Effects of nutrient loading on *Anabaena flos-aquae* biofilm: biofilm growth and nutrient removals

Xiaowei Li, Qun Wei, Xiaojie Tu, Yuxuan Zhu, Yanfei Chen, Lina Guo, Jun Zhou and Hongyun Sun

ABSTRACT

Effects of three different nutrient loadings (low nutrient loading, medium nutrient loading and high nutrient loading, denoted as LNS, MNS and HNS, respectively) on the structure and functions of algal biofilm using *Anabaena flos-aquae* were investigated using synthetic wastewater. Nutrients removal efficiencies, biofilm thickness, microalgae dehydrogenase activity (DHA) and exopolysaccharide (EPS) productions were examined. Results showed that the changes of nutrient concentration were insignificant after 4 days of experiment for the case of HNS condition; 9 days for the case of MNS condition, and 6 days for the case of LNS condition, respectively. The biofilm thickness, nutrient removal efficiencies, algae DHA and EPS productions increased with the increase of nutrient loadings in synthetic wastewater. For the case of HNS condition, the microalgal biofilm exhibited the best performance in terms of C, N and P removal efficiencies, reaching the removal rates of 68.45, 3.56 and 1.61 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for C, N, P, respectively. This was likely because, fact with the high nutrient loading, the high biological activity could be achieved, thus resulting in high nutrient removals. The thickness of the biofilm in HNS condition was 75 μm, which was closely related to EPS production. DHA and EPS concentrations were 7.24 and 1.8×10^{-2} $mg{\cdot}mm^{-2}$, respectively. It was also shown that apart from the nutrient loading, the structure and functions of microalgal biofilm were also influenced by other factors, such as illumination and temperature.

Key words | dehydrogenase activity, exopolysaccharide, microalgal biofilm, nutrient loading, thickness

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INTRODUCTION

Excessive nitrogen (N) and phosphorus (P) contained in municipal wastewaters or generated from agricultural and industrial production could lead to eutrophication (Cai *et al.* 2013). It is a tough challenge for human beings to find environmentally, technically and economically feasible solutions to solve the problem. Microalgae-based wastewater treatment is particularly attractive due to its photosynthetic capability, assimilating N and P compounds and converting solar energy into useful biomass, which can achieve high nutrient removal rate and effective use of microalgae resource (Abdel-Raouf *et al.* 2012). Since more complex microbial communities could be formed within the microalgal biofilm, it has higher nutrient removal ability; which makes it have great advantages in wastewater treatment (Arbib *et al.* 2014). Comparing to suspended microalgae cultivation system, microalgal biofilm technique is a time-saving and cost-effective approach for wastewater remediation, in terms of biomass harvesting and phototrophic biofilm cultivation. Microalgal biofilm is composed of two parts: immobilized bacteria–algae system and cell-free carrier system (Kesaano & Sims 2014).

Microalgal biofilm technology is markedly effective in overcoming the major challenges for microalgae biomass production and harvest, which can easily separate microalgae from effluent successfully. It has been demonstrated that if sufficient surface area is provided, microalgal biofilm can grow faster than suspended microalgae (Yabur *et al.* 2007). Microalgal biofilm, a kind of phototrophic biofilm, commonly occurs on most surfaces exposed to light, and usually consists of microalgae, bacteria and other

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microorganisms (Schnurr *et al.* 2014). During the microalgal biofilm acclimation, various microbial colonies continually attach on the surface of carriers. Differentiated biofilm architecture can be developed in different ecological habitats. The colonization process is not only affected by interactions of physical factors, namely, pH, light and temperature, but also closely related to nutritional resource availability (Kesaano & Sims 2014).

