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FALL 1977

MASTER OF

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STREUFERT,

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THE GRADUATE SCHOOL

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WE HEREBY RECOMMEND THAT THE THESIS BY

Jonathan Mark Streufert

ENTITLED SOME EFFECTS OF TWO PHTHALIC ACID ESTERS ON THE LIFE CYCLE OF THE MIDGE (Chironomus plumosus)

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF Master of Science

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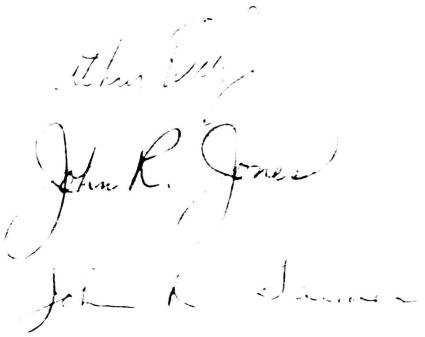
SOME EFFECTS OF TWO PHTHALIC ACID ESTERS ON THE LIFE CYCLE OF THE MIDGE (Chironomus plumosus)

presented by Jonathan Mark Streufert

a candidate for the degree of Master of Science

and hereby certify that in their opinion it is worthy of acceptance.



SOME EFFECTS OF TWO PHTHALIC ACID ESTERS ON THE LIFE CYCLE OF THE MIDGE (*Chironomus plumosus*)

A Thesis

Presented to

The Faculty of the Graduate School

University of Missouri - Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Jonathan M. Streufert

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John R. Jones Thesis Supervisor

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INTRODUCTION

Phthalic acid esters (PAEs) are organic chemicals that have caused environmental concern because of the variety of their application and annual production rates (Great Lakes Water Quality Board 1975). PAEs are the esters of the ortho form of benzenedicarboxylic acid. About 95% of the PAEs produced are used as plasticizers, primarily of polyvinylchloride (U.S. Tariff Commission 1974). As such, they lend flexibility and extensibility to the original resin, which may contain up to 60 parts per hundred of these PAEs in the final formulation (Nematollahi et al. 1967). Two PAE plasticizers of considerable interest are di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DBP). DEHP has been used as an orchard acaricide, while DBP has been used as an insect repellent (Farm Chemicals 1977) and in pesticide formulations to retard volatization (Brooks and Schoof 1964). Total phthalate anhydride ester production in the United States for 1972 was 519.8 x 10^6 kg of which DEHP accounted for 197.5 x 10^6 kg and DBP amounted to 13.2 x 10^6 kg (U.S. Tariff Commission 1974). Projected annual PAE production for 1981 is 705 x 10^6 kg (Chemical and Engineering News 1976).

Phthalate esters have been reported by several sources to occur in many segments of the environment. PAE residues have been detected in fish, water, and sediments (Table 1).

Table 1. Locations and types of PAE contaminated samples.

Location	Sample Type	DBP (ng/g)	DEHP (ng/g)	PAE (ng/g)	Source
Mississippi River, mouth	Water	-	600	-	Corcoran 1973
Gulf of Mexico	Water	-	Detectable	-	Ibid.
Great Lakes Area	Waste Treatment Effluent	-	-	1-1200	Gt. Lks. Water Qual. Bd. 1975
	Sewage	-	884,000	-	Ibid.
	Fishes	-	-	0-1300	Ibid.
Black Bay, L. Superior Ontario	Water	-	300	-	Mayer et al. 1972
	Sediment	100	200	-	Ibid.
	Walleye	-	800	-	Ibid.
Missouri River, McBaine, Missouri	Water	0.09	4.9	-	Ibid.
Mississippi and Arkansas (Indust. and Agri. area)	Channel Catfish	-	3200	-	Ibid.
Lake Huron, Michigan	Water	-	5.0	-	Ibid.
Iowa Fish Hatchery	Channel Catfish	200	400	-	Ibid.
	Dragonfly Naiad	200	200	-	Ibid.
	Tadpoles	500	300	-	Ibid.
---	Commercial Fish Food	-	2000-7000	-	Ibid.
Charles River, Massachusetts	Water	-	-	1.9	Hites 1973
Tama River, Japan	Water	3.14	4.4	-	Morita et al. 1974
	Sediment	350	-	-	Ibid.

Streams and effluents in the Great Lakes Region had PAE concentrations ranging from 1 to 1200 µg/l, while sewage sludge and fishes contained up to 884,000 and 1300 ng/g (dry wt), respectively (Great Lakes Water Quality Board 1975). Mayer et al. (1972) found DEHP concentrations of 300 µg/l in water, 3200 ng/g in fishes, 200 ng/g in invertebrates, and 200 ng/g in sediment. DBP concentrations as high as 100 ng/g were found in sediment and up to 500 ng/g in tadpoles.

Sources of PAEs are most likely municipal and industrial effluents (Hites 1973; Lake Michigan Toxic Substances Committee 1974). Monitoring surveys by several agencies in the Great Lakes states showed that effluents of industrial and municipal waste treatment facilities contained PAEs in concentrations ranging from less than 1 to 1200 µg/l and tributaries to Lake Michigan contained 1 µg/l or less (Great Lakes Water Quality Board 1975). The fate of PAEs discharged into these tributaries is not well defined, but analyses of settleable solids showed residues ranging from 1 to 75 µg/g (dry wt), which suggests that PAEs may be adsorbed to particulate matter in streams and ultimately deposited in bottom sediments.

Data on toxicity of PAEs to aquatic organisms are limited, but there is some indication that acute toxicity of DEHP and DBP is low. The 96-h LC50s of DBP to two invertebrates and four fishes ranged from 0.73 mg/l for the bluegill (Lepomis macrochirus) to > 10.0 mg/l for the crayfish (Orconectes nais). The 96-h LC50 for DEHP was above 10.0

mg/l for all organisms tested (Mayer and Sanders 1973). Less information is available concerning chronic effects of DEHP and DBP on aquatic organisms. Mayer and Sanders (1973) reported that dietary exposure of zebrafish (Brachydanio rerio) to DEHP could reduce fry survival. These authors also noted that a DEHP concentration of 3 µg/l caused a 60% decrease in the production of Daphnia magna.

