possible to chromogenically determine the presence of compounds in less than 2 µg quantities on the thin layer plate. Therefore, it was necessary to remove the Silica Gel in the area determined by visible standards and elute the material into a vial with hexane for gas chromatography.

RESULTS

Bioassay tests were run to determine the range between lethal and sublethal DDT concentrations. Static bioassays of an aqueous suspension of DDT in acetone showed that the 48 hour effective concentration, when the crayfish was inverted and unable to right itself, was approximately 0.1 ppm. Fourteen of the 18 animals exposed to 0.4 ppm were either dead or unable to maintain a normal crawling position, while one of the five animals exposed to 0.1 ppm was dead in 48 hours. The crayfish followed the expected symptomology when exposed to DDT. In the last stages the crayfish usually lies on its back unable to right itself.

It was suspected that the DDT-acetone mixture was not distributed evenly in the bioassay chambers, so a 72-hour static bioassay was run exposing 5 crayfish to each of the following DDT concentrations: 0.003, 0.006, 0.012, 0.025, and 0.049 ppm. To facilitate DDT dispersion in the water the DDT was dissolved in a 3% Triton X-100 in acetone solution. An amount of 3% Triton X-100, equal to the highest amount in the experimental group (200 ppm), was used in the control. One crayfish died in the 0.049 ppm solution.

In 72 hour bioassays of 100, 200, 400 and 800 ppm Triton X-100, one of the crayfish died while molting in the 800 ppm solution.

Therefore, emulsifier alone at concentrations in the 200 ppm range for the DDT bioassays above were considered non-toxic to the crayfish.

Short-term continuous-flow bioassay experiments were undertaken with calculated DDT concentrations of 0.033, 0.016, 0.008 and 0.004 ppm in the exposure chambers. Results from this test are recorded in Tables 2 and 3. In most cases when a crayfish was on its back, it did not survive.

After removal of the test animals and debris, a second bioassay was started. The results are given in Tables 4 and 5. Visible effects of DDT toxicity and death occurred earlier in the second than in the first test. No DDT analyses were done on the water during these tests, but after 12 hours of operation at 0.06 ppm during a preliminary test, GLC analysis indicated that the DDT concentration in the test chambers was 83% of the amount added.

The reliability of the serial dilution apparatus was tested after it had been operating for more than thirty days. It was calibrated to deliver 6, 3, 1.5 and 0.75 ppb DDT to the exposure chambers. Five hundred ml of water was removed from each of the following locations: meter cells #1, #2, #3, #4, from beneath the inflow tube, the middle chamber and near the outflow standpipe of one of the 0.75 ppb exposure troughs. Results from the hexane extracts of the water samples showed 0.56, 0.25, 0.19, 0.08, 0.126, 0.136 and 0.136 ppb DDT. These samples follow the flow of water through the apparatus as they were described above.

TABLE 2

Percent immobilization of Orconectes rusticus exposed to four DDT concentrations for 24, 48, 72, and 96 hours, first run.

	ppm DDT				
Hours	0.033	0.016	0.008	0.004	Control
24	80	20	-	-	-
48	80	60	40	-	-
72	100	100	60	20	-
96	100	100	100	60	-

TABLE 3

Percent mortality of Orconectes rusticus exposed to four DDT concentrations for 24, 48, 72, and 96 hours, first run.

	ppm DDT				
Hours	0.033	0.016	0.008	0.004	Control
24	-	-	-	-	-
48	-	-	-	-	-
72	60	20	20	-	-
96	80	80	60	-	-

TABLE 4

Percent immobilization of Orconectes rusticus exposed to four DDT concentrations for 24, 48, and 72 hours, second run.

	ppm DDT				
Hours	0.033	0.016	0.008	0.004	Control
24	100	100	100	40	-
48	100	100	100	100	-
72	100	100	100	100	-

TABLE 5

Percent mortality of Orconectes rusticus exposed to four DDT concentrations for 24, 48, and 72 hours, second run.

	ppm DDT				
Hours	0.033	0.016	0.008	0.004	Control
24	25	40	-	-	-
48	50	80	20	-	-
72	75	100	20	20	-

DDT concentrations less than those used in short term bioassays were used for long term exposures. These exposures were conducted in charcoal filtered tap water with 8.9 pH, 60-88 ppm calcium hardness, 85-104 ppm total hardness and no residual chlorine. Because the exposure concentration approached the 1.2 ppb solubility of DDT in water (Bowman, Acree and Corbett, 1960), emulsifier was no longer used. A concentrate of fifteen mg. DDT/320 ml acetone in the injector should have provided 2.0, 1.0, 0.5, and 0.25 ppb DDT in the exposure chambers, but hexane extracts of water samples showed much lower quantities: 0.8, 0.13, 0.07, and 0.05 ppb DDT. The organs analyzed for DDT residues came from crayfish exposed to the calculated DDT concentrations indicated above. Results of the DDT and DDE levels from these various crayfish organs are presented in Table 6.

