Effects of Fe(III) on biofilm and its extracellular polymeric substances (EPS) in fixed bed biofilm reactors

Xuewei Hu, Kai Chen, Xinke Lai, Siping Ji and Kevin Kaiser

ABSTRACT

The effects of Fe(III) on the biofilm mass and activity, the biofilm micromorphology as well as the composition and functional groups characteristics of extracellular polymeric substances (EPS) in biofilm were investigated in laboratory-scale fixed bed biofilm reactors. The results showed that 2 mg/L of Fe(III) promoted the biofilm mass and improved the biofilm activity, but 16 mg/L of Fe(III) adversely affected biofilm development. Scanning electron microscopy (SEM) study indicated a high concentration (16 mg/L) of Fe(III) led to significant reduction of the filaments, great promotion of the EPS secretion in biofilm. The result of the EPS composition suggested 2 mg/L of Fe(III) increased soluble EPS and loosely bound EPS which contributed to the microbial aggregation, while 16 mg/L of Fe(III) promoted tightly bound EPS production unfavourable for substrate mass transfer. Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy analysis demonstrated that Fe(III) exerted a significant influence on the –CONH– groups of proteins and the C–O groups of polysaccharides in EPS. This study reveals that Fe(III) influences biofilm development and activity not only by directly impacting the microbial physiology but by indirectly affecting the EPS constituents, and it helps to provide theoretical guidance for iron ion containing wastewater treatment.

Key words | biofilm, extracellular polymeric substances (EPS), Fe(III), microbial physiology

INTRODUCTION

Biofilm processes are attractive in wastewater treatment with their high biomass concentrations, high organic loading rates, and strong anti-shock loading capabilities (Karadag et al. 2015). Extracellular polymeric substances (EPS), the representative components of microbial aggregates, are not only responsible for the biofilm formation and stability of the biofilm structure, but play a crucial role in the functions of the adhesion ability, flocculation ability of biofilm as well as mass transfer in biofilm matrix (Sheng et al. 2010).

In recent years, various external conditions including dissolved oxygen (DO), shear rate, metal cations and toxic substances have been demonstrated to affect the EPS characteristics in the microbial aggregates in the wastewater (Sheng et al. 2010). Among these factors, the possible effects of monovalent and divalent metal ions have been considered important and investigated by many researchers, but the role of trivalent cations such as ferric ions in the properties of EPS is not well understood.

Currently, ferric salts have been widely used in the coagulation-flocculation process in sewage pretreatment.

The addition of iron ions in wastewater treatment is a common practice for phosphorus removal (De Gregorio et al. 2010). Moreover, iron is normally found in spent pickle and from plating shops, steel mills, foundries, chemical milling, and wire drawing operations (Anusha 2011). The application of the zero-valence iron process and Fenton oxidation technology also results in the presence of iron ions in wastewater treatment. For the above reasons, municipal and some industrial wastewater may contain various concentrations of iron ions which may exert an influence on the bioreactor properties.

Since Fe is importantly involved in the microbial aggregates, its possible impact on sludge characteristics (Yu et al. 2000; Li 2005; Oikonomidis et al. 2010; Li et al. 2012), membrane fouling (Wang et al. 2014), and biosorption (Dong et al. 2005) has been investigated by many researchers. For instance, Yu et al. (2000) found that introduction of high Fe dosages enhanced the granulation process, but reduced bacterial specific activity due to the possible toxicity of high-level Fe. Li (2005) considered that concentration of
ferric ions > 23.8 mg/L led to a shift in the size distribution of activated sludge from large to small flocs. Moreover, Li et al. (2012) suggested the binding affinity of Fe(III) for the activated sludge plays a crucial role in the content of loosely bound EPS (LB-EPS) which is important for governing bioflocculation.

However, there has been little information about the effects of ferric ions on the characteristics of biofilm and EPS in biofilm reactors. Huang et al. (2011) considered that iron ore sinter particles used as biofilm carrier enhanced the bioreactor performance. But both the effect of high Fe concentrations and the role of EPS in biofilm are not well understood. Thus, further research is required to clarify the interaction of iron with biofilm development and EPS characteristics. The effects of ferric ions have been studied on the biofilm mass, the micromorphology and EPS characteristics of biofilm were investigated in laboratory-scale fixed bed biofilm reactors (FBBRs). It may reveal the influences of Fe(III) on the bioreactor properties and help to provide theoretical guidance for iron ion containing wastewater treatment.

