Inorganic fractions in extracellular polymeric substance extracted from activated sludge and biofilm samples by different methods
Leiyan Zhang, Jinju Geng, Lili Ding and Hongqiang Ren

ABSTRACT
This study highlighted the inorganic fractions in the extracellular polymeric substance (EPS) extract from two activated sludges and one biofilm. Nine EPS extraction methods (centrifugation, sonication, cation exchange resin (CER) + sonication, CER, heating, formaldehyde + heating, formaldehyde + NaOH, ethanol and EDTA) were used in the study. The EPS extracts had large inorganic fractions, which ranged from 28% to 94% of the EPS dry weight. The EPS inorganic fraction was dependent on the source of the sludge and wastewater, the kinds of bacteria and the extraction method. The EPS extracts obtained by heating and sonication had smaller inorganic fractions than those obtained by centrifugation. The compositions of the inorganic fraction of EPS extracts obtained with CER and sonication + CER showed similar trends. The chemical extraction methods could contaminate the inorganic composition of EPS extracts by impurities, carrying over of the extractant itself or by changing the pH of the solution. Ethanol was the most effective extractant for obtaining inorganic ions.

Key words | activated sludge, biofilm, extracellular polymeric substances, extraction methods, inorganic fraction

INTRODUCTION
Organic and inorganic pollutants, such as heavy metals and metalloids, can be removed from wastewater by biosorption with activated sludges and biofilms. This method is widely used for the purification of industrial effluents and domestic wastewater (Aksu & Yener 1998; Aksu 2005). The extracellular polymeric substances (EPS) can be produced by activated sludge and biofilms, which play an important role in biosorption (Flemming & Wingender 2001).

EPS are composed of organic macromolecules such as polysaccharides, proteins, nucleic acids, lipids and other polymeric compounds (Flemming & Wingender 2001). These substances have various functions in biological systems, including consolidating biomass, supplying nutrition, and preventing toxic shock (Jang et al. 2001; Gerbersdorf et al. 2007). EPS contain a variety of negatively charged functional groups (e.g., carboxyl, sulfate, and hydroxyl), and can adsorb onto and chelate with organic matter, nutrients, metals, and non-metallic compounds (Bhaskar & Bhosle 2006; Raszka et al. 2006). EPS account for about 80% of sludge (dry weight (DW)), and are important for the flocculation (Frolund et al. 1996; Liu et al. 2010), sedimentation and dewatering of the activated sludge (Neyens et al. 2004; Li & Yang 2007), and membrane fouling of the membrane bioreactor (Chang & Lee 1998; Drews et al. 2006).

EPS extracted from anaerobic granular sludge and activated sludge samples contain an important mineral fraction (D’Abzac et al. 2010; Bourven et al. 2011). The inorganic fraction of EPS affects its physicochemical properties, such as absorption and chelation. For example, Guibaud et al. (2009) showed that some inorganic ions such as aluminum, iron, and manganese in EPS extracted from activated sludge could increase binding of the EPS to Cd$^{2+}$ or Pb$^{2+}$. However, to date, most studies have focused on the organic fraction of EPS extracts from activated sludge, and studies on the EPS inorganic fraction from activated sludge or biofilms treating wastewater from a pharmaceutical factory and municipal sewage are limited. The composition of the EPS inorganic fraction is greatly affected by the source of the activated sludge.
or biofilm and the extraction method (D’Abzac et al. 2010).

However, a standard extraction procedure has not been established, which makes it difficult to compare published results. For instance, the use of heat to extract EPS could induce hydrolysis of a part of EPS and decrease the amount of EPS (Jorand et al. 1995). Liu & Fang (2002) demonstrated that formaldehyde + NaOH was most effective in extracting EPS, and the effectiveness of the EPS extraction protocol depended on the different extraction matters (e.g., carbohydrate or protein).