Results from a second experiment showed the differences in DDT residues acquired by individual crayfish in 2 and 3 week exposures to various concentrations of DDT. Although the same concentrate was used as in the previous exposure, GLC analyses for DDT in the water indicated only 0.44, 0.16, 0.13, and 0.005 ppb for the 2, 1, 0.5, and 0.25 ppb calculated amounts respectively. Single whole crayfish from this exposure were analyzed for DDT and its metabolites. The results are shown in Table 7 and in Figure 8. None of the standards of DDT metabolites were altered by the concentrated acid clean-up procedure, but a 5 µg dieldrin standard was completely removed by the normal quantities of acid used for cleaning.

TABLE 6

DDT and DDE (ppb) in organs of Orconectes rusticus exposed to four DDT concentrations for two and four weeks.

Organ	Residue	2 ppb 2 wks	2 ppb 4 wks	1 ppb 2 wks	1 ppb 4 wks	.5 ppb 2 wks	.5 ppb 4 wks	.25 ppb 2 wks	.25 ppb 4 wks	Control 2 wks	Control 4 wks
Gill	DDT	412.9	346.8	113	156.6	79	65	51.9	39	28.9	*
	DDE	36.1	42.7	19.6	27.2	18.2	22.5	14.5	7.2	13.3	13.2
Antennal Gland	DDT	646.4	161.6	266.7	2066.7	400	333.3	745	258	33.3	*
	DDE	154	97	186.7	226.7	T	160	522	T	41.6	38.7
Alimentary Tract	DDT	226.4	130.4	155.8	62.9	53.8	57.3	209.4	35	111.3	22.9
	DDE	18.1	14.8	22.4	10.5	T	68.8	38.3	6.3	24.4	6.9
Muscle	DDT	172.9	95.7	65.1	65.2	39.4	26.9	22.6	11.5	15.6	14.1
	DDE	30.2	17.3	15.0	10.7	11.0	9.6	8.0	2.9	7.1	6.4
Hepatopancreas	DDT	1552.6	451.4	440.1	3296.7	129.1	227.3	141.4	103	53.1	61.0
	DDE	281.4	95.0	61.7	129.1	22.4	64.9	37.8	38.2	18.4	20.6
Ovary	DDT	3651.7	3358.0	1520.5	1931.8	879.1	893	417.9	583	187.3	124.5
	DDE	365.2	404.9	328	443.2	139.2	260.2	133.9	251.8	86.0	70.7

*interfering substance
T denotes trace amounts

TABLE 7

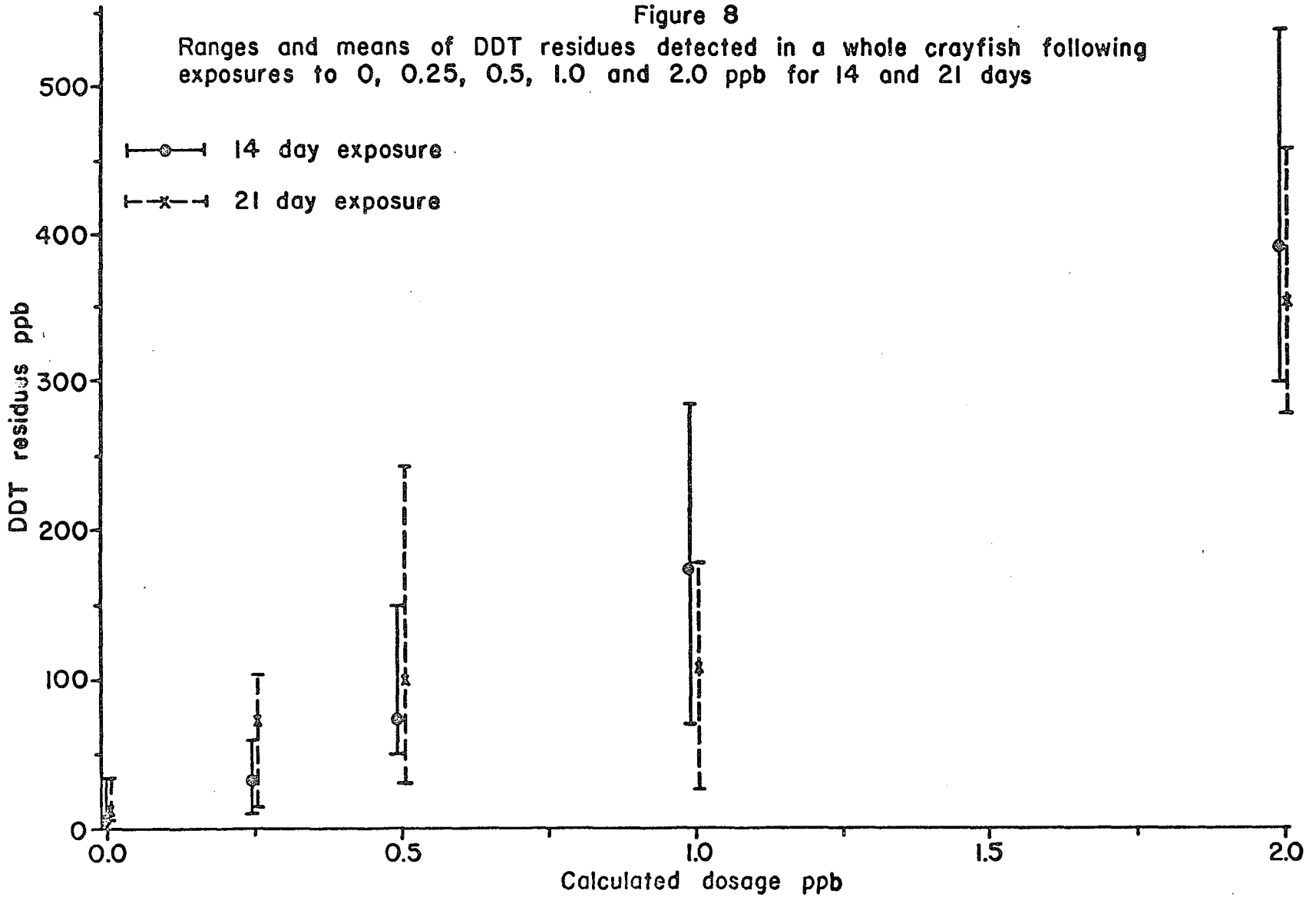
DDT and DDE in Orconectes rusticus after 14 and 21 day exposures to four DDT concentrations

