Comparative studies of aerobic and anaerobic biodegradation of methylparaben and propylparaben in activated sludge

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ABSTRACT

The biodegradability of two typical parabens (methylparaben and propylparaben) in activated sludge, at initial concentrations of 1 mg L⁻¹ or 10 mg L⁻¹, was investigated under aerobic and anaerobic conditions. The results showed that microorganisms played a key role in degradation of parabens in WWTPs, especially in aerobic systems. The half-lives of methylparaben and propylparaben under aerobic conditions have been estimated to range between 15.8 and 19.8 min, and benzoic acid was found to be one of the major biodegradation products. The calculated biodegradation efficiency of methylparaben and propylparaben in activated sludge under aerobic conditions was significantly higher than that observed under anaerobic (nitrate, sulfate, and Fe (III) reducing) conditions, as methylparaben and propylparaben exhibited comparatively higher persistence in anaerobic systems, with half-lives ≥ 43.3 h and ≥ 8.6 h, respectively. Overall, the results of this study imply that the majority of these parabens can be eliminated by aerobic biodegradation during conventional wastewater treatment processes, whereas minor removal is possible in anaerobic systems if an insufficient hydraulic retention time was maintained.

1. Introduction

Parabens, which are esters of *p*-hydroxybenzoic acid, have frequently been used as preservatives in food, cosmetics and pharmaceuticals since the mid-1920s (Andersen, 2008; Ma et al., 2016; Błędzka et al., 2014). Common esters include methyl-, ethyl-, propyl-, butyl-, and benzyl- parabens. Parabens have long been considered safe preservatives because of their good antimicrobial properties, low cost, low toxicity, and high stability (Błędzka et al., 2014; Haman et al., 2015; Andersen, 2008). However, recent studies have shown that parabens might possess endocrine disrupting effects and be associated with breast tumors in women and sperm DNA damage (Darbre and Charles, 2010; Meeker et al., 2011). Since parabens have the potential to harm human health, investigation of their occurrence, exposure, toxicity and removal from different environmental matrices has received global attention (Albero et al., 2012; Wu et al., 2016; Evgenidou et al., 2015; Xue et al., 2015; Schlumpf et al., 2010; Gorga et al., 2015; Liao et al., 2013; Petala et al., 2015). These substances are mainly discharged into the environment through urban sewage, and studies conducted to date have shown that trace concentrations of parabens and their halogenated by-products are ubiquitous in the aqueous phase

and sewage sludge of WWTPs (Wang and Kannan, 2016; Matamoros et al., 2016; Canosa et al., 2006; Li et al., 2015; Sun et al., 2016).

Methylparaben (MP) and propylparaben (PP), which are the most commonly used parabens, have been detected in different environmental matrices with high frequency. Wang et al. (2013) found the median concentration of urinary parabens measured for Chinese adults was 33.2 ng mL⁻¹. Notably, 1000–10,000 ng mL⁻¹ of parabens were found in urine from some Chinese adult females. They also indicated that parabens were present predominantly (> 90%) as conjugated species in urine, especially, MP and PP were the predominant compounds. A significant positive correlation was found between the concentrations of MP and PP in urine samples. In order to further investigate the sources of Chinese exposure to parabens, Guo et al. (2014) measured concentrations of six parabens in five categories of personal care products (N=52) collected from Tianjin, China, and estimated human exposure doses to these compounds. Their study showed that hand and body lotions were the major contributors to exposures, and the daily exposure doses for MP, and PP from these products were 10,200 and 4890 µg, respectively.

Widespread use of personal care products can make parabens leaking into aquatic environment, mainly through wastewater treat-

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ment discharges. Molins-Delgado et al. (2016) determined the occurrence of MP and PP in influent and effluent wastewater from the 19 major WWTPs of Catalonia, Spain. A significant positive correlation also was observed for those parabens in their study, strongly indicating a similar source of pollution. Although parabens are expected to be eliminated using conventional biological treatment, they are ubiquitous in effluent of WWTPs. Matamoros et al. (2016) assessed the seasonal performance of four different full-scale wastewater technologies on elimination of emerging contaminants, including MP. They found that the highest removal efficiency was only $89 \pm 3\%$ during the warm season when waste stabilization pond technology was employed. Notably, the MP removal efficiency of the other three technologies investigated, namely, an extended aeration system, rotating biological contactor and constructed wetland, was significantly lower than previously reported (Madsen et al., 2001; Hernández Leal et al., 2010; Li et al., 2015). Madsen et al. (2001) reported that MP and PP are readily biodegraded under aerobic conditions, with biodegradability (biological oxygen demand) of about 90% of the theoretical oxygen demand. Li et al. (2015) also indicated that biodegradation led to significant elimination of parabens during conventional wastewater treatment. However, they deduced that parabens were mainly degraded in the anaerobic tank based on mass balance results, which is in accordance with the results reported by Yu et al. (2011), who found that levels of parabens decreased significantly during anaerobic processes.

