Adsorption of Cu\(^{2+}\) and mechanism by natural biofilm
Xiaoying Cheng, Wenjia Xu, Ningyuan Wang, Yanan Mu, Jiatai Zhu and Jiaqi Luo

ABSTRACT

The biofilm culturing device fixed on the slides was vertically placed in the commonly called small Li Lake of Jiangnan University. The adsorption experiment of Cu\(^{2+}\) was carried out by mature biofilm. Besides, scanning electron microscope (SEM), polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy-energy spectrum (SEM-EDX) were used to analysis the effect of Cu\(^{2+}\) on the morphological structure of biofilm. The result indicated that when the initial concentration of Cu\(^{2+}\) was 5 mg·L\(^{-1}\), the adsorption capacity of Cu\(^{2+}\) by unit mass biofilm is the maximum. More extracellular polymeric substances (EPS) were released by biofilm due to the stimulation of Cu\(^{2+}\). EPS was beneficial to the adsorption of Cu\(^{2+}\) by biofilm. After the adsorption of Cu\(^{2+}\), the bacterial diversity index decreased, while there were no significant differences in microbial communities on biofilm. Moreover, the main groups combining Cu\(^{2+}\) were the hydroxyl groups and amide groups in S-EPS and B-EPS. Ion exchange is a mechanism of the adsorption of Cu\(^{2+}\) by EPS.

Key words | adsorption, biofilm, Cu\(^{2+}\), microbial communities

HIGHLIGHTS

- The adsorption experiment of Cu\(^{2+}\) by biofilm culturing device could actually simulate the adsorption of metal elements in the water ecological environment.
- The scanning electron microscope (SEM), polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy-energy spectrum (SEM-EDX) were used to analyse the effect of Cu\(^{2+}\) on the morphological structure of biofilm.
- The stimulation of Cu\(^{2+}\) establishing a positive feedback effect on releasing more extracellular polymeric substances (EPS) by biofilm.

INTRODUCTION

In recent years, the river contamination in China was serious, which led to degradation of the river ecosystems and deterioration of water quality. Heavy metal contamination occupied a large proportion (Chen 2015). Copper is an indispensable trace element in the life activities of organisms. However, it will be harmful to the creature and become a typical heavy metal pollutant in water when it reaches a certain concentration. Recently, natural biofilm has become one of the research hotspots in dealing with heavy metal contamination because it is the composite solid phase medium that can effectively combine heavy metals (Hua et al. 2013). The existing studies are as follows. Firstly, biofilm play a role of indication. Tien & Chen (2013) indicated that the extent of contamination of water by investigating the accumulation of Cr, Ni, Cu and Pb in the river biofilm. Secondly, environmental factors have an effect on the adsorption of heavy metals by natural biofilms. Huang et al. (2000) investigated that pH had a significant effect on the adsorption of biofilms. It indicated that the adsorption capacity of Cu by biofilms reached the maximum (161.80 μg/g) when the pH value was 5. Thirdly, biofilm has the ability of purification. Dong et al. (2005) investigated that the cultured biofilm had obvious adsorption on heavy metals such as Pb, Cd, Cu and Zn in water.

A large number of studies has shown that the EPS had a strong adsorption capacity of heavy metal ions, Liu et al. (2001) indicated that removal efficiency of Zn\(^{2+}\), Cu\(^{2+}\) and Cr\(^{2+}\) by EPS was above 95%. Wei et al. (2007) indicated that the maximum adsorption capacity of EPS, respectively, was 0.81, 0.62
and 0.50 mg/g EPS for the three heavy metal ions, which investigated the adsorption efficiency of EPS on Cu$^{2+}$, Cr$^{3+}$ and Ni$^{2+}$. Yin et al. (2013) indicated that the bio-absorption of Cd$^{2+}$, Pb$^{2+}$ and Cu$^{2+}$ was closely related to EPS and the adsorption efficiency was Cu$^{2+}$ > Pb$^{2+}$ > Cd$^{2+}$. The reasons for the removal of heavy metals are as follows. Firstly, the heavy metals were adsorbed by EPS which is on the surface of the biofilm. Then, the heavy metals which were partially fixed on the surface of the biofilm fell off with the biofilm. While another part of heavy metals were inside the cell through the penetration of biofilms, etc., for the trace elements required for microbial life activities or caused the death of microorganisms.