Nutritional condition is a limiting factor for the formation of microalgal biofilm among the factors mentioned above. Since autotrophs and heterotrophs show differential responses to nutrients supply, N and P concentrations could greatly affect the structure of microalgal biofilm. Moreover, nutritional conditions have some influences on metabolic activities (Jin *et al.* 2011) (directly) and biofilm communities (Gainswin *et al.* 2006) (indirectly), resulting in varied functions of microalgal biofilm. The structure and function of microbial biofilm play an essential role in the wastewater treatment process; hence, extensive studies, especially on the effects of nutrition enrichment, have been conducted since the 1990s (Sabater *et al.* 2005). Driving factors affected the function of microbial biofilm can be divided into two types: one is the community composition of microbial biofilm (Ma *et al.* 2014) and the other is the quantity and quality of extracellular organic polymeric materials produced by microbes (Ashok *et al.* 2014). Considering that the blooms of *Anabaena* species, which belong to blue-green algae, possess high capacity to utilize nutrients for their growth and reproduction, as well as their well geographically distributed and easy availability, *Anabaena flos-aquae* was selected as the model algae.

Most previous researches were focused on the removal efficiency improvements of nitrogen for treating the secondary effluent by single or mixed algal biofilm (Kesaano & Sims 2014), or on exploring the removal mechanisms of phototrophic biofilms in the wastewater treatment system (Ashok *et al.* 2014). However, limited information was available on the range of nutrient loading the algal biofilm is capable of processing (Cabije *et al.* 2009). As a matter of fact, it is crucial to study the relationship between the nutritional workload and carbon, nitrogen and phosphorus removals in the microalgal wastewater treatment system.

The objective of the present work mainly involves the following aspects: (i) to explore the effects of nutrient loading on the growth and the removal rate of nutrients by *Anabaena flos-aquae* biofilm; (ii) to observe the algal biofilm thickness and structure on different nutrient loadings; and (iii) to investigate the effect of dehydrogenase activity (DHA) and exopolysaccharide (EPS) production on the removal of nutrients by algal biofilm. For this purpose, several physio-biochemical parameters, including biofilm thickness, nutrient removal efficiencies, activity (microalgae DHA) and structure (EPS) were examined.

MATERIALS AND METHODS

Algae and carriers

The freshwater prototroph *Anabaena flos-aquae* was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB-Collection), the Chinese Academy of Sciences. The dimensional elastic carriers were purchased from Tiancheng Environmental Equipment Manufacturing Co., Ltd in Yixing City, China. The size of the carrier adopted in the experiment was 1 millimeter in diameter and 10 centimeters in length. Features and technical parameters of carriers are shown in Table 1.

Culture medium and wastewater

Stock culture of the species was maintained in Blue-Green Medium (i.e. BG11), which is commonly used for blue-green algae. The alga was grown in BG11 liquid medium at 25 °C and illuminated with cool-white fluorescent lights which provide a continuous light intensity of 3,000 lx. The culture was shaken thrice every day. Synthetic wastewater was prepared as follows (per litre): $CaCl_2$ 0.211 mg; $MgSO_4$ 0.382 mg; NaCL 1.078 mg; $FeSO_4$ 0.014 mg; $NaHCO_3$ 1.119 mg. Glucose, $(NH_4)_2SO_4$ and KH_2PO_4 were added

Table 1 | Features and technical parameters of carriers

Structure unit	Proportion	Breaking strength (N)	Tensile strength (MPa)	Continuous heat temperature (°C)	Brittle temperature (°C)	Acid–alkali stability	Specific surface area ($m^2\ m^{-3}$)	Porosity (%)	Film weight ($kg\ m^{-3}$)
Silking	0.93	120	≥30	800–100	−15	Stable	50–300	> 99	50–110
Axial cord	0.95	71.4	≥15	80–100	−15	Stable			

into synthetic wastewater as organic carbon, nitrogen and phosphorus resource, respectively. Studies (Ylla *et al.* 2009) have demonstrated that glucose is good for the removal of nitrogen and phosphorus by algal biofilm. Three different nutrient loading conditions in our experiment were obtained by changing the amount of glucose, $(NH_4)_2SO_4$ and KH_2PO_4 while other components of the wastewater were maintained constant. Three nutrient loading conditions (Table 2) were defined as low nutrient loading (LNS), medium nutrient loading (MNS) and high nutrient loading (HNS). The ratios of chemical oxygen demand (COD) to ammonia nitrogen (NH_4^+-N) to phosphate (PO_4^{3-}-P) in all media were maintained around 20:2:1. The initial pH of the growth media was adjusted to about 7.0 by adding sodium hydroxide and hydrochloric acid solution, whose concentration was 0.1 mol·L^{-1} and 0.1 mol·L^{-1}, respectively.

