ACCELERATED AROMATIC COMPOUNDS DEGRADATION IN AQUATIC ENVIRONMENT BY USE OF INTERACTION BETWEEN *SPIRODELA POLYRRHIZA* AND BACTERIA IN ITS RHIZOSPHERE

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ABSTRACT

Accelerated degradation of organic chemicals by aquatic plant-bacterial associations was reported for the first time with elucidation of the role and contribution of aquatic plant and bacteria in its rhizosphere using a fast-growing giant duckweed, *Spirodela polyrrhiza*. The results clearly showed the accelerated degradation of all the 3 aromatic compounds (phenol, aniline and 2,4-dichlorophenol (2,4-DCP)) tested by aquatic plant-bacterial associations. In phenol degradation system, phenol degrading bacteria indigenous to the rhizosphere fraction of *S. polyrrhiza* mainly contributed, while in aniline degradation system *S. polyrrhiza* mainly contributed by stimulating aniline degrading bacteria both in the rhizosphere and balk water fraction. On the other hand in 2,4-DCP degradation system, *S. polyrrhiza* itself mainly contributed to its removal by uptake and degradation. Thus, the mechanisms for accelerated removal of aromatic compounds were quite different depending on the substrates. *S. polyrrhiza* showed selective accumulation of phenol degrading bacteria in its rhizosphere fraction, while aniline and 2,4-DCP degrading bacteria were not much accumulated. *S. polyrrhiza* secreted peroxidase and laccase. However, both of the enzymatic activities increased with the addition of aromatic compounds, degrading ability of *S. polyrrhiza* itself should be owing to the production of peroxidase rather than laccase because the change of peroxidase activity and concentration of each aromatic compound well concurred. From the results obtained in the present study, it can be concluded that the feasibility of the use of aquatic plant-bacterial associations to accelerate the degradation of organic chemicals especially recalcitrant compounds in aquatic environment was shown.

KEYWORDS

phytoremediation, rhizosphere, aquatic plant-microbial association, *Spirodela polyrrhiza*, aromatic compound, accelerated degradation, laccase, peroxidase

INTRODUCTION

Phytoremediation, which is the use of plants to cleanup the polluted environments, has been mainly applied to remove radionuclides (Zhu and Smolders, 2000) or heavy metals (Salt *et. al.*, 1995) in soil environments and to remove nitrogen or phosphorus (Trhipathi *et al.*, 1991) in water environments using accumulation and transformation ability of plants themselves for the last several decades. Recently, accelerated degradation of organic chemicals including recalcitrant xenobiotics such as petroleum hydrocarbons (Radwan *et al.*, 1995), chlorinated solvents (Walton and Anderson, 1990), polycyclic aromatic hydrocarbons (Miya and Firestone, 2001), and pesticides

(Singh *et al.*, 2004) has been reported in rhizosphere or rhizoplane of terrestrial plants by certain plant-microbial associations. This accelerated degradation is mainly attributable to the ability, so called "rhizosphere effect", of plants to transport oxygen and to secrete exudates physiologically active substances such as carbohydrates, amino acids and organic acids into rhizosphere, so that the microbial activity in the rhizosphere are stimulated (Shaw and Burns, 2003). Moreover, it is reported that plants could recruit microbes that contain phenotypes or genotypes specific for herbicides (Shaw and Burns, 2005) and petroleum hydrocarbons degradation (Siciliano *et al.*, 2001) into the rhizosphere and root interior, and this selection occurred depending on the assortment of both plants and contaminants.

In some aquatic plants, this rhizosphere effect has been also reported, but most of the studies focused on the acceleration of the nitrification and the degradation of easily biodegradable substrates (Reddy *et al.*, 1989; Eriksson and Weisner, 1999). There is only one report on the accelerated mineralization of surfactants, linear alkylbenzene sulfonate (LAS), linear alcohol ethoxylate (LAE), and mixed amino acid (MAA) by microbiota of aquatic plants (Federle and Schwab, 1989). However, this report showed only the fact that the accelerated mineralization occurred and the mechanisms who contributed in what way to what extent were obscure. Thus the feasibility of the use of aquatic plant-microbial associations to accelerate the degradation of organic chemicals especially recalcitrant compounds is unclear.

The present study focused on a fast-growing giant duckweed, *Spirodela polyrrhiza*, which is worldwide distributed and often used for wastewater treatment (Caicedo *et al.*, 2000), and performed aromatic compounds degradation tests using pond water microcosms to confirm the accelerated degradation and to elucidate the role and contribution of both *S. polyrrhiza* and microbes in the accelerated aromatic compounds degradation. The change of the total and aromatic-compound degrading bacteria in bulk water and in rhizosphere was monitored by plate count method during the degradation tests. In addition, peroxidase and laccase enzymatic activities of the *S. polyrrhiza* were analyzed to evaluate the contribution of *S. polyrrhiza* itself for the degradation of the tested compounds.

MATERIALS AND METHODS

Aromatic compounds Three aromatic compounds, phenol, aniline and 2,4-dichlorophenol (2,4-DCP), were used in this study. These three compounds were selected by priority to investigate for the formulation of environmental water quality standards for the conservation of aquatic lives in 2003 in Japan.

