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Characteristics of extracellular polymeric substances of phototrophic biofilms at different aquatic habitats

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ABSTRACT

Three different phototrophic biofilms obtained from a natural lake (Sample 1), drinking water plant (Sample 2) and wastewater treatment plant (Sample 3) were investigated. Diatoms and green algae were the dominant algae of three biofilms, and the biomass was highest in biofilm of Sample 2. The three phototrophic biofilms also had variable extracellular polymeric substances (EPS) concentrations and compositions. Total EPS concentration of 14.80 mg/g DW was highest in biofilm of Sample 2, followed by biofilms of Samples 3 and 1 (13.11 and 12.29 mg/g DW). Tightly bound EPS (TB-EPS) were the main fraction, and polysaccharides and protein were the main components of total EPS in all three biofilms. However, the compositions of loosely bound EPS (LB-EPS) and TB-EPS were different in three biofilms. Fourier-transform infrared and fluorescence spectra indicated different structure and compositions of LB-EPS and TB-EPS. These results demonstrated the characteristics of EPS produced by phototrophic biofilms varied and had compact relation to their growth environmental conditions.

1. Introduction

Phototrophic biofilms are matrix-enclosed attached microbial communities of phototrophs (cyanobacteria and microalgae) and chemotrophs (archaea, bacteria, fungi and protozoa) driven by light energy (Di Pippo et al., 2012; Guzzon, Bohn, Diociaiuti, & Albertano, 2008; Roeselers, van Loosdrecht, & Muyzer, 2008; Staal et al., 2007). Generally, oxygenic phototrophs, such as cyanobacteria, green algae and diatoms, are typically inhabited in the surface of phototrophic biofilms, which utilizing light energy and reducing carbon dioxide, providing organic substrates and oxygen (Roeselers, van Loosdrecht, & Muyzer, 2007; Roeselers et al., 2008). The produced organic substrates and oxygen would support the growth of the heterotrophs (Roeselers et al., 2007, 2008). These communities play a significant ecological role in the cycling of carbon and nutrients. For example, phototrophic biofilms can be utilized in polishing nutrient-containing effluents from wastewater treatment plant or in algal pond systems, which are generally absence in organic carbon (Roeselers et al., 2008; Wolf, Picioreanu, & van Loosdrecht, 2007). It is also a suitable candidate for bioremediation, aquaculture, etc. (Bender & Phillips, 2004; Chaillan, Gugger, Saliot, Coute, & Oudot, 2006; Di Pippo et al., 2012; Guzzon et al., 2008).

Phototrophic biofilm growth is promoted by the excretion of extracellular polymeric substances (EPS) by the cells, which serves as an adhesive agent enabling cellular attachment and form the biofilm matrix embedding the cells (Wolf et al., 2007). EPS is a complex high-molecular-weight mixture of polymers, which mainly comprise polysaccharides, proteins and humic substances. Its production and composition is influenced by nutrient, growth conditions and environmental conditions (Kavita, Mishra, & Jha, 2013; Sheng, Yu, & Li, 2010). The formed EPS was important to phototrophic biofilms. It can afford a stable environment, promoting the growth of organisms in phototrophic biofilms. Thus, it prevents the losses of organisms and retains bacterial diversity over a long period of time, allowing the development of synergistic relationships in phototrophic biofilms (Ras, Lefebvre, Derlon, Paul, & Girbal-Neuhauser, 2011). Tightly bound EPS (TB-EPS) and loosely bound EPS (LB-EPS) are considered to be responsible for cell adhesion and attachment onto the carrier or cells through strong interactions (Liu et al., 2010; Nielsen & Jahn, 1999). Moreover, EPS could be used as carbon source and energy for the growth

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Table 1
The number and species of algae in three phototrophic biofilms.