Experimental design

To establish the microalgal biofilm, 80 mL of exponential growth phase of algae solution was inoculated into culture flasks containing 800 mL of non-sterile synthetic wastewater in 1,000 mL flasks. In all flasks, carriers with surface area of about 6.0×10^6 mm^2 were placed. Two 30 W fluorescent lamps were set up along the sides of the flasks to provide continuous illumination of 3,500 lx. After a week of static culturing, the microalgae were attached to the surface of carriers effectively. Then, 200 mL of culture medium was replaced by an equal amount of synthetic wastewater (LNS, MNS and HNS) each day to assimilate algal cells during the first 4 days; on the fifth day, the culture solution was completely replaced by equivalent fresh synthetic wastewater. Subsequently, all the algal biofilms formed on the surfaces of elastic packing were cultured statically for half a month to form stable biofilm in static condition at room temperature with continuous illumination of 3,500 lx. To ensure a similar living environment for algae, other ingredients in the synthetic wastewater of the three load conditions were maintained consistent with each other. During the static cultivation of microalgal biofilm, indicators including COD, ammonia nitrogen (NH_4^+-N) and (phosphate) PO_4^{3-}-P were measured in order to determine the critical time point at which the nutrient concentrations were changeless. All the experiments in static condition were conducted in triplicate. The carriers were taken out for detecting DHA and EPS contents within the microalgal biofilm when the nutrient concentrations were constant, as well as characterizing their surface structures and calculating biofilm thicknesses according to cross-section structures by a field emission scanning electron microscope (FESEM) (SU8020, Hitachi, Japan) in high vacuum mode (working distance 10 mm, acceleration voltage 10 kV).The thickness of biofilm was estimated by the scan imagery and image scale.

Table 2 | Three conditions of nutrient loading unit (mg·L^{-1})

Nutrient loading conditions	COD	NH_4^+-N	PO_4^{3-}-P
LNS	275	15.16	6.41
MNS	575	31.19	13.51
HNS	1,175	64.72	30.51

Physical and chemical parameters

The value of pH and dissolved oxygen were determined by PHS-25 (Shanghai, China) and OXI3310 (WTW, Germany) meters, respectively. The light intensity was measured by an IL-HB light illuminometer (Shenzhen, China). A microwave digestion colorimetric method was adopted to measure COD at an absorbance of 600 nm. NH_4^+-N and PO_4^{3-}-P concentrations were analyzed according to the standard methods (in China) (Ministry of Environmental Protection 1989, 2009): based on the absorbance at 420 nm and 700 nm for Nassler's reagent colorimetric method and ammonium molybdate spectrophotometry, respectively, the concentrations can be obtained.

To detach the microalgal cells from the carriers, a large amount of de-ionized water was used to rinse the algae cells attached on the carriers. The microalgal cells from the detachment process were used for determining the DHA activity and the EPS production from the microalgal biofilm. The 3,5-triphenyltetrazolium chloride (TTC) method was employed for assaying DHA activity (Xie *et al.* 2008) and the phenol-sulfuric acid method was used to estimate the EPS production of microalgal biofilm (Liu *et al.* 2014).

FESEM of the algal biofilm

The biofilm samples for the surface and cross-section structures characterization (about 2–4 mm in length, cutting from the carriers) were prepared as follows: firstly, drying at 40 °C for about 2 hours in a constant temperature oven; and then cutting into appropriate segments (usually less than 1 mm); afterwards, the samples were coated with a nanometer-thin gold layer by sputtering before observing with FESEM at different magnifications (Irving & Allen 2011). Images were obtained with a scanning electron microscope (S-3400 N, Japan).

Data analysis

The data reported in this study are average value of the triplicate experiments.

