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Biodegradation of Three Phthalic Acid Esters by Microorganisms from Aquatic Environment

MASANORI FUJITA[1], MICHIHIKO IKE[1], TOMONORI ISHIGAKI[1,2], KAZUNARI SEI[1], JIN-SUK JEONG[1], NAOHISA MAKIHIRA[1], and RUTTAPOL LERTSIRISOPON[1*]

[1]Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University/ 2-1, Yamada-oka, Suita, 565-0871 Japan

[2]Present address: Department of Environmental Solution Technology, Faculty of Science and Technology, Ryukoku University/ 1-5, Yokotani, Seta-Oe, Otsu, 520-2194, Japan

Abstract

We assessed the primary and ultimate biodegradability in aquatic environments of three phthalic acid esters (PAEs) — di-*n*-butyl phthalate (DBP), butyl benzyl phthalate (BBP) and di (2-ethylhexyl) phthalate (DEHP) — and phthalic acid (PA) by using microbes obtained from four river-water, three pond-water and four activated sludge samples. Though none of the tested samples had been acclimatized to PAEs, all of them showed an ability to biodegrade the PAEs and PA, suggesting the ubiquitous existence of PAE-degrading microbes in aquatic environments. The PAEs underwent rapid primary biodegradation: 40 and 10 mg·l^{-1} TOC of the PAEs disappeared within 2 weeks via biodegradation by activated sludge samples and by river- and pond-water samples, respectively. However, ultimate biodegradation reached only 40%-80% by activated sludge and 15%-70% by river- and pond-water samples within the 2-week experimental period, and metabolites accumulated, including monoalkyl phthalates, PA, protocatechuate and β-carboxy-*cis*-muconate. According to analyses of the biodegradation kinetics, the investigated PAEs can be ranked by their primary and ultimate biodegradability as DBP ≥ BBP > DEHP and BBP ≥ DBP > DEHP, respectively.

Key words: Activated sludge, pond-water microorganisms, primary biodegradation, river-water microorganisms, ultimate biodegradation

INTRODUCTION

Phthalic acid esters (PAEs) have been produced in large quantities and widely used as plasticizers. PAEs are served as important additives which impart flexibility in polyvinylchloride, polyvinyl acetates, cellulosics, and polyurethanes. These plasticizers, however, do not incorporate in the plastic polymer matrix but are only physically bound to the plastic structures. Hence, they are released from the plastics to the external environment under certain use or disposal conditions. Therefore, PAEs have been identified as the most ubiquitous contaminants in all kinds of environments, including river water, lake water and seawater, and have been found in fish, the food supply, medical devices and toys.

Concerns for the possible health effects of PAEs have been reported, especially of those in medical devices, where PAEs could easily be transferred intravenously to patients. Although the relatively low acute toxicity of most of PAEs quieted these fears, in the last decade the concern has shifted to their long-term, chronic effects such as mutagenicity and carcinogenicity. Dibutyl phthalate (DBP)

*Corresponding author

and butyl benzyl phthalate (BBP) possess estrogenic activity[1-3]. The possibility of these compounds entering biological systems has aroused great concern about their reproductive and developmental toxicities[4-8].

The environmental fates of these widespread pollutants depend significantly on their biodegradability. Studies have reported the biodegradation of PAEs by microorganisms derived from natural waters[9-11], wastewater[11-13] and soils[10, 14, 15]. Several types of aerobic and anaerobic microorganisms were found to degrade PAEs[16-21]. The majority of these studies, however, described the PAE biodegradation in acclimatized microcosms or identified isolates; relatively little is known about the biodegradability of PAEs in native microcosms. To assess the biodegradation behaviour of PAEs in the environment, questions of whether PAE-degrading microbes are distributed ubiquitously and/or evenly and whether PAEs can be completely mineralized in native microcosms should be answered.

In this study we examined three PAEs which have been used heavily in Japan: di-*n*-butyl phthalate (DBP), butyl benzyl phthalate (BBP) and di (2-ethylhexyl) phthalate (DEHP). DBP and BBP are suspected to be estrogenic, acutely and chronically toxic, and mutagenic[7, 22, 23]. DEHP is one of the most frequently used additives in the world and is suspected to have mutagenic and carcinogenic effects[4, 23]. To evaluate the PAEs' degradation potentials in aquatic environments, we subjected them to primary and ultimate biodegradation tests using a variety of native aquatic samples. For reference, *o*-phthalic acid (PA), which is one of the fundamental metabolites of PAEs[5, 23], was also used in the biodegradation tests.

