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Fluorescent quenching for biofilm extracellular polymeric substances (EPS) bound with Cu(II)

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ABSTRACT

The Cu(II) binding properties of loosely bound extracellular polymeric substances (LBEPSs) and tightly bound EPSs (TBEPSs) extracted from biofilm samples at two apparent molecular weight (AMW) ranges, >14 kDa and 1–14 kDa, were investigated using three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy. The protein-like and aromatic protein fluorescence peaks were identified in EEM fluorescence spectra as peaks A and B, respectively. The intensities of peaks A and B were generally quenched when Cu(II) was bound with LBEPSs or TBEPSs with AMWs > 14 kDa at various pH levels. Conversely, for 1–14 kDa EPSs, fluorescence intensities of peaks A and B were not quenched when Cu(II) was bound with LBEPSs at pH 4 or with both LBEPSs and TBEPSs at pH 8. The Stern–Volmer constant ($\log K_{sv}$) for the Cu(II)–LBEPSs and Cu(II)–TBEPSs binding processes were 2.38–4.37. The capability of EPSs to bind with Cu(II) increased as pH increased. At pH > 4, the protein-like substances and aromatic proteins in TBEPSs had greater Cu(II) binding capability than LBEPSs. Additionally, the EPSs with AMWs > 14 kDa had stronger binding capability with Cu(II) than EPSs with AMWs of 1–14 kDa. The difference in Cu binding behavior of LBEPS and TBEPS significantly affect the mobility, bioavailability, and toxicity of Cu in aquatic environments.

1. Introduction

Biofilms, which exist in both natural and artificial environments, comprise microbial cells embedded in a matrix of extracellular polymeric substances (EPSs). These EPSs may account for 50–90% of total carbon in a biofilm principally composed of carbohydrates, proteins, lipids, nucleic acids, and heteropolymers [1,2]. Lee *et al.* [3] labeled EPSs in aerobic granules using fluorochromes and confocal laser scanning microscopy. Rosenberger and Kraume [4] differentiated EPSs into extractable, tightly bound (TB) and soluble EPSs. Other classification paradigms classified EPSs as "loosely bound" (LB) and TB fractions [5–9].

Li and Yang [10] demonstrated that the quantity of LBEPSs in activated sludge correlated with activated sludge flocculation and sedimentation. Ramesh *et al.* [11] noted that the fouling potential of membrane filtration of wastewater sludge was mainly attributable to the LBEPS fraction in sludge. Chen *et al.* [12] noted that removal of >90% of heavy metals by biofilms was attributed to the binding capacity of EPSs. This metal binding capacity can result from several physico-chemical and biological processes, such as ion exchange, complexation, and surface microprecipitation, due to various metal binding functional groups on the surfaces of EPSs [13,14]. The hydroxy, carboxyl, phosphoric amine, hydroxyl groups, and amidocyanogen of proteins, polysaccharides and phospholipids, which can negatively charge a surface, act as strong metal binding sites to form organo-metal complexes [15]. Three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy was utilized to examine the chemical nature of dissolved organic matter and its interaction with metals [16–19].

The mobility and availability of heavy metals in environments are affected by the presence, forms (LB or TB), and quantities of EPSs as they are ubiquitous and have an excellent metal binding capacity [2]. Furthermore, compared with TBEPSs, LBEPSs are labile and have a liquid phase that releases bound metals more readily back into an aqueous surrounding. This study investigates the fluorescence properties of TBEPSs and LBEPSs with two apparent molecular weight (AMW) extracted from a natural algal–bacterial biofilm and their fluorescence quenching titration with Cu(II) under various pH levels. The Stern–Volmer constants for the Cu(II)–LBEPS and Cu(II)–TBEPS binding processes were estimated. The difference in Cu binding behaviors of LBEPSs and TBEPSs was discussed.