Residues in ppb	Calculated exposure concentration in ppb DDT													
	2.0		1.0		0.5		0.25		0.25		Control		Control	
Da:	14	21	14	21	14	21	14	21	14	21	14	21	14	21
DDT	375	461	68.8	74.2	149	46.5	57	24	32.3	75	12	7.6	T	22.5
DDE	112	50.3	T	18.4	32	5	20	3.2	2.5	12	4.2	T	6.3	12.8
DDT	539*	409	162	140	57	246.6	12	80.4	35.2	42.4	T	28	33.5	7
DDE	25	8.7	11	28	3.1	20.2	3.1	16.4	3.5	3.8	ND	12.8	14.8	2.4
DDT	312	312*	233	151	53	31.7	40	52.9	33	35.5	T	11.2	9.6	9.9
DDE	22	T	26	14	5	4.9	12	6.8	3	7.1	T	ND	T	3.9
DDT	288	302.5	124	24.1	48	74.9	30	26.8	58	16	T	27	7	12.5
DDE	7.7	37	T	2.2	1.6	8.6	3	6.1	20	3.2	ND	15.7	T	4.5
DDT	457*	279.8	284	178*	67.5	90.5	39	37.1	10.6	105.6	T	7.6	ND	10.9
DDE	54	11.1	27	16.8	11	7.3	3.4	2.9	1.7	20.6	149	T	ND	2.6

T denotes trace amounts
* This crayfish died before experiment was completed
ND None detectable

Figure 8

Ranges and means of DDT residues detected in a whole crayfish following exposures to 0, 0.25, 0.5, 1.0 and 2.0 ppb for 14 and 21 days



Retention time of peaks which occurred on the chromatogram of a sample were compared with those of DDD, DDMU, o,p-DDT and bis(p-chlorophenyl) trichloroethanol (Kelthane) as well as with DDT and DDE. Only DDT and DDE were identified in the samples by gas-liquid chromatography. Several unidentified materials were present in most of the residue analyses, but no attempt was made to identify them as they also occurred in control crayfish.

When the crayfish molted, the exuvia was left in the chamber to be eaten. The day the experiment was terminated exuvia were available from newly molted crayfish which had been exposed to 0.25, 0.25 for 14 days and 2.0 ppb DDT for 21 days. Extracts of these three exuviae had 55, 35, and 244 ppb DDT respectively.

Thin-layer chromatograms confirmed the presence of DDT and DDE in a composite sample of whole crayfish extracts. Gas chromatography of elutants of the silica gel, taken from areas of the thin-layer chromatogram where other metabolites should be, proved negative. No spot was detected with the same Rf value (0.66) as chat of DDA standards. However, the lowest quantity that could be detected was 2 µg. No attempt was made to elute any DDA from the silica get since this polar compound cannot be detected with electron capture techniques. Similar results were obtained from the thin-layer chromatography of the various organs.

