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ORIGINAL PAPER

Biodegradation of di-*n*-butyl phthalate in sequencing batch reactor bioaugmented with *Micrococcus* sp. and the bacterial community analysis

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Abstract Di-*n*-butyl phthalate is widely used as plasticizer, which has been listed as priority pollutant due to its toxic and ubiquitous characteristics. It is difficult to remove by the conventional wastewater treatment processes. In this paper, the feasibility of using *Micrococcus* sp. to bioaugment a sequencing batch reactor for degrading di-*n*-butyl phthalate (DBP) was investigated. The terminal restriction fragment length polymorphisms (T-RFLP) were used to analyze the variation of microbial community in the reactor. The experimental results showed that for the bioaugmented reactor, the removal efficiency of DBP was about 85 % as compared to 25 % of the control reactor when initial DBP concentration was 100 mg/L. The bioaugmentation not only enhanced the removal efficiency of target compound, but also shortened the start-up time of the reactor. The kinetics of DBP degradation conformed to the first-order model in both reactors. The T-RFLP analysis indicated the bacterial community changes in the acclimated activated sludge and the introduced *Micrococcus* sp. during the operational process.

Keywords Bioaugmentation · Priority pollutant · Plasticizer · Kinetics

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Introduction

Phthalic acid esters (PAEs) are a group of endocrine disruptors commonly used as plasticizers. These pollutants are persistent in the environment and highly recalcitrant to the conventional biological treatment processes (Staple et al. 1997). Therefore, they have been found in sediments, water body and soils. Di-*n*-butyl phthalate (DBP) is one of the most widely used PAEs, which have shown increasing environmental concerns worldwide. DBP is a reproductive toxicant which had effects on animals exposed pre- and postnatal. DBP has been listed as priority pollutant by several regulatory institutions, such as the US Environmental Protection Agency, the European Union and the China National Environmental Monitoring Center (Wang 2002).

Efficient removal of phthalate esters in wastewater treatment plants is becoming an increasing priority in many countries (Roslev et al. 2007; Wang et al. 1996, 1997a, b, 1998, 2000, 2003, 2004; Wang and Qian 1999). The degradation of DBP in water and wastewater has received increasing attention in recent years. Roslev et al. (2007) investigated the fate of dimethyl phthalate (DMP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and di-(2-ethylhexyl) phthalate (DEHP) in a full-scale activated sludge WWTP with biological removal of nitrogen and phosphorus. He et al. (2013) investigated the biodegradation of di-*n*-butyl phthalate by a stable bacterial consortium enriched from the activated sludge and identified the dominant microorganisms in the consortium by denaturing gradient gel electrophoresis (DGGE), and they found the existence of *Gordonia* sp., *Burkholderia* sp. and *Achromobacter* sp. The consortium could mineralize approximately 90 % of 1,200 mg/L DBP after 48 h of cultivation. Wu et al. (2013) studied the biodegradation of di-*n*-butyl

phthalate esters by *Bacillus* sp. SASHJ under simulated shallow aquifer condition. Dulazi and Liu (2011) investigated the removal of phthalate esters from water using immobilized lipase on chitosan beads. The removal efficiency for dimethyl phthalate and diethyl phthalate was 100 and 93.86 %, respectively. It is an efficient and cost-effective method for removing phthalate esters (PAEs) from water. Microbial degradation of DBP is one of the major routes for environmental degradation of this widespread pollutant due to its low rate of the hydrolysis and the photolysis. Xu et al. (2005) studied the biodegradation of an endocrine-disrupting chemical di-*n*-butyl phthalate ester by *Pseudomonas uorescens* B-1. Patil et al. (2006) investigated the degradation of DBP by *Delftia* sp. TBKNP-05. Luo et al. (2009) studied the degradability of the three dimethyl phthalate isomer esters (DMPEs) by a *Fusarium* species isolated from mangrove sediment. Pradeep et al. (2013) reported the complete degradation of the plasticizer di(2-ethylhexyl)phthalate (DEHP) bound to PVC blood storage bags (BB) in simple basal salt medium (BSM) by *Aspergillus japonicus*, *Penicillium brocae* and *Purpureocillium lilacinum*, isolated from heavily plastics-contaminated soil.

Bioaugmentation has been used to promote the start-up of a reactor, to protect the existing microbial community against adverse effects and to accelerate the removal of xenobiotics as a promising strategy for wastewater treatment (Wang et al. 2002; Abdulsalam et al. 2011; Sogani et al. 2012). Wang et al. (2002) studied the bioaugmentation for the removal of quinoline in coke plant wastewater by inoculating a quinoline-degrading bacterium *Burkholderia pickettii* in an anaerobic–anoxic–oxic system and found that bioaugmentation was an efficient strategy for increasing the removal of recalcitrant compounds from industrial wastewater. Bai et al. (2010) investigated the bioaugmentation treatment of coking wastewater containing pyridine and quinoline in a sequencing batch reactor.

