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The dual roles of extracellular polymeric substances (EPSs) in the microbial nitrification process in the presence of copper ions

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ABSTRACT

Extracellular polymeric substances (EPSs) can protect microorganisms from the toxicity and harm posed by potential toxicants; meanwhile, the changes of content and composition of EPSs may also affect the activity of microorganisms under different conditions. In this study, the impact of copper (Cu) on the microbial nitrification process and the effect of EPSs (LB-EPSs and TB-EPSs) on the nitrification activity of sludge were investigated. At the same time, the dynamic changes in the concentration and composition of EPSs were analyzed using three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy and fluorescence regional integration (FRI). The content and composition of fluorescent substances in EPSs were affected by the Cu(II) content of the influent water. When the concentration of Cu(II) increased from 0 to 9 mg·L^{-1}, the content of PN, PS and the primeval excitation-emission area volume in LB-EPSs and TB-EPSs decreased first, then increased and decreased again. For the TB-EPSs and LB-EPSs, when the concentration of Cu(II) was 5 mg·L^{-1}, the primeval excitation-emission area volume of substances in aromatic protein region I (Region I), aromatic protein region II (Region II), soluble microbial byproduct-like material region (Region IV), and humic acid-like organics region (Region V) reached maximum value to resist the toxic effects of Cu(II). Cu(II) indirectly affected the sludge nitrification activity by changing the composition of the EPSs. When the concentration of Cu(II) increased from 0 to 9 mg·L^{-1}, the inhibitory effect of EPSs on sludge nitrification activity gradually increased, and the effect of LB-EPSs was greater than that of TB-EPSs. This paper provides a deeper understanding of the effects of Cu(II) on the nitrification process.

Keywords: Copper; Extracellular polymeric substances; SOUR

1. Introduction

Heavy metal pollution has become increasingly serious with the rapid onset of global industrialization. Heavy metals are produced in mining, metal finishing, metallurgical processing, and battery manufacturing among other processes, and metal-containing waste products are discharged into the environment. The problem of heavy metal contamination has received widespread attention because heavy metals not only are highly toxic and nondegradable, but also accumulate in the food chain [1]. With increasing industrialization, many heavy metals have entered wastewater treatment systems and interacted with functional microorganisms [2]. Copper (Cu) is widely used in biocides, herbicides, electroplating and leather manufacturing, which results in industrial wastewater that containing a large amount of Cu(II) [3]. Cu is a toxic heavy metal, which has been listed as one of the most worrying and harmful metals by the World Health Organization [4]. The inhibition and toxic effects of Cu on activated sludge systems have

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been extensively studied [3,5–7]. Cu(II) is commonly found in many nitrogen-rich wastewaters [8]. It has been reported that Cu(II) significantly affects various nitrogen removal-related systems, such as stormwater bioretention, partial nitrification, nitrification-denitrification, denitrifying phosphorus removal and the anammox system [3,9–12].

In a biological wastewater treatment system, extracellular polymeric substances (EPSs) are secreted by active bacteria and are produced by the shedding of cell surface material and cell lysis. Ultimately, EPSs accumulate on the surface of the sludge, and they are the primary active component of flocculent sludge [13]. The primary components of EPSs are proteins (PN), polysaccharides (PS), humic acids and nucleic acids [14]. EPSs have a strong binding capacity with metals because they have many surface functional groups, including carboxyl, phosphate, amine and hydroxyl groups [15], therefore they play an important role in protecting biomass from potential inhibition of heavy metals [16]. EPSs are divided into loosely bound EPSs (LB-EPSs) and tightly bound EPSs (TB-EPSs) based on their different locations and appropriate extraction methods [17]. Due to the differences in composition, LB-EPSs and TB-EPSs influence bioflocculation, sedimentation and contaminant removal to different extents [18,19]. The influence of Cu(II) on the growth, reproduction rates and metabolic activity of microorganisms has attracted widespread attention. It is widely accepted that Cu(II) can stimulate microbes to secrete additional EPSs that protect the biomass from the potential inhibition of heavy metals [12,20]. However, Cu(II) stimulation will change the content and composition of EPSs, which may indirectly affect their biological activity. Studies on the effect of EPSs on the sludge activity have been primarily focused on the activity of anaerobic ammonia oxidation bacteria, but few studies have reported the effect of EPSs on the nitrification processes. In some studies, an appropriate dose of EPSs and the N-acyl-L-homoserine lactones in EPSs increases anaerobic ammonia oxidation activity [21]. The denitrification of sludge EPSs and perchlorate-reducing sludge EPSs can improve the activity of anaerobic ammonia oxidation bacteria [22]. A comprehensive understanding of the effects of Cu(II) on the nitrification processes in biological treatment systems remains elusive.

The major objective of this study is to reveal the role of EPSs in the presence of Cu(II). EPSs not only protect microorganisms from the toxicity and harm posed by potential toxicants but also may influence the nitrification activity of sludge under different Cu(II) content conditions. In this study, the impact of Cu on the microbial nitrification process was investigated, the dynamic changes in the concentration and composition of EPSs were analyzed using three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy and fluorescence regional integration (FRI). At the same time, the effect of EPSs (LB-EPSs and TB-EPSs) on the nitrification activity of sludge was investigated under different Cu(II) content conditions. This study improves our understanding of the effect of Cu(II) on the nitrification process in biological treatment systems.

