Environmental Toxicology and Chemistry, Vol. 4, pp. 167–179, 1985
Printed in the USA. Pergamon Press Ltd.

0730-7268/85 $3.00 + .00
1985 SETAC

0739

CHRONIC TOXICITY OF DI-*N*-BUTYL AND DI-*N*-OCTYL PHTHALATE TO *DAPHNIA MAGNA* AND THE FATHEAD MINNOW

J. F. McCarthy* and D. K. Whitmore
Environmental Sciences Division, Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37831

(*Received* 26 *January* 1984; *Accepted* 17 *May* 1984)

Abstract – The toxicities of di-*n*-butyl phthalate (DBP) and di-*n*-octyl phthalate (DOP) were assessed by measuring the effect of exposure to these compounds on the fecundity of *Daphnia magna* and on the hatching and survival of the early life stages of the fathead minnow *Pimephales promelas*. For *D. magna*, exposure to 1.8 mg/L DBP or 1.0 mg/L DOP caused a significant reduction in reproduction. Doses of 0.56 mg/L DBP or 0.32 mg/L DOP had no significant effect in decreasing reproduction. Survival of fathead minnow embryos was decreased by exposure to 1.8 mg/L DBP; none of the embryos exposed to this dose hatched successfully. Hatching and larval survival were affected by exposure to 1.0 mg/L DBP, but not to 0.56 mg/L. Exposure to DOP did not affect survival of either early embryos or larvae of the fathead minnow at doses up to 10 mg/L (the highest dose tested). Hatching of the embryos was significantly decreased at 10 mg/L, but not at 3.2 mg/L DOP.

Keywords – Phthalate esters Di-*n*-butyl phthalate Di-*n*-octyl phthalate *Daphnia magna* Fathead minnows *Pimephales promelas* Bioassay

INTRODUCTION

The diesters of phthalic acid have been identified by the U.S. Environmental Protection Agency (EPA) as priority pollutants because of their large annual production (in the range of 20 million kg/yr). The use and disposal patterns for these compounds tend to favor their eventual entry into the environment, and because of their potential for bioaccumulation and their resistance to biodegradation, they pose a potential environmental hazard. The primary use of phthalate esters is in the plastics industry, where they are used as plasticizers to soften resins. The phthalates tend to migrate slowly out of the plastic, either into the air by volatilization or into water or other solvents by dissolution. In view of the large production and ubiquitous use of phthalate esters, it is not surprising that phthalates have been identified in water, sediment and biota in many parts of the world.

To provide information on the toxicity of phthalate esters, the effect of chronic exposure to two phthalates on the early life stages of the fathead minnow *Pimephales promelas* and on the reproductive capacity of the cladoceran *Daphnia magna* was examined. The study was conducted using two phthalates of differing molecular weight and aqueous solubility, di-*n*-butyl phthalate (DBP) and di-*n*-octyl phthalate (DOP).

MATERIALS AND METHODS

The stock of *D. magna* used in these tests was procured from Miami University (Ohio) and has been maintained in our laboratory since April 1977. The *D. magna* stock was maintained on a diet of Purina Trout Chow and yeast. Test animals for both the acute immobilization tests (range-finding test) and the reproduction test were collected when the animals were less than 24 h old. The range-finding tests were run for 48 h; the reproduction tests were run for 15 to 16 d, which was the time required for production of at least three broods [1].

*To whom correspondence may be addressed.

Although the research described in this article has been funded wholly or in part by the U.S. Environmental Protection Agency (EPA) through Interagency Agreement Number EPA-79-D-X0856 to the U.S. Department of Energy under contract DE-AC05-840R21400 with Martin Marietta Energy Systems, Inc., it has not been subjected to EPA review and therefore does not necessarily reflect the views of EPA and no official endorsement should be inferred.

Publication No. 2374, Environmental Sciences Division, ORNL.

9381

The fathead minnows (*P. promelas*) used in the early life stage tests were obtained from a long-term laboratory culture maintained in large flow-through tanks (approximately 0.5 × 2.1 × 0.6 m). Minnows were maintained at 23°C and fed frozen brine shrimp five times a week. Freshly fertilized eggs were obtained by permitting the minnows to deposit eggs on the inside of sections of plastic pipe; the pipes were coated with sand to facilitate removal of the embryos.

DBP was obtained from Fluha-Garantre, Switzerland. The compound was 99.5% pure (T. Ho, Oak Ridge National Laboratory [ORNL], personal communication). DOP was synthesized to greater than 98% purity (T. Ho, ORNL, personal communication). The phthalate esters were dissolved in acetone, used as a carrier for their introduction into test exposure water. In all tests, two controls were run: an uncontaminated water control (no toxicant or carrier, designated "control" in the figures and tables) and a carrier control containing acetone at the same concentration as in the toxicant exposures (designated "0.0" in the figures and tables). Water quality characteristics of the dechlorinated tap water used during the experiments are listed in Table 1.

The concentrations of each phthalate ester in samples of the exposure water were measured twice a week during toxicity tests with both chemicals. The water was extracted twice with methylene chloride after addition of an internal standard [benzylbutyl phthalate (BBP), 100 μg]. The volume of aqueous sample extracted depended on the expected concentration. The methylene chloride extract was evaporated to dryness and the residue redissolved in 1.0 ml benzene. Two microliters of sample was analyzed on a Hewlett-Packard 5840 gas chromatograph equipped with a Model 7671A automatic liquid sampler, using a 3 m × 3.1 mm glass column packed with 3% SP-2250 DB on 100/20 mesh Supelcoport. The carrier gas was helium at a flow rate of 30 ml/min. The column oven temperature program ran from 200°C (held at 200°C for 2 min) to 250°C at a rate of 4°C/min, and held at 250°C for 10 min. The injector temperature was 250°C and the flame ionization detector was maintained at 300°C. The precision of the analysis was ±10%. Standards of 100 mg/L of DBP or DOP and BBP were analyzed with each group of samples.

Dissolved oxygen (DO) and temperature were measured with a Yellow Springs Instruments Model 51B oxygen meter, and pH was measured with a Corning Model 109 pH meter.

Table 1. Water quality characteristics of water used in toxicity tests

Characteristic	Value (mg/L)
Total alkalinity	82
Total hardness	85
Dissolved organic carbon	1.1
Total organic carbon	1.3
Ca	23.9
Mg	6.5
K	0.46
Na	0.64
Cl	1.50
NO_3-N	0.025
SO_4	4.32
Total phosphorus	0.020
F	0.1
	(μg/L)
Pb	0.1
Zn	0.40
Cr	0.35
Cd	0.01
Cu	7
Hg	0.003

Tests for the *D. magna* reproduction experiment followed the guidelines recommended by Organization for Economic Cooperation and Development [1]. For the immobilization test (range-finding test), animals were exposed to six doses of each phthalate ester, with two replicates per dose. Five animals were exposed in each replicate in 100-ml glass beakers for 48 h.