Samples	Total number	Diatoms		Green algae		Cyanobacteria	
	cell/cm^2	cell/cm^2	%	cell/cm^2	%	cell/cm^2	%
Sample 1	156,561	87,146	56	50,930	33	9431	6
Sample 2	161,277	60,833	38	70,264	44	4716	3
Sample 3	257,617	170,289	66	43,664	17	35,804	14

of heterotrophs in phototrophic biofilms (Wolf et al., 2007). These characteristics of EPS had significant influence on carbon and nutrients removal by phototrophic biofilms.

Phototrophic biofilms developing highly differentiated architectures are grown on surfaces in a range of terrestrial and aquatic environments, including marine and brackish near-shore environments (Zippel, Rijstenbil, & Neu, 2007), monumental fountains (Cuzman et al., 2010), lithic surface of monuments (Rossi et al., 2012), building stone (Hallmann et al., 2013), etc. The different environmental conditions with variable light, temperature and nutrients have shown to influence microbial community and microbial metabolism of phototrophic biofilms, resulting in the variations of microbial diversity, EPS production and composition, and physicochemical and biological properties of the whole biofilm (Di Pippo et al., 2012).

Therefore, the characteristics of EPS produced by phototrophic biofilms from a natural lake, drinking water plant and wastewater treatment plant were investigated in this work. In addition, the information about the structure and functional properties of EPS was obtained using Fourier-transform infrared (FTIR) spectrophotometer and three-dimensional excitation-emission-matrix (EEM) fluorescence spectrometry. Hopefully, the results of this work would provide a better understanding of the characteristics of phototrophic biofilm and their significant metabolic substrates of EPS under different environmental conditions. With the study of EPS of phototrophic biofilms, we hope to provide useful information on tertiary water treatment with phototrophic biofilms.

2. Materials and methods

2.1. Resources of the phototrophic biofilms

Three different phototrophic biofilms were sampled in spring of 2011 when the biofilms grown vigorously. Sample 1 was collected from a natural lake in Nanjing city, Jiangsu province of China (N 32.03637°, E 118.76111°), which was coated in rock along the shore under water about 10–20 cm. Sample 2 was obtained from the tank wall of clarifier under water 10–20 cm in a drinking water plant of Nanjing city (N 32.11796°, E 118.77004°). Sample 3 was collected from the wall of secondary settler under water 10–20 cm in a wastewater treatment plant (N 32.01975°, E118.69340°). Biofilms were transported to the laboratory and stored at 4 °C for analysis and EPS extraction.

2.2. EPS extraction

The EPS extraction process was performed according to the protocol described by Liang, Li, Yang, and Du (2010). Biofilm LB-EPS was extracted and harvested by using the regular centrifugation method described by Zhang and Bishop (2003) with modification. About 1.0 g biofilm (dried weight) was put into a centrifugation tube along with 10 mL MilliQ water and 0.06 mL formamide (37%) was added into suspension. The formamide was used to enhance efficiency of LB-EPS extraction and decrease contamination by intracellular substances (Liang et al., 2010). The tube was shaken gently in shaking incubator at 150 rpm and 4 °C for 1 h and then centrifuged at 5000 × *g* and 4 °C for 15 min. The supernatant was filtered through 0.45 μm acetate cellulose membranes to represent LB-EPS.

The biofilm pellet was re-suspended again with 10 mL buffer (2 mM $Na_2PO_4{\cdot}12H_2O$, 4 mM $NaH_2PO_4{\cdot}H_2O$, 9 mM NaCl, 1 mM KCl, pH = 7), followed by the cation exchange resin (CER) addition with a dosage of 70 g/g volatile suspended solids (VSS). These suspensions were stirred for 1 h at 600 rpm and 4 °C. After removing CER by settlement, the solutions were centrifuged at 10,000 × *g* and 4 °C for 15 min to remove remaining biofilm components. The supernatants were then filtered through 0.45 μm cellulose acetate membranes to represent TB-EPS.

2.3. Analytic methods

The biofilm biomass was determined with biofilm lipid phosphorus method (Wu et al., 2010), which was expressed as the number of cells. The algae number was measured with microscope count method (Jiang & Tu, 1990). The concentrations of chlorophyll a, chlorophyll b and chlorophyll c were measured using the standard methods (APHA, 1998). The content of carbohydrates in EPS was measured with the anthrone method (Raunkjaer, Hvitved-Jacobsen, & Nielsen, 1994), and the contents of proteins and humic substances were determined using the modified Lowry methods (Frolund, Palmgren, Keiding, & Nielsen, 1996).

