ISOLATION OF MICROALGAE TOLERANT TO POLYBROMINATED DIPHENYL ETHERS (PBDES) FROM WASTEWATER TREATMENT PLANTS AND THEIR REMOVAL ABILITY

D. DENG¹, H.X. CHEN¹,² and N.F.Y. TAM¹

¹ Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Kowloon, Hong Kong SAR, China, ²Nanjing College of Information Technology, 99 Wenlan Road, Qixia District, Nanjing, 210046, China

e-mail: bhntam@cityu.edu.hk

EXTENDED ABSTRACT

Polybrominated diphenyl ethers (PBDEs), widely used as flame retardants, are persistent contaminants which cause adverse effects to environments and pose health risks to human. The present study aims to isolate PBDE-tolerant microalgae from wastewater treatment plants (WTPs) and compare the PBDE removal ability of different isolates. Primary influent collected from four WTPs in Hong Kong was spiked with a mixture of PBDEs consisted of a commercial penta-BDE product (DE-71) and BDE-209 (5:1) at a concentration of 6 μg L⁻¹. From the influent, nine microalgal species, belonged to five genera, Chlorella (STCh and SICh), Parachlorella (STPa1 and STPa2), Scenedesmus (STS, TPSc1 and TPSc2), Nitzchia palea (YLBa) and Mychonastes (TPMy), were isolated and identified based on morphological features and phylogenetic analysis of 18S rRNA genes. Among these isolates, the growth (cell numbers and chlorophyll a content) of four, SICh, STCh, STPa1 and TPSc1, were not affected when exposed to the mixture of DE-71 and BDE-209 (5:1) at low (6 μg L⁻¹), medium (60 μg L⁻¹) and even high (600 μg L⁻¹) levels for seven days, suggesting that these four were resistant to PBDEs. On the contrary, the growth of YLBa was significantly inhibited even at the low level of PBDEs while the inhibition to the other three species TPSc2, STPa2, and TPMy only found at the high level. The removal of PBDEs by the isolate SICh was the highest, with percentages ranged from 82 to 90 % after seven days of exposure to the high level of PBDE mixture, while TPSc2, STCh and STPa2 were the poorest species and removed only 56% of total PBDEs. SICh and TPSc1 also accumulated more PBDEs than the other seven isolates. In conclusion, this study is the first time isolated and identified PBDE-tolerant microalgae from WTPs in Hong Kong. The study also successfully obtained a Chlorella isolate SICh with a high tolerance to PBDEs and a high removal ability, which could be used for the removal of PBDEs from contaminated wastewater.

Keywords: PBDES, microalgae, tolerant, removal

1. INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) commonly used as brominated flame retardants in household items are potentially leaching out into surrounding environments. Due to the physical and chemical properties, PBDEs are persistent, bioaccumulative and cause adverse effects on ecosystems and human health (Verreault et al., 2007; Athanasiadou et al., 2008). Although PBDEs have been gradually phased out in many countries (U.S. EPA, 2010), old products containing significant quantities of PBDEs may still release these pollutants (Hirai et al., 2006). Industrial sewage discharge, urban runoff following atmospheric deposition, household domestic sewage from rinsing of cleaning rags, carpet cleaning, laundry and shower gray water, human waste, etc., are dominant sources of
PBDE contamination (Environment Canada, 2006; Gevao et al., 2008). PBDEs in wastewater must be treated prior to its discharge.

Microalgae have been employed as secondary and tertiary treatment processes to remove toxic pollutants such as heavy metals (Han et al., 2007) and endocrine disrupting chemicals (Liu et al., 2010). It has been reported that microalgae grown in industrial sewage or contaminated water were more tolerant to contaminants such as nickel, copper and zinc and had higher removal abilities than those obtained from clean and pristine environments (Stokes et al., 1973; Chong et al., 2000). It is possible that the sewage from WTPs with PBDE contamination is a potential source of PBDE-tolerant microalgal species, and the tolerant isolates may also be employed for PBDE removal. The present study therefore aims to (i) isolate the PBDE-tolerant microalgae from sewage in WTPs and (ii) compare the ability of different isolates to remove PBDEs, aiming to obtain the most effective isolate for the removal of PBDE from contaminated water.

