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## Long-term Performance and Community Analysis of *Spirodela Polyrrhiza*–bacteria Association Treating Phenol-contaminated Water

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### ABSTRACT

We investigated the stability of the phenol-removal ability of an association between *Spirodela polyrrhiza* and bacteria. We carried out 21-cycle repeated batch experiments (equivalent to 21 days where each cycle was done for 24 hours) on phenol-contaminated water treatment using *S. polyrrhiza*–pond water bacteria association and pond water bacteria alone. The rate of phenol degradation by the *S. polyrrhiza*–bacteria association was higher than that by the pond water bacteria alone through the 21 cycles. The phenol degradation ability of the *S. polyrrhiza*–bacteria association rapidly increased after exposure to phenol, along with a notable increase in the density of catechol 2,3-dioxygenase (C23O) gene in the bacterial community. *S. polyrrhiza*–bacteria association rapidly gained an enhanced phenol degradation ability compared with the pond water bacteria alone. After phenol acclimation, this enhanced phenol degradation ability of *S. polyrrhiza*–bacteria association was maintained in the long term, with a high density of catechol 1,2-dioxygenase (C12O) gene and C23O gene in the bacterial community. Bacteria harboring a diverse range of C12O gene and C23O gene accumulated on the root of *S. polyrrhiza*, and bacterial diversity was stable under conditions of phenol contamination. The findings in this study demonstrate the possibility of using an aquatic plant treatment system as an effective and stable treatment technology for organic pollutants.

**Keywords:** aquatic plant treatment system, *Spirodela polyrrhiza*, phenol, rhizosphere

### INTRODUCTION

Treatment of secondary effluent from wastewater treatment plants and remediation of polluted aquatic environments using aquatic plants (aquatic plant treatment system; APTS) is a cost-effective and environmentally friendly technology for the conservation of the aquatic environment. Over the last few decades, APTS has mainly been applied to the removal of nitrogen and phosphorus (Tripathi *et al.*, 1991; Greenway, 2003; Li *et al.*, 2009), easily biodegradable organic compounds (Körner *et al.*, 1998; Al-Nozaily *et al.*, 2000), and heavy metals (Keskinan *et al.*, 2004; Miretzky *et al.*, 2004; Mishra and tripothi, 2008).

Recently, we found that the floating aquatic plants *Spirodela polyrrhiza* (giant duckweed) and *Pistia stratiotes* L. (water lettuce) can accelerate the biodegradation of synthetic surfactants (Mori *et al.*, 2005) and aromatic compounds (Toyama *et al.*, 2005; Toyama *et al.*, 2006; Hoang *et al.*, 2009) in the rhizosphere. Another emergent aquatic plant *Phragmites australis* (reed) can accelerate the biodegradation of pyrene (Jouanneau *et al.*, 2005) and bisphenol A (Toyama *et al.*, 2009a) in the rhizosphere

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sediment. These studies indicate that APTS may also be useful for the removal of recalcitrant organic pollutants in the finishing process of secondary effluents and in the remediation of aquatic environments. Our previous studies (Toyama *et al.*, 2006; Toyama *et al.*, 2009b) using *S. polyrrhiza* as a model plant also revealed that the stimulation of bacterial metabolism in the rhizosphere by oxygen and exudates released from the plant's roots is likely to be the main mechanism for the accelerated removal of recalcitrant compounds. In addition, we found that *S. polyrrhiza* can release root exudate containing highly concentrated and diverse phenolic compounds and that this ability results in selective accumulation of aromatic compound-degrading bacteria in its rhizosphere (Toyama *et al.*, 2009b). If this aquatic plant–bacteria association could effectively remove recalcitrant organic pollutants, the APTS strategy would be an ideal technology. Investigating the stability in removing recalcitrant organic compounds by this plant–bacteria association is, therefore, essential.

In this study, we aimed to show the stability of accelerated phenol removal by an *S. polyrrhiza*–bacteria association. We treated phenol-contaminated water by using an *S. polyrrhiza*–pond water bacteria association and pond water bacteria alone through a 21-cycle repeated batch experiments (equivalent to 21 days where 1 cycle was done in 24 hours). We examined the *S. polyrrhiza*-associated bacterial community over the 21-cycle experiments using the quantification of gene copy numbers of 16S rRNA gene, catechol 1,2-dioxygenase (C12O) gene and catechol 2,3-dioxygenase (C23O) gene by the most probable number-polymerase chain reaction (MPN-PCR) and PCR-denaturing gradient gel electrophoresis (DGGE). We determined how the phenol-removal ability of the *S. polyrrhiza*–bacteria association and the *S. polyrrhiza*-associated bacterial community change during long-term treatment of phenol-contaminated water.

