Bull. Environ. Contam. Toxicol. (2005) 75:775–782

DOI: 10.1007/s00128-005-0818-5

Bulletin of Environmental Contamination and Toxicology

Anaerobic Degradation of Di-*n*-butyl Phthalate and Di-(2-ethylhexyl) Phthalate in Sludge

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Received: 2 April 2005/Accepted: 25 July 2005

Phthalate acid esters (PAEs) are used as plasticizers, giving plastics flexibility and durability (Fatoki and Ogunfowokan 1993). They are widely distributed in sediment, natural water, wastewater and soil (Atlas and Giam 1981; Michael et al. 1984). PAEs are present in sludge through several non-point sources such as domestic and commercial discharges, street runoff, and aerial deposition, and through point sources such as industrial discharges (Alatriste-Mondragon et al. 2003). The United States Environmental Protection Agency and some of its international counterparts have classified di-*n*-butyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) as priority pollutants and as endocrine-disrupting compounds.

A number of studies have reported on the aerobic degradation of PAEs in natural water, wastewater, and soils (Walker et al. 1984; Wang et al. 1996). Few studies have concentrated on the anaerobic degradation of PAEs in sludge. For this study, we observed the effects of the following factors on the anaerobic degradation of DBP and DEHP in sludge: pH value, temperature, substrate concentration, and the addition of electron donors, electron acceptors, surfactants, heavy metals, or microbial inhibitors. We also isolated and identified PAE-degrading anaerobic bacteria from our sludge samples.

MATERIALS AND METHODS

DBP and DEHP, all with 99.0 percent analytical standards, were obtained from Chem Service (West Chester, PA, USA). Solvents were purchased from Mallinckrodt, Inc. (St. Louis, MO, USA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). Individual PAE stock solutions dissolved in acetone were established at a concentration of 100,000 mg/L, and then diluted to 10,000 mg/L before use. The chemical structures of DBP and DEHP are presented in Figure 1.

Sewage sludge samples were taken from the wastewater treatment plant at the Presidential Enterprise Co., Taoyuan, Taiwan (total solids 0.87 g/L, pH 6.7). Petrochemical sludge samples were collected from the wastewater treatment plant at the Chinese Petroleum Corp., Taoyuan, Taiwan (total solids 0.45 g/L, pH 6.5). The test material was the sediment formed after 1 h of settling.

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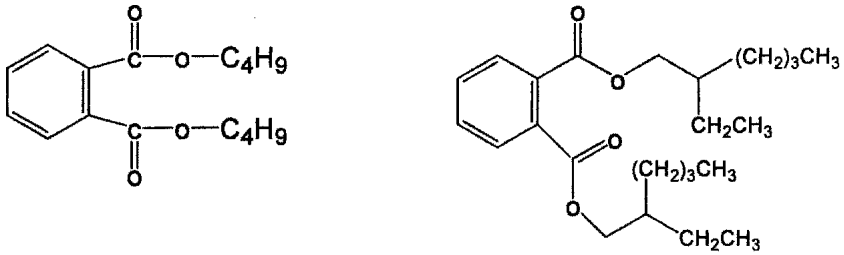

DBP DEHP

Figure 1. Chemical structures of DBP and DEHP.

Our experimental anaerobic medium consisted of (concentrations in g/L): NH_4Cl, 2.7; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_2 \cdot 4H_2O$, 0.02; K_2HPO_4, 0.27; KH_2PO_4, 0.35; resazurin, 0.001. Medium pH was adjusted to 7.0 following autoclaving; 0.9 mM titanium citrate was added as a reducing reagent. For isolation of pure cultures, yeast nitrogen base (YNB) agar containing DBP or DEHP (2 mg/L) was used. YNB consists of $(NH_4)_2SO_4$ as the nitrogen source, other salts (KH_2PO_4, $MgSO_4$, NaCl and $CaCl_2$), trace metals, and very small amounts of vitamins, as described in the Difco manual. DBP and DEHP are considered to be nearly the sole carbon source in the medium.