The accumulation of DDT by crayfish during its development from the egg stage through hatching was studied by exposing recently

collected ovigerous females to toxicant delivered by the serial diluter. To increase the measurable DDT in water, a higher concentrate (15 mg DDT/100 ml ethyl alcohol) was injected into the system. Calculated DDT concentrations were 6, 3, 1.5 and 0.75 ppb. The quantities of DDT in the water found by GLC analyses are presented in Table 8. The alcohol was used because of the presence of aquatic fungus in the chambers. It did not alleviate the fungus difficulty, however, and when the water temperature rose a few degrees the chambers required daily cleaning or the system became clogged. When 2 g of fungus from 3 ppb DDT water were anaylzed, 4500 ppb DDT was found.

After 11 days exposure, all 18 ovigerous females in the three highest concentrations were dead and covered with fungus. So that only DDT in the eggs would be measured an attempt was made to remove the fungus before the eggs were analyzed. The results of DDT residue analyses of the eggs are presented in Table 9 and Figure 9. The exposure factor is the product of exposure concentration (ppb) and exposure time in days.

Only one of the 12 crayfish exposed to the lowest concentration (0.75 ppb) died during the 37 day test. Many of the eggs hatched so that there were young crayfish present on exposure day 14. Thirteen young were removed from both the 0.75 ppb DDT concentrations and the control chambers on exposure day 36 and analyzed for DDT residues. These results are included in Table 9.

TABLE 8

Measured DDT (ppb) in the water during exposure of ovigerous crayfish

days exposure	Calculated DDT concentration (ppb) 6	3	1.5	0.75	control
-3*	1.36	0.55	0.27	0.17	NS
0**	1.4	0.93	0.48	0.18	NS
2	2.2	0.70	0.34	0.10	NS
8	2.23	0.38	0.12	0.13	NS
24	NS	NS	NS	0.09	NS
29	NS	NS	NS	0.10	NS
35	NS	NS	NS	0.09	0
36	NS	NS	NS	0.13	0

* 3 days before exposures were started
** chamber cleaned 8 hours previous to sampling
NS no sample collected