## **MATERIALS AND METHODS**

### **Pond water sample**

Natural pond water sample was collected from Inukai Pond of Osaka University. About 1.2 L of the water sample was filtered (Isopore membrane filter, pore size 10.0 µm, Millipore, Tokyo, Japan) and then stored at 4°C prior to usage.

### **Plant material**

Sterile *S. polyrrhiza* were obtained as described previously (Toyama *et al.*, 2006). They were maintained in Inukai Pond water and statically grown in an incubation chamber at  $28 \pm 1^\circ\text{C}$  under fluorescent light at 8,000 lux (16 h:8 h light–dark) for 2 months. The plants were then transferred to 500 mL Erlenmeyer flasks containing 300 mL of sterile modified Hoagland nutrient solution (Toyama *et al.*, 2006) for 7 days before being used in the experiments. The plants were used as *S. polyrrhiza*–pond water bacteria association in this study. In addition, sterile plants were maintained (until required for experiments) in 500 mL Erlenmeyer flasks containing 300 mL of sterile Hoagland nutrient solution.

### **Phenol degradation experiments**

We simulated three test systems in 500 mL Erlenmeyer flasks containing 300 mL of sterile modified Hoagland solution. Test systems A and B contained 30 fronds of *S. polyrrhiza* that had been maintained in Inukai Pond water (*S. polyrrhiza*–bacteria

association). Test system C contained suspended bacteria in 300 mL of Hoagland solution (pond water bacteria alone). The bacteria were obtained by filtering 300 mL of Inukai Pond water through a 0.2  $\mu\text{m}$  pore-sized Isopore membrane filter (Millipore). Phenol was added to test systems A and C at a final concentration of 10 mg/L. The test system B was used as control (without the addition of phenol). All the test systems were prepared in triplicate and statically incubated in an incubation chamber at  $28 \pm 1^\circ\text{C}$  under fluorescent light at 8,000 lux (16 h:8 h light–dark) for 1 day. Subsequently, *S. polyrrhiza* in test systems A and B were repeatedly transferred to fresh media (with/without phenol) every day for 15 days, that is, we repeated 15 cycles of 1-day batch cultures. In addition, suspended bacteria in the 300 mL of Hoagland solution in test system C were repeatedly filtered and then dispersed by moderate sonication (20 kHz, 80w,  $4^\circ\text{C}$ ) for 20 min in fresh medium with phenol everyday for 15 days. We have confirmed that almost all of the bacteria were recovered (more than 80%) by this method in the preliminary experiment. The concentrations of phenol in test systems A and C were periodically monitored and analyzed by high-performance liquid chromatography (HPLC) over the 15 cycles. After the 15th cycle, the addition of phenol to test systems A and C was stopped, and the process of transferring to fresh phenol-free media was repeated for an additional six (total 21) cycles. At the 18th and 21st cycles, 30 fronds of *S. polyrrhiza* from test system A were transferred into a 500 mL Erlenmeyer flask containing 300 mL of Hoagland solution with 10 mg/L phenol. Also, at the 18th and 21st cycles, suspended bacteria were collected from 300 mL of solution of test system C by filtration and then transferred into a 500 mL Erlenmeyer flask containing 300 mL of Hoagland solution with 10 mg/L phenol. The concentration of phenol was periodically monitored and analyzed by HPLC in each cycle. For test systems A and C, the first 15 cycles were the period of acclimation for phenol and the last six cycles were the period of de-acclimation for phenol. To evaluate the contribution of *S. polyrrhiza* to the phenol removal, we also performed sterile control test. The sterile control test system contained 300 mL of sterile Hoagland solution with 10 mg/L phenol and 30 fronds of sterile *S. polyrrhiza*.

#### **DNA extraction from the *S. polyrrhiza*–bacteria association and suspended bacteria in bulk water**

*S. polyrrhiza*-associated bacteria on the root surfaces were collected from three fronds of *S. polyrrhiza* in test systems A and B at the beginning of each cycle, and suspended bacteria were collected from 30 mL of Hoagland solution in test system C at the beginning of each cycle as described in a previous study (Toyama *et al.*, 2006). DNA was extracted from each sample by the proteinase K method (Sei *et al.*, 2000) and purified using a QIAquick PCR purification kit (Qiagen, California, USA).