MATERIALS AND METHODS

Chemicals Analytical grades of PA (Wako Pure Chemical Ind., Osaka), DBP and DEHP (Kishida Reagent Co., Osaka), and BBP (Tokyo Chemical Ind., Tokyo) were used for the biodegradation tests as received without any further purification. Ethanol was utilized as the solvent carrier for all the compounds investigated in the primary biodegradation test.

Media Microorganisms collected from aquatic samples were inoculated in artificial river water. The chemical composition of the artificial river water used as the basal medium in the primary biodegradation test was as follows: K_2HPO_4 21.8 mg, KH_2PO_4 8.5 mg, $Na_2HPO_4 \cdot 12H_2O$ 44.6mg, NH_4Cl 17 mg, $MgSO_4 \cdot 7H_2O$ 22.5 mg, $CaCl_2$ 27.5 mg, $FeCl_3 \cdot 6H_2O$ 0.25 mg; $MnSO_4 \cdot 5H_2O$ 0.71 mg, $ZnSO_4 \cdot 7H_2O$ 0.01 mg, $CuSO_4 \cdot 5H_2O$ 5 mg, $CoCl_2 \cdot 6H_2O$ 5 mg, and 1 liter of deionized water. The basal medium used in the ultimate biodegradation test was prepared with the same components of the artificial river water but $MnSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 6H_2O$ were omitted. The pH value of the media was adjusted to 7.2 by HCl or NaOH after supplemented with PAEs/PA for the degradation tests.

Aquatic samples Various aquatic samples were used for the biodegradation tests. Activated sludge samples were collected from a laboratory unit and three sewage treatment plants which receive mainly domestic wastewater. The activated sludge from laboratory unit (LS: mixed liquor suspended solids [MLSS] = ca. 4400 $mg \cdot l^{-1}$) had been acclimated to the synthetic wastewater mainly contain meat extract, peptone and urea for more than 6 years in a sequencing batch mode[24]. MLSS concentrations of activated sludge samples from sewage treatment plants (AS1, AS2, and AS3) were approximately 1200 $mg \cdot l^{-1}$, 1100 $mg \cdot l^{-1}$ and 1000 $mg \cdot l^{-1}$, respectively. River-water samples were collected from Sugawara Shirokita Ohashi, Yodo River, Osaka (RW1: suspended solids [SS] = ca. 5 $mg \cdot l^{-1}$), Kamisu Bridge, Kanzaki River, Osaka (RW2: SS = ca. 6 $mg \cdot l^{-1}$), Miyajima Bridge, Ai River, Ibaraki (RW3: SS = ca. 5 $mg \cdot l^{-1}$) and Uji Bridge, Uji River, Uji (RW4: SS = ca. 5 $mg \cdot l^{-1}$), while pond water samples from Zuion Pond, Suita (PW1: SS = ca. 6 $mg \cdot l^{-1}$), Numata Pond, Suita (PW2: SS = ca. 9 $mg \cdot l^{-1}$) and Inukai Pond, Suita (PW3: SS = ca. 8 $mg \cdot l^{-1}$). None of the aquatic environment samples had been acclimatized to PAEs, and they were used in the degradation tests within 12 h after sampling.

Primary biodegradation test Microor-

ganisms in the sludge and water samples were collected by centrifugation (15 000×*g*, 10 min) or filtration (0.22- μm-pore size), washed three times with ultra-pure water and inoculated into 150 m*l* of the artificial river water in a 300-m*l* Erlenmeyer flask at MLSS = ca. 100 mg·l^{-1} for activated sludge samples and SS = ca. 25 mg·l^{-1} for river- and pond-water samples, respectively. They were supplemented with 1m*l* PAEs/PA solution and the initial PAEs/PA concentration was adjusted to ca. 40 mg·l^{-1} TOC for activated sludge samples and 10 mg·l^{-1} TOC river- and pond-water samples. Flasks without PAEs/PA were prepared as blank tests for each inoculum, and flasks without inoculum served as the control tests for each PAE/PA. The flasks were plugged and incubated at 28 ℃ on a rotary shaker (120 rpm) for 2 weeks in the dark. Aliquots (0.75 m*l*) were periodically withdrawn and mixed with 0.75 m*l* acetonitrile to extract PAEs/PA followed by centrifugation (15 000×g, 10 min). The supernatant fractions were used for PAEs/PA analysis by high performance liquid chromatography (HPLC) as described below. Primary biodegradation was expressed as the percentage of removed PAEs/PA to the initial concentration.

