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Toxicity, Residue Dynamics, and Reproductive Effects of Phthalate Esters in Aquatic Invertebrates

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Aquatic invertebrates were exposed to di-*n*-butyl and di-2-ethylhexyl phthalate esters in water to determine toxicity, accumulation, and reproductive effects of these compounds. The acute toxicities were low and ranged from 2.1 mg/liter to greater than 32 mg/liter. Residue accumulation was rapid resulting in body residues 70–13,600 times that of the water concentration. Phthalate residues were essentially gone after 10 days in fresh water. A reproductive impairment of 60% occurred in *Daphnia magna* exposed continuously to 3 μg/liter of di-2-ethylhexyl phthalate.

Plasticizers are presently being used in amounts and products that could easily, although inadvertently, contribute to environmental pollution. Plasticizers are chemicals that are added to synthetic plastic resins to impart flexibility to the finished product, improve workability during fabrication, and extend or modify properties not present in the original resins (U. S. Tariff Commission, 1971). Plastic formulations may contain up to 60 parts per hundred of plasticizers (Nematollahi, Guess, and Autian, 1967). Phthalic acid esters are the most widely used plasticizers, particularly in polyvinyl chloride plastics. Production of these plasticizers was over 800 million pounds in 1969 (U. S. Tariff Commission, 1971), with di-2-ethylhexyl (355×10^6 lb.), diisodecyl (137×10^6 lb.), diiso-octyl (83×10^6 lb.), *n*-octyl-*n*-decyl (55×10^6 lb.), and di-*n*-butyl (34×10^6 lb.) phthalate esters representing the bulk of production. Di-2-ethylhexyl and di-*n*-butyl phthalates are also used as an orchard acaricide and an insect repellant, respectively (Farm Chemicals, 1971).

The acute toxicities of various phthalates have been determined in mammals and the compounds were found to have a very low order of toxicity with an LD_{50} range of 1.58–128 g/kg (Hodge, 1943; Shaffer, Carpenter, and Smyth, 1945; Calley, Autian, and Guess, 1966; Nematollahi, Guess, and Autian, 1967; Guess, 1968). Singh, Lawrence, and Autian (1971) demonstrated the teratogenic effects of phthalate esters in rats. The problems of phthalates leaching from plastics used in human medical practices have also been reported (Guess, Jacob, and Autian, 1967; Jaeger and Rubin, 1970; Marcel and Noel, 1970; Neergaard *et al.*, 1971).

Phthalate esters have been identified as an environmental contaminant by virtue of their discovery in soil (Ogner and Schnitzer, 1970), a deep sea jellyfish (Morris, 1970), fish, aquatic invertebrates, and water (Mayer, Stalling, and Johnson, 1971), and bovine tissues (Nazir, Beroza, and Nair, 1967; Taborsky, 1967). The biological significance of phthalate ester residues in aquatic organisms is unknown

at present and this report describes the toxicology of di-2-ethylhexyl and di-*n*-butyl phthalate in some freshwater invertebrates.

MATERIALS AND METHODS

The invertebrates used as test animals consisted of five species of crustacea, scud (*Gammarus pseudolimnaeus* Bousfield), glass shrimp (*Palaemonetes kadiakensis* Rathbun), crayfish (*Orconectes nais* Faxon), waterflea (*Daphnia magna* Strauss), aquatic sowbug (*Asellus brevicaudus* Forbes), and three species of immature aquatic insects, damselfly nymphs (*Ischnura verticalis* Say), burrowing mayfly (*Hexagenia bilineata* Say), and midge larvae (*Chironomus plumosus* Linnaeas). Waterfleas were obtained from laboratory reared cultures, and all other invertebrates were collected from various streams and ponds in central Missouri.

Acute toxicity tests were conducted under static conditions using the method previously described by Sanders (1969) with the exception that Triton X-100 was used as an emulsifier in preparing phthalate stock solutions. Toxic effects were measured in terms of the median tolerance limit (TL_{50}), the toxicant concentration in water which produces a 50-percent response in the test organisms under the test conditions.

