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Surfactants Degrading Activities in the Rhizosphere of Giant Duckweed (*Spirodela polyrrhiza*)

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Abstract

Surfactants removal capabilities and mechanisms around the rhizosphere of giant duckweed were investigated. From the roots of the weeds cultivated with various environmental water, almost 10^{10} CFU/g-roots d.w. of microorganisms were gained and the microbial flora were different from that of water samples. The continuous batch cultivation test showed that the acclimation of the rhizo-communities enhanced (or improved) the rhizodegradation and the symbiotic systems removed the artificial surfactants (LAS, SDS, AE, NPE) immediately. The bacterial degradation activities around the rhizosphere were accelerated by the oxygen release from the plant roots. These results indicate that the plant-microbe systems play an important role in the biodegradation, bioconversion and removal of chemical compounds in the water environment.

Key words: rhizosphere, duckweed, surfactant, degradation

INTRODUCTION

In Japan, the sewer systems cover 60 and over percent people. However, various kinds of chemical compounds used in the fields directly or contained in the nontreated wastewater are flowing into the water environments. These compounds may be degraded by various kinds of degrading microbe of the ecosystems in the water environments. But the behavior of these chemical compounds and the participation of various micropopulation in the biomembrane, rhizosphere, wetland soil, bottom soil or environmental water against the degradation of these chemicals are not clear. The ecological impacts of some xenobiotics and toxic compounds flown into the natural water systems are also under the apprehension and investigating¹⁻⁶⁾. And useful environmental

cleaning techniques applicable to environmental water available for decreasing the total impact derived from such chemical compounds including toxic or xenobiotic compounds are under construction. We paid attention to the activities of rhizosphere of water plants. The water purification activities of over 60 kinds of water plants have been studied from 1960's⁷⁾. Efficiencies of water plants to remove BOD and/or inorganic nutrients like nitrogen and phosphorus from domestic wastewater and eutrophied water environments of rivers, ponds and lakes have been confirmed⁸⁻¹³⁾. Even in Japan, 20 water treatment systems, at least, have been constructed before 1998⁷⁾. The water purification activities of these systems are due to the function of plants and the microorganisms in the rhizosphere¹³⁾. The typical functions of the plants are

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summarized to the absorption or adsorption and accumulation. Those of rhizo-micro-organisms are also summarized to the biodegradation or bioconversion. Combination of various kinds of plants and cultivation conditions produced several types of ecological treatment systems for the domestic wastewater or eutrophied environmental water using higher plants combined with wetland or hydroponic systems coming into practical use. But the efficiencies of water plants systems against such chemicals above-mentioned including xenobiotics are not studied well. And biodegradation activities of bacteria in the rhizosphere of water plants and the responses of rhizo-bacterial populations against such chemical loading are not clear. In case of cleaning of polluted soil, efficiencies of phytoremediation techniques against not only inorganic matters of heavy metals and nutrients but also xenobiotic organic compounds including hydrocarbons of aromatics and aliphatic compounds in the petroleum or agricultural chemicals have been revealed¹⁴⁻¹⁸. Detailed information about the capabilities and mechanisms of removal of xenobiotic and toxic compounds by water plants-microbe systems would be useful to develop the ecological water cleaning systems in the near future. In this study we elucidate the surfactants degrading activities of rhizosphere microorganisms and the plant-microbe symbiotic systems of giant duckweed as a model floating water plants.

MATERIALS AND METHODS

Plant culture medium Hutner solution¹⁹ consists of (mg/l) K_2HPO_4 (400), EDTA (500), KOH (200), NH_4NO_3 (200), $Ca(NO_3)_2 \cdot 4H_2O$ (354), $MgSO_4 \cdot 7H_2O$ (500), $MnCl_2 \cdot 4H_2O$ (17.9), $ZnSO_4 \cdot 7H_2O$ (65.9), $CuSO_4 \cdot 5H_2O$ (3.95), H_3BO_3 (14.2), $Na_2MoO_4 \cdot 2H_2O$ (25.2), $Co(NO_3)_2 \cdot 6H_2O$ (0.2), and $FeSO_4 \cdot 7H_2O$ (24.9) pH 7.0. Ten times dilution of this solution (1/10 Hutner solution) was used as plant culture medium provided that only EDTA was diluted by 1/100 of Hutner solution.

