Sustainable biodegradation of phenolic endocrine-disrupting chemicals by \textit{Phragmites australis}–rhizosphere bacteria association

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**ABSTRACT**

The efficacy of two rhizobacteria (\textit{Sphingobium fuliginis} TIK1 and \textit{Sphingobium} sp. IT4) of \textit{Phragmites australis} for the sustainable treatment of water polluted with phenolic endocrine-disrupting chemicals (EDCs) was investigated. Strains TIK1 and IT4 have recently been isolated from \textit{Phragmites} rhizosphere and shown to degrade various 4-alkylphenols – TIK1 via phenolic ring hydroxylation and meta-cleavage and IT4 via ipso-hydroxylation. The two strains also degraded bisphenol A (BPA), bisphenol B, bisphenol E, bisphenol F, bisphenol P and bisphenol S (BPS). Thus, strains TIK1 and IT4 have wide degradation spectra for phenolic EDCs. The two strains utilized \textit{Phragmites} root extracts as a sole carbon source and sustainably colonized \textit{Phragmites} roots, where they degraded phenolic EDCs. In sequencing batch reactor experiments using \textit{Phragmites} in association with TIK1 or IT4, both associations repeatedly removed phenolic EDCs from polluted secondary effluent water (BPA, BPS, 4-tert-butylphenol, 4-tert-octylphenol and 4-nonylphenol) from polluted secondary effluent water. The results suggest that hydroponic systems using \textit{Phragmites}–TIK and \textit{Phragmites}–IT4 associations would be useful for sustainable treatment of polluted waters containing various phenolic EDCs.

**Key words** | biodegradation, phenolic endocrine-disrupting chemicals, \textit{Phragmites australis}, polluted water treatment, rhizoremediation

**INTRODUCTION**

4-Alkylphenols – including 4-nonylphenol (4-NP), 4-tert-octylphenol (4-tert-OP) and 4-tert-butylphenol (4-tert-BP) – have been used in the production of nonylphenol polyethoxylate surfactants, phenolic resins or polycarbonate resins. 4-Alkylphenols are also persistent, toxic compounds classified as endocrine-disrupting chemicals (EDCs) capable of interfering with the hormonal systems of numerous organisms (Ying \textit{et al.} 2002; Soares \textit{et al.} 2008). Bisphenol A (BPA: 2,2-bis[4-hydroxyphenyl]propane) is a high-production-volume chemical used in the production of polycarbonates and epoxy resins (Staples \textit{et al.} 1998; Crain \textit{et al.} 2007). Other bisphenol analogs (bisphenols), such as bisphenol B (BPP: 2,2-bis[4-hydroxyphenyl]butane), bisphenol E (BPE: bis[4-hydroxyphenyl]ethane), bisphenol F (BPF: bis[4-hydroxyphenyl]methane), bisphenol P (BPP: 2,2-bis[4-hydroxy-3-methylphenyl]propane) and bisphenol S (BPS: bis[4-hydroxyphenyl]sulfone), are also used in the generation of resins and plastics (Chen \textit{et al.} 2002). These bisphenols are well known EDCs with estrogenic activity (Staples \textit{et al.} 1998; Chen \textit{et al.} 2002; Crain \textit{et al.} 2007). The widespread and extensive use of these phenolic EDCs has led to their release into aquatic environments. Phenolic EDCs are often found in effluents of sewage and wastewater treatment plants and in surface waters and sediments (Heemken \textit{et al.} 2001; Ko \textit{et al.} 2007; Jonkers \textit{et al.} 2009, 2010). It is therefore necessary to establish technologies to decontaminate waters polluted with phenolic EDCs.

Rhizoremediation – the degradation and removal of pollutants by microbial activity in the rhizosphere of plants – is a cost-effective and environmentally friendly remediation technology (Shaw & Burns 2001; Chaudhry \textit{et al.} 2005). Recently, an emergent aquatic plant, \textit{Phragmites australis} (common reed), and the floating aquatic plants \textit{Spirodela polyrhiza} (giant duckweed) and \textit{Lemna aouikikusa} (lesser duckweed) have been shown to accelerate the
biodegradation of single aromatic compounds (Toyama et al. 2006; Yamaga et al. 2010), BPA (Toyama et al. 2009) and 4-NP (Toyama et al. 2011), in the rhizosphere in both water and sediment. In the rhizosphere, oxygen and organic compounds released from plant roots are known to support bacterial growth and accelerate biodegradation (Shaw & Burns 2001; Chaudhry et al. 2005). However, rhizoremediation processes are often limited by the presence of persistent and toxic pollutants. The introduction of specific pollutant-degrading bacteria to the rhizosphere has great potential to enhance the efficacy of rhizoremediation (Shaw & Burns 2001; Chaudhry et al. 2005; Yamaga et al. 2010).