Ultimate biodegradation test Materials were prepared as above (except for inoculation into 200 mL of test medium). Flasks were sealed tightly with rubber stoppers and incubated at 28 ℃ with mixing by magnetic mixers (900 rpm) on an automatic biochemical oxygen demand (BOD) analyzer (DKK, Tokyo). Oxygen consumption in the flasks was automatically monitored by the BOD analyzer (DKK, Tokyo). Oxygen consumption in the flasks was automatically monitored by the BOD analyzer so we could evaluate the breakdown or mineralization of PAEs/PA. Ultimate biodegradation or mineralization is expressed as the percentage of oxygen consumption relative to the theoretical BOD of added PAEs/PA in each flask.

Analytical Procedures The HPLC apparatus consisted of a CCPE solvent delivery pump with a PX-8010 solvent controller and a UV-8010 spectrophotometric detector (Tosoh, Tokyo) connected to an advanced computer interface for the analyses by a chromatography work station (AI-450, ver.3.320J: Dionex, USA). Samples (0.5 m*l*) were mixed with ethanol (0.5 m*l*) and injected into TSK ODS 80-TM column (Tosoh, Tokyo) with the mobile phase of acetonitrile and water mixture (90:10 v/v) at a flow rate of 0.5 m*l*·min^{-1}. PAEs/PA were detected by the UV detector at a wavelength of 254 nm. A liquid chromatography-mass spectrometry (LC-MS) analysis was also performed to tentatively identify the biodegradation metabolites. The LC-MS used was LC/MS QP8000*α* system composed of a LC-10ADvp solvent delivery pump with a SCL-10Avp solvent programmer and a SPD-10Avp UV-VIS spectrophotometric detector connected to a CLASS-8000 work station (Shimadzu, Kyoto). A VP-ODS 150 *l*×2.0 column was used for the analyses, with the same conditions as the HPLC analyses.

RESULTS

Primary biodegradation Results of the primary biodegradation tests of PAEs and PA are shown in Fig. 1, where the percentage of remaining parent compounds relative to the initial concentration is plotted against incubation time. No significant amount of PAEs/PA was detected from the blank tests, and no significant change of PAEs/PA concentrations were observed in any control tests during the experimental period. Therefore, the results were not shown.

All the tested samples degraded the PAEs and PA (Fig. 1). The activated sludge samples biodegraded all the PAEs very efficiently without any lag to below the detection limits within 10 days. The activated sludge samples biodegraded DEHP significantly more slowly than the other PAEs and PA. The river- and pond-water samples showed nearly the same capacity of PAE biodegradation as each other, but the biodegradation rate was slower than that by the activated sludge samples on the whole. Microorganisms within the water samples completely removed PAEs/PA during the 2-week experimental period.

Ultimate biodegradation Results of the ultimate biodegradation tests of PAEs and PA are shown in Fig. 2. Mineralization, expressed as the percentage of oxygen