Firstly, the purpose of this study is to investigate systematically and discuss the inorganic fractions (especially the metallic elements) of EPS extracted from activated sludge and biofilm samples by nine common extraction procedures. Secondly, the study compares the difference between the EPS inorganic fractions from the activated sludge and biofilm samples. Thirdly, the study compares the extracting efficiencies of the nine different methods.

**MATERIALS AND METHODS**

**Activated sludge and biofilm samples**

EPS were extracted from two activated sludge samples and one biofilm sample. One of the activated sludge samples (sample PSS) was sampled from a moving bed biofilm reactor (MBBR) that treated wastewater from a pharmaceutical factory, and the biofilm sample (sample PBF) was obtained from the fillers of this reactor. MBBR had 25 days of sludge age and 48 h of hydraulic retention; the process removed 66–75% chemical oxygen demand (COD) from wastewater. The other activated sludge sample (sample MS) was from a sequencing batch reactor (SBR) that treated wastewater from municipal sewage. SBR had 10 days of sludge age and 18 h of hydraulic retention; the process removed 75–86% COD from wastewater. Two samples were collected from each reactor at the same time. The main characteristics of the sludge and biofilm samples are shown in Table 1.

**EPS extraction methods**

The EPS procedure was carried out according to methods developed by Liu & Fang (2002), and D’Abzac et al. (2010). Five physical and four chemical extraction procedures were used (Figure 1). The physical extraction procedures used centrifugation as the control method, sonication, sonication coupled with cation exchange resin (CER), CER, or heating. The chemical extraction procedures used formaldehyde and heating, formaldehyde and NaOH, ethanol, or EDTA.

**Table 1** Characteristics of the influent and effluent for the activated sludge and biofilm samples used for EPS extraction

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PSS</th>
<th>PBF</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average influent total N (mg/L)</td>
<td>34</td>
<td>\</td>
<td>2</td>
</tr>
<tr>
<td>Average influent total suspended solid (mg/L)</td>
<td>505</td>
<td>\</td>
<td>380</td>
</tr>
<tr>
<td>Total suspended solid in the reactor (mg/L)</td>
<td>2,105 ± 360</td>
<td>\</td>
<td>1,880 ± 176</td>
</tr>
<tr>
<td>Total N (mg/L)</td>
<td>150 ± 0.6</td>
<td>135 ± 0.7</td>
<td>117 ± 0.2</td>
</tr>
<tr>
<td>Color</td>
<td>Black</td>
<td>Black</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

Note: PSS — suspended sludge sample obtained from a pharmaceutical factory, PBF — biofilm obtained from the same pharmaceutical factory as PSS, MS — sludge sample obtained from municipal sewage. Mean ± standard deviation.

**Determination of the EPS inorganic fraction**

The EPS DW was determined after drying 20 mL of the EPS solution at 105 °C for 24 h. The residue obtained at 105 °C was then burnt at 550 °C for 4 h to obtain the EPS inorganic fraction (Figure 2).

**Analysis of the inorganic composition of the EPS**

Hydrogen peroxide (30%, 2 mL), nitric acid (65%, 3 mL), and hydrochloric acid (36%, 1 mL) were added to wet sludge (2 mL) or EPS extract (5 mL) for digestion in a microwave (XT-9900, Shanghai Xintuo Analytical Instruments Co., China). The digestion was performed in three microwave phases as follows: 80 s at 5 MPa, 50 s at 10 MPa, and 170 s at 170 MPa. After digestion, samples were made up to 50 mL with deionized water. Duplicate samples were mixed together. The elemental composition was determined by inductively coupled plasma atomic emission spectrometry (OPTIMA 5300DV, Perkin Elmer, CO, USA). The concentrations of 15 elements (Al, Ba, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Si, Sr, Zn) were above the detection limit of the instrument.