2.4. EEM and FTIR spectrometry

Three-dimensional excitation-emission-matrix (EEM) fluorescence spectrometry was used to characterize the EPS of biofilm. All EEM spectra were measured using a luminescence spectrometry (F7000, Hitachi, Japan). The EEM spectra were collected with subsequent scanning emission spectra from 200 to 450 nm at 0.5 nm increments by varying the excitation wavelength from 200 to 600 nm at 10 nm increments. Excitation and emission slits were both maintained at 10 nm, and the scanning speed was set at 1200 nm/min for all measurements. The spectrum of the deionized water was recorded as the blank (Sheng & Yu, 2006). The software MatLab 7.0 (MathWorks Inc., USA) was employed for handling EEM data.

The EPS from all extracted samples was dried at 60 °C. The compositions of the EPS were compared using a Fourier-transform infrared (FTIR) spectrophotometer. The powders were mixed with IR grade KBr powders at a mass ratio of 1:100 (Liang et al., 2010). The prepared pellets were placed in a sample cell that was fitted to the sample compartment of the FTIR spectrometer (Nexus870, Nicolet, USA).

3. Results and discussion

3.1. Characteristics of phototrophic biofilms

A large number of rod-shaped, spherical, rhombic, navicular, slug and filamentous bacteria, algae and organic, inorganic debris were observed in three biofilms through optical microscope. The dominant algae species in different biofilms are listed in Table 1 by microscopic counting. Diatoms, green algae, cyanobacteria,

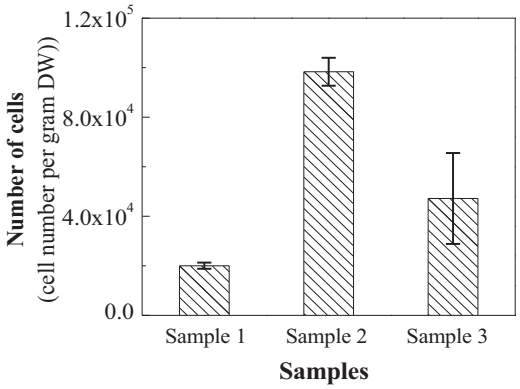

Fig. 1. The biomass of three different phototrophic biofilms.

and a small amount of yellow algae, euglena and charophytes composed of the algae community. The phototrophic biofilm of Sample 3 contained the largest number of algae, mainly dominating by diatoms, while the phototrophic biofilm collected from natural lake (Sample 1) contained the least number of algae. Both phototrophic biofilms of Samples 1 and 2 were dominated by diatoms and green algae, which play an important role in the light energy capture of phototrophic biofilms to maintain bacteria and algae stable in symbiotic environment (Wilhelm et al., 2006).

Fig. 1 presents the biomass results of three phototrophic biofilms. The biomass in phototrophic biofilm of Sample 2 was higher than that in phototrophic biofilms of Samples 1 and 3. Due to the presence of flocculant, which played an important role in the early period of biofilm development and provided as a good artificial substrate for microbial growth, the microbial community was more likely to be attached to the wall of clarifier tank in drinking water plant. Thus, most biomass would be retained in phototrophic biofilm of Sample 2. The biomass in phototrophic biofilm of Sample 1 was least. This sample was extracted from a natural lake, which had the low concentrations of N, P and organic matter. The oligotrophic environment led to least biomass among the three phototrophic biofilms. Furthermore, the attached biofilm on rock along the shore was susceptible to external interference, resulting in biofilm lost.

Table 2
The concentrations of chlorophyll in three phototrophic biofilms.