The experiments on the purification and adsorption of heavy metals by biofilms were mostly concentrated on deleterious Pb and Cd (Duong et al. 2008), while the trace element copper was less studied. In this study, the biofilm was vertically placed in the commonly called small Li Lake of Jiangnan University to cultivate, and the adsorption experiment of Cu$^{2+}$ by mature biofilm was carried out. Additionally, SEM, PCR-DGGE, FTIR and SEM-EDX were used to discuss the effect of Cu$^{2+}$ on the morphological structure of biofilm.

**MATERIALS AND METHODS**

Cultivation of biofilm

The slide glass (25.4 mm × 76.2 mm) (Stewart et al. 2013; Wang et al. 2014a) was used as a carrier of microorganisms in natural water bodies. The improved biofilm culture device based on Dong et al. (2003) and other patents were used as an experimental device (Figure 1). The slides were pretreated by the methods of Yu et al. (2004). The clean slides were fixed on a biofilm plate and placed vertically 30 cm below the surface of the water for 21 days to obtain mature biofilm (Matar et al. 2017). The characteristics of water quality during biofilms growth is shown in Table 1.

The experiments of static adsorption

1.8875 g Cu(NO$_3$)$_2$·3H$_2$O was dissolved with 500 mL ultrapure water, and the concentration of standard storage solution was 1,000 mg·L$^{-1}$. The Cu$^{2+}$ solution was prepared with miracle mineral solution (MMS) and divided into the same values of Cu$^{2+}$ solution in conical flasks (Zhang et al. 2014). On the basis of our previous research, the best Cu$^{2+}$ adsorption condition was set. The pH = 6 of the solution was regulated by HNO$_3$ and NaOH. The temperature was 20°C. After 24 h, the adsorption reached equilibrium. The glass slides with mature biofilms were put into the conical flasks. The flasks were oscillated (120 r·min$^{-1}$) for absorption of Cu$^{2+}$ (Chao et al. 2013; Liu et al. 2014). In order to exclude the adsorption of Cu$^{2+}$ by slides, blank slides in the same condition was carried out (Rešlin ’ski et al. 2013). The result indicated that the maximum removal ratio was 1.1% which was negligible in subsequent experiments. All experiments were performed in three

![Figure 1](https://iwaponline.com/wst/article-pdf/78/4/721/487399/wst078040721.pdf)
parallel samples, and the average of the measured data was used for the analysis.

The analysis of biofilm structure before and after the adsorption of Cu²⁺

The analysis of scanning electron microscopy

In order to analyze and compare the differences of the morphological structure of biofilm before and after adding 5 mg/L Cu²⁺, we have adopted SEM technology. The sample of biofilm was glued to the sample stage after it had dried. Then, the sample was placed in the vacuum sprayer plating instrument, sprayed gold and plated with a conductive layer. The sample of biofilm was scanned with Quanta 200 environmental scanning electron microscopy.

The analysis of denaturing gradient gel electrophoresis (PCR-DGGE)

We used PCR-DGGE to analyze the changes of species diversity and microbial community structure before and after adding 5 mg·L⁻¹ Cu²⁺.

Extraction of DNA. DNA was extracted using Ezup column bacterial genomic DNA purification kit (Sangon Biotech, Shanghai, China).

The PCR amplification of DNA. The amplification of V3 hypervariable region of the 16SrRNA gene of bacteria was performed using the universal primer set F357 and R518 (Table 2) (Tong et al. 2004; Susmita 2015). The reaction system is shown in Table 3. The PCR amplification was conducted at 94 °C for 4 min (pre-denaturation), 94 °C for 0.5 min, 56 °C for 1 min and 72 °C for 0.5 min, a total of 30 cycles and extended at 72 °C for 7 min. The amplified product was tested by 1.5% agarose gel electrophoresis (Singh et al. 2009).