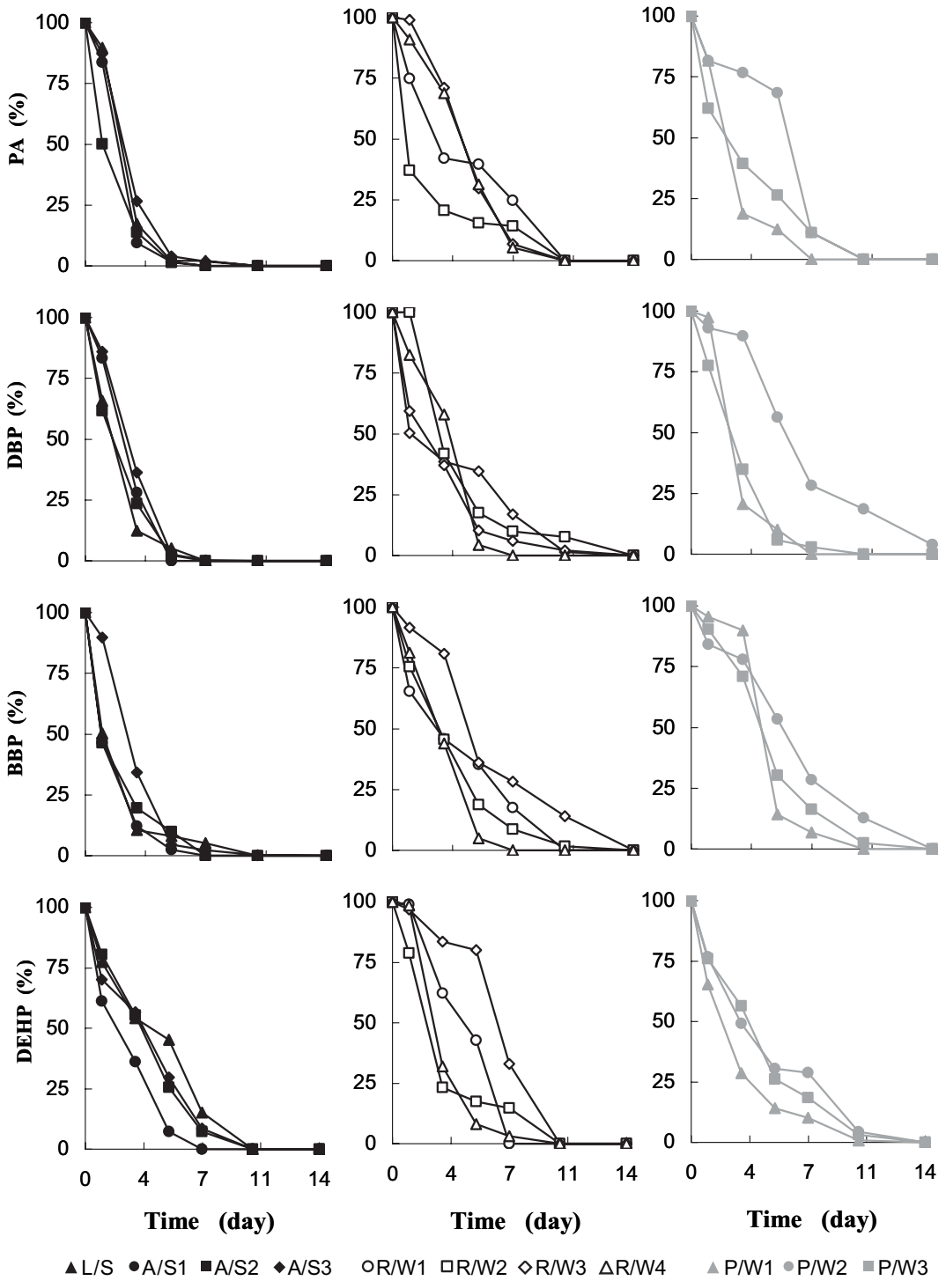

Fig. 1. Primary biodegradation of the PAEs by activated sludge microbes (left), river-water microbes (middle) and pond-water microbes (right)