Direct effects of DEHP on aquatic organisms are not the only interaction to be considered. An aquatic contaminant accumulated by organisms near the base of the food chain may indirectly affect organisms higher in the food chain. Metcalf et al. (1973) found that invertebrates accumulated DEHP several thousand times that of the water concentration to which they were exposed. Mayer and Sanders (1973) also observed rapid initial uptake of DEHP and DBP by aquatic invertebrates, but noted that PAE residues were lost rapidly when the organisms were transferred to uncontaminated water.

The lack of aquatic toxicity data on PAEs, their possible reproductive impairment of certain aquatic species, and their accumulation by other species indicates a need for further examination of their effects on aquatic organisms. One organism important in the diets of many fishes is the aquatic larva of the midge, Chironomus plumosus. The objectives of this study were to determine the acute toxicities of DEHP and DBP to midge larvae; to determine the effects of these chemicals on midge emergence and reproduction; to determine the potential of the larvae to accumulate these PAEs; and to develop a method for using hydrosoil in the

chronic exposure of benthic macroinvertebrates to aquatic contaminants. Because PAEs have been found at relatively high concentrations in hydrosoil, it was desirable to examine the effect of sand and hydrosoil substrates on the interactions between the PAEs and midges.

MATERIALS AND METHODS

Test organism and water quality

Larvae and egg cases of the midge Chironomus plumosus were obtained from cultures maintained at the CNFRL, Columbia, Mo. Rearing techniques described by Biever (1965) were used to maintain a continuously reproducing population of midges. Water used for cultures and all toxicity tests was from a deep well. The water had a pH of 7.5, a hardness of 272 mg/l as $CaCO_3$ and alkalinity of 237 mg/l. Other chemical characteristics of this water have been summarized by Mayer et al. (1975). Dissolved oxygen (DO) concentrations of inflow water were measured weekly and averaged 8.5 mg/l; DO levels in chronic test chambers were maintained above 2.0 mg/l. All toxicity tests were conducted at 22 $\pm$ 1 C in a photoperiod controlled for 16 h light and 8 h dark.

Chemicals tested

The following phthalate compounds and metabolites were tested: DEHP, DBP, mono-2-ethylhexyl phthalate (MEHP), phthalic acid (PA), and 2-ethylhexanol (2-EH). DEHP, 2-EH, and PA were provided by Monsanto Chemical Co., St. Louis, Missouri. DBP was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. The ^{14}C-ring-labelled DEHP (specific activity 10.52 mCi/mM = 55 dpm/ng) used in residue dynamic studies was purchased from Pathfinder Laboratories Inc.,

St. Louis, Missouri. DDT [1,1,1,-trichloro-2,2-bis(p-chlorophenyl)-ethane] was from City Chemical Corp., New York, N.Y., and it was used as a base line chemical for both acute and chronic tests. Purities of all chemicals were over 90%.

Ethanol and acetone were used as solvents to increase the solubility of the chemicals. Solvent concentrations never exceeded 1.8 ml/l in acute tests, a level which did not affect larval survival, and 0.12 ml/l in chronic exposures. Solvent limits suggested by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975) are 0.5 ml/l for acute and 0.1 ml/l for flow-through tests. It was necessary to exceed these recommendations somewhat due to the insolubility of the PAEs tested.

Hydrosoil

Hydrosoil used as larval substrate in the chronic tests and in the ^{14}C-DEHP uptake tests was taken from Little Dixie Reservoir, Callaway County, Mo. This is a typical Midwestern hardwater reservoir similar in water chemistry to those described by Jones (1977). The hydrosoil was obtained from a depth of 1.0 to 1.5 m with an Ekman dredge and only the top three cm of each sample were used. The sediment was oven-dried at 37 C and ground to a powder.

Hydrosoil used in initial range-finding tests was taken from 0.1 ha ponds at the CNFRL and prepared in the manner described.

Eight soil samples from Little Dixie Reservoir and

three from the CNFRL ponds were analyzed for organic matter, pH, Ca, and other chemical parameters relevant to the interactions between midge larvae, the PAEs, and the substrate. The results of these analyses are presented in Table 2. Soil chemistry tests were conducted by the School of Agronomy in the College of Agriculture, University of Missouri - Columbia, according to methods described by Brown et al. (1977). The chemical parameters of the Little Dixie hydrosoil indicate a close similarity to and derivation from the surface soils of surrounding farms in the reservoir's watershed (Brown, personal communication). The cation exchange capacity (CEC) of this soil is intermediate between the CECs of kaolinite and montmorillonite clays (Weber and Coble 1968).

Acute toxicity tests

Acute toxicity tests were conducted according to procedures recommended by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). In static tests 10 late-third and early-fourth instar larvae were exposed to concentrations of phthalate compounds for 48 h in 250 ml of solution in glass jars. The dead and affected larvae were recorded at 24 and 48 h. Defining larval mortality was difficult and death was recorded when larvae became turgid, turned pale, or no longer responded to touch. Other investigators have used similar criteria (Augenfeld 1967; Karnak and Collins 1974). Affected larvae were defined as those unable to make coordinated swimming motions when touched

Table 2. Chemical characteristics of hydrosoils used in chronic and uptake studies.

Soil Source	n	kg P_2O_5/ha P-I	kg P_2O_5/ha P-II	OM* (%)	pH_w	me H^+ /100g	me Ca /100g	me Mg /100g	me K /100g	me Na /100g
Little Dixie Reservoir	8	18.6** (4.14)	178 (21)	1.6 (0.14)	7.3 (0.04)	0.2 (0.14)	11.3 (0.74)	1.9 (0.14)	0.54 (0.03)	0.15 (0.01)
CNFRL Ponds	4	63.9 (1.65)	183 (8)	3.0 (0.10)	7.0 (0.05)	1.5 (0.30)	15.1 (0.25)	2.6 (0.05)	0.44 (0.01)	0.22 (0.01)

*Organic matter (dry weight basis).