The electrophoresis of DGGE. It was sampled that the PCR products of the V3 region were about 400 ng. The analysis of DGGE was performed on D-Code mutation detection system (Lv et al. 2017). The concentration of the polyacrylamide gel was 8% (acrylamide: acrylamide polymers = 37.5:1). The denatured concentration ranged from 30% to 60% (the 100% denaturing agent was 7 mol·L⁻¹ urea and 40% formamide) (Wang et al. 2014b). The gel was conducted at 60 °C, 60 V for 16.0 h on 1×TAE. After electrophoresis, the gel was rinsed with ultrapure water. The gel was put in an ethidium bromide (EB)-containing dye solution and stained on an oscillator for 30 min. After that, the sample was detected in the UVI imaging system.

The recycle of DGGE band and the clone and sequence of TA. The typical band was selected to completely cut into the 1.5 mL centrifuge tube. Then it was recycled according to the method of SK8131 to reserve.

Fourier transform infrared spectroscopy

The method (Fang et al. 2002) was used to extract EPS (Chaupart & Serpe 1998). The freeze-dried EPS samples were examined using pressed potassium bromide (KBr) pellets containing 1% of the samples on a Fourier transform infrared spectrum analysis spectrometer (Thermo IS10) in the scanning range of 4,000–400 cm⁻¹ (Li & Kobayashi 2016).

The analysis of scanning electron microscope energy dispersive X-ray spectroscopy

The fully dried blank EPS and EPS adsorbed Cu (II) were plated gold as preparation for electron microscope samples (Hayakawa & Matsuoka 2016; Wang et al. 2017). The sample was set in Quanta 200 environment SEM and the morphology was observed. The samples were determined by transformation of the surface element by EDX (Falona).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Amplify the size of the fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>F357-GC</td>
<td>5'- CCTACGGGAGGCAGCAG -3'</td>
<td>Around 230 bp</td>
</tr>
<tr>
<td>R518</td>
<td>5'- ATT ACC GCG GCT GCT GG -3'</td>
<td></td>
</tr>
</tbody>
</table>

GC: CCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCC.
RESULTS AND DISCUSSION

The morphology of biofilm

The biofilm samples cultured for 21 days were observed the morphology of biofilm under optical microscope and transmission electron microscope (TEM). It can be seen in Figure 2 that the heterogeneous and rough biofilm with diverse microorganisms was attached on the slide surface. Optical microscope and TEM observation showed that diatoms were the dominated algae on the biofilm, while bacillus and globular bacteria were the main bacteria.

Influence of initial concentration of Cu$^{2+}$ on adsorption of biofilm

The influence of the change of initial concentration of Cu$^{2+}$ on the adsorption of Cu$^{2+}$ by biofilm at pH 6 and 20 °C oscillated (120 r·min$^{-1}$) for 24 h. The unit of adsorption capacity was mg/g, which meant the mass of Cu$^{2+}$ corresponding to the mass of biofilm. The results are shown in Figure 3.

As we can see in Figure 3, the adsorption capacity on Cu$^{2+}$ of the sample increased with the initial concentration of Cu$^{2+}$ when it was 1–5 mg·L$^{-1}$, while it decreased when the initial concentration of Cu$^{2+}$ was more than 5 mg·L$^{-1}$. The results indicated that the biofilm could secrete EPS such as polysaccharides under the stimulation of lower Cu$^{2+}$ concentration, which was beneficial to the adsorption of heavy metals. The high concentration of Cu$^{2+}$ could lead to acute toxicity of biofilm, affect the activities of biofilm and reduce the capacity of biofilm adsorption. In natural water, Cu$^{2+}$ adsorbed on the natural biofilm gradually accumulated and caused biofilm to die and fell off. The Cu$^{2+}$ absorbed on biofilm was deposited into the river sediment and the Cu$^{2+}$ in water was removed. The removal rate decreased with the increase of Cu$^{2+}$ initial concentration, because the adsorbent lacked sufficient adsorption sites to adsorb Cu$^{2+}$ in the solution, as the concentration of Cu$^{2+}$ increased. When the initial concentration of Cu$^{2+}$ was 5 mg·L$^{-1}$, the adsorption capacity of Cu$^{2+}$ per unit mass biofilm reached its maximum. In which case, 5 mg·L$^{-1}$ Cu$^{2+}$ was chosen for subsequent adsorption mechanism experiments.