The aim of this study was to treat waters polluted with phenolic EDCs by using *Phragmites australis* in association with its rhizobacteria. Recently, *Sphingobium fuliginis* TIK1 (Toyama et al. 2010) and *Sphingobium* sp. IT4 (Toyama et al. 2011) were isolated from the *Phragmites* rhizosphere. Strain TIK1 can utilize 4-tert-BP as a sole carbon source and degrade various 4-alkylphenols via phenolic ring hydroxylation and a meta-cleavage pathway (Toyama et al. 2010). Strain IT4 can utilize branched 4-NP and 4-tert-OP as a sole carbon source and degrade various 4-alkylphenols via an ipso-substitution mechanism (Toyama et al. 2011). First, the biodegradation activities of the two strains for bisphenols were assessed. Second, the survival and biodegradation activities of the two strains on the root surfaces of sterilized *Phragmites* were examined. Third, flask-scale sequencing batch reactors (SBR) containing single-strain-inoculated *Phragmites* (i.e. *Phragmites*-TIK1 association and *Phragmites*-IT4 association) were prepared and used to treat water polluted by phenolic EDCs.

**MATERIALS AND METHODS**

**Chemicals**

4-Alkylphenols (4-tert-BP, 4-tert-OP and technical NP [tNP; a mixture of branched NP isomers]) and bisphenols (BPA, BPB, BPE, BPF, BPP and BPS) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

**Bacterial strains and culture media**

4-tert-BP-utilizing *Sphingobium fuliginis* TIK1 (Toyama et al. 2010) and 4-tert-OP-utilizing *Sphingobium* sp. IT4 (Toyama et al. 2011) were pre-cultured in liquid basal salts medium (BSM; pH7.2) containing 1.0 g/L (NH₄)₂SO₄, 1.0 g/L K₂HPO₄, 0.2 g/L NaH₂PO₄, 0.2 g/L MgSO₄·7H₂O, 0.05 g/L NaCl, 0.05 g/L CaCl₂, 8.3 mg/L FeCl₃·6H₂O, 1.4 mg/L MnCl₂·4H₂O, 1.17 mg/L NaMoO₄·2H₂O and 1 mg/L ZnCl₂. Strain TIK1 was pre-cultured in liquid BSM containing 1 mmol/L 4-tert-BP and 2 mmol/L glucose (BP-G-BSM) and strain IT4 was pre-cultured in liquid BSM containing 5 mmol/L 4-tert-OP and 2 mmol/L glucose (OP-G-BSM). Solid agar plates were prepared with 2.0% (w/v) agar.

**Plant samples**

To obtain sterile (bacteria-free) *Phragmites* plants, the seeds were sterilized by a 1-min wash in 70% ethanol, a 5-min wash in sodium hypochlorite solution and three 1-min rinses in sterilized water. Each sterilized young plant was aseptically grown in a 500-mL flask containing sterile Hoagland solution (36.1 mg/L KNO₃, 293 mg/L K₂SO₄, 3.87 mg/L NaH₂PO₄, 103 mg/L MgSO₄·7H₂O, 147 mg/L CaCl₂·H₂O, 5.33 mg/L FeSO₄·7H₂O, 0.95 mg/L H₃BO₃, 0.39 mg/L MnCl₂·4H₂O, 0.035 g/L CuSO₄·5H₂O, 0.08 mg/L ZnSO₄·7H₂O and 0.254 mg/L H₂MoO₄·4H₂O; pH 7.0) in an incubation chamber (28 ± 1°C, fluorescent lamps at 8,000 lux, 16-h light and 8-h dark photoperiod). Non-sterile *Phragmites* plants used in the SBR experiment were grown in the incubation chamber for 3 months in 1,000-mL bottles containing non-sterilized Hoagland solution. The Hoagland solution was changed every 2 weeks.

