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Interactions Between Dibutyl Phthalate and Aquatic Organisms

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Dibutyl phthalate (DBP) is an important phthalic ester, its production is second only to di-2-ethylhexyl phthalate. DBP extensively exists in air, water, soil and organisms in China (Yie 1993). We have detected DBP pollution in several lakes in Tianjin City, subsurface water ranging: 9.25 to 23.94 ng/mL; water surface microlayer ranging: 10.89 to 89.78 ng/mL. Hence, study of the mutual effects, including toxicity, bioconcentration and biodegradation between DBP and aquatic organisms is important. The study of the toxicity of DBP and other phthalic esters (PAEs) has focused on mammals such as rats in view of their potential carcinogenic and other toxic effects on humans (Abra and Satya 1992; Agarwal et al. 1985). The toxic effects of PAEs on aquatic organisms have been studied to some extent (David et al. 1990; Thuren and Per 1991; Adams et al. 1995; Jon et al. 1995). Compared to acute toxicity test, chronic tests that stress the changes of physiology and behavior of aquatic organisms are more meaningful and more sensitive to indicate the status of pollution (Fernandez 1994; Janssen 1993). The change in physiology and behavior may be the initial response of aquatic organisms to pollutants and also may explain some poisoning mechanisms. Chronic toxicity test on DBP included effects on motive ability and reproduction of water fleas *Daphnia magna*, incubating of salted shrimp *Artemia salina* and growth rate of grass shrimp *Palaemonetes pugio* larvae (Huntzinge 1984; Bravn and Thompson, 1982). These studies showed that the EC,, values of DBP were at several mg/L level. The study of Thuren and Per (1991) concern the effect of DBP on motion of *Gammarus pules* revealed that there was no significant effect at 0.1 mg/L of DBP, and a significant effect appeared in 20 days at 0.5 mg/L of DBP.

Studies have shown that microalgae have the ability to degrade several organic and organometallic pollutants (el-Sheekh et al. 1994; Lee et al. 1989; Klekner and Kosaric 1992). Little is known on the degradation of DBP and other PAEs by algae. Recently, Yan et al. (1995) reported the biodegradation kinetics of dimethyl phthalate, diethyl phthalate and dibutyl phthalate by fresh algae *Chlorella pyrenotdosa*. The kinetics did not followed first-order kinetics. However, the initial concentrations of the PAEs used in their experiments were 7-100 mg/L, far higher than the actual concentrations of PAEs in the natural environment which are only several μg/L. Hence the degradation of PAEs by algae need further research.

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In the present study, toxic effects of DBP, including acute toxicity and effects on feeding behavior and reproduction, on *Daphnia magna* and effects on the growth of green algae *Scenedesmus obliquus* were determined. Effects of environmental factors (temperature, humic acid and hardness) on the acute toxicity were observed. Meanwhile the bioconcentration and biodegradation of DBP by *Scenedesmus obliquus* was also studied.

MATERIALS AND METHODS

Green algae *Scenedesmus obliquus* was purchased from Institute for Hydrobiology, Academy of Science, China. The cultural medium consisted of 0.200g $(NH_4)_2SO_4$, 0.300g $Ca(H_2PO_4)_2$, 0.080g $MgSO_4$, 0.100g $NaHCO_3$, 0.025g KCl, 0.15 mL 1% $FeCl_3$ solution, and 1 mL soil extraction solution in 1L distilled water. The initial pH value was 7.40.

The single clone strain of *Daphnia magna* was continually cultured for more than three generations. *Daphnia magna* was cultured in tap water, which had been aerated for 72 hr, at 22 ± 2°C under twilight (1500-2500 Lux at daytime). *Scenedesmus obliquus* was added every day ($5x10^5$ cell/mL). The cultural medium was renewed twice a week. Pregnant water fleas were transferred to dilution solution containing 5.75 mg/L KCl, 294 mg/L $CaCl_2$, 123.25 mg/L $MgSO_4$, 2.5 mg/L $NaHCO_3$, and allowed to acclimate at test condition. The hardness of dilution solution is 360 mg/L $CaCO_3$. After 24 hr, the 6-24 hr active larvae were selected for experiments.

Considering that organic solvent may have adverse effect on organisms, stock solution of DBP was made directly in the water solution at concentration near the solubility (10 mg/L). To make PAEs dissolve completely, DBP solution in a capped flask were shaken for two hours immediately before experiment.

