

# Biosorption of Cu(II) to extracellular polymeric substances (EPS) from *Synechocystis* sp.: a fluorescence quenching study

Xiangliang PAN (✉)<sup>1</sup>, Jing LIU<sup>2</sup>, Wenjuan SONG<sup>1</sup>, Daoyong ZHANG<sup>2</sup>

<sup>1</sup> State Key Laboratory of Desert and Oasis Ecology,

Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China

<sup>2</sup> State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550002, China

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**Abstract** Biosorption of extracellular polymeric substances (EPS) from *Synechocystis* sp. (cyanobacterium) with Cu(II) was investigated using fluorescence spectroscopy. Three fluorescence peaks were found in the excitation-emission matrix (EEM) fluorescence spectra of EPS. Fluorescence of peak A (Ex/Em = 275/452 nm) and peak C (Ex/Em = 350/452 nm) were originated from humic-like substances and fluorescence of peak B (Ex/Em = 275/338 nm) was attributed to protein-like substances. Fluorescence of peaks A, B, and C could be quenched by Cu(II). The effective quenching constants ( $\lg K_a$ ) were 2.8–5.84 for peak A, 6.4–9.24 for peak B, and 3.48–6.68 for peak C, respectively. The values of  $\lg K_a$  showed a decreasing trend with increasing temperature, indicating that the quenching processes were static in nature. The binding constants ( $\lg K_b$ ) followed the order of peak A > peak B > peak C, implying that the humic-like substances in EPS have greater Cu(II) binding capacity than the protein-like substances. The binding site number,  $n$ , in EPS-Cu(II) complexes for peaks A, B, and C was less than 1. This suggests the negative cooperativity between multiple binding sites and the presence of more than one Cu binding site.

**Keywords** biosorption, conditional binding constant, extracellular polymeric substances (EPS), fluorescence quenching

## 1 Introduction

Extracellular polymeric substances (EPS), excreted by microorganisms, are mixture of macromolecules including

polysaccharides, proteins, nucleic acids, and humic substances [1]. EPS contain a number of negative functional groups such as carboxyl, phosphoric, amine and hydroxyl groups [2] so that they have strong adsorptive capacity for heavy metals [3–6]. Liu et al. showed that 1 mg of EPS could adsorb 0.25–1.48 mg of heavy metals [3]. The interaction of EPS and heavy metal ions can form complexes and consequently contribute much to biosorption of heavy metals.

Copper is an essential micronutrient for synthesis of various proteins and plays an important role in numerous metabolic processes. However, Cu in excess is highly toxic to organisms by modifying membrane permeability, protein synthesis, enzymatic activities, photosynthetic and respiratory processes [7,8].

There are a few reports on Cu(II) biosorption of EPS [9,10]. The maximum Cu(II) biosorption capacity of EPS in literature varies greatly, depending on the sources of EPS, from 7.81 mg·g<sup>-1</sup> for *Paenibacillus jamilae* [11] to 1602 mg·g<sup>-1</sup> for *Paenibacillus polymyxa* [9]. Recently, the binding constants of EPS for heavy metals including Cu(II) have also been calculated using polarographic method [2,12–14]. However, limited quantitative information was available about conditional stability constants and binding constants of EPS from cyanobacteria toward heavy metals.

Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy was well known for its speediness, good selectiveness and high sensitiveness in detecting fluorophorous substances [15]. The EEM fluorescence spectrum provides information on composition of organic matter and the relative content of the fluorescent components [16,17]. EEM fluorescence spectroscopy has been extensively used for studying dissolved organic matter (DOM) [18]. Recently, EEM fluorescence spectroscopy has also been proved to be a useful tool for studying EPS. Limited studies showed that there were several peaks in EEM spectra of EPS from activated sludge, being

assigned to protein-like substances, humic-like substances and fluvic-like substances [15,17,19].