The removal efficiency for COD and nutrients could be calculated by Equation (1):

$$R_i = \frac{S_0 - S_t}{S_0} \times 100\% \quad (1)$$

where R_i is the removal efficiencies of substrate, S_0 is the initial substrate concentrations of COD, NH_4^+-N and PO_4^{3-}-P, respectively; S_t is the corresponding concentration of substrate at time t at which the concentration of the substance did not change significantly (Rasoul-Amini *et al.* 2014).

In order to describe the removal capacity of microalgal biofilm more accurately, nutrient removal rate (μ), that is, nutrient removal by the unit initial algae biomass per day, was adopted. The calculation of μ was according to Equation (2):

$$\mu = \frac{(S_i - S_e)/t}{X_0} \quad (2)$$

where S_i represents the initial concentrations of COD, NH_4^+-N and PO_4^{3-}-P, S_e is the corresponding COD and nutrient concentrations at time t at which the nutrient concentrations did not change significantly, X_0 is the initial microalgal biomass or chlorophyll *a* (Chl*a*) content of microalgae biofilm.

RESULTS AND DISCUSSION

The surface and cross-section structures of microalgal biofilm

The morphology of algal biofilm is shown in Figure 1. As can be seen, the algal film of a certain thickness wrapped on the surface of the carrier was smooth. The surface and cross section structure of microalgal biofilm in LNS was characterized with the FESEM at magnifications of 1,000 and 10,000 times, respectively. The results showed that the microalgal cells attached tightly on the carriers, and similar surface structures of microalgal biofilms from the three different nutrient loading conditions were observed (Figure 3), indicating that *Anabaena flos-aquae* cells can grow well on the surface of the carriers. Usually, apart from microalgal cells, there could also be some bacteria and extracellular secretions on the surface of carriers

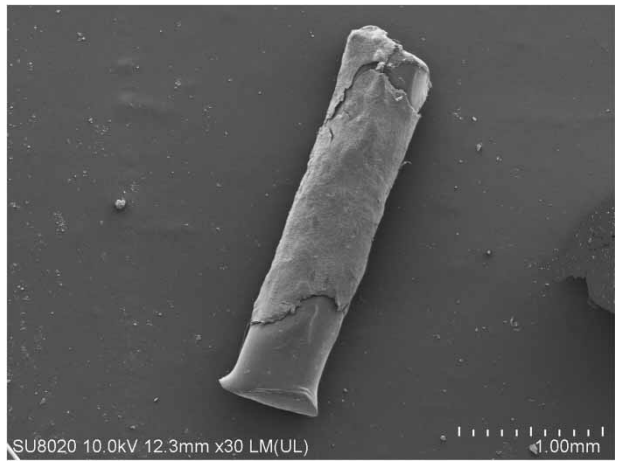

Figure 1 | Algal biofilm morphology with magnification of 100 times.

(Artigas *et al.* 2012). Microalgal cells attached on the carriers were wrapped with EPS (Figure 2(b)). Due to the adhesion force of EPS secreted by microalgae, microalgal cells and other microorganisms were easily and preferentially immobilized on the surface of carriers in various nutrient loading conditions (Arbib *et al.* 2014).

Although the surface structures of microalgal biofilm were similar in different nutrient loading conditions, their thickness varied significantly (Figure 3). The average thickness of microalgal biofilms was 25 μm in LNS, 35 μm in MNS, and 75 μm in HNS, respectively. It implied that the more nutrients supplied in synthetic wastewater, the thicker the microalgal biofilm could grow. A similar phenomenon was observed previously by Cabije *et al.* (2009). In their study, high growth rates in terms of the thickness of biofilm with an average of 7.71 μm at high organic loading rate was achieved, while the value for low organic loading was only 2.81 μm. However, the increase of thickness of microalgal biofilm may cause the vertical gradient changes of light quantity and quality. In that case, insufficient sunlight could reach the microalgae in the deeper biofilm layer, resulting in slowing down or even stopping of anabolism rates, but speeding up of catabolism rates. As a result, microalgae and other microorganisms detach from immobilized carriers. Therefore, the thickness of microalgal biofilm could not be increased proportionally by the increase of the nutrients in the medium.