Plant materials and treatments Intact plants of *S. polyrrhiza* were obtained from an existing laboratory stock culture. They were maintained in containers containing 10 *l* of pond water obtained from Inukai Pond in Osaka University Suita Campus (Suita, Osaka, Japan) and acclimated to pond water for 2 months before used for experiments. The pond water was changed twice a week to prevent from nutrient shortage and algae growth during the acclimation. To obtain the sterile *S. polyrrhiza*, the winter bud (turion) of *S. polyrrhiza* were sterilized by a 5 min-wash in sodium hypochlorite solution (0.5 % available chlorine), rinsed twice with sterile water and then germinated on sterile Hoagland nutrient solution (Arnon and Hoagland, 1940) with modification (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* $CaCl_2 \cdot 2H_2O$, 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* $ZuSO_4 \cdot 7H_2O$, 0.254 mg/*l* $H_2MoO_4 \cdot H_2O$, pH 7.0). They were aseptically transferred to 300 ml Erlenmeyer flasks containing 200 ml of sterile modified Hoagland nutrient solution and maintained until used for experiments. All plant materials were statically grown in an incubation chamber at 28 ± 1 °C under fluorescent lamp at 8,000 lux (16 h-light and 8 h-dark condition).

Design of aromatic compounds degradation test Five test systems were constructed for each aromatic compound as shown in Table 1. The test system A was made up of natural pond water

Table 1 Design of the five test systems to evaluate the accelerated aromatic compound degradation in the rhizosphere of *S. polyrrhiza* by plant-microbial associations

Test systems	Component of microcosms			Aromatic compounds (Initial concentration)
	Bacteria in bulk water fraction	Bacteria in rhizosphere fraction	*S. polyrrhiza*	
A	○	○	○	
B	○		○	Phenol (10 mg/*l*)
C	○	○		Aniline (10 mg/*l*)
D	○			2,4-DCP (5 mg/*l*)
E			○	

with 30 fronds of intact and non-sterile *S. polyrrhiza* to evaluate the accelerated degradation effect by whole plant-microbial associations. The test system B was made up of natural pond water with 30 fronds of sterile *S. polyrrhiza* to exclude the effect of microbes in the rhizosphere (rhizobacteria) of *S. polyrrhiza* so that the contribution of *S. polyrrhiza* to accelerate the aromatic compound degradation by stimulating pond water microbes could be evaluated. The test system C was made up of natural pond water with rhizobacteria of 30 fronds of *S. polyrrhiza* to exclude the effect of *S. polyrrhiza* so that the contribution of rhizobacteria of *S. polyrrhiza* to the aromatic compound degradation could be evaluated. For the collection of rhizobacteria, 30 fronds of mature *S. polyrrhiza* were collected, gently washed twice for 1 min in 50 ml of sterile 5 mg/*l* sodium tripolyphosphate (TPP) to remove microbes in the bulk water fraction. Then the roots were cut, transferred to a test tube containing 10 ml of TPP, and subjected to vortex for 60 s, ultrasonication (20 kHz, 200w, 5 s interval, 4 °C) for 60 s and vortex for additional 60 s. The test system D was made up of only natural pond water so that the contribution of microbes in pond water to the aromatic compound degradation could be evaluated. This was referred as a control of other test systems. The test system E was made up of sterile pond water with 30 fronds of sterile *S. polyrrhiza* to exclude the effect of microbes both in pond water and in rhizosphere of *S. polyrrhiza* so that the contribution of *S. polyrrhiza* to the aromatic compound degradation could be solely evaluated. These test systems were constructed using 300 ml of pond water from Inukai Pond (pH 7.4, dissolved oxygen; DO 8.93 mg/*l*, PO_4-P 0.066 mg/*l*, NH_4-N 0.05 mg/*l*, NO_2-N 0.005 mg/*l*, NO_3-N 0.38 mg/*l* and dissolved organic carbon; DOC 3.12 mg/*l*) in 500 ml Erlenmeyer flasks. Each aromatic compound was amended to give a final concentration of 10 (phenol and aniline) or 5 mg/*l* (2,4-DCP). Control experiments without addition of the aromatic compounds were also performed. In order to block the light from side or bottom, the flasks below the water level were covered with aluminum foil. All the microcosms were statically incubated in an incubation chamber at 28 ± 1 °C under fluorescent lamp at 8,000 lux (16 h-light and 8 h-dark condition) for 3 (phenol and aniline) or 5 days (2,4-DCP). During the experiment, the concentration of the aromatic compounds, the behaviors of the bacterial populations were monitored periodically.

