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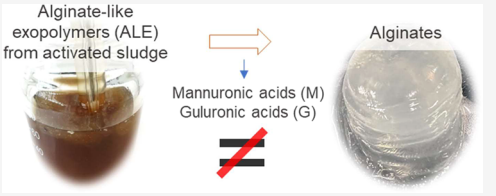
ABSTRACT: Over the past decade, a significant amount of research work on extracellular polymeric substances has been done on the "alginate-like exopolysaccharides" (ALE, also called "alginate-like exopolymers"). The term was used based on the FAO (Food and Agriculture Organization) biopolymer identification test. Although various chemical analyses have been conducted to characterize extracted ALE, it remained unclear whether ALE contains the two sugar monomers of alginates. Aiming to obtain a direct answer to the question: are there alginates in the ALE extracted from sludge, activated sludge was collected from two wastewater treatment plants in two different countries, where the ALE was previously extracted, characterized, and reported in the literature. The extracellular polymers were extracted from these sludges and fractionated according to the standard protocol. The sugar monomer composition of each fraction was analyzed, with special attention to the presence of mannuronic acid (M) and guluronic acid (G), which compose alginate biopolymers. None of these monomers were found in the extracted EPS, indicating there are no alginates resembling polymers extracted from the sludges. The possibility of the presence of other glycan components, such as lipopolysaccharides in EPS, was investigated and confirmed.



KEYWORDS: *extracellular polymeric substances (EPS), alginate-like exopolymers (ALE), mannuronic acid (M) and guluronic acid (G), lipopolysaccharides (LPS), sugar monomers*

1. INTRODUCTION

Extracellular polymeric substances (EPSs) form a complex matrix in sludge, providing structural support to sludge flocs and playing a crucial role in biological wastewater treatment processes. To identify the diverse components of EPS, analyses of its composition and chemical structures have been widely conducted in many studies. In 2010, Lin et al. extracted the EPS from sludge according to the same protocol as used for alginate extraction from brown seaweed.[1] Following the FAO/WHO biopolymer identification, together with further analysis of fractionation and spectroscopic measurement, it was suggested that the EPS resembled alginates, leading to the proposal that the EPS might be considered a potential substitute for commercial alginates.[1−4] Consequently, the EPS extracted with such a method was termed "alginate-like exopolysaccharides" (ALE, also as "alginate-like exopolymers" afterward). Gradually, the "ALE" gained increasing attention. As of 2024, there are about 70 studies associating the EPS investigation with ALE extraction and characterization (search in *Web of Science* with keywords, sludge and alginate-like-). These studies covered a broad range of fields, including the production and functions of ALE in sludge,[5−8] as well as their chemical/physical properties and potential applications.[3,9−11] Among them, over 40 papers directly considered the ALE as exactly the same as alginates in the chemical composition.

Alginates are mainly produced by seaweed and also by two bacterial genera, *Azotobacter vinelandii* and *Pseudomonas spp.*[12,13] They are linear polysaccharides composed of mannuronic acid (M) and guluronic acid (G). Although ALE was frequently considered as alginates, until now there was hardly any direct evidence showing the presence of the two sugar monomers in the literature. Instead, analytical methods, such as colorimetric assays for total carbohydrates and proteins,[14] spectroscopic techniques like Fourier transform infrared (FTIR),[9,15] and nuclear magnetic resonance (NMR)[16] related to functional groups, along with fractionation by partial acid hydrolysis[5,17] and the hydrogel formation tests,[4,6,10] have been employed to characterize the ALE. Based on the results from these indirect analyses, conclusions about the resemblance to alginates were drawn. Moreover, there is an increasing number of studies that simply assumed that ALE was identical to alginates and used it as the basis for further investigation. It is worth pointing out that alginates are a family of polysaccharides that have a specific chemical composition and properties; taking ALE as alginates for granted may hinder the EPS research in unraveling the new components, understanding its physical and chemical properties, recovering

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it as valuable biomaterials, and further developing the EPS-based sustainable products. Hence, before such a general conclusion is drawn, it is significantly important to obtain a direct answer to the following question: are there always alginates in the ALE extracted from sludge?