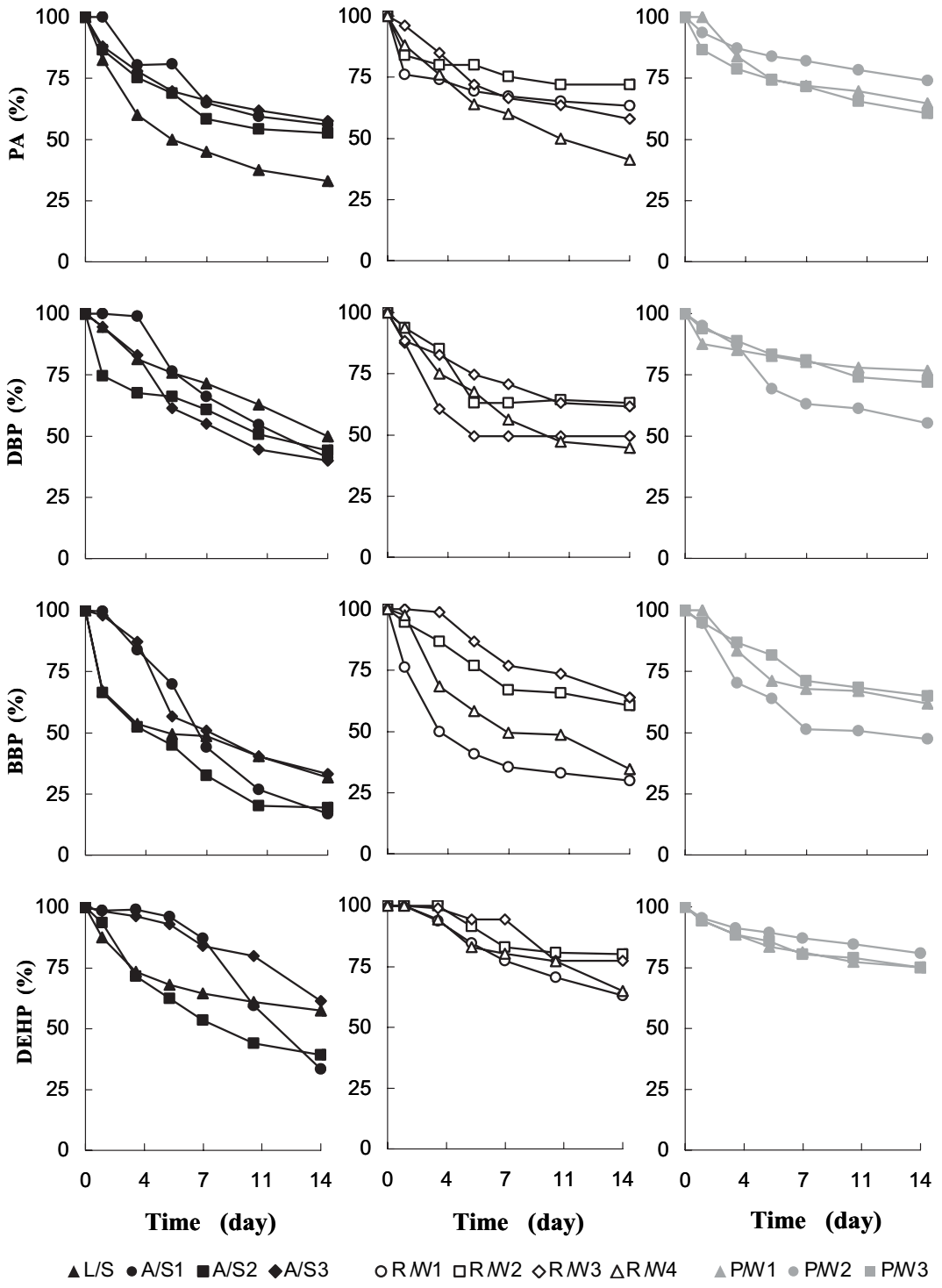

Fig. 2 Ultimate biodegradation of the PAEs by activated sludge microbes (left), river-water microbes (middle) and pond-water microbes (right)

consumption relative to the theoretical BOD derived from the test compounds, is plotted against time, adjusted for the results of the corresponding control tests.

All the tested samples showed a tendency to mineralize the examined PAEs and PA to varying degrees. However, no single sample achieved complete degradation or mineralization (100 % removal of BOD) during the experimental period. The activated sludge samples showed a slightly higher mineralization rate (40%-80%) than the river- and pond-water samples (15%-70%). Unlike in the primary degradation tests, there was often a clear lag phase before mineralization of the PAEs began. Mineralization of BBP proceeded most effectively, followed by DBP and then DEHP by both activated sludge samples and river- and pond-water samples. The activity of PAE mineralization depended considerably on the source of samples.

The LC-MS analyses of the test media after the 2-week ultimate degradation tests revealed that common metabolites of PAEs/PA were accumulated although the parent PAEs/PA were not detected. The common metabolites tentatively identified were protocatechuate (PCA) and β-carboxy-*cis*-muconate (β-CM) from PA; mono-butyl phthalate (MBuP), PA, PCA and β-CM from DBP; mono-benzyl phthalate (MBeP), MBuP and PCA from BBP; and mono-ethylhexyl phthalate (MEHP), MBuP, PA, PCA and β-CM from DEHP (mass spectra not shown).