Nutrients removal

The removal efficiency of nutrients by the algal biofilm, to a large extent, reflected the wastewater treatment ability of the algal biofilm. It was found that the time needed for reaching

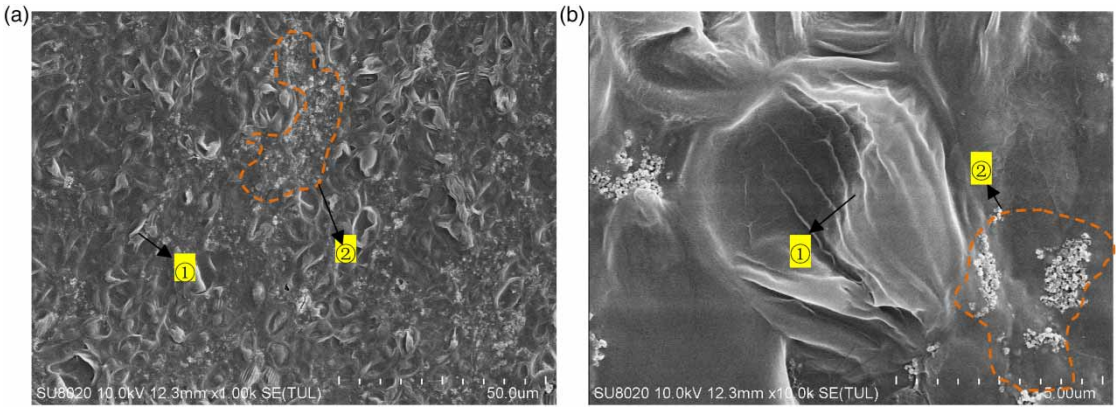

Figure 2 | Surface structure of microalgal biofilm in LNS: (a) magnification of 1,000 times, (b) magnification of 10,000 times (① microalgae ② EPS secreted by microalgae).

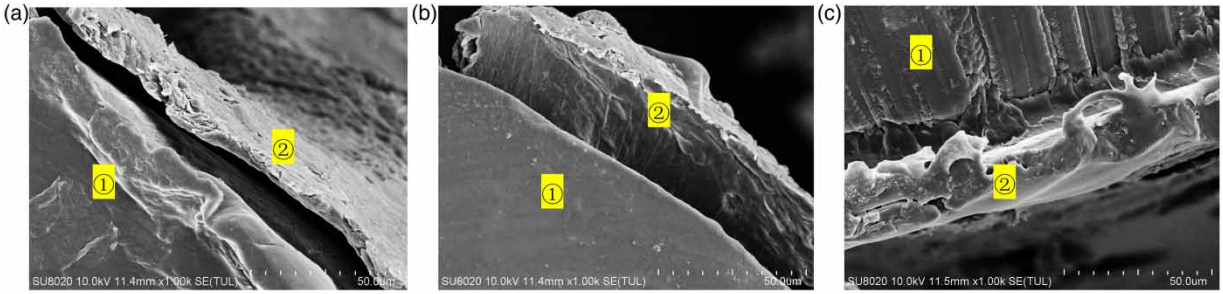

Figure 3 | The thickness of microalgal biofilm in LNS (a), MNS (b) and HNS (c): ① carrier, ② microalgal biofilm.

steady state (during which the concentrations of nutrients remain unchanged) varied for the LNS, MNS and HNS conditions. It took 6 days for the LNS condition to reach steady state, and 9 days for MNS condition and 4 days for HNS condition, respectively. Results showed that the removal efficiencies of these three nutrients exhibited a decreasing trend with increasing initial nutrient concentrations in synthetic wastewater (Figure 4). More specifically, the highest COD removal efficiency was obtained from LNS condition (93.9%), followed by that from MNS condition (85.5%), and the lowest COD removal efficiency was achieved from HNS condition, only 81.6%. For the case of NH_4^+-N removal, removal efficiencies from LNS, MNS and HNS conditions were 91.2%, 86.6% and 77.7%, respectively. For PO_4^{3-}-P removal, the average removal efficiencies were 94.2%, 88.5% and 74.1% for LNS, MNS and HNS conditions, respectively. MNS results were comparable to other studies of the microalgae system previously published. COD removal efficiency in MNS condition in our study was higher than that of a previous report with the same initial COD concentration (Kumar *et al.* 2011). N and P removal efficiencies in this study were low as compared with other reported results. For example, over 97.0% nitrogen and