**Scanning electron microscopy (SEM)**

The EPS extracts were filtered through 0.22 μm cellulose nitrate membrane filters and washed with 50 mL of deionized water to remove soluble organic matter and soluble...
ions, which may cause precipitation during sample drying. After the membrane was dried at room temperature, it was analyzed by SEM coupled with energy dispersive X-ray spectroscopy (SEM-EDX) (S-3400N II, Hitachi, Tokyo, Japan).

RESULTS AND DISCUSSIONS

Inorganic fractions per cent and inorganic cation concentrations in the activated sludge and biofilm samples

The inorganic fraction in the activated sludge and biofilm samples (PSS, PBF and MS) was 49–61% of the DW (Figure 2). The inorganic fraction in PSS (61%) was higher than that in PBF (49%).

Many different inorganic cations were present in the sludge and biofilm samples (Figure 3-D1, 3-D2). Those with the higher concentrations were Na, Ca and Al. The highest cation concentrations in the PSS and PBF samples were 904 mg/g DW and 294 mg/g DW for Na, respectively, and that in the MS sample was 171 mg/g DW for Ca.

Inorganic fractions percent in EPS extracts

The inorganic fractions in the EPS extracts obtained from PSS, PBF and MS, using the nine different methods ranged from 28% to 94% of the DW (Figure 2). For the physically extracted methods, sonication and centrifugation produced EPS extracts with similar inorganic fractions for the PSS, PBF and MS samples. Similarly, the EPS extracts obtained by CER and CER + sonication had comparable inorganic fractions. For the PSS and PBF samples, the EPS extracts obtained by chemical methods had smaller inorganic fractions than those obtained by physical methods. The inorganic percent of the EPS extracts obtained with EDTA was the smallest among the nine extraction methods. Except for the EPS extracts obtained with formaldehyde + NaOH, the inorganic fractions of the EPS extracts from the PSS and PBF samples were higher than those from the MS samples. The EPS extracts with the largest inorganic percent for the PSS, PBF and MS samples were obtained with the CER + sonication, ethanol, and formaldehyde + NaOH, respectively.
Figure 3 | Major and trace elements contents in EPS from PSS (A1, A2), PBF (B1, B2) and MS (C1, C2) extracted by nine methods, and major and trace elements contents in influent wastewater, biofilm and sludge (D1, D2). (Centrif. – centrifugation, Sonic. – sonication, Form. – formaldehyde, Eth. – ethanol, MIW – influent wastewater of MBBR, PSS – sample PSS, PBF – sample PBF, SW – influent wastewater of SBR, MS – sample MS) (Note: Because the contents of the different elements in EPS have large difference, the blank rows in the figures represent parts of the values in y-axis omitted).
Inorganic cation concentrations in EPS extracts obtained by centrifugation

The extraction by centrifugation was used as a control. The major elements present in the EPS extracts obtained from the PSS, PBF and MS samples by centrifugation showed different trends (Figure 3). The concentrations of the major elements in the EPS extract from the PSS sample were in the order Na > Ca > K > Mg > Al > Fe > P > Si. The high levels of Na and Ca could be attributed to the influent wastewater for this reactor. The high Mg concentrations were found in the EPS extracts obtained from the PSS, PBF and MS samples. Ca and Mg are important bridging ions that increase the stability of the sludge floc (Flemming & Winger 2001; Sobeck & Higgins 2002).

The trace elements contents were very different among the EPS extracts obtained from the PSS, PBF and MS samples (Figure 3). Compared with other trace elements, the Mn concentration (4.60 mg/g DW for PSS, 5.92 mg/g DW for PBF) was the highest in the EPS extracts from the PSS and PBF samples. For the PSS sample, the concentrations of the trace elements, except for Mn, were in the following order Ni > Sr > Zn. For the PBF sample, the Zn concentration was higher than those of Cr, Sr, Ba, Ni and Cu. The Zn concentration (1.69 mg/g DW) in the EPS extract from the MS sample was the highest among the trace elements, followed by Mn, Ba, Sr, Cr, Cu and Ni. The high Zn and Mn concentrations in the EPS extracts may be related to preferential absorption of these trace elements by bacteria (Ghiorse & Hirsch 1979; Bourven et al. 2011).