However, the species used for bioaugmentation sometimes fail to compete with indigenous microorganisms and may also cause process inhibition and even system breakdown. Therefore, it is important to monitor the microbial community dynamics during bioaugmentation process (Zhang et al. 2011). Many fingerprinting techniques have been used for analyzing the microbial community structure dynamics, such as denaturing gradient gel electrophoresis (DGGR), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphisms (T-RFLP) and fluorescent in situ hybridization (FISH). Among these, T-RFLP is one of the most frequently used high-throughput fingerprinting methods (Marsh 1999; Schütte et al. 2008), and it has been widely used to monitor the changes in the structure and composition of microbial communities. The objective of this study was to investigate the bioaugmentation of a sequencing batch reactor for enhancing the removal of DBP through introducing *Micrococcus* sp. and to analyze the microbial community structure dynamics by T-RFLP. This study was carried out between November 2011 and December 2012 at Tsinghua University, China.

Materials and methods

Microorganism and activated sludge

The strain used in this study was selected from the coking wastewater treatment plant. The microorganisms were purified by successive streak transfers on agar plate medium. The strain was identified to be *Micrococcus* sp. using Biolog system. *Micrococcus* occurs in a wide range of environments, including water and soil. Micrococci have Gram-positive spherical cells ranging from about 0.5–3 μm in diameter and typically appear in tetrads. They are catalase positive, oxidase positive, indole negative and citrate negative. They have been used for degradation of pyridine, 2-nitrotoluene, dibutyl phthalate and the like.

Micrococcus sp. strain was cultivated in 50 mL LB medium (1 L contains 10 g tryptone, 5 g yeast extract and 10 g NaCl) containing 100 mg/L DBP at 30 °C and 200 rpm for 24 h, then the free cells were centrifuged (10 min at 2,656×*g*) and washed with the mineral salt solution, and 30 % (v/v) of cell suspension was used as inoculum for bioaugmentation.

Activated sludge was collected from Gaobeidian Sewage Treatment Plant. The concentration of the activated sludge was approximately 6,000 mg MLSS/L at the start of acclimation to DBP. The sludge was acclimated by a fill-and-draw operation of the cycle in every other day in a reactor of 2.0 L at 25°C. In each cycle, half of the supernatant settled for 30 min in the reactor was drawn before the fresh basic medium of the same volume was added. Air was supplied by a sparger at the bottom of the reactor (Wang 2004).

Reactor operation

Two SBRs (1,000-mL) with 400 mL working volume were used for bioaugmentation experiments and kept at 30 °C and 150 rpm. Two hundred milliliter fresh activated sludge taken from a local wastewater treatment plant was centrifuged (15 min at 1,180×*g*), washed and added to the reactors.

Reactor 1 only inoculated with the activated sludge. Reactor 2 inoculated with the activated sludge and *Micrococcus* sp. to establish a bioaugmentation system.

The initial biomass concentration of the activated sludge in each reactor was 3 g/L. Different DBP concentration (10.0, 50 and 100.0 mg/L) was fed with the synthetic wastewater.

The SBR was operated in cycles of 8 h, 2–3 cycles each day. The system was controlled with a timer system. The cycle comprised of four phases: filling 0.5 h, reaction 6.0 h, settling 1.0 h and decanting 0.5 h. The discharge ratio in the reactor was 50 % in each cycle. The pH was not adjusted. The DO of the reactor was controlled by the aeration rate using air flow meter. The DBP concentration was measured in each cycle.

Analytical methods

DBP concentration was analyzed by GC at following conditions: helium as the carrier gas, flow rate of 1 mL/min, injector temperature 260 °C, detector temperature 285 °C, oven temperature initially 80 °C for 5 min and increased to 260 °C by 6 °C/min for 5 min, increased to 280 °C by 6 °C/min and held for 5 min, increased again to 300 °C by 10 °C/min and held for 3 min. The effluent from the GC column was connected to MS, and the spectra were obtained by EI mode, 70 eV ionization energy and 50–400 amu scan for 2 s. For GC/MS analysis, PCNB was added as internal standard.

T-RFLP analysis

The microbial biomass was collected from SBR at 0 h and the final cycle and immediately frozen at −20 °C for DNA extraction. Total DNA of the samples was extracted by 3S spin DNA isolation kit for environmental sample K717 (Shanghai Biocolor Bioscience & Technology Company, China). Total DNA of *Micrococcus* sp. was extracted by using TIANamp Bacteria DNA Kit (TianGen, Beijing, China). The extracted DNA was stored with TE in a −20 °C freezer.

PCR amplification of 16S rRNA gene from the extracted DNA was performed with the universal primers 63F (5′CAGGCCTAACACATGCAAGTC3′) and 1404R (5′GGGCGGWGTGTACAAGGC 3′). The 63F primer was labeled with 6-carboxyfluorescein at the 5′ end. The PCR program was performed in a thermocycler (LongGen, Hongzhou, China) with the following profile: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min, and then kept at 16 °C.