Given this background, microbial biodegradation is considered as an important environmental mechanism of parabens removal when they are discharged into wastewater treatment plants (WWTPs). Accordingly, it is necessary to investigate differences in the aerobic and anaerobic biodegradation of parabens. Therefore, in this study, the biodegradation of different levels of MP and PP in activated sludge collected from a WWTP in Xiamen, China, were investigated. The kinetics of the indigenous microbes-initiated oxidation of MP and PP under different redox conditions was systematically compared, as well as their possible transformation products. These experimental and theoretical data will be useful for further interpretation of the mechanism of parabens biodegradation in WWTPs.

2. Materials and methods

2.1. Chemicals

Methylparaben (methyl 4-hydroxybenzoate, $C_8H_8O_3$, MP) and propylparaben (propyl 4-hydroxybenzoate, $C_{10}H_{12}O_3$, PP) with purities

above 99% were purchased from Acros Organics. The molecular structures and chemical properties of MP and PP are listed in Table 1 (<http://www.drugbank.ca>, <http://www.chemspider.com>). All organic solvents were of HPLC grade and purchased from Tedia Co. (Fairfield, Ohio, USA). Conical flasks were deep cleaned with detergent and Milli-Q water, then baked at 400°C prior to use. Stock solutions of MP and PP were prepared in methanol and stored at -20°C .

2.2. Sample collection

Grab samples of return activated sludge were collected on July 24, 2015 from a municipal WWTP in Xiamen, China. This WWTP is equipped with a primary treatment tank, an Orbal oxidation ditch and UV disinfection before effluent discharge. The mixed samples were collected in amber bottles and transported to the laboratory immediately for preliminary incubation under aerobic and anaerobic conditions.

2.3. Biodegradation experiments

Biodegradation experiments were conducted in duplicate individually under different redox conditions. Prior to spiking with parabens, the activated sludge was pre-incubated for one week under aerobic and anaerobic conditions. Incubation medium with 10% activated sludge (v/v, MLSS: 6910 mg L^{-1}) was prepared in minimum mineral salt (MMS) containing peptone (32 g L^{-1}), yeast extract (22 g L^{-1}), urea (6 g L^{-1}), NaCl (1.8 g L^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8 g L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 g L^{-1}), K_2HPO_4 (55.6 g L^{-1}), KH_2PO_4 (16 g L^{-1}) (Lozada et al., 2004) and amended with trace vitamins (Yang and McCarty, 1998; Liu et al., 2011). For anaerobic treatment, the incubation media was purged with nitrogen to deoxygenate. Sterile controls containing the individual compounds were also analyzed to establish variations in concentration changes and verify that any hydrolysis metabolites formed were detected during the experiment. Prior to this study, our research group detected the concentrations of various types of PPCPs in the influent and sludge of the studied WWTP, which included MP and PP. The background concentration in sludge ranged from 1.0 ± 0.6 to $6.8 \pm 0.9\text{ }\mu\text{g kg}^{-1}$, while the average concentration of MP and PP in the influent was 247.4 ng L^{-1} and 311.3 ng L^{-1} , respectively (data not shown).

For sterile control samples, the conical flasks with incubation media were autoclaved (120°C , 20 min) for three consecutive days. The half-life of MP and PP degradation was calculated based on the first-order equation as follows:

Table 1
CAS NO., molecular structure, pKa, logKow, logKoc of the target compounds.

Compounds	CAS No.	Structure	pKa ^a	LogK _{ow} ^b	LogK _{oc} ^b
Methylparaben (MP)	99-76-3		8.3	1.9	2.1
Propylparaben (PP)	94-13-3		n.a	3.0	2.6

n.a. – Not available.

^a <http://www.drugbank.ca/>.

^b <http://www.chemspider.com>.

Table 2
Gradient of mobile phase for HPLC analysis of MP and PP.

Eluent	0 min	2 min	5 min	7 min	14 min
A	70%	70%	50%	70%	70%
B	30%	30%	50%	30%	30%

$$\ln(C_t) = \ln(C_0 e^{-kt}),$$

where, C_t represents the concentration of MP or PP at time t , C_0 represents the initial concentration and k is the kinetic rate constant.