#### **Quantification of 16S rRNA gene and C12O gene and C23O gene by MPN-PCR**

The copy numbers of 16S rRNA gene, C12O gene and C23O gene in each bacterial community were measured by MPN-PCR as described in a previous study (Sei *et al.*, 2004). Briefly, PCR for 16S rRNA gene was conducted using the universal eubacterial primers EUB-933f and EUB-1387r (Iwamoto *et al.*, 2002). PCRs for C12O gene and C23O gene were conducted using the C12Of/C12Or or C23Of/C23Or primer sets (Sei *et al.*, 1999). The copy number of 16S rRNA gene was used as the index of total bacteria, whereas the copy numbers of C12O gene and C23O gene were used as indices of phenol-degrading bacteria. The copy numbers in total DNA in the test systems were

calculated as follows:

$$\frac{\text{MPN-DNA copies}}{\text{frond}} \times \text{total number of frond of } S. \text{ polyrrhiza} \text{ in test systems A and B}$$

or

$$\frac{\text{MPN-DNA copies}}{1 \text{ mL}} \times 300 \text{ mL water in test system C}$$

### **PCR-DGGE analyses of 16S rRNA gene, C12O gene and C23O gene**

PCR-DGGE analyses of 16S rRNA gene, C12O gene and C23O gene were conducted as described in a previous study (Toyama *et al.*, 2009b). Briefly, for PCR-DGGE analysis of 16S rRNA gene, PCR was conducted using the universal eubacterial primers GC-clamped-EUB-933f and EUB-1387r (Iwamoto *et al.*, 2002). For PCR-DGGE analyses of C12O gene and C23O gene, a two-step PCR was performed. The first-step PCR was conducted with the C12Of/C12Or or C23Of/C23Or primer sets. The second-step PCR was conducted with the GC-clamped-CO12f/C12Or or GC-clamped-C23Of/C23Or primer sets using the first-step PCR products as template. The PCR products were confirmed using agarose gel electrophoresis, and 10 µL of PCR products were loaded onto a 6% (w/v) polyacrylamide gel with a denaturing gradient ranging from 25% to 50% (for 16S rRNA gene) or from 20% to 70% (for C12O gene and C23O gene) in 0.5× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The 100% denaturant consisted of 7 M urea and 40% (v/v) formamide. Electrophoresis was conducted using the D-Code system (Bio-Rad Laboratories, California, USA) at 60°C and 200 V for 5 h in 0.5× TAE buffer. After electrophoresis, the gel was stained with 0.5 µg/mL ethidium bromide solution and analyzed for the PCR-DGGE banding pattern.

The PCR-DGGE banding pattern was scanned using a Scion Image beta 4.02 (Scion Corp., Maryland, USA). Bacterial community similarities were analyzed by principal component analysis (PCA) using NTSYS-pc 2.1 software (Exeter Software, New York, USA). Only the clearly detected bands were selected for analysis. The lower limit for band detection was set by a relative intensity per lane of 0.5% of the total band intensity. The presence or absence of bands in fingerprints of test systems A and B was used for the PCA.

### **Calculation of the phenol degradation rate**

The phenol degradation rate was calculated with the assumption that the phenol degradation reaction follows zero-order kinetics.

## **RESULTS AND DISCUSSION**

### **Removal of phenol from contaminated water by the *S. polyrrhiza*-bacteria association**

Typical phenol degradation profiles of the experiments are shown in Fig. 1, and the calculated phenol degradation rates are shown in Fig. 2. In test system A, 44.3% of

phenol was removed from the Hoagland solution within the first cycle, and complete phenol removal was observed after the second cycle. The phenol degradation rate for test system A showed a rapid increase of about five times from the first (0.183 mg/L/h) to the third (0.964 mg/L/h) cycle. After the 15th cycle, the phenol degradation rate decreased slightly from the 15th (1.16 mg/L/h) to the 21st (0.748 mg/L/h) cycle, but the rate at the 21st cycle was about four times that of the first cycle. In test system C, 12.9% of phenol was removed from the Hoagland solution within the first cycle. The phenol degradation rate for test system C increased about six times from the first (0.0647 mg/L/h) to the fifth (0.412 mg/L/h) cycle. After the 15th cycle, the phenol degradation rate decreased markedly from the 15th (0.486 mg/L/h) to the 18th (0.0800 mg/L/h) cycle, suggesting that the enhanced phenol degradation rate returned rapidly to the initial level.