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2. Materials and methods

2.1. EPS extraction

Natural algal–bacterial biofilm was collected from a pond in Guiyang City, China. The biofilm was rinsed thoroughly with deionized water and Milli-Q water. The LBEPSs and TBEPSs were extracted using a physical and a chemical method, respectively [20–22]. That is, the biofilm suspension was extracted using 20,000 × *g* centrifugation for 20 min at 4 °C; the collected supernatant was the LBEPS fraction. The residue was first resuspended in 2% (w/w) ethylenediaminetetraacetic acid (EDTA) solution for 3 h at 4 °C; the suspension collected following 20,000 × *g* centrifugation for 20 min at 4 °C was the TBEPS fraction. The so-collected LBEPS and TBEPS fractions were filtered through a 0.22-μm acetate cellulose membrane at 25 °C. Filtrates were individually dialyzed at 4 °C with dialysis bags with a molecular weight cut-off of 14 kDa. The solutions remaining in dialysis bags were EPSs with AMWs > 14 kDa. The dialyzed solutions were further dialyzed using dialysis bags with a molecular weight cut-off of 1 kDa; those remaining in the dialysis bags were EPSs with AMWs of 1–14 kDa. All EPS samples were immediately lyophilized and stored at −20 °C.

2.2. Fluorescence measurements

The fluorescence spectra of EPS solutions were identified using an F-4500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). A 450-W Xenon lamp was the excitation source. The EEM was collected every 5 nm over an excitation range of 200–400 nm, with an emission range of 250–550 nm increased incrementally by 2 nm. Excitation and emission slits were 5 and 10 nm of band-pass, respectively. Scan speed was 1200 nm/min. The fluorometer's response to a Milli-Q water blank solution was subtracted from fluorescence spectra collected for samples containing EPSs and Cu(II) under the same condition. All experiments were conducted in triplicate with mean values reported.

In total, 10 ml EPSs (7.5 mg/l) in a 25-ml beaker were titrated with μl addition of 0.1 M Cu(II) at 25 °C. After each addition of Cu(II), the solution was mixed fully by magnetic stirring for 15 min. Fluorescence quenching titration curves of EPSs by Cu(II) at pH 4, 6, and 8 were reported. Solution pH was maintained unchanged during titration.

2.3. Chemical analysis

A stock Cu(II) solution (0.1 M) was prepared by dissolving $CuCl_2$ into Milli-Q water. Total organic carbon (TOC) content in samples

