Cleaning and regeneration of periphyton biofilm in surface water treatment systems
Yonghong Wu, Lizhong Xia, Nian Liu, Shuai Gou and Bibhash Nath

ABSTRACT
The fouling of periphyton biofilm is a common problem associated with surface water treatment systems. In this study, sulfuric acid (H2SO4), sodium acetate (CH3COONa) and ethylenediamine tetra-acetic acid (EDTA) solutions were sequentially used to clean periphyton biofilms collected from a surface water treatment system. The results showed that the sequential addition of H2SO4 and CH3COONa solutions could accelerate the exfoliation of the fouled periphyton biofilm, while the addition of EDTA solution could regenerate the periphyton biofilm. However, the addition of H2SO4 and CH3COONa solution might negatively affect the bacterial community structure, while the addition of EDTA solution facilitated improvement of the community structure. The combined effect of cleaning and regeneration of periphyton biofilm has significantly improved the removal efficiencies of chemical oxygen demand (COD), total phosphorus (TP), total nitrogen (TN) and ammonia (NH4-N), by 19, 20, 23 and 22%, respectively. The removal processes of COD, TP, TN and NH4-N by the cleaned biofilm were fitted to power regression curves, while those by the control biofilm during the removal process were fitted to polynomial regression curves. These systemic results indicate that the sequential addition of H2SO4, CH3COONa and EDTA solution is able to clean fouled periphyton biofilm and to enhance the efficiency of surface water treatment systems.

Key words | cleaning, periphyton biofilm, power regression, regeneration, surface water

INTRODUCTION
The fouling of biofilm is a widespread problem in wastewater treatment and reclamation applications that severely limits the performance of biofilters and increases operating costs (Subramani & Hoek 2010; Filloux et al. 2012). So far, fouling of biofilm in membrane-based system (e.g., nanofiltration) has attracted considerable attention in order to minimize the effect of fouling on water quality (Al-Amoudi & Lovitt 2007; Simon et al. 2013; Zaviska et al. 2013). However, few studies have been performed on similar types of surface wastewater treatment systems, such as biopond and activated sludge treatment systems, where biofouling normally occurs.

In biopond and activated sludge treatment systems, the biofilm is comprised of heterotrophic and autotrophic microbial cells. The colonies are embedded in a polymer matrix whose structure and composition is a function of biofilm age and environmental conditions (Lazarova & Manem 1995; Khatoon et al. 2007). The surface water treatment system based on periphyton biofilm uses periphyton to capture pollutants such as phosphorus and nitrogen (Rectenwald & Drenner 1999). Additionally, the periphyton biofilm can remove pollutants such as microcystins by adsorption (Wu et al. 2010). Therefore, to improve its performance, it is necessary to renew microbial aggregates and to develop microporous periphyton biofilm.

Periphyton biofilm is sensitive to changes of the surrounding aquatic environment (Meylan et al. 2005). It is easily enwrapped by sludge and suspended particles in wastewater treatment systems (Kulkarni et al. 2005). This most likely causes plugging of the pores and channels to the inner biofilm (Schafer et al. 2005). Moreover, due to the presence of both heterotrophic and autotrophic microorganisms, the periphyton biofilm can be irreversibly polluted by the deposition of dead microorganisms (Sladeckova 1962). As a result, the activated cavities of periphyton biofilm can fill with debris, leading to a decrease in the adsorption capacity of the pollutants (Wu et al. 2010). Additionally, aging and desquamating of biofilm may occur when the flora and fauna in/on the biofilm are mature or the biofilm ‘active thickness’ is up to a specific
level (Lazarova & Manem 1995). These detriments to biofilm nature (including structure and constituent) may result in a decrease in removal efficiency because biofilm activity is associated with the biomass and ‘active thickness’ (Chung et al. 2007; Khatoon et al. 2007). Therefore, fouling of biofilm in surface water treatment systems will be much more complicated than in membrane-based water treatment systems.