**Secondary effluent sample**

A sample of secondary effluent for use in the SBR experiment was collected from a sewage treatment plant in Kofu, Yamanashi, Japan. The chemical properties of the effluent were pH 7.4; 2.26 mg/L ammonium-N; 0.52 mg/L nitrite-N; 9.40 mg/L nitrate-N; 2.09 mg/L phosphate-P; 21.2 mg/L total dissolved organic carbon (TOC); and 1.15 × 10⁵ colony-forming units/mL total heterotrophic bacteria. Bacterial counts were performed by using 0.1 × LB agar plates (1.0 g/L Bacto Peptone, 0.5 g/L yeast extract, 1.0 g/L NaCl [pH 7.0]; 2.0% [w/v] agar). The effluent sample was stored at 4°C until used.

**Bisphenols degradation assay using pure cultures of strain TIK1 or IT4**

Six bisphenols (BPA, BPB, BPE, BPF, BPP and BPS) were used for the degradation assay. Strain TIK1 was pre-cultured in liquid BP-G-BSM and strain IT4 was pre-cultured in liquid OP-G-BSM. Cells of each strain were harvested by
centrifugation (9,600 g at 20 °C for 10 min) and washed twice with liquid BSM. The cells were suspended at a cell density (600-nm wavelength, optical density [OD₆₀₀]) of 0.5 in 10 mL BSM containing 0.5 mmol/L of one of the six bisphenols. The whole-cell mixtures were incubated in vials at 28 °C and 120 rpm. Triplicate vials were prepared for each sampling time, and the substrate concentrations were determined 0, 3 and 24 h after the start of the experiment. Degradation products of bisphenols were also analyzed. A sterile control experiment without cells was conducted in parallel.

**Growth experiment of strains TIK1 and IT4 using Phragmites root extract**

For preparation of Phragmites root extract, the roots of 2-month-old sterile Phragmites plants were cut into pieces about 5 mm long. About 2 g wet-weight roots and 10 mL pure water were crushed (10,000 rpm at 4 °C for 3 min) by a crusher. The crushed root mixture was centrifuged (8,000 g at 4 °C for 10 min) and then the supernatant was sterilized by filtration through a membrane filter (0.2 μm pore size, Millipore, Tokyo, Japan). Cells of each strain were inoculated at OD₆₀₀ = 0.02 into 100 mL of BSM containing the sterilized root extract (TOC of 140 mg/L) in 500-mL flasks. The flasks were then incubated at 28 °C and 120 rpm in triplicate. After a 36-h incubation, the TOC concentration and dry-weight of cells were determined. Then, the yield coefficient for cell mass was calculated using the weight of removed substrate carbon and dry-weight of increased cells.

**Phenolic EDC degradation assay using Phragmites–TIK1 or Phragmites–IT4 associations**

Sterilized young Phragmites plants were grown for 2 months in a 500-mL flask containing 200 mL of sterilized Hoagland solution. Inoculation of TIK1 or IT4 to Phragmites roots was performed as follows. Cells of each strain were suspended at OD₆₀₀ = 0.3 in 200 mL of sterilized Hoagland solution in the 500-mL flask with a 2-month-old plant (about 0.05 g dry root biomass and 0.14 g dry above-ground biomass). The flask containing the plant and cells of each strain was incubated statically for 1 h. After that the cell suspension was removed from the flask and the roots were gently washed three times using sterilized Hoagland solution to remove the cell suspension. The Phragmites plant thus treated was then used for either Phragmites–TIK1 or Phragmites–IT4 association, depending on the strain with which it had been incubated. One Phragmites–TIK or Phragmites–IT4 association was then placed in a 500-mL flask with 200 mL of Hoagland solution containing phenolic EDCs (5 mg/L BPA, 5 mg/L BPS, 5 mg/L 4-tert-BP, 2.5 mg/L 4-tert-OP and 2.5 mg/L tNP). Then, the flask was statically incubated in a chamber (28 ± 1 °C, 8,000 lux, 16-h light and 8-h dark). After 24 h, the EDC–Hoagland solution was removed completely from the flask and replaced with 200 mL of fresh EDC–Hoagland. This 24-h cycle was repeated a total of five times in triplicate. The concentration of EDCs in the solution was monitored during the whole experiment. The cell numbers of each strain present on the roots were measured at the beginning and end of the whole experiment. A control experiment using a 2-month-old sterile plant without inoculation of the two strains was also conducted under otherwise identical conditions.