**MATERIALS AND METHODS**

**Experimental set-up**

Experiments were performed in parallel in two identical column-type FBBRs (inner diameter 20 cm, height 50 cm) with an effective volume of 8 L (Figure S1 in the Supplementary Material, available with the online version of this paper) to assess the effects of Fe(III) on the initial and already developed biofilms. The biomass carrier was removable square plates composed of polyvinyl chloride. A control reactor (R1) was left free of iron concentration all the time, while an experimental reactor (R2) was left free of iron in the first week and third week, augmented with FeCl₃·6H₂O at 2 mg/L of Fe(III) during the second week (W₂) and 16 mg/L of Fe(III) during the fourth week (W₄). The operational timing period in reactors was 24 h, including 10 min of influent filling, 1.5 h/0.5 h of aeration on/off (a total of 23 h), 30 min of settling and 20 min of effluent withdrawal. The reactor temperature (18.8 ± 2.1 °C), pH (7.47 ± 0.42) and DO (4.5 ± 0.9 mg/L) in R1 and R2 were controlled.

Activated sludge was used for inoculation for one week for biomass stabilization with synthetic wastewater in FBBRs. The activated sludge was taken from the aeration tank at wastewater treatment station in Kunming University of Science and Technology (Yunnan, China) with mixed liquid suspended solids (MLSS) and sludge volume index (SVI) of 1.93 g/L and 83 mL/g, respectively. Table S1 in the Supplementary Material (available online) gives the details of the synthetic wastewater composition.

**EPS analysis**

A physical extraction process (Chen 2013) was modified to extract the soluble EPS (SEPS), LB-EPS and tightly bound EPS (TB-EPS) of biofilm samples. The detailed protocol of extraction is described in Figure S2 in the Supplementary Material (available online). The polysaccharides (PS) content in EPS was measured with the phenol-sulfuric acid method (Lazarova & Manem 1995) using glucose as the standard, while the content of proteins (PN) was determined using a modified Lowry method (Frolund et al. 1996) using bovine serum albumin (Shanghai Yuanye Bio-Technology Co., Ltd, China) as the standard. The DNA content was determined with the diphenylamine colorimetric method (Burton 1956) using calf thymus DNA (Shanghai Yuanye Bio-Technology Co., Ltd, China) as the standard.

**Chemical analysis**

Biofilms were scraped using a blade, washed and then diluted with deionized water to form suspensions. The biofilm dry weight (DW) was determined by the mass obtained after drying the biofilm solution at 105 °C for 24 h. The residue obtained at 105 °C was burned at 600 °C for 1 h to determine the mineral fraction of the biofilm. The organic fraction (volatile dry weight, VDW) was then calculated from the loss of mass. The chemical oxygen demand (COD), MLSS and SVI contents of sludge were determined according to Standard Methods (APHA 1998).

The temperature, pH and DO were measured by a multi-parameter water quality analyzer (DZS-707, Shanghai Precision & Scientific Instrument Co., Ltd, China).

**Micromorphology analysis**

Micromorphology of the biofilm samples was investigated using a scanning electron microscopy (SEM) (VR2A 3 SBU, Czech Republic). Prior to SEM analysis, the samples were fixed with an aqueous 2.5% glutaraldehyde solution for 24 h, and washed three times with PBS, then followed by a six-step dehydration with an ethanol/water gradient (50–100%), all at 4 °C. The dehydrated specimens were dried in a desiccator at 25 °C for 24 h and then gold-coated with for SEM.
**FTIR and XPS analysis**

Before Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) analysis, the wet biofilm samples were freeze-dried at −50 °C. A mixture of dry powdered EPS sample (about 1 mg) and spectrometry grade KBr (about 100 mg) was pressed into a pellet, and analyzed between 4,000 and 400 cm⁻¹ using an FTIR spectrometer (BRUKER TENSOR 27, Germany). The XPS analysis was performed using an XPS (PHI 5000 Versaprobe, Japan) with an AlKα X-ray source. XPS high resolution spectra of C1 s and O 1 s scans were analyzed with XPS peak 4.1 software, using the containment carbon (284.6 eV) for calibration.