**$\bar{x} \pm$ SE.

(Sanders, personal communication). Mortality data were plotted on log-arithmetic probability paper and the 48-h EC50s, LC50s, and 95% confidence limits were calculated by the Litchfield and Wilcoxen (1949) method. EC50s are those concentrations which immobilize or affect 50% of the organisms, while LC50s are those concentrations lethal to 50% of the organisms.

Second instar (96 h old) larvae were also exposed in static tests with DEHP and DBP to determine if toxicities of the chemicals changed with age of the larvae. These tests were conducted as described above, except that exposure was in 10 ml of solution in the cells of commercial jelly trays.

Acute tests were also performed with the base line chemical DDT.

Chronic tests

The methods for chronically exposing midge larvae to phthalate compounds followed the procedures recommended by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). Chronic tests were conducted by exposing 100 first instar larvae to the chemicals in flow-through systems modified after Mount and Brungs (1967) and Chandler et al. (1974). Exposure chambers measured 10 x 20 x 20 cm with screened drain pipes adjusted so that each chamber contained 2 l of solution. The containers were made from glass cemented together with Dow-Corning 781 building sealant. Chemical stock solutions in stock bottles drained approximately 1 ml of solution into mixing chambers. The

dilution apparatus delivered an additional 9 ml of well water to each mixing chamber. The resulting 1:10 diluted solutions were delivered to the chambers every 7.5 min, which resulted in a turnover rate of approximately one volume per day.

Solvent concentrations in control chambers equalled the highest solvent concentration used in test chambers. DO levels were monitored weekly during the tests to determine proper feeding rates. The larvae were fed 0.12 g of Hartz Mountain Dog Kisses® every four days for the first 12 days and 0.15 g every four days thereafter up through day 24. Substrate for the larvae was either 200 g (dry wt) of hydrosoil from Little Dixie Reservoir or 200 g of sand.

Effects of DEHP and DBP on midge emergence

Effects of the PAEs on the emergence of adult midges were determined by exposing 100 first instar larvae to selected concentrations of DEHP and DBP. Pupation and emergence started about day 15 and continued for 20 to 25 days. Tests were terminated when no exuviae were present in any of the eight exposure chambers for two consecutive days following the onset of emergence. The exuviae were removed and recorded daily. The effects of PAEs on midge emergence were determined by conducting analyses of variance on the arcsin transformation for proportions (angle = arcsin $\sqrt{\text{proportion}}$) followed by a least significant difference test (Snedecor and Cochran 1974). Significance in these analyses and throughout this study were taken at the $P < 0.05$ level.

As in the acute studies, base line tests were performed using DDT.

Effects of DEHP on midge reproduction

Effects of DEHP on the reproductive aspects of the midge life cycle were examined to determine if the chemical affected either the hatchability of eggs produced by PAE-exposed adults or the average number of eggs in each egg case. Screened covers, consisting of two 7 x 22 cm pieces of glass connected by a 8 x 22 cm piece of netting, were placed over exposure chambers at day 16. They allowed for DO determinations and food introduction at appropriate intervals. Egg cases were laid by emerging adults that had been exposed to DEHP solutions. The egg cases were removed daily and placed in separate exposure containers. Within six days all viable eggs had hatched. It was not determined whether the failure of eggs to hatch was due to non-mating or to a chemical effect. Some virgin female midges raised alone laid normal appearing egg cases, a fact also noted by Hilsenhoff (1965). Only those egg cases which hatched were used for calculating hatchabilities and average numbers of eggs per egg case. After six days the unhatched eggs were counted.

Larvae hatching from the tests were further exposed to DEHP. The experimental design was previously described and only the emergence effects were investigated.

Determination of chronic concentrations

The chemical concentration delivered to each chamber in

chronic tests was calculated from the amounts of stock solution and water delivered within three day time intervals, from day 0 to day 3, then for day 2 to day 5, and so on until the last day of the test. The calculations were performed using the formula:

$$\text{Concentration} = \frac{(\text{Liters of stock delivered})\ (\text{Stock Conc.})}{(\text{Liters stock delivered}) + (T)\ (K_{cell})}$$

where T is the number of times the diluter delivered water to the mixing chamber and K_{cell} is the average number of milliliters delivered to a particular mixing chamber. The resulting concentrations for a chamber were averaged to obtain calculated toxicant and solvent concentrations. Toxicant concentrations were measured once during the DEHP and DDT chronic tests, as recommended by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975).

In the DEHP tests triplicate 40 ml aliquots from the highest PAE concentration chamber were extracted according to the method of Johnson (1977). Triplicate 10 ml aliquots of methylene chloride were used to extract the DEHP from the 40 ml. The methylene chloride was evaporated to 3 to 5 ml, 5 ml of isooctane added, and this was then evaporated to about 4 ml. The isooctane was brought up to 5 or 10 ml by the addition of petroleum ether. DDT measurements were taken from one high, one medium, and one low concentration test chamber and extracted in the manner previously described.

Gas chromatograph analyses were performed using a Packard 803 oven. Column length was 180 cm with an internal diameter of 2 mm. It was packed with Chromasorb W-HP 80 to

100 mesh Corning glass beads coated with 3% OV-7. Column temperature was 260 C at the inlet, 280 C at the outlet, and 235 C at the detector. The detector was a ^{63}Ni electron capture cell. The flow rate of the N_2 carrier gas was 30 ml/min. DEHP's retention time was about 27 min at a chart speed of 8 mm/min, and its detection limit was 100 ng/l (Whitener, personal communication). Concentrations were determined by comparison against known standards.

By correcting for extraction efficiency the concentrations in the 40 ml aliquots could be derived. Dividing the average measured concentration by the calculated concentration for the preceding three day period, correction factors (CFs) were obtained (Table 3). The CF for a particular test was then used to adjust downward the calculated values.

Extraction efficiencies were determined using radiolabelled DEHP and DDT. Spiked samples were extracted according to the aforementioned method but using toluene instead of isooctane. Radiometric analyses determined an extraction efficiency of 0.72 for DEHP and 0.94 for DDT.

