Bull. Environm. Contam. Toxicol. 20, 149-154 (1978)

The Toxicity of Phthalates to the Marine Dinoflagellate *Gymnodinium breve*

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INTRODUCTION

The acute toxicity levels of seven phthalate compounds to Gymnodinium breve (the fish-killing blooms of which are popularly known as "red tide") were determined by subjecting culture portions of this organism to various concentrations of these compounds. The compounds tested were dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-propyl phthalate (DPP), di-n-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), potassium hydrogen phthalate and phthalic acid, disodium salt. Over one billion pounds of phthalic acid esters (PAE's) were produced in 1976 (CEKIS 1976), and these substances have become widely-dispersed in the environment (GIAM 1975).

METHODS

Cultures of Gymnodinium breve that were grown in an artificial sea water medium--NH-15 (GATES and WILSON 1960) were used in all assays and were transferred 14 days prior to use so that they would be in an active logarithmic growth phase. The cultures contained between 1000 and 5000 G. breve per milliliter at the time the assays were started. A 25°C constant temperature room with approximately 1000 foot-candles of light supplied by 40-watt, cool-white, fluorescent lights was utilized for culture growth.

Saturated solutions of four compounds--di-n-propyl, dibutyl, diethyl and dimethyl phthalate were prepared by placing 10.0 ml of each compound and 90.0 ml of NH-15 medium in a separatory funnel, shaking for five minutes and allowing the mixture to remain undisturbed for a ten-minute period. The more dense, undissolved phthalates settled to the bottom and were removed. The aqueous supernatant was considered to be a saturated solution and was used in the assays. In addition, a portion of each of the four saturated solutions was frozen and later analyzed (GIAM 1975) to determine the phthalate concentrations of the saturated solutions (Table 1).

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0007-4861/78/0020-0149 $01.20

TABLE 1

CONCENTRATION OF PHTHALATES IN SATURATED AQUEOUS SOLUTIONS

Compound	Measured Concentration (ppm)
DMP	1744
DEP	210
DPP	56
DBP	10.6

Dilutions of the saturated phthalate solutions were made with NH-15 medium as detailed in Table 2. Five ml of each of these dilutions (right column, Table 2) was added to 5 ml of G. breve culture, in a 16 x 125 mm Pyrex brand, disposable test tube with a polypropylene cap. This resulted in test concentrations expressed as "percent saturated solutions" as follows: 50, 25, 10, 5, 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01. The phthalate concentrations (ppm) of each of these dilutions are shown in Table 3, and are based on the measured values for saturated solutions (Table 1). "No add" controls consisted of 5.0 ml of culture and 5.0 ml of NH-15. Control 2 consisted of 5.0 ml of the saturated solutions (undiluted) and 5 ml of NH-15. All test portions and controls were set up in triplicate.

TABLE 2

STOCK DILUTIONS OF SATURATED SOLUTIONS

Milliliters of Saturated Solutions	Milliliters of NH-15	Resulting "Percent Saturated Solutions"
20	0	100
10	10	50
5	20	25
2	18	10
0.4	19.6	2
2 ml of 10%	18	1
0.1	24.9	0.4
2 ml of 2%	18	0.2
2 ml of 1%	18	0.1
2 ml of 0.4%	18	0.04
2 ml of 0.2%	18	0.02

TABLE 3

% Saturated Solution	CONCENTRATION (ppm) OF PHTHALATES			
	DMP	DBP	DPP	DEP
0.01	0.17	0.001	0.006	0.02
0.02	0.34	0.002	0.012	0.04
0.05	0.85	0.005	0.03	0.1
0.1	1.7	0.01	0.06	0.2
0.2	3.4	0.02	0.11	0.4
0.5	8.5	0.05	0.28	1.05
1.0	17	0.11	0.57	2.1
5.0	85	0.53	2.85	10.5
10.0	174	1.06	5.7	20.9
25.0	435	2.65	14.25	52
50.0	872	5.3	28	105

The fifth compound tested, diethylhexyl phthalate (DEHP), was added directly to appropriate amounts (9.0 to 10.0 ml) of G. breve culture, i.e. without preparation of a saturated solution. This was done because preliminary experiments indicated that the toxicity of this compound was low and would possibly not be detectable in a saturated solution. The percentages of this compound tested and the method of preparation were as shown in Table 4 (dilutions made with NH-15 medium).