For acute toxicity test for *Daphnia magna*, five concentrations were used, each was replicated three times. Toxicity tests were conducted with 100 mL test medium in 100 mL beakers. The beakers were cultured at 25 ± 0.6°C under darkness. After 24 hr, dead larvae were counted, and 24 hr-EC_{50} was calculated.

In order to investigate the effects of environmental factors, 24 hr acute toxicity tests were conducted under different conditions. Cultural temperature of 15, 20, 25, 30°C were used. Concentrations of humic acid adopted in this experiment were 2, 10, 25, 50 mg/L. Hardness was adjusted to 90, 180, 360, 540 mg/L $CaCO_3$ by adjusting the concentrations of Ca^{2+} and Mg^{2+} with the ratio of Ca^{2+} and Mg^{2+} maintaining at 1:4. Other conditions were the same as above.

Five concentrations (0, 2.59, 5.18, 6.90, 10.35 mg/L) of DBP were used in feeding behavior test based on 24 hr-EC_{50}, each was replicated Five times. This test was carried out in 50 mL test medium in 100 mL beaker containing 10 larvae (6-24 hr). The system was allowed to stand for 5 hrs in darkness, then algae were added in

the test medium, and the exact initial density of algae was recorded under a microscope (about 5 X 10^5 cell/mL). The system was allowed to stand for another 5 hrs, and density of algae in each beaker was counted under a microscope again. Filter rate (F) and digest rate (I) were calculated as follows:

$$F=V(\ln C_0-\ln C_t)/Nt \quad (1)$$

$$I=F(C_0C_t)^{1/2} \quad (2)$$

where F(μ1/ind·h) was filter rate, I (ce1l/ind·h) was digestive rate, V(μ1) was the solution volume, N was the number of *Daphnia magna* in test water, C_0 (cell/mL) was the initial density of algae, C, (cell/mL) was the final density of algae, t (hr) was the test time. Because the test was conducted in darkness, increase of density of the algae due to growth was omitted.

For reproduction test, one larva (6-24 hr) was added in each 100 mL test medium. The test conditions were: 25 ± 0.6°C, and 12 hr light (4000 ± 100 Lux): 12 hr dark photoperiod. The concentrations of DBP were 10.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0 mg/L, each replicated three times. *Daphnia magna* was fed once a day with *Scenedesmus obliquus* (5 X 10^5 cell/mL). The test medium was renewed every 48 hours. Every day, the tested organisms were observed and new-borne larvae were counted and picked out. The test lasted for 21 days. The body length of each tested *Daphnia magna* was determined at the end of the test. Net fertility (R_0) and intrinsic growth rate (r) were calculated as follows:

$$R_0=\Sigma\, l_x\cdot m_x \quad (3)$$

$$r=\ln R_0/T \quad (4)$$

$$T=\Sigma\, x\cdot 1_x\cdot m_x/\Sigma\, l_x\cdot m_x \quad (5)$$

where l_x was the survival percent, m_x was the number of new-borne larvae per water flea, T was the generation time, and x was the age of water flea.

Toxicity test for *Scenedesmus obliquus* was conducted with 100 mL test medium containing different concentrations of DBP in 250 mL flasks under sterilized condition. The initial density of the algae was 5-6 X 10^5 cell/mL. Six concentrations of toxicant were chosen based on pre-experiments, and each concentration including control was done duplicate. The inoculated flasks were kept in a culture room at 25 ± 0.5°C under the light intensity of 4000 Lux with 12 hr light: 12 hr dark photoperiod. The flasks were shaken at three-hour intervals during the light period. The experiments lasted for 96 hr. Every day the number of the algae was counted using hemocytometer under a microscope. 96 hr-EC_{50} was calculated according to the correlated equation between probability of inhibition (PI=(log N_0 - log N_c/log N_0) and 1ogC.

For bioconcentration and biodegradation test, the algae was cultured to 5L step by step under sterilized condition. Two L cultural medium containing 5% formaldehyde was used as control. The initial concentration of DBP was 50 μg/L. One L algae culture and 0.5L control were taken out at 8 hr, 48 hr, 96 hr and 168 hr. The algae were separated by 0.45 μm film, and weighed. DBP concentrations in water and algae phase were determined by GC-FID. GC conditions were as follows: GC column: capillary column(OV-101, 5m x 0.53mm id); Temperature

programming: 165° C (5 min)— 15 °C/min → 200 °C; Injector temperature: 250 °C; Detector (FID) temperature: 240 °C. Carrier gas: Nitrogen (99.999%); Flow rate: 13.9 mL/min; Flow rate of air : 200 mL/min; Flow rate of hydrogen: 20 mL/min; Injection without split stream.