2. Materials and methods

2.1. Reactor setup and operation

The test equipment consisted of four 3-L sequencing batch reactors (SBRs, reactors numbered R_1, R_2, R_3, and R_4). These SBRs ran 3 cycles per day, consisting of 5 min of influx, 300 min of aeration, 45 min of settling, 10 min of draining, and 60 min of idling for each cycle. The water exchange rate was 50%, the sludge age was 15 days, and the dissolved oxygen (DO) content was controlled at 6–7 $mg \cdot L^{-1}$.

2.2. Test wastewater and sludge

The influent test water used was an artificial, simulated wastewater. Ammonium chloride was used as the nitrogen source, potassium dihydrogen phosphate provided the phosphorus source, glucose and sodium acetate were used as carbon sources, and $CuSO_4 \cdot 5H_2SO_4$ was used as the Cu(II) source. Detailed characteristics of the sewage were as follows: 140–160 $mg \cdot L^{-1}$ chemical oxygen demand (COD), 480–500 $mg \cdot L^{-1}$ ammonium nitrogen (NH_4^+-N), 10 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 20 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, pH 7.0–7.5, and 0.6 $mL \cdot L^{-1}$ trace elements [23]. The Cu(II) concentrations in the four reactors were set at 0, 1, 5, and 9 $mg \cdot L^{-1}$ to investigate the influence of Cu(II) on the sludge treatment effect and activity.

The test inoculation sludge was flocculated sludge that was transferred from an SBR in the laboratory to the four SBRs after cultivation and domestication for a period of time. The initial mixed liquor suspended solids (MLSS) was 3300±100 $mg \cdot L^{-1}$, and the sludge volume index (SVI) was 100±5 $mL \cdot g^{-1}$.

2.3. Extraction of TB-EPSs and LB-EPSs

Mixed solution samples of the SBRs were collected 30 min before the end of aeration. Each sample was centrifuged (4000× g, 5 min) and then diluted to its original volume with deionized water. Afterward, the LB-EPSs and TB-EPSs were extracted according to procedures in the literature [24].

When measuring the nitrification activity of sludge, care was taken to avoid influence from the extraction method on the sludge activity and the concentration of Cu(II) in the EPSs. In this experiment, high-speed centrifugation [25] was used, and some improvements were made in the extraction of the EPSs. (i) A certain amount of sludge-water mixture was centrifuged (4000× g, 5 min), the supernatant was discarded, and the sample was diluted to its initial volume with deionized water. The dilute mixture was centrifuged (4°C, 16000× g, 40 min), and the excess sludge was the active sludge without EPSs. (ii) A certain amount of sludge-water mixture was centrifuged (4000× g, 5 min), the supernatant was discarded, and the samples were diluted to their initial volume with deionized water. The dilute mixture was further centrifuged (5000× g, 15 min), and the remaining sludge was the active sludge without LB-EPSs. (iii) After removing the LB-EPSs, a certain amount of the sludge-water mixture was diluted to its initial volume with deionized water. The mixture was centrifuged (4°C, 16000× g, 40 min), and the excess sludge was the active sludge without the LB-EPSs and TB-EPSs. (iv) Another samples of untreated sludge was taken as a reference.

2.4. 3D-EEM and FRI analysis

The 3D-EEM analyses of the LB-EPSs and TB-EPSs solutions were conducted using a fluorescence spectrophotom-

eter (F-4600; Hitachi, Japan) equipped with a 10-mm quartz cuvette. The excitation wavelength was increased from 220 to 450 nm in increments of 5 nm; the emission wavelength was increased from 220 to 550 nm in 1 nm increments, and the widths of the excitation and emission slits were both 5.0 nm throughout the experiments, which were conducted at a scanning speed of 1200 nm/min.

FRI proposed by [26] based on the traditional peak method. Chen uses FRI to divide the EEM into five consecutive areas: aromatic protein region I (Region I, 200–250/280–330), aromatic protein region II (Region II, 200–250/330–380), fulvic acid-like region (Region III, 200–250/380–550), soluble microbial byproduct-like material region (Region IV, 250–450/280–380), and humic acid-like organics region (Region V, 250–450/380–550). The solution was diluted to give a total organic carbon (TOC) concentration of 10 mg·L^{-1} before 3D-EEM analysis, and the blank used during the analysis of the samples was deionized water. The volume (Φ) beneath region "i" of the EEM can be calculated as follows [27]:

$$\Phi_i = \int_{ex}\int_{em} I\left(\lambda_{ex}\lambda_{em}\right) d\lambda_{ex} d\lambda_{em} \quad (1)$$

The normalized excitation-emission area volume ($\Phi_{i,n}$) is calculated with Eqs. (2) and (3)

$$\Phi_{i,n} = MF_i \times \Phi_i \quad (2)$$

$$\Phi_{p,n} = D_m \times \Phi_{i,n} \quad (3)$$

$\Phi_{p,n}$ is the primeval excitation-emission area volume, D_m is dilution multiplier.