2.3.1. Aerobic biodegradation

To investigate aerobic degradation, 50 mL of the incubation medium (10% activated sludge (v/v) in MMS and trace vitamins) was spiked with 1 mg L⁻¹ or 10 mg L⁻¹ of each paraben, then placed in a dark incubator shaker (25 °C, 150 rpm). Aerobic conditions were maintained by sealing with a ventilative film. For aerobic experiments, preliminary experiments showed a rapid dissipation of selected parabens; thus, aerobic samples were collected at 0, 5, 10, 20, 30, 60, and 90 min.

2.3.2. Anaerobic biodegradation

Anaerobic samples were prepared under the continuous flow of nitrogen. Briefly, parabens were spiked into 50 mL incubation medium (10% activated sludge (v/v) in MMS and trace vitamins) in an anaerobic bottle in amounts sufficient to give 1 or 10 mg L⁻¹ of each paraben. Following solvent evaporation, the bottles were sealed with rubber stoppers and plugged with aluminum rings to prevent air loss. For anaerobic experiments, samples were collected to detect the concentration of parabens at 0, 2, 4, 6, 9, 18, 24, 48, and 72 h based on the results of a preliminary experiment. The incubation medium was unamended with any of electron acceptors as the anaerobic control. Nitrate-reducing, sulfate-reducing and Fe-reducing conditions were achieved by amending with 20 mM of NaNO₃, 20 mM of Na₂SO₄, and 20 mM of Fe (III) citrate, respectively. 1 mM of Na₂S was added to three reducing treatments as reducing agent (Liu et al., 2011). All anaerobic bottles were incubated in the dark at 25 °C.

2.4. Analytical methods

To monitor the concentration of MP and PP, 2.0 mL samples were quickly moved from each experimental conical flask at the designated time and amended with 8 µL of concentrated o-phosphoric acid to stop biodegradation (Oie et al., 2007; Larcher and Yargeau, 2011). Samples were then centrifuged at 10,000 rpm for 10 min at 4 °C (Thermo Legend Micro 21), after which 1 mL of supernatant was passed through a

0.22 µm PVDF filter and transferred to an amber vial for HPLC analysis. Additionally, the samples prepared at sampling end points to determine the formation product of aerobic degradation were performed by gas chromatography-mass spectrometry (GC-MS) analysis (Agilent 7890-5975c), equipped with Agilent 19091S-433 HP-5MS capillary column (30 m × 250 mm × 0.25 mm, 5% phenyl methyl silox). The aerobic degradation product was extracted from the supernatant with an equal volume of *n*-hexane three times. The total extracts were collected and evaporated under fume cupboard, followed by redissolving in *n*-hexane. The oven temperature program was set from 60 °C at initial 2 min, and then raised to 280 °C at 10 °C min⁻¹ followed by 2 min hold at 280 °C. Helium was used as the carrier gas at a flow rate of 1.0 mL min⁻¹. Full scan mode was used with mass scan range of 40–300 amu, and electron ionization mode was conducted at 70 eV with 1 µL of injection volume.

Determination of MP and PP was conducted using a Dionex Ultimate 3000 HPLC system equipped with a UV detector at a wavelength of 270 nm and a BDS HYPERSIL C18 column (150 × 2.1 mm, 3 µm). The mobile phase consisted of water: acetic acid (A; 100:0.1, v/v) and acetonitrile (B). The gradient is presented in Table 2. The flow rate was set at 0.2 mL min⁻¹ and an injection volume of 10 µL. The limit of detection (LOD) of the HPLC method for MP and PP was 5 and 2 µg L⁻¹. Recoveries (%) of the selected parabens in the experimental activated sludge were calculated from the data obtained by analysis of three replicates with MP and PP at 1 mg L⁻¹. The recovery of the spiked concentration of MP and PP was 90 ± 5%, 94 ± 7%, respectively.

2.5. Statistical analyses

Data were collected and analyzed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Significant difference was undertaken using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Aerobic biodegradation

The biodegradation of MP and PP at two levels for the activated sludge as a percentage of the initial concentration across an interval of 90 min under aerobic conditions are presented in Fig. 1. The results revealed rapid biodegradation of MP and PP in non-sterile sludge, whereas little loss was observed in sterile sludge within 90 min ($P < 0.05$). Abiotic hydrolysis of MP and PP to *p*-hydroxybenzoic acid (4-HB) in liquid media has previously been reported (Valkova et al., 2001). In this study, the lack of an obvious mass loss of parent compound indicated that hydrolysis did not occur in the aerobic sterile samples, which may have been a result of the low initial concentration compared

Fig. 1. Aerobic biodegradation of MP and PP using 10% activated sludge as inoculum (1, 10-initial concentration of 1 mg L⁻¹ or 10 mg L⁻¹; S-sterile; all data points are mean values).

