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Accelerated degradation of a variety of aromatic compounds by *Spirodela polyrrhiza*-bacterial associations and contribution of root exudates released from *S. polyrrhiza*

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Abstract

Removal experiments of phenol, aniline, 2,4-dichlorophenol, nonylphenol and bisphenol A (BPA) using *Spirodela polyrrhiza*-bacterial associations revealed that all compounds but BPA underwent accelerated removal. The mechanisms differed depending on the substrates. It was found that *S. polyrrhiza* has a great ability to release phenolic compound-rich root exudates, and the exudates seem to stimulate bacterial degradation of a variety of aromatic compounds.

Key words: accelerated biodegradation; aromatic compounds; rhizosphere; root exudates; *Spirodela polyrrhiza*
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Introduction

Treatment of secondary effluent from wastewater treatment plants and remediation of polluted aquatic environments by using aquatic plants (aquatic plant treatment system, APTS) is a cost-effective, environmentally friendly technology for the conservation of the aquatic environment. Over the last few decades, ATPS has been mainly applied for the removal of nitrogen and phosphorus (Tripathi et al., 1991; Greenway, 2003; Li et al., 2009) and heavy metals (Keskinkan et al., 2004; Miretzky et al., 2004; Mishra and Tripathi, 2008) because plants have ability to uptake such compounds through their growth. It has also been reported that aquatic plants can contribute to the degradation/removal of easily biodegradable organic compounds (Körner et al., 1998; Al-Nozaily et al., 2000). In these reports, the ability of the aquatic plants to supply oxygen into their root zone by photosynthesis or to provide bacterial growth surface area were the proposed mechanism.

In recent years, we have found that synthetic surfactants (Mori et al., 2005) and aromatic compounds (Toyama et al., 2006, 2009b; Hoang et al., 2009) in the aquatic phase can undergo accelerated degradation/removal in the rhizosphere of a floating aquatic plant, *Spirodela polyrrhiza*. Accelerated degradation of bisphenols and pyrene in the rhizosphere sediment of *Phragmites australis* (Jouanneau et al., 2005; Toyama et al., 2009a) was also reported by our group and other research groups. Results from these studies indicated that APTS may also be used for the removal of recalcitrant compounds in the finishing process of secondary effluents and in the remediation of aquatic environments. Our studies using *S. polyrrhiza* as a model aquatic plant have revealed that stimulation of bacteria in the rhizosphere by oxygen supply and exudates secreted from plant roots would be a main mechanism for the acceleration of degradation/removal of recalcitrant compounds in the aquatic plant rhizosphere. In addition, we found that aromatic compound-degrading bacteria are selectively accumulated in the *S. polyrrhiza* rhizosphere, suggesting that *S. polyrrhiza* has a high potential to degrade aromatic compounds.

This study aimed to determine what kinds of aromatic compounds can be removed effectively by the *S. polyrrhiza*-bacterial association. To this end, we examined the effect of planting *S. polyrrhiza* on the accelerated degradation of phenol, aniline, 2,4-dichlorophenol (2,4-DCP), nonylphenol (NP) and bisphenol A (BPA), compounds which have often been detected in the aquatic environment and are of concern due to their toxicity. We also characterized the root exudates of *S. polyrrhiza* as a possible factor which could accelerate the degradation of these aromatic compounds.

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1 Materials and methods

1.1 Chemicals

Phenol and aniline were purchased from Kishida Chemical (Osaka, Japan). 2,4-DCP, NP and BPA were purchased from Tokyo Chemical Industry (Japan). *n*-Hexane and ethyl acetate were purchased from Sigma-Aldrich (USA). Acetonitrile was purchased from Kanto Kagaku (Tokyo, Japan).

1.2 *Spirodela polyrrhiza*

Intact plants of *S. polyrrhiza* were obtained from an existing laboratory stock culture. They were maintained in pond water collected from Inukai Pond without significant contamination by chemicals in Osaka University Suita Campus (Japan). Sterile *S. polyrrhiza* were obtained and maintained as described previously (Toyama et al., 2006). *S. polyrrhiza* were statically grown in an incubation chamber at (28 ± 1)°C under a fluorescent lamp at 8000 lux (16 hr-light and 8 hr-dark condition).

