Sustainable biodegradation of phenolic endocrine-disrupting chemicals by *Phragmites australis*–rhizosphere bacteria association

T. Toyama, T. Ojima, Y. Tanaka, K. Mori and M. Morikawa

ABSTRACT

The efficacy of two rhizobacteria (*Sphingobium fuliginis* TIK1 and *Sphingobium* sp. IT4) of *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 *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 *Phragmites* root extracts as a sole carbon source and sustainably colonized *Phragmites* roots, where they degraded phenolic EDCs. In sequencing batch reactor experiments using *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 *Phragmites*–TIK and *Phragmites*–IT4 associations would be useful for sustainable treatment of polluted waters containing various phenolic EDCs.

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

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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 *et al.* 2002; Soares *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 *et al.* 1998; Crain *et al.* 2007). Other bisphenol analogs (bisphenols), such as bisphenol B (BPB: 2,2-bis[4-hydroxyphenyl]butane), bisphenol E (BPE: bis[4-hydroxyphenyl] ethane), bisphenol F (BPF: bis[4-hydroxylphenyl]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 *et al.* 2002). These bisphenols are well known EDCs with estrogenic activity (Staples *et al.* 1998; Chen *et al.* 2002; Crain *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 *et al.* 2001; Ko *et al.* 2007; Jonkers *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 *et al.* 2005). Recently, an emergent aquatic plant, *Phragmites australis* (common reed), and the floating aquatic plants *Spirodela polyrrhiza* (giant duckweed) and *Lemna aoukikusa* (lesser duckweed) have been shown to accelerate the

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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 $(NH_4)_2SO_4$, 1.0 g/L K_2HPO_4, 0.2 g/L NaH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L NaCl, 0.05 g/L $CaCl_2$, 8.3 mg/L $FeCl_3{\cdot}6H_2O$, 1.4 mg/L $MnCl_2{\cdot}4H_2O$, 1.17 mg/L $NaMoO_4{\cdot}2H_2O$ and 1 mg/L $ZnCl_2$. 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 KNO_3, 293 mg/L K_2SO_4, 3.87 mg/L NaH_2PO_4, 103 mg/L $MgSO_4{\cdot}7H_2O$, 147 mg/L $CaCl_2{\cdot}H_2O$, 3.33 mg/L $FeSO_4{\cdot}7H_2O$, 0.95 mg/L H_3BO_3, 0.39 mg/L $MnCl_2{\cdot}4H_2O$, 0.03 mg/L $CuSO_4{\cdot}5H_2O$, 0.08 mg/L $ZnSO_4{\cdot}7H_2O$ and 0.254 mg/L $H_2MoO_4{\cdot}4H_2O$; pH 7.0) in an incubation chamber ($28 \pm 1\,^{\circ}C$, 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 1.15×10^5 colony-forming units/mL total heterotrophic bacteria. Bacterial counts were performed by using $0.1 \times$ 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 $4\,^{\circ}C$ 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 [OD_{600}]) 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 $OD_{600} = 0.02$ into 100 mL of BSM containing the sterilized root extract (TOC of 140 mg/L) in 300-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 $OD_{600} = 0.3$ 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 $OD_{600} = 0.3$. 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

8-h dark photoperiod). After 12 h, the EDC-polluted effluent was completely removed from the flask and replaced with 500 mL of fresh EDC-polluted effluent. This 12-h cycle was repeated 10 times in triplicate. At the end of each cycle, the EDCs were extracted from the whole discharged effluent sample (about 500 mL) and analyzed. In order to simplify the preparation of the *Phragmites*-bacteria association system for practical use, non-sterile *Phragmites* was adopted in this experiment. A control experiment using a 3-month-old non-sterile plant without inoculation was also conducted under the same conditions.

