Nutrient, Linear Alkyl Benzene Sulfonate, and Caffeine Removal from Synthetic Wastewater with an Algal–bacterial Culture and an Activated Sludge Culture in Batch Mode

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Abstract
An algal–bacterial culture and an activated sludge culture were cultivated for simulating wastewater treatment in an algal pond and an aerated pond, respectively. Linear alkyl benzene sulfonates (LAS) and caffeine were selected as model pollutants. In 7-day batch treatment of synthetic wastewater (dissolved organic carbon (DOC) 106 mg/L, dissolved nitrogen (DN) 57 mg/L, dissolved phosphorous (DP) 6.7 mg/L, LAS 16 mg/L, caffeine 0.03 mg/L), the degradation kinetics for LAS and sulfophenyl carboxylic acids as the intermediates, and caffeine suggested that the bacterial species and population in the activated sludge culture and the algal–bacterial culture were quite different from each other. Finally, the algal–bacterial culture showed balanced removals for the pollutants (DOC 82%, DN 32%, DP 72%, LAS 100%, caffeine 50%). The activated sludge culture showed rapid degradation of LAS and caffeine but it only insufficiently removed nutrients (DOC 73%, DN –20%, DP 23%, LAS 100%, caffeine 63%). These results suggest that the algal pond is a promising technology for simple and low-cost wastewater treatment in warm countries.

Keywords: algae, bacteria, caffeine, linear alkyl benzene sulfonate, wastewater treatment

INTRODUCTION
In some countries in warm climates, simple and low-cost pond (lagoon) systems such as aerated ponds and algal ponds are used for wastewater treatment. The aerated pond is aerated mechanically to remove biochemical oxygen demand (BOD) from wastewater by suspended sludge with a residence time of several days. The algal pond is shallow to support algal growth for the nutrient removal with a residence time of more than 10 days. Aside from the high numbers of bacteria in the algal pond, photosynthetic microorganisms such as green algae, diatoms, cyanobacteria uptake nutrients into their biomass. A balanced purification process in the algal pond is achieved by combining the ability of algae to assimilate nutrients with the ability of bacteria to degrade organic matter.

Recently, the presence of various chemicals such as detergents and pharmaceuticals in wastewater presents an important concern. Only a few studies examining the removal of specific chemicals in the pond systems have been reported. Because even the activated
sludge process in a modern wastewater treatment plant is not designed to remove contaminants of this type, many of these compounds and their biodegradation intermediates are discharged to natural water bodies, where they can exert ecotoxicological effects, even at low concentrations. The objective of this study was evaluation of macro-pollutant and micro-pollutant removal in an algal–bacterial culture in laboratory-scale batch experiments. Linear alkyl benzene sulfonates (LAS) and caffeine were selected as model pollutants. In fact, LAS are biodegradable anionic surfactants used as key components in household detergents, dishwashing products, and multipurpose cleaners. Caffeine is a chemical marker for urban wastewater contamination in freshwater systems.

**MATERIALS AND METHODS**

**Microorganisms** Seed activated sludge collected from municipal wastewater treatment plant in Shiga Prefecture, Japan was acclimated to synthetic wastewater (Bonito Extract 120 mg, Hipoly Peptone S 120 mg, NaH₂PO₄ 25.2 mg, KH₂PO₄ 4.2 mg, NaCl 4.5 mg, KCl 2.1 mg, CaCl₂·2H₂O 3.0 mg, MgSO₄·7H₂O 3.3 mg in tap water 1 L) for half a year at 25°C in the dark. A seed algal–bacterial culture collected from a small pond (N34° 58′ 34.806″, E135° 58′ 8.1876″) in Shiga Prefecture was cultivated with a 1000-fold diluted solution of a commercial liquid fertilizer (Hyponex 6–10–5, Hyponex Japan Corp., Ltd., Japan) with 35 mg/L sodium silicate for a few weeks at room temperature under electric lighting (12:12 light–dark cycle) with quantum flux of 32 µmol/m²/s. These microbial cultures had not been exposed to LAS or caffeine.

**Wastewater** Synthetic municipal wastewater used for the batch experiment was composed of glucose 250 mg, NH₄Cl 190 mg, K₂HPO₄ 40 mg, KH₂PO₄ 15 mg, NaHCO₃ 275 mg, Na₂SiO₃·9H₂O 100 mg, FeCl₃·6H₂O 12 mg, sodium LAS (Fujifilm Wako Pure Chemical Corp., Japan) 20 mg, caffeine (Fujifilm Wako Pure Chemical Corp.) 0.03 mg, and pure water 1 L. The initial values of dissolved organic carbon (DOC), dissolved nitrogen (DN), and dissolved phosphorous (DP) were 106 ± 4.5, 57 ± 2.2, and 6.7 ± 0.25 mg/L, respectively. The distribution of the LAS homologues was C₁₀ (13%), C₁₁ (38%), C₁₂ (31%), and C₁₃ (18%). The pH of the synthetic wastewater was adjusted to 7.5–7.8.