Peroxidase and laccase production by *S. polyrrhiza* during the aromatic compounds removal Twenty fronds of sterile *S. polyrrhiza* were inoculated into 200 ml of sterile modified Hoagland nutrient solution, and phenol, aniline or 2,4-DCP was added to be final concentration of 5 mg/*l* to monitor the removal of amended aromatic compounds by *S. polyrrhiza* and the change of its enzymatic activities. For the control experiments, 20 fronds of sterile *S. polyrrhiza* were inoculated into 200 ml of sterile modified Hoagland nutrient solution to monitor the enzymatic activities without aromatic compounds. Two hundred milliliter of sterile modified Hoagland nutrient solution with each aromatic compound (5 mg/*l*) but *S. polyrrhiza* were also prepared to check that the abiotic removal of amended aromatic compounds.

Analysis of the aromatic compounds Aliquot (1 ml) of the sample from each microcosm was centrifuged (20,000 × *g*, 15 min) and the supernatant was subjected to HPLC equipped with Shim-pack VP-ODS column (150 × 4.6 mm i.d., particle size 5 μm, Shimadzu, Kyoto, Japan) and UV/VIS detector. As mobile phases 50 % and 70 % of acetonitrile were used for phenol and aniline

analyses, respectively, while 2 % acetic acid in 60 % acetonitrile was used for 2,4-DCP under the flow rate of 1.0 ml/min. The detection was carried out at the wavelengths of 270, 254 and 225 nm for phenol, aniline and 2,4-DCP, respectively.

Sampling for microbial monitoring Microbes were collected from the bulk water and the rhizosphere fractions separately. For the bulk water fraction, 10 ml of the bulk water sample was simply collected. For the rhizosphere fraction, 3 fronds of mature *S. polyrrhiza* were collected, gently washed twice for 1 min in 50 ml of sterile 5 mg/*l* TPP to remove microbes in the bulk water fraction. Then the roots were cut, transferred to a test tube containing 10 ml of TPP, and subjected to vortex for 60 s, ultrasonication (20 kHz, 200w, 5 s interval, 4 °C) for 60 s and vortex for additional 60 s.

Enumeration of total culturable and aromatic compound-degrading bacteria The numbers of total culturable and aromatic compound-degrading bacteria in the bulk water and rhizosphere fractions were determined by plating serially diluted samples in triplicate. For the total culturable bacteria, 1/10 diluted Luria Bertani medium (1.0 g/*l* bacto-peptone, 0.5 g/*l* yeast extract, 1.0 g/*l*, pH 7.0) was used while for the aromatic compound-degrading bacteria, basal salt medium (1.0 g/*l* K_2HPO_4, 1.0 g/*l* $(NH_4)_2SO_4$, 0.2 g/*l* $MgSO_4 \cdot 7H_2O$, 0.01 g/*l* $FeCl_3$, 0.05 g/*l* NaCl, 0.01 g/*l* $CaCl_2$, pH 7.2) supplemented with 100 mg/*l* of phenol, aniline or 2,4-DCP was used. The bacterial numbers were determined as colony forming units (CFU) per ml for bacteria in bulk water and CFU per frond of *S. polyrrhiza* for bacteria in the rhizosphere, respectively. The results were shown as CFU per test system calculated as follow: (CFU/ml) × (total volume of bulk water in the test system) for bacteria in the bulk water and (CFU/frond of *S. polyrrhiza*) × (total number of *S. polyrrhiza* in test system) for bacteria in the rhizosphere, respectively.

Enzymatic analysis Peroxidase and laccase activities in bulk water and root tissue fractions were determined. For the analysis of the enzymatic activity in root tissue fraction, roots from 3 fronds of *S. polyrrhiza* were subjected to ultrasonication (20 kHz, 200w, 5 s interval, 4 °C) in 6 ml of phosphate buffer (50 mM potassium phosphate buffer [pH 7.0], 2 mM EDTA, 1 % polyvinylpyrrolidone) for 5 min, centrifuged (20,000 × *g*, 15 min, 4 °C), and the supernatant was used for analysis. For the peroxidase activity, 2.0 ml of sample (bulk water or root tissue fraction), 0.6 ml of 3.0 mM guaiacol, 0.6 ml of 500 mM potassium phosphate buffer [pH 7.0], 0.12 ml of 25 mM EDTA, 0.6 ml of 45 mM H_2O_2 and 2.08 ml of MilliQ water were mixed and the increase in absorbance at 436 nm (A_{436}) was measured in triplicate. The enzymatic reactions were followed for 1, 5 to 10 min. As for laccase, 2.0 ml of sample, 0.12 ml of 25 mM 2,2'-azinobis-(3-methylbenzothiazoline-6-solfonic acid) diammonium salt (ABTS), 0.6 ml of 500 mM malonic acid, 0.12 ml of 25 mM EDTA and 3.16 ml of MilliQ water were mixed and the increase in absorbance at A_{420} was measured in triplicate. The enzymatic reactions were followed for 20, 40 to 60 s. One unit of peroxidase and laccase activities (U) represented the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol and ABTS in 1 min, respectively. Results were shown as enzyme activity per g-dry root weight. The dry root weight was determined after roots were dried at 105 °C for 2 h.

Statistical analysis All the experiments were performed in triplicate and the results were indicated as the mean values with standard deviations (± 95 % confidence interval). Significant differences were determined by Student's *t* test with $P < 0.05$.