Extraction of DEHP from hydrosoil

DEHP extractions were performed according to the method of Hesselberg and Johnson (1972) on triplicate 20 g samples of wet hydrosoil which had been decanted for 30 minutes. Eighty grams of Na_2SO_4 were thoroughly mixed with each sample and packed into an extraction column. Four 25 ml aliquots of diethyl ether were used to extract the DEHP from the hydrosoil. After evaporation of the diethyl

Table 3. Correction factors used to adjust calculated concentrations downward in DEHP and DDT chronic tests.

Compound	Substrate	Calculated Concentration (µg/l)	Measured Concentration (µg/l)	Correction Factor
DEHP	Hydrosoil	645	245	0.38
DEHP	Sand	725	522	0.72
DDT	Hydrosoil	5.8	0.16	0.027
DDT	Hydrosoil	10	0.41	0.041
DDT	Hydrosoil	20	0.54	0.027
DDT	Sand	5.1	0.39	0.08
DDT	Sand	11	1.44	0.13
DDT	Sand	21	2.10	0.10

ether to 2 ml, 4 ml of cyclohexane was added. This was further evaporated to 2 ml and rinsed with 2 ml of diethyl ether into an extraction column containing 5 g of Florisil. A 40 ml aliquot of 10% diethyl ether in petroleum ether was poured into the column. The first 15 ml of eluent were discarded. A second 40 ml aliquot of 20% diethyl ether in petroleum ether was added and collected with remaining 25 ml from the preceding aliquot. This was then evaporated to 4 ml and rinsed into a graduated centrifuge tube. Adjustment of the eluent to an appropriate volume with petroleum ether was followed by gas chromatographic analysis. Extraction efficiency of three spiked soil samples averaged 0.94.

DEHP uptake and elimination by midge larvae

The study of midge larvae uptake and elimination of ^{14}C-DEHP was conducted in a flow-through system after the method of Mount and Brungs (1967). Third instar larvae were placed in 12 x 12 x 15 cm screened containers which in turn were suspended in a 30 x 30 x 60 cm chamber containing 40 l of well water. ^{14}C-ring-labelled DEHP at a concentration of 202 ng/l (11,110 dpm/l) was delivered to the chamber at a rate of three chamber volumes per day. Half of the larvae were exposed up to nine days. For elimination studies, the remaining larvae were removed after four days of exposure and placed into a similar system in which only fresh well water was delivered. Triplicate 50 $\pm$ 7 mg samples of larvae were taken at hours 0, 1, 3, 7, and 24 of exposure for both the uptake and the elimination studies.

Samples were taken every 24 hours thereafter, up to nine days in the uptake study and for five days in the elimination study. The larvae were homogenated on a Sorvall omni-mixer tissue grinder, and mixed with 15 ml of a scintillation cocktail consisting of 6 ml of Triton-X 100®:toluene (2/3 v:v) and 9 ml of a toluene-fluor mixture (Rodgers and Stalling 1972; Sanders and Chandler 1972).

Duplicate 500 ml water samples were taken every other day from the exposure chamber. Radiometric determinations of the ^{14}C-DEHP content of tissue and water samples were made on a Beckman 230 scintillation counter. Extraction efficiency of the 500 ml water samples averaged 0.85.

Larval uptake of DEHP in hydrosoil versus sand

To determine whether the hydrosoil or sand had an effect on the accumulation of DEHP by midge larvae, an eight day uptake experiment was carried out in the flow-through system described in the Uptake-Elimination section. Larvae were placed into each of the four screened containers, two containing 200 g (dry wt) of the hydrosoil used in chronic tests and the other two 200 g of sand. Two tenths of a gram of Dog Kisses® were added to each container. Within 24 h the larvae had constructed dwelling tubes and disappeared from view. The addition of ^{14}C-DEHP stock started at this time. Twenty four hours later the water contained 293 ng DEHP/l. Duplicate water samples taken every other day averaged 267 ng DEHP/l. Four days after the exposure had begun triplicate 50 mg samples of larvae were taken from one

hydrosoil and one sand container. Four days later samples were taken from the two remaining containers. The amount of ^{14}C-DEHP in the tissue samples was determined radiometrically.

Quench curve

A quench curve was made by homogenating triplicate 0, 20, 40, 50, 60, 80, and 100 mg samples of larval tissues with 15 ml of the scintillation cocktail. One ml of ^{14}C-DEHP (14 ng/ml = 780 dpm/ml) in acetone and PBBO was added to each homogenate in scintillation vials. The samples were counted, corrected for background, and averaged. The quench correction factor for any particular larval weight was obtained by the formula:

$$CF = \frac{(\bar{x} \text{ counts for the 0 mg weight class})}{(\bar{x} \text{ counts for the x mg weight class})}$$

For the 50 mg samples this correction factor was 0.95.

RESULTS

Acute toxicity tests

Acute toxicities of DEHP and DBP to midge larvae were low compared with that of the organochlorine DDT. The 48-h EC50 of DBP for the larvae was 0.76 mg/l, while for DDT it was 0.023 mg/l, a 33-fold difference in toxicity (Table 4). The 48-h LC50 of DBP was 5.4 mg/l, compared to the 48-h LC50 of 0.1 mg/l for DDT (Table 5). Both the 48-h EC50 and the LC50 for DEHP were greater than 18 mg/l. This value is well above the solubility of DEHP in water.

To determine any change in the PAEs' toxicity to different life stages of the midge, second instar (96 h old) larvae were also tested, in addition to late third and early fourth instar larvae. DBP's 48-h LC50 for second instar larvae was 4.0 mg/l, a value not significantly ($P < 0.05$) different from that of the later instars, while DEHP's 48-h LC50 was greater than 18 mg/l.

The toxicities of MEHP and PA were greater than 72 mg/l while 2-EH had a 48-h EC50 of 34 mg/l and a 48-h LC50 of 56 mg/l.