Some studies have showed that by mechanical means, such as airing, scraping or vacuuming, the fouling of periphyton biofilm can be successfully removed (Chen et al. 2007; Jin et al. 2013). This can act as an alternative to biologically harvesting periphyton from invertebrates such as fish and shrimp (Rectenwald & Drenner 1999; Khatoon et al. 2007). However, in most cases wastewater does not meet the required standard beneficial to invertebrate growth. Moreover, the use of mechanical and biological measures are less feasible in large-scale applications such as in domestic wastewater treatment plants because of the relatively high-cost and low-efficiency (Al-Amoudi & Lovitt 2007). Thus, the development of chemical measures may offer alternative solutions for removing the fouling from periphyton biofilm.

The primary objectives of this study were to assess the effects of cleaning and regeneration of periphyton biofilm on improving water quality in a surface water treatment system. The study also aimed to explain pollutant removal kinetics by using mathematic models (e.g., power and polynomial regression curves). The findings of this study will (i) provide promising bio-measures (efficient and easily-deployed) to remove fouling from periphyton biofilm (or other similar microbial aggregates) in surface water treatment systems, (ii) provide an insight into improved water quality parameters (e.g., nutrient and pH) of surface water treatment systems together with improved bacterial growth, and (iii) present a fundamental understanding of the kinetics of pollutants removal by periphyton biofilm and other similar microbial aggregates.

**MATERIALS AND METHODS**

**Periphyton biofilm culture**

To obtain specific desirable native microorganisms and to facilitate large-scale industrial application, the periphyton biofilms were collected from the surfaces of stones in a pond in Kunming, southwestern China, where water was hyper-eutrophic (total nitrogen (TN) 2.0–6.0 mg L$^{-1}$, total phosphorus (TP) 0.2–1.5 mg L$^{-1}$, dissolved oxygen (DO) 4.0–7.6 mg L$^{-1}$, pH 6.5–8.0). The annual average air temperature around the pond was about 15–30°C. The periphyton biofilms were carefully peeled off by hand. The collected periphyton biofilms were cultured in an aerobic tank where eutrophic water was being treated. The physicochemical parameter of the eutrophic water is shown in Table 1. The inert substrates (Length 80 cm; Dia. 10 cm, Density 0.85 kg m$^{-3}$) made of polyurethane (Wuhan Zhongke Hydrobiology Environmental Engineering Co., Ltd) were washed using 0.1 M HCl solution and then rinsed three times with double-distilled water. The washed substrates were fixed under the water surface, with a density of 0.3 m$^{-3}$. The periphyton biofilm cultures were incubated at 25–30°C with aeration (the dissolved oxygen level was kept at 7.0–9.5 mg L$^{-1}$) until dense periphyton (green and gray in color) was formed after 40 days. The dense periphyton biofilms with their substrates were then used in the following trials. The detailed description on the wastewater treatment system and the main constituents of the periphyton biofilm has been reported in our previous work (Wu et al. 2011).

**The cleaning of periphyton biofilm**

During the episodes of cleaning periphyton biofilm, the eutrophic water was filtered (0.22 μm) to avoid any fouling. The periphyton biofilms with substrates were put into 2.5 L plastic bottles with 2.0 L eutrophic water. For the control sample, 2.0 L double-distilled water was used instead of eutrophic water. The biofilm biomass was estimated to be 1,600 g/m$^3$ (moisture content 85%) at 25–30°C. For the cleaning treatment, 0.5% sulfuric acid (H$_2$SO$_4$) (v/v) 1.0 mL, 0.5% sodium acetate (CH$_3$COONa) (v/v) 1.0 mL, and 0.5% ethylenediamine tetraacetic acid (EDTA) (v/v) 1.0 mL solutions were sequentially added into the plastic bottles at an interval of 24 h. For control, no cleaning agent was added. All bottles, including treatment and control samples, were aerated to keep the dissolved oxygen level ranging from 7.0 to 9.5 mg L$^{-1}$. All bottles were placed in an incubator with stable illumination (3,250 Lux) and temperature (28°C). The experiment was conducted in triplicate.