1.3 Aromatic compound removal experiments

Pond water used in the aromatic compound removal experiments was collected from Inukai Pond. Four test systems were constructed for each aromatic compound. Test system A was made up of natural pond water with 30 fronds of intact *S. polyrrhiza*, to evaluate the accelerated degradation effect by whole plant-bacterial associations. Test system B consisted of natural pond water with 30 fronds of sterile *S. polyrrhiza*, to exclude the effect of bacteria in the rhizosphere (rhizobacteria) of *S. polyrrhiza*, so that the contribution of *S. polyrrhiza* to the accelerated degradation of the aromatic compounds by the stimulation of pond water microbes could be evaluated. Test system C was made up of only natural pond water, so that the contribution of pond water bacteria to the aromatic compound degradation could be evaluated. This test system was used as a control for other test systems. Test system D was made up of sterile pond water with 30 fronds of sterile *S. polyrrhiza*, so that the contribution of *S. polyrrhiza* alone to the aromatic compound removal could be evaluated. All test systems were constructed in duplicates using 300 mL of pond water in 500 mL Erlenmeyer flasks. Phenol, aniline and BPA were amended to a final concentration of 10 mg/L, while 2,4-DCP and NP were amended to 5 mg/L. Control microcosms without the addition of the aromatic compounds were also prepared. All microcosms were statically incubated in an incubation chamber at (28 ± 1)°C under a fluorescent lamp at 8000 lux (16 hr-light and 8 hr-dark condition) for 3 days (phenol and aniline) or 5 days (2,4-DCP, NP and BPA).

During the experiments, the concentrations of the aromatic compounds were monitored periodically by high-performance liquid chromatography (HPLC). For phenol, aniline and BPA amended systems, an aliquot (1 mL) of sample from each microcosm was centrifuged (20,000 ×*g*, 4°C, 10 min), and the supernatant was subjected to HPLC analysis. For NP amended systems, 0.5 mL of the bulk water fraction was mixed with an equal volume of acetonitrile and centrifuged, and then the supernatant was subjected to HPLC analysis. In addition, three fronds of *S. polyrrhiza* were shaken (300 r/min, 10 min) in 5 mL of acetonitrile and NP adsorbed on the surface of *S. polyrrhiza* was dissolved in acetonitrile. The solution was then centrifuged (20,000 ×*g*, 4°C, 10 min), and the supernatant was subjected to HPLC analysis. The HPLC analysis was conducted using a Shimadzu LC-10A*vp* HPLC system (Japan) equipped with a Shim-pack VP-ODS column (150 (phenol, aniline, 2,4-DCP and BPA) or 250 (NP) × 4.6 mm (i.d.); particle size, 5 μm, Shimadzu, Japan). Mobile phases of 50%, 70%, 90% and 50% acetonitrile were used for phenol, aniline, NP and BPA analyses, respectively, while 2% acetic acid in 60% acetonitrile was used for 2,4-DCP analysis. The flow rate of the mobile phase was 0.7 mL/min for NP analysis and 1.0 mL/min for other compounds. Detection was carried out at 270, 254, 225, 277 and 254 nm for phenol, aniline, 2,4-DCP, NP and BPA, respectively.