Effects of DEHP and DBP on midge emergence

Chronic exposure of midge larvae to DBP and DEHP using hydrosoil as a substrate did not significantly ($P < 0.05$) reduce adult emergence. DBP was tested at concentrations

Table 4. Acute toxicities of PAEs, metabolites of PAEs, and DDT to midge (Chironomus plumosus) larvae.

Compound	48-h EC50 and 95% Confidence Interval (mg.l)	Slope
DDT	0.023 (0.019-0.028)	1.37
DBP	0.76 (0.52-1.10)	1.46
DEHP	> 18	-
2-EH	34 (28-41)	1.25
MEHP	> 72	-
PA	> 72	-

Table 5. Acute toxicities of PAEs, metabolites of PAEs, and DDT to midge (Chironomus plumosus) larvae.

Compound		48-h LC50 and 95% Confidence Interval (mg/l)	Slope
DDT		0.100 (0.065-0.152)	2.31
DBP	2nd instars	4.0 (3.0-5.4)	2.60
	3rd-4th instars	5.4 (3.8-7.5)	1.40
DEHP	2nd instars	> 18	-
	3rd-4th instars	> 18	-
2-EH		56 (42-75)	1.77
MEHP		> 72	-
PA		> 72	-

up to 695 µg/l for 40 days, while DEHP was tested up to 240 µg/l (Tables 6 and 7).

Midge larvae were also exposed to concentrations of DEHP using sand as a substrate. DEHP concentrations as high as 362 µg/l produced no significant ($P < 0.05$) reduction in midge emergence within the first generation (Table 8), and concentrations up to 552 µg/l did not affect emergence during the second generation (Table 9).

Effects of DEHP on midge reproduction

Studies using both sand and hydrosoil substrates showed DEHP had no effect on midge reproduction. DEHP concentrations up to 193 µg/l in the hydrosoil substrate test affected neither egg hatchability nor average numbers of eggs per egg case when compared with reproductive parameters from control midges (Table 10). Likewise, neither reproductive parameter was affected by DEHP concentrations up to 362 µg/l when sand was used as substrate (Table 11).

Effects of DDT on midge emergence

Chronic studies were conducted with DDT to determine the effect of this chemical on midge emergence using different substrates. Larvae in hydrosoil were exposed to DDT concentrations in water of 0.15, 0.30, and 0.65 µg/l (Table 12). Larvae in sand were exposed to DDT concentrations of 0.44, 1.4, and 2.0 µg/l (Table 13). Calculated concentrations for the two tests were similar but, due to the hydrophobic nature of DDT, water concentrations were 2.7 to 4.1% of

Table 6. Cumulative percentages of midges (Chironomus plumosus) emerging after continuous exposure of the larvae to DBP using hydrosoil substrate.

Days of Exposure	DBP (µg/l)*			
	0	274	465	695
20	16**	8	20	16
25	41**	30	55	37
30	60**	62	76	62
35	72**	72	82	80
40	85**	81	88	85

*Calculated water concentrations.

**Mean of two controls.

Table 7. Cumulative percentages of midges (Chironomus plumosus) emerging after continuous exposure of the larvae to DEHP using hydrosoil substrate.

Days of Exposure	DEHP (µg/l)*			
	0	109	196	240
20	16**	4	13	13
25	41**	25	36	45
30	60**	50	57	53
35	72**	63	70	65
40	85**	67	76	71

*Calculated water concentrations adjusted downward by a correction factor of 0.38.

**Mean of two controls.

Table 8. Cumulative percentages of midges (Chironomus plumosus) emerging after continuous exposure of the larvae to DEHP using sand substrate. Generation 1.

Days of Exposure	DEHP (µg/l)*							
	0	0	138	138	199	199	362	362
20	34	48	40	49	49	41	45	44
25	68	68	74	72	72	61	69	74
30	75	77	78	81	83	67	70	80
35	76	79	79	82	83	67	75	80

*Calculated water concentrations adjusted downward by a correction factor of 0.72.

Table 9. Cumulative percentages of midges (Chironomus plumosus) emerging after continuous exposure of the larvae to DEHP using sand substrate. Generation 2.

Days of Exposure	DEHP (µg/l)*							
	0	0	169	169	296	296	552	552
20	18	4	9	20	18	6	1	9
25	58	37	43	46	54	49	30	46
30	79	65	63	55	79	72	64	69
35	89	78	72	60	82	82	77	76
40	93	81	78	68	84	85	84	81

*Calculated water concentrations adjusted downward by a correction factor of 0.72.

Table 10. Reproductive parameters from DEHP exposed midges (*Chironomus plumosus*) using hydrosoil substrate.

DEHP Conc. (µg/l)*	Egg Case Hatchability (%)	Average No. of eggs/egg case	No. of egg cases
0	91.5 (3.3)**	396 (16)	18
89	92.8 (4.5)	375 (19)	13
144	89.3 (4.9)	390 (44)	6

*Calculated water concentrations adjusted downward by a correction factor of 0.38.

**Mean with SE in parentheses.

Table 11. Reproductive parameters from DEHP exposed midges (*Chironomus plumosus*) using sand substrate.

DEHP Conc. (µg/l)*	Egg Case Hatchability (%)	Average No. of eggs/egg case	No. of egg cases
0	89.7 (1.9)**	198 (11)	35
139	81.1 (3.8)	237 (18)	26
199	86.6 (2.8)	241 (16)	45
362	88.2 (3.7)	211 (20)	28

*Calculated water concentrations adjusted downward by a correction factor of 0.72.

**Mean with SE in parentheses.

Table 12. Cumulative percentages of midges (Chironomus plumosus) emerging after continuous exposure of the larvae to DDT using hydrosoil substrate.

Days of Exposure	DDT (µg/l)*							
	0	0	0.15	0.15	0.30	0.30	0.65	0.65
20	1	13	34	3	23	1	19	0
25	27	47	49	12	27	22	22	1
30	50	72	51	12	27	22	22	1
35	69	82	51	12	27	22	22	1
40	71	90	51	12	27	22	22	1

*Calculated water concentrations adjusted downward by correction factors from Table 3.

Table 13. Cumulative percentages of midges (Chironomus plumosus) emerging after continuous exposure of the larvae to DDT using sand substrate.