Samples	Chl-a (μg/g)	Chl-b (μg/g)	Chl-c (μg/g)
Sample 1	193.74 ± 21.57	2.64 ± 0.37	51.70 ± 1.36
Sample 2	264.34 ± 16.89	14.52 ± 6.33	55.11 ± 10.38
Sample 3	287.56 ± 14.96	11.90 ± 2.80	55.49 ± 7.94

The concentrations of chlorophyll in the three phototrophic biofilms were listed in Table 2. The results demonstrated that the content of chlorophyll was highest in phototrophic biofilm of Sample 3 and lowest in phototrophic biofilm of Sample 1. The results were in consistent with algal number listed in Table 1. Chlorophyll b content was a bit difference from chlorophyll a, which was highest in phototrophic biofilm of Sample 2. To chlorophyll c, the content was about the same level in three phototrophic biofilms, which was about 55 μg/g.

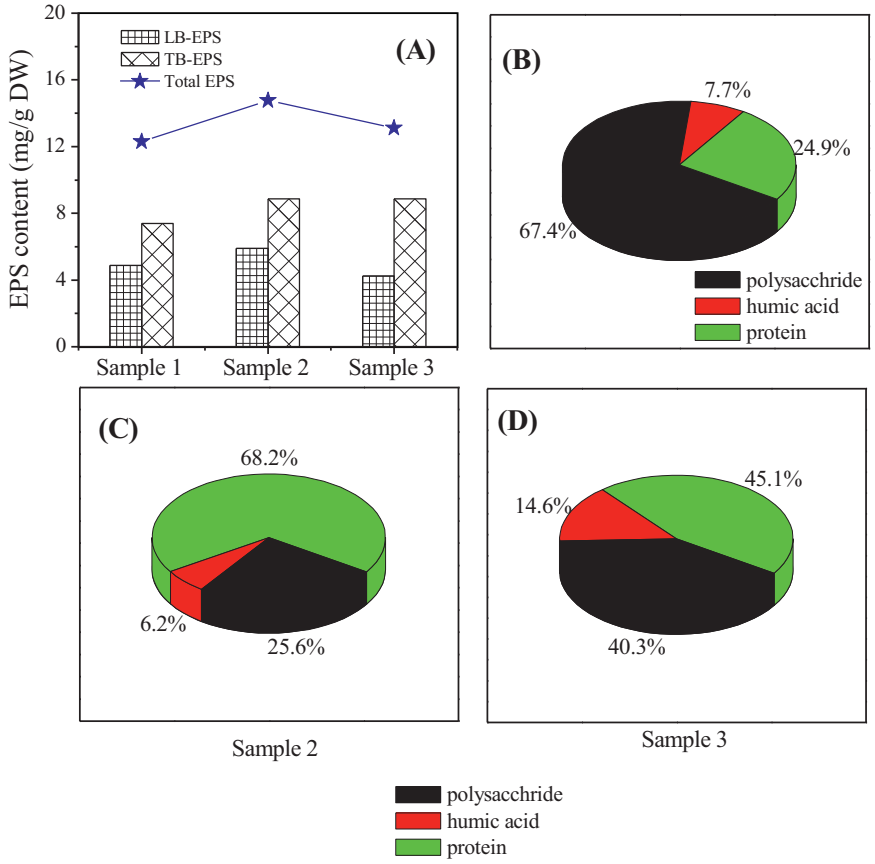

Fig. 2. The contents of EPS (A), and the fractions of polysacchrides, proteins and humic acids of EPS extracted from three phototrophic biofilms: (B) Sample 1; (C) Sample 2; and (D) Sample 3.

3.2. Contents and constituents of EPS in phototrophic biofilms

Fig. 2 presents the contents and constituents of EPS extracted from the three different phototrophic biofilms. The EPS amount (total contents of LB-EPS and TB-EPS) of phototrophic biofilm grown in Sample 2 was 14.77 mg/g DW, which was higher than those of phototrophic biofilms collected from Samples 3 and 1 (13.11 and 12.29 mg/L, respectively). It is reported the main components of EPS are polysaccharides, proteins and humic substances (Sheng et al., 2010). In phototrophic biofilm of Sample 3, polysaccharides and proteins were major composition compared to humic acid (Fig. 2). In phototrophic biofilm of Sample 1, polysaccharides were the main constituent and their fractions were 67.5%, while the key compositions in phototrophic biofilm of Sample 2 were proteins with their fractions of 68.3%. The different environmental conditions would give rise to variable microbial community and microbial metabolism of phototrophic biofilms, leading to heterogeneous content and constituent of EPS (Di Pippo et al., 2012; Wingender, Neu, & Flemming, 1999).