The aim of the present study was to investigate interaction of Cu(II) with EPS from *Synechocystis* sp., a common cyanobacterial species in various aquatic environments, using EEM fluorescence spectroscopy.

## 2 Materials and methods

### 2.1 Culture of cyanobacterium

The cyanobacterium *Synechocystis* sp., supplied by Institute of Hydrobiology, Chinese Academy of Sciences, was cultured photoautotrophically in BG-11 growth medium at 25°C and about  $25 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  [20].

### 2.2 Extraction of EPS

EPS of the cyanobacterial cells was extracted by centrifugation [5,21]. This EPS extraction method would not cause cell lysis [21]. The cyanobacterial cells were centrifuged at  $4300 \times g$  for 10 min at 4°C in order to remove medium and other solved substances [22]. The residue was suspended in high-purity Milli-Q water and EPS were separated by centrifugation at  $20000 \times g$  at 4°C for 20 min [5]. The supernatants were filtered through 0.22  $\mu\text{m}$  acetate cellulose membranes and stored at 4°C for use.

### 2.3 EEM fluorescence spectroscopy and fluorescence titration

The EEM spectra of the EPS solution were recorded with a fluorescence spectrophotometer (F-7000, HITACHI, Japan). The EEM spectra were collected at 5 nm increments over an excitation range of 230–400 nm, with an emission range of 250–500 nm by every 2 nm. The

excitation and emission slits were set to 5 and 10 nm of band-pass, respectively. The scan speed was  $1200 \text{ nm} \cdot \text{min}^{-1}$ . The Milli-Q water blank was subtracted from the sample's EEM spectra and EEM data was processed using the software SigmaPlot 2000 (Systat, US).

EPS solution in a 1 cm  $\times$  1 cm quartz cuvette was titrated with the incremental additions of 5  $\mu\text{L}$   $0.1 \text{ mol} \cdot \text{L}^{-1}$  Cu(II) at room temperature.  $0.01 \text{ mol} \cdot \text{L}^{-1}$   $\text{KNO}_3$  was used as the ionic strength adjustment solution. After each addition of Cu(II) solution, the mixed solution was stirred using a magnetic stirrer for 15 min. Fluorescence quenching titrations of EPS by Cu(II) at various temperatures were conducted.