1.4 Characterization of root exudates

To characterize the root exudates of *S. polyrrhiza* exposed to aromatic compounds, root exudates were collected from triplicate sterile cultures of *S. polyrrhiza* with and without exposure to phenol, aniline, 2,4-DCP or BPA. Twenty fronds of sterile *S. polyrrhiza* gently washed in sterile MilliQ water to flush initial root exudates were statically incubated in 200 mL of sterile modified Hoagland nutrient solution (Toyama et al., 2006) which was amended with 5 mg/L of phenol, aniline, 2,4-DCP or BPA for 3 days. Then *S. polyrrhiza* was washed twice with sterile MilliQ water and statically incubated in 50 mL of sterile MilliQ water for 1 day. Root exudates were also obtained from sterile *S. polyrrhiza* without any chemical exposure. The root exudates of *S. polyrrhiza* in bulk water and rhizosphere fractions were collected and analyzed separately. For the analysis of the bulk water fraction, a 200-mL sample of bulk water was collected, and 10 mL of the sample was subjected to total organic carbon (TOC) analysis. The remaining 190 mL was freeze-dried, dissolved in 9.5 mL of MilliQ water and subjected to total phenolic compounds analysis. For the analysis of the rhizosphere fraction, 20 fronds of *S. polyrrhiza* were shaken in 20 mL of sterile MilliQ water on a rotary shaker (120 r/min) for 3 min. Ten milliliters of the sample was subjected to TOC analysis, and the remaining 10 mL was subjected to total phenolic compounds analysis. The TOC concentration was measured using a TOC analyzer (TOC-5000A, Shimadzu, Japan). The total phenolic compounds concentration was measured by the 4-aminoantipyrine method (APHA, 1998) with minor modifications, using a calibration curve drawn for the standard phenol solution. The ability of *S. polyrrhiza* to secrete phenolic compounds was shown as milligrams of TOC or phenol per gram of wet root per day (mg-C or mg-phenolic compounds/(day·g wet root)). The root wet weight was measured after dewatered the root using Kimtowels (Nippon Paper Crecia, Japan). For HPLC analysis of the root exudates, 50 mL of root exudates mixture (1:1, *V/V*) of rhizosphere and

bulk water fractions acidified to pH 3.0 with 1 mol/L HCl was passed through an Oasis HLB cartridge (500 mg/6 cc, Waters, USA), which was conditioned by 6 mL of *n*-hexane, ethyl acetate and methanol in sequence, at a flow rate of 5–10 mL/min. After the sorbent bed was air dried, the concentrated root exudates were eluted with 6 mL of *n*-hexane, ethyl acetate and methanol in sequence. The eluent was dried under a gentle stream of nitrogen, dissolved in 500 μL of acetonitrile and subjected to HPLC analysis with ten Chromolith RP-18 columns (100 × 4.6 mm (i.d.) each; particle size, 2 μm; Merck, USA) connected in series. The mobile phase was 20% acetonitrile with a flow rate of 1.0 mL/min. The detection was carried out at 254 nm.

2 Results and discussion

2.1 Aromatic compound degradation

Degradation profiles of the studied five aromatic compounds in the test systems are shown in Fig. 1. Accelerated removal of all the aromatic compounds except BPA was confirmed in test systems A and B, where intact and sterile *S. polyrrhiza*, respectively, were planted, in comparison to the bacterial degradation in the natural pond water (test system C) which served as a control. That is, the *S. polyrrhiza*-bacterial association can effectively remove a wide variety of aromatic compounds.

For phenol and aniline removal, test system B showed more effective removal than test system C (Fig. 1a, b). Because no significant removal was observed in test system D, where sterile *S. polyrrhiza* was planted in sterile pond water over the experimental period (72 hr), this accelerated removal should not be ascribed to the adsorption or enzymatic degradation by *S. polyrrhiza* but to activation of phenol and aniline degrading bacteria in pond water and rhizospheres by *S. polyrrhiza*. Interestingly, more effective phenol and aniline degradation was confirmed in test system A, where the intact rhizobacteria were maintained in the rhizosphere, than in test system B, where only indigenous bacteria in the pond water were stimulated by *S. polyrrhiza*. Thus, a high degradation ability of rhizobacteria with respect to these compounds was suggested.

In the case of 2,4-DCP, the removal tendency differed from that for phenol and aniline (Fig. 1c). No degradation was confirmed in the natural pond water (test system C), whereas a linear decline in the 2,4-DCP concentration was observed in test system D. This suggests that significant removal of 2,4-DCP was caused by *S. polyrrhiza* itself. Here, because it was confirmed that the decrease in the