RESULTS

Degradation of aromatic compounds Degradation profiles of three aromatic compounds were shown in Fig. 1. In phenol-amended systems, phenol was immediately and completely removed in the test systems A and C. There was no difference in the phenol removal rate between these 2 test systems. In the test systems B and D, phenol was also rapidly and completely removed at almost the same rate as test systems A and C after the lag period of 24 and 48 h, respectively. On the other hand, about 36 % of the amended phenol was removed in test system E after 72 h of the

experimental period. In aniline-amended systems, the complete removal of aniline was observed in test systems A, B and C after the lag period of about 40 h. The removal rates were in the order A, B and C. About 25 % of the amended aniline was removed in test systems D and E after 72 h of the experimental period. In 2,4-DCP-amended systems, about 80 % of 2,4-DCP was removed in test system A, while about 57 % of 2,4-DCP was removed in test systems B and E at the same rate after 120 h of experimental period. Through the experiment, only a slight and no removal of 2,4-DCP were observed in test systems C and E, respectively. Any adverse effect of 3 aromatic compounds on the growth of *S. polyrrhiza* was not observed during degradation tests.

Degradation mechanisms of aromatic compounds Change of the total culturable and aromatic compound-degrading bacteria were summarized in Fig. 2 and Table 2. During 3 days experimental periods, the total culturable bacteria in both bulk water and rhizosphere fractions similarly increased even among different test systems within 3 days. In test systems A and B, total culturable bacteria tended to be slightly larger than in test systems C and D. In 2,4-DCP amended systems, the number of total culturable bacteria tended to be diminished in comparison to phenol or aniline amended systems.

In phenol amended systems, phenol-degrading bacteria in bulk water fraction were only 10.1 % of total culturable bacteria, while those in rhizosphere fraction accounted for 40.6 % at the beginning of the experiment in test system A. After 3 days of experimental periods, phenol degrading bacteria increased to 51.6 % of total culturable bacteria in rhizosphere fraction. Even when sterile *S. polyrrhiza* was used (test system B), bacteria in bulk water fraction were accumulated in rhizosphere fraction immediately and 57.9 % of bacteria in rhizosphere were phenol degrading bacteria after 3 days. When bacteria in rhizosphere fraction were recovered and inoculated into bulk water fraction (test system C), 39.8 % of initial phenol degrading bacteria increased to occupy 85.2 % of total culturable bacteria while phenol degrading bacteria in bulk water (test system D) occupied only 19.5 % of total culturable bacteria even after 3 days.

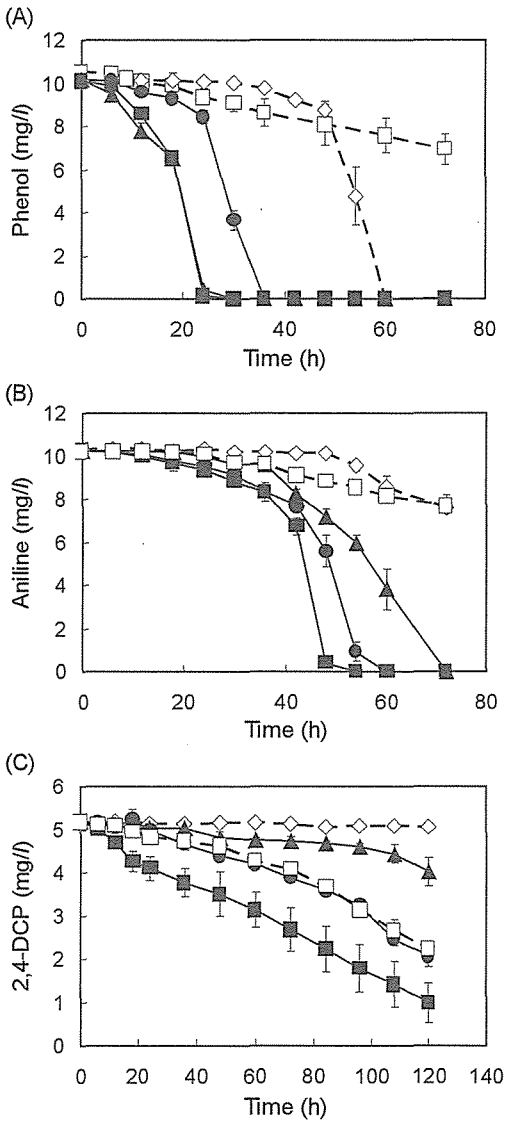

Fig. 1 Degradation profiles of phenol (A), aniline (B) and 2,4-dichlorophenol (C) in test systems A (closed squares), B (closed circles), C (closed triangles), D (open diamonds) and E (open squares), respectively. Error bars represent 95% confidence intervals.