2.5. Method of measuring the sludge nitrification activity

The toxic effects of Cu on the nitrification process are often studied by measuring substrate removal efficiency or respiratory activity (the specific oxygen uptake rate (SOUR) method is the most common [28–30]). The SOUR method was used as the index of sludge activity. The specific method was conducted according to the following published literature [31], and some improvements to the procedure were made. The substrate solution (ammonium chloride concentration of 150 mg·L^{-1} and trace element concentration of 0.6 mL·L^{-1}) was prepared before the test, and the pH of the solution was adjusted to 7.5 using a phosphate buffer solution. Then, the solution was aerated for 30 min to reach the DO saturation state. The substrate solution was then mixed with the above-mentioned treated sludge in an Erlenmeyer flask, and measured the DO concentration. The time between each SOUR measurement was 20 min or more. The oxygen uptake rate (OUR) was determined as the slope of the linear relationship between the DO and the time curve. The SOUR was calculated by dividing the OUR by the MLSS. The SOUR inhibition rate was calculated according to the following formula:

$$SOUR_{inhibition}(\%) = [(SOUR_{absence} - SOUR_{presence}) \times 100] / SOUR_{presence} \quad (4)$$

where $SOUR_{absence}$ (mg DO·g MLSS^{-1}·h^{-1}) is the SOUR of the active sludge without EPSs (TB-EPSs, LB-EPSs), and $SOUR_{presence}$ (mg DO·g MLSS^{-1}·h^{-1}) is the SOUR of the active sludge with the EPSs present (before testing).

2.6. Analytical methods

The COD, NH_4^+-N, SVI, and MLSS in the samples were determined by standard methods [32]. The PN content was measured according to the modified Lowry method with bovine serum albumin as the standard [33], and the anthrone-sulfuric acid method was used to measure the content of PS with glucose as the standard [34]. The TOC was measured by using a TOC analyzer (muliN/C2100; Analytikjena, Germany). The DO was measured by using a dissolved oxygen meter (inoLab Oxi 7310; WTW Company, Germany). The EEM data were analyzed with MATLAB 2014a software (MathWorks, United States). Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Inc., USA), and $p < 0.05$ was considered to be statistically significant. All tests were performed in triplicate and the results were expressed as the mean values ± standard error.

3. Results and discussion

3.1. Effect of Cu(II) on contaminant removal and sludge properties

Table 1 shows the effects of Cu(II) on contaminant removal, including the COD and NH_4^+-N removal capabilities.

As shown in Table 1, the COD removal ability decreased after the addition of Cu(II). When the Cu(II) concentration was 1 mg·L^{-1}, the COD removal ability was reduced to 2.15 mg·g^{-1}·h^{-1}, which was 8.12% lower than that of the control

Table 1
Sludge parameters

Parameters	Cu(II) concentration (mg·L^{-1})			
	0	1	5	9
COD removal ability (mg COD·g MLSS^{-1}·h^{-1})	2.34±0.39	2.15±0.42	2.05±0.40	1.09±0.42
NH_4^+-N remove ability (mg NH_4^+·g MLSS^{-1}·h^{-1})	3.00±0.39	2.82±0.37	2.05±0.39	1.23±0.33
MLSS (g·L^{-1})	3.53±0.32	3.76±0.19	2.42±0.32	2.92±0.37
SVI (mL·g^{-1})	95.25±10.55	90.79±13.24	167.66±29.49	94.59±18.17
Partical size (μm)	200.77	151.03	217.25	548.60

group. When the Cu(II) concentrations were 5 and 9 mg·L^{-1}, the COD removal ability decreased to 2.05 and 1.09 mg·g^{-1}·h^{-1}, which were 12.39% and 53.42% lower than that of the control group, respectively. Cu(II) significantly inhibited the removal of organic matter [35].

Also shown in Table 1, the addition of Cu(II) affected the NH_4^+-N removal capacity of the sludge. Similar to COD, when the Cu(II) concentration was 1 mg·L^{-1}, the NH_4^+-N removal capacity slightly decreased to 2.82 mg·g^{-1}·h^{-1}, a decrease of 6.00% compared with that of the control group. The Cu(II) accumulation in the biofilm exceeded the resistance capacity of the nitrifying bacteria, and as a result, the ammonia removal ability was reduced. When the Cu(II) concentration was 5 and 9 mg·L^{-1}, the NH_4^+-N removal capacity decreased to 2.05 and 1.23 mg·g^{-1}·h^{-1}, which were 31.67% and 59.00% lower than that in the control group, respectively. The high load of Cu(II) exceeded the resistance capacity of the biofilm or EPSs during nitrification, causing some Cu(II) to bind with the functional enzymes after entering the cell, which inhibited the activity of the microorganisms [36]. The presence of Cu(II) can affect the productivity and composition of EPSs, which will in turn affect the growth rate of the microorganisms. EPSs contain a large number of charged groups, such as –COOH, –NH, –OH, and –CO– groups as well as nonpolar groups. These groups can initially react with pollutants through electrostatic interactions and ion exchange [34–39]. The delivery of the Cu(II) into the cells has negative impacts on the growth rate of the microorganisms and the substrate conversion rate.

The effects of different concentrations of Cu(II) on the MLSS, SVI and particle size of sludge are shown in Table 1. When Cu(II) was added, both the MLSS and SVI changed. The MLSS initially increased, then decreased and then increased again, whereas SVI tended to decrease first, then increase, and then decrease again. The MLSS value in the activated sludge at a Cu(II) concentration of 1 mg·L^{-1} increased from approximately 3353.65 to 3761.71 mg·L^{-1}, and the SVI value decreased from approximately 95.25 to 90.79 mg·L^{-1}. The good sedimentation of sludge and a slight increase in MLSS were due to the role of coprecipitation between Cu(II) and the activated sludge [40,41]. When the Cu(II) concentration was 5 mg·L^{-1}, the MLSS decreased to approximately 2416.56 mg·L^{-1}, and the SVI increased to approximately 167.66 mg·L^{-1}. These changes were due to the toxicity of the heavy metal ions toward the microorganisms in the activated sludge, which caused the MLSS and the sludge sedimentation to decrease. However, when the Cu(II) concentration was 9 mg·L^{-1}, the sludge particle size increased to 548.6 μm. At this concentration, the sludge changed from flocculated sludge to granular sludge, resulting in increased values of MLSS and SVI.

