Bull. Environ. Contam. Toxicol. (2003) 71:602–608

DOI: 10.1007/s00128-003-8559-9

Bulletin of Environmental Contamination and Toxicology

Toxicity of Dibutyl Phthalate to Algae

Q. J. Kuang,[1] W. Y. Zhao,[1,2,3] S. P. Cheng[1]

[1] Institute of Hydrobiology, The Chinese Academy of Sciences, Wuhan 430072, People's Republic of China
[2] Graduate School, The Chinese Academy of Sciences, Beijing 100039, People's Republic of China
[3] Guilin University of Electronic Technology, Guilin 541000, People's Republic of China

Received: 18 March 2002/Accepted: 9 May 2003

Phthalate acid esters (PAEs, phthalates) are mainly used as plasticizers and constituents in many commercial products such as paper coatings, cosmetics, adhesives, inks, paints, and so on (Yan et al. 1995). Capacity and consumption of PAEs in China and the world is increasing. Due to being ubiquitous environmental contaminants and to their effects on organisms' behavior, they have been of concern. The research group of environmental priority pollutants in China (1989) and Keith and Telliad (1979) have listed PAEs as 'priority pollutants'. There have been reports of toxic effects on phytoplankton, zooplankton, arthropods and fish (Huang et al. 1999; Thuren 1991; Defoe 1990; Yan et al. 1995). Accumulation of PAEs in organisms may be irreversible, being adverse for natural growth and reproduction and even harmful for continuation of the species.

Dibutyl phthalate (DBP) is one of the most commonly used PAEs in many countries, including China. In recent years, some of PAEs, like DBP, have been reported to have weak carcinogenic and estrogenic effects (Pederson and Larson 1996) in many kinds of aquatic animals (Patyna and Cooper 2000) and hydrophytes (Staples et al. 1985). DBP may even affect male reproductive health (Foster et al. 2000), and, therefore, threaten the freshwater ecosystem. This study evaluated the toxicity of DBP on *Scenedesmus obliquus* and natural mixed algae in order to assess potential environmental hazard of this PAE.

MATERIALS AND METHODS

The Unicellular Algal Toxicity Test (UAT), in the laboratory, and the Natural Mixed Algae Test (NAT), outdoors, were conducted, following the standard method [APHA, 1980] and Kuang et al. (1997), respectively.

Dibutyl phthalate, made in Shanghai, China (purity$\geqslant$99%), was prepared with acetone at a final concentration of 400 mg/L as a stock solution. Six definitive test concentrations (0, 10, 20, 40, 60, 80 mg/L) of DBP were selected for the

Correspondence to: Q. J. Kuang

UAT, and five for the NAT (0, 10, 20, 40, 60 mg/L) based on a preliminary range-finding test.

For the UAT, the test algae was *Scenedesmus obliquus*, obtained from the Department of Pathology, the Institute of Hydrobiology (Academia Sinica, China) and batch cultured by Kuang et al. (1997). Culture medium HB-4 is a common medium for unicellular green algae. It consists of $(NH_4)_2SO_4$ 0.20g, $MgSO_4.7H_2O$ 0.08g, $NaHCO_3$ 0.30g, $Ca(H_2PO_4)_2$ 0.030g, KCl 0.025g, 1% $FeCl_3$ 0.15 mL, soil solution 1.0 mL, distillate water 1000 mL, pH 7.30. Culture vessels were 150-mL glass flasks, which were autoclaved at 180℃ for 2hr before use. The flasks contained 25 mL of double HB-4 medium, 5 mL of *S. obliquus* batch cultures and DBP at the selected concentration. Distilled water was used to reach 50 mL volume. The final cell density of *S. obliquus* was $5\text{-}7\times10^5$/mL. Triplicate algae cultures were exposed in the test medium to each DBP concentration. The experiment was repeated three times. The flasks were cultivated in a constant temperature room at 24±2℃ with the light intensity of about 4000 μ Es^{-2} m^{-2} for four days. The light and dark cycle was 14:10 hr.

For the NAT, the test algae were natural mixed algae which were collected with a 25# plankton net from the East Lake (Wuhan, China), and cultured in the same lake water enriched with $Ca(NO_3)_2$ (0.04 mg/L, as NO_3^-) and K_2HPO_4 (0.01 mg/L, as PO_4^{3-}) in order to avoid weak concentrations of algal growth. Culture vessels were five unenclosed buckets of 50-L volume, cleaned completely before using. Enriched lake water (50 L) was filled into the culture buckets. Natural mixed algae were added to the buckets as much as possible. The initial pH, NH_4-N and PO_4-P concentrations of the lake water samples were 7.55, 0.103 mg/L and 0.052 mg/L. Initial cell densities were determined for each bucket as described below, prior to addition of the appropriate amount of DBP to all but the control container. These buckets were open to the atmosphere. Air temperature varied between 16-24℃ with natural light during the testing. Tap water was added every two days to replace evaporated water. The culture period was seven days.