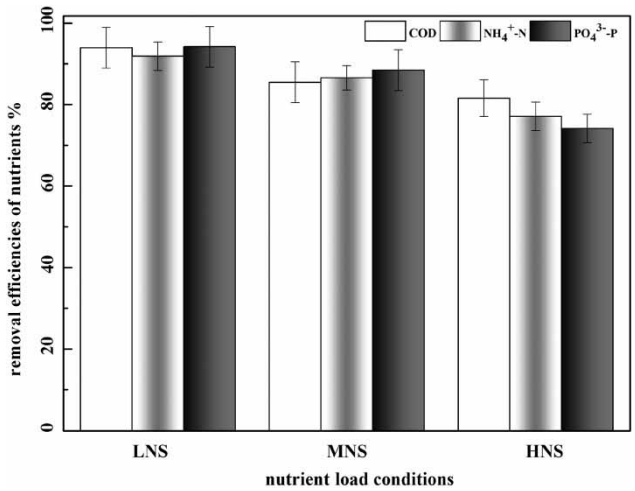

Figure 4 | Removal efficiencies of nutrients in synthetic wastewater by algal biofilm in three nutrient loading conditions.

phosphorus removal efficiencies were obtained by *Scenedesmus obliquus* in the nutrient concentrations of 27.4 $mg \cdot L^{-1}$ and 11.8 $mg \cdot L^{-1}$, respectively; *Desmodesmus communis* was shown to assimilate almost 100% nitrogen and phosphorus at any N/P ratio characterizing wastewater

nutrient composition in view of civic wastewater treatment (Samori *et al.* 2013). These differences may be attributed to different algae and different operational conditions that were adopted in our study. It is believed that the N and P removal efficiencies could be improved by optimizing the operational conditions. Further studies should be carried out in this regard. In spite of the lower N and P removal efficiencies in our study, adopting the microalgal biofilm for wastewater treatment has superiority in separating algae from effluent as compared with the above-mentioned system, which makes it hold great potential in wastewater remediation.

On account of different initial nutrient concentrations in three nutrient loading conditions, removal efficiency was not a scientific indicator to express nutrient removal ability by microalgal biofilm. Therefore, a more precise parameter of removal rate was evaluated. Nutrients removal efficiencies declined with the increase of initial nutrient concentrations. In contrast, nutrient removal rate showed an increasing trend from LNS condition to HNS condition (Figure 5). For instance, removal rate of COD increased from 23.27 $mg{\cdot}mg^{-1}$ Chl*a*$\cdot d^{-1}$ (LNS condition) to 45.03 $mg{\cdot}mg^{-1}$ Chl*a*$\cdot d^{-1}$ (HNS condition). For N removal, it increased by 49% (from 1.57 $mg{\cdot}mg^{-1}$ Chl*a*$\cdot d^{-1}$ to 2.34 $mg{\cdot}mg^{-1}$ Chl*a*$\cdot d^{-1}$) from LNS to HNS conditions. P removals also increased significantly from 0.68 $mg{\cdot}mg^{-1}$ Chl*a*$\cdot d^{-1}$ in LNS condition to 1.06 $mg{\cdot}mg^{-1}$ Chl*a*$\cdot d^{-1}$ in HNS condition. A previous study (Aslan & Kapdan 2006) reported that the maximum removal rates of N and P by suspended *C. vulgaris* reached 3.0 (N) and 0.52 (P) $mg{\cdot}mg^{-1}$ Chl*a*$\cdot d^{-1}$, respectively. Compared with their results, *Anabaena flos-aquae* biofilm had a higher P removal rate but lower N removal rate.