Accumulation experiments were conducted in an intermittent-flow system modified from Mount and Brungs (1967) which provided a constant concentration of phthalates over extended periods of time. Exposure vessels were two-liter glass aquaria containing one liter of well water (pH 7.4, total hardness 270 mg/liter as $CaCO_3$) maintained at 21 ± 1°C. Stock solutions of [^{14}C]carbonyl-labeled di-2-ethylhexyl (1.64 mCi/mmole) and di-*n*-butyl (1.53 mCi/mmole phthalates were prepared in water and further diluted in the intermittent-flow system. The test system was operated for at least 24 hours prior to addition of organisms to allow for concentration equilibrium. The organisms were not fed during the experiments.

Invertebrate samples were taken in triplicate for radiometric analyses. Individual samples were prepared directly for analysis by homogenizing the whole organism in a tissue grinder. The homogenate was obtained by adding 6 ml of Triton X-100–toluene (2:3 v/v) emulsifier to each sample prior to grinding. The homogenate was then transferred to a scintillation vial and a toluene-fluor mixture was used to bring the total volume to 15 ml. The radioactivity of the sample was measured with a Beckman 200-L liquid scintillation counter.

The concentration of phthalate residues in water was monitored radiometrically by extracting triplicate 100-ml water samples with three 10-ml portions of diethyl ether. The extract was prepared by the aforementioned scintillation method. Residue concentrations and magnification factors presented in the text and tables were computed on whole body, dry weight basis. Dry weight conversion factors were obtained by drying the organisms to a constant weight at 50°C. Magnification was expressed as the ratio of concentration in the organism to concentration in water.

Reproductive studies with waterfleas were conducted in an intermittent-flow system as described in the accumulation studies. Ten first-instar organisms up to 24 hours old were placed in exposure vessels. Stock solutions of di-2-ethylhexyl phthalate were prepared in ethyl alcohol and then further diluted with water to

concentrations of 30, 10, and 3 μg/liter. All concentrations and controls contained 0.1 ml/liter of alcohol. Test organisms were fed a suspension of yeast in sufficient amounts to support a stable population. The offspring produced in each concentration were counted after the parent waterfleas had been exposed for 2 weeks and at the end of the test (3 weeks).

RESULTS

Acute Toxicity of Phthalates

Static 96-hour bioassays indicated that phthalate esters have a relatively low acute toxicity to scud. The toxicity of di-*n*-butyl phthalate increased with time of exposure from 7 mg/liter at 24 hours to 2.1 mg/liter at 96 hours. Di-2-ethylhexyl phthalate appeared relatively nontoxic to scud with a 96-hour TL_{50} value greater than 32 mg/liter. The higher toxicity of di-*n*-butyl phthalate was probably due to its greater solubility in water. The 96-hour TL_{50} of di-*n*-butyl phthalate to crayfish was greater than 10 mg/liter.

Accumulation of Phthalate Residues

All invertebrates exposed to ^{14}C-labelled di-*n*-butyl and ^{14}C-labelled di-2-ethylhexyl phthalate showed an initial rapid uptake and magnification of radioactive residues several thousand times greater than the concentration in water (Tables 1 and 2). With the exception of some invertebrates, the accumulation of di-2-ethylhexyl phthalate was greater than that of di-*n*-butyl phthalate. For example, di-2-ethylhexyl phthalate residues were accumulated and stored by scud during a 14-day exposure at levels 13 400 times greater than the 0.1 μg/liter concentrations in the surrounding water. Under similar experimental conditions, di-*n*-butyl phthalate was accumulated only 6700 times greater than the water concentrations.

TABLE 1
"BIOLOGIC MAGNIFICATION" OF ^{14}C-LABELLED DI-*n*-BUTYL PHTHALATE FROM WATER BY SIX SPECIES OF AQUATIC INVERTEBRATES AT 21°C

Organism	No. per sample	Water concentration (μg/1 ± SE[a])	Magnification factor[b] after (days) 1	3	7	14
Midge larvae *Chironomus plumosus*	18	0.18 ± 0.015	3500	3900	6600	—
Waterflea *Daphnia magna*	180	0.08 ± 0.005	2200	3500	5000	5000
Scud *Gammarus pseudolimnaeus*	18	0.10 ± 0.010	1700	3700	6500	6700
Mayfly *Hexagenia bilineata*	9	0.08 ± 0.001	500	980	1900	—
Glass shrimp *Palaemonetes kadiakensis*	9	0.08 ± 0.001	1500	5000	—	—
Damselfly *Ischnura verticalis*	9	0.10 ± 0.005	1000	1600	2700	—

[a] Samples taken in triplicate and expressed as mean value ± SE ($P = 0.05$).