Bacterial culture medium Ten times dilution of LB broth²⁰ consisting of (mg/l) bacto peptone (10), yeast extract (5), NaCl (10)

pH7.0 were used as bacterial culture media. Basal medium supplemented with agar (1.7%) were used for bacterial colony forming.

Plants materials Giant duckweeds (*Spirodela polyrrhiza*) were recovered from a pond in the campus of University of Yamanashi. This plant material were cultured in a vessel containing 1 l of 1/10 Hutner solution kept in a greenhouse at 25 °C. Sufficient light exceeding 7000 lx were supplied over 16-h photoperiod. The biomass was subcultured every 7 days. Aseptic plant materials were also used in the study. Winter buds (turion) of this plant were surface-sterilized with 70% ethanol for 5min, then in 0.5% NaOCl for 15min and rinsed with sterilized water 5 times. The aseptic buds were inoculated to the culture solution and the seedlings were used as aseptic plant materials. Any bacterial colony was not formed from the whole plant extracted solution spread on the colony forming medium. Water lettuce (*Pistia stratiotes*) used in some experiments were purchased from Tojakuengei co. (Kyoto, Japan) and cultivated in the greenhouse above mentioned.

Monitoring CFU number in the rhizosphere Colony forming units per gram bulk plant roots were determined. Roots samples were cut off from 5 plants with knife in a petri dish and washed with 9 ml of sterilized washing solution (5 mg/l of tri-poly-phosphate) in a test tube on the test tube mixer for 3min. The washed root samples were homogenized with 9ml of washing solution under the condition of 15000rpm for 5min. The washing solution after first washing (bacterial samples weakly attaching to the roots) and homogenized extraction (bacterial samples tightly attaching to the roots) were serially diluted (10-fold), and 0.1 ml aliquots were spread on colony forming media in triplicate respectively. Colony forming units of culturing solutions were also measured. Bacterial solution prepared from the roots were also used for the PCR-DGGE analysis.

Surfactant removal, degradation and absorption/adsorption tests

Surfactant removal tests were performed in plant-microbe system, which contain 1000 ml of the plant culture medium supplemented with surfactant (the final concentration of 5 mg-TOC/l) and 100 plants of duckweed and the remaining TOC derived from the surfactants were measured periodically. Continuous surfactant removal tests were also performed under the same condition. After cultivation for 5 days, a few duckweeds were withdrawn as excess plant to keep 100 plants of duckweed in the testing system, and then the total of medium was discharged as the effluent. Then, 1000 ml of new surfactant medium was added to the test system as influent. Surfactant degradation tests were performed using micorbial samples according to the method to evaluate the biodegradability of chemicals in the environmental water⁹. The microbial samples (the final concentration of $4.2\text{--}6.2 \times 10^5$ CFU/ml) were inoculated to 500 ml Erlenmeyer flask containing 200 ml of the inorganic artificial river water ((mg/l) ; K_2HPO_4 (21.8), KH_2PO_4 (8.5), $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (44.6), NH_4Cl (1.7), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (22.5), CaCl_2 (27.5) and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.25)) supplemented with each surfactants (final concentration of 15 mg-TOC/l) and TOC concentrations were measured periodically. Surfactants absorption/adsorption by plants were also tested using aseptic giant duckweed which contain 500 ml of the sterile basal culture medium and 50 plants of aseptic duckweed.

Detection of oxygen transport by plants

Just 90 plants of aseptic plant materials were cultivated in a vessel with the 900 ml of deoxygenated culture medium (the final DO concentration of 1.4–1.8 mg- O_2 /l) and the dissolved oxygen concentrations were measured periodically.

Analytical methods

Total organic carbon (TOC), methylene blue active substances (MBAS), cobaltothiocyanate active substances (CTAS), and metabolites of nonylphenol polyethoxylates (NPE) were analyzed in the several degradation tests. TOC concentrations of various water samples

were measured by total organic carbon analyzer (TOC-5000A, Shimazu co., Kyoto). MBAS and CTAS were analyzed according to the tap water analytical methods²¹. Concentrations of the metabolites of NPE including NPnEOs (n; number of ethoxy units (EOs)), NPnEC (n; 1–3, EO plus a teminal acetate), and nonylphenol were determined by AP ELISA KIT (Takeda Pharmaceutical company limited, Osaka). Dry weight, dissolved oxygen concentration were analyzed according to the Sewage Wastewater Examination Methods²².