Kinetics of biodegradation Numerous models have been used to describe the biodegradation kinetics of organic pollutants. The PAEs biodegradation data obtained in this study were fitted to simple models which have been frequently used, and the first-order kinetic model (eq. 1) was selected as a best one to properly describe the degradation kinetics:

$$\ln C = -Kt + \ln C_0 \qquad \text{(eq.1)}$$

where C is the concentration of the PAE (BBP, DBP or DEHP), K is first-order kinetic constant, t is time, and C_0 is the initial concentration of the PAE. According to the parameters obtained from the kinetics, the biodegradation half-life ($t_{1/2}$) of the PAEs can be expressed as:

$$t_{1/2} = \frac{\ln 2}{K} \qquad \text{(eq.2)}$$

The K and $t_{1/2}$ values for PAEs/PA primary and ultimate degradation were obtained from the parameter fitting to the experimental data of this study and depicted in Fig. 3. The $t_{1/2}$ for the primary degradation of PAEs were determined to be less than 5 days, while that for the ultimate degradation were much longer (3-15 times). As a whole, the $t_{1/2}$ values for the primary degradation were determined as DBP ≤ BBP < DEHP, while those for the ultimate degradation were BBP ≤ DBP < DEHP.

DISCUSSION

All the tested aquatic microorganism samples, which had never been acclimatized to PAEs/PA, were capable of primary biodegradation of the selected PAEs and the typical intermediate PA, indicating that microbes responsible for the degradation of PAEs exist ubiquitously in a variety of aquatic environments. However, the biodegradation rate of the PAEs varied considerably depending on the sample source; for example, microbes in sample PW1 were able to remove about 80% of DBP within 3 days, while PW2 took 10 days to achieve the same level of removal. Thus, the population density and/or the catabolic activity of the PAE-degrading microbes appear to vary significantly even in similar aquatic environments.

Previous studies have shown that acclimatization can accelerate the degradation of PAEs. Graham[25] showed that acclimatized activated sludge could remove up to 99% of BBP and up to 91% of DEHP within 48 h. O'Grady *et al.*[20] reported that acclimatized activated sludge could primarily degrade more than 95% of DBP and 81.5% of DEHP within 1 day. For ultimate degradation, acclimatized activated sludge could degrade about 75%-85% of BBP and 45%-50% of DEHP within 7 days[13]. Thus, both primary and ultimate biodegradation of PAEs could be accelerated by acclimatization, particularly of natural microbes, and