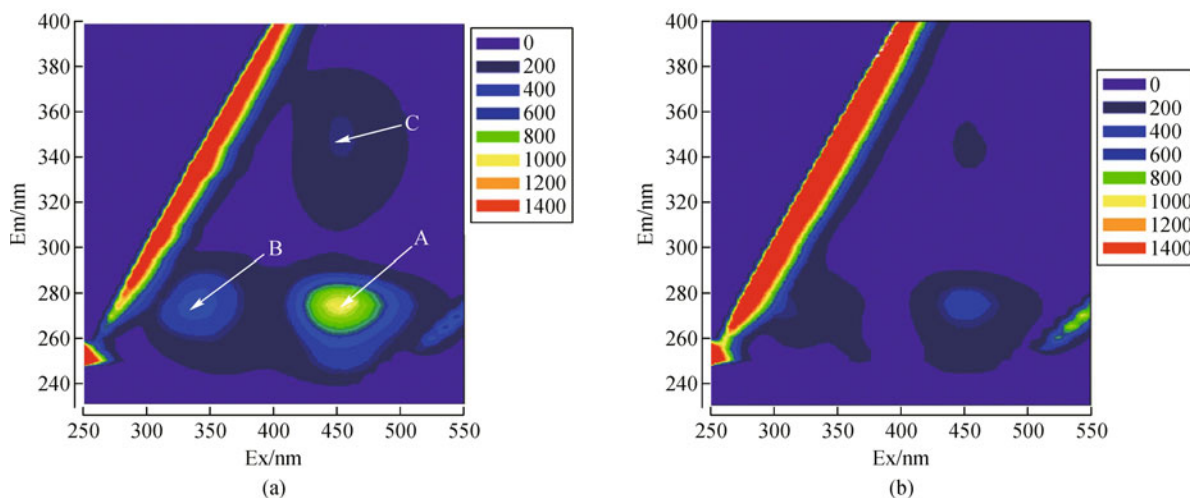
## 3 Results and discussion

### 3.1 Fluorescence characterization of EPS

Three fluorescence peaks (peaks A, B, and C) were identified in the EEM spectra of EPS from *Synechocystis* sp. (Fig. 1(a)). The peak A (Ex/Em = 275/452 nm) and peak C (Ex/Em = 350/452 nm) could be assigned to humic-like fluorescence [16,17]. The peak B (Ex/Em = 275/338 nm) could be identified as protein-like substances [17–19]. Sheng et al. showed that the protein-like fluorescence peak for EPS from activated sludge was found at Ex/Em: 225/340–350 nm [17]. The location of peak B showed blue-shift compared with their results [17]. However, peak A was not found in EEM fluorescence spectra of EPS from activated sludge [15,17,19]. These results suggest that composition of EPS from *Synechocystis* sp. is significantly different from that of activated sludge.

### 3.2 Fluorescence titration of EPS with Cu(II)

It is shown that fluorescence of peaks A, B, and C were



**Fig. 1** (a) EEM fluorescence spectra of *Synechocystis* sp. in absence Cu(II) at 298 K; and (b) EEM fluorescence spectra of *Synechocystis* sp. in presence of  $0.0003 \text{ mol} \cdot \text{L}^{-1}$  Cu(II) at 298 K

clearly quenched by Cu(II) at all experimental temperatures (278 K, 288 K, and 298 K) (Fig. 1(b) and Fig. 2). Peaks B and C disappeared in presence of 0.0003 mol·L<sup>-1</sup> Cu(II). This indicates that the fluorescent components in the EPS from *Synechocystis* sp. reacted strongly with Cu(II). The strong quenching effect of Cu(II) may be due to its paramagnetic effect. Cu(II), with unpaired electron spins (d9), increases intersystem crossing and thus the chealation of a fluorescent system with Cu(II), which significantly decreases the fluorescence [23].

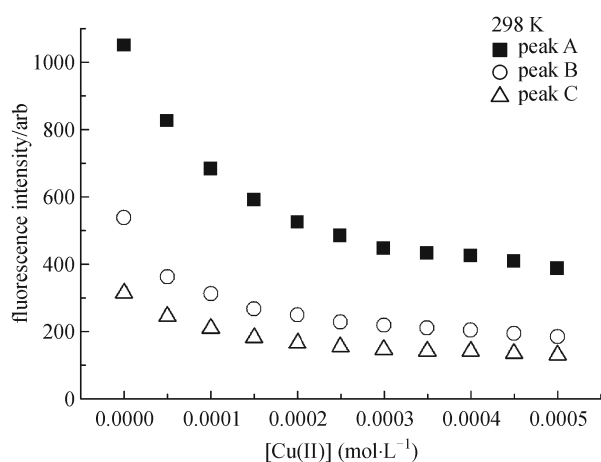


Fig. 2 Variation of the fluorescence intensities of the peaks A, B, and C with increasing Cu(II) concentration

To quantify the stability of EPS-Cu(II) complexes and understand the mechanisms involved in the fluorescence quenching of EPS by Cu(II), the modified Stern–Volmer model was used to fit the fluorescence titration data [24,25]:

$$F_0/\Delta F = F_0/(F_0 - F) = 1/(f_a K_a [Cu(II)]) + 1/f_a, \quad (1)$$

where  $F_0$  and  $F$  are the fluorescence intensity in absence and presence of quencher (Cu(II)), respectively.  $K_a$  is the Stern–Volmer quenching constant of the accessible fraction,  $f_a$  represents the fraction of the initial fluorescence, which is accessible to quencher (Cu(II)), and  $[Cu(II)]$  is the concentration of Cu(II).