The *D. magna* reproduction tests were run as semistatic exposures with a 12-h light:12-h dark cycle. Solutions were renewed three times a week during the experiment with DOP. During the reproduction test with DBP, solutions were renewed daily because the concentration of this phthalate ester decreased to very low levels if solutions were renewed only three times a week. In the reproduction test with DBP, 40 animals less than 24 h old were placed individually into 100-ml glass beakers (80 ml of solution) for each dose. Before solutions were renewed each day, the beakers were inspected for dead animals, presence of eggs and released young. The first-instar daphnids were counted and discarded. The reproduction test with DOP was run with five 400-ml glass beakers per dose (8 animals and 320 ml per beaker). Animals were examined three times a week, and the number of surviving adult daphnids and the number of released, viable juvenile daphnids was recorded.

In reproduction tests with both phthalate esters, temperature, DO and pH were recorded for each

dose when solutions were renewed. Animals were fed a mixture of Trout Chow and yeast (2 mg/80 ml or 8 mg/320 ml) when solutions were renewed.

Since DOP and DBP are hydrophobic compounds capable of binding to the particulate organic matter introduced as the food source for the *D. magna*, the fraction of the phthalate ester bound to the particulates was measured under the conditions of these toxicity tests. Phthalate ester in carrier was dissolved in water and the mixture of Trout Chow and yeast added. The solution was stirred for 4 h and filtered through precombusted glass fiber filters (Whatman GF/F) to retain the particulates. The filters were extracted with methylene chloride and analyzed for phthalate levels by gas chromatography. The fraction bound to particles was calculated as the ratio of the amount of phthalate retained on the filter to the total amount of compound in the suspension.

The protocol for the fathead minnow early life stage test followed the standard test protocol developed by the U.S. EPA [2]. Embryos that were between 24 and 48 h old were pooled from several egg masses; they were separated from each other by finger-rolling and gentle teasing with forceps. Any dead or unhealthy eggs were discarded after inspection under a dissecting microscope. For initial range-finding tests, groups of embryos were allowed to hatch in uncontaminated water, and the new fry were divided into 4-liter aquaria containing different doses of phthalate ester. The percent survival of the fry over 96 h was recorded.

For the early life stage tests, 30 to 50 embryos were randomly distributed into glass embryo cups, with Nitex screening on the bottom, and placed in finger bowls (90 × 170 mm). Two replicates (one embryo cup per finger bowl per replicate) were prepared to test the effect of each concentration. The organisms were exposed to the phthalate esters in a flow-through exposure system (Environ-Tox diluter system, Specialized Environmental Equipment, Inc., Easley, South Carolina). All containers and tubing were constructed of glass, stainless steel or Teflon. The acetone solutions of the phthalate esters were injected into water in a toxicant mixing bottle using Frazer injecters (George Frazer, Duluth, Minnesota). Acetone with no toxicant was injected at the same rate into the diluent water so that the concentration of carrier was constant across all doses (0.1 ml/L water). A set of replicates was also exposed to carrier-free uncontaminated water. A 12-h light:12-h dark cycle was maintained during all exposures.

During the embryonic exposure period, the finger bowls were fitted with self-starting siphons, which partially drained the exposure chambers when they filled to the top of the siphons; this promoted flushing of the toxicant through the embryo cups and increased aeration of the embryos. When the embryos hatched, they were released into the finger bowls for the remainder of the test. After the swim-up stage, fry were fed freshly hatched brine shrimp. Records were kept of the number of embryos hatched, and the number of normal and deformed fry. Dead animals were removed daily.

DO was monitored daily, and temperature and pH were monitored weekly in each replicate; one replicate was monitored continuously for fluctuations in temperature.

Statistical analysis of the results of all tests used the procedures in the Statistical Analysis System (Statistical Analysis Institute, Cary, North Carolina). Range-finding tests were analyzed by probit analysis when possible. Differences in survival, hatching or reproduction among doses were determined by an analysis of variance; a Duncan's multiple range test was used to separate means that were significantly different. Results are reported based on nominal concentrations of the phthalate esters.

RESULTS

D. magna *reproduction tests*

Effect of DBP. In the acute mortality test (range-finding test), all *D. magna* were dead after 48 h of exposure to nominal concentrations of 7.5 and 10.0 mg/L DBP. At the lower doses of 3.0, 1.0 and 0.5 mg/L DBP and in controls, all animals survived, except for one individual at 3.0 mg/L. The LC50 (lethal concentration to 50% of the test population) is between 3.0 and 7.5 mg/L DBP. Although a probit analysis cannot be performed, because this procedure requires two responses that are between 0 and 100% mortality, a nonparametric analysis was developed for steep dose–response bioassays (Schmoyer, Beauchamp and McCarthy, manuscript in preparation). The LC50 was estimated using this method and was equal to 5.2 mg/L, with 95% confidence limits of 4.7 and 5.6 mg/L.

Temperature, DO and pH remained relatively constant throughout each test and across all concentrations (Table 2). The measured concentrations of DBP in the freshly prepared solutions (before animals were added) were fairly close to the nominal doses; however, after 24 h, the concentrations dropped by about one-third (Table 2).

Table 2. Water quality and phthalate concentrations during exposure of *Daphnia magna* to di-*n*-butyl phthalate

Nominal concentration (mg/L)	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Observed concentration (mg/L)	
				Fresh solutions	After 1 d with *D. magna*
Control	21.1 ± 0.1	6.8 ± 0.1	8.1 ± 0.03	<0.001	<0.001
0.0	21.1 ± 0.1	6.0 ± 0.1	7.9 ± 0.04	<0.001	<0.001
0.056	21.1 ± 0.1	6.0 ± 0.1	7.9 ± 0.02	0.044 ± 0.004	0.029 ± 0.006
0.18	21.3 ± 0.1	5.9 ± 0.1	7.9 ± 0.02	0.15 ± 0.02	0.059 ± 0.013
0.56	21.2 ± 0.1	5.9 ± 0.1	7.9 ± 0.03	0.50 ± 0.05	0.33 ± 0.04
1.8	21.2 ± 0.1	5.6 ± 0.1	7.9 ± 0.02	1.56 ± 0.15	0.92 ± 0.17
3.2	21.3 ± 0.1	5.7 ± 0.1	7.9 ± 0.03	2.80 ± 0.25	2.08 ± 0.11

Values are means ± SE for all recorded measurements.

The results of the filtration experiment designed to determine the fraction of DBP bound to the suspended particulates in the food demonstrated that only 2.5% (±0.2%) of the DBP was bound to the suspended particulate matter.

Survival of *D. magna* exposed to DBP exceeded 80% in all concentrations except 1.8 and 3.2 mg/L and in the carrier-free control. The reason for the poor survival (and poor reproduction) of the control group is not clear; the same problem was evident in the DOP experiment (see below). By the end of the experiment (day 16), 70% of the *D. magna* were alive at 1.8 mg/L and 18% were alive at 3.2 mg/L DBP (Fig. 1 and Table 3).