Days of Exposure	DDT (µg/l)**							
	0	0	0.48	0.48	1.4	1.4	2.0	2.0
20	23	27	18	8	2*	3*	0*	0*
25	51	55	20*	10*	4*	4*	0*	0*
30	77	79	20*	10*	4*	4*	0*	0*
35	77	87	20*	10*	4*	4*	0*	0*

*Values significantly ($P < 0.05$) different from controls.

**Calculated water concentrations adjusted downward by correction factors from Table 3.

calculated values in hydrosoil chambers, compared with 8 to 13% of the calculated values from sand substrate chambers.

All DDT concentrations were above the no effect level. In only one hydrosoil chamber did emergence exceed 50%. Emergence variation within concentrations in the test using hydrosoil was great, and LSD tests could not be used to show statistical difference between treatments and controls. It should be noted, however, that with one exception, no emergence from treatment chambers occurred after day 25. Slime mold growth was severe in these chambers, possibly due to uneaten food. No midge emerged after day 25 in the sand substrate test, and all emergence values except one were significantly ($P < 0.05$) different from controls.

Effects of DDT on midge reproduction

Reproduction in midges reared in hydrosoil was not affected by DDT concentrations up to 0.65 µg/l. The few egg cases produced in these tests indicated that neither egg hatchability nor the average number of eggs per egg case were significantly ($P < 0.05$) affected by increasing DDT concentrations (Table 14). However, about half of the fully developed embryos failed to hatch from two egg cases laid by DDT exposed midges. In other reproduction tests eggs which did not hatch did not normally contain developed embryos, presumably due to non-fertilization. Hatching failure of eggs from DDT exposed midges could indicate possible DDT interference. Because no egg cases were obtained from the sand substrate test, in which higher DDT concentrations were

Table 14. Reproductive parameters from DDT exposed midges (*Chironomus plumosus*) using hydrosoil substrate.

DDT Conc. (µg/l)*	Hatchability (%)	Average No. of egg/egg case	No. of egg cases
0	91.2 (6.4)**	386 (28)	5
0.15	97.8 (1.1)	435 (16)	5
0.30	87.3 (5.8)	533 (53)	7
0.65	83.0 (16)	596 (23)	2

*Calculated water concentrations and adjusted downward by correction factors from Table 3.

**Mean with SE in parentheses.

attained, it was not possible to verify this suspicion.

Because all DDT concentrations tested were above the no effect level and yet reproductive parameters were not affected, it is possible that midge emergence is a more sensitive indicator of chemical toxicity than is midge reproduction.

DEHP uptake and elimination by midge larvae

Midge larvae exposed to 0.202 µg ^{14}C-DEHP/l showed a rapid initial uptake. After one day of exposure the larvae concentrated DEHP 193 times (wet wt) the concentration in water (Table 15). The 3 and 7 day accumulation factors of 302 and 411, respectively, agree quite well with Mayer and Sander's (1973) values of 330 and 350 when larvae were exposed to 0.3 µg ^{14}C-DEHP/l.

Accumulation of DEHP by midge larvae did not plateau until after eight days of uptake (Figure 1). After nine days the larvae had a total body residue (TBR) of 90 ng/g, representing an accumulation factor of 446X. Adults emerging after nine days of exposure had a TBR of 59 ng/g, considerably less than the larvae from which they had emerged.

The accumulation curve was computer fitted to the asymptotic regression:

$$Y = A - B\ (\rho)^X$$

where Y is TBR, X is hours of exposure, and A, B, and ρ are constants for a particular asymptotic curve. Analysis of uptake data yielded the formula:

$$Y = (89.613) - (74.388)(0.988)^X$$

which fit the data with a $r^2 = 0.93$.

Table 15. Uptake of ^{14}C-DEHP by midge (Chironomus plumosus) larvae.

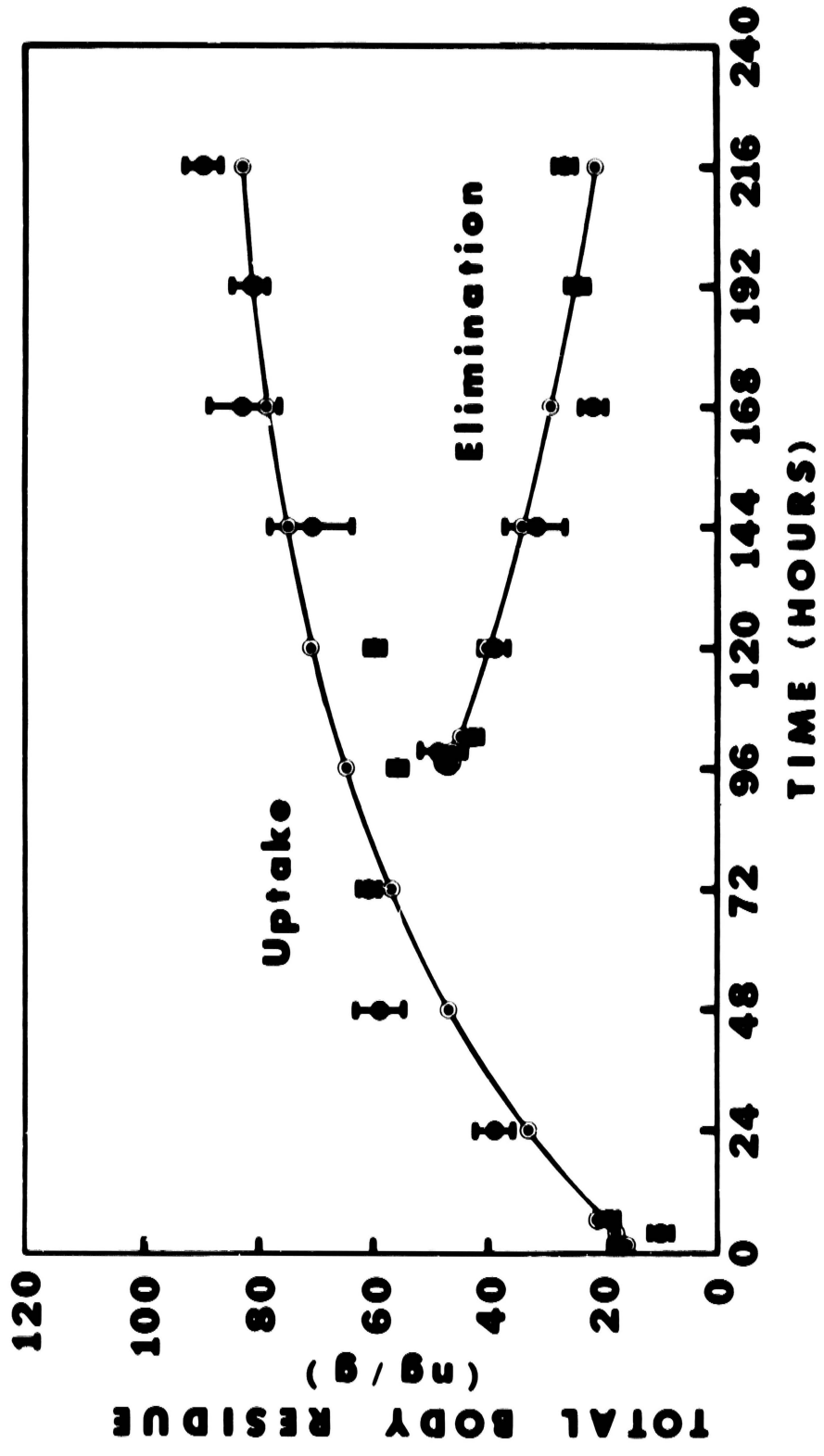
Hours of Uptake	Total Body Residue (ng/g)	Accumulation Factor
1	18 (16)**	89
3	10 (18)	50
7	19 (21)	94
24	39 (33)	193
48	59 (47)	292
72	61 (57)	302
96	56 (65)	277
120	60 (71)	297
144	71 (76)	351
168	83 (79)	411
192	82 (82)	406
216	90 (83)	446
Adults*	59	-