We compared the accelerated removal of phenol from the Hoagland nutrient solution by the *S. polyrrhiza*–bacteria association (test system A) to the removal by the pond water bacteria alone (test system C) through the 21 cycles. In sterile control experiment with the sterile *S. polyrrhiza*, about 10.2% of the phenol was removed from the Hoagland solution within 1 day. The result indicates that *S. polyrrhiza* alone has the ability to degrade/adsorb phenol but to a lesser extent than that contributed by its association with bacteria. Thus, the accelerated removal of phenol in test system A through the 21 cycles largely resulted from the biodegradation of phenol by *S. polyrrhiza*-associated bacteria.

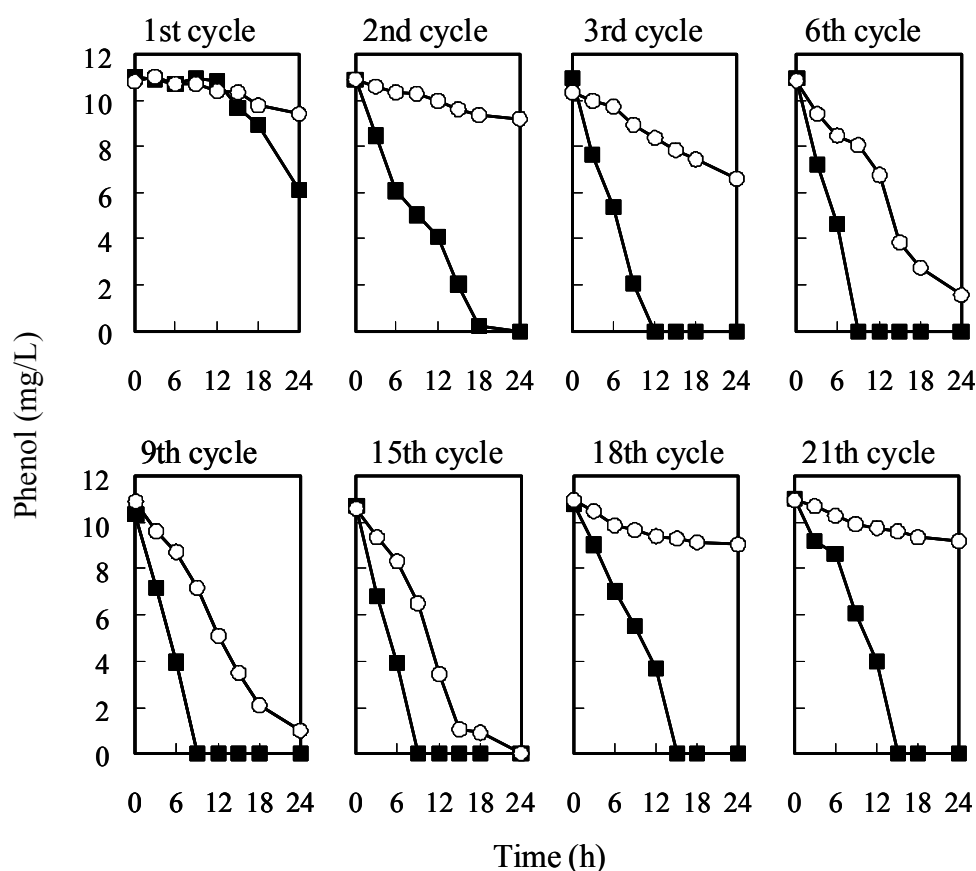


Fig. 1 - Typical phenol degradation profiles during 21-cycle repeated cultures in test system A (closed squares) and C (open circles). The results are indicated as the mean values.

### **Changes in bacterial communities based on 16S rRNA gene, C12O gene and C23O gene during phenol-contaminated water treatment experiment**

Changes in the numbers of 16S rRNA gene, C12O gene and C23O gene in *S. polyrrhiza*-associated bacterial communities in test systems A and B and those in the suspended bacterial community in test system C are shown in Fig. 2.