PCR products were digested with *Rsa*I (Promega, WI, USA) according to manufacturer's protocol. The salt of all digested products was removed by using purification column. The mixture of the eluted products and the GS500 Liz internal size standards was denatured at 95 °C for 5 min and immediately chilled on ice for 3 min prior to electrophoresis with the automated DNA sequencer (ABI 3730XL, USA) in the GeneScan mode. The scan results were inspected with the Genemapper 3.0 software.

Results and discussion

Comparison of DBP biodegradation performance in two SBRs

DBP at the concentration of 10.0 mg/L was fed with the synthetic wastewater for the start-up of the reactors. At first, the removal percentage of DBP maintained at approximately 20 % during the first two cycles in Reactor 1 (Fig. 1a), on the contrary, about 50 % DBP was degraded at first cycle in Reactor 2 (Fig. 1c). As for Reactor 1, the removal percentage of DBP increased from 25.0 % at third cycle to 68.5 % at sixth cycle, and it maintained at about 70 % since seventh cycle (Fig. 1a).

When initial DBP concentration increased to 100 mg/L, the removal efficiency of DBP was very low at first for Reactor 1, <10 %. After acclimation process, it could reach 25 % at sixth cycle. However, the removal efficiency of DBP in Reactor 2 was higher than 50 % at first cycle, and it reached more than 70 % at third cycle (Fig. 1c). When initial DBP concentration increased to 50 mg/L, the removal efficiency of DBP was fast in both Reactor 1 and Reactor 2 (Fig. 1b).

The sludge was acclimated by a fill-and-draw operation of the cycle in every other day at 25 °C. In each cycle, half of the supernatant settled for 30 min in the reactor was drawn before the fresh basic medium of the same volume was added. Air was supplied by a sparger at the bottom of the reactor. The acclimation processes of activated sludge were according to Wang (2004). The acclimated activated sludge could degrade DBP effectively (Fig. 1a), indicating that the activated sludge used in this experiment had a DBP-degrading potential, and was capable of degrading DBP after acclimation. The removal rate of DBP by the acclimated activated sludge reached about 70 %, lower than that of bioaugmented system in which more than 90 % of DBP removal efficiency was reached at lower initial DBP concentrations (10.0 mg/L), showing that the enhancing effect of bioaugmentation on DBP degradation was effective even at low initial DBP concentration. Used butane-utilizing microorganisms to

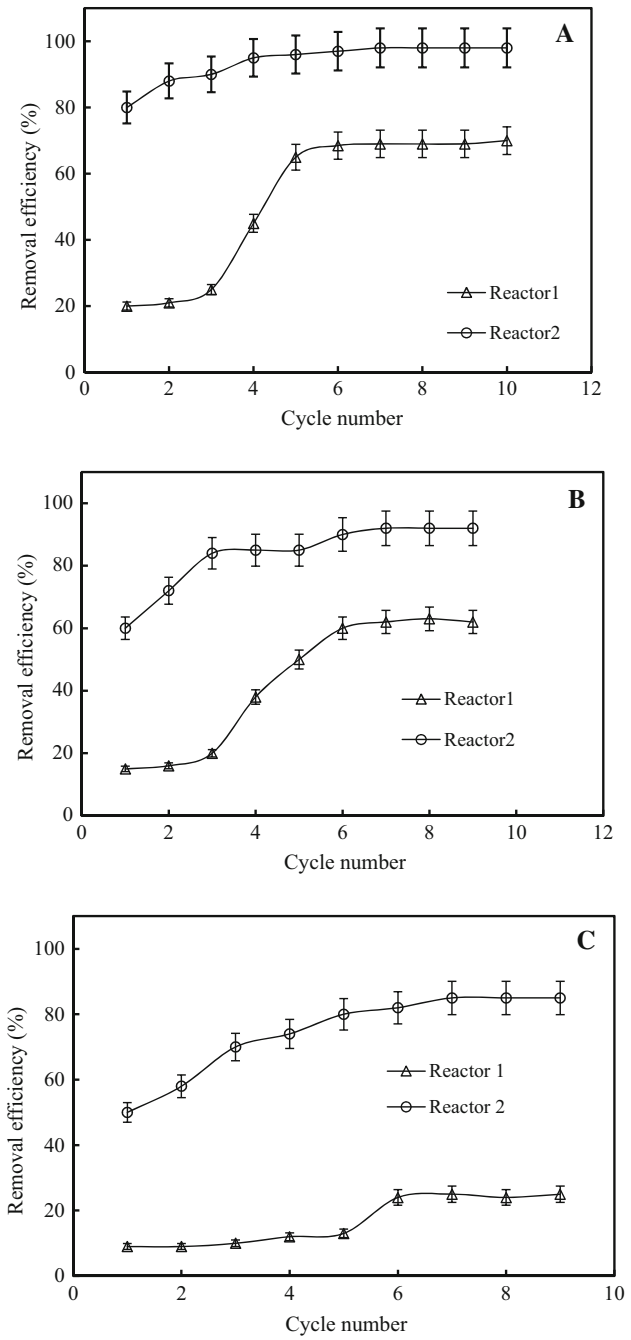

Fig. 1 DBP degradation at different cycles with the initial concentration of 10.0 mg/L (**a**), 50.0 mg/L (**b**) and 100.0 mg/L (**c**)