[b] The extracted radioactivity was assumed to be all ^{14}C-labeled di-*n*-butyl phthalate and the concentrations were derived from the original specific activity (1.64 mCi/mmole).

TABLE 2
"BIOLOGIC MAGNIFICATION" OF ^{14}C-LABELLED DI-ETHYLHEXYL PHTHALATE FROM WATER BY FIVE SPECIES OF AQUATIC INVERTEBRATES

Organism	No. per sample	Water concentration ($\mu g/1 \pm SE^a$)	Magnification factor[b] after (days)				
			1	3	7	14	21
Scud *Gammarus pseudolimnaeus*	18	0.1 ± 0.01	2800	5300	13600	13400	—
	18	62.8 ± 3.31[c]	30	100	116	270	260
Midge larvae *Chironomus plumosus*	18	0.3 ± 0.04	2400	2600	3100	—	—
Waterflea *Daphnia magna*	180	0.3 ± 0.04	1200	2500	5200	—	—
Mayfly *Hexagenia bilineata*	9	0.1 ± 0.01	850	1000	2300	—	—
Sowbug *Asellus brevicaudus*	4	1.9 ± 0.12[c]	—	—	80	71	70
	4	62.3 ± 3.31[c]	—	—	20	230	250

[a] Samples taken in triplicate snd expressed as mean ± SE ($P = 0.05$).
[b] The extracted radioactivity was assumed to be all ^{14}C-labelled di-ethylhexyl phthalate and the concentrations were derived from the original specific activity (1.64 mCi/mmole).
[c] Temperature = 25°C.

Accumulation of di-2-ethylhexyl phthalate by scud was directly related to water concentration, whereas, magnification was inversely related. After a 14-day exposure to 62.8 μg/liter, scud concentrated di-2-ethylhexyl phthalate 270 times (18.1 μg/g) the level in water. However, when they were exposed to 0.1 μg/liter, they accumulated total body concentrations 13 400 times (1.34 μg/g) that of water.

In some experiments, we were able to continue the tests until an equilibrium concentration was reached within the organism. Waterfleas exposed to 80 ng/liter of di-*n*-butyl phthalate in water reached an equilibrium at 7 days. The total body residues at this time were 0.4 μg/g or a concentration 5000 times that in water. Once this equilibrium was reached within the organisms, no further residue magnification was observed after an additional 7 days of exposure. With the exception of midge larvae, aquatic insects appear to accumulate phthalate residues at a slower rate than crustacea.

Retention of Phthalate Residues

To determine the time required for biological elimination of phthalate residues by invertebrates, waterfleas were exposed to 0.1 μg/liter of di-*n*-butyl phthalate for 7 days. This exposure was previously shown to result in maximal residue accumulation of 0.4 μg/g. The organisms were transferred to phthalate-free flowing water and, after 3 days, 50 percent of the total radioactivity remained. Twenty-five percent of the activity was still present in the organisms after 7 days in fresh water. In similar experiments, scud exposed to 0.1 μg/liter of di-2-ethylhexyl phthalate accumulated total body residues of 5.4 μg/g in 3 days. Residual radioactivity decreased rapidly during 4 days in phthalate-free water to 20 percent of the beginning activity. After 10 days, only 6 percent of the total activity remained in the organisms. The loss in radioactive residues may have been due to degrada-

TABLE 3
WATERFLEA (*Daphnia magna*) REPRODUCTION AS AFFECTED BY CONTINUOUS EXPOSURE TO DI-2-ETHYLHEXYL PHTHALATE FOR 21 DAYS AT 21° C

Concentration (μg/liter)	Offspring produced per ten adults			
	2-week sample		3-week sample	
	$\bar{X} \pm$ SD[a]	% inhibition	$\bar{X} \pm$ SD	% inhibition
Control	71.5 ± 13.4	—	114.0 ± 8.5	—
3	26.5 ± 7.8	62.9	45.5 ± 14.8	60.1
10	30.0 ± 8.5	58.1	34.0 ± 8.5	70.2
30	12.5 ± 3.5	82.5	19.5 ± 3.5	82.9

[a] Mean ± standard deviation.

tion and/or excretion of the parent compound. Techniques to determine phthalate ester degradation in invertebrates are not complete. However, preliminary results indicate that the degradation products may consist of phthalic acid, the monoester, and a glucuronide.