The number of young produced per surviving adult at each dose is indicated in Table 3. Reproduction in *D. magna* was stimulated at low levels and inhibited at higher concentrations of DBP. Exposure to 0.056 to 0.56 mg/L DBP increased the total numbers of young produced. Reproduction was significantly impaired at 1.8 mg/L, compared with that at either the 0.56 mg/L dose or the carrier control. Although eggs were occasionally observed in the brood sacs of animals exposed to a dose of 3.2 mg/L DBP, no viable young were produced. If the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) are assumed to reflect only inhibition of reproduction (as opposed to the apparent stimulation observed at moderate doses), then the NOEC for DBP is 0.56 mg/L and the LOEC is 1.8 mg/L (nominal concentrations).

Since the animals exposed to DBP were cultured individually, information was obtained on the effect of DBP on the total number of broods (as defined by appearance of eggs in the brood sac, rather than by release of viable young) and on the number of days and number of molts required to achieve reproductive maturity (primiparous instar). DBP had relatively little effect on any of these parameters, although the highest dose did result in a significant decrease in the total number of broods and in the instar at which eggs were first observed in the brood sac (primiparous instar, Table 4).

Effect of DOP. The results of the acute immobilization test (range-finding test) for *D. magna* exposed to DOP indicated that this phthalate ester was not toxic to the animals at any dose up to and including 10 mg/L, the highest dose tested; this dose exceeds the aqueous solubility of DOP by three to four orders of magnitude (M. Maskarinec, ORNL, personal communication).

Temperature, DO and pH remained constant

Table 3. Effect of di-*n*-butyl phthalate on the survival and reproduction of *Daphnia magna*

Nominal concentration (mg/L)	Survival of adults (%)	Number of young per adult
Control	70.0 ± 1.1 (A)	56.0 ± 4.8 (AB)
0	88.0 ± 0.8 (B)	69.4 ± 14.1 (AB)
0.056	88.0 ± 0.8 (B)	89.3 ± 6.9 (A)
0.18	80.0 ± 1.0 (C)	78.9 ± 9.9 (A)
0.56	95.0 ± 0.5 (D)	85.0 ± 8.8 (A)
1.8	70.0 ± 1.1 (A)	32.7 ± 10.4 (B)
3.2	18.0 ± 9.6 (E)	0 ± 0 (C)

Values are means ± SE. For each variable, means with the same letter (in parentheses) are not significantly different ($p = 0.05$).

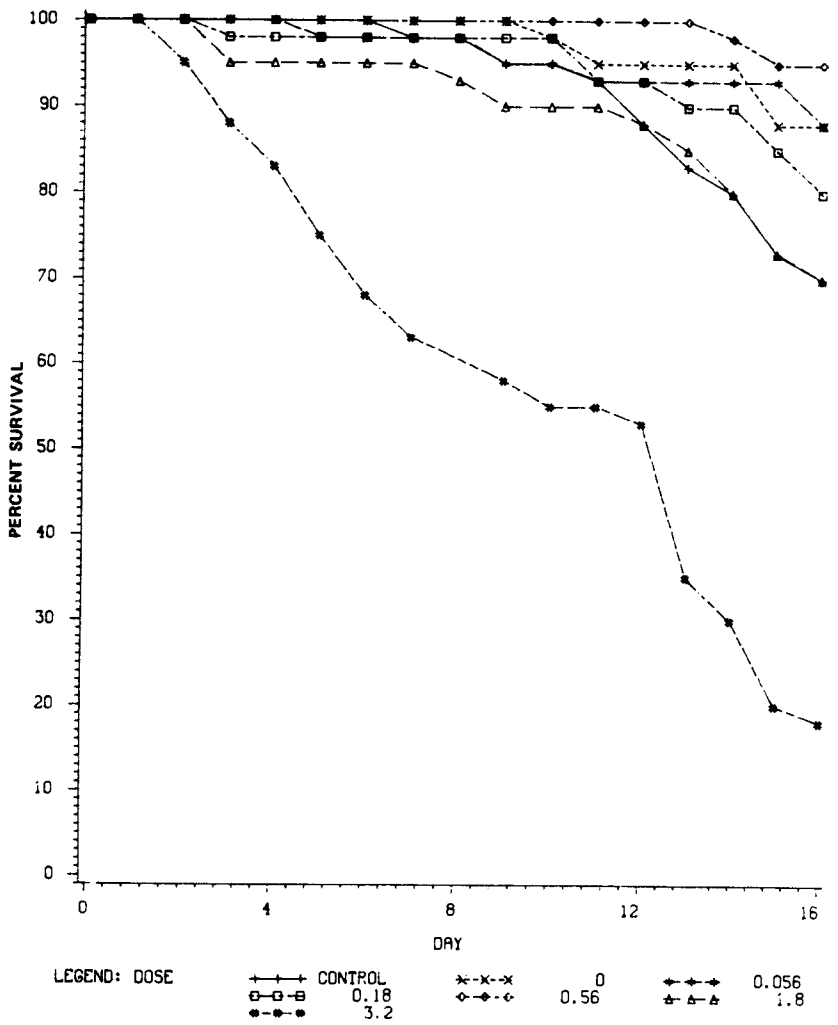

Fig. 1. Survival (%) of *Daphnia magna* exposed to di-*n*-butyl phthalate for 16 d.

Table 4. Effect of di-*n*-butyl phthalate on three biological measurements of reproductive success in *Daphnia magna*

Nominal concentration (mg/L)	Primiparous instar	Days to primiparous instar	Total number of broods
Control	6.0 ± 0.2 (A)	7.4 ± 0.2 (AB)	3.1 ± 0.1 (BC)
0	5.9 ± 0.1 (A)	7.2 ± 0.2 (AB)	3.3 ± 0.1 (ABC)
0.056	6.1 ± 0.2 (A)	7.5 ± 0.3 (AB)	3.2 ± 0.1 (ABC)
0.18	5.9 ± 0.2 (A)	6.9 ± 0.2 (B)	3.4 ± 0.1 (AB)
0.56	6.2 ± 0.1 (A)	7.1 ± 0.2 (B)	3.5 ± 0.1 (A)
1.8	6.2 ± 0.2 (A)	7.7 ± 0.3 (AB)	3.0 ± 0.1 (C)
3.2	5.3 ± 0.2 (B)	8.0 ± 0.2 (A)	2.2 ± 0.3 (D)

Values are means ± SE. For each variable, means with the same letter (in parentheses) are not significantly different ($p = 0.05$).