enhance the transformation of 1,1,1-trichloroethane in groundwater microcosms. The results demonstrated that initially the augmented microcosms were more effective in transformation of 1,1,1-trichloroethane than the non-augmented microcosms; however, after long-term incubation (about 440 days), both the non-augmented and augmented microcosms produced almost the same transformation yields and owned the same microbial composition.

Comparison of DBP degradation kinetics in two SBRs

The rate of biological reactions could be described using a hyperbolic saturation function in terms of substrate concentration, which can be expressed by following equation (Wang et al. 1997a):

$$r = \frac{r_m \cdot c}{k + c} \tag{1}$$

where r is the biodegradation rate, r_m is the maximum specific biodegradation rate, c is the substrate concentration and k is the half-saturation coefficient.

If $c \ll k$, Eq. (1) can be reduced to the following form:

$$r = \frac{r_m \cdot c}{k} \tag{2}$$

The degradation process conforms to a first-order reaction kinetic. Assuming $k_1 = (r_m/k)$ and integrating Eq. (1), the following relation of substrate concentration to time can be obtained:

$$\ln c = a + k_1 t \tag{3}$$

where c is DBP concentration, t is time and k_1 is the first-order rate constant.

The half-life of first-order reaction can be calculated according to following equation:

$$t_{1/2} = \frac{0.693}{k_1} \tag{4}$$

If $c \gg k$, Eq. (1) can be simplified to following equation:

$$r = r_m \tag{5}$$

The biodegradation process conforms to a zero-order reaction kinetic, and the biodegradation rate constant $k_0 = r_m$. Thus, the relation of substrate concentration to time can be expressed as:

$$c = b + k_0 t \tag{6}$$

where c is DBP concentration, t is time and k_0 is the zero-order rate constant.

The DBP biodegradation either by the acclimated activated sludge or by the bioaugmented sludge conformed to the first-order kinetics when the initial DBP concentration was in the range of 10.0–100.0 mg/L. Figure 2 reveals the kinetic analysis of DBP degradation when the initial DBP concentration was 10.0, 50 and 100.0 mg/L, respectively. The first-order rate constant is calculated and listed in Table 1.