Table 3
Kinetic parameters for the biodegradation of MP and PP under aerobic conditions.

Compound	Condition	Concentration (mg L ⁻¹)	k (mg L ⁻¹ min ⁻¹) ^a	$T_{1/2}$ (min) ^b	R^{2c}
MP	Aerobic	1	0.036	19.3	0.9911
		10	0.044	15.8	0.9996
PP	Aerobic	1	0.041	16.9	0.9827
		10	0.035	19.8	0.9887

^a Kinetic rate constant (k), which was calculated using the first-order reaction equation (mean value from duplicate experiments).

^b Half-life ($T_{1/2}$), calculated as $(\ln 2)/k$.

^c Coefficient of correlation (R), which represents the fitness of the experimental data.

with the levels in liquid culture (initial concentration of 500 mg L⁻¹ of MP, or PP) in Valkova et al.'s study (2001). The aerobic degradation data for MP and PP were well explained by the first order kinetic model. The aerobic degradation kinetics parameters of MP and PP are listed in Table 3. The calculated kinetic rate constant (k) of MP and PP showed the same dissipation trend under aerobic conditions. The k values of MP and PP ranged from 0.036 to 0.044, and 0.035–0.041, respectively. The half-lives of MP and PP at initial concentrations of 1 mg L⁻¹ or 10 mg L⁻¹ were estimated to range between 15.8 and 19.8 min, suggesting the parent compounds of MP and PP may quickly and largely biodegraded and eliminated from the WWTPs during the aerobic biological phase. The removal efficiencies of MP and PP over 90 min ranged from 96.7% to 98.2%, and 96.6–98.3%, respectively. The results were generally in accordance with those reported by González-Mariño et al. (2011), in their study, the test of aerobic biodegradability and stability in raw wastewater also showed that parabens degrade rapidly in real sewage. Notably, after 90 min of

Table 4
Kinetic parameters for the biodegradation of MP and PP under different anaerobic conditions.

Compound	Condition	Concentration (mg L ⁻¹)	k (mg L ⁻¹ h ⁻¹) ^a	$T_{1/2}$ (h) ^b	R^{2c}
MP	Anaerobic	1	0.010	69.3	0.9909
		control	0.008	86.6	0.9889
	Nitrate reducing	1	0.016	43.3	0.9921
		10	0.015	46.2	0.9919
	Sulfate reducing	1	0.009	77.0	0.9906
		10	0.008	86.6	0.9834
	Fe(III) reducing	1	0.009	77.0	0.9875
		10	0.007	99.0	0.9799
PP	Anaerobic	1	0.073	9.5	0.9992
		control	0.071	9.8	0.9977
	Nitrate reducing	1	0.081	8.6	0.9968
		10	0.078	8.9	0.9923
	Sulfate reducing	1	0.058	12.0	0.9991
		10	0.063	11.0	0.9869
	Fe(III) reducing	1	0.072	9.6	0.9922
		10	0.077	9.0	0.9803

^a Kinetic rate constant (k), which was calculated using the first-order reaction equation (mean value from duplicate experiments).

^b Half-life ($T_{1/2}$), calculated as $(\ln 2)/k$.

^c Coefficient of correlation (R), which represents the fitness of the experimental data.

incubation, trace residues of MP and PP were still detected in the activated sludge; hence, additional studies should be conducted to address their removal. Indeed, this might be the reason for the occurrence of these types of parabens in the following treatment phase and their subsequent spread to different environmental matrices (Jonkers et al., 2009; Hernández Leal et al., 2010).

Fig. 2. Anaerobic biodegradation of MP and PP using 10% activated sludge as inoculum (1, 10-initial concentration of 1 mg L⁻¹ or 10 mg L⁻¹; S-sterile; all data points are mean values).

Fig. 3. Mass spectra of benzoic acid, a major biodegradation product, identified by GC-MS analysis under aerobic conditions for MP(a) and PP(b), respectively.