TABLE 4

DILUTIONS OF DEHP

	Amounts of DEHP	Milliliters of Culture	Resulting Percentage of DEHP
(No add)	0	10	0
Control 2	1.0	0 (9 ml of NH-15)	10
	1.0 ml	9	10
	0.5	9.5	5
	0.2	9.8	2
	0.1	9.9	1
	0.1 of 50% dil.	9.9	0.5
	0.1 of 20% dil.	9.9	0.2
	0.1 of 10% dil.	9.9	0.1

Potassium hydrogen phthalate and the disodium salt of phthalic acid were assayed by preparing 1% solutions of these compounds in distilled water, i.e. 1.0 g of the compound in 100 ml of distilled water. The schedule for these assays was as shown in Table 5.

TABLE 5

DILUTIONS OF POTASSIUM HYDROGEN PHTHALATE AND PHTHALIC ACID, DISODIUM SALT

Dilution of 1% Solution	Milliliters of Dilution	Milliliters of Culture	Resulting Conc. of Compound (ppm)
(No add)	1.0 ml*	9.0	0
Control 2	1.0	9.0 (NH-15)	1000
--	1.0	9.0	1000
--	0.5	9.5	500
--	0.2	9.8	200
--	0.1	9.9	100
1 in 2	0.1	9.9	50
1 in 4	0.1	9.9	20
1 in 10	0.1	9.9	10
1 in 100	0.1	9.9	1

*Distilled water

Test portions were placed in a single row in test tube racks which were placed 2 inches from 40-watt, cool-white fluorescent lights (incident light ca. 1000 foot-candles) in a 25°C, constant-temperature room. Special care was taken to insure uniform light on test portions to avoid variability of relative chlorophyll a values. These conditions were basically the same as conditions employed for culture growth. Microscopic examinations and relative chlorophyll a measurements were made as soon as the portions were set up, and 24, 48, 72 and 96 hours thereafter. Initial counts of the culture population level were made prior to setting up the test portions and counts were made of each test portion after 96 hours.

RESULTS AND CONCLUSIONS

The TLM_{96} (median tolerance limit, 96 hour exposure) values were determined by plotting on semilog paper the concentration of test material (ppm) versus the percent survival (linear axis of

graph) and recording the concentration responsible for 50 percent survival. These data for two replicate tests of the four most toxic compounds are listed in Table 6.

TABLE 6

MEDIAN TOLERANCE LIMITS (TLM_{96}, ppm) FOR FOUR PHTHALATES

Compound	First Assay	Second Assay
DBP	0.6	0.02
DPP	6.5	1.3
DEP	33.0	23.5*
DMP	125.0	185.0

*This is a TLM_{24} value.

The EC_{50} (median growth limit concentration) values were obtained by plotting on semilog paper the concentration of test material (ppm) versus the growth rate (linear axis of graph), expressed as a percentage of the growth of a "no-add" control. The concentration values (ppm) which caused a 50 percent growth reduction are shown in Table 7.

TABLE 7

MEDIAN GROWTH LIMIT CONCENTRATIONS (EC_{50}, ppm) FOR FOUR PHTHALATES

Compound	First Assay	Second Assay
DBP	0.2	0.0034
DPP	2.4	0.9
DEP	6.1	3.0
DMP	96.0	54.0

TLM_{96} and EC_{50} data indicate DBP to be the most toxic of the four substances listed in Tables 6 and 7. DEHP and the disodium salt of phthalic acid exhibited little acute toxicity even at the higher test concentrations (Tables 4 and 5). The highest concentration of DEHP tested (10%) did not reduce the culture populations significantly, but they did not grow in high concentrations of this phthalate (EC_{50} = 3.1%). A significant toxicity of potassium hydrogen phthalate was evident at concentrations of 500 and 1000 ppm. We suspected the pH of these media (4.2) to be responsible, thus an additional assay was carried out in which the pH

of the one percent solution of potassium hydrogen phthalate was adjusted to 7.6 with potassium hydroxide (yielding the dipotassium salt *in situ*) before adding aliquots to the culture portions. This adjustment alleviated the observed toxicity. Additional studies of the type described herein are in progress, the results of which will be described in due course.

ACKNOWLEDGEMENT

The financial support of the National Science Foundation, International Decade of Oceanographic Exploration (NSF-IDOE) is gratefully acknowledged.

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