**RESULTS AND DISCUSSION**

**Effect of Fe(III) on the biofilm mass and activity**

The DW and biomass of biofilm in R1 and R2 from the end of week 2 and week 4 are shown in Figure S3 in the Supplementary Material (available with the online version of this paper). It suggests the biofilm biomass in reactors accounts for the majority of the total biofilm DW, and measured in the range from 88.3 to 91.4%. The DW and the organic matter content in biofilm from R2 were both slightly higher than those in R1 from the end of week 2. In contrast, the DW and biomass of biofilm in R1 from the end of week 4 were 14.9% and 17.8% higher than those in R2, respectively. The results indicate 2 mg/L of Fe(III) accelerates biofilm formation, while 16 mg/L of Fe(III) significantly reduces the biofilm mass ($p < 0.05$) and adversely affects biofilm development. Additionally, the degradation performance of COD affected by Fe(III) augmentation was investigated in both reactors. The result shows that R2 has better efficiency of organic matter removal during the second week (R1 with 77.1 ± 3.1%; R2 with 80.2 ± 3.4%) which is associated with the relatively higher biomass and enhanced biofilm activity. However, better performance of COD removal was found in R1 (85.7 ± 5.2%) compared with that in R2 (77.2 ± 4.7%) at week 4, which corresponded with the result of biofilm mass.

Prior study showed Fe was involved in the synthesis of many important PN and serves as enzymatic cofactors increasing the bacterial metabolism activity (Hintze & Theil 2006). Huang et al. (2011) found that the dissolution of around 0.5 mg/L of iron ions in the biological aerated filter enhanced both the adhered biomass and the organic matter removal. However, the high dosage of ferric ions can generate stress condition, which decreases the number of organisms (e.g., filaments and nitrifying bacteria) and brings an immediate shift in the microbial ecology (Oikonomidis et al. 2010). Since EPS play an important role in the aggregation of bacterial cells, EPS production affected by Fe(III) dosing may influence the biofilm mass. Above all, further research was done to confirm the reasons for the changes of biofilm mass and biofilm activity in this study.

**Effect of Fe(III) on the biofilm micromorphology**

Figure 1 shows the micromorphology of the biofilms sampled from both reactors at the end of week 4. It reveals marked alteration in their morphology when 16 mg/L of Fe(III) was added to the reactor. On the whole, the biofilm in R1 (Figure 1(a)) was characterized by a more compact structure than that in R2 (Figure 1(b)). Additionally, the biofilm in R2 appeared to scatter with few filamentous (Figure 1(b)) but abound with EPS (Figure 1(d)) compared with the biofilm in R1 (Figures 1(a) and (c)), indicating 16 mg/L of Fe(III) significantly influences the microbial ecology and stimulates EPS production.

Bacterial cells were usually intertwined with filaments which could be significantly involved in stabilization of the aggregates structure (Oikonomidis et al. 2010; Julien et al. 2014). However, a high Fe concentration would lead to significant reduction of filaments, which resulted in the looseness of biofilm structure. Furthermore, one natural response of microbes upon exposure to an adverse environment is to increase the production of EPS to form a protective shield for the cells against the adverse influences (Sheng et al. 2005).

**Effect of Fe(III) on the composition of EPS**

Table 1 shows the contents and constituents of EPS extracted from the initial and mature biofilms, respectively, growing in both reactors from the end of week 2 and week 4. It was found that the contents of PN were always higher than those of PS, which may be attributed to the low C/N ratio of influent (Ye et al. 2011). The results reveal soluble EPS (SEPS) and loosely bound EPS (LB-EPS) extracted from the initial biofilms had various yield but similar fractions of tightly bound EPS (TB-EPS), whereas the mature biofilms show an opposite result.

For the initial biofilms, the total EPS content was 152 mg/g VDW in R1, and the fractions of SEPS, LB-EPS and TB-EPS were 7.9, 6.0 and 86.1%, respectively. However, the content of the total EPS from the initial biofilms growing in R2 was 187.7 mg/g, with the SEPS, LB-EPS and TB-EPS...
Table 1 | The contents of PS, PN and DNA in EPS extracted