Without considering the initial microalgal biomass, the highest average removal rates of nutrients from LNS condition to HNS condition were 34.44, 54.63 and 68.45 (COD) $mg{\cdot}L^{-1}{\cdot}d^{-1}$, 2.32, 3 and 3.56 (N) $mg{\cdot}L^{-1}{\cdot}d^{-1}$, and 1.01, 1.33 and 1.61 (P) $mg{\cdot}L^{-1}{\cdot}d^{-1}$, respectively. In consideration of nutrient removal efficiency for the same initial concentration, the results of this study were consistent with other researches. For instance, Cabanelas *et al.* (2013) reported that the highest removal rates were 6.63 (N) and 0.68 (P) $mg{\cdot}L^{-1}{\cdot}d^{-1}$ in the pretreated urban wastewater with $N_0 = 72$ and $P_0 = 8.91$ $mg{\cdot}l^{-1}$ using *Chlorella vulgaris* suspended in the system. Jimenez-Perez *et al.* (2004) showed nutrient removal rates of 0.66 (N) and 0.42, (P) $mg{\cdot}L^{-1}{\cdot}d^{-1}$ using a suspension culture of *Scenedesmus obliquus* and 0.33 (N) and 0.18 (P) $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for *Nannochloris* sp. Yet, when cultured in immobilization mode, 0.27 (N) and 0.36 (P) $mg{\cdot}L^{-1}{\cdot}d^{-1}$ were achieved by *Scenedesmus obliquus*; and 0.18 (N) $mg{\cdot}L^{-1}{\cdot}d^{-1}$ and 0.27 (P) $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for *Nannochloris sp.* Results showed that algal biofilm had a much better effect for removing of nutrients.

Changes of DHA and EPS contents in the algal biofilms

The DHA of algae can reflect the quantity and activity of living algae (Xie *et al.* 2008). In this study, DHA was determined to characterize the number of living microalgal cells and analyze the biological activities of microalgal biofilm in the wastewater treatment process. EPS plays a vital

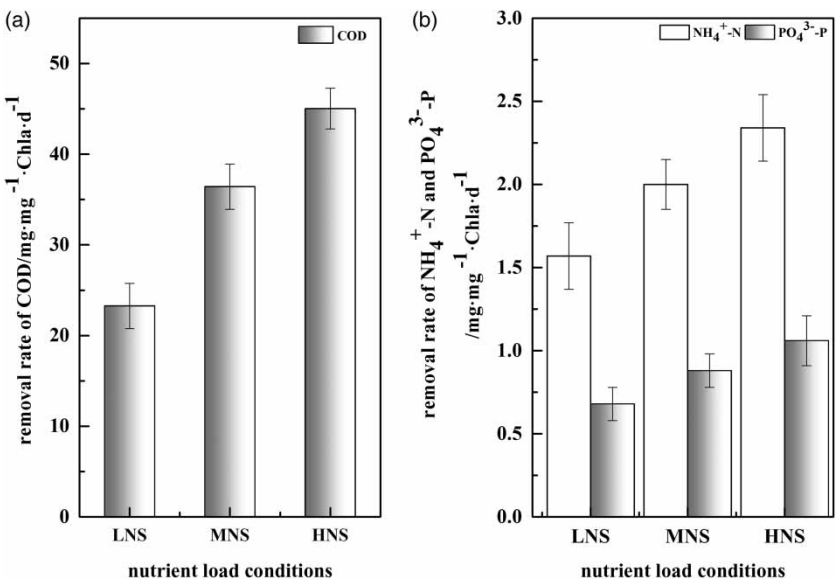

Figure 5 | Removal rates of nutrients in synthetic wastewater by algal biofilm in three nutrient loading conditions: (a) COD, (b) NH_4^+-N and PO_4^{3-}-P.

role in the formation of microalgal biofilm (Irving & Allen 2011). DHA and EPS contents in microalgal biofilm in three nutrient loading conditions are shown in Figure 6.