In test systems A and B, the copy numbers of 16S rRNA gene in the *S. polyrrhiza*-associated bacterial communities remained between  $5.6 \times 10^6$  and  $5.6 \times 10^8$  MPN-DNA copies/flask through the 21 cycles. The copy numbers of C12O gene in test systems A and B increased gradually from  $2.6 \times 10^3$  to  $2.8 \times 10^5$  MPN-DNA copies/flask and from  $2.6 \times 10^3$  to  $6.6 \times 10^4$  MPN-DNA copies/flask, respectively, through the 21 cycles. The copy number of C23O gene in test system A increased from  $2.7 \times 10^2$  to  $4.2 \times 10^5$  MPN-DNA copies/flask in the first three cycles, while this increase did not occur in test system B. In test system A, the copy number of C23O gene drastically increased in parallel with a rapid increase in the phenol degradation rate after phenol exposure. This increase did not occur with 16S rRNA gene and C12O gene. The findings indicate that the *S. polyrrhiza*-bacteria association enhances the phenol degradation ability after phenol exposure, and C23O gene-harboring bacteria likely function actively during this phenol adaptation period. After the 15th cycle of test system A, although the copy number of C23O gene decreased rapidly, the number at the 21st cycle was about four times that of the first cycle. Interestingly, after phenol acclimation in test system A, we found that the number of C12O gene and an elevated phenol degradation rate were maintained for at least six more cycles. Thus, the findings demonstrate that an *S. polyrrhiza*-bacteria association can continue to enhance the phenol degradation rate gained through the acclimation period, and C12O gene and C23O gene-harboring bacteria likely contribute to the stability of this enhanced phenol degradation ability.

In test system C, the copy number of 16S rRNA gene in the suspended bacterial community remained between  $8.2 \times 10^6$  and  $1.5 \times 10^8$  MPN-DNA copies/flask through the 21 cycles. C12O gene and C23O gene were not detected during the first cycles but were detected after the 2nd and 6th cycles, respectively. The copy numbers of C12O gene and C23O gene then increased up to the 15th cycle. The increases in the numbers of C12O gene and C23O gene were observed simultaneously with the increase in the phenol degradation rate. After the 15th cycle, the copy numbers of C12O gene and C23O gene decreased rapidly, as did the phenol degradation rate.

The bacterial communities in test systems A, B, and C were analyzed by PCR-DGGE (Fig. 3). The PCR-DGGE banding patterns of 16S rRNA gene, C12O gene and C23O gene in test system A were notably different from those in test system C. The PCR-DGGE profiles of C12O gene and C23O gene showed a relatively higher number of bands of the *S. polyrrhiza*-associated bacteria in test systems A and B compared to test system C. These results clearly show that *S. polyrrhiza* can accumulate bacteria harboring a diverse range of C12O gene and C23O gene on its root surfaces and the diversity of these bacteria can be maintained in the long term under natural conditions and under conditions of phenol contamination.