All the fluorescence titration data were satisfactorily represented with the modified Stern–Volmer equation ( $R^2 > 0.975$ ). The parameter  $\lg K_a$  of fluorophores at

peaks A, B, and C at various temperatures was calculated and listed in Table 1. The values of  $\lg K_a$  were close to those for complexation of EPS with heavy metals determined using other methods [2,12–14]. For example, using polarographic methods, Guibaud et al. [12,14] determined the relative stability constants of metal complexation of EPS from activated sludge for Cd, Pb, Cu, and Ni to be 1.54–3.35, 0.45–1.28, 3.0–4.4, and 2.6–3.0, respectively. Comte et al. [2] showed that the conditional binding constants for Cu(II) and Cd(II) and Pb(II) to EPS from two different activated sludges were 3.2–4.5 and 3.7–5.0, respectively.

Generally, the fluorescence quenching process is divided into static quenching process and dynamic quenching process. Dynamic quenching is attributed to the collision between fluorophore and quencher at excited state whereas the static quenching is due to the formation of a complex between fluorophore and quencher with the external forces [26,27]. To distinguish static quenching from dynamic quenching, the changes in  $K_a$  with temperature were examined. Because higher temperature causes larger diffusion coefficient and thus larger amounts of collisional quenching and the dynamic quenching is dependent on the diffusion of the fluorophore and the quencher,  $K_a$  increases with increasing temperature for dynamic quenching [28]. On the contrary,  $K_a$  for static quenching decreases with increasing temperature because higher temperature typically causes dissociation of weakly bound complexes. In the present study,  $K_a$  for peaks A, B, and C showed decreasing trends with increasing temperature, indicating the quenching processes at peaks A, B, and C were static [27]. A few previous studies also reported that Cu(II) fluorescence quenching of fulvic acids was governed by static processes [24,29].

### 3.3 Binding site number

To quantify the binding affinity between EPS and Cu(II), the Hill Eq. (2) was used to calculate the binding constant ( $K_b$ ) and the binding site number ( $n$ ) [30]:

$$\lg[(F_0 - F)/F] = \lg K_b + n \lg [Cu(II)], \quad (2)$$

where  $F_0$  and  $F$  are the fluorescence intensities of fluorophore in absence and presence of Cu(II), respectively;  $K_b$  is the binding constant, describing the bonding affinity between EPS and Cu(II) at equilibrium, and  $n$  is the binding site number.

Table 1 Calculated conditional stability constants of peaks A, B, and C at various temperatures

T/K	peak A		peak B		peak C	
	$\lg K_a$	$R^2$	$\lg K_a$	$R^2$	$\lg K_a$	$R^2$
278	4.25	0.997	4.36	0.976	4.22	0.995
288	3.92	0.999	4.20	0.991	4	0.999
298	3.84	0.997	4.21	0.989	3.94	0.998

**Table 2** Binding constants ( $\lg K_b$ ) and binding site number ( $n$ ) for EPS-Cu(II) system at various temperatures

T/K	peak A			peak B			peak C		
	$\lg K_b$	$n$	$R^2$	$\lg K_b$	$n$	$R^2$	$\lg K_b$	$n$	$R^2$
278	2.42	0.634	0.997	1.75	0.46	0.976	2.12	0.56	0.991
288	2.93	0.786	0.995	2.18	0.59	0.997	2.56	0.70	0.991
298	2.85	0.783	0.990	2.25	0.59	0.992	2.47	0.70	0.978

There was a good linear relationship ( $R^2 > 0.975$ ) between  $\lg[(F_0 - F)/F]$  and  $\lg[\text{Cu(II)}]$ . The values of binding constant, binding site number  $n$  and the correlation coefficients were listed in Table 2. The binding constant,  $\lg K_b$ , followed the order of peak A > peak B > peak C, indicating that the humic-like substances have greater binding capacity than the protein-like substances. The binding site number,  $n$ , for complexation EPS with Cu(II) for peaks A, B, and C was less than 1, implying the presence of more than one Cu binding site and the negative cooperativity between multiple binding sites. In addition, the values of  $\lg K_b$  and  $n$  for all the three peaks were higher at 288 K and 298 K than those at 278 K. This shows that the Cu(II) binding affinity of EPS increased with increasing temperature because of the increasing binding sites in the EPS for Cu(II) at higher temperatures.