*13 adults which emerged on day 9.

**Measured TBRs with theoretical TBRs in parentheses.

Fig. 1. Uptake and elimination of ^{14}C-DEHP by midge (*Chironomus plumosus*) larvae exposed to 0.202 µg DEHP/l).

Solid dots with bars represent mean and SE of three samples.
Circles with dots in the center represent theoretical values.


TOTAL BODY RESIDUE
(ng/g)
120
100
80
60
40
20
0
0
24
48
72
96
120
144
168
192
216
240
TIME (HOURS)
Uptake
Elimination

After four days of exposure larvae were removed to fresh water to determine the rate of elimination of DEHP residues. The larvae eliminated the ^{14}C-DEHP fairly rapidly, losing 30% of the body burden in the first 24 hours (Table 16). The elimination data was fit to the equation:

$$\log Y = A + B\ (X)$$

where Y is TBR, X is hours of elimination, and A and B are constants. The equation:

$$\log Y = (-0.00284)\ (X) + (1.6748)$$

produced the elimination curve in Figure 1 ($r^2 = 0.80$). The theoretical half life of the accumulated DEHP was 3.4 days. However, 48% of the initial radioactivity was still present on day 5. This indicates continual, but extremely decelerated, loss of DEHP by the larval tissues.

Larval uptake of DEHP--in hydrosoil versus sand

Larval uptake of ^{14}C-DEHP varied according to the substrate. Larvae in sand exposed to 267 ng DEHP/l for 4 days accumulated 153 ng DEHP/g, compared with a TBR of only 113 ng/g for larvae exposed in hydrosoil (Table 17), a 26.1% difference. Likewise, after eight days of exposure those larvae in hydrosoil carried a body burden which was 26.4% less than that for the larvae in sand.

Hydrosoil uptake of DEHP during chronic exposures

DEHP extractions were performed on hydrosoil samples from one chronic test chamber to determine uptake of DEHP by hydrosoil in flow-through experiments. The concentration of

Table 16. Elimination of ^{14}C-DEHP by midge (Chironomus plumosus) larvae.

Hours of Elimination	Total Body Residue (ng/g)
0*	56 (47)**
1	48 (47)
3	49 (46)
7	43 (45)
24	39 (40)
48	32 (35)
72	22 (30)
96	25 (25)
120	27 (22)

*Larvae exposed to DEHP for 96 hours.

**Measured TBRs with theoretical TBRs in parentheses.

Table 17. Total body residues and accumulation factors of midge (Chironomus plumosus) larvae exposed to 267 ng DEHP/l in hydrosoil and in sand.

Days of Exposure	TBRs (ng/g) of larvae exposed in: Hydrosoil	Sand
4	113 (423)*	145 (543)
8	153 (573)	197 (738)

*TBR with accumulation factor in parentheses.

Table 18. Amounts of DEHP remaining in solution after three days when 327 ng DEHP was added to 700 ml of water containing different substrates.

Jars Containing	Ng DEHP remaining in solution	% DEHP remaining in solution
Water only	211	64.5
Sand	154	47.1
Hydrosoil	118	36.0

DEHP in the water averaged 302 µg/l over 35 days, while the concentration in the soil at day 35 averaged 29.2 µg/g, a 97-fold increase.

Substrate reduction of DEHP concentrations

To determine if sand and hydrosoil substrates adsorb DEHP at different rates, 327 ng of ^{14}C-DEHP was added to 700 ml samples of water. Triplicate jars contained 200 g of sand, 200 g of hydrosoil, and water only. After three days aliquots of water from each jar were extracted. An average of 64.5% of the initial DEHP remained in solution in the jars with no substrate, 47.1% in the jars containing sand, and 36.0% in the jars with hydrosoil (Table 18).