3.2. Anaerobic biodegradation

As shown in Fig. 2, comparatively slow biodegradation of MP and PP over the incubation period (72 h for MP, 48 h for PP) was observed at both concentrations under different anaerobic reducing conditions, whereas little loss was observed from sterile activated sludge within 72 h for MP and 48 h for PP ($P < 0.05$). Nitrate-reducing, sulfate-reducing and Fe-reducing conditions commonly occur in anaerobic environments (Liu et al., 2011); however, among these three reducing conditions, only nitrate addition significantly accelerated the anaerobic biodegradation of MP or PP, when compared to that of the anaerobic control ($P < 0.05$). These findings indicate that the biodegradation of these two compounds was relatively slow in the anoxic conditions compared to aerobic degradation. Similar to the aerobic sterile controls, there was no obvious loss of mass of the parent compounds, indicating that hydrolysis did not occur in the anaerobic sterile samples as well. The anaerobic degradation data for MP and PP also fit the first order kinetic model well. The anaerobic degradation kinetics parameters of MP and PP are listed in Table 4. The removal efficiencies of were

estimated to range from 43.2% to 70.1% for MP over 72 h, and 94.1–97.8% for PP over 48 h, respectively. However, Li et al. (2015) and Yu et al. (2011) adopted mass balance to calculate the overall removal efficiency, and their mass flow results showed that the high mass change in the anaerobic tank suggested that parabens were mainly biodegraded in this unit, this may be due to the theoretical calculation only based on the concentration difference between each treatment unit, which may not accurately describe the actual biodegradation process; additionally, in their study, the sampling of wastewater was just conducted on a separate weekday, not for longer consecutive days, this also should be addressed. In this study, half-lives of MP and PP under anaerobic conditions have been estimated to range between 8.6 and 99.0 h, showing that parabens with longer alkyl chains (PP) underwent faster degradation than those with short alkyl chains (MP) under anaerobic conditions, while no difference in the rate of degradation was observed under aerobic conditions. Therefore, based on the calculated half-lives of MP and PP from anaerobic biodegradation test, we further speculate that elimination of parabens does not occur in the anaerobic phase of actual WWTPs if a sufficient hydraulic retention

Fig. 4. Possible methylparaben and propylparaben pathway of degradation by aerobic mixed microbes (Valkova et al., 2001; Amin et al., 2010).

time is not achieved.

3.3. Possible biodegradation products of MP and PP

In the case of aerobic incubation (initial concentration of 10 mg L^{-1}), biodegradation products of MP and PP were tentatively investigated by GC-MS analysis. A significant peak with a retention time 8.70–8.75 min appeared in the *n*-hexane extract of aerobic incubation samples. The *m/z* value of 121.9 of the peak was clearly extracted (Fig. 3) and identified as benzoic acid (BA) by comparison to a GC-MS library, suggesting that biodegradation of the selected parabens occurred via cleavage of a series of carbon bonds by mixed aerobic microbes. Benzoic acid is recognized as a nonspecific marker of parabens (Ye et al., 2006). In the present study, *p*-hydroxybenzoic acid, the reported common metabolite of all paraben esters (Amin et al., 2010; Wang et al., 2015; Moos et al., 2015; Wang and Kannan, 2016), was not detected in the samples upon GC-MS analysis, while we did not detect the metabolite in the MP and PP controls as well. Therefore, it is possible that *p*-hydroxybenzoic acid was further oxidized to benzoic acid by mixed microbes during the rapid aerobic degradation process. Based on a study conducted by Valkova et al. (2001), paraben degradation originally occurred through hydrolysis of the ester bond to produce *p*-hydroxybenzoic acid via esterase activity, followed by a decarboxylation step under aerobic conditions, and phenol was reported as one of the major products (Amin et al., 2010). A possible MP and PP degradation pathway by aerobic mixed microbes based on the results of previous studies combined with those of our study is shown in Fig. 4. However, additional research is needed to clearly and accurately interpret the mechanism of biodegradation. In addition, these above results demonstrate that the ability of mixed microbes to degrade parabens does not precisely reflect the roles of individual microorganisms; thus, further research to evaluate individual microorganisms isolated from WWTPs should also be initiated.

4. Conclusions

The results of the biodegradation experiments demonstrated that microbes could readily degrade MP and PP under aerobic conditions, with half-lives of less than 20 min for both compounds, regardless of their original concentration. However, comparatively longer period of time was needed for anaerobic degradation. The shortest biodegradation half-lives for MP and PP of 43.3 h and 8.6 h, respectively, were both observed under nitrate reducing conditions. Anaerobic biodegradation tests also clearly demonstrated that paraben species with longer alkyl chains (e.g., PP) underwent faster degradation than those with shorter alkyl chains (e.g., MP). Benzoic acid was identified as the biodegradation product of MP and PP under aerobic conditions. These results suggest that biodegradation of parabens would be an important removal mechanism in WWTPs, whereas trace concentrations of parabens were still detected in the final samples of activated sludge, and additional work to optimize biological removal techniques is still needed.

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