DNA extraction and PCR-DGGE

Total DNA samples were extracted from the bacterial solution prepared from plant roots by DNA extraction method for water environmental samples²³ modified from proteinase K method²⁴. DNA fragments encoding the 16S rRNA gene were amplified by PCR. Touch down PCR²⁵ amplification were performed for 30 cycles using Program Temp Control System PC-800 (ASTEC Fukuoka) with the primer sets of EUB-933f-GC-clamp and EUB-1387r²⁶ under the following conditions : 20 cycles at 94 °C for 1min, 65–55 °C ($\Delta -0.5^\circ\text{C}/\text{cycle}$) for 1min, 72 °C for 3min; 9 cycles at 94 °C for 1min, 55 °C for 1min, 72 °C for 3min; and a final cycle at 94 °C for 1min, 55 °C for 1min, 72 °C for 10min. DGGE were performed using D-code system (Bio-Rad Lab. Inc., USA). The PCR products (10 μl) were electrophoresed in 6.0% (w/v) polyacrylamide (37.5:1 acrylamide/bisacrylamide) in 0.5xTAE buffer. The denaturing gradient ranged from 25–45% (100% denaturant agent was defined as 7 M urea and 40% formamide). Electrophoresis was performed for 5h at 200V, at 60 °C. Gels were stained with ethidium bromide and visualized on an UV transilluminator. The photographing and detection of DNA bands were performed with a DegiDoc-It System (UVQ Inc., USA).

RESULTS**Colony forming units detected on the roots**

The microorganisms were extracted from the roots of giant duckweed cultivated with various environmental water or Hutner nutrient solution. The numbers of colony

forming units (CFU) of each sample were shown in Table 1. Almost same amount of viable counts were detected on the roots of giant duckweeds and water lettuce cultivated with each water samples. About 80-90% of the total viable cells on the roots detected by colony forming techniques were easily recovered from the root surface by agitation in the water and another 10-20% adhered to the root organization tightly recovering only by smashing the root tissue. In case of duckweed cultivated with pond water (1.8×10^5 CFU/ml), bacterial counts of 1.2×10^{10} CFU/g dry root were detected in the root and 90% of them were attached on the roots weakly and another 10% were tightly linked to the tissue. We selected the plants-microbe system cultivating with Hutner solution as a model system for the following degradation tests because the number of the bacteria detected on the roots in this condition were almost same as another cultivating system using various kinds of environmental water.

PCR-DGGE analysis of the microbial communities The microbial diversity of the microflora on the roots was compared with that in environmental water samples using PCR-DGGE (Figure 1). Aseptic weeds were planted into the river, pond and secondary treated water and the microbial diversity on the roots were analyzed periodically. The PCR-DGGE analysis of initial water samples detected 8 fragments in the river water, 13 fragments in the lake water, 16 fragments in the secondary treated water. The DNA fragments of the microflora

weakly attaching to the roots constructed during the 10 days cultivation were different from the fragments detected in the water samples used for the microbial inoculation. Same results were obtained from the tests for the microflora tightly attaching to the roots. Almost 70-90% of the fragments detected on the roots (weakly attaching to the roots: river water 12/14; pond water 12/13; secondary treated water 8/12, tightly attaching to the roots: river water 9/12; pond

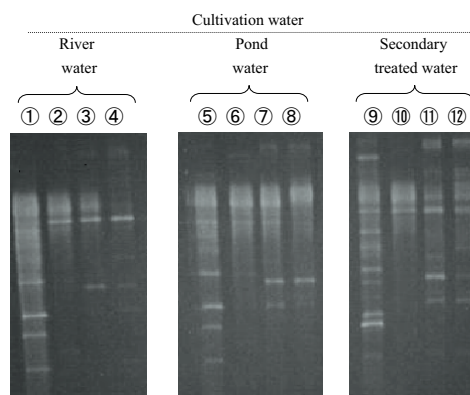


Fig. 1 Variation of the microbial community analyzed by 16SrDNA fragment patterns.