Algal growth was determined by absorbance (A_{650}), algal cell counts and chlorophyll *a* content for the UAT, and by cell density and species numbers for the NAT. A_{650} was determined daily using a spectrophotometer at 650nm. Algal cells were counted with a hemocytometer every other day. Chlorophyll *a* content was measured at 96hr when the acute toxicity test was ended. One flask of culture medium among the triplicate cultures with the same DBP concentration was used for extracting the pigments from the test algae concentrate with aqueous acetone, and the absorbance of the extract was determined with a spectrophotometer. The other two culture flasks were combined together and maintained continually under

the same conditions mentioned above to examine chronic effects. The average 96hr-EC_{50} of DBP on *S. obliquus* was calculated by statistical methods (SGMI 1979) for each endpoint. The cell densities of natural mixed algae were counted by a 0.1 mm^3 plankton counting chamber under a microscope once every two days. Limiting percentage was calculated by the ratio between treated cells and the initial cells in each bucket.

RESULTS AND DISCUSSION

Table 1 shows the exact values of A_{650}, cell counts and chlorophyll *a* content of *S. obliquus* exposed to different DBP concentrations. The inhibition percentages of DBP on A_{650} are shown in Figure 1. Figure 2 compares the effects of DBP on the A_{650}, cell counts and chlorophyll *a* content at 96hr. The data reveal

Table 1. Changes of A_{650}, cell counts ($\times 10^5$ cells/mL) and chlorophyll *a* content (mg/m^3) of *S. obliquus*

DBP	A_{650}				Cell counts		Chl. *a*
mg/L	24hr	48hr	72hr	96hr	48hr	96hr	96hr
0.0	0.714	1.128	1.482	1.884	13.71	20.3	26.53
10.0	0.636	0.822	1.039	1.269	9.79	13.47	20.07
20.0	0.580	0.736	0.940	1.162	8.51	10.89	16.17
40.0	0.513	0.660	0.768	0.956	7.70	9.28	14.27
60.0	0.459	0.576	0.723	0.815	6.69	8.22	10.23
80.0	0.390	0.522	0.603	0.644	6.01	6.23	7.90

that the three parameters of *S. obliquus* were declining (Table 1), while inhibition percentages were increasing with prolonged exposure time and increasing concentration. The 96hr-EC_{50} of DBP on cell densities, A_{650} values, and chlorophyll *a* content of *S. obliquus* were 30.2 mg/L, 39.8 mg/L and 44.7 mg/L, respectively. Cells were most sensitive to DBP possibly because the dead cells could be distinguished when counting cells under a microscope, while dead cells still contributed to absorbance.

It was reported that acute toxicity values of DBP to green algae, *Daphnia magna* and *Lepomis macrochiru* were between 1-10 mg/L (Adams et al. 1995), and to *S. obliquus* was 0.21 mg/L (Huang et al. 1999). However, in this study DBP was less toxic, perhaps due to differences in experimental protocols. This requires further research.

In order to prove the reliability of the experiment, our data (shown in Fig. 2) were fit to the following equations: algal cell count inhibition $Y = 15.78\ln(X)-2.5056$, $r^2 = 0.9747$; chlorophyll *a* inhibition $Y = 20.797\ln(X) - 24.509$ $r^2 = 0.9622$; and