**Table 1** Properties of the eutrophic water ($n=4$)

<table>
<thead>
<tr>
<th>DO (mg L$^{-1}$)</th>
<th>pH</th>
<th>COD (mg L$^{-1}$)</th>
<th>TP (mg L$^{-1}$)</th>
<th>TN (mg L$^{-1}$)</th>
<th>NH$_4$-N (mg L$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>6.3 ± 0.35</td>
<td>7.46 ± 0.08</td>
<td>20.2 ± 3.46</td>
<td>0.5 ± 0.12</td>
<td>13.2 ± 2.16</td>
<td>0.6 ± 0.15</td>
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The regeneration of periphyton biofilm

To support the growth of periphyton biofilm, the eutrophic water from the wastewater treatment system was used for the regeneration of periphyton biofilm. Moreover, to avoid the effect of substrates, the periphyton biofilm was peeled off by hand and then used in the following trials. The cleaned periphyton biofilms from the above cleaning experiment (from treatment groups) were rinsed using double-distilled water three times. Afterward, the periphyton biofilms were put into 2.5 L plastic bottles containing 2.0 L filtered eutrophic water. For control, uncleaned periphyton biofilms from the above cleaning experiment (from control) were rinsed using double-distilled water three times and were added, along with 2.0 L filtered eutrophic water, in 2.5 L plastic bottles. The samples (both treated and control) were aerated to keep the dissolved oxygen level at 7.0–9.5 mg L$^{-1}$. All of the bottles were kept in an incubator with stable illumination (3,250 Lux) and temperature (28°C). During the cultural period, the periphyton biofilm in the treatment groups renewed with time and the color of the biofilm started to become green and then gray. This regeneration experiment was also conducted in triplicate.

Sampling and analyses

Water samples in triplicate were collected daily or at an interval of two days. Chemical oxygen demand (COD) in the water was measured by the potassium dichromate method (APHA-AWWA-WEF 1998). TP, TN and ammonia (NH$_4$-N) in the water were determined according to the standard method (APHA-AWWA-WEF 1998). DO and pH in the water were determined by DO and pH meters (YSI Inc., USA).

Periphyton biofilm samples were collected daily in triplicate. The morphology of the periphyton was characterized by optical microscope, with 100-fold magnification. The Shannon diversity index (Eichner et al. 1999) was also calculated to evaluate the bacterial community diversity in the biofilm. The use of the Shannon diversity index to evaluate bacterial diversity was detailed in a previous report (Miura et al. 2007). Total DNA extraction and purification of the biofilms was conducted for the enterobacterial repetitive intergenic consensus sequence-polymerase chain reaction (ERIC-PCR) analyses. The total DNA was isolated from the biofilm samples following a procedure modified from Hill et al. (Hill et al. 2002). Biofilm sample aliquots (1 mL) were thawed in an ice-bath, and the cells were harvested by centrifugation for 5 min. DNA was then purified by sequential extraction with Tris-equilibrated phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform isoamyl alcohol (24:1) followed by precipitation with two volumes of ethanol. DNA was collected by centrifugation, air-dried and dissolved in 50 μL sterile TE buffer. The detailed procedures were described in a previous report (Wei et al. 2004).

Community fingerprints were obtained for bacteria in the biofilms by using total bacterial DNA as templates for ERIC-PCR. The sequence of the ERIC primers and the detailed procedures are described in previous work (Li et al. 2006):

E1 (ERIC-PCR): 5′-ATGTAAGCTCTGGGGATTCCAC-3′,
E2 (ERIC-PCR): 5′-AAGTAAGTGACTGGGGTGAGCG-3′.

Data analysis was performed using SPSS statistical software (version 15.0), with the level of statistical significance set at $p < 0.05$. Statistically significant differences between the data were evaluated by determination of standard deviation and analysis of variance (one-way ANOVA).