<table>
<thead>
<tr>
<th>Component</th>
<th>R1</th>
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<th>R2</th>
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<tr>
<td></td>
<td>SEPS</td>
<td>LB-EPS</td>
<td>TB-EPS</td>
<td>SEPS</td>
<td>LB-EPS</td>
<td>TB-EPS</td>
</tr>
<tr>
<td>Week 2 (W2)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Proteins (mg/g VDWa)</td>
<td>10.18 ± 0.75</td>
<td>8.58 ± 1.51</td>
<td>79.52 ± 0.75</td>
<td>31.37 ± 1.38</td>
<td>15.87 ± 4.75</td>
<td>79.59 ± 0.35</td>
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<tr>
<td>Carbohydrates (mg/g VDW)</td>
<td>1.89 ± 0.88</td>
<td>0.50 ± 0.15</td>
<td>47.29 ± 1.31</td>
<td>1.55 ± 0.34</td>
<td>0.70 ± 0.27</td>
<td>48.32 ± 1.58</td>
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<tr>
<td>PN/PS</td>
<td>5.4</td>
<td>17.1</td>
<td>1.7</td>
<td>20.2</td>
<td>22.6</td>
<td>1.6</td>
</tr>
<tr>
<td>DNA (mg/g VDW)</td>
<td>0</td>
<td>0</td>
<td>4.0 ± 0.12</td>
<td>0</td>
<td>0</td>
<td>4.12 ± 0.05</td>
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<tr>
<td>Week 4 (W4)</td>
<td></td>
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<tr>
<td>Proteins (mg/g VDW)</td>
<td>6.48 ± 0.70</td>
<td>6.48 ± 0</td>
<td>76.54 ± 0.70</td>
<td>6.37 ± 1.28</td>
<td>6.07 ± 0</td>
<td>99.59 ± 0.21</td>
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<tr>
<td>Carbohydrates (mg/g VDW)</td>
<td>1.98 ± 0.14</td>
<td>1.06 ± 0.05</td>
<td>34.74 ± 0.84</td>
<td>1.75 ± 0</td>
<td>1.39 ± 0.13</td>
<td>48.48 ± 1.69</td>
</tr>
<tr>
<td>PN/PS</td>
<td>3.3</td>
<td>6.1</td>
<td>2.2</td>
<td>3.6</td>
<td>4.4</td>
<td>2.1</td>
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<tr>
<td>DNA (mg/g VDW)</td>
<td>0</td>
<td>0</td>
<td>5.00 ± 0.03</td>
<td>0</td>
<td>0</td>
<td>5.46 ± 0.26</td>
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aVDW: volatile dry weight of biofilm.

Figure 1 | SEM images of biofilms in (a), (c) R1 and (b), (d) R2 from the end of week 4 (R1 was without Fe(III)) and R2 was augmented with 2 mg/L of Fe(III) in the second week and 16 mg/L of Fe(III) in the fourth week.)
fraction measuring 17.5, 12.1 and 70.4%, respectively. It suggests the biofilms from R2 possesses more SEPS and LB-EPS but less TB-EPS in contrast to those from R1. EPS has a role of nutrition for microorganisms reducing macromolecules in metabolizable low molecular-weight nutrients with an ‘enzyme-like’ reaction (Nielsen & Jahn 1999). It suggests that Fe(III) influences microbial metabolism activity by acting as cofactors for enzymatic reactions, accordingly, increase LB-EPS and SEPS utilized to serve as energy source by microorganisms under the nutrient-deprived conditions (Laspidou & Rittmann 2002). Furthermore, the increase of SEPS and LB-EPS resulted in bacterial adhesion but TB-EPS showed no positive effect on the microbial aggregation (Zhao et al. 2015), which might influence the biofilm development.

For the mature biofilms, the PN/PS ratios of SEPS, LB-EPS and TB-EPS ranged from 3.3 to 3.6, from 4.4 to 6.1 and from 2.1 to 2.2, respectively, which agrees with the finding of Liu & Fang (2003). The contents of SEPS, LB-EPS and TB-EPS were in the range of 8.1–8.5 mg/g, 7.4–7.5 mg/g and 116.3–153.5 mg/g VDW, respectively. The PN and PS contents in TB-EPS from R2 were 30.1 and 39.6% higher than those from R1, severally, which is consistent with the SEM result. Nielsen & Jahn (1999) claimed that EPS could either delay or prevent toxicants from reaching microbes by diffusion limitation and/or by chemical reactions to reduce the adverse influences. That is to say, the abrupt increase of TB-EPS in R2 stimulated by the high-iron environment forms a protective shield for microbes. However, high TB-EPS contents could impose a steric hindrance in microbial aggregates, which is not beneficial for substrate mass transfer (Sheng et al. 2010). Consequently, the significant decrease (p < 0.05) of biofilm mass in R2 is importantly associated with the sharp increase of the TB-EPS production in biofilm.