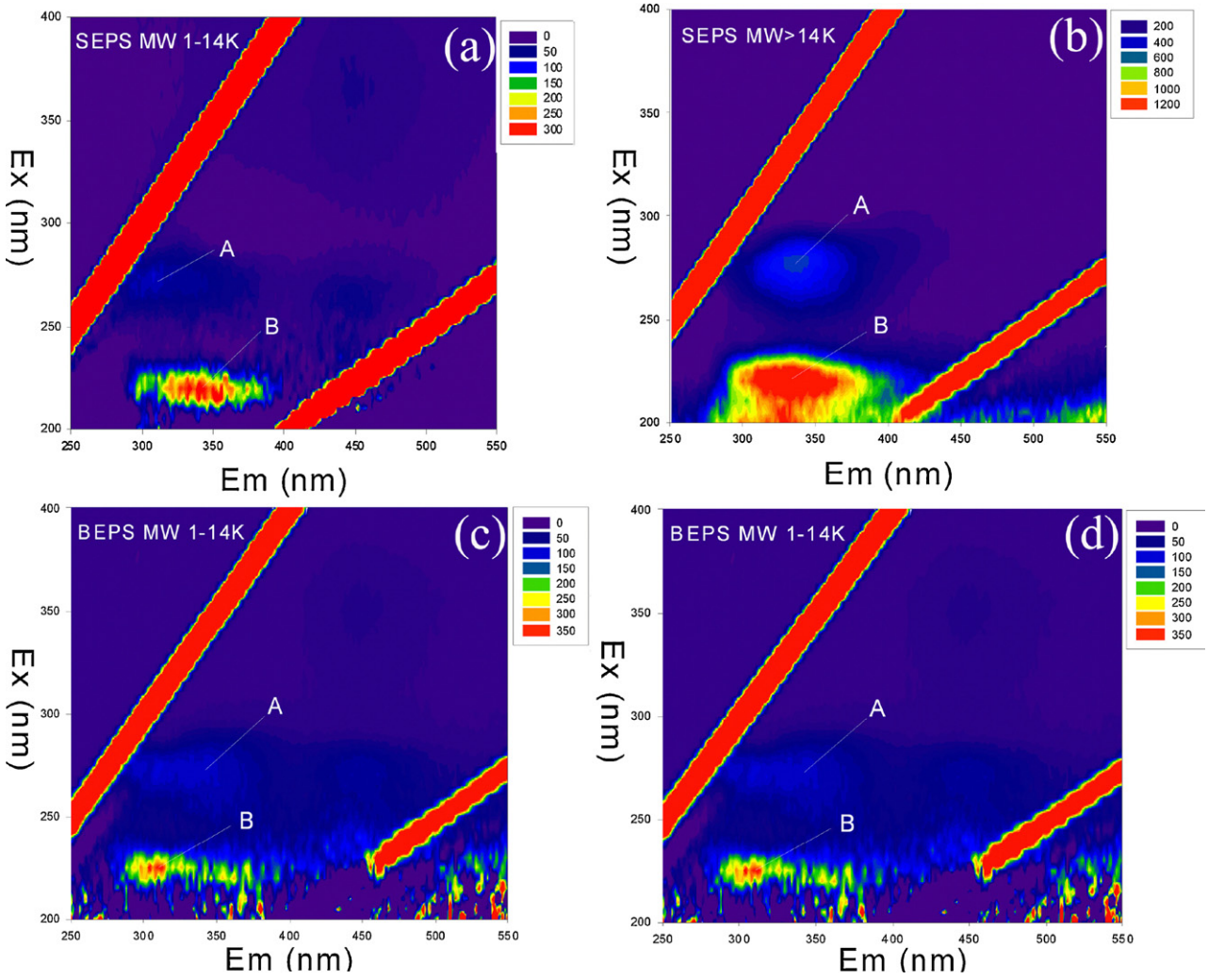

Fig. 1. EEM fluorescence spectra of EPS at pH 6. (a) LBEPS with AMW of 1–14 kDa, (b) LBEPS with AMW of >14 kDa, (c) TBEPS with AMW of 1–14 kDa, and (d) TBEPS with AMW of >14 kDa.

was measured by a TOC analyzer (TOC 5000; Shimadzu, Tokyo, Japan).

3. Results and discussion

3.1. The EEM spectra

Fig. 1 shows the EEM fluorescence spectra of LBEPSs and TBEPSs with two AMWs at pH 6. Two peaks, A (Ex/Em = 270–280/302–344 nm) and B (Ex/Em = 205–240/296–340 nm), existed on EEM spectra of all EPS samples and were identified as protein-like substances and aromatic proteins, respectively. Peak A for LBEPSs with AMWs > 14 kDa was further identified as tryptophan-like substances, and peak A for TBEPSs with AMWs > 14 kDa was attributed to tryptophan and tyrosine-like substances [23]. Similarly, peak A for LBEPSs of 1–14 kDa was assigned to tryptophan and tyrosine-like substances, and peak A for TBEPSs belonged to tyrosine-like substances.

Peaks A and B for LBEPSs and TBEPSs in the biofilm were located at similar positions (Fig. 1). Peaks A and B for 1–14 kDa EPSs had greater blue shifts than those for >14 kDa EPSs. Additionally, all EEM peaks for the biofilm EPSs had greater blue shifts than those for activated sludge samples [18,24]. Restated, the molecular structures of fluoropheres in biofilm EPSs were stiffer than those for activated sludge EPSs.