After the phenolic EDC degradation assay, the Phragmites roots were picked, cut into 10-mm fragments and stained in the dark for 15 min with the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes–Invitrogen, San Diego, CA) in a 1.5-mL tube containing 250 μL each of SYTO 9 and propidium iodide solutions. Bacterial cells attached to the roots were observed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Simultaneous application of both dyes results in green fluorescence of viable cells and intense red fluorescence of dead ones.

**SBR experiment using Phragmites–TIK1 or Phragmites–IT4 association for simultaneous treatment of phenolic EDC-polluted effluent**

Laboratory-scale SBR experiments using the Phragmites associations to treat 500 mL of effluent sample polluted with EDCs (12-h reaction time/cycle, 10 cycles) were conducted. The EDC-polluted effluent was prepared by dissolving 5 mg/L BPA, 5 mg/L BPS, 5 mg/L 4-tert-BP, 2.5 mg/L 4-tert-OP and 2.5 mg/L tNP into the previously described secondary effluent sample. The Phragmites–TIK1 and Phragmites–IT4 associations were prepared by inoculating roots of 3-month-old non-sterile Phragmites (about 2.5 g dry root biomass and 10 g dry above-ground biomass) with cells of each of the strains TIK1 and IT4 at OD₆₀₀ = 0.3. The roots were gently washed three times using sterilized Hoagland solution to remove the cell suspension in the same manner as previously described. EDC-polluted effluent (500 mL) was placed in the flask with one Phragmites–TIK or one Phragmites–IT4 association. The flask was statically incubated in a chamber (28 ± 1 °C, 8,000 lux, 16-h light and
RESULTS AND DISCUSSION

Abilities of strains TIK1 and IT4 to degrade phenolic EDCs

The abilities of strains TIK1 and IT4 to degrade bisphenols are summarized in Table 1. The results of previous studies of the 4-alkylphenol degradation ability of the strains (Toyama et al. 2010, 2011) are also shown in Table 1. Strain TIK1 can degrade various 4-alkylphenols via phenolic ring hydroxylation followed by a meta-cleavage pathway, resulting in the production of 4-alkylcatechols (hydroxylated products of the 4-alkylphenols) and metabolites on the meta-cleavage pathway of 4-alkyl catechols as intermediate metabolites (Toyama et al. 2010). On the other hand, strain IT4 can degrade 4-alkylphenols by the ipso-substitution mechanism, resulting in the production of hydroquinone (Toyama et al. 2011). Strain TIK1 degraded all six of the bisphenols tested (BPA, BPB, BPE, BPF, BPP, and BPS), and hydroxylated products of the bisphenols and meta-cleavage products of them were detected. The electron impact–mass spectrometry (EI–MS) spectral characteristics of the TMS derivatives of these metabolites were consistent with hydroxylated bisphenols and meta-cleavage products of them produced by Sphingobium fuliginis OMI; strain OMI can degrade 4-alkylphenols and bisphenols via phenolic ring hydroxylation and the meta-cleavage pathway (Ogata et al. 2013). Also, strain IT4 degraded the six bisphenols, and hydroquinone or p-benzoquinone were detected as intermediate metabolites of them. These metabolites were found to have the same peak retention time and EI–MS data as authentic hydroquinone or p-benzoquinone. The results suggest that the six bisphenols were presumably degraded by the respective pathway of each strain. Thus, the two strains have wide degradation spectra for phenolic EDCs and are potentially useful for the treatment of waters polluted with multiple species of phenolic EDCs.

Growth of strains TIK1 and IT4 using Phragmites root extract

The two strains grew using Phragmites root extract as carbon source. The yield coefficients of the cell mass were 0.74 mg dry-weight (mg carbon of root extract)$^{-1}$ for strain TIK1 and 0.61 mg dry-weight (mg carbon of root extract)$^{-1}$ for strain IT4. Thus, organic compounds in the Phragmites root extracts were shown to support cell growth of strains TIK1 and IT4.
Colonization of strains TIK1 and IT4 on Phragmites roots and degradation of phenolic EDCs by the Phragmites–TIK1 or Phragmites–IT4 associations

To determine whether the two strains can colonize and express degradation activities on the root surfaces of sterile Phragmites, phenolic EDC degradation assay (24-h reaction time/cycle, five cycles) using the Phragmites–TIK1 or Phragmites–IT4 associations was conducted, and populations of both strains on the roots were monitored. After inoculation, both strains easily colonized the roots, TIK1 at a density of 9.08 ± 2.32 × 10⁹ CFU (g dry root)⁻¹ and IT4 at a density of 3.13 ± 0.83 × 10¹⁰ CFU (g dry root)⁻¹. At the end of five cycles, the populations on the roots remained at 5.37 ± 0.83 × 10¹¹ CFU (g dry root)⁻¹ for TIK1 and 3.24 ± 0.24 × 10¹¹ CFU (g dry root)⁻¹ for IT4. When the roots of Phragmites–TIK1 or Phragmites–IT4 associations were stained with LIVE/DEAD BacLight kit and observed by fluorescence microscopy, many green fluorescent spots, indicating small colonies or biofilms of living bacteria, were also observed on surface of each root after five cycles (Figure 1).