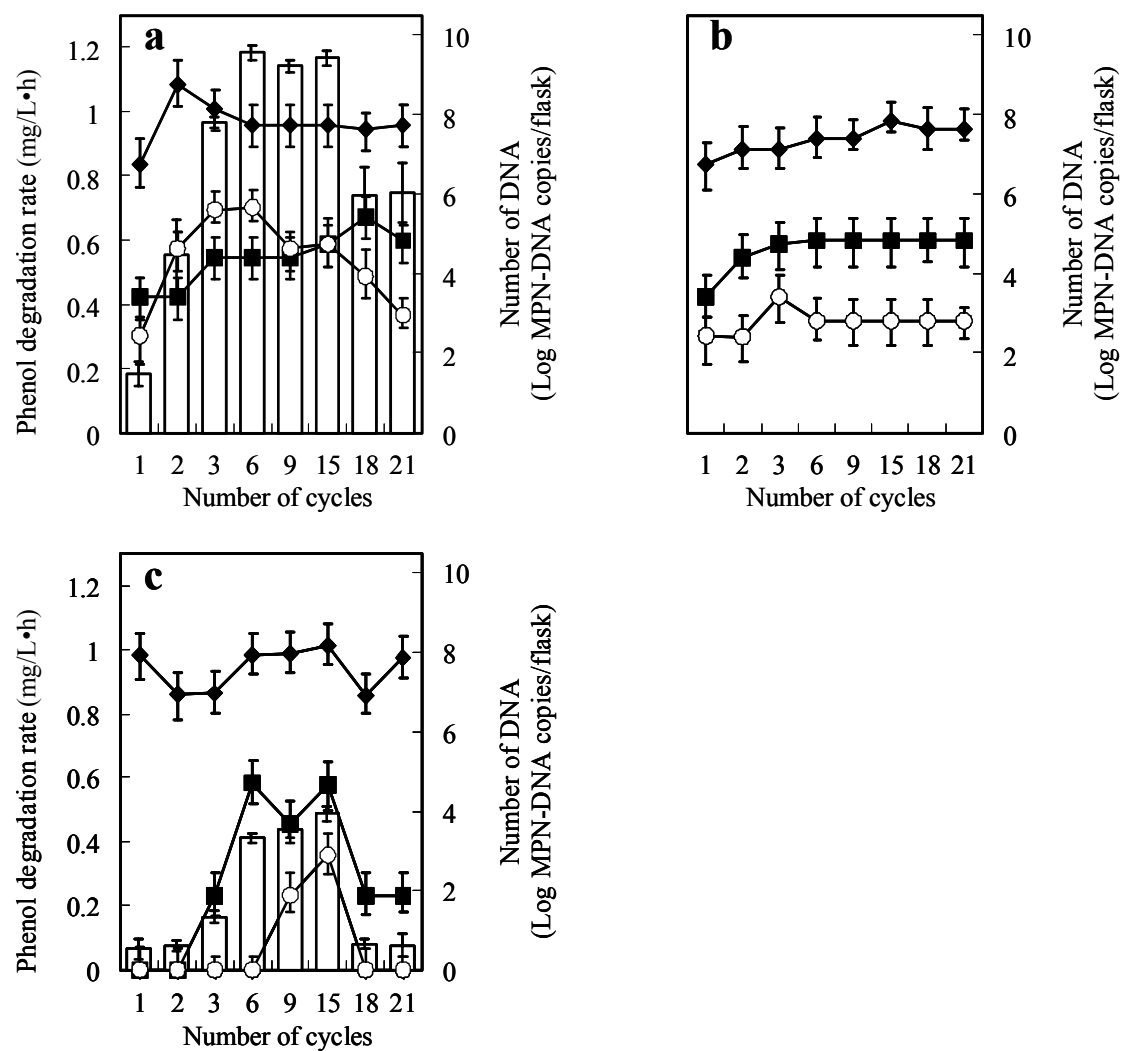


Fig. 2 - Calculated phenol degradation rates (open bars) and the copy numbers of 16S rRNA gene (closed diamonds), C12O gene (closed squares), and C23O gene (open circles) in test system A (a), B (b), and C (c) during 21-cycle repeated cultures. Error bars represent 95% confidence intervals.