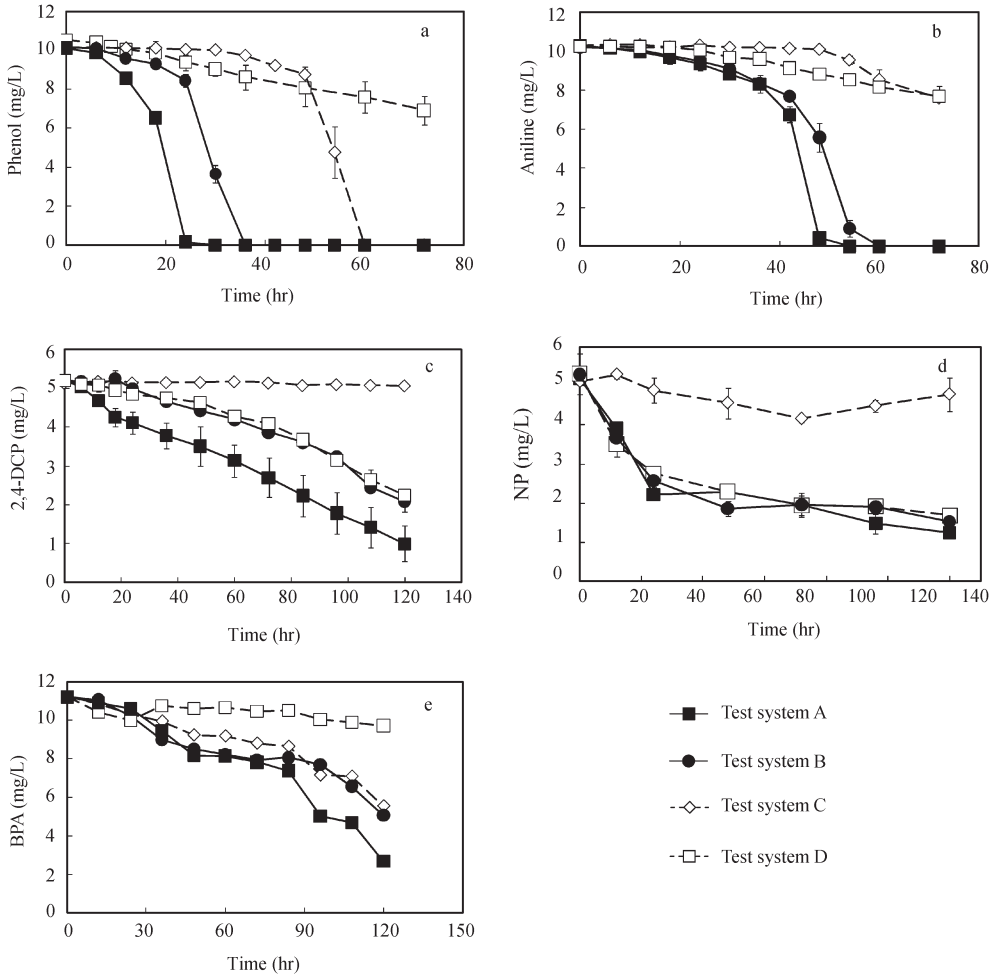

Fig. 1 Degradation profiles of phenol (a), aniline (b), 2,4-dichlorophenol (2,4-DCP) (c), nonylphenol (NP) (d) and bisphenol A (BPA) (e) in test systems A (natural pond water + 30 fronds of intact *S. polyrrhiza*), B (natural pond water + 30 fronds of sterile *S. polyrrhiza*), C (natural pond water) and D (sterile pond water + 30 fronds of sterile *S. polyrrhiza*). Error bars represent 95% confidence intervals.

2,4-DCP concentration did not depend on the adsorption by *S. polyrrhiza*, the enzymatic degradation or uptake by *S. polyrrhiza* could be the reasons for 2,4-DCP removal. The removal rate of 2,4-DCP in test systems B and D was the same, while it was a little faster in test system A, suggesting the ability of rhizobacteria to degrade 2,4-DCP to a certain extent.

In the NP removal test, 48%–57% of the initial NP was removed after 24 hr in test systems A, B and D, which contained sterile or non-sterile *S. polyrrhiza*. On the other hand, no significant decrease in the NP concentration in test system C was observed over the experimental period (120 hr) (Fig. 1d). Because the adsorptive removal of NP was suggested, the distribution of NP in bulk water and rhizosphere fractions was also analyzed to evaluate the removal of NP by adsorption. No obvious NP degradation by natural pond water (test system C) was observed during the experimental period (120 hr), whereas slight (13%) NP removal by *S. polyrrhiza* itself (test system D) was confirmed (Table 1). This suggests that the enzymatic degradation or uptake of NP by *S. polyrrhiza* also occurred after adsorption. When bacteria were also present (test systems A and B), the NP removal ratios from the test system were considerably higher (42% and 21% for test systems A and B, respectively) than for test system D. Thus, it was confirmed that *S. polyrrhiza* activated bacterial NP degradation activity in pond water and rhizospheres, and indigenous rhizobacteria seem to have a higher ability to degrade NP.