The microbial community on the plants roots (weakly attaching on the roots) were analyzed by DGGE fragments pattern analysis of PCR amplified 16SrDNA fragment periodically. Aseptically precultured duckweed were inoculated to each environmental water sample and fragment patterns of the inoculum (①,⑤, ⑨) and the roots microbial communities were analyzed 1day (②,⑥,⑩), 5days(③,⑦,⑪), 10days (④,⑧,⑫) after inoculation.

Table 1 Colony forming units detected on the roots.

	attaching to the roots(CFU/g-root d.w.)	
	weakly	tightly
Giant duckweed (lake water)	4.1×10^{10}	9.2×10^9
Giant duckweed (pond water)	1.1×10^{10}	1.4×10^9
Giant duckweed (river water)	3.9×10^{10}	7.8×10^9
Giant duckweed (secondary treated water)	3.4×10^{10}	5.2×10^9
Giant duckweed (Hutner solution)	3.4×10^{10}	2.8×10^{10}
Water lettuce (pond water)	6.3×10^9	1.8×10^9

water 10/11; secondary treated water;11/13) after 10 days cultivation were not detected in the initial water samples.

Surfactants removal in the rhizosphere of active growing giant duckweed Surfactants removal capability around the rhizosphere of giant duckweed was investigated. Four kinds of surfactants, liner alkylbenzene sulfonate (LAS), alcohol ethoxylate (AE), sodium dodecyl sulfate (SDS) and nonylphenol polyethoxylate (NPE) were added to the plant-microbe system. As shown in Figure 2, Greater parts of TOC derived from LAS, NPE or AE in the testing vessel were maintained through the cultivation period in case of plants-microbe systems pre-cultivated with normal basal medium without any surfactants. The anionic or nonionic surfactants detected by MBAS or CTAS assay were also remained after this removal tests. But after pre-cultivation with each surfactant, the acclimated system removed the each compounds from the solution rapidly and high percentage of TOC (90-100%) and surfactants (100%) removal

were achieved in testing period of five days. In the other hands, SDS was easily removed from the solution even in case of non-acclimated plants system.

Surfactants degradation capabilities of microorganisms recovered from the roots

The surfactant degradation capabilities of the microbial consortia recovered from roots were tested. Figure 3 shows the microbial degradation capabilities of each microbial consortia. As shown in the Figure 3, each compounds were degraded completely by microorganisms extracted from the roots of acclimated systems with the removal efficiency of over 90% although lower removal efficiency of 50% were shown in case of microorganisms prepared from non-acclimated plants in the testing period of 5 days.

Surfactants absorption/adsorption by aseptic plants

It is estimated that surfactants removal in the rhizosphere was due to the microbial degradation and plant uptake. The degradation activities of

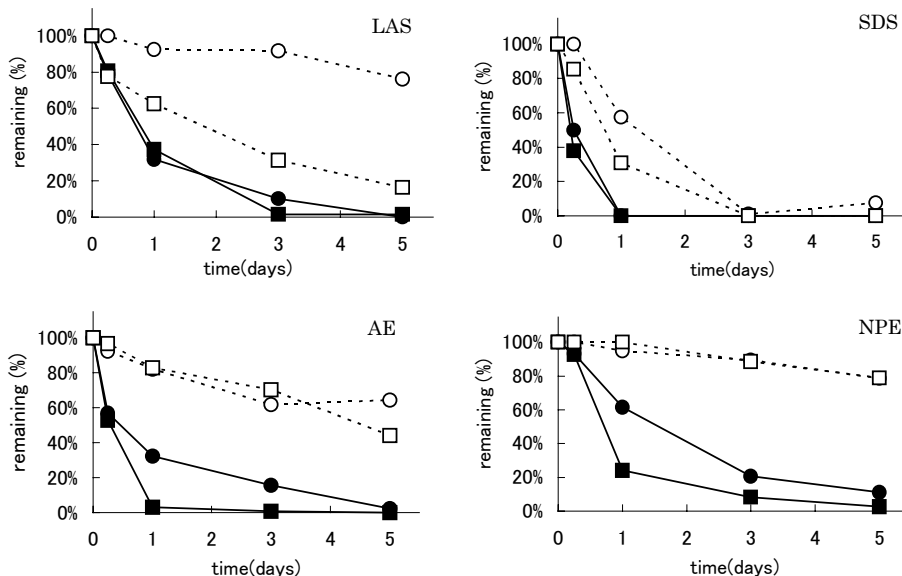


Fig. 2 Surfactant removal around the rhizosphere of giant duckweed. Plants-microbe systems were cultivated with the Hutner solution supplemented with LAS, AE, SDS, NPE. Remaining TOC (●, ○) and MBAS (LAS and SDS) or CTAS (AE and NPE) (■, □) were measured periodically. The closed symbols indicate the data of acclimated systems and open symbols indicate those of normal system.