S. polyrrhiza could also accelerate the phenol degradation by selectively accumulating phenol degrading bacteria; more than 50 % of total culturable bacteria were phenol degrading bacteria in its rhizosphere fraction (Figs. 1, 2 and Table 2). It should be noted that even the sterile *S. polyrrhiza* could selectively accumulate phenol degrading bacteria within 3 days. Considering the fact that phenol degradation rate was almost the same in test systems A, B, C and D, the role of the *S.*

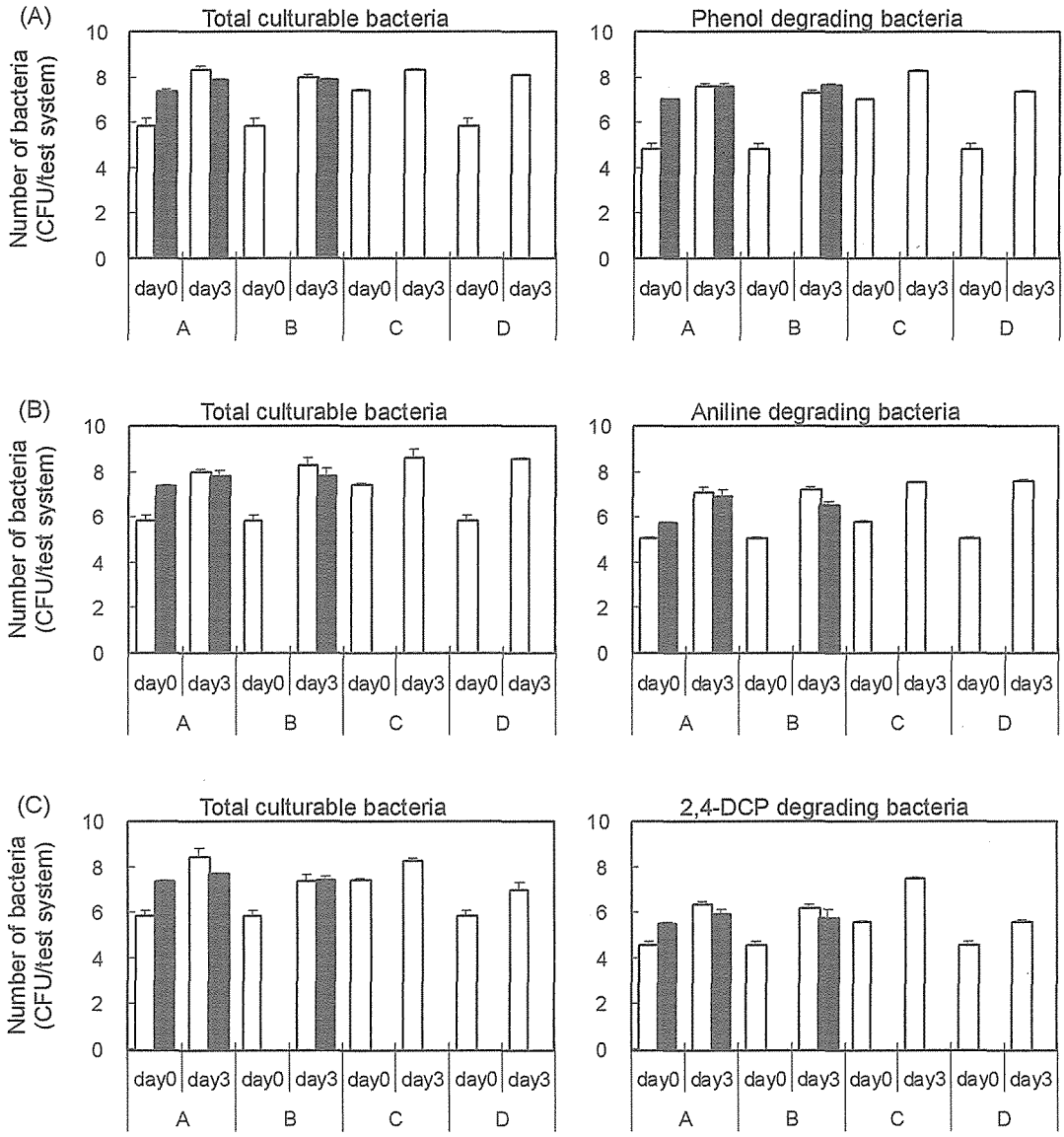

Fig. 2 The numbers of total culturable and aromatic compound-degrading bacteria in the bulk water (open bars) and rhizosphere (closed bars) fractions in phenol (A), aniline (B) and 2,4-dichlorophenol (C) amended systems, respectively. Error bars represent 95% confidence intervals.

polyrrhiza for the phenol degradation was to accumulate and stimulate phenol degrading bacteria in its rhizosphere fraction. *S. polyrrhiza* itself could degrade phenol but the contribution was much lower than the degradation by bacteria (Fig. 1). Thus, bacteria in the rhizosphere fraction played an important role for the phenol degradation.