Inorganic cation concentrations in EPS extracts obtained by physical methods

Figure 3 shows the concentrations of the major elements and trace elements in the EPS extracts obtained by the nine methods from the PSS, PBF and MS samples. The concentrations of the major and trace elements in the EPS extracts obtained from the PSS and PBF samples by heating were lower than those in the extracts obtained by centrifugation. Expect for the Cu concentration in the EPS extract from the PSS sample, all the concentrations of the major and trace elements in the EPS extracts obtained from the PSS, PBF and MS samples by sonication were lower than those in the extracts obtained by centrifugation. Inorganic ions will be released by heating or sonication because their binding to the organic fraction of the EPS will be disrupted and the extracellular matrix will dissolve (Jorand et al. 1995).

The concentrations of the major elements in the EPS extracts obtained from the PSS, PBF and MS samples by CER and sonication + CER showed similar trends, with Na > Ca > Mg > K > Mn > Zn. D’Abzac et al. (2010) explained that the resin exchanges Na for divalent ions – mainly Ca$^{2+}$ and Mg$^{2+}$, which bind to EPS or the bacterial cell – but not monovalent or trivalent cations.

Inorganic cation concentrations in EPS extracts obtained by chemical methods

The element concentrations in the inorganic fraction of the EPS extracts obtained from the PSS, PBF and MS samples with the control, heating, and formaldehyde + heating were different, but the trends were similar. The concentrations of Na and Ca were higher than those of the other inorganic cations (Figure 3). Except for Cu, Ba and P, the concentrations of the cations in the PSS and PBF extracts obtained with the heating method were lower than those obtained with the formaldehyde + heating. For the MS sample, all of the cation concentrations were higher in the extract obtained using the heating method than in that obtained using the formaldehyde + heating.

The Na concentrations in the EPS extracts obtained from the PSS, PBF and MS samples with the formaldehyde + NaOH method were higher than those in the extracts obtained with the control method. The EPS extracts obtained from the PSS, PBF and MS samples with the EDTA extraction also had higher Na concentrations than those in the control extracts. The extractants in these cases (NaOH and disodium EDTA) would have contributed to the high Na concentrations in the extracts.

The Al concentrations in the extracts obtained with the formaldehyde + NaOH method were one to three times higher than those in the extracts obtained with the control method. The maximum Al concentration (20.2 mg/g DW) was obtained with this method for the PBF sample. By contrast, the concentrations of Ca, Fe, Mn and Mg were lower in the formaldehyde + NaOH extracts than in the control extracts. The high Al concentrations could be explained by addition of NaOH, which would increase the pH of the solution and induce dissolution of phosphate compounds, such as aluminum phosphate. By contrast, the increase in the pH would increase precipitation of Ca, Fe, Mn and Mg as carbonates or hydroxides. These precipitates would be removed from the EPS extracts by centrifugation, which would decrease the concentrations of these cations in the EPS extracts obtained by the formaldehyde + NaOH method.
The Ca concentrations in the extracts obtained using the ethanol method were 4–100 times higher than those in the extracts obtained by the other methods. This could be attributed to quicklime (CaO), which was often used to dehydrate commercial ethanol (D’Abzac et al. 2010). The K, Mg and Si concentrations were higher in the ethanol extracts than those obtained with other extraction methods.

The results showed that chemical extraction methods could contaminate the inorganic component in EPS extracts with impurities from the extractant itself or by changing the pH of the solution.

**SEM-EDX analysis of the EPS samples**

Figure 4 shows SEM images for samples extracted with centrifugation, sonication, heating, formaldehyde + NaOH and EDTA from the PSS, PBF and MS samples. Inorganic aggregates (size 10 μm) were observed in the SEM images of these extracts.