RESULTS AND DISCUSSION

Morphology of the periphyton biofilm

Acid solutions are expected to form micelles around the organic macromolecules and help in solubilizing and/or removing them from the biofilm surface (Ang et al. 2006). Similarly, alkaline solutions are known for the cleaning agent which can solubilize organic foulants that are deposited on the biofilms (Ang et al. 2006). Therefore, to investigate the removal of foulants from the biofilm, the periphyton biofilms with their substrates were cut off to observe the morphology after the addition of each cleaning agent.

Figure 1(a) shows that the control sample of the periphyton biofilm is dense in structure. However, with the addition of H$_2$SO$_4$ solution, the loose matter (scales) attached to the ribs of the dense periphyton biofilm started to exfoliate. As a result, the cavities in the dense periphyton biofilm became larger (Figure 1(b)). With the addition of CH$_3$COONa, the micropores of the substrate became very clear as a result of the loss of enclosed fauna and flora (Figure 1(c)).

The fouling of biofilm can be accumulated in the external surface (build-up of a cake/gel-like layer on the upstream face of a membrane) or in the pores of the biofilm (Knyazkova & Maynarovich 1999). Figure 1 shows that sequential cleaning of the periphyton biofilm with H$_2$SO$_4$ and CH$_3$COONa solutions results in exfoliation of the fouling biofilm and
subsequent removal of foulants from external surfaces and pores. This is because the surfactants or aggregates on the biofilm were transformed at low and high pH conditions (Childress & Elimelech 2000; Zhang et al. 2007).

After the addition of EDTA solution, the biofilm started to form and spread to the center of the micropores of the substrate (Figure 1(d)). This is mainly due to the chelation reaction between the micelles in the biofilm, trace metals and EDTA (Li et al. 2008). This indicates that the application of EDTA solution could renew the periphyton biofilm. It is likely that EDTA has reduced the intermolecular Ca\(^{2+}\)–humic acid complexes. These reduced individual humic acid molecules can then be easily rinsed off the biofilm surface (Li & Elimelech 2004), thereby providing beneficial conditions for biofilm regeneration.

**Response to the bacterial community structure**

Fluorescent *in situ* hybridization (FISH) and 16S rRNA gene sequence analyses revealed that a specific phylogenetic group of bacteria, the betaproteobacteria, plays a major role in the development of mature biofilms, which lead to severe irreversible biofouling (Miura et al. 2006). Considering the complex nature of periphyton biofilm, the evaluation of the structural change of the bacterial community based on the ERIC-PCR fingerprint was proposed. In Figure 2, 1 and 2 are the repetitive samples for each sample. There were some differences between 1 and 2 for each sample. This is because the bacterial composition and mass in the

![Figure 1](image1.png)  **Figure 1** | Morphological characterization of the periphyton biofilm: control sample (a), and response to the morphology of periphyton biofilm after the addition of 0.5% H\(_2\)SO\(_4\) (b), 0.5% CH\(_3\)COONa (c) and 0.5% EDTA (d).

![Figure 2](image2.png)  **Figure 2** | ERIC-PCR fingerprint of bacteria. M is the reference band. c\(_1\) and c\(_2\) are the sample bands of the control, d\(_1\) and d\(_2\) are the sample bands after the addition of H\(_2\)SO\(_4\) solution, e\(_1\) and e\(_2\) are the sample bands after the addition of CH\(_3\) COONa solution and f\(_1\) and f\(_2\) are the sample bands after the addition of EDTA solution.
periphyton biofilms were sensitive to environmental changes such as different collecting time and operation processes. When only the bacteria were quantified using ERIC-PCR measures, the results from repetitive samples of the ERIC-PCR fingerprint might be affected by dominant phototrophics (i.e., diatom and cyanobacteria).

There are typically 10 major bands in the control samples (Figure 2, c1 and c2, from 200 to 2,000 bp). However, with the addition of H2SO4 solution, the major bands decreased to eight (Figure 2, d1 and d2, from 300 to 1,600 bp). Meanwhile, six major bands were observed in the samples after the addition of CH3COONa solution (Figure 2, e1 and e2). These indicate that the addition of acid (H2SO4) and alkali (CH3COONa) solution lead to decreases in bacterial species and changes in their community structure. Indeed, acid and alkali solutions could negatively affect the DNA extract, thereby shaping the ERIC-PCR fingerprint (Sinden et al. 1998).