In aniline amended systems, aniline degrading bacteria in bulk water fraction were 15.4 % of total culturable bacteria and those in rhizosphere fraction were only 2.2 % at the beginning of the experiment in test system A. Even after 3 days of experimental periods, aniline degrading bacteria accounted for only 12.6 % and 11.9 % in bulk water and rhizosphere fractions, respectively. When sterile *S. polyrrhiza* was used (test system B), aniline degrading bacteria in both bulk water (8.6 %) and rhizosphere (5.0 %) fractions again could not occupy large populations even after 3 days. Also,

aniline degrading bacteria in bulk water fraction could not occupy large populations regardless of with (test system C) / without (test system D) inoculating bacteria recovered from rhizosphere. When considering the fact that 10 mg/*l* of aniline was degraded within 60 h in test system B while more than 70 % of the amended aniline was still remained after 72 h in test system D (Fig. 1), there was no obvious effect of *S. polyrrhiza* to selectively accumulate aniline degrading bacteria in its rhizosphere fraction because those in both bulk water and rhizosphere fractions were around 5-10 % (Fig. 2 and Table 2). Bacteria in the rhizosphere fraction also had aniline degradation ability to some extent although complete degradation of the amended aniline was confirmed after 72 h. Thus, *S. polyrrhiza* was considered to play an important role for the aniline degradation by stimulating the aniline degrading bacteria in both bulk water and rhizosphere fractions.

Table 2 Distribution and change of the total culturable and aromatic compounds degrading bacteria in bulk water and rhizosphere fractions during degradation tests

Test systems		day	Number of total culturable and aromatic compounds degrading bacteria (CFU/test system) [a]			
			Total culturable bacteria		Aromatic compounds degrading bacteria (% of aromatic compounds degrading bacteria) [b]	
			Bulk water	Rhizosphere	Bulk water	Rhizosphere
Phenol						
	A	0	$(7.2\pm2.0)\times10^5$	$(2.4\pm0.7)\times10^7$	$(7.2\pm1.9)\times10^4$ (10.1%)	$(9.9\pm2.6)\times10^6$ (40.6%)
		3	$(8.6\pm2.3)\times10^8$	$(3.1\pm1.0)\times10^8$	$(1.4\pm0.3)\times10^8$ (16.7%)	$(1.6\pm0.2)\times10^8$ (51.6%)
	B	0	$(7.2\pm2.0)\times10^5$	– [c]	$(7.2\pm1.9)\times10^4$ (10.1%)	–
		3	$(3.9\pm1.2)\times10^8$	$(2.9\pm1.0)\times10^8$	$(8.4\pm2.8)\times10^7$ (21.3%)	$(1.7\pm0.3)\times10^8$ (57.9%)
	C	0	$(2.5\pm0.8)\times10^7$	–	$(1.0\pm0.3)\times10^7$ (39.8%)	–
		3	$(2.1\pm0.2)\times10^8$	–	$(1.8\pm0.3)\times10^8$ (85.2%)	–
	D	0	$(7.2\pm2.0)\times10^5$	–	$(7.2\pm1.9)\times10^4$ (10.1%)	–
		3	$(1.2\pm0.2)\times10^8$	–	$(2.4\pm0.5)\times10^7$ (19.5%)	–
Aniline						
	A	0	$(7.2\pm2.0)\times10^5$	$(2.4\pm0.7)\times10^7$	$(1.1\pm0.5)\times10^5$ (15.4%)	$(5.3\pm1.0)\times10^5$ (2.2%)
		3	$(3.7\pm1.3)\times10^8$	$(2.7\pm0.9)\times10^8$	$(4.6\pm1.9)\times10^7$ (12.6%)	$(3.2\pm1.5)\times10^7$ (11.9%)
	B	0	$(7.2\pm2.0)\times10^5$	–	$(1.1\pm0.5)\times10^5$ (15.4%)	–
		3	$(7.1\pm2.3)\times10^8$	$(2.6\pm0.9)\times10^8$	$(6.1\pm2.2)\times10^7$ (8.6%)	$(1.3\pm0.4)\times10^7$ (5.0%)
	C	0	$(2.5\pm0.8)\times10^7$	–	$(6.4\pm1.1)\times10^5$ (2.5%)	–
		3	$(3.9\pm1.2)\times10^8$	–	$(3.3\pm1.6)\times10^7$ (8.4%)	–
	D	0	$(7.2\pm2.0)\times10^5$	–	$(1.1\pm0.5)\times10^5$ (15.4%)	–
		3	$(3.4\pm0.8)\times10^8$	–	$(3.8\pm0.9)\times10^7$ (11.1%)	–
2,4-DCP						
	A	0	$(7.2\pm2.0)\times10^5$	$(2.4\pm0.7)\times10^7$	$(3.4\pm0.9)\times10^4$ (4.7%)	$(3.2\pm1.3)\times10^5$ (1.3%)
		3	$(5.2\pm1.8)\times10^8$	$(9.3\pm0.7)\times10^7$	$(4.7\pm0.7)\times10^6$ (0.9%)	$(1.5\pm0.3)\times10^6$ (1.6%)
	B	0	$(7.2\pm2.0)\times10^5$	–	$(3.4\pm0.9)\times10^4$ (4.7%)	–
		3	$(4.7\pm1.3)\times10^7$	$(5.1\pm1.6)\times10^7$	$(3.2\pm0.7)\times10^6$ (6.9%)	$(1.1\pm0.7)\times10^6$ (2.2%)
	C	0	$(2.5\pm0.8)\times10^7$	–	$(3.6\pm1.4)\times10^5$ (1.4%)	–
		3	$(1.9\pm0.2)\times10^8$	–	$(3.1\pm0.9)\times10^7$ (16.1%)	–
	D	0	$(7.2\pm2.0)\times10^5$	–	$(3.4\pm0.9)\times10^4$ (4.7%)	–
		3	$(9.0\pm2.0)\times10^6$	–	$(3.7\pm0.9)\times10^5$ (4.1%)	–