Table 5. Water quality and phthalate concentrations during exposure of *Daphnia magna* to di-*n*-octyl phthalate

Nominal concentration (mg/L)	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Observed concentration (mg/L)	
				Fresh solutions	After 2-3 d with *D. magna*
Control	22.6 ± 0.08	7.2 ± 0.07	8.0 ± 0.02	<0.0001	0.007 ± 0.007
0.0	22.4 ± 0.06	5.1 ± 0.18	7.7 ± 0.01	<0.0001	0.002 ± 0.002
0,01	22.5 ± 0.04	5.3 ± 0.15	7.7 ± 0.01	0.008 ± 0.008	0.004 ± 0.002
0.03	22.4 ± 0.04	5.3 ± 0.16	7.7 ± 0.02	0.027 ± 0.004	0.033 ± 0.010
0.10	22.4 ± 0.05	5.3 ± 0.15	7.7 ± 0.01	0.093 ± 0.020	0.147 ± 0.043
0.32	22.6 ± 0.05	5.2 ± 0.14	7.7 ± 0.02	0.31 ± 0.09	0.44 ± 0.13
1.00	22.8 ± 0.05	5.3 ± 0.17	7.7 ± 0.02	1.24 ± 0.120	1.48 ± 0.43
3.20	22.9 ± 0.06	4.0 ± 0.15	7.7 ± 0.02	4.50 ± 0.64	3.44 ± 1.26

Values are means ± SE for all recorded measurements.

throughout the course of the exposure and was constant between treatments, except for the slightly higher DO levels in the carrier-free control solutions (Table 5). The concentration of DOP in the exposure beakers remained relatively constant over the course of the study; the nominal and observed concentrations agreed well, except at the highest dose in which the actual concentration was higher than the nominal. Solutions in which *D. magna* were maintained for 2 to 3 d were also analyzed for the levels of DOP remaining. The level of DOP was slightly higher after incubation of the animals, although the differences are not statistically significant (Table 5).

The results of the filtration experiment designed to measure the fraction of DOP bound to suspended food particles demonstrated that all of the DOP was bound to the food particles. Little, if any, of the phthalate ester was available for uptake from the dissolved phase.

Survival of the test animals exceeded 80%, except at the highest concentration (3.2 mg/L) in which only 45% remained alive through the end of the experiment (Table 6 and Fig. 2).

The number of young produced per surviving adult at each dose is indicated in Table 6. Reproduction in the carrier-free control was significantly lower than in the acetone control. The reason for this effect is not clear; however, a similar effect was observed in the reproduction test with DBP. Since the carrier control was identical in composition to the toxicant solutions, except for the presence of the DOP, this control seems the more appropriate one for comparison with the treated groups.

The number of young produced per surviving adult was not significantly different in *D. magna* exposed to doses of DOP from 0 (acetone control) to 0.32 mg/L, but reproduction was significantly reduced at 1.0 and 3.2 mg/L. The NOEC is 0.32 mg/L and the LOEC 1.0 mg/L.

Fathead minnow early life stage test

Effect of DBP. The results of the preliminary range-finding test with newly hatched fathead minnow larvae indicated that the LC50 for DBP was 2.02 mg/L (lower and upper 95% fiducial limits of 1.32 and 2.85 mg/L).

The embryo-larval early life stage test was conducted with doses of DBP ranging from 0.1 to 1.8 mg/L. Temperature, DO and pH remained constant over the exposure period (Table 7). The

Table 6. Effect of di-*n*-octyl phthalate on the survival and reproduction of *Daphnia magna*

Nominal concentration (mg/L)	Survival of adults (%)	Number of young per adult
Control	82.1 ± 11.6 (A)	19.1 ± 3.5 (B)
0	82.5 ± 9.3 (A)	79.4 ± 8.3 (A)
0.01	95.0 ± 5.0 (A)	84.5 ± 6.3 (A)
0.03	95.0 ± 5.0 (A)	82.3 ± 7.1 (A)
0.10	92.5 ± 5.0 (A)	77.6 ± 7.4 (A)
0.32	97.5 ± 2.5 (A)	71.2 ± 10.1 (A)
1.0	77.5 ± 10.7 (A)	19.9 ± 3.3 (B)
3.2	45.0 ± 12.2 (B)	18.3 ± 3.5 (B)

Values are means ± SE. For each variable, means with the same letter (in parentheses) are not significantly different ($p = 0.5$).

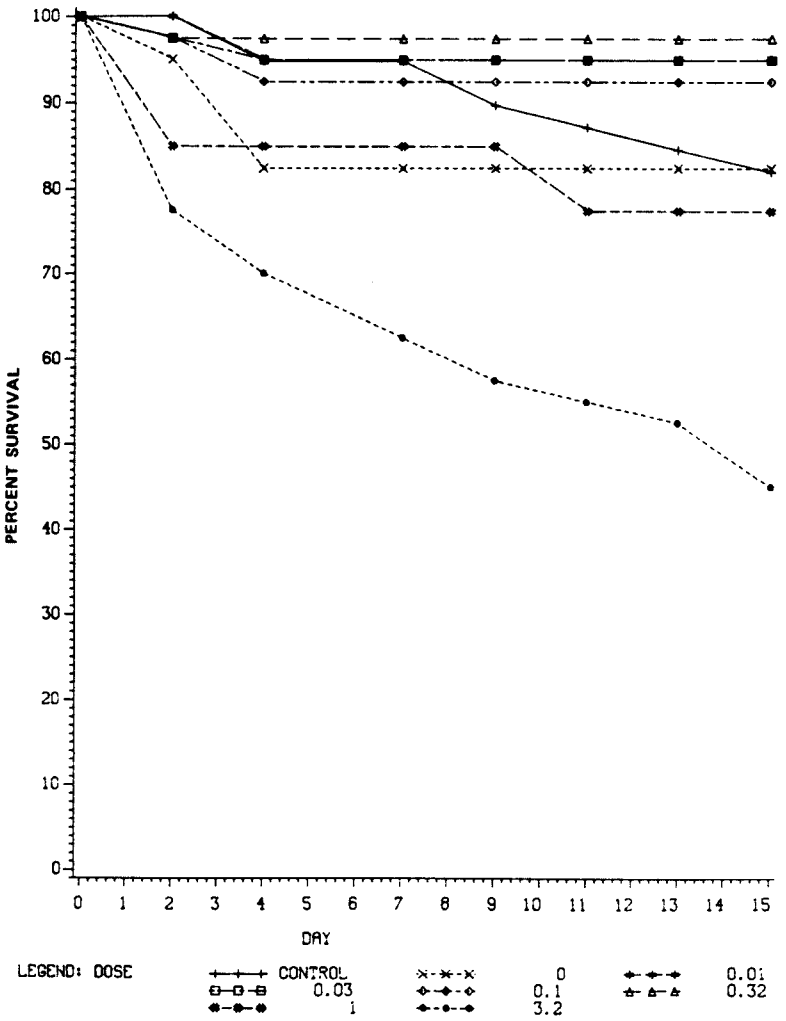

Fig. 2. Survival (%) of *Daphnia magna* exposed to di-*n*-octyl phthalate for 15 d.