The nature of the solid phase was determined by EDX analysis. Table 2 shows the chemical compositions of some particles present in the EPS extracts. The oxygen concentration is higher than that of any other elements in the aggregates. Aggregates rich in Ca, Mn and S (Figure 4-1-a and Figure 4-2-b) or Ca and Si (Figure 4-4-f) are also identified. Several aggregates have high Cu, N, and S concentrations (Figure 4-6-i, Figure 4-6-j, Figure 4-8-l, Figure 4s9-n, Figure 4-9-o). Some elements present in the EPS extracts are not detected by EDX analysis of the aggregates. This indicates the inorganic fractions of EPS extracts contain two forms, one being soluble form while the other being solid particles.

**Comparison of the activated sludge and biofilm samples from the same reactor**

The inorganic percent in the PSS sample was smaller than that in the PBF sample using centrifugation, but the inorganic percent in the EPS extract from the PSS sample was higher than that in the extract from the PBF sample (Figure 2). This was because the extraction yield by centrifugation was four times greater for the PSS sample than the PBF sample (Table 3).
The EPS extracts from the PSS and PBF samples showed similar trends with high concentrations for Na, Ca, K, Mn and Ni (Figure 3). However, the actual concentrations for these elements were very different for the PSS and PBF samples. This could be related to the types of bacteria present in the samples. Zita & Hermansson (1991) showed that microorganisms (mainly bacteria), inorganic particles and exocellular polymers caused activated sludge flocculation. EPS were produced by bacterial secretion and cell lysis (Wingender et al. 1999). The activated sludge sample and the biofilm sample contained planktonic bacteria and attached bacteria, respectively. The different metabolisms of these bacteria could produce the different EPS extracts (Flemming & Wingender 2001). The size and compositions of the inorganic fractions of the EPS extracts varied largely.

The extraction methods also greatly affected the inorganic fraction concentrations in EPS extracts from the PSS and PBF samples, and had different effects on different inorganic elements. Except for Na, Fe and Al, the concentrations of the major elements were highest in the ethanol EPS extracts from the PSS and PBF samples. For Al, formaldehyde + NaOH and EDTA were the most effective extraction method for the PBF and PSS samples, respectively.

### Table 2

| Extraction yield for each extraction method expressed as a percentage (DW EPS/DW sludge or biofilm, mean ± SD) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Extraction methods | PSS | PBF | MS |
| Centrifugation | 48.4 ± 0.9 | 12.4 ± 0.5 | 1.76 ± 0.2 |
| Sonication | 51.7 ± 2.1 | 15.9 ± 0.5 | 2.17 ± 0.2 |
| CER + Sonication | 47.1 ± 1.5 | 11.4 ± 0.3 | 1.51 ± 0.0 |
| CER | 43.8 ± 0.9 | 10.4 ± 0.7 | 1.18 ± 0.0 |
| Heating | 56.0 ± 1.7 | 15.1 ± 0.5 | 1.92 ± 0.1 |
| Formaldehyde + Heating | 48.9 ± 2.8 | 10.3 ± 0.2 | 2.57 ± 0.5 |
| Formaldehyde + NaOH | 130 ± 4.5 | 76.1 ± 5.2 | 29.4 ± 2.3 |
| Ethanol | 26.5 ± 1.1 | 7.89 ± 9.0 | 1.05 ± 0.0 |
| EDTA | 67.5 ± 6.3 | 16.8 ± 9.6 | 4.38 ± 0.9 |

### Table 3

The main element concentrations (% w/w) of the EPS extracts for the following samples in Figure 4: PSS treated with centrifugation (1, a), sonication (2, b), and EDTA (3, c, d); PBF treated with centrifugation (4, e, f), heating (5, g, h) and formaldehyde + NaOH (6, i, j); and MS treated with centrifugation (7, k), heating (8, l, m), and formaldehyde + NaOH (9, n, o).