## 4 Conclusions

Biosorption of Cu(II) to *Synechocystis* sp. EPS was examined using EEM fluorescence spectroscopy. Two groups of humic-like fluorophores and one group of protein-like fluorophores were observed. Fluorescence of these three groups of fluorophores could be statically quenched by Cu(II), indicating formation of Cu(II)-EPS complexes. The humic-like substances in EPS have greater Cu(II) binding capacity than the protein-like substances. The cooperativity between multiple binding sites was negative and more than one Cu(II) binding site was present.

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## References

- Guibaud G, Bordas F, Saaïd A, D'abzac P, van Hullebusch E. Effect of pH on cadmium and lead binding by extracellular polymeric substances (EPS) extracted from environmental bacterial strains. *Colloids and surfaces. B, Biointerfaces*, 2008, 63(1): 48–54
- Comte S, Guibaud G, Baudu M. Biosorption properties of extracellular polymeric substances (EPS) towards Cd, Cu and Pb for different pH values. *Journal of Hazardous Materials*, 2008, 151(1): 185–193
- Liu Y, Lam M C, Fang H H P. Adsorption of heavy metals by EPS of activated sludge. *Water Science and Technology: A Journal of the International Association on Water Pollution Research*, 2001, 43(6): 59–66
- Bhaskar P V, Bhosle N B. Bacterial extracellular polymeric substance (EPS): a carrier of heavy metals in the marine food-chain. *Environment International*, 2006, 32(2): 191–198
- Zhang D Y, Wang J L, Pan X L. Cadmium sorption by EPSs produced by anaerobic sludge under sulfate-reducing conditions. *Journal of Hazardous Materials*, 2006, 138(3): 589–593
- Zheng L, Ding AZ, Wang JS, Tian Y. Adsorption of Cd(II), Zn(II) by extracellular polymeric substances extracted from waste activated sludge. *Water Science and Technology: A Journal of the International Association on Water Pollution Research*, 2008, 58(1): 195–200
- Vinit-Dunand F, Epron D, Alaoui-Sosse B, Badot P. Effects of copper on growth and on photosynthesis of mature and expanding leaves in cucumber plants. *Plant Science*, 2002, 163(1): 53–58
- Yruela I. Copper in plants. *Brazilian Journal of Plant Physiology*, 2005, 17(1): 145–146
- Acosta M P, Valdman E, Leite S G F, Battaglini F, Ruzal S M. Biosorption of copper by *Paenibacillus polymyxa* cells and their exopolysaccharide. *World Journal of Microbiology and Biotechnology*, 2005, 21(6–7): 1157–1163
- Zheng Y, Fang X L, Ye Z L, Li Y H, Cai W M. Biosorption of Cu(II) on extracellular polymers from *Bacillus* sp. F19. *Journal of Environmental Sciences-China*, 2008b, 20(11): 1288–1293
- Pérez J A M, García-Ribera R, Quesada T, Aguilera M, Ramos-Cormenzana A, Monteoliva-Sa'nchez M. Biosorption of heavy metals by the exopolysaccharide produced by *Paenibacillus jamilae*. *World Journal of Microbiology & Biotechnology*, 2008, 24(11): 2699–2704
- Guibaud G, Tixier N, Bouju A, Baudu M. Use of a polarographic method to determine copper, nickel and zinc constants of complexation by extracellular polymers extracted from activated sludge. *Process Biochemistry*, 2004, 39(7): 833–839
- Guibaud G, Comte S, Bordas F, Dupuy S, Baudu M. Comparison of the complexation potential of extracellular polymeric substances (EPS), extracted from activated sludges and produced by pure bacteria strains, for cadmium, lead and nickel. *Chemosphere*, 2005, 59(5): 629–638
- Guibaud G, van Hullebusch E, Bordas F. Lead and cadmium biosorption by extracellular polymeric substances (EPS) extracted from activated sludges: pH-sorption edge tests and mathematical equilibrium modelling. *Chemosphere*, 2006, 64(11): 1955–1962
- Ni B J, Fang F, Xie W M, Sun M, Sheng G P, Li W H, Yu H Q. Characterization of extracellular polymeric substances produced by