2. MATERIALS AND METHODS

2.1. Isolation and identification of PBDE-tolerant microalgal species

Primary settled influent and dewatered sludge from four different WTPs in Hong Kong, namely Shatin, Stonecutters Island, Tai Po and Yuen Long, were collected from September to October 2011. The solid sample was extracted by accelerated solvent extraction system (ASE 200, Dionex, USA) with a surrogated standard, polychlorinated biphenyl (PCB-209), while liquid sample was done by solid phase extraction using Discovery DSC18 SPE cartridge (Supelco, Bellefonte, PA, USA), according to the U.S.EPA method 1614. The extract was concentrated and stored at 4°C, then analyzed by gas chromatography-mass spectrometry (GC-MS).

The primary influent after filtered through a 100 μm mesh net was spiked with a mixture of commercial penta-BDE product (DE-71) (Wellington laboratories, Canada) and BDE-209 (J&K Scientific, China) dissolved in dimethyl sulfoxide (DMSO) at a ratio of 5:1 with a total concentration of 6 μg L⁻¹. Two controls, both did not have any PBDE addition, were also set-up, with the solvent control contained DMSO (0.1%, v/v) and the positive control did not have any solvent. The cultures were incubated at 22±1°C on a rotary shaker with a speed of 150 rpm, illuminated with cold fluorescent lamps at a light intensity of 40 μmol s⁻¹ m⁻² and a 16/8 h light/dark cycle in an environmental chamber. After enrichment for two weeks, 1 mL of the culture was serially diluted and inoculated on the Bristol medium (BM) (James, 1978) agar plates with 30-300 cells per plate. After incubation for two weeks, the visible algal colonies with distinct morphology were picked and sub-cultured for the next round of selection until an axenic culture was obtained.

The morphology of each isolated strain was observed under microscope (Zeiss, Axioskop, Germany) at a magnification of 400x for morphological identification (Bellinger and Sigee, 2010; Algaebase, 2012). 1 mL of the cells at the exponential phase was harvested and the genomic DNA of the re-suspended pellet was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., USA). Two pairs of oligonucleotide primers prepared by Life Technologies were used for the amplification of microalgal 18S rRNA gene. Forward 1 (GTCAGAGGTGAAATTCTTGGATTTA) and reverse 1 (AGGGCAGGGACGTAATCAACG) were universal eukaryotic primers (Rasoul-Amini et al., 2009), while forward 2 (GCCGAAATCCGACTTCTGGAAGG) and reverse 2 (ATGCCCCGGACTGTCCTTGAAGG) were designed from multiple alignment of the known 18S rRNA gene sequences of Chlorellaceae. The PCR amplification products were sequenced by Beijing Genomics Institute (BGI, China). Sequences were compared with that in the GenBank database by Blast. The sequences of most related species were
imported to ClastalW for alignment and the phylogenetic relationships were analyzed by MEGA version 5.1 (MacOSX 10.6).

2.3. Toxicity assessment

Cells of each isolate were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilized BM culture medium on a rotary shaker at 150 rpm for one week. 30 mg L\(^{-1}\) cells at the exponential growth phase were exposed to the medium containing mixtures of DE-71 and BDE-209 (5:1) at low, medium and high contamination levels, with respective concentrations of 6, 60 and 600 µg L\(^{-1}\), each in triplicate. This initial cell density was chosen to have sufficient viable cells and have the maximum sensitivity to PBDEs (Monteiro et al., 2011). The solvent control, also in triplicate, was prepared by growing cells in 0.1% DMSO culture medium but without PBDEs. On days 1, 3, 5 and 7, cultures were harvest from each flask and growth parameters, that is, cell number, chlorophyll a content and dry weight at 105\(^\circ\)C, were analyzed according to the standard methods (Gao and Tam, 2011). The growth rate (\(\mu\)) at the log phase and the percentages of growth inhibition (GI) at different levels of PBDEs were calculated as follows:

\[
\mu/(d) = (Chl_n - Chl_0) / t_n, \quad GI_{\mu_i}/(\%) = (\mu_c - \mu_i) \times 100 / \mu_c
\]

Where \(Chl_n\) and \(Chl_0\) are chlorophyll a concentrations at time \(t_n\) and \(t_0\) (initial), respectively, while \(\mu_c\) and \(\mu_i\) are growth rates of the control and treated group \(i\), respectively.