The structure of bound EPS is generally depicted by a two-layer model, which is composed of LB-EPS and TB-EPS (Nielsen & Jahn, 1999). In all three phototrophic biofilms, TB-EPS were the main fraction of total EPS. The LB-EPS and TB-EPS fractions were 32.4% and 67.6%, respectively, in phototrophic biofilm of Sample 3. The phototrophic biofilms grown in Samples 1 and 2 nearly have the same fractions of LB-EPS and TB-EPS (about 40% vs 60%). The results revealed that the EPS extracted from the three phototrophic biofilms had similar fractions of each EPS.

The compositions of LB-EPS and TB-EPS were shown to be different in the three phototrophic biofilms. As illustrated in Fig. 3, polysaccharides were the main constituent of LB-EPS and TB-EPS in phototrophic biofilm of Sample 1. Oppositely, protein became the main component of LB-EPS and TB-EPS in phototrophic biofilm of Sample 2. To phototrophic biofilm of Sample 3, protein was the main component of LB-EPS, however, polysaccharides was the main component of TB-EPS. Thus, the contents and compositions of EPS were varied significantly among the three phototrophic biofilms grown under different environmental conditions.

In three phototrophic biofilms, the EPS content of phototrophic biofilm in Sample 2 was highest, corresponding to the highest biomass in Sample 2. However, phototrophic biofilm of Sample 1 had the least biomass, resulting in lowest EPS content. These results demonstrated the EPS content of phototrophic biofilms was positive related to the biomass. The phototrophic biofilm of Sample 1 had the highest fraction of algae, leading to highest polysaccharides proportion in EPS of Sample 1. Oppositely, lowest polysaccharides proportion in EPS of Sample 2 was due to the lowest algae proportion in phototrophic biofilm of Sample 2. These results indicated that polysaccharides proportion in EPS was positive related with algae fraction in phototrophic biofilms. The result was consistent with the study of Xu, Cai, Yu, and Jiang (2013), who found the polysaccharides were the main composition in algae EPS and the content and proportion of polysaccharides was higher than that of proteins. Since the content and proportion of polysaccharides in EPS play a significant role in cell attachment and structure stabilization in biofilms (Dogsa, Brloznik, Stopar, & Mandic-Mulec, 2013) and its production was influenced by algae fraction, more effect of algae on the formation and structure stability of phototrophic biofilms should be investigated in further work. This will be important in tertiary wastewater treatments with phototrophic biofilms.

3.3. FTIR spectra and EEM analysis of EPS

The FTIR spectra of extracted LB-EPS and TB-EPS from phototrophic biofilm of Sample 3 was illustrated in Fig. 4. The spectra of extractants from the other two phototrophic biofilms are very