3.2. Effect of Cu(II) on changes in the EPSs content and composition

3.2.1. Changes in the contents of TB-EPSs and LB-EPSs

PS and PN are the primary components of EPSs [42], and this study examined the effect of the Cu(II) concentration on the PN and PS in the EPSs. As shown in Fig. 1a, the contents of TB-EPSs (the sum of PS and PN) fluctuated, first decreasing, then increasing and decreasing again as the concentration of Cu(II) in the influent increased. When the concentration of Cu(II) from the influent in the reactor was relatively low (1 mg·L^{-1}), Cu(II) inhibited bacterial metabolic activity in the sludge, decreasing the TB-EPSs content [43]. As the concentration of Cu(II) in the influent increased (5 mg·L^{-1}), microorganisms secreted more EPSs to compensate for the harsh environment, and these EPSs can bind to Cu(II) to form a barrier to protect cells from the adverse effects of the external environment, [12] which then contributes to the stable functioning of biological systems [16]. When the concentration of Cu(II) in the influent water was further increased (9 mg·L^{-1}), the Cu(II) load exceeded the capacity of the EPSs, then Cu(II) entered the cells and denatured enzymes to reducing enzyme activity; these all led to the death of some microorganisms and further reductions in the contents of EPSs [39].

Because the difference between the contents of LB-EPSs and TB-EPSs was caused by the differences in their location and the discrepancy in the effects of the extraction methods [23], the content of LB-EPSs was lower than that of TB-EPSs, but the trends in the changes in the contents of LB-EPSs and TB-EPSs were similar.

3.2.2. Changes in the compositions of TB-EPSs and LB-EPSs

The changes in the contents of PS and PN in the TB-EPSs that occurred with changes in the Cu(II) content are shown in Fig. 1a. The primary component of TB-EPSs was PN, which was much higher than that of PS. As the concentration of Cu(II) in the influent water increased, the contents of PN and PS in the TB-EPSs initially decreased, then increased and then decreased. Changes in the PN content were particularly dramatic; therefore, the contribution of PN was greater than that of PS in protecting microorganisms from the influence of heavy metal ions. The PN in the EPSs played a major role in protecting cells from damage caused by diffusion limitations and/or chemical binding [12].

When the concentration of Cu(II) in the influent water was 1 mg·L^{-1}, the content of PN was significantly reduced. Therefore, when the Cu(II) concentration was low, the decrease in the TB-EPSs content was primarily due to the decrease in the content of PN [44]. When the Cu(II) concentration was 5 mg·L^{-1}, Cu(II) caused the microbes to secrete additional PS and PN. However, when the concentration of Cu(II) was 9 mg·L^{-1}, the toxicity increased beyond the capacity of the microorganisms, which resulted in decreases in both the PS and PN contents.

As shown in Fig. 1b, the primary component of LB-EPSs was PS. As the content of Cu(II) in the influent increased, the overall content of PS in the LB-EPSs tended to decrease gradually, whereas the PN in the LB-EPSs initially increased and then decreased. Comparing Fig. 1a and Fig. 1b, the LB-EPSs were more sensitive to the effect of the Cu(II) content in the influent water, and the LB-EPSs may play a major protective role under low Cu(II) content conditions.

3.2.3. Analysis of the protein and polysaccharide ratios (PN/PS values) in TB-EPSs and LB-EPSs

Also shown in Fig. 1b, the PN/PS value of the TB-EPSs gradually decreased as the Cu(II) concentra-

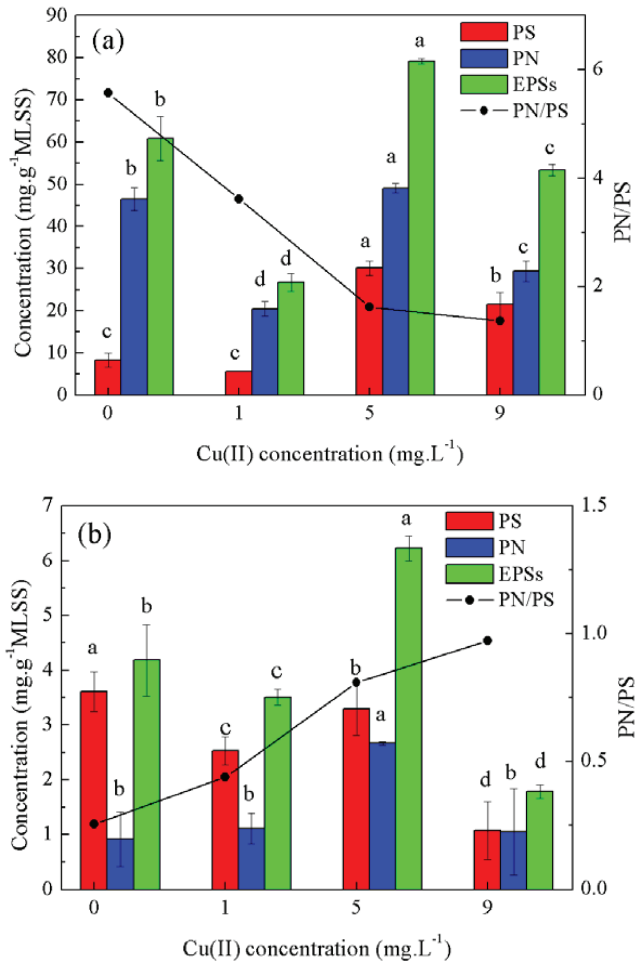

Fig. 1. The proteins content, polysaccharides content and PN/PS ratio in TB-EPSs (a) and LB-EPSs (b) at different Cu (II) concentrations. The letters (a, b, and c) represent that the same index has significant difference under different Cu(II) concentrations ($P < 0.05$).