**Experimental design** The activated sludge sample and the algal–bacterial culture sample were inoculated separately into synthetic wastewater of 100 mL in a conical beaker for simulating an aerated pond and an algal pond, respectively. The initial biomass concentration of all beakers was set to 2000 mg/L as mixed liquor suspended solids (MLSS). Synthetic wastewater with no microbial culture was also prepared as a control system. All cultures were kept on an orbital shaker at 100 rpm for 7 days at 25°C with a 12/12 h light-dark cycle with light intensity of 15 µmol/m²/s.

**Analytical procedures** A 2 mL sample was collected periodically from the beaker. The chlorophyll concentration in the sample was measured using the standard methods. Before analysis of DOC, DN, DP, LAS, and caffeine, all samples were filtered through 0.45-µm-pore-size filter paper. DOC and TN were measured using a total organic carbon analyzer (TOC-V; Shimadzu Corp., Japan). The TP concentration was analyzed using the APHA standard methods.

The respective concentrations of LAS, sulfophenyl carboxylic acids (SPCs), and caffeine were analyzed using liquid chromatography mass spectrometry (LC–MS 8030; Shimadzu Corp.) with a multiple reaction monitoring mode. The LC column (Shim-pack VP–ODS, 2.0 × 50 mm i.d., 5 µm) was maintained at 40°C. The flow rate of the mobile phase was 0.2 mL/min with isocratic elution (20:80=7.5 mM ammonium acetate: methanol). Quantification was done by measuring the intensity of protonated molecular ion at m/z for LAS, SPCs, and caffeine. Quantitative analysis for LAS and caffeine was conducted through external calibration. Because of the lack of a commercially available standard, SPCs were quantified on a semi-quantitative basis by the ratio of their peak areas to the peak area of 0.1 mg/L C₁₀ LAS homologue.

**Degradation kinetics** The primary degradation of LAS and caffeine with time in
the microbial cultures was fitted to a second-degree polynomial model\textsuperscript{10}.

\[ \frac{dC}{dt} = -k_2C^2 - k_1C \]

In that equation, \( C \) is the concentration of chemicals (C\textsubscript{13}LAS, C\textsubscript{12}LAS, C\textsubscript{11}LAS, C\textsubscript{10}LAS, and caffeine); \( k_1 \) and \( k_2 \) respectively denote the degradation coefficients of the first-order and second-order terms. The term \( k_2 \) is a correction factor reflecting the lag phase in the activity of the bacteria while they adapt to the substrate to be degraded. Values for \( k_1 \) and \( k_2 \) were estimated using least-squares method. The coefficient of the determination (\( r^2 \)) was calculated for fitness of the model to the experimental data.

**RESULTS AND DISCUSSION**

**Macro-pollutant removal in microbial cultures** Figure 1 portrays the change in the chlorophyll concentration in the algal–bacterial culture arranged to simulate an algal pond. The initial chlorophyll concentration was only 0.3 mg/L in the algal–bacterial culture of the MLSS concentration of 2000 mg/L. Microscopic images of the algal-bacterial culture are presented in Fig. 2. Many diatoms of about 0.1 mm were observed in the culture. Diatoms and bacteria

![Figure 1](image1.png)  
**Fig. 1** Growth curve of algae in the activated sludge culture and the algal–bacteria culture (25°C, 12 h photoperiod of 15 µmol/m\(^2\)/s).

![Figure 2](image2.png)  
**Fig. 2** Microscopic images of the algal–bacterial culture.
are well known to colonize and form biofilms containing extracellular polymeric substances\textsuperscript{11}. The chlorophyll concentration increased until day 1 and reached a steady state at 3.0–3.5 mg/L. Subsequently, it decreased slightly after day 3, possibly because of a lack of nutrients. The chlorophyll concentration in the activated sludge simulating an aerated pond was below 0.1 mg/L in 7 days, indicating that the algal population was negligible.

Figure 3 shows removal of macro-pollutants in the microbial cultures. The control system showed a slight decrease in the macro-pollutant concentration in 7 days, suggesting adsorption to the glass wall. The DOC concentration in all microbial cultures dropped from about 110 mg/L to 30–33 mg/L within 0.5 days. Afterward, the activated sludge culture showed a gradual increase in the DOC concentration to 55 mg/L on day 3.5. The DN and DP concentrations in the activated sludge culture were also higher than those in the control system, suggesting release of organic matter and the nutrients by endogenous respiration. By contrast, the DOC concentration in the algal–bacterial culture was kept less than 30 mg/L. The DN removal reached 30% in the algal pond. The algal–bacterial culture showed a higher DP removal at 72% than the activated sludge culture at 23% in 7 days.