However, with the addition of EDTA solutions, the number of major bands was increased to eight (Figure 2, f1 and f2, from 300 to 2,000 bp). In addition, three obvious bands (indicated by red arrows) appeared from 300 to 700 bp and from 1,600 to 2,000 bp (Figure 2). This implies that the addition of EDTA solution facilitates bacterial growth and improvements in the bacterial community structure. These results are consistent with those obtained from the morphological observation of periphyton biofilm (Figure 1). Therefore, a community-based structural fingerprint is capable of evaluating the cleaning of periphyton biofilm.

Based on the relative ERIC-PCR fingerprint area, the Shannon-Weaver diversity index of the bacterial community was calculated. In the control sample, the Shannon-Weaver diversity index was 0.73 ± 0.03, while the Shannon-Weaver diversity indices decreased to 0.66 ± 0.06 and 0.57 ± 0.03, respectively, after the addition of H2SO4, and CH3COONa solutions to the periphyton biofilm. However, the Shannon-Weaver diversity index was increased to 0.65 ± 0.23 after the addition of EDTA solution. This suggests the regeneration of periphyton biofilm after cleaning the fouling biofilm following the sequential addition of H2SO4, CH3COONa and EDTA solutions.

**Effects of periphyton biofilm cleaning on water quality**

The concentrations of COD, TP, TN and NH4-N were determined during the cleaning episode of the periphyton biofilm. The result shows that the concentrations increased from 26.04 to 65.54 mg L⁻¹ for COD and 1.41 to 1.75 mg L⁻¹ for TP after the periphyton biofilms were cleaned sequentially (at an interval of 24 h) by H2SO4, CH3COONa and EDTA solutions (Figure 3). The concentrations of COD and TP in the control samples were lower than those in the treated samples (Figure 3). The deteriorating water quality of the treated wastewater might have resulted due to the removal of scales from the periphyton biofilm during cleaning, leading to an increase of organic matter in the water. Periphytons often act as phosphorus sinks (McCormick et al. 2006) and play an important role in the removal, uptake or transformation of phosphorus (Bushong & Bachmann 1989; Sainto & Reddy 2003). Therefore, during cleaning, the adsorbed phosphorus was dissolved in the water column, resulting in the increase of TP concentrations. In natural water, some inverse solubility salts such as calcium carbonate (CaCO3), calcium sulphate hydrate (CaSO4·nH2O), silica and calcium phosphate are inclined to form inorganic scales (Schafer et al. 2005; Xu et al. 2010). Therefore, it is highly likely that chemical reactions involving cleaning solutions and the scales might have led to deteriorating water quality. Moreover, the cleaning agents' concentrations were below the critical micelle concentrations (Schafer et al. 2005), thereby reducing reaction intensity.

Before the start of the cleaning episode, the TN concentration in the double-distilled and eutrophic water was 8.64 and 21.76 mg L⁻¹, respectively. The differences in the TN concentrations with those in the source water (the initial TN concentration in the double-distilled and eutrophic water was 0 and 13.2 mg L⁻¹, respectively) indicate that the scales in the biofilm might have auto-exfoliated and released nitrogen, leading to the increase in TN concentration. However, after the addition of H2SO4, the TN concentrations were decreased to 6.89 and 18.65 mg L⁻¹, respectively for the culture of double-distilled and eutrophic water. However, with the sequential addition of CH3COONa and EDTA solutions, the TN concentrations were slightly increased to 8.18 and 8.57 mg L⁻¹, respectively, for the culture of double-distilled water and 19.87 and 20.23 mg L⁻¹, respectively, for the culture of eutrophic water (Figure 3).