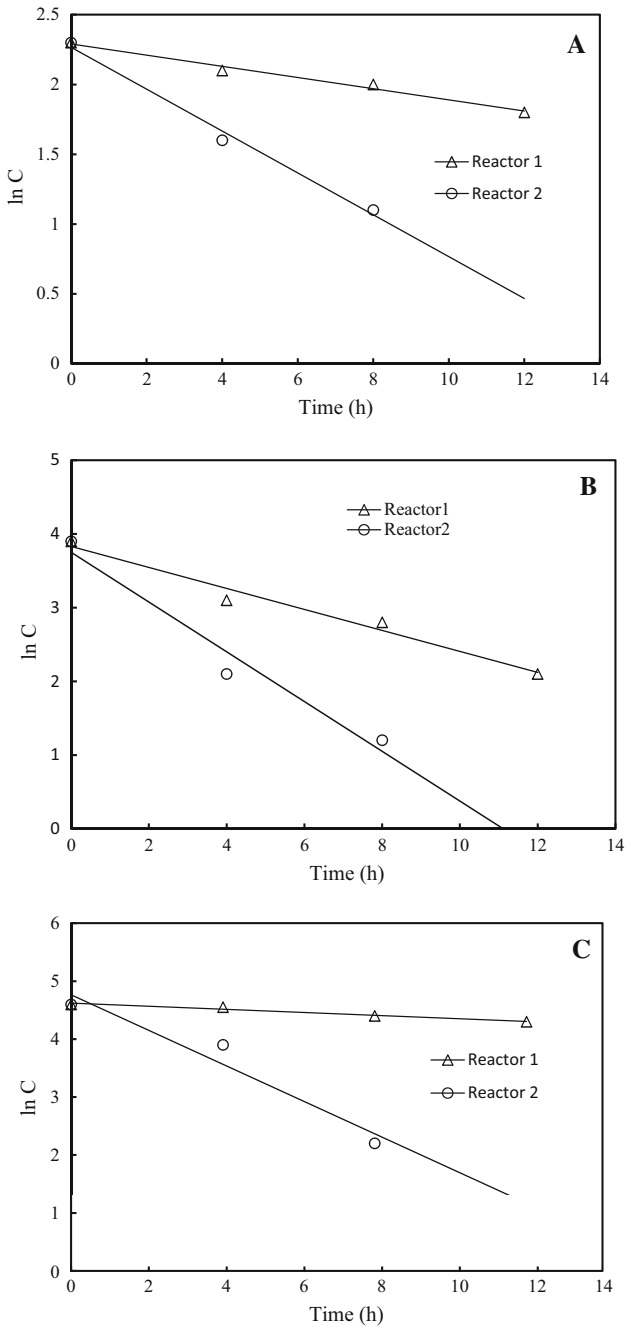

Fig. 2 Kinetic analysis of DBP degradation with the initial concentration of 10.0 mg/L (**a**), 50.0 mg/L (**b**) and 100.0 mg/L (**c**)

Table 1 Kinetic constants of DBP degradation

Initial DBP concentration (mg L^{-1})	K_1 (h^{-1})		R^2	
	Reactor 1	Reactor 2	Reactor 1	Reactor 2
10.0	0.040	0.150	0.984	0.990
50.0	0.014	0.337	0.974	0.964
100.0	0.026	0.302	0.969	0.945

The first-order rate constant of DBP degradation by the acclimated activated sludge was lower than the bioaugmented system by introducing *Micrococcus* sp. cells revealing that the enhancing effect of bioaugmentation on DBP degradation was significant. In particular, bioaugmentation had a positive effect on DBP degradation at high initial DBP concentration. The removal efficiency of DBP in Reactor 2 bioaugmented by *Micrococcus* sp. strain was always higher than that in Reactor 1 without bioaugmentation, indicating the advantages of bioaugmentation.

The DBP biodegradation in both reactors conformed to the first-order kinetics (Fig. 2), indicating that introduced *Micrococcus* sp. strain had no impact on the kinetics of DBP degradation by the activated sludge, probably because the DBP biodegradation by pure culture of *Micrococcus* sp. also conformed to the first-order kinetics. Furthermore, the DBP degradation rate constant decreased with the increase of initial DBP concentration in Reactor 1, suggesting that higher concentration of DBP had an adverse effect on the DBP-degrading activity of the activated sludge (Table 1). However, for Reactor 2, the DBP degradation rate constant increased with the increase of initial DBP concentration, indicating that higher concentration of DBP had not adverse effect on the DBP-degrading activity for the bioaugmented system.

Table 1 shows that the first-order rate constant of DBP degradation in bioaugmented system (Reactor 2) was higher than Reactor 1, suggesting that bioaugmentation by *Micrococcus* sp. strain really enhanced DBP degradation.

T-RFLP analysis

To relate the DBP degradation to the changes in the community structure and diversity and to simultaneously trace *Micrococcus* sp. strain in the reactor, terminal restriction fragment length polymorphisms (T-RFLP) of 16S rDNA of microbial consortium from the reactors were analyzed with the automated DNA sequencer in the GeneScan mode. Figures 3, 4 and 5 show the results of T-RFLP of 16S rDNA digested by *Rsa*I.

For the original activated sludge and the pure culture of *Micrococcus* sp. strain, Fig. 3a indicates the abundance and structure of the microbial community of the original fresh activated sludge. Nine significant peaks at 88, 173, 272, 276, 381, 384, 396, 430 and 449 bp were detected in the original fresh activated sludge. Figure 3b shows that the characteristic T-RFs of pure culture of *Micrococcus* sp. strain digested by *Rsa*I were 79 and 383, and 79 bp was main characteristic peak generated from pure culture of *Micrococcus* sp. strain.

In Reactor 1, three significant peaks at 94, 381 and 400 bp were detected when the initial DBP concentration was 10.0 mg/L (Fig. 4b), and eight significant peaks at 94,