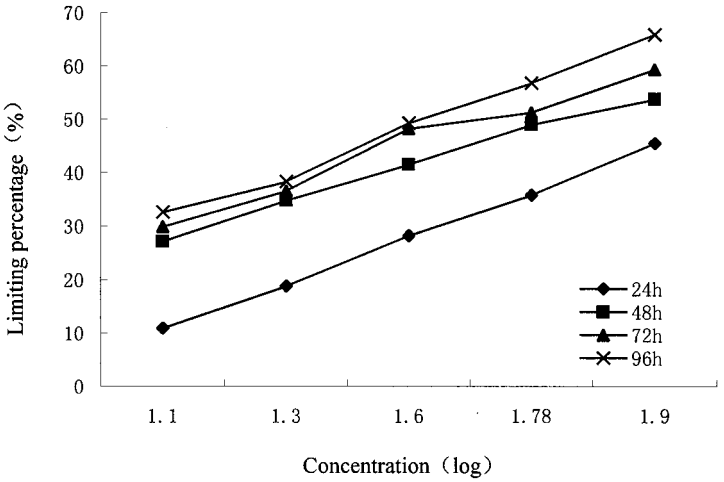

Figure 1. Inhibition curves of DBP on A_{650} of *S. obliquus.*

inhibition of A_{650}: Y = 0.4684Ln(X)+28.882 r^2 = 0.9946; where Y is the inhibition percentage and X is the algal cell count, chlorophyll *a* content, or A_{650}, respectively. Since the r^2 values all exceed 0.95, the toxicity data is of high quality.

The chronic test showed that the color of the culture medium in each treatment flask deepened gradually after the third week of incubation. No observed difference could be found between the treated flasks and control flasks until the fourth week by the naked eye. The value of A_{650} was only a little lower than the control. However, it was observed that the cell wall was thickened, with coarse, granular cell inclusions. This pathology was not observed in control cells. The reason for this was perhaps adsorption by the glass flasks, biodegradation by the test algae, as well as the photochemical action during the inoculation period. Actually, the concentration of DBP in each flask declined during treatment.

The intention of simulating natural conditions was to examine the effects of DBP on natural mixed algae. The changes of cell density of natural algae in each test bucket are shown in Table 2, and the inhibition percentage is shown in Figure 3. It can be seen clearly that the effects of DBP on natural algae was also marked. The cell density of algae exposed to DBP was decreased significantly with prolonged exposure. Cell density in the control bucket was also decreased because natural conditions were much more complex. Compared with the results obtained from the laboratory test, natural algae were more tolerant to DBP than pure unicellular algae. The inhibition percentage of DBP on natural algae was lower

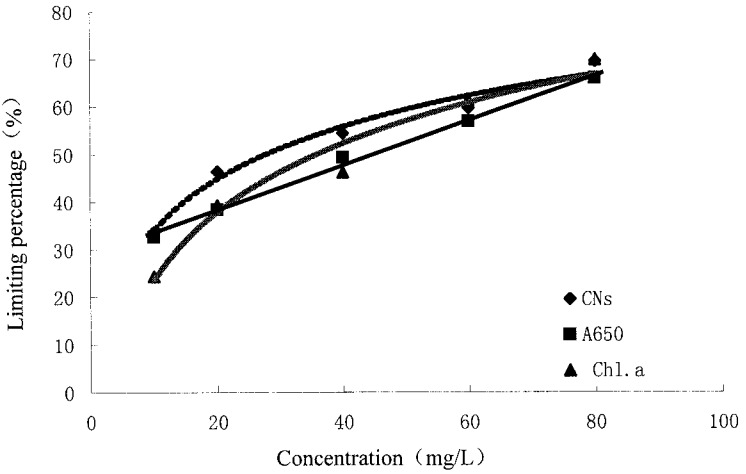

Figure 2. Effect of DBP on cell counts, A_{650} and chlorophyll a content of *S. obliquus* at 96hr.

than that on *S. obliquus* with the same DBP concentration and exposure time. This suggests that different algal species had varying reactions to DBP.

Table 2. Changes of cell density of natural mixed algae in the study outdoors

Bucket No.	DBP	Cell counts ($\times 10^4$/L)				%*		
	mg/L	Initial	48hr	96hr	144h	48hr	96hr	144h
1	0.0	8457	8360	8236	8195	1.15	2.61	3.10
2	10.0	8499	7161	6592	5295	15.74	22.44	37.70
3	20.0	8612	6730	5179	4233	21.85	39.86	50.85
4	40.0	8541	5438	4955	3763	36.33	41.99	55.94
5	60.0	8453	4774	4026	3316	43.52	52.37	60.77

*Notes: The inhibition percentages (%) were calculated using the treated value divided by the initial value for each concentration.

Algal species number declined by 26%, 39%, 46% and 51% at DBP concentrations of 10, 20, 40 and 60 mg/L, respectively, versus the species number in each treatment before DBP exposure. There was no species loss in the control. The original algal species in the test water belonged to 6 phyla and 34 genera. Green algae comprised the dominant group, followed by blue-green algae and diatoms. Other phyla, such as Euglenophyta, Cryptophyta and Xanthophyta had 3, 2 or 1 species present, respectively. The lost species mainly were from the following genera: *Chlamydomonas, Closterium, Cryptomonas, Dictyospharium, Dinobryon, Euglena, Oocystis, Ophiocytium* and *Trachelomonas.* Many of them are flagellated species, these are perhaps more sensitive to DBP.