The average NH4-N concentration in the double-distilled water was higher (0.32 mg L⁻¹) than in the eutrophic water. However, with the addition of the CH3COONa solution, the average NH4-N concentration in the double-distilled water was observed to be lower than that in the eutrophic water. It is possible that some matter (such as carbonate scales) exfoliated from the biofilm scales and reacted with NH4-N, resulting in the depletion of NH4-N in the water. The addition of CH3COONa solution, the reaction of OH⁻ with H⁺ in Eq (OH⁻ + NH4⁺ ↔ NH3↑ + H2O) favors the release of NH3.
Pollutant removal during biofilm regeneration

Figure 4 shows that COD, TP, TN and NH₄-N concentrations increased during the cleaning episode of the biofilm and peaked at the initial phase of the regeneration period of the biofilms. However, the water quality started to improve during a later phase of the regeneration and became steadier from the 14th day of the experiment. In the control samples, the concentrations of COD, TP and TN decreased initially until the 12th day, while the water quality started to deteriorate afterwards. However, in the case of NH₄-N concentration, the control samples peaked on the 6th day of the experiment, and overall, showed fluctuations in the level of NH₄-N concentration. The results suggest improvement of water quality after cleaning of the biofilm. This is mainly associated with the regeneration of biofilms during treatment.

During the experimental period, the overall removal loads of COD, TP, TN and NH₄-N in the treated samples were higher than those in the control samples. The removal efficiencies were significantly higher: 19% for COD, 23% for TN, 20% for TP and 22% for NH₄-N than those in the control samples (p < 0.05). This indicates the effectiveness of the applied cleaning agents (i.e., H₂SO₄, CH₃COONa and EDTA).

Kinetics of COD, TP, TN and NH₄-N removal

A power regression curve was employed to investigate the dynamics of COD, TP, TN and NH₄-N removal by the cleaned periphyton biofilm. Table 2 shows that the removal of COD, TP, TN and NH₄-N fitted to power regression curves (p < 0.05). This implies that removal efficiency increased rapidly during biofilm regeneration. It is likely that the rapid growth of biofilm accelerated the removal of COD, TP, TN and NH₄-N by assimilation. In addition, the pollutants such as COD, TP, TN and NH₄-N were adsorbed onto the activated cavities of the regenerated periphyton biofilm (McCormick et al. 2006).

However, in the case of the control samples, the removal kinetics of COD, TP, TN and NH₄-N by biofilm were fitted to polynomial regression curves (p < 0.05) (Table 2). This indicates that the COD, TP, TN and NH₄-N concentrations in the control samples increased in the late stage. It has been further tested that overall removal efficiency in the control sample was lower than that in the
treated samples. The rebounded COD, TP, TN and NH₄-N concentrations were mainly due to the aging and desquamating of biofilm, leading to the release of pollutants from the biofilm aggregates (Lazarova & Manem 1995).

CONCLUSIONS

This is the first study to report the effect of cleaning and regeneration of periphyton biofilm in a surface water treatment system. The sequential addition of H₂SO₄, CH₃COONa and EDTA solution was observed to be a highly cost-effective and easily-deployed measure to remove fouling from periphyton biofilm and to improve water quality (e.g., COD, TN, TP and NH₄-N concentration) in a domestic wastewater bio-treatment system. The community structure based on bacterial fingerprint can be employed to effectively assess the cleaning of periphyton biofilm. The regenerated periphyton biofilm was able to efficiently remove COD, TN, TP and NH₄-N from the wastewater and its removal efficiency was characterized by power regression curves. This study provides a promising and easily-deployed measure to improve the removal efficiency of pollutants in domestic wastewater bio-treatment system based on periphyton biofilm. It also gives an insight into the kinetics of removing pollutants by microbial aggregates. Similarly, with regard to the traditional domestic wastewater treatment system, e.g., technologies based on anoxic/oxic or active sludge processes, the current technology based on adjusting pH by mixing different types of wastewater or the sequential addition of H₂SO₄, CH₃COONa and EDTA solution could significantly improve the overall removal loads of pollutants.

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