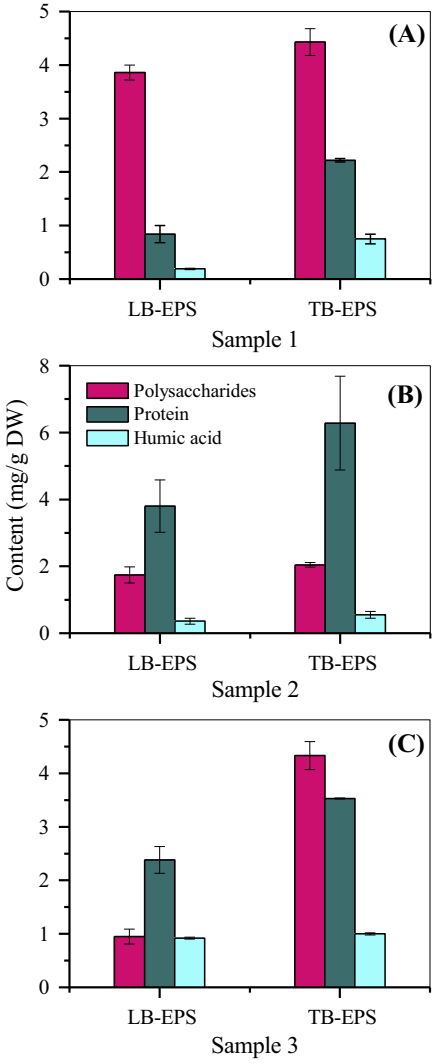

Fig. 3. The contents and compositions of LB-EPS and TB-EPS extracted from three phototrophic biofilms: (A) Sample 1; (B) Sample 2; and (C) Sample 3.

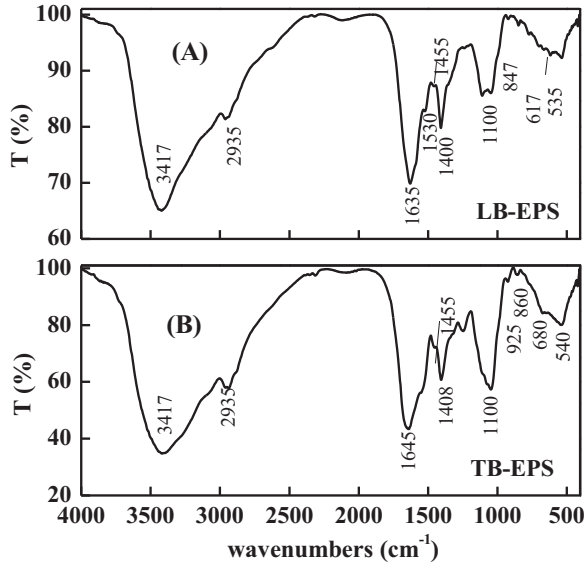

Fig. 4. FTIR spectra of LB-EPS (A) and TB-EPS (B) extracted from phototrophic biofilm of Sample 3.

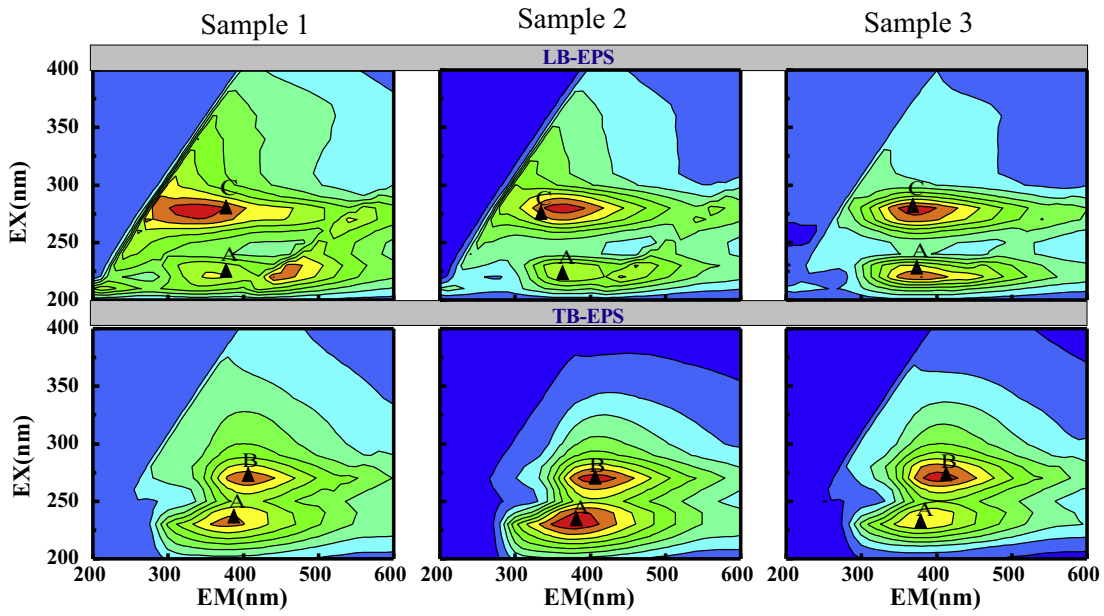

Fig. 5. Three-dimensional EEM spectra of extracted LB-EPS and TB-EPS from phototrophic biofilms of Sample 1, Sample 2 and Sample 3.