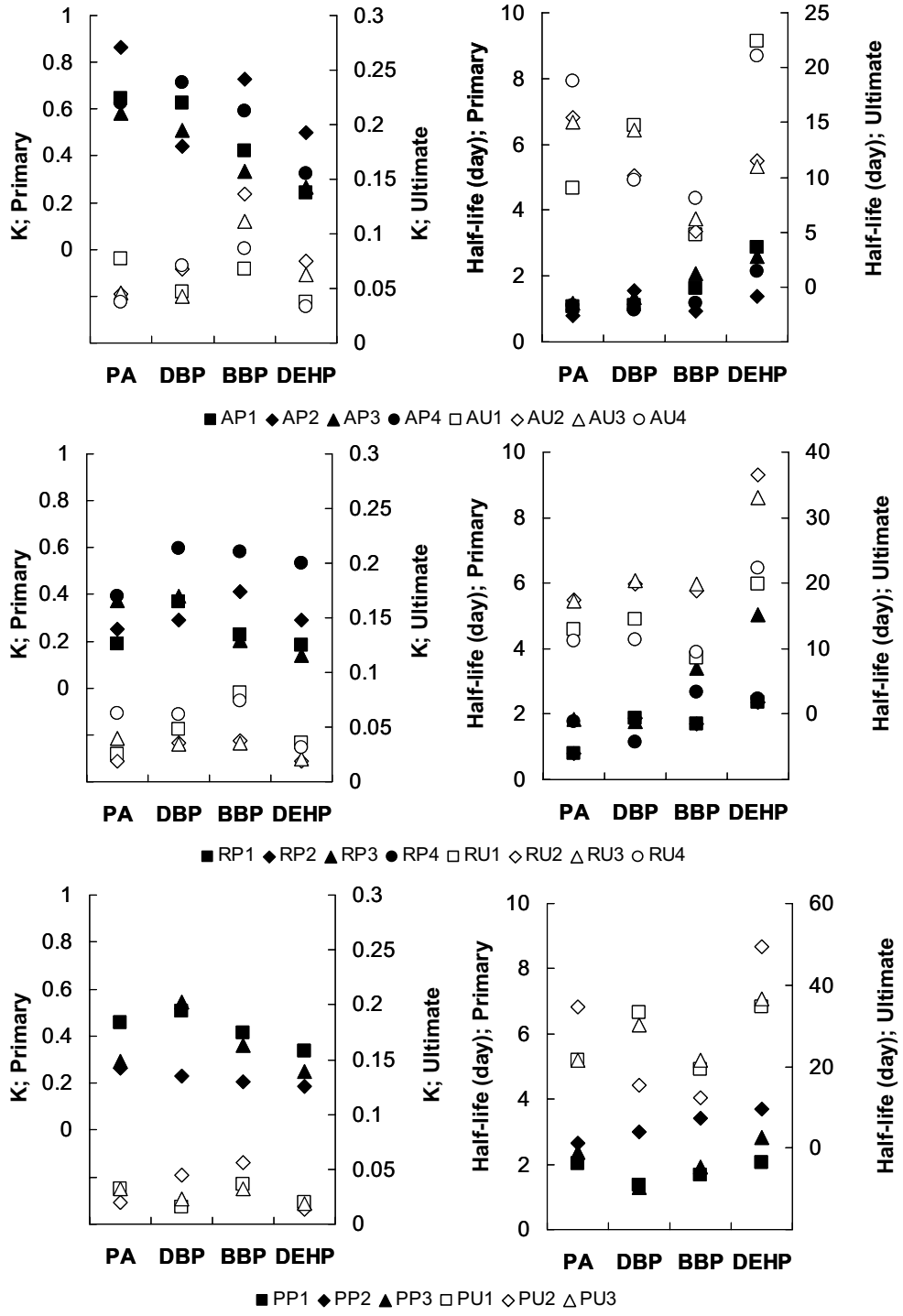

Fig. 3 Kinetic parameters of the PAEs: first-order kinetic constant (left) and biodegradation half-life ($t_{1/2}$; right)

especially for PAEs with higher molecular weights, such as DEHP.

Because the activated sludge samples commonly exhibited high potentials to degrade the PAEs, it appears that PAEs can be removed relatively easily by normal domestic wastewater treatment processes, such as conventional activated sludge processes. The native river- and pond-water samples also showed considerable PAE-degrading activity, although the degradation rates were lower than those by activated sludge samples. Therefore, PAE parent compounds would be degraded readily and disappear even if discharged into aquatic environments. PAEs have been found frequently in all kinds of aquatic environments, however, so this fact seems to contradict the above inference. PAEs with high hydrophobicity may be absorbed rapidly into the sediment or suspended matter, where oxygen is not sufficient for aerobic degradation, and biodegradation of PAEs hardly proceeds under anoxic or anaerobic conditions.

The rates of ultimate degradation or mineralization of the PAEs were much slower than that of primary degradation in all samples, and several metabolites were accumulated in the degradation tests even when the parent PAEs were removed below the detection limits. These findings suggest that discharge of PAEs into aquatic environments can cause secondary pollution by more refractory metabolites, including monoalkyl phthalates, PCA, PA and β-CM. Much less information is available on the ecological impacts caused by PAE metabolites compared with the parent PAEs. Thus, further intensive studies of their biodegradability, bioaccumulation and toxicity are necessary to assess all the risks of PAE pollution.

According to the K and $t_{1/2}$ values obtained from the kinetic analyses, the investigated PAEs may be ranked by their primary and ultimate biodegradability as DBP ≥ BBP > DEHP and BBP ≥ DBP > DEHP, respectively. PA, however, ranks between DBP and BBP depending on the source of the aquatic samples. The experimental results obtained in this study approximately followed this trend, and it may be concluded that BBP and DBP are easily biodegraded in the aquatic environments under aerobic conditions, while DEHP is relatively recalcitrant.

CONCLUSION

The ubiquitous existence of aerobic microcosms responsible for the degradation of PAEs in natural aquatic environments was established. Since the population density and/or the catabolic activity of the PAE-degrading microbes appear to vary significantly even in similar aquatic environments, the biodegradation rate of PAEs/PA varied considerably depending on the sample source. The complete primary biodegradation was demonstrated in all samples but the ultimate biodegradation achieved 40%-80% by activated sludge samples and 15%-70% by river- and pond-water samples. Thus, the metabolites such as monoalkyl phthalates, PA, PCA and β-CM are suspected to accumulate. According to the kinetic analyses, the investigated PAEs can be ranked by their primary and ultimate biodegradability as DBP ≥ BBP > DEHP and BBP ≥ DBP > DEHP, respectively. PA was ranking between DBP and BBP depending on the source of the aquatic samples.

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