tion increased. When the concentration of Cu(II) was 1 mg·L^{-1}, the decrease in the PN/PS value indicated that Cu(II) inhibited microorganisms by reducing the PN content. Simultaneously, Cu(II) combined with the amino acid side chains in PN via electrostatic interactions, effectively reducing the PN content [45]. When the Cu(II) concentration was 5 mg·L^{-1}, the PN/PS value decreased, indicating that the microorganisms secreted more PS (most likely because of the extracellular carbohydrates generated by metal-bound polymers [46]). Compared with the Cu(II) concentration of 5 mg·L^{-1}, the decrease in the PN/PS value was less dramatic when the Cu(II) concentration was 9 mg·L^{-1} because the granular sludge had a higher resistance to the toxic effects of Cu(II) (as Table 1 shows).

The change in the PN/PS value of LB-EPSs with increasing Cu(II) concentration was opposite of that seen with TB-EPSs; it increased. Therefore, the adjustments of the contents of PN and PS in the EPSs can be considered a defensive strategy for microbial cells to protect themselves from adverse external conditions [47].

3.3. EPSs fluorescence characteristics analysis

3.3.1. 3D-EEM analyses of EPSs at different Cu(II) concentrations

The EPSs were extracted after 45 days of stable reactor operation, and 3D-EEM analyses were performed. Fig. 2a shows the 3D-EEM spectra of the TB-EPSs under different Cu(II) concentrations. When the Cu(II) concentrations were 0 and 1 mg·L^{-1}, two peaks, A and B, were detected. The position of peak A was consistent with tryptophan and protein-like byproducts of soluble microorganisms, and peak B was indicative of an aromatic protein (Region II). When the influent Cu(II) concentration was increased to 5 and 9 mg·L^{-1}, two peaks C and D were also identified. The byproducts of soluble microorganisms changed from tryptophan and protein-like compounds to tyrosine and protein-like compounds, and the aromatic proteins of Region II changed to aromatic proteins of Region I [26].

Fig. 2b shows the 3D-EEM spectra of the LB-EPSs at different Cu(II) concentrations. As shown in Fig. 2, the fluorescent components in the LB-EPSs of the control group were different from those of the TB-EPSs. The two identified peaks in the spectrum of the LB-EPSs corresponded to the aromatic proteins of Region II and the tyrosine and protein-like byproducts of soluble microorganisms. After adding Cu(II), the aromatic protein of region II become the aromatic protein of region I in LB-EPSs.

The fluorescence peaks of the TB-EPSs and LB-EPSs were blueshifted. In the presence of Cu(II), the functional groups on the EPSs changed, and the EPSs and Cu(II) formed complexes, leading to fluorescence quenching of the EPSs and reduced absorption intensity of the fluorescent substances in the EPSs [12,48,49].

3.3.2. FRI analysis

As shown in Fig. 3, the Cu(II) content in the influent influenced the relative contents of the organic components in the TB-EPSs and LB-EPSs. For the TB-EPSs, as the concentration of Cu(II) increased, the percentage of aromatic protein I-type substances initially decreased, then increased and then decreased again. The percentage of aromatic protein II-type substances initially increased, then decreased and then increased again. The percentage of fulvic acid-like substances tended to decrease, whereas the byproducts of soluble microorganism substances and humic acid-like organics substances tended to increase. For the LB-EPSs, the percentage of aromatic protein I-type substances initially decreased and then increased, and the percentage of aromatic protein II-type substances initially increased and then decreased. The percentage of fulvic acid-like substances initially increased and then decreased. The percentage of byproducts of soluble microorganism substances tended to initially decrease and then increase, whereas the humic acid-like organics substances tended to increase. Considering that the different contents of EPSs in the samples used in the extraction process may affect the distribution of the relative contents of organic components, the relative contents were converted into relative concentration. The volume of the excitation-emission area of the substances can represent the relative concentration to a certain extent [50].