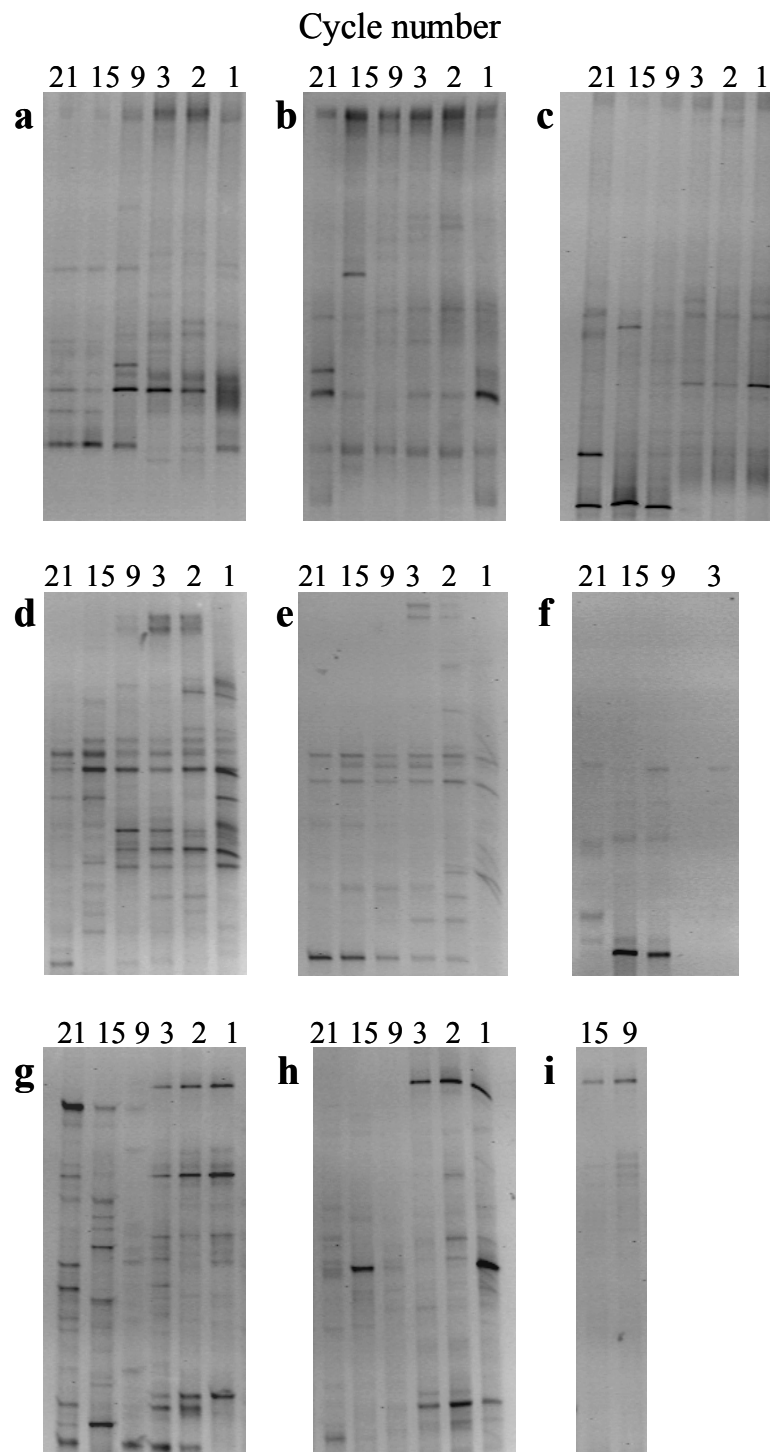


Fig. 3 - PCR-DGGE banding patterns of 16S rRNA gene, C12O gene and C23O gene of *Spirodela polyrrhiza*-associated bacterial communities in test systems A and B and that of suspended bacterial communities in test system C. The labels a, b, and c are PCR-DGGE banding patterns of 16S rRNA gene in test systems A, B, and C, respectively; d, e, and f are PCR-DGGE banding patterns of C12O gene in test systems A, B, and C, respectively; and g, h, and i are PCR-DGGE banding patterns of C23O gene in test systems A, B, and C, respectively. PCR products of C12O gene in test system C at the 1st and 2nd cycles were not detected. PCR products of C23O gene in test system C at the 1st, 2nd, 3rd, and 21st cycles were not detected.

PCA revealed differences in the dynamics of the DGGE banding patterns of 16S rRNA gene, C12O gene and C23O gene between systems A and B (Fig. 4). These differences are attributed to the influence of the addition of phenol. In test system A, in general, the PCR-DGGE banding patterns of each of the three types of DNA during the initial period of adaptation to phenol (2nd and 3rd cycles) formed a group distinct from banding patterns at the end of acclimation (15th cycle) and de-acclimation for phenol (21st cycle). In addition, banding patterns of 16S rRNA gene, C12O gene and C23O gene were relatively stable after the 15th cycle.