Experiments were performed using 125 mL serum bottles containing 45 mL of medium and 5 mL of sludge (total concentration of solids 5 g/L), to which we added 2 μg/g of a mixture of the two PAEs (DBP and DEHP). The DBP and DEHP degradation rates in sterile control sludge and non-sterile sludge were initially compared. Sterile controls were produced by autoclaving at 121℃ for 20 min. The following factors were manipulated to investigate their effects on DBP and DEHP degradation in the batch experiment: sludge source (sewage or petrochemical sludge); pH (5.0, 6.0, 7.0, 8.0, or 9.0); temperature (20, 30, 40, or 50℃); DBP or DEHP concentration (1, 2, or 5 μg/g); the presence of two PAEs (DBP and DEHP) individually or simultaneously; electron donors or acceptors (sodium acetate, 20 mM; sodium pyruvate, 20 mM; sodium lactate, 20 mM; manganese dioxide, 50 mM; or ferric chloride, 50 mM); methanogenic, sulfate-reducing, and nitrate-reducing conditions (sodium hydrogen carbonate, 20 mM; sodium sulfate, 20 mM; or sodium nitrate, 20 mM, respectively); treatment with a microbial inhibitor (BESA, 50 mM; vancomycin, 50 mM; or sodium molybdate-2-hydrate, 20 mM); nonionic surfactants (brij 35 or triton N101 at a concentration of 91 or 100 μM); heavy metals such as Pb, Cd, Cu, Zn (2 mg/L). Inoculated controls were treated with the sludge. Unless otherwise noted, sample bottles were incubated without shaking at 30℃ and pH 7.0 in darkness. Bottles were capped with butyl rubber stoppers and wrapped in aluminum foil to prevent photolysis. All experiments were conducted in an anaerobic glove box (Forma Scientific, model 1025 S/N, USA) filled with N_2 (85%), H_2 (10%), and CO_2 (5%) gases. Aqueous samples were periodically collected in order to measure residual DBP, DEHP, nitrate, phosphate, sulfate concentrations, methane production, and ORP values.

PAE extraction and analysis were performed as described in Yuan et al. (2002). Recovery percentages were 96.5 and 97.5 percent for DBP and DEHP, respectively. Detection limits were 100 μg/L for both compounds. Methane levels were measured as described in Chang et al. (2004). Measurements of nitrate, phosphate, and sulfate concentrations were made using procedures described by the American Public Health Association (APHA, 1998). Supernatant pH and ORP values were measured with a pH/ORP meter (HI 9017, Hanna). Pure culture was identified using the Biolog System (Biolog, USA). The PAE biodegradation data we collected fit well with first-order kinetics, $S = S_0\exp(-k_1 t)$, $t_{1/2} = \ln 2/k_1$, where S_0 is the initial concentration, S the substrate concentration, t the time period, and k_1 the biodegradation rate constant. Remaining percentages were calculated as the two PAE residue concentrations divided by their original concentrations. Significant differences were calculated using a standard variance F-test.

RESULTS AND DISCUSSION

The remaining percentages of DBP and DEHP were compared from the two sources of sludge within 28 days of incubation, as shown in Table 1. The results show that the remaining percentages were 95.3 and 94.1 for DBP, and 96.7 and 97.3 for DEHP in the sterile control samples of petrochemical sludge and sewage sludge respectively. Table 1 also shows that DBP and DEHP were degraded within 14 days and 28 days respectively in sewage sludge, and within 7 days and 21 days respectively in petrochemical sludge. This indicates that DBP and DEHP degradation in the non-sterile sludge samples occurred as a result of microbial action. These results also show that the degradation rates of DBP or DEHP were higher in the petrochemical sludge samples than in the sewage sludge samples. DBP and DEHP concentrations may be higher in petrochemical sludge than in sewage sludge. We will therefore restrict our discussion to results from the petrochemical sludge in the next experiment.

Table 1. Remaining percentages of DBP and DEHP in two kinds of sludge within 28 days of incubation.

Treatment	Remaining percentage (%)[a]			
	Petrochemical sludge		Sewage sludge	
	DBP	DEHP	DBP	DEHP
Sterile control	95.3±1.2	96.7±3.2	94.1±2.9	97.3±2.2
Non-sterile sludge	[b]	[b]	[c]	[c]

[a] Values are means ± standard deviations.

[b] DBP and DEHP were completely degraded within 7 and 21 days, respectively.

[c] DBP and DEHP were completely degraded within 14 and 28 days, respectively.