similar to those in Fig. 4 (results not shown). The broad band around 3417 cm^{-1} was related to the stretching vibration of both hydroxyl (of the carbohydrates) and amino groups (of the proteins) (Fig. 4) (Liang et al., 2010). The absorption bands at 1635–1645 cm^{-1} was formed by the stretching vibration of C=O and C=N (amide I), and the absorption bands around 1530 cm^{-1} was related to the stretching vibration of C—N and deformation vibration of N—H groups in the presence of proteins (amide II) (DAbzac, Bordas, van Hullebusch, Lens, & Guibaud, 2010). Furthermore, weak bands at 1240 and 1246 cm^{-1} were assigned to amide III vibration (Cheng et al., 2013). The band near 1400 cm^{-1} is the absorption of the C—H deformation vibrations in the methyl, while the weak absorption bands at 2935 cm^{-1} was assigned to the asymmetrical C—H stretching vibration of the aliphatic CH_2-group (Cheng et al., 2013; Liang et al., 2010). The band near 1100 cm^{-1} was correlated with the C—O—C stretching vibrations from the carbohydrates and aromatics (DAbzac et al., 2010). Bands at 600–900 cm^{-1} reflect the existence of unsaturated bonds in the sample (Guibaud, Comte, Bordas, Dupuy, & Baudu, 2005). The band results demonstrated that more carbohydrates were present in TB-EPS (Liang et al., 2010). The FTIR qualitative spectra of LB-EPS and TB-EPS were highly consistent as illustrated in Fig. 3. The amide III vibration represented β-sheet in both LB-EPS and TB-EPS. However, there were some differences between LB-EPS and TB-EPS in qualitative points. The amide I in LB-EPS was assigned to the β-sheet, while that in TB-EPS was attributed to random coils (Cai & Singh, 1999; Tretinnikov & Tamada, 2001).

The three-dimensional EEM spectra of extracted LB-EPS and TB-EPS from three phototrophic biofilms are shown in Fig. 5. Two peaks were identified from the EEM fluorescence spectra of the LB-EPS extracts. The first peak was identified at the excitation/emission wavelength (Ex/Em) of 230/375 nm (Peak A), which was attributed to the tyrosine protein-like fluorescence (Chen, Westerhoff, Leenheer, & Booksh, 2003; Li, Sheng, Liu, & Yu, 2008). The second peak at the Ex/Em of 280/360 nm (Peak C) was also identified as the fluorescence of protein-like substances. The TB-EPS extracts had a similar fluorescence peak at the excitation/emission wavelength (Ex/Em) of 230/375 nm as LB-EPS, which was the protein fluorescence. The other peak of TB-EPS at the Ex/Em of 270/410 nm (Peak B) was described as the fluorescence of humic-like substances (Pons, Bonté, & Potier, 2004).

4. Conclusions

The characteristics of EPS produced by three different phototrophic biofilms from a natural lake (Sample 1), drinking water plant (Sample 2) and wastewater treatment plant (Sample 3) were investigated. Different environments affected the algae number, species and biomass of the three phototrophic biofilms. The biomass in the phototrophic biofilm of Sample 2 was highest, while the phototrophic biofilm in Sample 3 contained the largest number of algae. The concentrations of total EPS, TB-EPS and LB-EPS varied due to the different aquatic habitats of the three phototrophic biofilms, and their structure and compositions of TB-EPS and LB-EPS also dependent on the living environments. These results demonstrated that the growth environmental conditions significantly influence the characteristics of EPS produced by phototrophic biofilms.

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