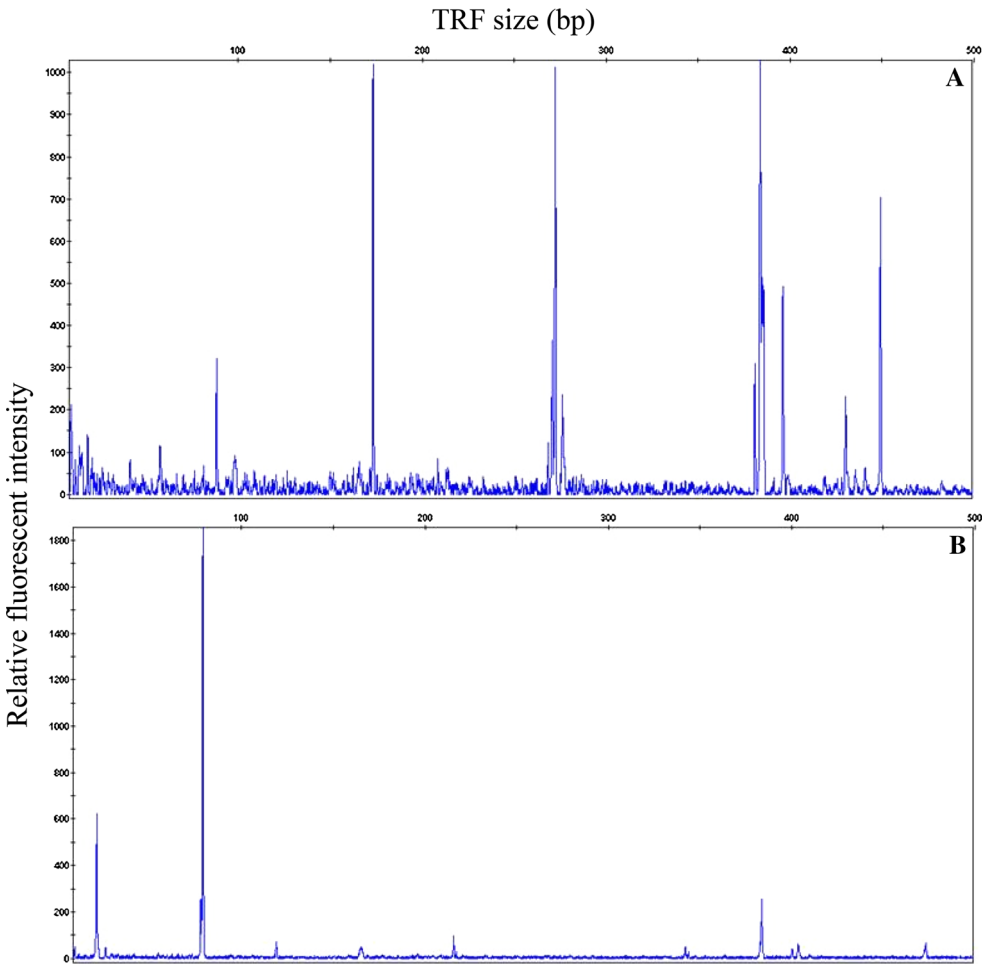

Fig. 3 Electropherograms of the 5′ T-RFLPs of *Rsa*I digested 16S rDNA amplified from the activated sludge (**a**) and pure culture of *Micrococcus* strain (**b**)

178, 272, 276, 381, 386, 399 and 435 bp were detected when the initial DBP concentration was 100.0 mg/L (Fig. 4c). Therefore, three T-RFs at 272, 276 and 381 bp always existed in Reactor 1 during the whole operation process; however, two T-RFs at 272 and 276 bp were not detected at the initial DBP concentration of 10.0 mg/L, while a T-RFs at 94 bp resulting from the acclimation occurred in Reactor 1 at the initial DBP concentration of 100.0 mg/L.

In Reactor 2, only two significant perks at 79 and 383 bp were detected at 0 h, showing that *Micrococcus* sp. was the sole dominant bacteria due to the addition of large amount of *Micrococcus* sp. (Fig. 5a). Subsequently, eight significant peaks at 66, 94, 178, 276, 381, 384, 399 and 435 bp were detected when the initial DBP concentration was 10.0 mg/L (Fig. 5b), and seven significant peaks at 94, 178, 276, 384, 386, 399 and 435 bp were detected when the initial DBP concentration was 100.0 mg/L (Fig. 5c). Six T-RFs at 94, 178, 276, 381, 399 and 435 bp were all detected in both Reactor 1 and Reactor 2 when the initial DBP concentration was 100.0 mg/L, while a T-RFs at 384 bp was only detected in Reactor 2 at the initial DBP concentration of 100.0 mg/L.

The introduced *Micrococcus* sp. was not detected any more when the initial concentration of DBP was 10.0 mg/L (Fig. 5b, c), which suggested that free *Micrococcus* sp. failed to compete with indigenous population or integrate in the sludge flocs under the condition of long-term performance, so that DBP degradation in Reactor 2 was attributed to utilization of the acclimated microorganisms originated from the activated sludge at high initial concentration range of DBP. That is, bioaugmentation by free *Micrococcus* sp. was effective but transient, which was also observed in the bioaugmentation reactors by other researchers. For example, an *o*-nitrobenzaldehyde-degrading strain *Pseudomonas putida* ONBA-17 was inoculated into a SBR for enhancing the degradation of *o*-nitrobenzaldehyde from synthetic water under the laboratory scale (Yu et al. 2010). They found that bioaugmentation really