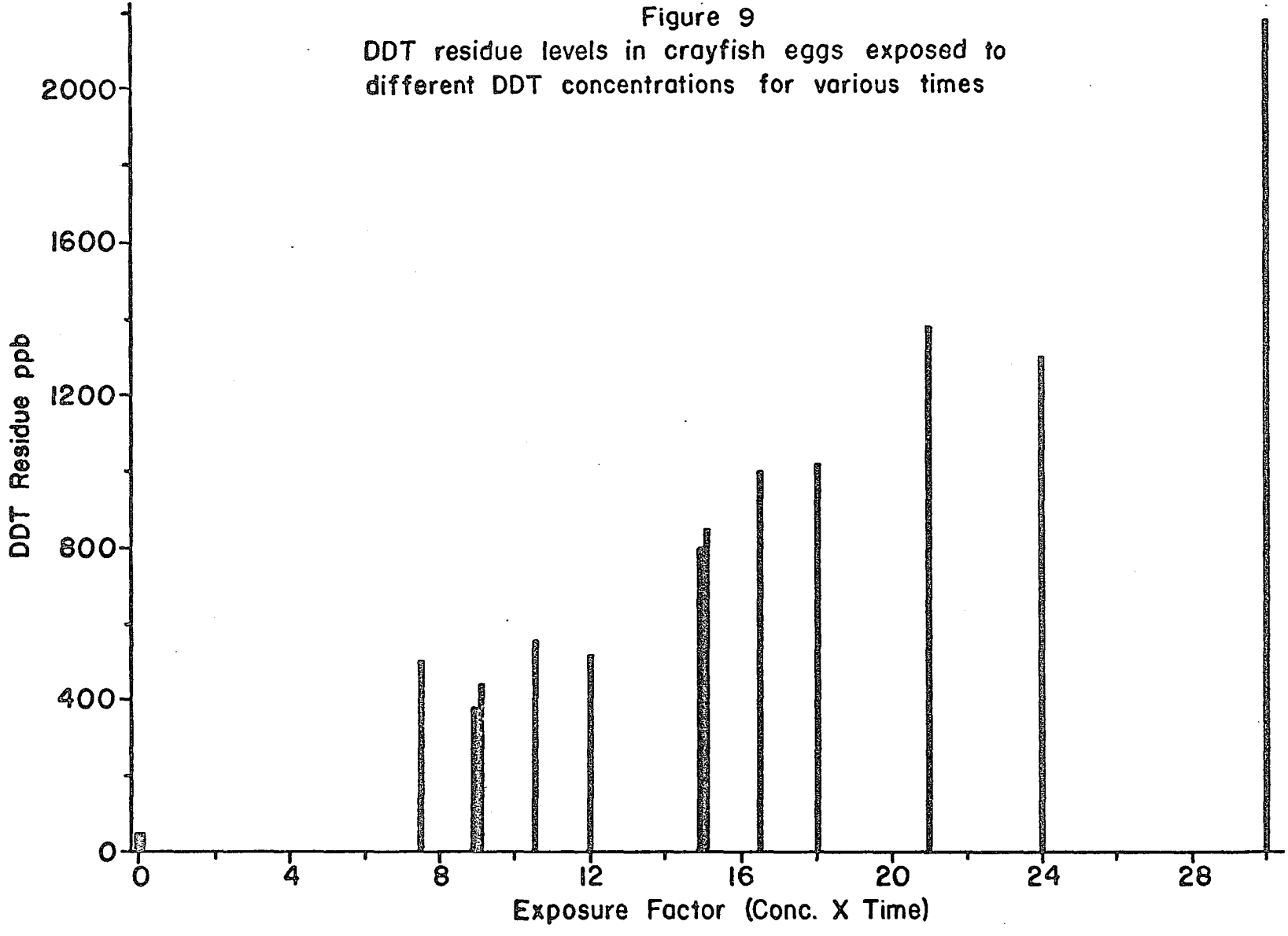
TABLE 9

DDT residues in eggs and young crayfish exposed to four DDT concentrations during their development.

Exposure factor da x conc	Number crayfish sampled	Exposure time (days)	Exposure conc (ppb)	Eggs number	Sample weight g.	Residues DDT (ppb)	Residues DDE (ppb)
0	1	8	C	60	0.17	ND	T
0	1	8	C	68	0.19	53	17
0	1	8	C	57	0.14	57	ND
7.5	3	5	1.5	246	1.45	508	17
9	2	3	3	449	2.86	382	15
9	1	6	1.5	128	0.63	439	T
10.5	1	14	0.75	113	0.68	552	31
12	2	4	3	339	2.0	514	T
15	1	5	3	261	1.84	801	25
15	1	10	1.5	292	1.63	858	44
16.5	1	11	1.5	224	1.46	999	45
21	1	7	3	229	1.44	1374	46
24	1	4	6	290	1.71	1293	T
30	2	5	6	345	2.05	2180	43
Young	11	19	0.75	-	0.17	1265	63
Young	11	19	C	-	0.56	38	23
Young	13	36	0.75	-	0.6	81	19
Young	13	40	C	-	0.2	ND	ND

T-denotes trace amounts
ND-none detectable
C-no DDT added

Figure 9
DDT residue levels in crayfish eggs exposed to different DDT concentrations for various times



Nine of the 12 adult females that were exposed for 37 days to 0.75 ppb DDT were dissected when the experiment was terminated. Many of these animals were about to molt so the new cuticles, as well as the other organs, were removed and extracted for GLC analysis. DDT residues found were: gill, 27 ppb; antennal gland, 0.0; alimentary tract, 37 ppb; muscle, 19 ppb; hepatopancreas, 76 ppb and new cuticle, 36 ppb. Females from higher concentrations were not analyzed for DDT residues.

DISCUSSION

The crayfish Orconectes rusticus used in these experiments is the dominant crayfish species found in the Scioto River watershed and is easily collected throughout the warmer months. Parts of its life cycle have been the topic for several studies (Langlois, 1936, Busch, 1940, and Dudrow, 1964).

Orconectes rusticus was particularly suitable as an animal in which to study DDT accumulation because it was readily available, large in size, adaptable to the laboratory apparatus and sensitive to small quantities of pesticides. With the limited facilities available, it was impossible to rear crayfish from one generation to the next or to maintain large numbers in the laboratory so that animals of similar physiological state would be available at one time for the test exposures. Therefore, differences had to be tolerated in the size, age, physiological state and previous accumulation of DDT of collected crayfish. In spite of these irregularities, some trend in DDT accumulation from continuous exposures to low levels of DDT in the test water should be discernible.

Short term static bioassays were conducted to determine the range for continuous-flow exposures. Comparing the results of the early static bioassays (without emulsifier) with those reported for Procambarus clarkii (Muncy and Oliver, 1963), O. rusticus has a lower

48-hour LC_{50} value of 0.1 - 0.4 ppm. Muncy and Oliver's report of the same 24-, 48-, and 72-hour TL_m value of 0.6 ppm was not explained, but it could have been due to the hydrophobic characteristic of DDT, thus causing adherence to the plastic liner used in their bioassay containers. These authors do suggest that the use of the plastic liner may have increased the TL_m values for DDT up to 0.5 ppm. The application of this factor would make our results similar.

Short-term bioassays of DDT with emulsifier were performed in the continuous-flow apparatus to determine the range of the chronic exposure experiments. From these results, it appears that the 96-hour LC_{50} is between 0.016 and 0.033 ppm. By extrapolating 72-hour results, the 96-hour LC_{50} for static bioassays (DDT + emulsifier) would be greater than 0.049 ppm. It is difficult to compare these flowing water bioassays with the static tests since few test animals were involved and the testing period was different. Other different factors were variable room temperature and photoperiod for the flowing water bioassays.