DISCUSSION

Midge larvae are an important food source for many game and non-game fishes (Johnson and Munger 1930; Novak and Estes 1974); therefore, it is important that such widespread aquatic contaminants like phthalate esters be examined for their toxicity to these aquatic organisms. Acute and chronic tests indicate that phthalate esters are relatively non-toxic to midges when tested at reported environmental concentrations. The EC50s and LC50s determined for both DEHP and DBP to midge larvae support the findings of Mayer and Sanders (1973) who also found these compounds to be realtively non-toxic to several species of aquatic invertebrates and fishes. Chronic tests showed no interference by DEHP with midge emergence, even when the larvae were exposed through two generations. Reproductive parameters also remained unaffected by DEHP exposure as high as 362 µg/l, in contrast to the 60% reduction in production of young Daphnia magna by adults exposed to a DEHP concentration of 0.3 µg/l (Mayer and Sanders 1973). Evidently there is a large variation in reproductive response by aquatic invertebrates to DEHP. Results of my experiments show no interference of DEHP with the life cycle of the midge.

Uptake by midge larvae of DEHP from water and from sediment is important because accumulation of PAEs by midge

larvae may transfer these residues to oragnisms higher in the food chain. Macek and Korn (1970) demonstrated the accumulation of DDT through the food chain, a process which might occur with DEHP. Uptake of DEHP appeared to level off after eight to nine days of exposure when the total body burden was about 450 times that of the exposure water. Comparison of the 4-day accumulation factor of 277 with the 4-day accumulation factor for Daphnia magna of 1500 (Johnson 1977) indicates that these larvae probably represent a poor avenue for transport of this aquatic contaminant through the food chain. This is also seen when the 7-day accumulation value of 411 is compared with the 7-day value of 3900 for the scud (Gammarus pseudolimnaeus) (Mayer and Sanders 1973).

The fact that emergent adult midges contained a smaller body burden of DEHP than the larvae from which they emerged is possibly explained by DDE studies performed by Derr and Zabik (1974). They found that adsorption rates of this relatively insoluble compound onto live and dead midge (C. tentans) were not significantly different. They also noted that DDE uptake was highly correlated with cuticular area of the midge. This indicates passive adsorption of the DDE onto the larval cuticle, a process probably applying to DEHP as well. Because this outer cuticle is left behind upon emergence, a drop in the body burden of adults would be expected.

If reliable tests for examining aquatic contaminants are developed, they must involve conditions which resemble

the natural environment as closely as possible. For this reason hydrosoil is the preferred substrate for chronic exposure of midge larvae and other organisms inhabiting sediment-type bottoms.

Interaction between the substrate and the contaminant will vary greatly from one type of substrate to another (Edwards 1966). For relatively insoluble compounds like DEHP and DDT the substrate particle size is of great importance. When DEHP was added to samples of water over sand and hydrosoil, more DEHP remained in solution over the sand substrate (Table 18). Likewise, the correction factors for DEHP and DDT chronic tests show that hydrosoil reduced water concentrations of the compounds half again as much as sand. DDT concentrations in the hydrosoil chambers were only 2.7 to 4.1% of the calculated values because of DDT's hydrophobic nature. Bridges et al. (1963) treated a pond with 0.02 mg DDT/l and within two days the water concentration was less than 5% of the concentration applied. The DDT had been adsorbed by the sediment and vegetation. Hydrosoil exposed to 302 µg DEHP/l for 35 days in one experiment accumulated 29.2 µg/g, a 97-fold increase.

The interaction between the waterborne contaminant and the test organism can be affected by this substrate-contaminant interplay. One example is the 26% greater residue uptake of DEHP by midge larvae exposed in sand than those in sediment. So even though hydrosoil is capable of accumulating high concentrations of DEHP, the DEHP is not

necessarily available to the larvae. Several workers have shown that clay mineral adsorption of organic compounds reduces their availability to microbes (Pinck and Allison 1951; Weber and Coble 1968) and thus, possibly, to benthic invertebrates. Edwards (1966) states that the most effective soil insecticides are those that are least adsorbed. Roberts (1963) found that greater concentrations of dieldrin were needed to kill test insects in muck or loam soils than in sand. Thus, hydrosoil can reduce the availability of certain compounds to benthic organisms.

Hydrosoil may influence the condition of midge larvae in chronic exposures. In the substrate suitability test, larvae developing in hydrosoil were observed to be larger, more pigmented, and more active than those developing in sand. While substrate type did not affect egg hatchability in reproduction studies, hydrosoil-reared midges produced egg cases containing an average of 396 eggs, compared with the 194 egg average from sand-reared midges. This may indicate that females developing in hydrosoil were in better reproductive condition.

Although larvae developing in hydrosoil may be in better condition than those raised in sand, the method for using hydrosoil in chronic exposure of midge larvae to aquatic contaminants has not been perfected. The feeding intervals and solvent concentrations, developed during DEHP exposures, are acceptable when none of the larvae are affected. The hydrosoil test with DDT, however, showed that

uneaten food, the result of larval mortality, can produce severe growth of slime mold and a blackening of the substrate surface. This interfered with successful midge emergence. By contrast, uneaten food in sand exposure chambers resulted in slime mold only toward the end of the test. In future tests using hydrosoil and midge larvae, dwelling-tube construction and DO levels will have to be carefully monitored to determine appropriate feeding rates. Insufficient food can result in delayed or reduced emergence. Further tests will demonstrate whether substrate type can directly influence the effect of a compound on midge emergence or reproduction.

Use of hydrosoil in chronic exposures should be investigated further. Choice of an appropriate hydrosoil source should be based on sediment purity and organic matter content. Hydrosoil from the CNFRL ponds, which contained more organic matter, developed slime mold problems earlier than hydrosoil with less organic matter from Little Dixie Reservoir. If an acceptable, practical hydrosoil method is eventually developed, hydrosoil standardization will be necessary to allow comparisons between different laboratories, investigators, and compounds. It is recommended that the standardization not be based on a set of synthesized (hydro)soils as these "may give results difficult to duplicate in the field or difficult to compare with natural soils" (Adams 1973). It would be better to use natural hydrosoils and to classify them with regard to proper parameters. Organic matter

content and cation exchange capacity would be appropriate for contaminant adsorption considerations as these reflect the surface area (Adams 1973). Organic matter and trace element contents would be important in nutritional considerations.

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