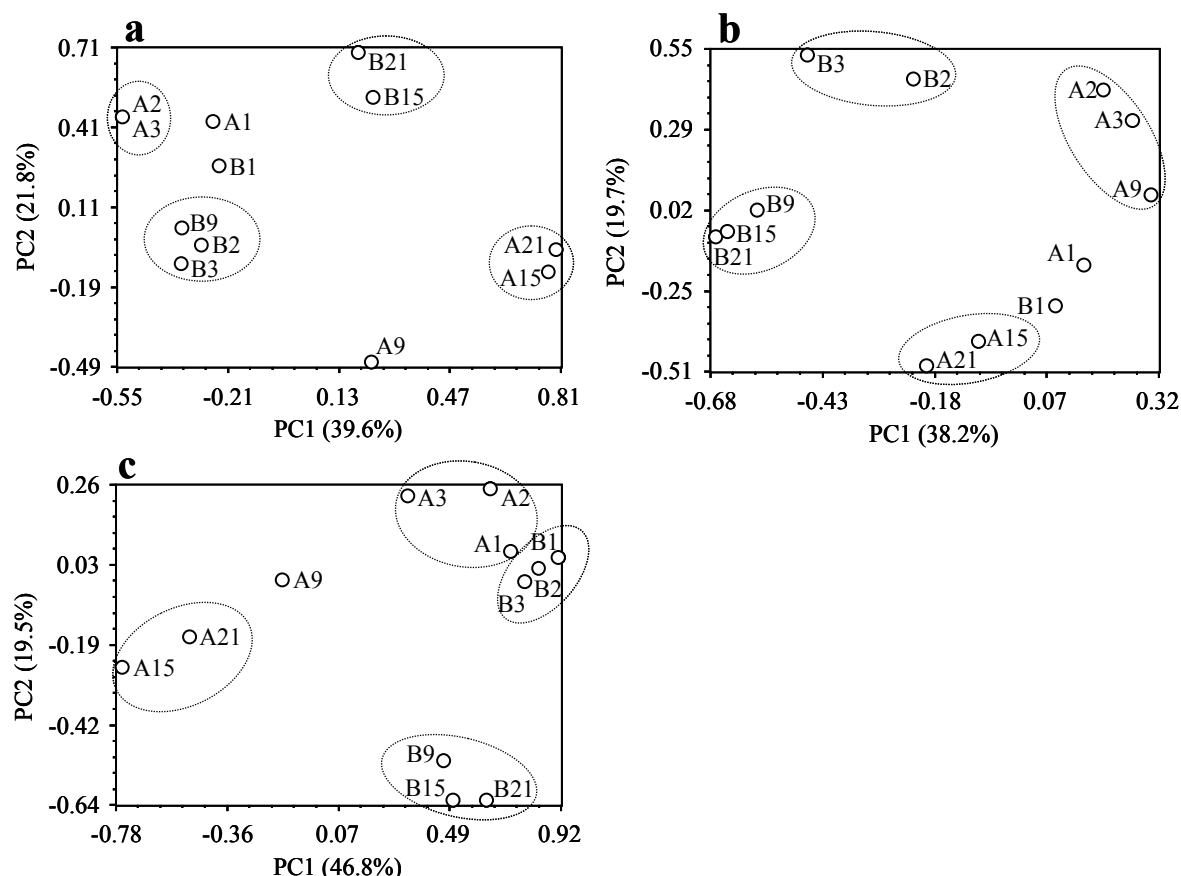


Fig. 4 - Principal component analysis based on the DGGE banding pattern of 16S rRNA gene (a), C12O gene (b) and C23O gene (c) of the *S. polyrrhiza*-associated bacterial communities in test systems A and B. The label A is for test system A and B is for test system B. Numbers represent the cycle number. Only clearly detected bands in PCR-DGGE banding patterns of test systems A and B in Fig. 3 were selected for analysis. The lower limit for band detection was set by a relative intensity per lane of 0.5% of the total band intensity. The presence or absence of bands in fingerprints was used for the PCA.

The phenol degradation rate for system A (*S. polyrrhiza*-pond water bacteria association) was higher than that for test system C (pond water bacteria alone) over the 21 cycles even though the difference between the copy number of 16S rRNA gene in test system A and that in test system C was minimal. Moreover, the *S. polyrrhiza*-bacteria association adapted rapidly to the addition of phenol and rapidly gained an enhanced phenol degradation ability compared with the pond water bacteria

alone. In addition, after the phenol acclimation period the enhanced phenol degradation ability of the *S. polyrrhiza*–bacteria association was maintained longer compared with that of the pond water bacteria alone. The density and behavior of C12O gene and C23O gene in the *S. polyrrhiza*–bacteria association differed substantially from those of the pond water bacteria alone, even though the *S. polyrrhiza*-associated bacteria originated from the same pond water. These characteristics of the *S. polyrrhiza*–bacteria association likely caused the release of phenol-rich root exudates of *S. polyrrhiza* (Toyama *et al.*, 2009b). We assume that the phenol compounds released by *S. polyrrhiza* accumulate C12O gene and C23O gene-harboring bacteria in the rhizosphere and stimulate their growth and metabolites. With the support of the phenol-rich root exudates of *S. polyrrhiza*, *S. polyrrhiza*-associated bacteria likely contribute to the enhanced phenol removal in the long term.

## CONCLUSIONS

We carried out experiments on phenol-contaminated water treatment over the long term by using an *S. polyrrhiza*–pond water bacteria association and pond water bacteria alone through a 21 cycle repeated batch cultures (equivalent to 21 days where 1 cycle was done in 24 hours). We investigated the changes in phenol removal performance and bacterial communities based on 16S rRNA gene, C12O gene and C23O gene of the *S. polyrrhiza*–bacteria association and pond water bacteria. Our conclusions are as follows:

- (1) Accelerated removal of phenol by an *S. polyrrhiza*–bacteria association in pond water was observed in comparison to the removal of phenol by the pond water bacteria alone.
- (2) The phenol degradation ability of *S. polyrrhiza*–bacteria association was rapidly enhanced by exposure to phenol compared with the pond water bacteria alone. The enhanced phenol degradation rate of *S. polyrrhiza*–bacteria association was maintained longer compared with the pond water bacteria alone.
- (3) Bacteria that harbor a diverse range of genes encoding C12O and C23O accumulated on the roots of the *S. polyrrhiza*. With the support of the phenol-rich root exudates of *S. polyrrhiza*, these *S. polyrrhiza*-associated bacteria probably contribute to the rapid enhancement and stability of phenol removal.

Our findings clearly indicate that the ability to degrade phenol in the APTS can be enhanced by exposure to phenol, and the enhanced ability will be stable in the long term. In this study we have therefore demonstrated the possibility of making the APTS an effective and stable treatment technology for organic pollutants.

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