Main component content and enrichment rate on Cu$^{2+}$ of biofilm

Fe oxides and Mn oxides are the main metal oxides in natural biofilm, which play important roles in the enrichment of trace metals (Dong et al. 2003). EPS is the main component of biofilm. A large number of functional groups such as carboxyl, amino, hydroxyl groups on EPS make it able to capture and hold tons of heavy metals in water. The content of above substances is in Table 4. When the initial concentration of Cu$^{2+}$ was 5 mg·L$^{-1}$ at pH 6 under 20 °C, the sample was shaken (120 r·min$^{-1}$) to absorb Cu$^{2+}$ for 24 h. Contributions of major components (the ratio of Mn oxide, Fe oxide, organic matter and other components in total biofilm adsorption) to Cu$^{2+}$ enrichment is in Figure 4.

It could be seen in Figure 4 that the contribution ratio of main components to Cu$^{2+}$ enrichment in biofilm was Mn oxides (46.03%) > organic matter (32.67%) > Fe oxides (15.69%) > others (5.61%). Fe oxides and Mn oxides took a little mass proportion in biofilm, while they had great influence on the adsorption of Cu$^{2+}$. The contribution ratio of organic matter to Cu$^{2+}$ enrichment was only inferior to
that of Mn oxides, which reflected the strong Cu²⁺ combining ability of organic matter. Other components in biofilm such as Al oxides contributed too little on the enrichment of Cu²⁺ to be negligible.

The analysis of SEM

The observation of SEM (Figure 5) showed that there were more particulate matters obviously around the cell after the adsorption than before, which indicated that the adsorption occurred. Biofilm with 5 mg·L⁻¹ Cu²⁺ had rougher surface than the control group. The secretion of EPS increased. In addition, more passageways and cavities appeared and the surface area enlarged. All these changes were beneficial to the transmission and transport of matter.

The ultrastructure of natural biofilm microbes

PCR amplification and analysis of DGGE profile

The length of the amplified products of 16S rDNA was about 230 bp according to the electrophoresis detection. Amplified products of each sample had a single band and their concentration met the demand of analysis of DGGE (Figure 6(a)). The amount, strength and migration position of the bands of DGGE separation were different before and after the absorption. The microflora diversity index is in Table 5.

As we can see in Figure 6(b), the amount of sample band (lane 1) before the absorption was more than that after the absorption. The bacterial species was much richer, and the Shannon index of lane 1 was higher (Table 5). After the adsorption of Cu²⁺, band 1, 2, 3, 6, 8, 9, 10, 13, 19, 20, 22, 29 and 30 became brighter, indicating that the bacteria represented by these bands became predominant bacteria by then and Cu²⁺ might be the prerequisite to their growth which promoted it. The enrichment of bacterial communities represented by bands 11, 14, 15, 16, 21, 23, 24, 25, 27, 28, 31, and 32 declined and the bands became dimmer. Besides, the disappearance of bands 4, 5, 7, 12, 17, 18, and 26 indicated that this part of bacterial community could not grow under 5 mg·L⁻¹ Cu²⁺ stress, because Cu²⁺ accumulated in bacteria and produced great toxicity which led to its elimination. Compared to lane 1, the Simpson’s diversity index of lane 2 declined, but the decreasing amplitude was not obvious. The decline of diversity index could have resulted from toxicity enhancement caused by the enrichment of Cu²⁺ in bacteria. The similarity of these two lanes was 73%, indicating that natural biofilm communities did not change significantly.

Retrieving and sequencing of characteristic bands and analysis of phylogenetic tree

Twenty significantly changed bands (3, 6, 8, 9, 10, 11, 12, 13, 14, 16, 19, 20, 24, 25, 27, 28, 29, 30, 31, and 32) were selected to retrieve, sequence and remark as 1–20. The electrophoretogram was as Figure 6(c) shown after PCR
amplification. By Blast comparative identification, the result of sequencing fabricated the phylogenetic tree.