We compared DBP and DEHP concentrations, ORP values, methane production, and nitrate, phosphate, and sulfate concentrations within 21 days of incubation. As shown in Figure 2, the anaerobic degradation rate constant calculated by first order kinetics was 0.581 1/day and the half-life was 1.2 day for DBP, and the figures were 0.111 1/day and 6.2 day for DEHP. The results showed that the

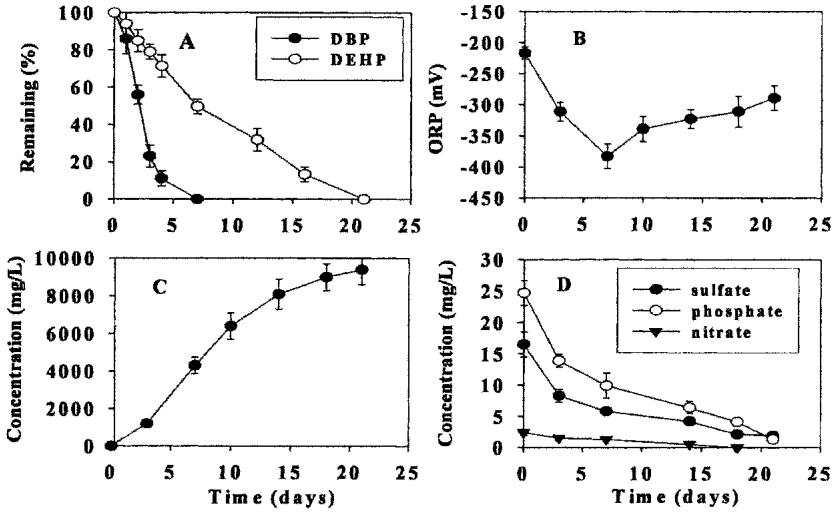

Figure 2. Changes in DBP and DEHP concentration (A), ORP value (B), methane production (C), and nitrate, phosphate, and sulfate concentrations (D) in sludge.

degradation rate of DBP was higher than that of DEHP. The PAEs with a lower molecular weight (i.e. DBP) were easier to degrade than those with a higher molecular weight (i.e. DEHP) (Alatriste-Mondragon et al. 2003). The ORP value was initially measured at -217 mV, decreased to -383 mV during the first 7 days of incubation, and then increased to -289 mV during the next 14 days of incubation. Methane production was increased to 9400 mg/L over the 21 days of incubation. After 21 days of incubation, the nitrate content decreased from 2.4 mg/L to non-detected, the phosphate content decreased from 24.7 to 1.3 mg/L, and the sulfate content decreased from 16.5 to 1.9 mg/L. Sludge may provide nutrients that support the growth of the consortium, which degrades DBP and DEHP.

Mass spectra of the biodegradation product of DEHP in sludge is shown in Figure 3. The characteristic ions were produced by cleavages of $[M-116]^+$, $[M-90]^+$, and $[M-62]^+$, corresponding to the ions of m/z 50, 76, and 104. The mass spectra of the metabolites showed a close match with data for the authentic sample, phthalic acid. The results are similar to those of Staples et al. (1997), who reported that phthalic acid was one of the biodegradation products of PAEs. We also detected biodegradation products of DBP. DBP may have higher degradation rate, and thus we did not detect phthalic acid in the experiment.

Data on the effects of various temperatures, pH levels, and substrate concentrations of DBP and DEHP on anaerobic degradation in the sludge are presented in Table 2. We found the optimal conditions for DBP and DEHP degradation were pH 7.0 and 30℃. Anaerobic microorganisms prefer an environment around 30℃ and pH 6.0~8.0. Microorganism growth is usually

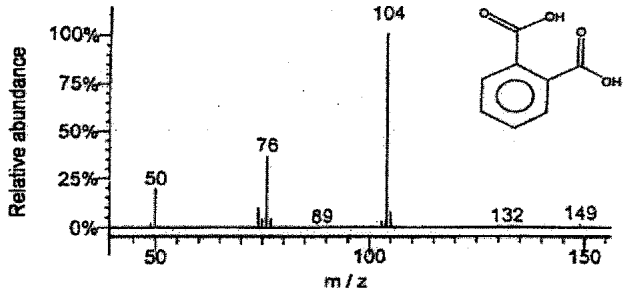

Figure 3. Mass spectra of the anaerobic degradation product of DEHP in sludge samples.