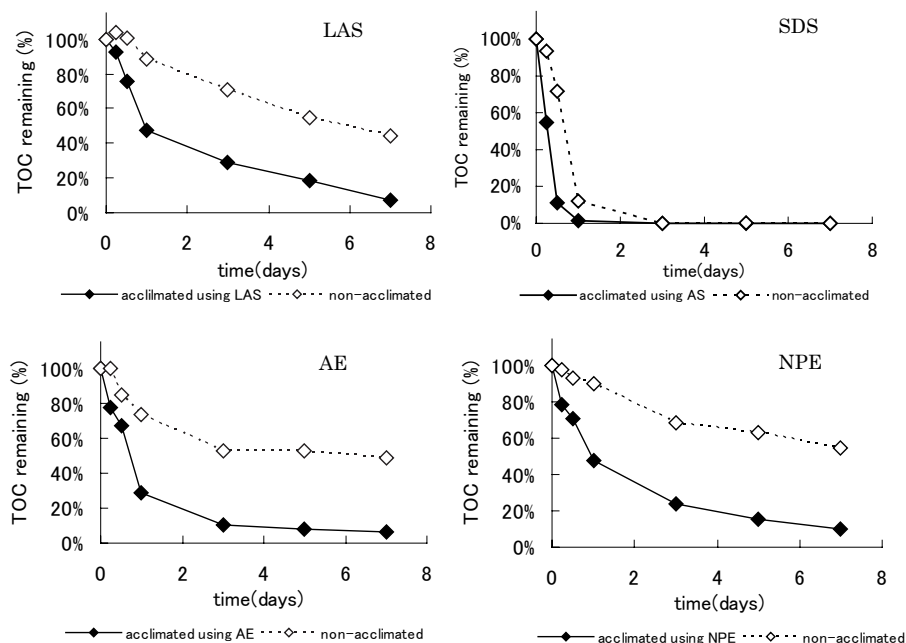


Fig. 3 Complete degradation of surfactants by microorganisms recovered from the roots of giant duckweed.

microorganisms were evaluated as shown in the Figure 3. Subsequently, the surfactants absorption or adsorption by plants were also tested using bacteria-free aseptic giant duckweed planting system. In the absorption/adsorption tests, LAS, SDS, NPE and AE were used as the initial compounds (the final concentration of 5 mg-TOC/l) and putative intermediary metabolites of NPE (NP5EO) were also tested. Figure 4 shows the results of absorption tests. The concentrations of TOC derived from LAS, SDS, NP10EO, NP5EO and AE did not decreased in the five days cultivation tests and the concentrations of non-ionic surfactants derived from NPE and AE showed the constant value. However, 11-25% of anionic surfactants concentrations were decreased in spite of the trends of constant value of TOC concentrations. These quantities of removed compounds were no match for the removed value obtained in the surfactants removal tests using plant-microbe systems shown in Figure 2. In case of absorption/adsorption tests using aseptic plants precultivated with each mother compounds, remaining TOC concentration

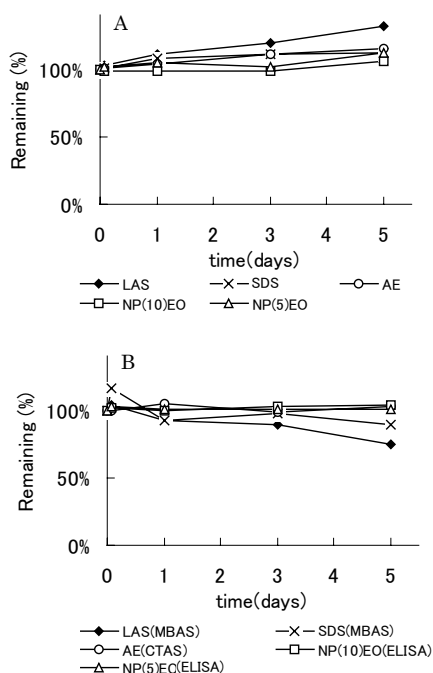


Fig. 4 Surfactant removal by the aseptic giant duckweed.