BPA was degraded in natural pond water (test system C) while *S. polyrrhiza* (test system D) did not remove BPA significantly over the experimental period (120 hr) (Fig. 1e). In addition, there was no obvious difference between test systems B and C, suggesting that *S. polyrrhiza* could not activate bacterial BPA degradation, although indigenous rhizobacteria might slightly degrade BPA (test system A). The first step of bacterial BPA degradation has recently been reported to require cytochrome P450 (Sasaki et al., 2005), while it is well known that hydroxylase or dioxygenase is the key enzyme for the degradation of phenol, aniline, 2,4-DCP and NP. Thus, the difference between the degradation pathways should be one possible reason why accelerative degradation of BPA did not take place.

It is interesting that the accelerative degradation of all tested aromatic compounds apart from BPA depended, at least partly, on the bacteria activated by *S. polyrrhiza*. These results indicate that *S. polyrrhiza* can contribute to the activation of the bacterial degradation of a variety of aromatic compounds.

2.2 Root exudates analysis

Table 2 shows the amounts of TOC and phenolic compounds in root exudates of *S. polyrrhiza* exposed to the different aromatic compounds. Corresponding HPLC chromatograms of the root exudates are shown in Fig. 2. The cases in which *S. polyrrhiza* was exposed to phenol and aniline are shown as examples of obvious activation of bacterial degradation by *S. polyrrhiza*, whereas those of 2,4-DCP and BPA are shown as examples of less and insignificant activation, respectively. Because NP was adsorbed on rhizoplane, it was difficult to clearly characterize the root exudates from *S. polyrrhiza* exposed to NP and the results are not shown here. The root exudates of *S. polyrrhiza* not exposed to aromatic compounds were also analyzed.

S. polyrrhiza has a great ability to release phenolic compound-rich root exudates and this seemed crucial for the accelerated degradation of aromatic compounds by stimulating the catabolic bacteria in the rhizosphere (Table 2). In the control system, while *S. polyrrhiza* secreted 90% of the root exudates into the bulk water fraction (126.5 mg TOC/(day·g wet root)), 95% (9.9 mg-phenolic compounds/(day·g wet root)) of the phenolic compounds in the root exudates were found in the rhizosphere fraction. When *S. polyrrhiza* was exposed to phenol and aniline, the specific release rates of phenolic compounds significantly increased in the rhizosphere fraction, and as a result, the specific release rates of root exudates (TOC) in the rhizosphere fraction also increased. The HPLC chromatograms

Table 1 Distribution of nonylphenol (NP) in bulk water and root surface fractions and efficiency of NP removal during experimental period

Test system	Time (day)	Distribution of NP in test system (mg-NP/L)		NP removal efficiency (%)		
		Bulk water fraction	Root surface fraction	Removal from water	Removal by adsorption	Removal by degradation
System A	0	5.2	0	0	0	0
	1	2.2	2.9	57.7	55.8	1.9
	3	2.0	1.9	61.5	36.5	25.0
	5	1.2	1.8	76.9	34.6	42.3
System B	0	5.3	0	0	0	0
	1	2.6	2.7	50.9	50.9	0
	3	2.0	2.9	62.3	54.7	7.6
	5	1.5	2.7	71.7	50.9	20.8
System C	0	5.1	–	0	–	0
	1	4.9	–	3.9	–	3.9
	3	4.2	–	17.6	–	17.6
	5	4.8	–	5.9	–	5.9
System D	0	5.3	0	0	0	0
	1	2.8	2.6	47.2	47.2	0
	3	2.0	2.9	62.3	54.7	7.6
	5	1.7	2.9	67.9	54.7	13.2