The biomass inhibition (\(Bl\)) after seven days of exposure was calculated as:

\[
Bl(\%) = (B_i - B_c) \times 100 / B_c
\]

Where \(B_c\) and \(B_i\) are microalgae biomass of control and treated group \(i\) on Day 7, respectively.

2.4. Removal of PBDEs by microalgal isolates

The nine microalgal isolates were exposed to 0.1% DMSO (v/v) culture medium containing a mixture of DE-71 and BDE-209 (5:1) at a concentration of 600 µg L\(^{-1}\). Culture medium with the same concentration of PBDEs but without microalgal was served as the control. The cultures were grown under the same condition as the toxicity test. The flasks were harvested at the end of seven days of exposure. The cells were separated by centrifugation, the supernatant and algal pellets were extracted with n-Hexane by a separating funnel and ultrasonic bath, respectively. The surrogate standard PCB-209 was spiked to the sample prior to extraction. The extract was concentrated to a final volume of 1 mL stored at 4\(^\circ\)C prior to GC-MS analysis.

The bioconcentration factor (BCF) was calculated as the ratio between the concentration of PBDEs accumulated in the algal cells (µg g\(^{-1}\) dry weight) and the residual in the medium (µg mL\(^{-1}\)). The removal efficiency (R) and unaccountable loss (L) were calculated according to the following equations:

\[
R(\%) = (N_c - N_m) \times 100 / N_c, \quad L = N_c - N_a - N_m
\]

Where \(N_0\), \(N_a\) and \(N_m\) are the amounts of PBDEs (µg) in control (without algae cell), accumulated in cells and remained in medium at the end of exposure, respectively.

2.5. GC-MS analysis

Eight congeners, including BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209, were analyzed by an Agilent gas chromatography-mass spectrometry 6890N/5975 (Palo Alto, CA, USA) equipped with an DB-5HT (crosslinked 5% phenyl methyl siloxane, 15 m x 0.25 mm, 0.1 µm film thickness) capillary column using negative chemical ionization (NCI) in the selected ion monitoring (SIM) mode. The
column temperature was initiated at 150°C (hold for two minutes) and increased to 300°C at 6°C min⁻¹ (hold for three minutes). The injector temperature was 240°C. Methane and helium (99.999%, Hong Kong Oxygen & Acetylene Co., Ltd.), both at a flow rate of 1.5 mL min⁻¹, were used as chemical ionization moderating and carrier gases, respectively. 2 µL of sample was injected in splitless mode. The purge flow rate was 46 mL min⁻¹. The ion source and interface temperature were 250°C. PBDEs were quantified by monitoring the ion fragments with mass-to-charge ratio (m/z) 79 and 81 for tri- to hepta-BDEs, m/z 488 and 489 for BDE-209, and m/z 498 for PCB-209 (surrogate standard). An analytical standard, PBDE congeners of primary interest (BDE-CSM) (Supelco, USA) was used for quantification. The limit of detection (LOD), defined as three times the standard deviations of referenced sample spiked with PBDEs, of liquid and solid samples were 63-77 pg L⁻¹ and 0.13-0.8 ng g⁻¹, respectively, and the respective recoveries were 54-101% and 111-130%. The final concentration was not adjusted for the recovery.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of PBDE-tolerant microalgae from WTPs

BDE-209 was the most dominated congeners followed by BDE-47 and BDE-99 in the influent collected from Hong Kong WTPs, ranging from 1.3-184, 0.3-62 and 0.1-26 ng L⁻¹, respectively, and the respective concentrations in the dewatered sludge were 0.8-572, <0.7-19 and <0.7-5.2 ng g⁻¹. These values were higher than those reported in Mainland China (Peng et al., 2009) and Hong Kong (Li, 2008) in previous years, indicating that deca- and penta-BDE are still the major application in commercial products although PBDEs have been gradually phased out since 2008 (U.S. EPA, 2010).