- mixed microorganisms in activated sludge with gel-permeating chromatography, excitation-emission matrix fluorescence spectroscopy measurement and kinetic modeling. *Water Research*, 2009, 43 (5): 1350–1358
16. Chen W, Westerhoff P, Leenheer J A, Booksh K. Fluorescence excitation-emission matrix regional integration to quantify spectra for dissolved organic matter. *Environmental Science & Technology*, 2003, 37(24): 5701–5710
  17. Sheng G P, Yu H Q. Characterization of extracellular polymeric substances of aerobic and anaerobic sludge using three-dimensional excitation and emission matrix fluorescence spectroscopy. *Water Research*, 2006, 40(6): 1233–1239
  18. Wu F, Tanoue E. Isolation and partial characterization of dissolved copper-complexing ligands in streamwaters. *Environmental Science & Technology*, 2001, 35(18): 3646–3652
  19. Adav S S, Lee D J. Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. *Journal of Hazardous Materials*, 2008, 154(1–3): 1120–1126
  20. Rippka R, Deruelles J, Waterbury J B, Herdman M, Stanier R Y. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology*, 1979, 111(1): 1–61
  21. Liu H, Fang H H P. Extraction of extracellular polymeric substances (EPS) of sludges. *Journal of Biotechnology*, 2002, 95(3): 249–256
  22. Comte S, Guibaud G, Baudu M. Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: soluble or bound. *Process Biochemistry*, 2006, 41(4): 815–823
  23. Ravat C, Dumonceau J, Monteil-Rivera F. Acid/base and Cu(II) binding properties of natural organic matter extracted from wheat bran: modeling by the surface complexation model. *Water Research*, 2000, 34(4): 1327–1339
  24. Esteves da Silva J C G, Machado A A S C, Oliveira C J S, Pinto M S. Fluorescence quenching of anthropogenic fulvic acids by Cu(II), Fe(III) and  $\text{UO}_2^{2+}$ . *Talanta*, 1998, 45(6): 1155–1165
  25. Lu X Q, Jaffe R. Interaction between Hg(II) and natural dissolved organic matter: a fluorescence spectroscopy based study. *Water Research*, 2001, 35(7): 1793–1803
  26. Papadopoulou A, Green R J, Frazier R A. Interaction of flavonoids with bovine serum albumin: a fluorescence quenching study. *Journal of Agricultural and Food Chemistry*, 2005, 53(1): 158–163
  27. Chen C Y, Gu X T, Zhou J H. Binding studies of paeonolum with bovine serum albumin using spectroscopic methods. *Spectroscopy*, 2007, 21(1): 53–60
  28. Lakowicz J R. *Principles of Fluorescence Spectroscopy*. 3rd ed. New York: Springer-Verlag, 2006
  29. Hays M D, Ryan D K, Pennell S. A modified multisite Stern-Volmer equation for the determination of conditional stability constants and ligand concentrations of soil fulvic acid with metal ions. *Analytical Chemistry*, 2004, 76(3): 848–854
  30. Hill T L. *Cooperativity: Theory in Biochemistry*. New York: Springer-Verlag, 1985