RESULTS AND DISCUSSION

The 24 hr-EC_{50} of DBP to *Daphnia magna* was 10.35 mg/L at 25 °C . Concentration of DBP in natural water is at μg/L level. Hence acute toxic effect of DBP in natural aquatic water can be omitted. Figures l(a)-l(c) show the effect of temperature, humic acid and hardness on 24 hr-EC_{50} of DBP. It can be seen that 24 hr-EC_{50} of DBP decreased with temperature. Enhancement of temperature may cause the increase of growth and metabolism rate of *Daphnia magna* and exchange rate of chemical with the ambient medium. Hence toxicity of DBP increased. Toxicity of DBP decreased with the increasing concentration of humic acid. Humic acid can complex DBP, thus bioavailability of DBP decreased with the decline of free, dissolvable DBP. The value of 24 hr-EC_{50} became low with increasing hardness. Hardness of water medium has been shown to affect toxicity of many chemicals including heavy metals and organic pollutants. The mechanisms for the effects of hardness on organic compounds have not been elucidated clearly. The possible reason is that the change of concentration of Ca^{2+} and Mg^{2+} ions caused the change of uptake and distribution of DBP in the organism, which results in the change of toxicity of DBP.

Figure 2 shows the effects of DBP on the filter rate (F) and the digestive rate (I) of *Daphnia magna.* When the concentration of DBP was higher than 5.18 mg/L, effects on F and I were significant (95% confidence). The EC,, of DBP for F was 6.25 mg/L and 6.62 mg/L for I.

Table 1 shows effect of DBP on the reproduction of *Daphnia magna.* At 8.0 mg/L, the development and reproduction of *Daphnia magna* was affected by DBP, only one of the three tested organisms reproduced two larvae within 21 days. Furthermore, *Daphnia magna* was easy to die or be hurt at this concentration. The second antenna of one water flea didn't develop well, hence the water flea could not move normally. The growth of *Daphnia magna* wasn't affected significantly at 4.0, 2.0, 1.0, 0.5 mg/L of DBP, but the reproduction was affected significantly at those concentrations. At low concentration (0.5 mg/L), DBP stimulated the reproduction of *Daphnia magna,* R_0, r and average reproduction number per water flea were higher than those of control groups. At high concentrations (1.0, 2.0 and 4.0 mg/L), DBP inhibited the reproduction of *Daphnia magna.* The inhibition increased with the increasing concentration of DBP. The EC,, of DBP for R_0 and r was 3.93 and 4.93 mg/L, respectively.

The 96 hr-EC_{50} of DBP to *Scenedesmus obliquus* was 0.210 mg/L. Algae was more sensitive to DBP than *Daphnia magna.* The results of bioconcentration and biodegradation test of DBP in algae are shown in Table 2. It can be seen from

Table 1. The influence of DBP on the growth and reproduction of *Daphnia magna*

Concentration (mg/L)	R_0	R	highest number of per embryo	body length (mm)	time for first reproduction (d)
4.0	*50.0	*0.58	17	3.47	11
2.0	*61.6	*0.65	24	3.50	11
1.0	*72.7	*0.66	26	3.59	10
0.5	99.3	1.02	39	3.68	8
0	92.3	0.97	29	3.79	8

* significant different at 95% confidence between control and given concentration compared with control