Nine isolates were obtained from influent which could be divided into spherical unicell, lanceolate unicell and colonies of 2-4 cells with sizes varied from 1-35 µm (Table 1). The phylogenetic tree, inferred by 18s rDNA sequences, revealed that STCh was most related to Chlorella sp. and SICh was closer to C. vulgaris (Fig. 1). STPa1 and STPa2 were closely related to each other, with 99% identity to Parachlorella kessleri. TPMy was linked to Mychonastes timauensis, M. homosphaera and M. rotundus. STSc was most associated with Scenedesmus obliquus; TPSc1 was related to S. bajacalifornicus and TPSc2 allied with S. deserticola. YLBa was most related to Nitzschia palea and Peridinium balticum; however, the morphological characteristics showed that YLBa was not a dinoflagellate and should not be P. balticum belonging to the class of Dinophyceae.

3.2. Toxicity of PBDEs

The solvent control did not have any significant effects on the growth of all microalgal isolates but the toxicity of PBDEs varied among isolates. The biomass of SICh and STCh was not inhibited by PBDEs; STPa1, TPSc1, TPMy and STSc were slightly affected by high level PBDEs with a 20% reduction on biomass, while the toxic effects on STPa2, YLBa and TPSc2 were most serious, with 24%, 40% and 55% reduction on biomass, respectively after seven days of exposure (Fig. 2). The growth curves of the four isolates, SICh, STCh, STPa1 and TPSc1 exposed to PBDEs were almost the same as the control, but the lag phase of TPMy and TPSc2 was lengthened (Fig. 3). The doubling time during the exponential phase of TPMy, YLBa, TPSc2 and STSc increased and the growth rate inhibition, in terms of chlorophyll a content, under the high level of PBDEs was 28%, 27%, 23% and 13%, respectively. These results indicated that SICh and STCh were the most tolerant isolates, followed by STPa1 and TPSc1. Such inhibition may be caused by the increased production of reactive oxygen and nitrogen species or by the depletion of protective antioxidants, leading to oxidative damages to critical macromolecules such as...
lipid membranes, cellular proteins and DNA which regulate cell division, differentiation, and growth (Foyer et al., 2009; Reisman et al., 2009).

### Table 1 Morphological characteristic of nine microalgal isolates

<table>
<thead>
<tr>
<th>Shape</th>
<th>Size (μm)</th>
<th>Chloroplast</th>
<th>Color</th>
<th>Motile</th>
</tr>
</thead>
<tbody>
<tr>
<td>STCh</td>
<td>2-3</td>
<td>single, cup shape</td>
<td>yellow green</td>
<td>N</td>
</tr>
<tr>
<td>SICCh</td>
<td>2-4</td>
<td>single, cup shape</td>
<td>light green</td>
<td>N</td>
</tr>
<tr>
<td>STPa1</td>
<td>5-7</td>
<td>single, cup shape</td>
<td>green</td>
<td>N</td>
</tr>
<tr>
<td>STPa2</td>
<td>4-5</td>
<td>single, cup shape</td>
<td>light green</td>
<td>N</td>
</tr>
<tr>
<td>TPMy</td>
<td>2-4</td>
<td>Invisible</td>
<td>green</td>
<td>N</td>
</tr>
<tr>
<td>STSc</td>
<td>3-4×11-14</td>
<td>single, parietal</td>
<td>green</td>
<td>N</td>
</tr>
<tr>
<td>TPSc1</td>
<td>3-6×11-15</td>
<td>single, parietal</td>
<td>green</td>
<td>Y</td>
</tr>
<tr>
<td>TPSc2</td>
<td>1-3×5-7</td>
<td>single, parietal</td>
<td>green</td>
<td>N</td>
</tr>
<tr>
<td>YLBa</td>
<td>5-7×33-35</td>
<td>Two, plate shape</td>
<td>brown</td>
<td>Y</td>
</tr>
</tbody>
</table>