Table 2. Effects of changes in various factors on DBP and DEHP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) in the sludge samples.

Temp (℃)	pH value	Concn (mg/kg)	DBP			DEHP		
			k_1 (1/day)	$t_{1/2}$ (day)	r [a]	k_1 (1/day)	$t_{1/2}$ (day)	r [a]
30 [b]	7.0 [b]	2 [b]	0.581	1.2	0.96	0.111	6.2	0.97
20	7.0	2	0.315	2.2	0.99	0.097	7.1	0.96
40	7.0	2	0.159	4.4	0.99	0.075	9.3	0.96
50	7.0	2	0.099	7.0	0.97	0.065	10.7	0.92
30	5.0	2	0.079	8.8	0.97	0.043	16.1	0.96
30	6.0	2	0.231	3.0	0.89	0.077	9.0	0.95
30	8.0	2	0.304	2.3	0.98	0.103	6.7	0.96
30	9.0	2	0.216	3.2	0.95	0.055	12.6	0.93
30	7.0	1	0.849	0.8	0.94	0.209	3.3	0.95
30	7.0	5	0.226	3.1	0.92	0.088	7.9	0.93
30 [c]	7.0 [c]	2 [c]	0.330	2.1	0.95	[e]	[e]	[e]
30 [d]	7.0 [d]	2 [d]	[e]	[e]	[e]	0.099	7.0	0.96

[a] r = correlation coefficient. [b] Inoculated control. [c] Only DBP present. [d] Only DEHP present. [e] Not measured. Each figure represents the mean of three measurements; in all cases, standard deviation was less than 10%. All inoculated control and treatment figures were significantly different at $p<0.05$.

inhibited at pH values lower than 6.0 or higher than 9.0 (Widdel 1988). We found that the higher the concentration of DBP and DEHP present, the slower the degradation rate observed. This is perhaps a reflection of increased levels of toxicity at higher concentrations of DBP and DEHP. We also noted that the degradation of PAEs was enhanced when the two compounds were present simultaneously. This result may reflect a cross-acclimation of degradation enzymes or increased PAEs bioavailability

Data on the effects of the addition of electron donors, electron acceptors, surfactants, and heavy metals on the anaerobic degradation of DBP and DEHP in the sludge samples are presented in Table 3. We noted an inhibition of DBP and DEHP degradation as a result of the addition of acetate, lactate, or pyruvate.

Addition of acetate, lactate, or pyruvate promotes the growth of methanogen, thus leading to an increased degradation rate (Oremland 1988; Chang et al. 2001). In this experiment, methanogen may not play a role in DBP and DEHP degradation, We also found that the addition of ferric chloride or manganese dioxide inhibited DBP and DEHP degradation in the sludge. It may be that ferric chloride and manganese dioxide do not serve as electron acceptors for ferric- and manganese-reducing bacteria respectively under anaerobic conditions (Lovely and Philips 1995). Sludge may contain heavy metals. We studied the effects of these pollutants on DBP and DEHP degradation. The data in Table 3 show that degradation was inhibited by the addition of heavy metals. The toxicity of heavy metals is known to decrease microorganism activity (Giller et al. 1998). Table 3 also presents data on the effects of nonionic surfactants on the anaerobic degradation of DBP and DEHP in sludge. The addition of brij 35 or triton N101 enhanced DBP and DEHP degradation. PAEs that have partitioned into a surfactant's micellar phase are accessible to microorganisms, thus enhancing their degradation rate.

Table 3. Effects of treatment with electron donors, electron acceptors, surfactant, or heavy metals on DBP and DEHP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) in the sludge samples.