Table 2 Specific release rates of total organic carbon (TOC) and phenolic compounds in the root exudates of *S. polyrrhiza*

	TOC[a,b] (mg C/(day·g wet root))			Phenolic compounds[a,b,c] (mg phenol/(day·g wet root))		
	Bulk water fraction	Root surface fraction	Total	Bulk water fraction	Root surface fraction	Total
Control	126.5 ± 12.4	12.5 ± 3.7	139.0 ± 16.1	0.5 ± 0.1	9.9 ± 1.5	10.3 ± 1.5
Phenol	137.0 ± 9.3	17.4 ± 2.2	154.4 ± 11.5	0.5 ± 0.1	14.2 ± 2.2	14.7 ± 2.3
Aniline	134.7 ± 15.0	19.6 ± 4.0	154.3 ± 19.0	0.7 ± 0.1	13.5 ± 1.6	14.2 ± 1.7
2,4-DCP	121.7 ± 5.9	15.4 ± 1.8	137.1 ± 7.7	0.5 ± 0.1	10.9 ± 1.2	11.4 ± 1.3
BPA	130.4 ± 5.4	9.4 ± 2.6	139.8 ± 8.0	1.7 ± 0.1	7.6 ± 1.6	9.3 ± 1.7

[a] Results are shown as mean ± 95% confidence interval (n = 3).
[b] Results are shown as mg carbon or phenolic compounds per g of wet root per day (mg/(day·g wet root), specific release rate).
[c] Total phenolic compounds are shown as mg of phenol equivalent.

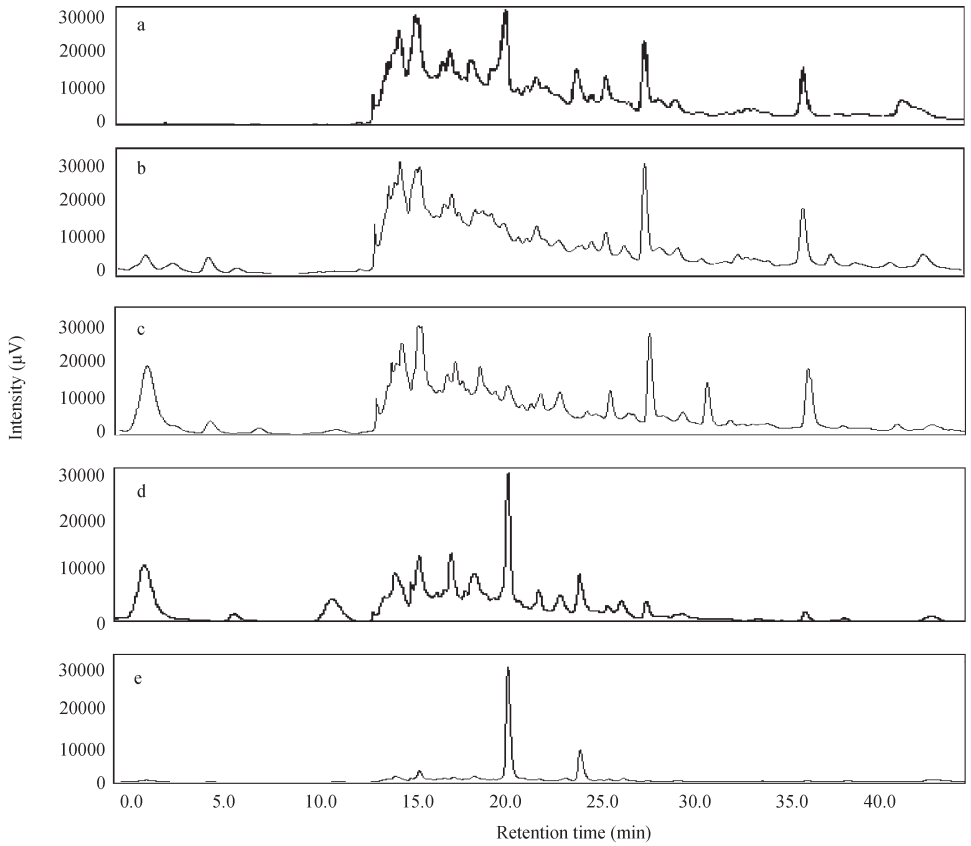

Fig. 2 HPLC chromatograms of phenolic compounds in root exudates. The root exudates released by sterile *S. polyrrhiza* free from chemical exposure (control) (a) and by *S. polyrrhiza* exposed to phenol (b), aniline (c), 2,4-DCP (d) or BPA (e), respectively.