**LAS and SPC removal in microbial cultures** Degradation profiles of LAS homologues in the microbial cultures are presented in Fig. 4. In the control system, the LAS removal was negligible in 7 days. Table 1 presents kinetic parameters obtained for the LAS degradation. The first step in the typical aerobic degradation of C\textsubscript{n}LAS is the \(\omega\)-oxidation of the terminal methyl group of the alkyl chain, releasing C\textsubscript{n-1}SPC. Then, lower molecular SPCs are produced by \(\beta\)-oxidation, by which the alkyl chain is shortened by two carbon atoms (C\textsubscript{n-2}SPC), and \(\alpha\)-oxidation as a minor pathway, by which the alkyl chain is shortened by a carbon atom (C\textsubscript{n-3}SPC). Finally, the resulting intermediates are mineralized or incorporated into the cells\textsuperscript{10,12}.

The primary degradation of LAS in the activated sludge culture is well-fitted by the first-order kinetics without lag time \((k_2 = 0)\). The LAS homologues with longer alkyl chain showed rapid removal \((k_1, C_{13}>C_{12}>C_{11}>C_{10} \text{LAS})\). Other researchers have also reported preferential biodegradation of LAS with longer alkyl chain when various homologues were present simultaneously in water\textsuperscript{13}. Removal of 99% was achieved in 2 days for C\textsubscript{13}LAS and C\textsubscript{12}LAS and 3 days for C\textsubscript{11}LAS and C\textsubscript{10}LAS. The detection of C\textsubscript{13}SPC, which is produced only from C\textsubscript{13}LAS, confirms the \(\omega\)-oxidation in the activated sludge culture. The major intermediates generated by C\textsubscript{13}LAS degradation are expected to have odd carboxylic chain length (C\textsubscript{13}, C\textsubscript{11}, C\textsubscript{9}, C\textsubscript{7}, and C\textsubscript{5}SPCs), whereas those from C\textsubscript{12}LAS have even chain length (C\textsubscript{12}, C\textsubscript{10}, C\textsubscript{8}, and C\textsubscript{6}SPCs). In the activated sludge culture, the C\textsubscript{7}SPC
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Fig. 4 Time courses of LAS and SPC concentrations in the microbial cultures: (A) activated sludge culture and (B) algal–bacterial culture. The SPC concentration was defined as the SPC area/ the C10LAS area (0.1 mg/L) on LC-MS analysis.

Table 1  Kinetic parameters of the second-degree polynomial model for primary degradation of LAS and caffeine in microbial cultures

|                  | C13LAS | C12LAS | C11LAS | C10LAS | Caffeine |
|------------------|--------|--------|--------|--------|----------|
| **Activated sludge** |        |        |        |        |          |
| $k_1$ (d$^{-1}$)  | 2.37   | 1.31   | 0.959  | 0.723  | 0.115    |
| $r^2$ (%)        | 0.976  | 0.981  | 0.993  | 0.999  | 0.939    |
| **Algal–bacterial culture** |        |        |        |        |          |
| $k_2$ (Lmg$^{-1}$d$^{-1}$) | -0.746 | -0.428 | -0.261 | -1.085 | 0        |
| $k_1$ (d$^{-1}$)  | 2.403  | 2.256  | 1.78   | 2.454  | 0.107    |
| $r^2$ (%)        | 0.999  | 0.995  | 0.995  | 0.999  | 0.989    |

$k_1$, first-order degradation coefficient; $k_2$, second-order degradation coefficient; $r^2$, coefficient of determination.
concentration was high on day 3, whereas C₇SPC as its precursor and C₈SPC as its successor were detected at low levels. The C₈SPC concentration was also high on days 3–5, whereas C₁₀SPC and C₆SPC were detected at low levels. These results suggest that the β-oxidation of C₇ and C₈SPCs are rate-limiting steps in the activated sludge culture.

The algal–bacterial culture required the lag phase for the elimination of the LAS homologues. The term $k_2$ with a negative sign represents the inhibitory effect of the substrate on the bacterial flora. The LAS homologues with longer alkyl chain showed stronger inhibitory effects but rapid degradation, except for C₁₀LAS ($k_2$, C₁₁L > C₁₂L > C₁₃L > C₁₀LAS; $k_1$, C₁₀L > C₁₁L > C₁₂L > C₁₃L). The 99% removal was achieved in 3 days for C₁₀LAS, 4 days for C₁₂LAS, and 5 days for C₁₃LAS and C₁₀LAS. Actually, C₁₁SPC generated by the ω-oxidation of C₁₁LAS and the β-oxidation of C₁₃SPC reached a maximum concentration on days 2–3. Then it converted rapidly to shorter-chain homologues: C₈SPC and C₆SPC. C₁₂SPC generated by ω-oxidation of C₁₂LAS and C₁₄SPC generated by the ω-oxidation of C₁₄LAS with the β-oxidation of C₁₃SPC respectively reached maximum concentrations on day 2 and day 3. These observations suggest that β-oxidation of C₁₂, C₁₁, and C₁₄SPCs were rate-limiting steps in the algal–bacterial culture.