Fluorescence quenching titration tests were conducted for peaks A and B of all EPS samples at pH 4, 6, and 8 (Fig. 2). For >14 kDa EPSs, fluorescence intensities of peaks A and B decreased

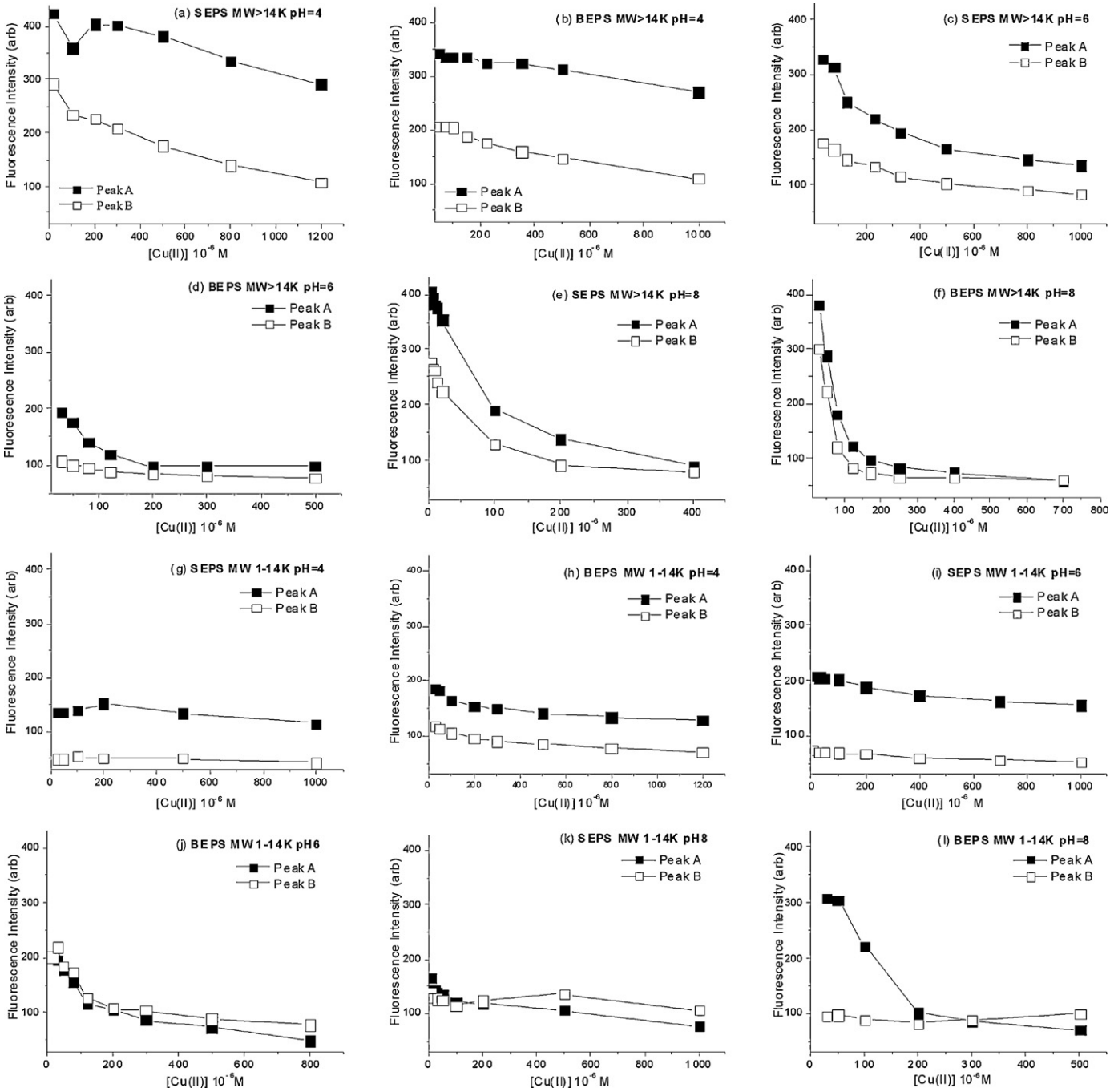

Fig. 2. Fluorescence quenching of EPS titrated with a Cu(II) solution under various pH conditions.