of the root exudates showed almost the same pattern as the control (Fig. 2a, b, c). When *S. polyrrhiza* was exposed to 2,4-DCP, no significant change was observed in the specific release rates of TOC and phenolic compounds in either the bulk water or the rhizosphere fractions. The HPLC chromatogram of the root exudates revealed a pattern that had some similarities to the control, but some major peaks which were completely different from the control were also observed (Fig. 2a, d). In contrast, BPA significantly lowered the specific release rate of phenolic compounds in the rhizosphere fraction, increasing that in the bulk water fraction instead. The specific release rate of root exudates (TOC) was almost the same as that of the control (Table 2). This suggests that *S. polyrrhiza* changed the components of the root exudates into more soluble substrates. The HPLC chromatogram of the root exudates was quite different from that of the control, which confirmed that the components of the root exudates secreted by *S. polyrrhiza* exposed to BPA were quite different from those under natural conditions.

These results indicate an interesting tendency, that the amount of phenolic compounds in the root exudates increased in the rhizosphere fraction when *S. polyrrhiza* was exposed to the aromatic compounds that were effectively degraded by bacterial activation. Meanwhile, they decreased when exposed to the aromatic compounds that resulted in less effective or insignificant bacterial activation. In addition, the HPLC chromatograms imply another interesting phenomenon: when *S. polyrrhiza* is exposed to aromatic compounds that can be effectively degraded by bacterial activation, it secretes root exudates with almost the same components that unexposed *S. polyrrhiza*, which is free from chemical stress, does. When *S. polyrrhiza* is exposed to aromatic compounds that result in less effective or insignificant bacterial activation, it secretes root exudates with different components from those secreted by

unexposed *S. polyrrhiza*.

These observations imply a significant contribution by the root exudates to the accelerated bacterial degradation of aromatic compounds. The identification of key components in the root exudates is desirable to open the way for the development of accelerated bacterial degradation technologies for a variety of aromatic compounds.

3 Conclusions

To determine what kinds of aromatic compounds can be effectively removed by using an *S. polyrrhiza*-bacterial association, we examined the effect of planting *S. polyrrhiza* on the accelerated degradation/removal of phenol, aniline, 2,4-dichlorophenol (2,4-DCP), nonylphenol (NP) and bisphenol A (BPA). We also characterized the root exudates of *S. polyrrhiza* as a possible factor that causes the accelerated degradation of these aromatic compounds. Accelerated removal of all the aromatic compounds except BPA by aquatic plant-bacterial associations was confirmed. This shows that an *S. polyrrhiza*-bacterial association can effectively remove a wide variety of aromatic compounds. The acceleration of bacterial degradation by *S. polyrrhiza* was confirmed for phenol and aniline. However, enzymatic degradation and adsorption were the main factors for 2,4-DCP and NP, respectively. In these two cases, accelerated bacterial degradation was less effective. Analysis of the root exudates revealed that *S. polyrrhiza* has a great ability to release phenolic compound-rich root exudates and this seemed crucial for the accelerated degradation/removal of various aromatic compounds by stimulating/recruiting the key-role bacteria in the rhizosphere. The amounts of phenolic compounds in the root exudates increased in the rhizosphere fraction when *S. polyrrhiza* was exposed to the aromatic compounds that were effectively degraded by bacterial activation. At the same time, the HPLC chromatograms implied that *S. polyrrhiza*, when exposed to the aromatic compounds that are effectively degraded by bacterial activation, secretes root exudates with almost the same components as unexposed *S. polyrrhiza*, which is free from chemical stress, secretes. Further analysis of the root exudates to identify the key substrates for the accelerated biodegradation of aromatic compounds can lead to a rational strategy for the development of an effective chemical compound degradation system using aquatic plant-bacterial associations.

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