The changes in EDC concentrations during the degradation assay experiments are shown in Figure 2. The Phragmites–TIK1 and Phragmites–IT4 associations both consistently removed all EDCs from the EDC-dosed Hoagland within the 24-h reaction time throughout all five cycles. In the control experiment using sterile Phragmites, the concentrations of BPA, BPS and 4-tert-BP did not decline at all, whereas 4-tert-OP declined by about 20% and tNP declined by about 30% within the reaction time throughout all five cycles (Figure 2). 4-tert-OP and tNP are hydrophobic compounds and easily adsorb onto the sediments and solids in aquatic environments (Ying et al. 2002; Soares et al. 2008), thus their concentration decline in the presence of the sterile plant might have resulted from adsorption on the roots. The results strongly indicate that phenolic EDCs were degraded by the two strains in the Phragmites–TIK1 and Phragmites–IT4 associations. Strains TIK1 and IT4 appear to be capable of sustainably colonizing sterilized Phragmites roots and degrading phenolic EDCs for at least 120 h, presumably with support from organic compounds exuded from the Phragmites roots.
Sustainable removal of phenolic EDCs from polluted effluent by SBR system using the Phragmites–TIK1 or Phragmites–IT4 associations

SBR experiments were conducted to evaluate the potential for using Phragmites–TIK1 and Phragmites–IT4 associations to treat secondary effluent sample polluted with EDCs (Table 2). Uninoculated Phragmites removed small amounts of BPA (1.9–10.3%), BPS (0.5–3.8%), 4-tert-BP (1.7–4.1%), 4-tert-OP (22.0–26.8%) and tNP (31.2–33.6%) from the effluent. Their removals from EDC-polluted effluent might have resulted from the adsorption on the roots and biodegradation by indigenous bacteria in the secondary effluent. In contrast, both Phragmites–TIK1 and Phragmites–IT4 repeatedly achieved complete removal of all phenolic EDCs from the polluted water throughout all 10 cycles. The results suggest that TIK1 and IT4 can thoroughly colonize on the surface of non-sterile Phragmites roots. Both Phragmites–TIK1 and Phragmites–IT4 could be used for long-term simultaneous removal of phenolic EDCs from polluted effluent. Therefore, the use of hydroponic systems using Phragmites–TIK1 and Phragmites–IT4 associations...
would be a successful strategy for the sustainable treatment of polluted water containing various phenolic EDCs. It will be necessary to evaluate the performance in a pilot system to construct practical systems for treating polluted waters.

**CONCLUSION**

This study showed that two bacterial species associated with the *Phragmites australis* rhizosphere, namely *Sphingobium fuliginis* TIK1 and *Sphingobium* sp. IT4, can degrade various bisphenols, in addition to their previously documented ability to degrade various 4-alkylphenols. Strains TIK1 and IT4 were shown to have wide degradation spectra for phenolic EDCs. The two strains sustainably colonized the roots of *Phragmites*, with the support of organic compounds exuded from the roots, and inoculated plants were able to simultaneously and repeatedly remove various phenolic EDCs from polluted water. We conclude that the use of hydroponic systems using *Phragmites*–TIK1 and *Phragmites*–IT4 associations would be an effective strategy for the sustainable treatment of polluted waters contaminated by various phenolic EDCs. This study suggests that field-scale trials to test the efficacy of such a strategy would be well justified.

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**Table 2** Phenolic EDC removal performances of *Phragmites*–TIK1, *Phragmites*–IT4 and uninoculated *Phragmites* in sequencing batch reactors. Values shown are the reduction in contaminant concentration at the end of a reaction cycle as a percentage of the concentration at the beginning of the cycle. The beginning concentration was 5.0 mg/L for BPA, BPS and 4-tert-BP and 2.5 mg/L for 4-tert-OP and tNP

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