The variability between the results of the first and second run short-term bioassay tests in continuous-flow conditions could be due to the longer stabilization period before the second run. This probably meant that a greater amount of the DDT going into the chambers was able to come in contact with the crayfish instead of being absorbed on the sides of the chamber. Although the use of an emulsifier had no apparent toxic effect on the crayfish, it could have made the DDT less hydrophobic, thus more available to the crayfish. The lower DDT

concentrations used for the chronic exposures did not require an emulsifier so it did not have to be considered in the ability of the crayfish to accumulate DDT.

The two-, three-, and four-week exposures, providing 70 whole crayfish for analyses and an equal number for organ analyses, were conducted during February and March. Water temperature, $20 \pm 1°C$; flow rate, 15 ml/min.; injected concentrate, 15 mg DDT/320 ml acetone were controlled and room temperature, $21 \pm 2°C$ was stable. Photoperiod was determined by manual control of the lights in the room which did not insure a specific dark period.

Previously attempts had been made to adjust the concentrate injected into the system so that the desired quantity of DDT would be present in the exposure water. Such a level was never achieved according to analysis of exposure water extracts. The losses of DDT which varied between 20 and 80% may be explained in several ways: crystallization of the concentrate at the site of mixing, adsorption to the surfaces of the glass and stainless-steel parts of the apparatus (Bowman et al., 1959), adsorption and uptake by microorganisms and test animals in the system and co-distillation from the water surface.

Weidhass, Bowman and Schmidt (1961) showed the loss of 78% of the DDT from 0.02 ppm aqueous suspensions in 24 hours suggesting that co-distillation is probably the cause of greatest loss. Bowman et al., (1964) reported over 50% loss of DDT from aqueous suspensions of 0.001 - 0.1 ppm in 24 hours. From the above reports, it appears that co-distilled quantities of DDT are not similar for all concentrations and

may be expected that a higher per cent of the DDT would leave the higher concentrated aqueous suspensions. The slight fluctuations in room temperature and barometric pressure are other factors which could influence co-distilled quantities from the exposure chambers. Although the above factors, in part, had an influence on the exposure concentration, they do not explain the large range of DDT residues (Table 7, Figure 8) found in individual crayfish exposed to any fluctuating conditions in the same troughs. Attention then should be given to the other component of the system, the experimental animal.

Physiological variation among individual crayfish used for each exposure is evident as several of the animals molted during the test period. Most decapod Crustacea continue to go through hormonal-induced molting cycles for their entire life, the duration of the intermolt period increasing with age.

Many studies have been conducted to correlate biochemical changes at the premolt stage with the action of the crustacean molting hormone. Changes in oxygen consumption, phases of carbohydrate, lipid and protein metabolism, exoskeleton formation, and water uptake have been followed during the molting cycle (Scheer, 1957 and Passano, 1960). Histochemistry of the integument of intermolt and premolt spiny lobsters (Panulirus argus) has been extensively studied by Travis (1955). During intermolt, reserve cells are predominant in the sub-epidermal connective tissue, and function in the storage of polysaccharides and fats. At the time of premolt, resorption from the old skeleton and building of the new progresses. The reserve cells, filled with break-

down products as well as substances for the new skeleton, are apparent in the connective tissue.

Changes in the cuticle and other body functions during the molting cycle probably could alter the DDT uptake by the organism. Richards and Cutkomp (1946) proposed that chitinous cuticles facilitate the entry of DDT into the animal body. They showed that the insecticide was actually concentrated by adsorption to chitinous cuticles similar to the concentrating action of charcoal. They suggest that DDT utilizes the surfaces of known intermicellar spaces in the chitin. No mention was made of lipid in the epicuticle as a possible concentrating mechanism for DDT. The epicuticular lipid substance is important in determining cuticular permeability (Dennell, 1960). If the lipid content of the epicuticle changes during the molt cycle, some fluctuations of DDT uptake could occur.

Travis (1955) suggests that some of the lipid materials conveyed to the integumental tissues preceding the molt are used in the formation of epicuticle. The occurrence of lipo-protein cells in the blood of the decapod crustacean Carcinus meanas was described by Sewell (1955). These cells show a cyclical activity and congregate directly beneath the epidermis at the time of the new cuticle formation, losing their lipid material to the new cuticle. Although the presence of such lipid-containing cells has not been shown for the crayfish decapods, similar cells have been described for insects (Wigglesworth, 1948).

In several insect studies (Munson and Gottlieb, 1953), susceptibility to DDT varied with the total lipid content of the American cockroach. In crayfish, total lipids are 2.96% of fresh body weight, but

the reliability of this figure depends on the molting stage (Vonk, 1960). Recognizing all of the variability and fluctuations of the DDT concentrations to which the crayfish were exposed, it is understandable that residue levels found should extend over a wide range.

As would be expected, DDE quantities from the analyzed whole crayfish fluctuated also. These quantities were not always directly correlated to DDT residues. Again, such differences could be due to the physiological state of the crayfish. The presence of DDT-dehydrochlorinase in crayfish has not been reported and its absence or availability may be a factor in the crayfish's susceptibility to low concentrations of DDT.