Table 7 Water quality and phthalate concentrations during exposure of fathead minnow embryos and larvae to di-*n*-butyl phthalate

Nominal concentration (mg/L)	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Observed concentration (mg/L)
Control	22.3 ± 0.2	7.5 ± 0.1	8.3 ± 0.2	<0.001
0.0	22.7 ± 0.2	7.0 ± 0.2	8.2 ± 0.03	<0.001
0.10	23.1 ± 0.2	6.7 ± 0.2	8.2 ± 0.03	0.06 ± 0.01
0.18	23.1 ± 0.3	6.9 ± 0.2	8.2 ± 0.03	0.14 ± 0.02
0.32	23.3 ± 0.2	5.7 ± 0.2	8.2 ± 0.04	0.27 ± 0.02
0.56	23.9 ± 0.2	6.9 ± 0.3	8.2 ± 0.02	0.53 ± 0.05
1.0	24.1 ± 0.2	6.7 ± 0.2	8.2 ± 0.02	0.97 ± 0.10
1.8	24.5 ± 0.4	6.7 ± 0.5	8.3 ± 0.08	1.74 ± 0.22

Values are means ± SE for all recorded measurements.

concentration of DBP did not vary greatly over the experiment; the observed concentrations were very close to the nominal doses (Table 7).

Two replicates containing approximately 50 freshly deposited embryos (<48 h old) were exposed to each concentration of DBP or to control solutions. The survival of the embryos during the first 48 h of exposure was not significantly reduced by doses of DBP up to 1.0 mg/L; however, survival was significantly reduced after 48 h of exposure to 1.8 mg/L (Table 8). Exposure to the higher doses of DBP had a significant effect on hatching success. None of the embryos exposed to 1.8 mg/L succeeded in hatching. Hatching rate was significantly reduced at 1.0 mg/L DBP. Lower doses had no significant effect (Table 8).

The survival of the fish fry was followed through day 20; the experiment was terminated at this time due to an accident in the laboratory. Although the test did not continue for the recommended 28 d, the percentage of larvae surviving was fairly constant throughout the exposure period, except at 1.0 mg/L in which a large number of sac fry died shortly after hatching (Fig. 3). There is no evidence that the additional exposure time would have produced any meaningful differences in the test results. Larval survival was greater than 80% for animals exposed to doses of 0.56 mg/L or less (Table 8), but was significantly reduced at 1.0 mg/L. Since none of the embryos hatched at 1.8 mg/L, larval survival was not measured at this dose.

The NOEC for the fathead minnow early life stage test with DBP is 0.56 mg/L and the LOEC is 1.0 mg/L.

Effect of DOP. The results of the preliminary range-finding test with newly hatched fathead minnow larvae indicated that DOP did not reduce larval survival at concentrations from 0.32 to 32 mg/L, the highest dose tested. The highest dose exceeded the water solubility by several orders of magnitude.

Temperature, DO and pH varied only slightly over the course of the 34-d exposure period (Table 9). The concentration of DOP remained fairly constant over the course of the experiment; observed concentrations were slightly lower than the nominal concentrations (Table 9).

Two replicates of 30 embryos (24 to 48 h old) were exposed to each dose of DOP or to control solutions. None of the embryos died during the first 48 h of exposure. Continued exposure to DOP did not significantly decrease the survival or hatching success of the embryos in controls or in DOP-exposed animals at doses from 0.1 to 3.2 mg/L (survival and hatching rates exceeded 95%). At a dose of 10 mg/L, hatching dropped to 65%, a statistically significant decrease (Table 10).

The survival of the larvae was followed through 28 d of exposure. There was no significant decrease in the survival of the larvae in the controls through DOP doses of 10 mg/L; survival equaled or exceeded 80% at all doses (Table 10 and Fig. 4).

The length and weight of the larvae that were alive at the end of the experiment were determined for each replicate. There were significant differences in the length and even larger differences in the weight of larvae exposed to DOP (Table 11); however, the pattern of change did not follow any dose-dependent relationship. Also, density did not play a part since there was no correlation between the number of larvae in the exposure chambers and their final length or weight. Since the amount of food supplied to each chamber was varied

Table 8. Effect of di-*n*-butyl phthalate on the early life stages of the fathead minnow

Nominal concentration (mg/L)	Embryo survival at 48 h (%)	Hatching rate (%)	Larval survival (%)
Control	73.4 ± 9.0 (A)	68.7 ± 8.8 (A)	93.8 ± 0.4 (AB)
0	61.1 ± 6.9 (A)	56.5 ± 2.5 (AB)	98.2 ± 1.8 (A)
0.10	72.5 ± 3.9 (A)	66.0 ± 3.0 (A)	82.9 ± 4.0 (B)
0.18	74.5 ± 3.6 (A)	68.5 ± 6.5 (A)	85.9 ± 3.6 (AB)
0.32	67.5 ± 3.8 (A)	68.0 ± 7.0 (A)	94.0 ± 0.5 (AB)
0.56	54.0 ± 17.3 (A)	54.5 ± 16.5 (AB)	85.4 ± 9.2 (AB)
1.0	54.1 ± 8.7 (A)	28.0 ± 15.0 (CB)	20.8 ± 2.6 (C)
1.8	10.0 ± 10.0 (B)	0.0 ± 0 (C)	—

Values are means ± SE. For each variable, means with the same letter (in parentheses) are not significantly different ($p = 0.05$).

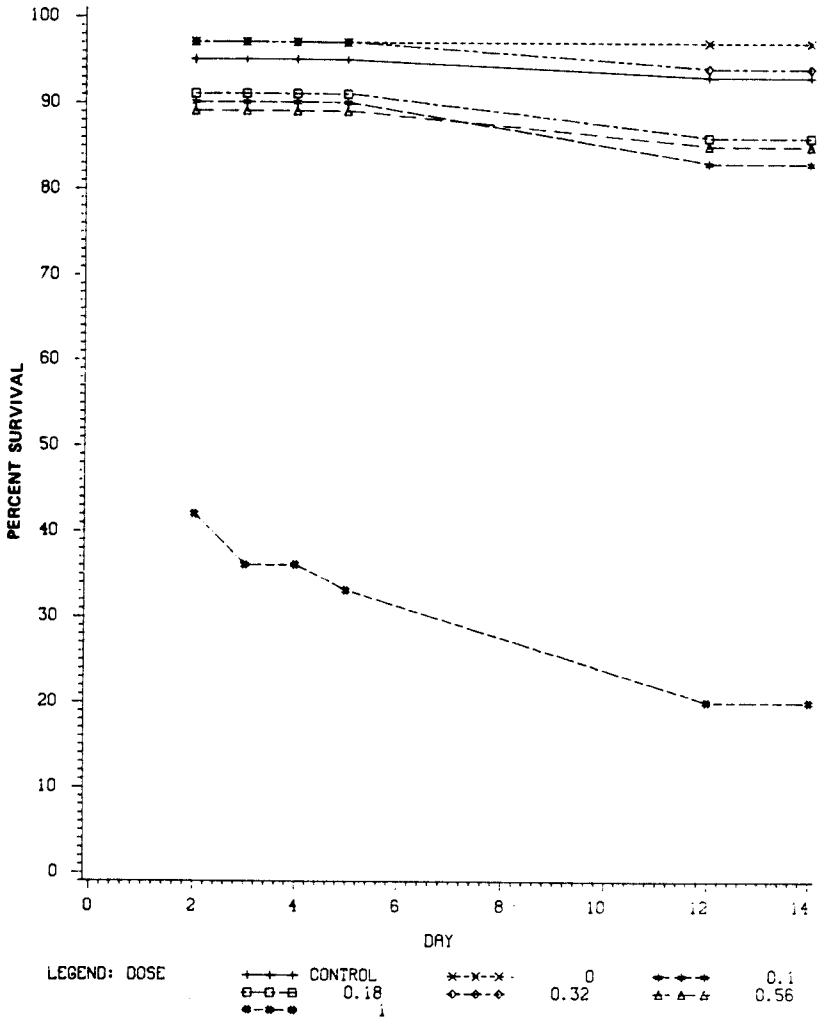

Fig. 3. Survival (%) of fathead minnow larvae exposed to di-*n*-butyl phthalate since the embryo stage. Day 0 is the day that larvae were released from the embryo cups.