Effect of Fe(III) on functional groups characteristics of EPS

Since TB-EPS are closely associated with the bacterial cells and account for the majority of EPS, the FTIR spectra of TB-EPS extracted from both biofilm samples were recorded and are shown in Figure S4 in the Supplementary Material (available with the online version of this paper). The assignments of IR bands were according to the existing literature (Maquelin et al. 2002; Comte et al. 2006). Based on the characteristic bands in the IR spectra, EPS samples contained various molecules, including PS, PN, lipids, nucleic acids and humic-like substances. The large band near 3,420 cm⁻¹ was attributed to the stretching vibration of both the O–H groups of carbohydrates and the N–H groups of PN. The weak band at 2,957 cm⁻¹ was assigned to the asymmetrical C–H stretching vibrations of the aliphatic CH₃– group. The absorption bands around 1,650 cm⁻¹, 1,560 cm⁻¹, and at 1,234–1,244 cm⁻¹ region were, respectively, correlated with the CONH– groups of Amide I, Amide II and Amide III of PN. The peak around 1,400 cm⁻¹ was attributed to the symmetrical stretching of the carboxylate anion. The bands at 1,000–1,132 cm⁻¹ were the C=O and C=C stretching, C=O–H and C–O–C deformation vibrations of PS. Bands in the regions of lower than 1,000 cm⁻¹ might be assigned to the phosphate groups for nucleic acids. The bands at 2,359 and 2,397 cm⁻¹ were attributed to the interference peak caused by atmospheric CO₂.

EPS are bound with cells mainly through the ion bridging with metals, and metal cations such as Fe(III) may function by bridging negatively charged functional groups in EPS. Compared with the IR spectra of EPS samples in R2 at week 2, the spectra of EPS samples at week 4 exhibited significant changes. An obvious phenomenon was the sharp decrease in the band intensity of 3,431, 1,649 and 1,082 cm⁻¹ for EPS samples in R2 from week 4 compared with that in R1. For the broad overlapping region for N–H and O–H stretching near 3,431 cm⁻¹, it is hard to distinguish which group causes the shift. And it indicates the binding of iron ions with the –CONH– groups of Amide I from PN and the C–O groups from PS. Moreover, slight shifting of peaks at around 2,957 and 1,388 cm⁻¹ reveals the binding character of iron ions with the aliphatic CH₃– groups and the carboxylate anion. The results shows Fe(III) has a significant influence on the functional groups in EPS, especially the –CONH– groups of PN and the C–O groups of PS.

The functional groups in TB-EPS are also identified depending on the parameters of the XPS C1 s and O1 s spectra in Figure S5 and Figure S6 in the Supplementary Material, respectively (available online). Additionally, the ratios of various components in EPS are summarized in Table S2 in the Supplementary Material (available online). Changes in the relative proportions of EPS constituents suggests that the functional groups in EPS are biochemically active (Dufène et al. 1997). The changes in C1 s spectra showed that the relative abundance of C–(O, N) decreased, whereas the relative abundance of C–(C, H) and C = O or O–C–O increased in EPS from R2 at week 2 compared with R1. This is consistent with the change of abundance ratio of O = C and C–OH or C–O–C in O1 s spectra, where that of O = C increased from 0.414 to 0.475, and that of C–O from 0.586 to 0.545 in EPS in R2. However, except for C–(C, H), the abundance ratio of other components from week 4 showed an opposite conclusion attributed to
the high Fe(III) content. The result indicates the possible bonding reaction between C–(O, N) and C = O or O–C–O with possible products of C–(C, H). It is worth mentioning that the changes in the functional groups in TB-EPS of the XPS spectra correspond with the FTIR result.

CONCLUSION

The influences of Fe(III) on the biofilm mass and activity, the biofilm micromorphology as well as the components and functional groups characteristics of EPS in biofilm were investigated in two laboratory-scale FBBRs. The study indicated that 2 mg/L of Fe(III) promoted biofilm formation and improved the biofilm activity through the SEPS production, and 16 mg/L of Fe(III) inhibited the biofilm development and activity due to the high production of TB-EPS. The following conclusions could be drawn from the results:

- A low concentration (2 mg/L) of Fe(III) contributed to the increase of the biofilm mass and the better efficiency of organic matter removal, but a high concentration (16 mg/L) showed an obviously opposite result.
- SEM observation indicated a high Fe(III) content (16 mg/L) led to significant reduction of filaments, but promoted the secretion of EPS in biofilm.
- The result of the EPS composition suggests that 2 mg/L of Fe(III) increases LB-EPS and SEPS both of which contribute to the microbial aggregation. However, 16 mg/L of Fe(III) promotes TB-EPS as a protective shield for microbes but imposes a steric hindrance in biofilm.
- Analysis of FTIR and XPS spectra demonstrated that Fe(III) exerted a significant influence on the functional groups in EPS, especially the –CONH– groups of PN and the C–O groups of PS.

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