A: Remaining surfactants measured by TOC
 B: Remaining surfactants measured by MBAS, CTAS or ELISA methods

were also maintained constant value (data not shown). These results indicate that plant systems have a little effects on the removal of each compounds used in this study.

Stability of the removal efficiency We also examined stability of the removal efficiency and the number of the microorganisms on the roots. The continuous batch cultivation tests showed that the degrading activities around the rhizosphere were improved through acclimation process for 15 days (Figure 5). And after acclimated sufficiently, the symbiotic systems attack the substrates and the surfactant concentrations measured by TOC and CTAS or MBAS could be decreased immediately. The improved biodegradation activities were maintained during the testing period with three times water exchange. The profiles of viable cell counts through the continuous batch cultivation were summarized in Figure 6. The numbers of bacteria attached on the roots and suspended in the water were maintained stably. In our testing condition whole of the cultivating solution were exchanged to fresh one periodically but one day after changing the solution CFU number were completely recovered.

Oxygen transport from roots to the rhizosphere

Oxygen supply is one of the major rate limiting factors in the BOD or COD removal in the water. The oxygen released to the rhizosphere from plants play an important role in the purification around rhizosphere^{13, 27, 28}. So, the transporting activities of giant duckweed were examined under lightening and dark conditions using aseptic plants. The DO concentrations increased immediately while an insignificant DO upward tendency were observed under the dark condition. The oxygen transportation rate under the lightening condition of $385 \text{ mg-O}_2/\text{h/g-root d.w.}$ ($176 \text{ mg-O}_2/\text{h/m}^2$) were observed after correcting the oxygen transport from the water surface. Effect of the oxygen supply from the plants roots on the rhizodegradation was investigated using SDS as the model compound, which was easily biodegraded in the previous tests. SDS removal tests were carried out under the same lightening conditions consecutively. The TOC removal and the oxygen consumption rate obtained from the tests were summarized on Table 2. Under the lightening condition the TOC removal rate were double as high as that of dark condition. And the oxygen consumption rate of the lightening

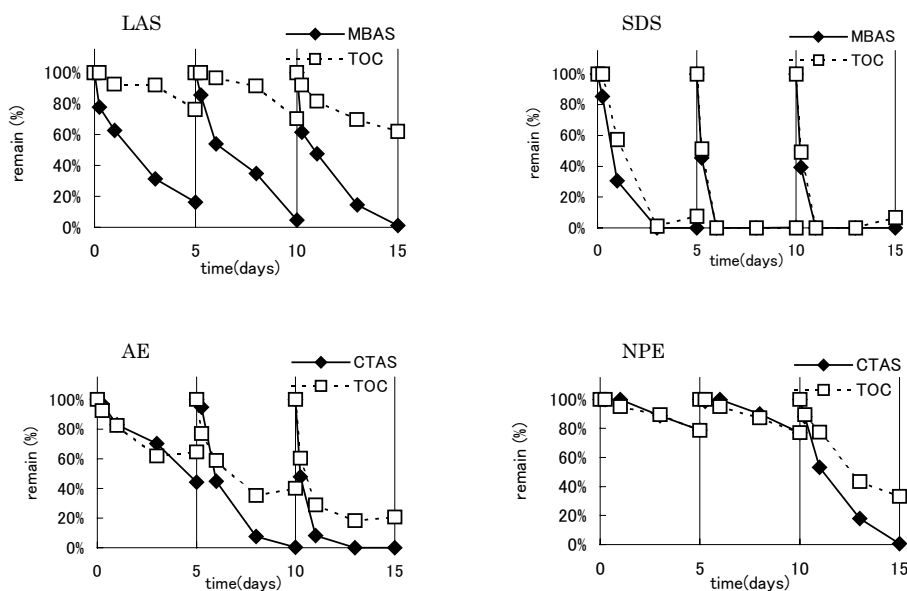


Fig. 5 Surfactants removal around the non-acclimated rhizosphere under the condition of continuous batch cultivation.