Reproduction

Continuous exposure of waterfleas for a complete life cycle (21 days) to 3, 10, and 30 μg/liter of di-2-ethylhexyl phthalate in an intermittent-flow system significantly ($P < 0.01$) reduced reproduction (Table 3). Total production of offspring was inhibited 60, 70, and 83 percent in the three concentrations respectively. The degree of reproductive inhibition remained relatively constant during the 21-day exposure period in all concentrations except for the 10 μg/liter treatment where reproduction declined further by 12 percent between 14 and 21 days.

DISCUSSION

Di-2-ethylhexyl and di-*n*-butyl phthalates are not acutely toxic to aquatic invertebrates. The toxicity of DDT (96-hour TL_{50} = 1.0 μg/liter) to scud is much greater than that of the two phthalate esters. These essentially nontoxic effects in acute mortality studies have also been reported for fish (Mayer, Stalling, and Johnson, 1971), birds (McLaughlin *et al.*, 1963; Verrett *et al.*, 1969) and mammals (Hodge, 1943; Shaffer, Carpenter, and Smyth, 1945; Calley and Autian, 1967; Guess, 1968). However, dimethyl and diethyl phthalates were lethal to mouse fibroblasts grown in tissue culture, and di-2-ethylhexyl phthalate was found to retard growth in mice (Calley, Autian, and Guess, 1966).

Invertebrates exposed continuously to phthalate esters in water rapidly accumulated total body residues many times greater than the concentrations in water. However, phthalate residues were not magnified in invertebrates to the same degree as found with organochlorine insecticides (Johnson *et al.*, 1971). The process of biological magnification of chemical residues in an organism is largely dependent on the balance existing between the process of accumulation and elimination. In accumulation studies, the magnification of di-2-ethylhexyl phthalate residues in scud were much less when they were exposed to a higher phthalate

concentration and increased water temperature. This suggests that phthalate accumulation in scud may be favored at low concentrations (0.1 μg/liter) and uptake exceeds elimination. At the higher phthalate concentration (67 μg/liter) and with increased water temperature, the factors which contribute to metabolism and elimination may be stimulated and the elimination rate may equal or exceed the accumulation rate, resulting in decreased magnification. Although radioactive residues accumulated by scud and waterfleas were not identified, these organisms rapidly lost radioactivity when they were transferred to fresh water. This loss in radioactive residues may be related to either metabolism and/or excretion of residues by the organisms.

The observed toxicity values (TL_{50}) for aquatic organisms are 700 to 11 000 times that which inhibited reproduction in waterfleas. The toxic insecticide DDT reduced reproduction by 40 percent at concentrations of 0.1 μg/liter and this is only 1/10 the TL_{50} value for DDT. Predictions from regression analyses indicated that a 50 percent inhibition of reproduction could occur at 0.13 and 2.5 μg/liter for DDT and di-2-ethylhexyl phthalate, respectively.

The low degree of toxicity and the high excretion rate of di-*n*-butyl and di-2-ethylhexyl phthalates suggest that these compounds might be relatively safe as far as aquatic organisms are concerned, provided exposure is not constant. However, successful growth and reproduction are essential for the maintenance of animal populations. Aquatic invertebrates are the main food source of many fishes and wildlife, and thus growth and reproduction of these predatory vertebrates could be adversely affected in an indirect manner whether or not phthalate esters directly affected fish and wildlife. The present evidence indicates that phthalate esters in small amounts are detrimental to ecologically important aquatic invertebrates, and these compounds should therefore be considered as environmental pollutants.

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REFERENCES

CALLEY, D., AUTIAN, J., AND GUESS, W. L. (1966). Toxicology of a series of phthalate esters. *J. Pharm. Sci.* **5**, 158–162.

Farm Chemicals (1971). "Dictionary of pesticides." Meister Publishing Company, Willoughby, Ohio.