Although DDT metabolites other than DDE were sought, none were found. Since the electron capture GLC analysis is sensitive to tenths of nanogram quantities, the apolar metabolites should have been seen. DDE, DDA, and Kelthane are known metabolites of DDT in some insects and these can be derived directly from DDT (Perry, 1965). The other metabolites thought to be associated with DDT must go through a chemical step with DDD as an intermediate. At this writing there is no report of the DDD intermediate from any insect species.

Bridges, Kallman and Andrews (1963) report the presence of DDD as well as DDE from crayfish collected 3 weeks after treatment of the pond with 0.02 ppm DDT. The residues continued to decrease up to 16 months when the sampling was stopped. Because these crayfish were kept in a natural environment, it is difficult to be sure that the DDD was really metabolized from DDT by the crayfish or whether it was a breakdown

product from some other source. Miskus, Blair and Casida (1965) demonstrated that natural lake waters would convert DDT to DDD. They found up to 95% conversion in lake plankton. Other reports (Kallmann and Andrews, 1963; Barker, Morrison and Whitaker, 1965) provide evidence for the DDT breakdown to DDD by microorganisms.

The fifty hexane extracts of whole crayfish exposed to DDT were combined for thin-layer chromatography. The previous GLC analysis of these samples showed that there would be 15.6 μg DDT and 2.2 μg DDE quantities which would be visible by TLC techniques. Unfortunately, many interferring substances inhibited the flow of the spotted residue and most of the sample moved only as far as the DDD area. The eluted material from this area was respotted on another plate, but the DDE that was left was not sufficient to be seen with the chromagenic agent. It is understandable then that other metabolites present in even lower quantities than DDE could not be observed with thin-layer chromatography. Composite samples representing larger numbers of crayfish exposed to DDT and cleaned up by column chromatography might provide enough metabolite for identification by other analytical techniques. Metabolites might also be discovered by autoradiography of thin-layer chromatograms of crayfish exposed to radioactive DDT.

Analysis of the various organs for DDT and its apolar metabolites show that the greatest concentration of DDT and DDE is to be found in the ovary. These animals exposed in February had ova developing, and in some cases, they occupied a large portion of the cephalothorax. The presence of large quantities of yolk material, high in lipid content,

probably is the reason for the large DDT concentrations found in this organ. The testes were not dissected because they became very soft as the crayfish thawed. Therefore, no comparison of DDT residues in the gonad is possible between the sexes.

The hepatopancreas is another expected site of DDT concentration. Its recognized role in most of the metabolic activity would suggest that dehydrochlorination might occur there. McWhinnie and Kirchenberg (1962) showed that the respiratory rate of O. virilis hepatopancreas tissue was dependent upon the stage of the intermolt cycle. Respiration was lowest during intermolt and highest early in postmolt. These authors even suggest a shift of an intermediate pathway in carbohydrate metabolism during the premolt period, so it is evident that the hepatopancreas is undergoing continuous biochemical changes which might influence DDT uptake during the molt cycle. Lipids in the hepatopancreas are almost depleted for a short time after the molt and gradually build up to 14.25% of fresh body weight during the intermolt period (Vonk, 1960). Often during the intermolt stage, lipid, in the form of droplets, is very abundant throughout the epithelial tissues. During the premolt stage, the hepatopancreas becomes an important storage organ for fat, glycogen and calcium reserves (Travis, 1955). If the concentration of lipid in any organ is correlated to DDT concentration, the DDT residues in the hepatopancreas could be expected to fluctuate according to the particular molt stage. Comparatively large and similar quantities of DDT and DDE were found in the four replicates of control hepatopancreas suggesting that DDT residue levels reach a stabilization concentration which is not greatly affected by molting.

The relatively large quantities found in the antennal gland could suggest that the organ acts as a concentrating area for subsequent excretion. However, the total quantity of DDT found in the antennal gland was usually much less than that found in the other organs. Because of their light weight (0.03-0.06 g), the computation of DDT residues in antennal glands is complicated by the possibility of magnifying any error in analytical procedure.

Variability among DDT residues in alimentary tracts could be due to the weight added to the tissue by various gastrolith sizes. In O. virilis gastrolith formation is dependent on animal size, duration of premolt stage and temperature (McWhinnie, 1962). DDT residues resulting from ingestion could come about by adsorption of DDT to the food while it remained on the bottom of the exposure chambers for 24 hours. The relatively high analysis of 111.3 ppb DDT in 2-week control animals is the result of the inclusion of one unusually high replicate.