**Fig. 1** Phylogenetic relationships of microalgal isolates inferred by 18S rDNA sequence data. The tree was produced by MEGA 5.1 (Arizona State University, Tempe, USA). Values above branches are confidence levels estimated by 1000 bootstrap replicates.
Fig. 2 Inhibition on the biomass of nine microalgal isolates at high level of PBDEs after seven days exposure. Mean and standard deviation of three replicates are shown. Bars with different letters indicate significant difference among isolates according to one-way analysis of variance (ANOVA) followed by Tukey’s tests (p<0.05).

Fig. 3 Cell numbers and chlorophyll a content of microalgal isolates after exposed to high (◆, ◇), medium (■, □) and low (▼, ▽) levels of PBDEs, and the control (●, ○) for seven days. Solid and open symbols are cell numbers and chlorophyll a content, respectively. Mean and standard deviation of three replicates are shown.

3.3. Removal of PBDEs by microalgal isolates

The tolerant isolate (SICh) displayed the highest removal ability of BDE-47, -99, -209 and total PBDEs, with more than 80% removal in seven days, followed by TPSc1 (Table 2). Although the growth of YLBa was suppressed by PBDEs, its removal efficiencies of BDE-47, -99, -209 and total PBDEs were still comparable to that by TPSc1. TPSc2 and STCh
were the isolates having the poorest removal ability and only 56% of total PBDEs were removed in seven days. The mass balance calculation showed that the residual amount of PBDEs in the medium of SICh was significantly lower (accounted 15% of total spiked PBDEs) but had more accumulation of PBDEs in cells (48% of total spiked PBDEs) than other isolates (Table 3). The lowest cellular accumulation was found in YLBa which also had lower amount of PBDEs remained in the medium than the other six isolates, but it had the second highest amount of unaccountable loss. The unaccountable loss may be caused by the transformation of PBDEs to other compounds, which could not be detected by current method. The bioconcentration factors (BCF) of nine isolates were very high (Log BCF ranged from 2.9 to 4.1), with SICh and TPMy had the highest Log BCF (around 4.0) while TPSc2 had the lowest value of 2.9. The isolates SICh, TPSc1, YLBa, TPMy and STPa2 were considered as high potential bioaccumulation species to PBDEs according to the screening level established by United Nations Environmental Program (UNEP) (WWF, 2005).

Table 2 Removal of major congeners and total PBDEs by microalgal isolates at the end of seven days exposure (mean and standard deviation of three replicates are shown; values in the same column with different letters indicated significant difference according to one-way ANOVA and Tukey’s tests at p<0.05)

<table>
<thead>
<tr>
<th>Genera</th>
<th>Isolates</th>
<th>BDE-47 (μg)</th>
<th>BDE-99 (μg)</th>
<th>BDE-209 (μg)</th>
<th>ΣPBDEs (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyceae</td>
<td>SICh</td>
<td>86±2.7a</td>
<td>82±3.9a</td>
<td>91±1.9a</td>
<td>85±3.2a</td>
</tr>
<tr>
<td></td>
<td>STCh</td>
<td>68±4.3c</td>
<td>49±0.8d</td>
<td>44±40bc</td>
<td>56±2.6c</td>
</tr>
<tr>
<td>Parachlorella</td>
<td>STPa1</td>
<td>68±3.5bc</td>
<td>52±0.9bc</td>
<td>50±7.7bc</td>
<td>60±1.0bc</td>
</tr>
<tr>
<td></td>
<td>STPa2</td>
<td>64±1.0bc</td>
<td>50±3.7cd</td>
<td>34±1.8bc</td>
<td>57±2.2bc</td>
</tr>
<tr>
<td>Mychonastes</td>
<td>TPMy</td>
<td>70±3.0bc</td>
<td>45±0.9d</td>
<td>66±11abc</td>
<td>58±0.9bc</td>
</tr>
<tr>
<td>Scenedesmus</td>
<td>STSc</td>
<td>70±3.0bc</td>
<td>50±1.9cd</td>
<td>18±24c</td>
<td>58±1.9bc</td>
</tr>
<tr>
<td></td>
<td>TPSc1</td>
<td>73±5.8b</td>
<td>58±3.2bc</td>
<td>71±2.3ab</td>
<td>64±4.2b</td>
</tr>
<tr>
<td></td>
<td>TPSc2</td>
<td>63±0.8c</td>
<td>55±0.1bc</td>
<td>66±6.3abc</td>
<td>56±0.1c</td>
</tr>
<tr>
<td>Nitzschia</td>
<td>YLBa</td>
<td>68±3.5bc</td>
<td>55±3.0bc</td>
<td>79±16ab</td>
<td>62±3.0bc</td>
</tr>
</tbody>
</table>