markedly as the Cu(II) concentration increased (Fig. 2(a)–(f)), indicating that protein-like and aromatic substances with AMWs > 14 kDa can bind to Cu(II) at all tested pH levels. Comparably, the fluorescence quenching effects by Cu(II) were relatively much weaker for 1–14 kDa EPSs; in some tests no quenching existed (Fig. 2(g)–(l)). For instance, at pH 4, the fluorescence intensities of peaks A and B in LBEPSs changed slightly as the Cu(II) concentration changed, implying that low-AMW protein-like substances and aromatic proteins in LBEPSs did not interact with Cu(II) under acidic conditions. Conversely, at pH 6, the fluorescence intensities of peaks A and B for both LBEPSs and TBEPSs were quenched by Cu(II), indicating that the low-AMW protein-like substances and aromatic proteins in both LBEPSs and TBEPSs bound to Cu(II) at pH 6. At pH 8, the fluorescence intensities of peak A for both LBEPSs and TBEPSs decreased markedly as the Cu(II) concentration increased; no marked fluorescence quenching of peak B was noted. This finding suggests that, under a slightly alkaline condition, low-AMW protein-like substances in both EPS fractions strongly bound to Cu(II); however, low-AMW aromatic proteins did not. Generally, 1–14 kDa EPSs in TBEPSs have stronger binding capability with Cu(II) than those in LBEPSs.

3.2. Fluorescence quenching processes

The following Stern–Volmer equation was used to correlate the fluorescence quenching data:

$$\frac{F_0}{F} = 1 + K_{sv}[\mathrm{Cu(II)}] = 1 + k_q \tau_0 [\mathrm{Cu(II)}] \quad (1)$$

where F_0 and F are fluorescence intensities in the absence and presence of Cu(II), respectively; K_{sv} the Stern–Volmer constant; [Cu(II)] is the Cu(II) concentration, and

$$K_{sv} = k_q \tau_0 \quad (2)$$

where k_q is the quenching rate constant of the biological macromolecule, and τ_0 is the average lifetime of the molecule without a quencher, taken as 10^{-8} s in this study [25]. Table 1 lists the best Stern–Volmer constants based on fluorescence titration data fitting (r^2 = 0.921 to 0.998). The Stern–Volmer constants ($\log K_{sv}$) for 1–14 kDa LBEPSs at pH 4 and those for peak B for both LBEPSs and TBEPSs were not calculated as no fluorescence quenching was observed.

The values of $\log K_{sv}$ for complexation of biofilm LBEPSs and TBEPSs with Cu(II) (2.38–4.37) were very close to those reported in literature for complexation of activated sludge EPSs and heavy metals [26–29]. According to Zhang *et al.* [19], the $\log K_a$ values of a biofilm EPS–Hg(II) complex were 3.28–4.48. The relative stability constant log (K) values of the complex between metals (*i.e.*, Cu, Pb, Cd, and Ni) and activated sludge EPSs were 3.0–4.4, 0.45–1.28, 1.54–3.35 and 2.6–3.0, respectively [26,27]. Comte *et al.* [28] demonstrated that the conditional binding constants for Cu(II), Cd(II), and Pb(II) to sludge EPSs were 3.2–4.5, 3.7–5.0 and 3.9–5.7, respectively.

Except for >14 kDa EPSs at pH 4, the $\log K_{sv}$ values for peaks A and B for TBEPSs were higher than those for LBEPSs, indicating that protein-like substances and aromatic proteins in TBEPSs can bind more Cu(II) than those in LBEPSs. This analytical observation conflicts with that by Pan *et al.* [18], who noted that activated sludge LBEPSs bind more dicamba than TBEPSs. The $\log K_{sv}$ value generally increased as solution pH increased, indicating that the surface charge of functional groups of EPSs, such as carboxylates and phosphates, has an important role in Cu(II) binding capability [13]. Moreover, the >14 kDa EPSs had greater $\log K_{sv}$ than 1–14 kDa EPSs, which is attributable to excess functional groups inhibited by high-AMW EPSs as binding sites for Cu(II).

The k_q values for peaks A and B in both LBEPSs and TBEPSs were in the range of 2.39×10^{10} to 2.33×10^{12} l/(M s), exceeding the maximum scattering collision quenching constant of various quenchers (2.0×10^{10} l/(M s)) (Ware [30]). This observation suggests that the fluorescence quenching process of peaks A and B in both EPS fractions is likely initiated by a static quenching process.