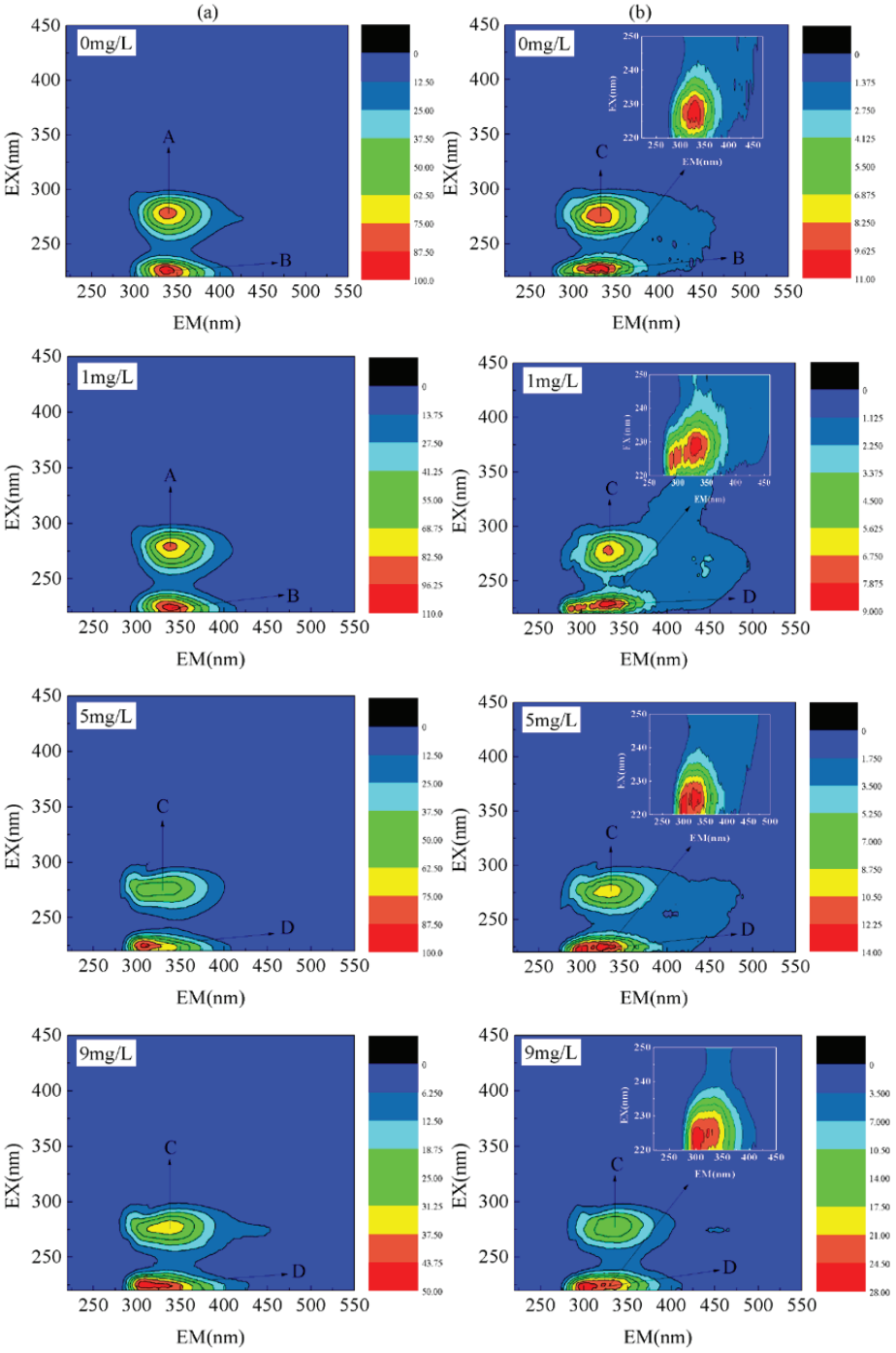

Fig. 2. 3D-EEM of TB-EPSs (a) and LB-EPSs (b) in different Cu (II) concentrations.

As shown in Fig. 4, the primeval excitation-emission area volume of compounds in the five regions changed with the concentration of Cu(II) increased.

For the TB-EPSs, comparing with the control group, when the concentration of Cu(II) was 1 mg·L^{-1}, the primeval excitation-emission area volume of substances in the five regions decreased. When the concentration of Cu(II) was 5 mg·L^{-1}, the primeval excitation-emission area volume of substances in aromatic protein region I (Region I), aromatic protein region II (Region II), soluble microbial byproduct-like material region (Region IV), and humic acid-like organics region (Region V) clearly increased. When the concentration of Cu(II) was 9 mg·L^{-1}, the primeval excitation-emission area volume of the substances in the five regions did not change significantly. The content of substances in each region of the EPSs was related to the amount of relevant substances secreted by the microorganisms, the amount bound to Cu(II), and the content of

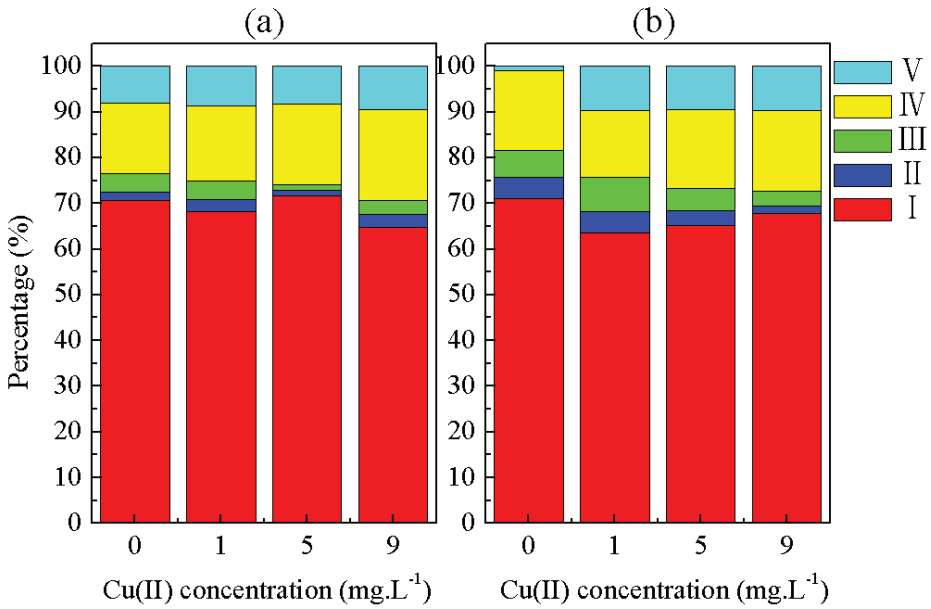

Fig. 3. FRI distribution of TB-EPSs (a) and LB-EPSs (b) at different Cu (II) concentrations.