Treatment	DBP			DEHP		
	K_1 (1/day)	$t_{1/2}$ (day)	r^a	k_1 (1/day)	$t_{1/2}$ (day)	r^a
Inoculated control	0.581	1.2	0.96	0.111	6.2	0.96
Acetate	0.321	2.2	0.97	0.081	8.6	0.92
Pyruvate	0.220	3.2	0.98	0.075	9.2	0.97
Lactate	0.119	5.8	0.93	0.064	10.8	0.94
Ferric chloride	0.059	11.8	0.89	0.033	21.0	0.91
Manganese dioxide	0.112	6.2	0.91	0.057	12.2	0.93
Brij 35	0.786	0.9	0.94	0.165	4.2	0.96
Triton N101	0.921	0.8	0.93	0.136	5.1	0.97
Pb	0.080	8.7	0.87	0.049	14.1	0.91
Cd	0.110	6.3	0.94	0.064	10.8	0.89
Cu	0.211	3.3	0.96	0.119	5.8	0.94
Zn	0.095	7.3	0.95	0.051	13.6	0.96

[a] r = correlation coefficient. Each figure represents the mean of three measurements; in all cases, standard deviation was less than 10%. All inoculated control and treatment figures were significantly different at $p<0.05$.

In comparison to the inoculated control, DBP and DEHP anaerobic degradation were inhibited under sulfate-reducing conditions, methanogenic and nitrate-reducing conditions (Table 4). Data on the effects of adding various microbial inhibitors on DBP and DEHP degradation in sludge are presented in Table 5. The results show that in all three cases, degradation rates were inhibited by the addition of molybdate (a selective sulfate-reducing bacteria inhibitor), BESA (a selective methanogen inhibitor), or vancomycin (a selective eubacteria inhibitor) (Lovely and Philips 1988; Distefano et al. 1992). The results for the three

anaerobic conditions and the addition of each of the three microbial inhibitors indicate that methanogen, vancomycin, and sulfate-reducing bacteria are involved in DBP and DEHP degradation.

We also isolated pure microbial strains from sludge samples and found 15 anaerobic bacterial strains capable of degrading DBP and DEHP as carbon sources. The four isolates showing the greatest degrading power were strains OG1, OM1, OM4 and OM5. The order of degradation rates were strain OM1 > strain OM5 > strain OG1 > strain OM4. The strain OM1 was identified as an *Enterococcus* sp. The strains completely degraded DBP within 21 days of incubation; remaining percentage of DEHP within the same incubation period was 6.2 percent. The OM1 strain was gram-negative and cocci-shaped. The stable degrading power of the OM1 strain was retained after several generations.

Table 4. Effects of treatment with microbial inhibitors on DBP and DEHP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) in the sludge samples.

Treatment	DBP			DEHP		
	k_1 (1/day)	$t_{1/2}$ (day)	r [a]	k_1 (1/day)	$t_{1/2}$ (day)	r [a]
Inoculated control	0.581	1.2	0.96	0.111	6.2	0.96
BESA	0.119	5.8	0.93	0.056	12.4	0.94
Vancomycin	0.321	2.2	0.97	0.086	8.1	0.89
Molybdate	0.180	3.9	0.98	0.073	9.5	0.97

[a] r = correlation coefficient. Each figure represents the mean of three measurements; in all cases, standard deviation was less than 10%. All inoculated control and treatment figures were significantly different at $p<0.05$.

Table 5. Effects of three reducing conditions on DBP and DEHP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) in the sludge samples.

Treatment	DBP			DEHP		
	k_1 (1/day)	$t_{1/2}$ (day)	r [a]	k_1 (1/day)	$t_{1/2}$ (day)	r [a]
Inoculated control	0.581	1.2	0.96	0.111	6.2	0.97
Nitrate reducing conditions	0.121	5.7	0.95	0.053	13.1	0.93
Sulfate reducing conditions	0.170	4.1	0.93	0.057	12.2	0.94
Methanogenic conditions	0.098	7.1	0.92	0.046	15.1	0.96

[a] r = correlation coefficient. Each figure represents the mean of three measurements; in all cases, standard deviation was less than 10%. All inoculated control and treatment figures were significantly different at $p<0.05$.

In summary, the sludge samples used in these experiments were all capable of degrading DBP and DEHP within 28 days of incubation. Degradation rates were affected by alterations in pH value, temperature, substrate concentration, electron donors, electron acceptors, surfactants, and heavy metals. Our next goal is to monitor the mineralization of PAEs and characterize the structure of the microbial community involved in the anaerobic degradation of PAEs in sludge.

Acknowledgments. This research was supported by the National Science Council, Republic of China; grant number NSC90-2211-E-031-001.

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