Analytical procedures

Three methods of measuring bacterial cell density were used: OD_{600}, the dry-weight of cells, and colony forming units on BP-G-BSM or OP-G-BSM plates. Concentrations of 4-alkylphenols and bisphenols were determined by high-performance liquid chromatography (HPLC). In the bisphenol degradation assay using pure-culture and the EDC degradation assay using *Phragmites*–strain association, each collected sample was acidified with 2 mol/L HCl to pH 2–3, shaken for 3 min with an equal volume of an ethyl acetate–*n*-hexane mixture (2:1, v/v), and centrifuged (3,200 *g*, 4 °C, 10 min). The organic layer was collected, 500 μL of the organic phase was dried under nitrogen flow, and the dry extract was dissolved in 500 μL of acetonitrile. The sample was analyzed by HPLC. In the SBR experiment, the collected discharged water (about 500 mL) was acidified with 2 mol/L HCl to pH 2–3 and then shaken twice for 3 min with 25 mL ethyl acetate–*n*-hexane mixture (1:1 v/v). The organic layer was collected and dried with anhydrous sodium sulfate; the organic phase was dried under nitrogen flow and the dry extract was dissolved in 10 mL of acetonitrile. The sample was analyzed by HPLC. Detection limits for BPA, BPS, 4-*tert*-BP, 4-*tert*-OP and tNP in the SBR experiment were 0.01–0.05 mg/L. In the HPLC analysis, acetonitrile and water were used at ratios of 1:1 (v/v) as the mobile phase, and detection was performed at a wavelength of 280 nm. In bisphenol degradation assay, degradation products of bisphenols were also analyzed by gas chromatography–mass spectrometry (GC–MS). For GC–MS analysis, the above ethyl acetate–*n*-hexane extract and extract with trimethylsilylation (TMS) derivatization were analyzed by GC–MS as described previously (Toyama *et al.* 2011). TOC was measured with a TOC analyzer. The collected sample was centrifuged (8,000 *g*, 4 °C, 10 min) twice; the supernatant was filtered (0.2 μm) and then the filtrate was analyzed for TOC.

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-alkylcatechols 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.

Table 1 | *Sphingobium fuliginis* strain TIK1 and *Sphingobium* sp. strain IT4 display exceptionally strong ability to degrade phenolic EDCs

	Strain TIK1			Strain IT4	
		Main degradation products			Main degradation products
Phenolic EDC	Transformation ratio (%)[a]	Catechol products	Metabolites on *meta*-cleavage pathway	Transformation ratio (%)[a]	Quinone products[d]
BPA	100	+	+	100	Hydroquinone
BPB	99.0	+	+	100	Hydroquinone
BPE	100	+	+	100	Hydroquinone
BPF	100	+	+	100	Hydroquinone
BPP	78.2	+	Not detected	91.1	Hydroquinone
BPS	100	+	Not detected	100	*p*-Benzoquinone
4-*tert*-BP	100[b]	+[b]	+[b]	100[c]	Hydroquinone[c]
4-*n*-BP	100[b]	+[b]	+[b]	70.2[c]	Hydroquinone[c]
4-*sec*-BP	100[b]	+[b]	+[b]	95.5[c]	Hydroquinone[c]
4-*tert*-PenP	100[b]	+[b]	+[b]	98.9[c]	Hydroquinone[c]
4-*tert*-OP	100[b]	+[b]	+[b]	100[c]	Hydroquinone[c]
4-*n*-OP	100[b]	+[b]	+[b]	91.9[c]	Hydroquinone[c]
tNP	64.0[b]	+[b]	+[b]	92.6[c]	Hydroquinone[c]
4-*n*-NP	100[b]	+[b]	+[b]	44.7[c]	Hydroquinone[c]

[a]Transformation ratio (%) refers to the percent reduction in the concentration of the phenolic EDC in cultures after 24-h incubation relative to that in a sterile control. Concentrations were obtained from HPLC chromatograms.
[b]Toyama *et al*. (2010).
[c]Toyama *et al*. (2011).
[d]*ipso*-Substitution of phenolic compounds that have *para*-substituents with electron-donating (BPA, BPB, BPE, BPF, BPE) or electron withdrawing (BPS) properties can produce hydroquinone or *p*-benzoquinone, respectively (Gabriel *et al*. 2007).

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 $9.08 \pm 2.32 \times 10^{10}$ CFU (g dry root)$^{-1}$ and IT4 at a density of $3.13 \pm 0.83 \times 10^{10}$ CFU (g dry root)$^{-1}$. At the end of five cycles, the populations on the roots remained at $5.37 \pm 0.83 \times 10^{11}$ CFU (g dry root)$^{-1}$ for TIK1 and $3.24 \pm 0.24 \times 10^{11}$ 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.