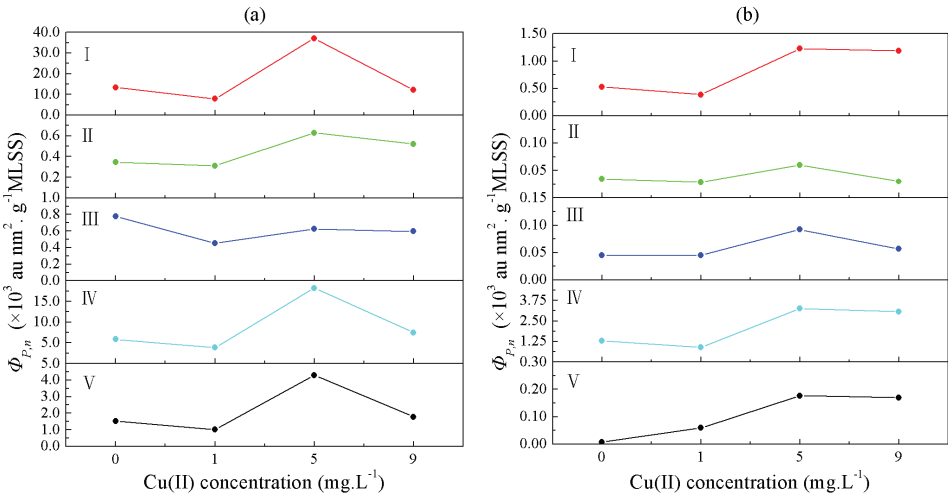

Fig. 4. Substance concentrations in five fluorescence regions of TB-EPSs (a) and LB-EPSs (b) at different Cu (II) concentrations.

Cu(II) in the influent water. The substances in each region of the EPSs had a certain binding ability for Cu(II). When the Cu(II) content in the influent water was low (1 mg·L^{-1}), the low concentration of Cu(II) was not enough to promote the secretion of five substances by microorganisms; at the same time, the combination of Cu(II) with five substances resulted in a reduction of the primeval excitation-emission area of substances in the five regions. With the increase of Cu(II) content in the influent water, a high concentration of Cu(II) (5 mg·L^{-1}) stimulated the microorganisms to secrete substances that were aromatic protein I-like (Region I), aromatic protein region II (Region II), byproducts of soluble microorganisms (Region IV) and humic acid-like organics (Region V). The increase in the content of aromatic protein I-like (Region I) and aromatic protein region II (Region II) species was due to the anionic nature of the proteins and the ability of the amide group of the proteins to react with the metal; thus, the protein-like substances in the EPSs have a strong ability to form complexes with metal cations [49]. The increases in the concentration of byproducts of soluble microorganisms (Region IV) and humic acid-like organics (Region V) were due to the many ionizable functional groups (carboxylic acid and phenolic groups) in these substances, indicating a strong ability to form complexes with heavy metals as well as a high affinity for heavy metals [12].

For the LB-EPSs, when the concentration of Cu(II) was 1 mg·L^{-1}, the change of primeval excitation-emission area volume of substances in the five regions was similar to TB-EPSs except for humic acid-like organics (Region V). When the concentration of Cu(II) was 5 mg·L^{-1}, the primeval excitation-emission area volume of substances in the five regions increased.

For TB-EPSs and LB-EPSs, when the content of Cu(II) in influent water was further increased to 9 mg·L^{-1}, the effect of toxicity and fluorescence quenching of Cu(II) [19] resulted in a decrease of substances in the five regions, compared with the observations at 5 mg·L^{-1}. At this time, it can also be seen that the reduction of substance content in five regions of LB-EPSs was lower than that in TB-EPSs. It can be inferred that TB-EPSs have stronger binding ability than LB-EPSs with Cu(II), which is consistent with the results of literature research [19].

3.4. The nitrification activity of the sludge

In this study, samples were extracted after 60 days of stable operation of the SBRs for sludge activity determination. Heavy metal ions induce microorganisms to secrete more EPSs to protect the biomass from the toxic effects of heavy metals, and the biological activity is affected by the change in content and composition of the EPSs. The effects of the inhibition of EPSs, TB-EPSs and LB-EPSs on the sludge nitrification activity are shown in Fig. 5.

As shown in Fig. 5a, in the absence of Cu(II), the EPSs significantly inhibited ($P < 0.05$) the nitrification activity of the sludge. EPSs protected the biological mechanisms from external hazards while also hindering the substrate diffusion rate [51] and inducing changes in substance morphology, which affect substrate utilization and conversion rates [37,38]. The diffusion coefficients of antibiotics in glycoprotein or polysaccharide gels are obviously lower than their values in water, and substance diffusion through the EPSs layer can be retarded [52]. This result is inconsistent with the conclusion [15] that the appropriate dose of EPSs promotes anaerobic ammonia oxidation activity. The reason for the discrepancy might be that the metabolic pathway of the anaerobic ammonia oxidation system is different from that of the functional microbial population and the components of EPSs in this study. As shown in Fig. 5b and Fig. 5c, in the absence of Cu(II), LB-EPSs increased the nitrification activity of sludge, whereas TB-EPSs had a negative effect on the nitrification activity, and these differences were related to the differences in the composition and content of the EPSs [21]. Further research on this phenomenon is necessary.