Table 9. Water quality and phthalate concentrations during exposure of fathead minnow embryos and larvae to di-*n*-octyl phthalate

Nominal concentration (mg/L)	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Observed concentration (mg/L)
Control	22.1 ± 0.15	7.2 ± 0.06	8.4 ± 0.03	<0.001
0.0	23.2 ± 0.37	7.0 ± 0.11	8.2 ± 0.04	<0.001
0.10	23.5 ± 0.54	8.1 ± 0.11	8.2 ± 0.06	0.10 ± 0.02
0.32	22.9 ± 0.24	8.1 ± 0.10	8.2 ± 0.06	0.21 ± 0.05
1.0	23.5 ± 0.20	7.7 ± 0.14	8.3 ± 0.06	0.94 ± 0.36
3.2	23.4 ± 0.40	7.9 ± 0.13	8.2 ± 0.05	1.76 ± 0.43
10.0	23.6 ± 0.54	7.0 ± 0.13	8.2 ± 0.06	8.30 ± 0.90

Values are means ± SE for all recorded measurements.

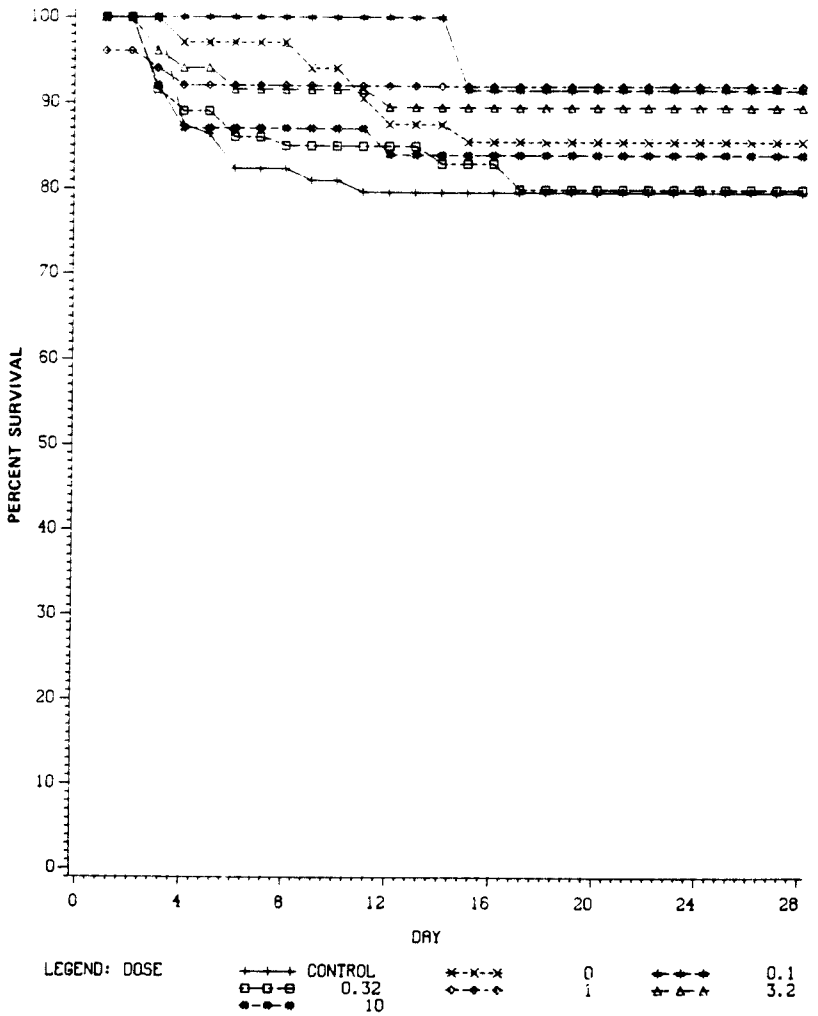

Fig. 4. Survival (%) of fathead minnow larvae exposed to di-*n*-octyl phthalate since the embryo stage. Day 0 is the day that larvae were released from the embryo cups.

Table 10. Effect of di-*n*-octyl phthalate on the early life stages of the fathead minnow

Nominal concentration (mg/L)	Embryo survival at 48 h (%)	Hatching rate (%)	Larval survival (%)
Control	100 ± 0 (A)	98.5 ± 1.5 (A)	79.7 ± 8.2 (A)
0	100 ± 0 (A)	100 ± 0 (A)	85.5 ± 105 (A)
0.10	98.5 ± 1.5 (A)	100 ± 0 (A)	91.5 ± 8.5 (A)
0.32	100 ± 0 (A)	98.5 ± 1.5 (A)	80.0 ± 7.0 (A)
1.0	96.5 ± 3.5 (A)	95.0 ± 5.0 (A)	92.0 ± 4.0 (A)
3.2	100 ± 0 (A)	100 ± 0 (A)	89.5 ± 10.5 (A)
10	100 ± 0 (A)	65.0 ± 22 (B)	84.0 ± 16.0 (A)

Values are means ± SE. For each variable, means with the same letter (in parentheses) are not significantly different ($p = 0.05$).

Table 11. Length and weight of fathead minnow larvae surviving exposure to di-*n*-octyl phthalate

Nominal concentration (mg/L)	Length (cm)	Weight (mg)
Control	1.5 ± 0.04 (A)	24.2 ± 2.0 (A)
0	1.5 ± 0.03 (A)	28.1 ± 2.1 (A)
0.10	1.3 ± 0.04 (CD)	15.5 ± 1.3 (BC)
0.32	1.2 ± 0.03 (D)	10.7 ± 1.3 (C)
1.0	1.4 ± 0.03 (AB)	18.6 ± 1.5 (B)
3.2	1.3 ± 0.03 (BC)	14.6 ± 1.1 (BC)
10.0	1.5 ± 0.05 (A)	25.1 ± 3.0 (A)

Values are means ± SE. For each variable, means with the same letter (in parentheses) are not significantly different ($p = 0.05$).

according to the number of larvae present, food availability should not have played a role in the observed differences.

Based on the hatching success of the fathead minnows, the most sensitive parameter in this experiment, the NOEC for DOP is 3.2 mg/L and the LOEC is 10 mg/L.