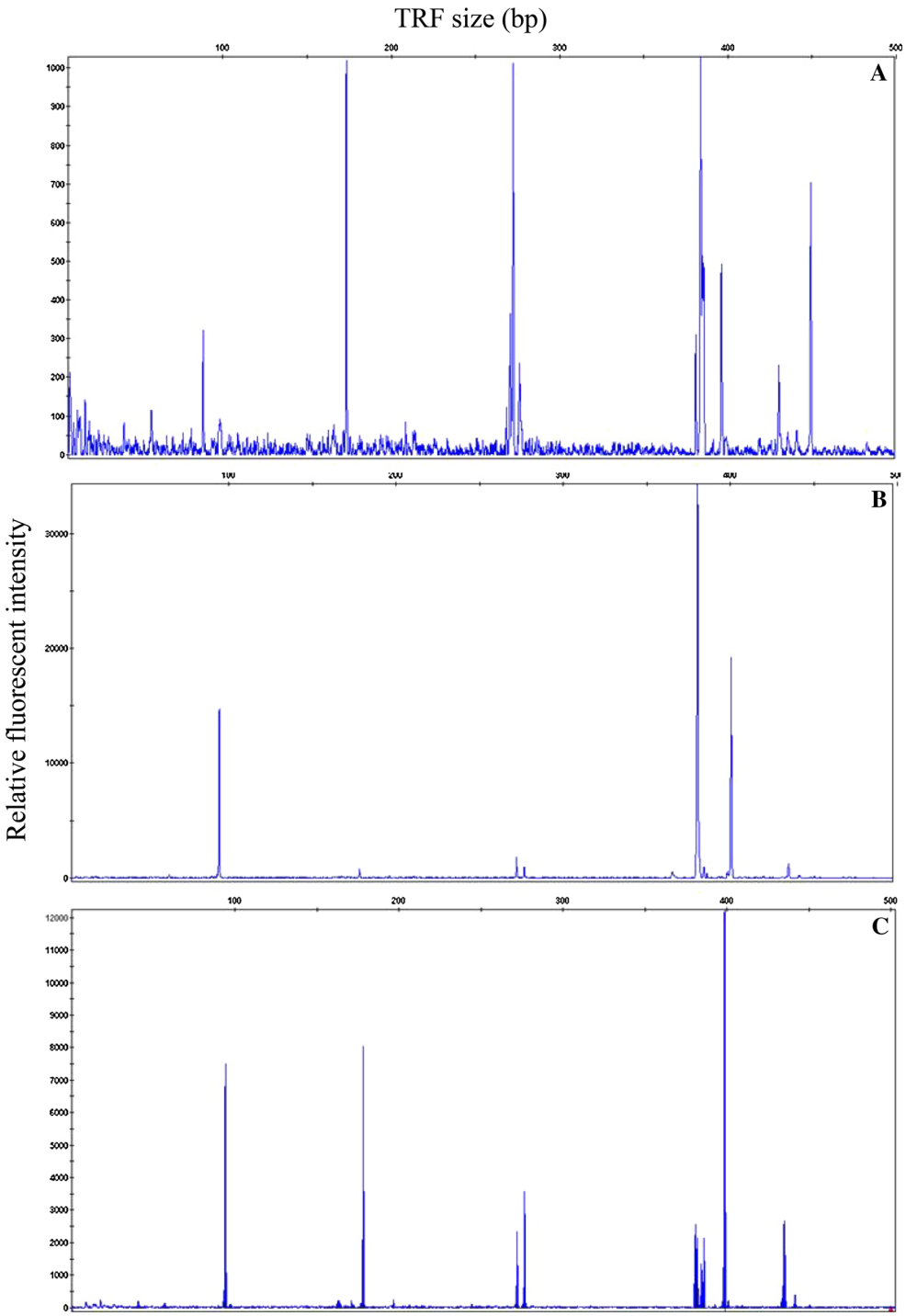

Fig. 4 Electropherograms of the 5′ T-RFLPs of *Rsa*I digested 16S rDNA amplified from the microbial biomass in Reactor 1, **a** at 0 h; **b** at the initial DBP concentration of 10.0 mg/L; and **c** at the initial DBP concentration of 100.0 mg/L

accelerated the start-up of the system, but lost the advantages in the long-term performance, compared with the acclimatized non-bioaugmentation system, due to the predation of introduced bacteria by protozoa.

In this study, the intermediate products during DBP degradation were identified by GC/MS analysis. According to the results, the DBP degradation pathway could be tentatively proposed as follows: di-butyl phthalate was firstly hydrolyzed to mono-butyl phthalate and then to phthalic acid (PA) which can be further degraded through TCA cycle to form carbon dioxide and water (Fig. 6).