Table 3 Mass balance of PBDEs in microalgal cultures at the end of seven days exposure (mean and standard deviation of three replicates are shown; values in the same column with different letters indicated significant difference according to one-way ANOVA and Tukey’s test at p<0.05)

<table>
<thead>
<tr>
<th>Genera</th>
<th>Isolates</th>
<th>Remained in medium (μg)</th>
<th>Accumulated in algae (μg)</th>
<th>Unaccountable loss (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyceae</td>
<td>SICh</td>
<td>5.8±1.2c</td>
<td>19.0±0.2a</td>
<td>14.5±1.1a</td>
</tr>
<tr>
<td></td>
<td>STCh</td>
<td>17.4±1.0a</td>
<td>14.9±1.8ab</td>
<td>7.0±1.0bc</td>
</tr>
<tr>
<td>Parachlorella</td>
<td>STPa1</td>
<td>15.7±0.4ab</td>
<td>17.5±3.2ab</td>
<td>6.2±3.0c</td>
</tr>
<tr>
<td></td>
<td>STPa2</td>
<td>16.8±0.9ab</td>
<td>16.9±1.9ab</td>
<td>5.6±1.1c</td>
</tr>
<tr>
<td>Mychonastes</td>
<td>TPMy</td>
<td>16.7±0.4ab</td>
<td>16.7±2.6ab</td>
<td>5.9±2.2c</td>
</tr>
<tr>
<td>Scenedesmus</td>
<td>STSc</td>
<td>16.4±0.8ab</td>
<td>15.2±1.2ab</td>
<td>7.7±1.5bc</td>
</tr>
<tr>
<td></td>
<td>TPSc1</td>
<td>14.3±1.7b</td>
<td>18.2±0.4ab</td>
<td>6.8±1.8bc</td>
</tr>
<tr>
<td></td>
<td>TPSc2</td>
<td>17.1±0.0a</td>
<td>16.4±1.4ab</td>
<td>5.8±1.4c</td>
</tr>
<tr>
<td>Nitzschia</td>
<td>YLBa</td>
<td>15.0±1.2ab</td>
<td>13.3±0.3b</td>
<td>11.1±1.0ab</td>
</tr>
</tbody>
</table>

4. CONCLUSIONS

The present study reveals that the concentrations of PBDEs in the influent and dewatered sludge of four WTPs in Hong Kong were high. Nine microalgae belonged to five genera, namely *Chlorella* (STCh and SICh), *Parachlorella* (STPa1 and STPa2), *Scenedesmus* (STSc, TPSc1 and TPSc2), *Nitzschia palea* (YLBa) and *Mychonastes* (TPMy), were isolated from the influent of the four WTPs according to morphological features and phylogenetic analysis of 18S rRNA genes. Some of these isolates were tolerant to PBDEs...
toxicity. One of the *Chlorella* isolates, SICh not only had high tolerance to PBDEs but also possessed a high ability to remove PBDEs, which could be used for removal purposes.

REFERENCES