The bacterial species and population in the activated sludge culture and the algal–bacterial culture were quite different from each other, as suggested by the degradation kinetics for LAS and SPC. Although the acclimation period for LAS degradation ($k_2 < 0$) was needed, the algal–bacterial culture showed higher $k_1$ values for all LAS homologues than the activated sludge culture (Table 1). The LAS-degrading bacteria in the algal–bacterial culture might have potentially higher degradation abilities than those in the activated sludge culture.

Caffeine removal in microbial cultures

Figure 5 shows the caffeine concentration profile in the microbial cultures. The caffeine removal was slow, but well fitted by first-order kinetics without lag time ($k_2 = 0$). In the control system, the caffeine removal over 7 days was negligible. The activated sludge culture showed a slightly higher degradation rate than the algal–bacterial culture (Table 1). After 7 days, the respective caffeine removal rates in the activated sludge culture and the algal–bacterial culture were 62% and 50%. Very few studies have been conducted to assess caffeine degradation by algal–bacterial cultures. Although the contribution of biosorption and biodegradation to this caffeine removal was unknown, biodegradation of caffeine typically initiates with the parallel conversion to paraxanthine and theobromine by demethylases. The oxidation of xanthine, mono and dimethylxanthine are followed by the purine catabolism pathway.

Advantage of algal–bacterial culture over bacteria-based culture

Figure 6 depicts the macro-pollutant and micro-pollutant removals in the microbial cultures. The activated sludge culture simulating the aerated pond showed high DOC removal and rapid degradation of LAS and caffeine within 1 day, but it insufficiently removed nutrients in 7 days. These results confirmed that the bacteria-based process needs complicated configuration and operation using bacteria of different types and combinations of oxic, anoxic, and anaerobic conditions for nutrient removal. The algal–bacterial culture simulating the algal pond showed high removals for DOC, DN, and DP in 7 days. The instant increase in the chlorophyll
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Concentration indicates that the algae used the nutrients in wastewater for their own growth. Although the algal–bacterial culture showed slower degradation of LAS, SPCs, and caffeine than the activated sludge culture, those capabilities are sufficiently high for the residence time of the typical algal pond.

For sustainable operation of the aerated and algal pond treatment systems, the excess biomass should be withdrawn from the pond for avoiding increasing effluent levels of BOD, nutrients, and suspended solids along with the terrible odor. Algae-based treatment is characterized by poor settleability of the biomass. An effective biomass harvesting strategy such as a settleable algal-bacterial culture is required. Algal biomass with carbohydrates, lipids, and proteins is an excellent candidate for bioenergy and biomaterial production.

Few studies of toxicological data have arrived at an effective concentration (EC50) of caffeine as 100 mg/L on algae. By contrast, the toxicity of LAS on algae has been well documented. Reportedly, the growth of an algal species was inhibited by 10–20 mg/L of LAS, but was promoted slightly by 5.0 mg/L of LAS. The C13 LAS homologue exhibited higher toxicity on an algal species than C11 LAS did. LAS-degrading bacteria, which decreased the LAS concentration in the algal–bacterial culture, were expected to be helpful for algal survival. The algal–bacterial culture might have expressed higher LAS removal rates without a lag phase if the bacteria had been acclimated to the synthetic wastewater.

Algae and their photosynthesis are extremely important for the removal of LAS and caffeine in the algal–bacterial culture. Sorption of LAS by algal cells was followed by release and degradation by bacteria. Although the contribution of direct photogradation was negligible, some reactive oxidants other than dissolved oxygen produced by photosynthesis might account for LAS degradation. It has also been reported that algae can increase caffeine removal by releasing exudates to stimulate biodegradation processes.

CONCLUSION

An algal–bacterial culture produced to simulate the waters of an actual algal pond showed high DOC, DN, and DP removal. The LAS and SPC removal rates in the algal–bacterial culture were slightly lower than the activated sludge culture, but were sufficiently high for the residence time of the typical algal pond. The caffeine removal up to 7 days was not as high as that of the activated sludge culture. Algal ponds are a promising technology for simple and low-cost wastewater treatment in warm countries if a sufficiently large area is available for the necessarily long residence time.

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