The results demonstrated that the higher the level of nutrients was, the more DHA and EPS contents were contained in the microalgal biofilm. This was likely due to the fact that when nutrients content in synthetic wastewater was higher, microalgae could grow better and faster, thus showing higher metabolic activity and secreting more metabolic intermediates. Therefore, DHA and EPS contents in the microalgal biofilm were higher in high nutrient loading condition. As nutrients were consumed constantly, microalgal cells suffered from nutrient stress, which led to the falling off of microalgal cells from the surface of carriers in the three nutrient loading conditions. This work showed that the ratio of EPS contents was 1:1.4:3 and the ratio of thickness was 1:1.8:3.5 of three nutrient loading conditions. It has been elucidated that the proportions of monosaccharide in many EPS produced by cultured or natural microbiota were mainly dependent on the specific biological communities of biofilm, which was used to form the complex anionic polymeride of EPS (Arbib *et al.* 2014).

However, the increasing proportions of DHA and EPS contents in microalgal biofilm did not always correspond to the concentrations of nutrients (i.e. COD, NH_4^+-N and PO_4^{3-}-P) in the three nutrient loadings, which revealed that the amount of microalgal biomass attached on the carrier was not only affected by nutrients in the culturing environment, but also subjected to other factors, e.g. light intensity and temperature. Light competition and environmental temperature may affect the relative abundances of phototrophic species, which would have an effect on the composition and production of EPS standard in the microalgal biofilm (Schnurr *et al.* 2014). Meanwhile, the illumination condition also affected DHA content. Without sufficient light, microalgae failed to produce adequate metabolites for adhesive attraction between microbes and carriers, particularly EPS (Schnurr *et al.* 2014).

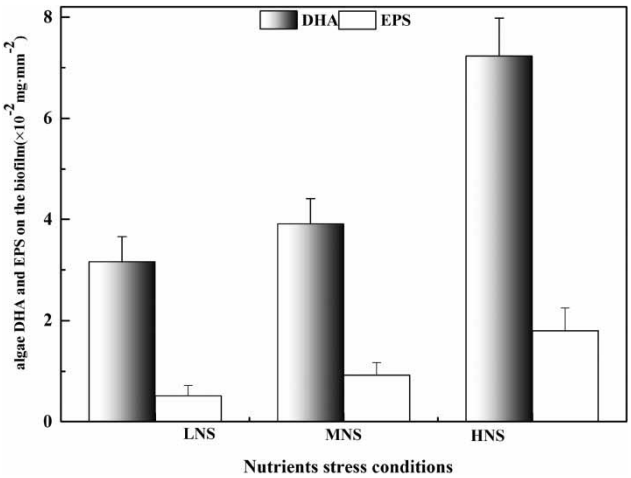

Figure 6 | DHA and EPS contents in microalgal biofilm in three nutrient loading conditions.

CONCLUSIONS

Nutrient loadings have great influence on algae growth as well as their nutrient removal capability. Understanding the relationship between nutrient loading and microalgal biofilm structures and functions could provide essential information for using algae for wastewater treatment. *Anabaena flos-aquae* was adopted as the model microalga and cultivated on carriers to form microalgal biofilm. The study was conducted under three nutrient loading conditions (low, medium and high nutrient concentration conditions, respectively). Results showed that *Anabaena flos-aquae* biofilm under three nutrient loading conditions all had high removal efficiencies of COD, NH_4-N and TP in synthetic wastewater. Nutrient loading had a significant effect on nutrient removal efficiencies of COD、NH_4^+-N and TP, which decreased with the increasing of nutrient loading. However, removal rate of unit cell in unit time, production of DHA and EPS, and thickness of algal biofilm increased with the increasing of nutrient loading. Due to the high nutrient removal rates and simplicity of harvesting microalgal biomass in wastewater treatment by microalgal biofilm, microalgal biofilm technology holds great potential in nitrogen and phosphorus removal and microalgal biomass production in the wastewater remediation process in the future.

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