Gill tissue showed residue levels which were correlated to the exposure concentration. This structure provides a large surface area for possible adsorption and would seem an ideal entrance site. Ventilation volumes vary considerably, but Wolvekamp and Waterman (1960) stated rates of 0.2-0.8 liters per hour for Astacus astacus. If such rates are considered for Orconectes rusticus, the flow rate in the exposure chambers, which would pass over the crayfish, is similar to the respiratory volume. Lack of large quantities of DDT in the gill may also have been due to its little mass as the structure is composed almost entirely of spaces filled with blood. Differences between the

cuticle of the gills and that of the rest of the exoskeleton have not been described; therefore, presence of an epicuticle and its lipid constituency is not known.

The abdominal muscle mass provided 1.5-2.5 g of tissue and shows residue levels which correlate closely with exposure concentration. Of the organs analyzed, the muscle probably undergoes the least biochemical changes during the molting cycle. Like the hepatopancreas, the four replicate control muscle samples have very similar residue levels (14.9 $\pm$ 1 ppb DDT and 6.4 $\pm$ 1 ppb DDE). From the consistency observed here, the crayfish abdominal muscle might make an ideal monitor for DDT in natural waters.

From the results given in Table 6, it is evident that residue levels did not often increase proportionately with increase in exposure time. It appears that for a given continuous exposure concentration, DDT levels are stabilized within 2 weeks and do not increase with longer exposure periods up to 4 weeks. How much the residue levels would decrease when exposure concentration was decreased would be of ecological significance and would determine how much influence the fluctuating exposures had on residue levels.

The effect of continuous exposures on the accumulation of DDT by various stages in the life cycle of the crayfish is of interest in considering the ecological implications of DDT in natural waters. Female O. rusticus were collected soon after they had released their eggs, brought to the laboratory and acclimated for 48 hours, and placed in the exposure chambers.

Normally this species mates in the fall and the female retains the sperm over winter. Egg laying occurs from late April through early June. Development of the young crayfish usually takes about 18 days (Dudrow, 1964) which was similar to the time observed in this laboratory study. Upon hatching the young crayfish remains attached to the mother by a telson thread and chaelae closed on the egg stalk until the first postembryonic molt. After the second post-embryonic molt, the young move freely about the parent and begin active feeding. At this stage, about 11 days after hatching, the cephalothorax length is approximately 4.5 mm. During the first growing season O. rusticus goes through at least 9 or as many as 13 molts and attains a mean cephalothorax length of 28 mm (Dudrow, 1964).

Before these exposures were started, it was necessary to move the dilution apparatus to another room. The temperature in the new location fluctuated greatly and the water temperature rose to 23°C on some occasions. An aquatic fungus became prominent in the chambers and undoubtedly placed the ovigerous crayfish under a stress. Because of these adverse conditions, including the presence of DDT, all of the crayfish died that were in the higher concentrations before any of their eggs hatched.

The eggs from females which were in the same DDT concentration and had died the same day were combined. Since there were only a few samples that represented a given exposure concentration and time period, an exposure factor was calculated in order to compare all of the egg samples. The graphed results (Figure 9) indicate a gradual increase

in residue level with exposure factor. Such an increase might be expected of a material which adsorbed DDT and thus was dependent on time and concentration. Unfortunately, not enough data are available to determine the carry-over of DDT from egg to young. There was a decrease of DDT in the young crayfish from the day of hatching until 17 days later when the experiment was terminated. The relatively small quantities of DDE present indicate a reduced DDT metabolism by the developing crayfish compared to adults.

Results from this study show that crayfish, during all periods in their life cycle, are able to accumulate large quantities of DDT from low level aqueous concentrations.

SUMMARY

1. Static and flowing water bioassay experiments of DDT on the crayfish Orconectes rusticus (Girard) showed that DDT is lethal to most crayfish at concentrations above 0.03 ppm.

2. A serial dilution apparatus was used to deliver various DDT concentrations continuously to chambers in which crayfish were kept for 2, 3, and 4 weeks.

3. Tests conducted to determine the reliability of the serial diluter showed losses of 20-80% from known DDT concentrations.

4. Seventy individual crayfish exposed for 2 and 3 weeks to 0.0, 0.025, 0.05, 0.1 and 0.2 ppb DDT were quantitatively analyzed for DDT and DDT metabolites by electron capture gas-liquid chromatography and thin-layer chromatography.

5. DDT residues were found in gills, muscle, antennal gland, alimentary tract, hepatopancreas and ovary from 70 crayfish exposed to 0.0, 0.025, 0.05, 0.1 and 0.2 ppb DDT.

6. A slight increase in residue level was correlated to increased exposure time and DDT concentration.

7. Hepatopancreas, ovary and antennal gland were the organs in which the highest amounts of DDT and its metabolite were found.

8. Several tests were made for DDT metabolites, but only DDE was found.

9. Ovigerous females exposed to 0.75, 1.5, 3.0, and 6.0 ppb DDT in the serial dilution apparatus provided evidence of DDT accumulation by adsorption during the embryonic development of the crayfish.

10. The variable results are discussed as a function of the physiological differences between individual crayfish in different molting stages and the fluctuations of the exposure system.

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