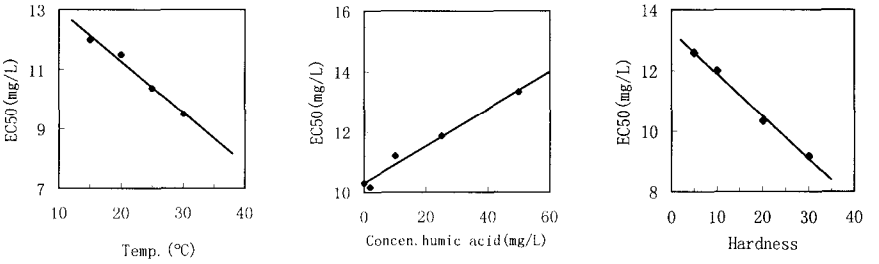

Figure 1. Effect of temperature, humic acid and hardness on 24 hr-EC_{50} of DBP

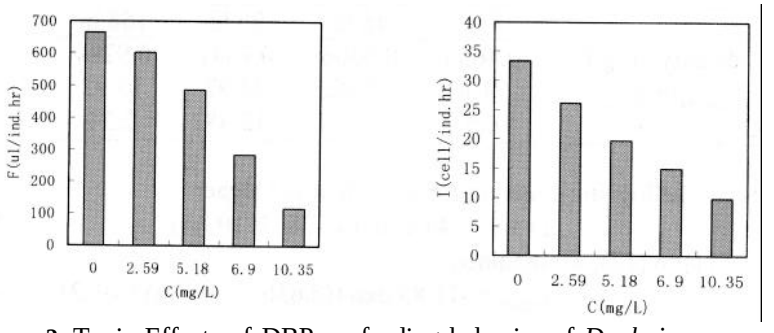

Figure 2. Toxic Effects of DBP on feeding behavior of *Daphnia magna*

Table 2 that *Scenedesmus obliquus* rapidly enriched DBP. After DBP concentration in algae reached a peak value at 8 hr, it declined due to the degradation of DBP by the algal cell, and to a small extent due to the growth dilution effect of the algae. BCF at 8 hr was 4.33 X 10^3. Total amounts of DBP in algae solution varied with time as illustrated in Figure 3. It can be seen that degradation rate of DBP was not constant with time. We used a model to describe the dynamic degradation course:

$$-dC/dt = KDU \quad (6)$$

$$U = k_2 r \quad (7)$$

$$r = dD/dt \quad (8)$$

where C is total concentration of DBP in the algae solution; K is degradation rate constant; D is algae density (g/L); U is activity of algae; r is the growth rate of algae; k_2 is an coefficients. Then equation 6-8 can combine into equation 9:

$$-dC/dt = KD\ dD/dt \quad (9)$$

Thus:

$$C = -1/2\ KD^2 + C_o \quad (10)$$

Through regression analysis we obtained:

$$C = -51.06D^2 + 61.21 \quad (11)$$

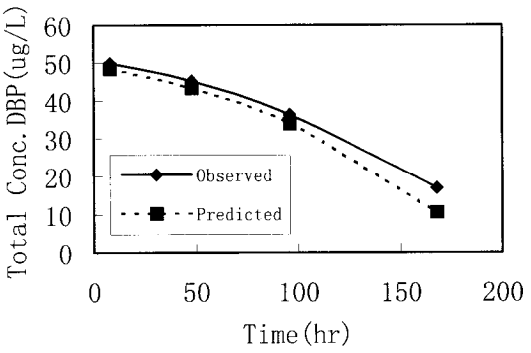

Figure 3. Observed and predicted total concentration of DBP in algae solution

Table 2. Results of enrichment and degradation test

	8 hr	48 hr	96 hr	168 hr
Algae density D (g/L)	0.4650	0.5606	0.7041	0.9284
C_{algae} (μg/g)	71.81	50.02	33.97	15.92
C_{water} (μg/L)	16.57	17.17	12.38	2.24

According to the logistic equation for growth of the algae:

$$D = 0.4544 \exp((4.328 \times 10^{-3})\ t) \quad (12)$$

Substitute (11) to (12), we obtained:

$$C_{DBP} = -11.85 \exp((8.656 \times 10^{-3})\ t) + 61.21 \quad (13)$$

The relative deviation between observed and calculated data is ≤ 7.59% (see Figure 3), which demonstrates that DBP degradation by algae *(Scenedesmus obliquus)* is satisfactorily described with equation 13. From the control test, we found that the loss of DBP was about 2.0%. This further proved that DBP concentration decline was mainly attributed to the biodegradation of algae.

According to those test data obtained, toxicity of DBP to *Daphnia magna* was not great, especially acute toxicity. The 24 hr-EC_{50} of DBP was 10.35 mg/L, approaching its solubility. The study of effect on behavior showed that for F the EC_{50} was 6.25 and for I the EC_{50} was 6.62 mg/L. The chronic test results showed that EC_{50} to R_0 was 3.93 and EC_{50} to r was 4.93 mg/L. The hardness, temperature and humic acid can affect the toxicity of DBP.

In addition, DBP was toxic for alage *Scenedesmus obliquus,* its 96 hr-EC_{50} is 0.21

µg/L, much lower than 24 hi-EC_{50} for *Daphnia magna.* DBP can be enriched and degraded by *Scenedesmus obliquus.* The degradation course of DBP can be satisfactorily described by the following equation:

$C_{DBP} = -11.85 \exp [(8.656X\ 10^{-3})\ t] + 61.21$

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