DISCUSSION

Although there have been several reports on the toxicity of DBP to aquatic organisms, almost nothing is known about the toxicity of DOP. In the range-finding test with DBP, acute mortality was observed at levels of 2 to 7 mg/L for both *D. magna* and the larval fathead minnows. The LOECs for chronic, sublethal effects on reproduction of *D. magna* or on survival of fathead minnow early life stages was 1 to 1.8 mg/L DBP. These results are within the range of toxic or chronic effect concentrations previously reported for other aquatic organisms. The 48-h EC50 for midge larvae was 0.76 mg/L [3] and the 96-h LC50 for amphipods was 2 mg/L [4,5]. The survival and development rate of larval grass shrimp were unaffected by 30-d exposures to levels of DBP up to 1 mg/L [6]. The grass shrimp did die rapidly at DBP concentrations of 10 to 50 mg/L; the effect was thought to be due to the presence of coalesced droplets of DBP at concentrations beyond the solubility limits of the phthalate [6]. The acute toxicity of DBP (96-h LC50) in fish ranged from 0.7 and 1.3 mg/L for bluegills and fathead minnows, respectively, to 6.5 mg/L for trout [4,5]. DBP was reported to decrease the hatchability of brine shrimp eggs at levels of 10 mg/L [7] and to decrease the heart rate of goldfish within minutes after exposure to 5 mg/L [8]. On a community level, 2 weeks of exposure to 3.7 mg/L of DBP decreased the abundance and diversity of laboratory-developed estuarine benthic communities; the next lowest treatment level of 0.34 mg/L had a small, but statistically significant, effect on community structure, and 0.04 mg/L had no effect [9]. DBP has also been reported to affect the growth of phytoplankton: Exposure to levels of 5 mg/L over 4 d decreased the growth rate of *Skeletonema* [10], whereas exposure to 0.2 mg/L over 4 d reduced the growth rate of *Gymnodinium* [11]. In general, both chronic and acutely toxic levels of DBP appear to fall in the range of 0.5 to 5 mg/L for a wide variety of aquatic organisms. This range corresponds to 5 to 50% of the aqueous solubility limit of DBP [12].

The only other phthalate ester that has been extensively studied is di-2-ethylhexyl phthalate (DEHP), and there is less agreement on levels of DHEP that cause toxic effects than there is for DBP, especially with respect to its effect on reproduction of *D. magna*. While Mayer and Sanders [4] reported reduced reproduction at 3 to 30 μg/L, Brown and Thompson [13] found that doses up to 100 μg/L DEHP (the highest dose tested) had no significant effect. The large difference in the reproductive rate observed in the two studies is notable: Mayer and Sanders reported an average of only 11 offspring produced per adult over 3 weeks, compared with 170 offspring per adult over the 3 weeks of the Brown and Thompson study. In the present study, an average of 70 to 80 offspring was produced per adult over 2 weeks, a reproductive rate comparable with that in the Brown and Thompson study. The greater tolerance to DEHP reported by Brown and Thompson is also in general agreement with our result for DBP and DOP (NOECs of 0.56 and 0.32 mg/L, respectively).

Mayer and Sanders [4] and Mehrle and Mayer [14] also reported that survival of trout sac fry was affected by very low levels of DEHP (5 to 14 μg/L). In contrast, long-term exposure to concentrations up to 100 μg/L did not affect the survival or growth of adult fathead minnows or rainbow trout (60-d exposures) or of *D. magna* (21-d exposures) or *Mytilus* (28-d exposures) [13–15], and 1 mg/L did not affect survival or growth of grass shrimp larvae [6]. The 48-h EC50 for midge larvae exposed to DEHP was greater than 18 mg/L, whereas chronic effects were seen at levels greater than 0.36 mg/L [3]. Acute toxicity to

DEHP was not observed below concentrations of 32 mg/L in amphipods [5] or in bass, trout or goldfish [16]. (Reference 16 identifies the toxicant as dioctyl phthalate, but the correct nomenclature is DEHP.) Since the aqueous solubility limit of DEHP is approximately 1 mg/L [6], reports of toxic levels far in excess of the solubility limit are difficult to interpret.

The problem of solubility limits versus apparent toxic levels of a contaminant is even more obvious in the present study with DOP. The aqueous solubility of DOP, based on retention time on reversed phase high performance liquid chromatography columns [17] and on the amount passing through a 0.45-μm silver filter, is in the low microgram per liter range (M. Maskarinec, ORNL, personal communication). Yet, acute toxicity to DOP was not seen even at levels of 10 to 32 mg/L in either *D. magna* or larval fathead minnows. The hatching success of fathead minnow embryos was not deleteriously affected at DOP concentrations below 10 mg/L. At this exposure level, an obvious surface slick of DOP was visible in the exposure chambers. It is unclear whether the reduced rate of hatching to viable larvae was due to uptake of DOP from the water, or due to the embryos or larvae coming in contact with the surface slick. Reproduction of *D. magna* was deleteriously affected at 1 mg/L; however, in this experiment, all of the DOP was bound to the food particles. In this case, the toxic effect could be due to uptake of the phthalate from the contaminated food.

These results raise questions about how to interpret structure-activity relationships for different phthalates that vary so widely in solubility. The toxicity may be controlled as much by the mode of exposure as by the direct toxic properties of the contaminant. For compounds that are toxic at levels below their solubility limit, such as DBP, the effect is probably due to the contaminant being taken up from the water and exerting a direct effect on the organism. Reports of acute or chronic toxicity under these conditions can be interpreted in a straightforward manner. When the deleterious effect is observed at concentrations above the solubility limit, interpretation of the results is confounded by the partitioning of the compound within the environment (e.g., sorption to particulate or dissolved organic matter in the water or to the sediment and the sides of exposure chambers) and the relative availability of the contaminant in these different phases for uptake by organisms. The sorption of phthalate esters to clay minerals, calcite and sediment is inversely proportional to the aqueous solubility of the phthalate [18]. This relationship has been observed for a wide variety of organic contaminants [19,20]. In a microcosm experiment with DEHP, 62% of the phthalate was recovered from the glass, sediment or surface microlayer [21]. In the present experiment with DOP, all of the contaminant was bound to the food particles during the exposure of the *D. magna*.

Since a basic toxicological assumption is that the effect of a contaminant will be related to the amount taken up by the animal (dose), the relative availability of contaminant from the various phases within the environment must be considered in interpreting the toxicity data. For polynuclear aromatic hydrocarbons (PAHs), the rate coefficient for uptake of the contaminant in true solution in the water is 10-fold greater than the uptake rate for contaminant sorbed to food particles [22]; i.e., for every mole of the chemical bound to particles, 90% is unavailable for uptake by filter-feeders. The interaction of phthalates with dissolved organic matter in the water [23,24] may also reduce their bioavailability in the same way that hydrophobic contaminants (PAHs) bound to dissolved organic matter are essentially unavailable for uptake by fish or by *D. magna* [25]. Uptake of PAH contaminants sorbed to the sediment is also much lower than is uptake of dissolved material [26].