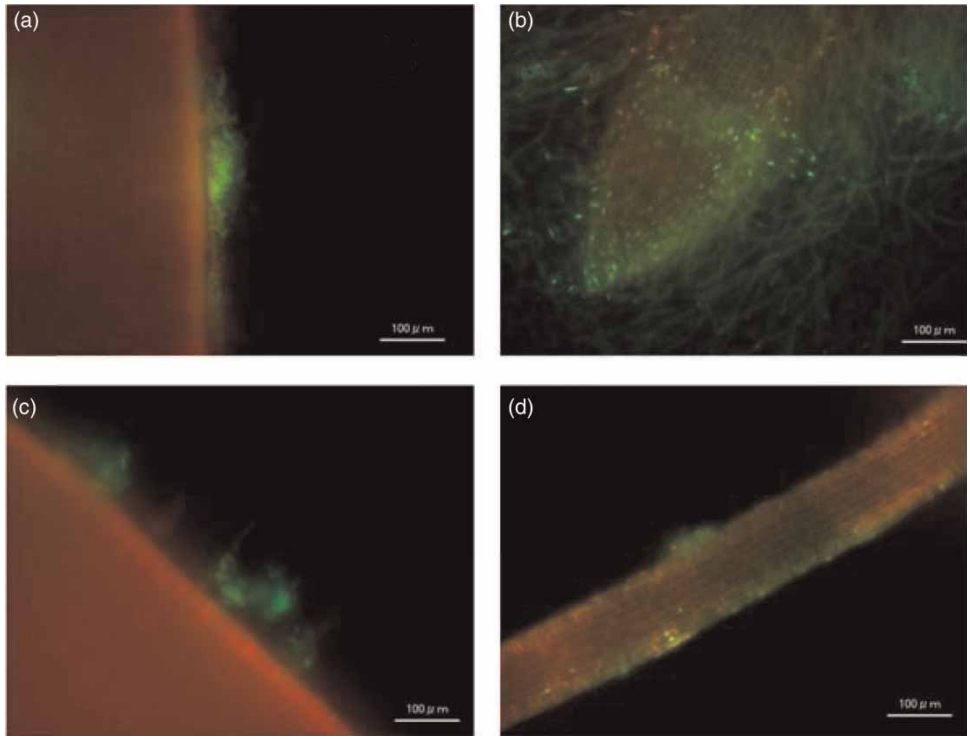

Figure 1 | Staining for live bacteria and observation by fluorescence microscopy of *Phragmites*–TIK1 or *Phragmites*–IT4 roots. (a) Main root of *Phragmites*–TIK1 association. (b) Main root tip of *Phragmites*–TIK1. (c) Main root of *Phragmites*–IT4 association. (d) Side root of *Phragmites*–IT4 association. The high intensity of green fluorescence (white areas in the B/W version) indicates living bacteria (the full colour version of this figure is available in the online version of this paper, at http://www.iwaponline.com/wst/toc.htm).

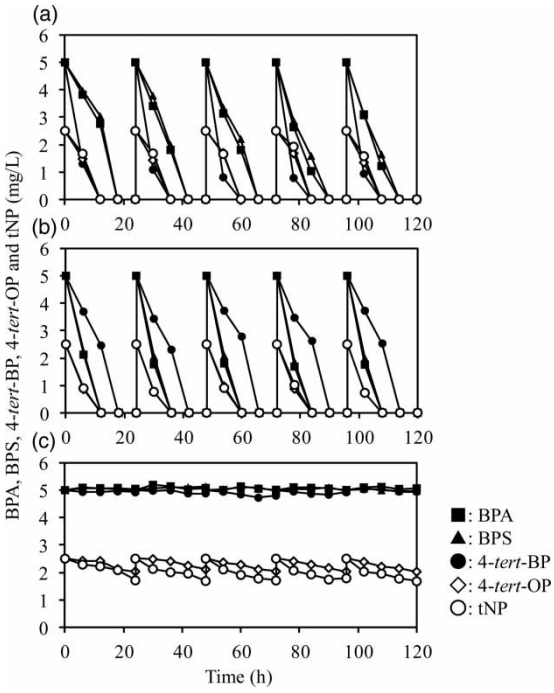

Figure 2 | Changes in concentration of the phenolic EDCs BPA, BPS, 4-*tert*-BP, 4-*tert*-OP and tNP in Hoagland solution, reflecting degradation in the presence of (a) *Phragmites*–TIK1 association, (b) *Phragmites*–IT4 association and (c) sterile and uninoculated *Phragmites*. Values are means from triplicate experiments. The Hoagland solution containing EDCs was replaced with fresh solution every 24 h.

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 in the presence of uninoculated *Phragmites* 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

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

	***Phragmites*–TIK1**			***Phragmites*–IT4**			**Uninoculated *Phragmites***		
	Cycle number			**Cycle number**			**Cycle number**		
Contaminant	**1**	**5**	**10**	**1**	**5**	**10**	**1**	**5**	**10**
BPA	100	100	100	100	100	100	1.9	2.6	9.0
BPS	100	100	100	100	100	100	1.7	3.5	1.4
4-*tert*-BP	100	100	100	100	100	100	2.1	2.5	4.1
4-*tert*-OP	100	100	100	100	100	100	23.4	26.8	25.2
tNP	100	100	100	100	100	100	31.8	33.6	33.2

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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