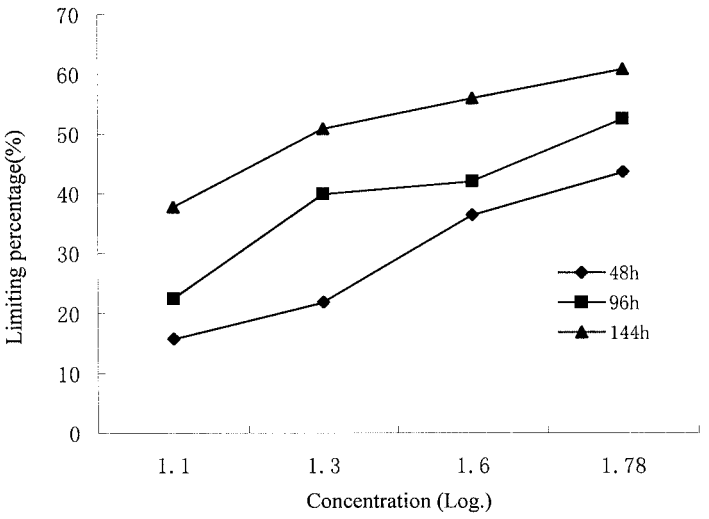

Figure 3. Effects of DBP on cells of natural algae.

Based on the results, it can be considered that the impacts of DBP on algal growth, chlorophyll *a* content and species composition are marked and increased with increasing DBP concentration, for both pure unicellular algae or natural mixed algae. Toxicity of DBP on *S. obliquus* reduced chlorophyll *a* content and prevented cell division. Natural mixed algae showed decreasing cell density and species numbers. Both were reduced to 50% when the algae were exposed to 60 mg/L DBP in NAT.

Acknowledgments. We thank Mr. Yonghong Bi and Guo-xiang Liu for their excellent assistance in the process of writing this paper.

REFERENCES

Adams WJ, Biddinger GR, Robillard KA, Gorsuch JW (1995) A summary of the acute toxicity of 14 phthalate esters to representative aquatic organisms. Environ Toxicol Chem 14: 1569-1574

APHA, AWWA, WPCF (1980) Standard methods for the examination of water and wastewater: Bioassay methods for aquatic organisms (15th Ed.). American Public Health Association, Washington, DC, pp 649-660

Defoe DL (1990) Solubility and toxicity of eight phthalate esters to four aquatic organisms. Environ Toxicol Chem 9: 623-636

Foster PMD, Cattley RC, Mylchreest E (2000) Effects of di-n-butyl phthalate (DBP) on male reproductive development in the rat: Implications for human

risk assessment. Food Chem Toxicol 38: 97-99
Huang GL, Sun HG, Song ZH (1999) Interactions between dibutyl phthalate and aquatic organisms. Bull Environ Contam Toxicol 63: 759-765
Keith LH, Telliard WA (1979) Priority pollutants. Environ Sci Technol 13: 416-419
Kuang QJ, Xia YC, Wu ZB, Qiu DR (1997) Study on the correlations between macrophytes and algae in artificial simulated ecosystem. ACTA Hydrobiologica Sinica 21: 90-93
Patyna P, Cooper KR (2000) Multigeneration reproductive effects of three phthalate esters in Japanese medaka (Oryzias latipes). Mar Environ Res 50: 191-199
Pederson F, Larson J (1996) Review of Environmental Fate and Effects of Diethylhexyl Phthalate. Working Report. No.54, Danish Environmental Protection Agency, Copenhagen
Research Group of Environmental Priority Pollutants in China (1989) Environmental priority pollutants. Chinese Environmental Publisher, Beijing, p 11-13
Staples CA, Werner AF, Hoogheem TJ (1985) Assessment of priority pollutant concentrations in the United States using STORET database. Environ Toxicol Chem 4: 131-142
Statistical Group of Mathematical Institute in China (1979) Mathematical Statistical Methods in Common Use. Scientific Publishing House, Beijing, p 19-32
Thuren A (1991) Effects of phthalate esters on the locomotor activity of the fresh water amphipod *Gammarus pulex*. Bull Environ Contam Toxicol 46: 159-166
Yan H, Ye CM, Yin CQ (1995) Kinetics of phthalate ester biodegration by *Chlorella pyrenoidosa*. Environ Toxicol Chem 14: 931-938