From the structure-activity perspective, these observations suggest that the activity of a phthalate may be a function of the chemical properties of the contaminant affecting its partitioning within the environment (related, for example, to the solubility of the compound) as much as a function of the toxicological properties acting within the organism.

Acknowledgements—The authors would like to express their appreciation to Dr. J. J. Beauchamp for his assistance in the statistical analysis and to Dr. M. Maskarinec for performing the chemical analyses. We also thank J. L. Keller and Dr. A.S. Bradshaw for supplying the *D. magna* cultures, Drs. B. G. Blaylock and J. M. Giddings for critical review of the manuscript, and P. T. Singley, Dr. B. D. Jimenez and C. P. Cirmo for technical assistance and advice during the experiments.

REFERENCES

1. **Organization for Economic Cooperation and Development.** 1981. OECD guidelines for testing of chemicals: *Daphnia* sp., acute immobilization test and reproduction test. No. 202.
2. **Benoit, D.A.** 1981. User's guide for conducting life-cycle chronic toxicity tests with fathead minnows

(*Pimephales promelas*), EPA-60018-81-011. U.S. Environmental Protection Agency, Washington, DC.

3. **Streufert, J.M., J.R. Jones** and **H.O. Sanders**. 1980. Toxicity and biological effects of phthalate esters on midges (*Chironomus plumosus*). *Trans. Missouri Acad. Sci.* **14**:33-40.
4. **Mayer, F.L.** and **H.O. Sanders**. 1973. Toxicology of phthalic acid esters in aquatic organisms. *Environ. Health Perspect.* **3**:153-157.
5. **Sanders, H.O., F.L. Mayer** and **D. F. Walsh**. 1973. Toxicity, residue dynamics, and reproductive effects of phthalate esters in aquatic invertebrates. *Environ. Res.* **6**:84-90.
6. **Laughlin, R.B., J.M. Neff, Y.C. Hrung, T.C. Goodwin** and **C.S. Giam**. 1978. The effects of three phthalate esters on the larval development of the grass shrimp, *Palaemonetes purgio* (Holthuis). *Water Air Soil Pollut.* **9**:323-336.
7. **Sugawara, N.** 1974. Effect of phthalate esters on shrimp. *Bull. Environ. Contam. Toxicol.* **12**: 421-424.
8. **Pfuderer, P.** and **A.A. Francis**. 1975. Phthalate esters: heartrate depressors in the goldfish. *Bull. Environ. Contam. Toxicol.* **13**:275-279.
9. **Tagatz, M.E., C.H. Dean, J.C. Moore** and **G.R. Plaia.** 1983. Alterations in composition of field- and laboratory-developed estuarine benthic communities exposed to di-*n*-butyl phthalate. *Aquat. Toxicol.* **3**:239-248.
10. **Medlin, L.K.** 1980. Effects of di-*n*-butyl phthalate and salinity on the growth of the diatom *Skeletonema costatum. Bull. Environ. Contam. Toxicol.* **25**:75-78.
11. **Wilson. W.B., C.S. Giam, T.E. Goodwin, A. Aldrich, V. Carpenter** and **Y.C. Hrung.** 1978. The toxicity of phthalates to the marine dinoflagellate *Gymnodinium breve. Bull. Environ. Contam. Toxicol.* **20**:149-154.
12. **Schwarz, F.P.** and **J. Miller**. 1980. Determination of the aqueous solubilities of organic liquids at 10.0, 20.0, and 30.0°C by elution chromatography. *Anal. Chem.* **52**:2162-2164.
13. **Brown, D.** and **R.S. Thompson**. 1982. Phthalates and the aquatic environment: Part I. The effect of di-2-ethylhexyl phthalate (DEHP) and di-isodecyl phthalate (DIDP) on the reproduction of *Daphnia magna* and observations on their bioconcentration. *Chemosphere* **11**:417-426.
14. **Mehrle, P. M.** and **F.L. Mayer**. 1976. Di-2-ethylhexyl phthalate: Residue dynamics and biological effects in rainbow trout and fathead minnows. In D. D. Hemphill, ed., *Trace Substances in Environmental Health-X.* University of Missouri, Columbia, MO, pp. 519-524.
15. **Brown, D.** and **R.S. Thompson.** 1982. Phthalates and the aquatic environment: Part II. The bioconcentration and depuration of di-2-ethylhexyl phthalate (DEHP) and di-isodecyl phthalate (DIDP) in mussels (*Mytilus edulis*). *Chemosphere* **11**:427-435.
16. **Birge, W.J., J.A. Black** and **D.M. Bruser**. 1979. Toxicity of organic chemicals to embryo-larval stages of fish. EPA-560/11-79-007. U.S. Environmental Protection Agency, Washington, DC.
17. **Veith, G.D.** and **R.T. Morris**. 1978. A rapid method for estimating Log P for organic chemicals. U.S. Environmental Protection Agency, Ecological Research Series EPA-600/3-78-049, Duluth, MN.
18. **Sullivan, K.F., E.L. Atlas** and **C.S. Giam.** 1982. Adsorption of phthalic acid esters from seawater. *Environ. Sci. Technol.* **16**:428-432.
19. **Karickhoff, S.W., D.S. Brown** and **T.A. Scott.** 1979. Sorption of hydrophobic pollutants on natural sediments. *Water Res.* **13**:241-253.
20. **Means, J.C., J.J. Hassett, S.G. Wood** and **W.L. Banwart.** 1979. Sorption of energy-related pollutants and sediments. In P. W. Jones and P. Leber, eds., *Polynuclear Aromatic Hydrocarbons.* Ann Arbor Science Publishers, Ann Arbor, MI, pp. 327-340.
21. **Sodergren, A.** 1982. Significance of interfaces in the distribution and metabolism of di-2-ethylhexyl phthalate in an aquatic laboratory model ecosystem. *Environ. Pollut.* **27**:263-274.
22. **McCarthy, J. F.** 1983. Role of particulate organic matter in decreasing accumulation of polynuclear aromatic hydrocarbons by *Daphnia magna. Arch. Environ. Contam. Toxicol.* **12**:559-568.
23. **Boehm, P. D.** and **J. G. Quinn.** 1973. Solubilization of hydrocarbons by the dissolved organic matter in sea water. *Geochem. Cosmochim. Acta* **37**:2459-2477.
24. **Ogner, G.** and **M. Schnitzer.** 1970. Humic substances: Fulvic acid-dialkyl phthalate complexes and their role in pollution. *Science* **170**:317-318.
25. **McCarthy, J. F.** 1983. Effect of environmental variables on the bio-availability and metabolic fate of polynuclear aromatic hydrocarbons. ORNL-6009. Oak Ridge National Laboratory, Oak Ridge, TN.
26. **Landrum, P. F.** and **D. Scavia.** 1983. Influence of sediment on anthracene uptake, depuration, and biotransformation by the amphipod *Hyalella azteca. Can. J. Fish. Aquat. Sci.* **40**:298-305.