Fig. 5a also shows that after the removal of the EPSs the inhibition rate of the nitrification activity of sludge increased with increasing Cu(II) concentration. The EPSs located on the cell surface can increase the mass transfer resistance of the substrate [37,38] and the nitrification activity of the sludge is associated with the mass transfer resistance. After adding Cu(II), the content and composition of EPSs also changed. It is speculated that in this case, Cu(II) induced the microorganisms to change the components of the EPSs for protection while also inhibiting the nitrification activity.

The changes in the nitrification activity with the removals of the TB-EPSs and LB-EPSs showed different trends. In the absence of TB-EPSs, the nitrification activity inhibition rate decreased when the Cu(II) concentrations were 1 and 5 mg·L^{-1}, whereas the nitrification activity inhibition rate increased significantly when the Cu(II) concentration

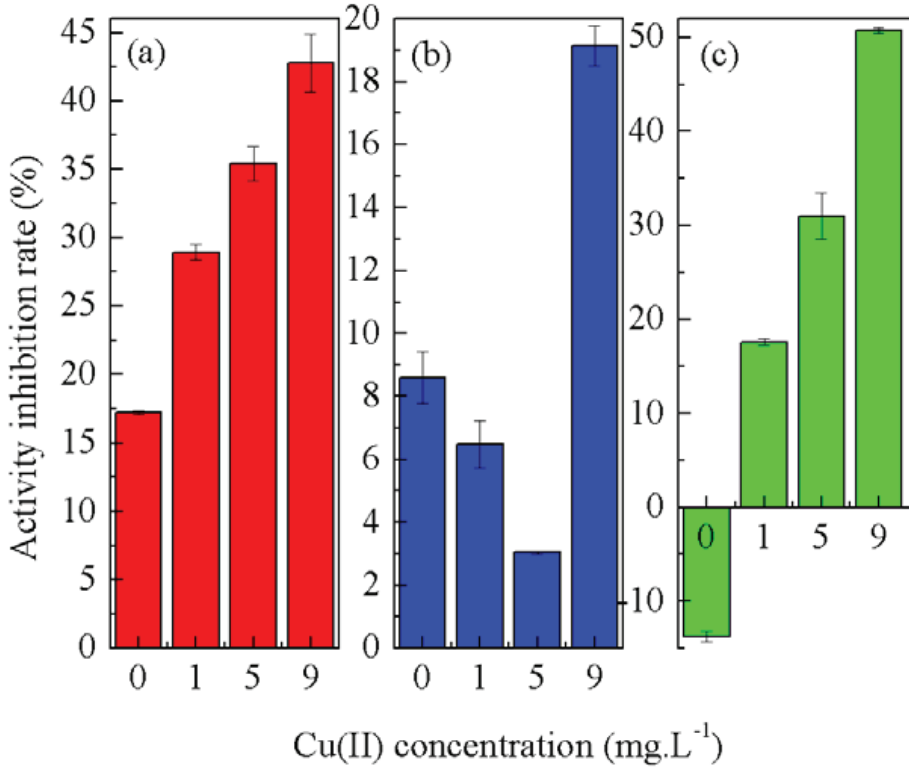

Fig. 5. The inhibition rate of nitrification activity of sludge without EPSs (a), TB-EPSs (b) and LB-EPSs (c).

increased to 9 mg·L^{-1}. The initial decrease and then increase in the nitrification activity inhibition rate may be related to the EPSs content and the influent Cu(II) content. When both the EPSs and Cu(II) contents are low, a small amount of Cu(II) penetrates the EPSs layer and enters the cells interior, inhibiting the nitrification activity of the sludge. As the EPSs content increases, more EPSs adsorb Cu(II), making it difficult for the copper to enter the cells. At this concentration, the effect on sludge nitrification activity is small. When the Cu(II) content further increased, a large amount of Cu(II) entered the cells because the adsorption point of the EPSs was exceeded, and the toxic effect of the Cu(II) increased [25]. Further research is needed to understand the mechanism of action of EPSs in protecting the microorganisms.

In the absence of LB-EPSs, the nitrification activity inhibition rate increased with increasing Cu(II) concentration, which was consistent with the trend seen with EPSs. In addition, Fig. 5 shows that in the presence of Cu(II), the LB-EPSs had a greater inhibitory effect on the nitrification activity of the sludge than did the TB-EPSs. The LB-EPSs show more absorption bands and play a major role in the adsorption process of EPSs [25].

4. Conclusions

The presence of Cu(II) affected the removal of organic matter, ammonia nitrogen and the content and composition of EPSs. When the content of Cu(II) influent increased from 0 mg·L^{-1} to 9 mg·L^{-1}, the removal capacity of COD and NH_4^+-N of sludge was reduced. The content of PN and PS in LB-EPSs and TB-EPSs decreased first, then increased and decreased again. Overall, for the TB-EPSs and LB-EPSs, when the concentration of Cu(II) was low (1 mg·L^{-1}), the primeval excitation-emission area volume of substances in the five regions decreased. A high concentration of Cu(II) (5 mg·L^{-1}) stimulated microbes to secrete more substances in aromatic protein region I, aromatic protein region II, the soluble microbial byproduct-like material region, and the humic acid-like organics region. When the content of Cu(II) in influent water was further increased to 9 mg·L^{-1}, the effect of toxicity and fluorescence quenching of Cu(II) resulted in the decrease of substances in the five regions, compared with the results at 5 mg·L^{-1}. In the presence of Cu(II), EPSs not only protected microorganisms from the toxicity and harm of Cu(II) but also influenced the nitrification activity of sludge due to the changes in content and composition. In comparison, LB-EPSs had a greater effect on the inhibition of the nitrification activity of sludge than TB-EPSs under different Cu(II) content conditions.

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