The EPS extracts from the PSS and PBF samples showed similar trends with high concentrations for Na, Ca, K, Mn and Ni (Figure 3). However, the actual concentrations for these elements were very different for the PSS and PBF samples. This could be related to the types of bacteria present in the samples. Zita & Hermansson (1997) showed that microorganisms (mainly bacteria), inorganic particles and exocellular polymers caused activated sludge flocculation. EPS were produced by bacterial secretion and cell lysis (Wingender et al. 1999). The activated sludge sample and the biofilm sample contained planktonic bacteria and attached bacteria, respectively. The different metabolisms of these bacteria could produce the different EPS extracts (Flemming & Wingender 2001). The size and compositions of the inorganic fractions of the EPS extracts varied largely.

The extraction methods also greatly affected the inorganic fraction concentrations in EPS extracts from the PSS and PBF samples, and had different effects on different inorganic elements. Except for Na, Fe and Al, the concentrations of the major elements were highest in the ethanol EPS extracts from the PSS and PBF samples. For Al, formaldehyde + NaOH and EDTA were the most effective extraction method for the PBF and PSS samples, respectively.
Extraction efficiencies

Table 3 summarizes the extraction yields calculated as a percentage from the DW of the EPS and the DW of the original sample (sludge or biofilm). The EPS extraction yields for the PSS sample were in the order formaldehyde + NaOH > EDTA > heating > sonication > formaldehyde + heating > centrifugation > CER + sonication > CER > ethanol. For the PSS, PBF and MS samples, the formaldehyde + NaOH method gave the highest extraction yields, and the ethanol method gave the lowest extraction yield. The efficiency of the extraction method was related to the extraction yield. However, the samples with higher extraction yields did not have higher inorganic ion concentrations. Among the extraction methods, the formaldehyde + NaOH method gave the highest Mg, Fe, Mn, Cu, Cr and Ba concentrations for the PSS, PBF and MS samples, and the ethanol method gave the highest Ca, Mg, K and Si concentrations. The ethanol extraction also gave the highest Cu and Cr concentrations for the PSS and MS samples. This could be because the inorganic ions combined with ethanol were released easily under the digestion conditions. Formaldehyde could react with the amino and hydroxyl groups of proteins and prevent cell lysis (Sutherland et al. 2008), and the increase in pH because of NaOH addition may allow extraction of exoenzymes surrounding the cells (Dignac et al. 1998; Wingender et al. 1999). This would make desorption of the inorganic ions in the EPS difficult.

CONCLUSIONS

High levels of Na, Ca, Mg, Mn and Zn were present in the EPS extracts obtained from the PSS, PBF and MS samples by centrifugation. But the Na concentrations in the EPS extracts obtained from the PSS, PBF and MS samples by centrifugation were higher than those obtained with the control method. The formaldehyde + NaOH method also extracted high Al concentrations. The Ca, Mg and Si concentrations were higher in the ethanol extracts than those obtained with other extraction methods.

The inorganic fractions in the EPS extracts obtained from samples ranged from 28% to 94% of the DW. The inorganic percent in the EPS sample was smaller than that in the PBF sample using centrifugation, but the inorganic percent in the EPS extract from the PSS sample was higher than that in the extract from the PBF sample. The EPS extracts from the PSS and PBF samples showed similar trends, with high concentrations for Na, Ca, K, Mn and Ni. However, the actual concentrations of these elements were very different for the PSS and PBF samples. Except for Na, Fe and Al, the concentrations of the major elements were highest in the ethanol EPS extracts from the PSS and PBF samples. For Al, formaldehyde + NaOH and EDTA were the most effective extraction method for the PBF and PSS samples, respectively.

The results indicated that ethanol was the most effective extractant for obtaining inorganic ions. The results also showed that chemical extraction methods could contaminate the inorganic component in EPS extracts with impurities from the extractant itself or by changing the pH of the solution.

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