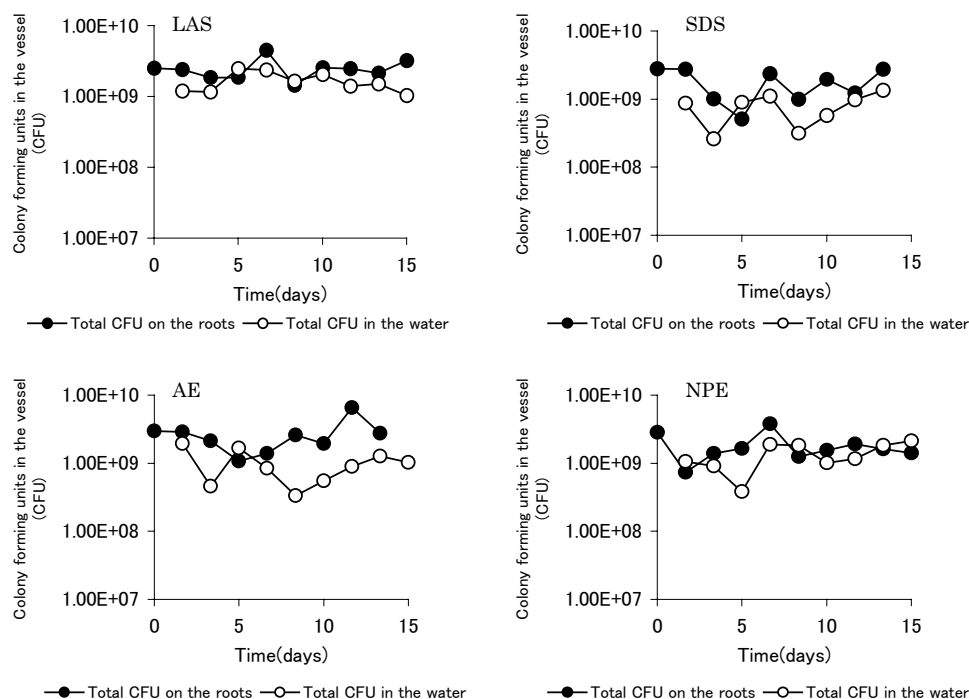


Fig. 6 Colony forming units in the rhizosphere during continuous batch cultivation of giant duckweed.

Table 2 Effect of the oxygen supply from the plants root on the rhizodegradation of SDS.

	TOC removal rate (mg-TOC/l/h)	DO consumption rate (mg-O ₂ /l/h)
Lightening condition	0.406	0.595
Dark condition	0.204	0.203

conditions was three times as high as that of dark condition. These results suggest that oxygen supply from plant roots to the rhizosphere accelerate the rhizodegradation evidently and they would be controlled by the intensity of illumination.

DISCUSSION

Biodegradation activities around the rhizosphere of floating weeds were investigated. Four kinds of artificial surfactants were used as the model compounds. Non-acclimated plant-microbe system showed weak degradation activities against each compounds. But after acclimation for at least 2 weeks with each compounds, efficient substrate removal caused by biodegrading

activities of microorganisms not by root absorption nor adsorption were observed (Figure 2, Figure 3 and Figure 4). The surfactant removal rate after acclimation were summarized on Table 3. In this one-batch cultivation condition, microorganism introduced into the testing vessels were originated from only roots surface. So, the degradation activities also derived from the microorganisms on the root surface and suspended in the water detached from the roots in the vessel. Actually over 50% of the detected total CFU in the testing vessel (plants roots and cultivating solution) were recovered from the root organization. The density of total CFU on the roots obtained in these testing vessel were equivalent to be 1.6

Table 3. Surfactant removal activities of giant duckweed system.

	removal rate constant* (h ⁻¹)		average removal rate** (mg//day)	
	TOC	Surfactant	TOC	Surfactant
LAS non-acclimated	5.04×10 ⁻²	3.49×10 ⁻¹	2.58×10 ⁻¹	1.34
LAS acclimated	6.69×10 ⁻¹	1.07	1.03	2.69
SDS non-acclimated	6.32×10 ⁻¹	1.22	1.77	6.55
SDS acclimated	2.86	3.88	5.33	1.82×10
AE non-acclimated	9.18×10 ⁻²	1.54×10 ⁻¹	6.88×10 ⁻¹	1.33
AE acclimated	6.71×10 ⁻¹	3.52	1.52	1.15×10
NPE acclimated	5.58×10 ⁻²	5.32×10 ⁻²	230×10 ⁻¹	3.68×10 ⁻¹
NPE acclimated	4.48×10 ⁻¹	9.20×10 ⁻¹	934×10 ⁻¹	2.71

* Removal rate constant were calculated on the supposition that surfactant degradation in the testing system used in this study obey the following equation.