3.3. Binding constants and binding sites

In the static quenching process, the binding constant (K_b) and number of binding sites (n) for EPS–Cu(II) systems can be calculated as follows:

$$\log\left[\frac{F_0 - F}{F}\right] = \log K_b + n\log[\mathrm{Cu(II)}] \quad (3)$$

where ($F_0 - F$) is the fraction of quenched fluorescence with Cu(II) binding; the binding constant, K_b, is the binding strength of fluorophore; and n represents the binding sites provided by fluorophore to Cu(II).

Table 2 lists the binding constants and binding sites of EPS–Cu(II). The values of $\log K_b$ for TBEPSs were greater than those for LBEPSs, except for EPS > 14 kDa at pH 4, indicating that TBEPSs have a higher binding strength than LBEPSs. The n values for the EPS–Cu system around or exceeding unity suggest the presence of only one interactive site type in EPSs for Cu(II). The n values for the 1–14 kDa EPS–Cu system were <1 (range, 0.43–0.74), indicating negative cooperation between multiple binding sites and the presence of more than one Cu binding site.

The LBEPSs can be used by invertebrates quickly and easily, whereas proteins in TBEPSs may be difficult to access [31]. Notably, EPSs may also be a pathway for the transfer of contaminants into food webs [32]. The different Cu(II) binding properties of LBEPSs and TBEPSs in natural biofilms may affect the transport, bioavailability, and toxicity of metals in aquatic environments. For instance, the heavy metals bound with TBEPS at >14 kDa AMW should have much lower mobility than those with LBEPS at 1–14 kDa AMW. To estimate the total sum of specific heavy metals gives incomplete information in toxicity risk assessment for environment.

Table 1
The calculated Stern–Volmer constants of peaks A and B at various pH levels.

pH	$\log K_{sv}$							
	AMW > 14 kDa EPS				1–14 kDa EPS			
	LBEPS		TBEPS		LBEPS		TBEPS	
	Peak A	Peak B	Peak A	Peak B	Peak A	Peak B	Peak A	Peak B
4	2.59	3.16	2.45	2.98	NA	NA	2.38	2.61
6	3.17	3.06	3.75	3.16	2.63	2.70	3.68	3.62
8	3.95	3.83	4.33	4.37	2.98	NA	3.90	NA

NA: not available.

Table 2
The binding constants $\log K_b$ and binding sites n for SEPS–Cu(II) system and BEPS–Cu(II) system at various pHs.

MW	pH	LBEPS Peak A			LBEPS Peak B			TBEPS Peak A			TBEPS Peak B		
		$\log K_b$	n	r^2	$\log K_b$	n	r^2	$\log K_b$	n	r^2	$\log K_b$	n	r^2
>14 kDa	4	3.47	1.32	0.965	3.11	0.99	0.996	3.09	1.23	0.945	3.63	1.23	0.995
	6	3.85	1.19	0.858	3.03	0.97	0.964	5.92	1.58	0.933	3.09	0.98	0.959
	8	4.51	1.12	0.989	4.70	1.18	0.974	5.99	1. 48	0.918	5.69	1.39	0.843
1–14 kDa	4	–	–	–	–	–	–	1.22	0.52	0.997	1.87	0.69	0.981
	6	3.81	1.32	0.975	1.87	0.74	0.962	5.08	1.39	0.926	4.94	1.37	0.898
	8	1.20	0.43	0.966	–	–	–	10.13	2.77	0.827	–	–	–

–: No significant fluorescence quenching at this pH so with no value of $\log K_b$ being reported.

4. Conclusions

Fluorescence properties and Cu(II) binding properties of biofilm LBEPSs and TBEPSs in two AMW ranges (>14 kDa and 1–14 kDa) were characterized using EEM fluorescence spectroscopy. Fluorescence quenching of LBEPSs and TBEPSs by Cu(II) was initiated by a static quenching process. The protein-like substances and aromatic proteins in TBEPSs had better Cu(II) binding capability than LBEPSs. The >14 kDa EPSs generally had stronger binding ability to Cu(II) than 1–14 kDa EPSs. Alkaline environments favor Cu(II) binding to EPSs. Differences in the binding capability of different EPS fractions affect the transport and bioavailability of Cu(II) in particular aquatic environments.

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