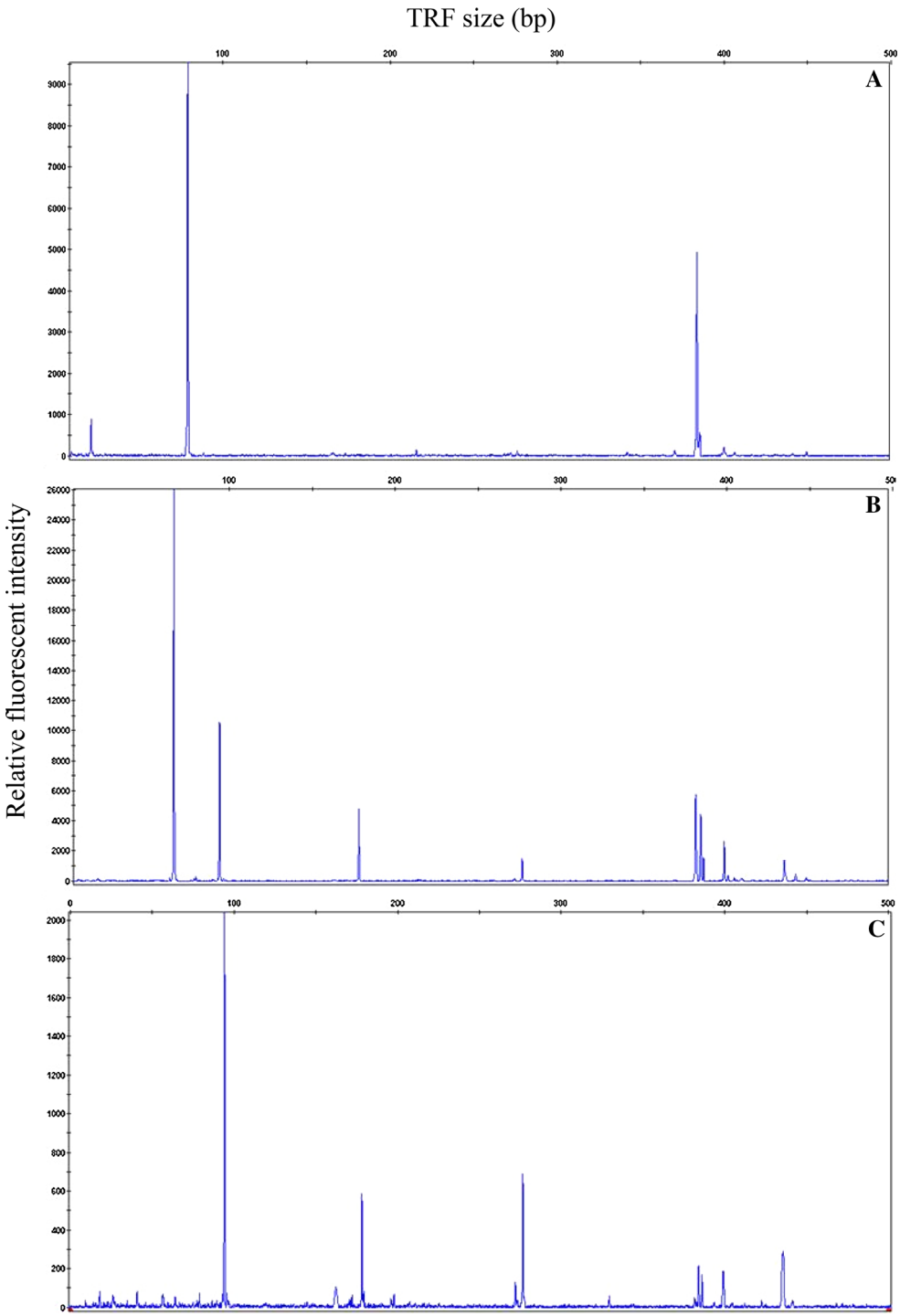

Fig. 5 Electropherograms of the 5′ T-RFLPs of *Rsa*I digested 16S rDNA amplified from the microbial biomass in Reactor 2, **a** at 0 h; **b** at the initial DBP concentration of 10.0 mg/L; and **c** at the initial DBP concentration of 100.0 mg/L

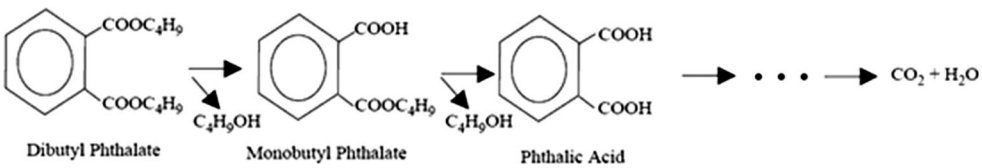

Fig. 6 Proposed DBP degradation pathway

Conclusion

This study demonstrated that inoculation with *Micrococcus* sp. strain capable of degrading DBP could speed up the start-up of the reactor, clearly indicating the feasibility of bioaugmentation technology for treatment of wastewater containing high concentration of DBP. The bioaugmentation not only enhanced the removal efficiency of target compound, but also shortened the start-up time of the reactor. The kinetics of DBP degradation conformed to the first-order model. The T-RFLP analysis indicated the bacterial community changes in the acclimated activated sludge and the introduced *Micrococcus* sp. during the operational process.

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