$\ln C = kt + \ln C_0$ where C is the remaining TOC or surfactant concentration, and k is the removal rate constant, and t is the time elapsed, and C_0 is the initial TOC or surfactant concentration.

**Average removal rate were calculated using the first and final concentrations.

$\times 10^{10}$ CFU on the roots of duckweeds cultivated in 1m² of the cultivation area. These results indicated that nearly 10% of the bacteria in the plant-microbe system are attaching on the roots with high cell density and the others are suspended in the water in case of the cultivation of duckweed using pond water (1.8×10^{11} CFU/m³) with the depth of water of 1m. Under the condition in which cultivation water are exchanged continuously or periodically, root microflora is an important resource of the degraders even in the water phase. Actually, the continuous batch test showed the stable surfactant removal activities as shown in Figure 5. The PCR-DGGE analysis pointed out the unique bacterial community on the root surface. It means that microbial florae attaching on the roots are constructed by minor populations in the environmental water. The diversity of the microbial florae expressed by Shannon-Weaver index⁽²⁹⁾ calculated using DNA band intensity as individual importance were maintained the value of 3-4. The roots microbial populations are rich in diversity as in case of environmental water although the constructions of the microbial populations are

different from each other. The high potential of biodegradability of this unique root microflora would be a important factor to improve the water purification activities of the systems using water plants.

These microbial activities around the rhizosphere would be limited by the environmental conditions. Dissolved oxygen is a major rate limited factor of microbial activities in the water environment. Around the rhizosphere, oxygen concentrations are highly affected by plants oxygen transport and release. We investigated the effects of oxygen transport on the rhizodegradation. As shown in the Table 2, lightening operation promoted TOC removal rate and DO consumption rate in the vessel supplemented with SDS. In the four surfactants, LAS and NPE were harder to degrade than another two compounds. The degradation rates of acclimated plant system were also lower as shown in the Figure 2 and Table 3. We confirmed the effect of the lightening operation on the degradation of such compounds (Figure 7). While significant effect by lightening on removal of the surfactants detected as MBAS or CTAS was not observed, TOC removal i.e. complete

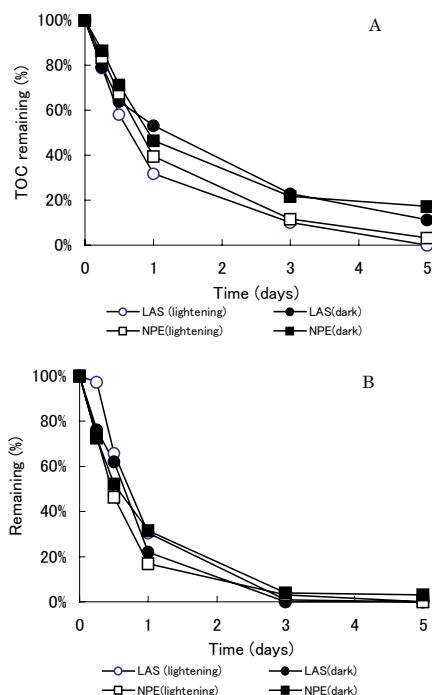


Fig. 7 Effect of the lightening condition on the surfactants removal in the rhizosphere.

A: TOC removal in the system

B: MBAS (LAS) and CTAS (NPE) removal in the system

degradation were accelerated by this operation. These results pointed out the possibilities of controlling the rhizodegradation activities against several xenobiotic compounds indirectly by optimizing the cultivating conditions like photo conditions besides optimizing the degrading microflora.

CONCLUSION

The results obtained in this study clarified the high degradation activities against surfactants around the rhizosphere of giant duckweed and also indicate that microbial flora on the roots play important roles in the fate of chemical compounds. It means that biostimulation and bioaugmentation techniques would be applicable to enhance the biodegradation or bioconversion and to improve the cleaning potential for the pollutants around the rhizosphere. In this study we paid attention to two controlling factors of DO concentration and microbial

community around rhizosphere. Acclimation of the microbial communities and controlling the cultivating condition were effective to accelerate rhizodegradation.

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