Bands 1, 3, 4, 7, and 8 were Gram-positive bacilli belonging to Proteobacteria phylum. Bands 2, 5, 9, 10, 13, 14, 15, 17, 18, 19, and 20 were Gram-negative bacilli belonging to Proteobacteria Phylum. Bands 6, 12, and 16 were uncultivable bacteria belonging to Chloroflexi phylum, Cyanobacteria phylum and Proteobacteria phylum, respectively. Band 11 belonging to Sphingobacteria. The result was similar to TEM that biofilm was mainly on bacillus.

Predominant bacteria were Acinetobacter beijerinckii, Pseudomonas sp. 3B_8, Pseudomonas plecoglossicida (T), Bacterium SRMC-31-2, Curvibacter lanceolatus (T), Enterobacter cloacae, Aeromonas sp. RC278, Enterobacter sp. Pptphilum, Aeromonas ichthiosmia (T) and Duganella violaceinigra before the absorption and changing to Acinetobacter

Figure 5 | The SEM images of biofilm before and after adsorption (1,200x and 2,400x).

Figure 6 | Map of PCR products in biofilm samples (a); DGGE profile of 16S rRNA in biofilm samples (b); phylogenetic tree based on the sequences of bacteria in the biofilm (c). (Continued.)
After the absorption. Only *Aeromonas ichthiosmia* (T) and *Duganella violaceinigra* were predominant bacteria before and after the absorption. Their bands became brighter after the absorption which indicated that Cu$^{2+}$ had a promoting effect on their growth.

**The analysis of FTIR**

Microorganisms accounted for only 10% of the biofilm dry weight, and the remaining 90% were EPS in most biofilms.
As is shown in Figure 7, there was no difference on the peaks of S-EPS and B-EPS before and after the adsorption. The conclusion that the whole structure of EPS was not destroyed could be obtained. Nonetheless, the position and intensity of some peaks had been changed. The phenomenon indicated that some reaction happened between functional groups and Cu²⁺. After the adsorption of Cu²⁺, the peak width of hydroxyl (Nwodo & Okoh 2013) of S-EPS and B-EPS at 3,200–3,600 cm⁻¹ increased while the intensity weakened. In the meantime, a slight deviation occurred, indicating that the oxygen atoms were involved in the complexation reaction which showed the differences of the bond length of the O-H bond (Lim et al. 2008). It was possible to be carbohydrates (Iyer et al. 2005) or proteins involved in adsorption leading to the asymmetric stretching vibration at 2,900–3,000 cm⁻¹ of C-H. After the adsorption of Cu²⁺, the carboxyl peak appeared at 1,760 cm⁻¹ and 1,770 cm⁻¹ for S-EPS and B-EPS, respectively. It was confirmed that humus acid was involved in the adsorption of EPS (Iqbal & Edyvean 2004). After the adsorption of Cu²⁺, the intensity of peak at 1,630–1,680 cm⁻¹ decreased obviously and shifted. Moreover, the intensity of peak at 1,350–1,450 cm⁻¹ was enhanced and shifted. The peak of S-EPS at 1,540 cm⁻¹ disappeared after the adsorption of Cu²⁺, indicating that several groups of proteins were involved in the adsorption of Cu²⁺. After the adsorption of Cu²⁺, the peak intensity of S-EPS and B-EPS at 1,100–1,000 cm⁻¹ was obviously weakened and shifted somewhat. At the same time, the peak intensity of sulfur groups and phosphorus groups in fingerprint area was obviously weakened. Functional groups of various substances in S-EPS and B-EPS were involved in the reaction. Besides, hydroxyl and amide groups played significant roles in the reaction.