GUESS, W. L. (1968). Toxicity profiles of vinyl and polyolefinic plastics and their additives. *J. Biomed. Mater. Res.* **2**, 313–335.

GUESS, W. L., JACOB, J., AND AUTIAN, J. (1967). A study of polyvinyl chloride blood bag assemblies. I. Alteration or contamination of ACD solutions. *Drug Intellig.* **1**, 120–127.

HODGE, H. C. (1943). Acute toxicity for rats and mice of 2-ethyl hexanol and 2-ethylhexyl phthalate. *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **53**, 20–23.

JAEGER, R. J., AND RUBIN, R. J. (1970). Plasticizers from plastic devices: extraction, metabolism, and accumulation by biological systems. *Science* **170**, 460–462.

JOHNSON, B. T., SAUNDERS, C. R., SANDERS, H. O., AND CAMPBELL, R. S. (1971). Biological magnification and degradation of DDT and aldrin by freshwater invertebrates. *J. Fish. Res. Bd. Canada.* **28**, 705–709.

MARCEL, Y. L., AND NOEL, S. P. (1970). Contamination of blood stored in plastic packs. *Lancet* **I**, 35–36.

MAYER, F. L., JR., STALLING, D. L., AND JOHNSON, J. L. (1971). Phthalate esters: an environmental contaminant. Presented at Midwest Regional American Chemical Society Meeting, October 28–29, St. Louis.

MCLAUGHLIN, J., JR., MARLIAC, J. P., VERRETT, M. J., MUTCHLER, M. K., AND FITZHUGH, O. G. (1963). The injection of chemicals into the yolk sac of fertile eggs prior to incubation as a toxicity test. *Toxicol. Appl. Pharmacol.* **5**, 760–771.

MORRIS, R. J. (1970). Phthalic acid in the deep sea jellyfish *Atolla. Nature (London)* **227**, 1264.

MOUNT, D. I., AND BRUNGS, W. A. (1967). A simplified dosing apparatus for fish toxicology studies. *Water Res.* **1**, 21–29.

NAZIR, D. J., BEROZA, M., AND NAIR, P. P. (1967). Isolation and identification of di (2-ethylhexyl) phthalate—a component of heart muscle mitochondria. *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **26**, 412.

NEERGAARD, J., NIELSEN, B., FAURBY, V., CHRISTENSEN, D. H., AND NIELSEN, O. F. (1971). Plasticizers in PVC and the occurrence of hepatitis in a haemodialysis unit. *Scand. J. Urol. Nephrol.* **5**, 141–145.

NEMATOLLAHI, J., GUESS, W. L., AND AUTIAN, J. (1967). Plasticizers in medical application. I. Analysis and toxicity evaluation of dialkyl benzenedicarboxylates. *J. Pharm. Sci.* **56**, 1446–1453.

OGNER, G., AND SCHNITZER, M. (1970). Humic substances: Fulvic acid-dialkyl phthalate complexes and their role in pollution. *Science* **170**, 317–318.

SANDERS, H. O. (1969). Toxicity of pesticides to the crustacean *Gammarus lacustris. Bur. Sport Fish. Wildl. (U. S.) Tech. Paper* **25**.

SINGH, A. R., LAWRENCE, W. H., AND AUTIAN, J. (1971). Teratogenicity of a group of phthalate esters in rats. *Toxicol. Appl. Pharmacol.* **19**, 372.

SHAFFER, C. B., CARPENTER, C. P., AND SMYTH, H. F., JR. (1945). Acute and subacute toxicity of di(2-ethylhexyl) phthalate with note upon its metabolism. *J. Ind. Hyg. Toxicol.* **27**, 130–135.

TABORSKY, R. G. (1967). Isolation studies on a lipoidal portion of the bovine pineal gland. *J. Agr. Food Chem.* **15**, 1073–1076.

U. S. Tariff Commission. (1971). Synthetic organic chemicals, U. S. production and sales of plasticizers, 1969. Superintendent of Documents, U. S. Government Printing Office, Washington, D. C.

VERRETT, M. J., MUTCHLER, M. K., SCOTT, W. F., REYNOLDS, E. F., AND MCLAUGHLIN, J. (1969). Teratogenic effects of captan and related compounds in the developing chicken embyro. *Ann. NY Acad. Sci.* **160**, 334–343.