[a] Results are shown as mean ±95% confidence interval.
[b] Results are shown as percentage of aromatic compound degrading bacteria in total culturable bacteria.
[c] –, not tested

In 2,4-DCP amended systems, the distribution and the behavior of 2,4-DCP degrading bacteria were almost the same as aniline degrading bacteria but the population was much smaller that only 0.9 – 16.1 % and 1.3 – 2.2 % of total culturable bacteria in bulk water and rhizosphere fractions, respectively. There seems a slight effect of *S. polyrrhiza* to stimulate the 2,4-DCP degrading bacteria in its rhizosphere fraction although there was no obvious effect of *S. polyrrhiza* to selectively accumulate 2,4-DCP degrading bacteria in this rhizosphere fraction and the bacterial 2,4-DCP degradation was much lower. Indeed, the population of 2,4-DCP degrading bacteria was less than 5 % of total culturable bacteria in most of the 2,4-DCP amended systems even after 3 days. Thus, *S. polyrrhiza* played an important role for the 2,4-DCP removal by absorption and degradation ability of itself (Fig. 1).

Removal of aromatic compounds by *S. polyrrhiza* To evaluate the ability of *S. polyrrhiza* to remove aromatic compounds, the time course of the removal of aromatic compounds and expression of enzymatic activity by *S. polyrrhiza* was shown in Fig. 3. Both the peroxidase and laccase activities of the *S. polyrrhiza* were constantly detected even in the control systems in which aromatic compounds were not amended. In phenol amended system, both the enzymatic activities increased to 13 units/g-dry root in first 48 h and 91 % of the amended phenol was removed constantly within 168 h. The peroxidase activity showed gradual decrease in accordance with the decrease of the phenol, while the laccase activity remained high even at 168 h. In aniline amended system, laccase activity increased immediately to 15 units/g-dry root, while that of peroxidase increased gradually to 10 units/g-dry root in first 72 h and 26 % of the amended aniline was removed within 168 h. The peroxidase activity showed slight decrease after 120 h in accordance with the decrease of the aniline, while laccase activity remained high even after 168 h. In 2,4-DCP amended system, peroxidase activity increased immediately to 12 units/g-dry root in first 24 h, while laccase activity increased to 15 units/g-dry root after 48 h of lag period. The rapid removal of 2,4-DCP was observed in first 24 h and amended 2,4-

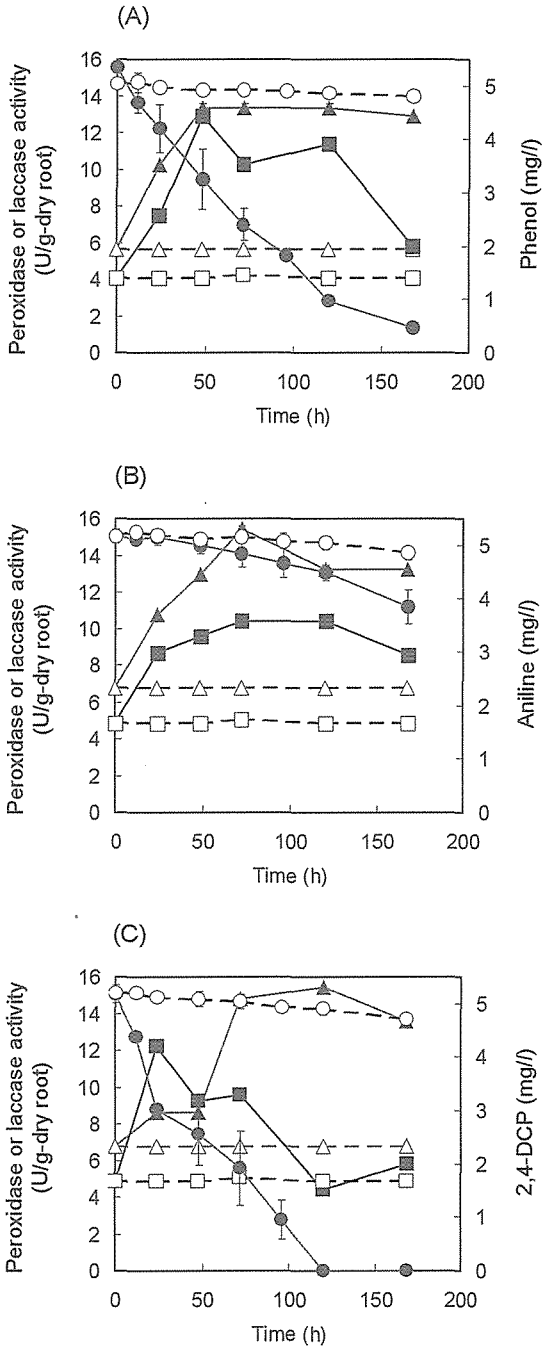

Fig. 3 Removal of phenol (A), aniline (B) and 2,4-dichlorophenol (C) by sterile *S. polyrrhiza*, and concomitant peroxidase and laccase activities in the root tissue. Concentration of aromatic compounds in nutrient solution with (closed circles) and without (open circles) sterile *S. polyrrhiza*, peroxidase activity (closed squares) and (open squares), and laccase activity (closed triangles) and (open triangles) in the aromatic compound amended systems and control, respectively.