The analysis of SEM-EDX

The differences of surface structure and element composition of EPS were investigated with SEM-EDX before and after adsorption of Cu²⁺ (Zhigang 2009). As is shown in Figure 8, the surface particles and the roughness of S-EPS and B-EPS increased after the adsorption of Cu²⁺. As is vividly shown in Table 6, it could be concluded that C, O, N, Na, Mg, Al, Si, P and Cl were the main elements of EPS. What is more, the elements on the surface of S-EPS were much more abundant than B-EPS. After the adsorption of Cu²⁺, an obvious -Cu peak appeared both in S-EPS and B-EPS, indicating that the adsorption of Cu²⁺ occurred. Besides, the peaks of Mg, Al, Si, K and Ca on the surface of S-EPS were weakened in different degrees. The peaks of Mg and Si in B-EPS had decreased, indicating that the ion exchange existed. The contents of C, O, N and P made differences before and after the adsorption. It could draw the conclusion that the oxygen functional groups, amino groups and phosphate groups in EPS were involved in the adsorption process, which was consistent with the results of FTIR. In addition, the presence of Cu and S elements indicated the possible existence of the CuS complex, contributing to the implementation of Cu²⁺ fixation.

CONCLUSION

(1) The experiment investigated the effect of Cu²⁺ on the morphology and structure of biofilm in spring. It could be summed up that the biofilm could secrete more
Figure 8 | SEM-EDX images of the S-EPS and B-EPS.
Table 6 | EDX analysis of S-EPS and B-EPS before and after adsorption

<table>
<thead>
<tr>
<th>Element</th>
<th>S-EPS (before adsorption)</th>
<th>S-EPS (after adsorption)</th>
<th>B-EPS (before adsorption)</th>
<th>B-EPS (after adsorption)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>32.26</td>
<td>24.68</td>
<td>26.08</td>
<td>28.53</td>
</tr>
<tr>
<td>N</td>
<td>6.83</td>
<td>4.21</td>
<td>3.29</td>
<td>5.91</td>
</tr>
<tr>
<td>O</td>
<td>33.28</td>
<td>32.5</td>
<td>30.73</td>
<td>28.62</td>
</tr>
<tr>
<td>Na</td>
<td>1.73</td>
<td>5.48</td>
<td>34.38</td>
<td>10.57</td>
</tr>
<tr>
<td>Mg</td>
<td>2.37</td>
<td>1.09</td>
<td>0.29</td>
<td>0.23</td>
</tr>
<tr>
<td>Al</td>
<td>0.41</td>
<td>0.39</td>
<td>0.05</td>
<td>0.21</td>
</tr>
<tr>
<td>Si</td>
<td>1.25</td>
<td>0.83</td>
<td>0.25</td>
<td>0.13</td>
</tr>
<tr>
<td>P</td>
<td>2.26</td>
<td>1.76</td>
<td>0.46</td>
<td>1.00</td>
</tr>
<tr>
<td>S</td>
<td>1.86</td>
<td>10.9</td>
<td>–</td>
<td>8.04</td>
</tr>
<tr>
<td>Cl</td>
<td>1.81</td>
<td>1.70</td>
<td>5.00</td>
<td>0.56</td>
</tr>
<tr>
<td>K</td>
<td>0.83</td>
<td>2.50</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ca</td>
<td>13.4</td>
<td>6.28</td>
<td>–</td>
<td>0.36</td>
</tr>
<tr>
<td>Cu</td>
<td>–</td>
<td>9.42</td>
<td>–</td>
<td>15.85</td>
</tr>
</tbody>
</table>

– represents not detected.

EPS under the stimulation of Cu$^{2+}$ at the concentration of 5 mg L$^{-1}$ through the analysis of SEM, which was beneficial to the adsorption of Cu$^{2+}$ by biofilm.

(2) After the adsorption of the Cu$^{2+}$, PCR-DGGE showed that the bacterial diversity index decreased in the biofilm, but Cu$^{2+}$ stress had a slight impact on the microbial community of the biofilm.

(3) The contribution ratio of main components to Cu$^{2+}$ enrichment in biofilm was Mn oxides (46.05%) > organic matter (32.67%) > Fe oxides (15.69%) > others (5.61%). Fe oxides and Mn oxides took a little mass proportion in biofilm, while they had great influence on the adsorption of Cu$^{2+}$.

(4) The FTIR indicated that the hydroxyl groups, amino groups and amide groups were the main groups of S-EPS and B-EPS leading to the combination with Cu$^{2+}$. Proteins were the dominant components of EPS. SEM-EDX indicated that ion exchange was one of the mechanisms about the adsorption of Cu$^{2+}$ on EPS.

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