DCP was completely removed within 120 h. The peroxidase activity showed gradual decrease in accordance with the decrease of the 2,4-DCP, while laccase activity remained high even after 168 h same as for phenol and aniline amended systems. These 3 aromatic compounds were not detected from root tissue of *S. polyrrhiza* used in the degradation tests. Thus there was no evidence that *S. polyrrhiza* took up these 3 aromatic compounds.

DISCUSSION

There are many reports on the experimental attempts and practical applications of accelerated degradation of organic chemicals using terrestrial plant-microbial associations. In such reports, the rhizosphere effect, transportation of oxygen and secretion of physiologically active substances, of the plants has been shown to activate the microbes in the rhizosphere of them, which enhance the degradation of various chemicals. Aquatic plant-microbial associations can be also cost-effective and attractive remedy for the aquatic environment contaminated with organic chemicals if the rhizosphere effect is confirmed, and applicable substrate range and the degradation/removal mechanisms are clarified. Within our reference search there is only one report that showed the accelerated degradation of LAE and MAA using microbiota associated with duckweed (*Lemna minor*) and the roots of cattail (*Typha latifolia*) (Federle and Schwab, 1989). However, no attempt was made to elucidate the roles of plant and bacteria in its rhizosphere.

The present study focused on a fast-growing giant duckweed, *Spirodela polyrrhiza*, and the accelerated degradation of phenol, aniline and 2,4-DCP with the role and contribution of both *S. polyrrhiza* and microbes was confirmed. This is the first report on the accelerated degradation of organic chemicals with elucidation of the role and contribution of aquatic plant and bacteria in its rhizosphere. The results clearly showed the accelerated degradation of all the 3 aromatic compounds by aquatic plant-bacterial associations (Fig. 1). In phenol degradation system, phenol degrading bacteria indigenous to the rhizosphere fraction of *S. polyrrhiza* mainly contributed, while in aniline degradation system *S. polyrrhiza* mainly contributed by stimulating aniline degrading bacteria both in the rhizosphere and balk water fraction. On the other hand in 2,4-DCP degradation system, *S. polyrrhiza* itself mainly contributed to its removal by uptake and degradation. Thus, the mechanisms for accelerated removal of aromatic compounds were quite different depending on the substrates. *S. polyrrhiza* showed selective accumulation of phenol degrading bacteria in its rhizosphere fraction (Table 2 and Fig. 2), while aniline and 2,4-DCP degrading bacteria were not much accumulated. This can be owing to the substrates which plant secrets to its rhizosphere. Indeed, plant roots secret phenols but nitrogen or chlorine containing aromatics such as aniline or 2,4-DCP (Peters and Verma, 1990; Kamath *et al.*, 2004), and therefore, aniline and 2,4-DCP degrading bacteria could not be accumulated in the rhizosphere of *S. polyrrhiza*. As is clear from Fig. 1 (B) and (C), aniline and 2,4-DCP degrading bacteria were also activated by the presence of *S. polyrrhiza* probably due to the oxygen and exudates from its root, which contributed to the accelerated degradation of aniline and 2,4-DCP to some extent. But in case of the 2,4-DCP the contribution of *S. polyrrhiza* itself was much higher than that of bacteria. Plants also secrete peroxidase and laccase from their roots (Adler *et al.*, 1994; Mayer and Staples, 2002). It is known that these enzymatic activities in plant roots increase in response to the stress of phenolic compounds and return to normal levels after the stress is depleted (Araujo *et al.*, 2002; Agostini *et al.*, 2003). They catalyze the oxidation and polymerization of a wide range of phenolic and anilinic compounds to give detoxification effect of such compounds. In the present study, both of the enzymatic activities increased with the addition of aromatic compounds (Fig. 3). Aromatic compounds degrading ability of *S. polyrrhiza* itself should be owing to the production of peroxidase rather than laccase because the change of peroxidase activity and concentration of each aromatic compound well concurred. The reason why the laccase activity remained relatively high level even after the decrease of the aromatic compounds is unclear.

From the results obtained in the present study, it can be concluded that the feasibility of the use of aquatic plant-bacterial associations to accelerate the degradation of organic chemicals especially recalcitrant compounds in aquatic environment was shown. The questions what kind of bacteria play an important role for the systems, how to construct and operate the system to maximize the ability of the aquatic plant-bacterial associations, and whether any kinds of chemicals can be treated in this system come in the next step.

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