Aiming at reinvestigating the composition of ALE, in current research, the activated sludge was collected from two wastewater treatment plants from two different countries, where the ALE was previously extracted, characterized, and reported in the literature.[2,18] The ALE was extracted from these sludges and fractionated according to the previously reported protocol.[2,18] The sugar monomer composition of each fraction was analyzed, and a direct search for the two sugar monomers, mannuronic acid (M) and guluronic acid (G), was performed in comparison to the commercial alginates. Furthermore, the presence of other carbohydrate components in the ALE was investigated. Research results will shed light on clarifying the composition of ALE and identifying the EPS components.

2. MATERIALS AND METHODS

2.1. Sludge Sample Collection and the Extracellular Polymeric Substance Extraction. Activated sludge was collected from two municipal wastewater treatment plants (WWTP): Delft (Harnaschpolder) WWTP located in The Netherlands (with a VS/TS ratio of sludge at 77.8 ± 0.7%) and Beijing (Gaobeidian) WWTP in China (60.1 ± 0.3%). Sludge samples were centrifuged at 3900 rpm for 15 min first, and the pellets were lyophilized for further analysis.

EPS extraction was done according to the previous protocol.[4,18] Lyophilized sludge samples (3.0 g from each WWTP) were heated at 80 °C in 100 mL of a sodium carbonate solution (0.5%, w/v) for 30 min. Afterward, the mixture was centrifuged at 3900 rpm for 15 min. The supernatant was collected, and its pH was adjusted to 2.2 with 1.0 M HCl. After centrifuging at 3900 rpm for 15 min, the pellet was solubilized and adjusted pH to 8.5 with a 1.0 M NaOH solution before being dialyzed in the dialysis bag (MWCO 3.5 kDa) against demi water overnight. The dialyzed EPS solution was lyophilized for further analysis.

The organic and ash content of both the sludge and EPS samples were determined according to the standard methods as specified by APHA.[19]

2.2. Partial Acid Hydrolysis of the Extracted Extracellular Polymeric Substances. Partial acid hydrolysis of the extracted EPS samples was performed according to Lin et al.[1] EPS (0.25 g) was dissolved into 9 mL of demi water. After the addition of 1 mL of 3.0 M HCl, it was heated at 100 °C for 0.5 h. Once cooled, the mixture was centrifuged at 3900 rpm for 30 min. The supernatant was collected, neutralized with 1.0 M NaOH, and poured into an ethanol solution with 80% (v/v) for being precipitated overnight. After centrifugation (3900 rpm for 30 min), both the precipitate and the ethanol supernatant were collected and lyophilized, which were referred to as fraction I and ethanol supernatant I, respectively. The previous nonsoluble material after heating at 100 °C in a HCl solution was dissolved in 1.0 M NaOH; the pH was decreased to 2.85 by the addition of 1.0 M HCl. The mixture was centrifuged at 3900 rpm for 30 min. The supernatant was collected, neutralized with 1.0 M NaOH, and precipitated by the addition of ethanol to a final concentration of 80% (v/v). After centrifugation (3900 rpm for 30 min), both the precipitate and the ethanol supernatant were collected and lyophilized, which were referred to as fraction II and ethanol supernatant II, respectively. Finally, the nonsoluble material at pH 2.85 was dissolved with 1.0 M NaOH and adjusted to pH 7. It was poured into an ethanol solution with 80% (v/v). Both the precipitate and the ethanol supernatant were collected and lyophilized, which were referred to as fraction III and ethanol supernatant III. The diagram of partial acid hydrolysis is included in Supporting Information Figure S1.

2.3. Sugar Monomer Detection by HPAEC-PAD. To analyze the sugar monomers, complete acid hydrolysis was performed according to Felz et al.[14] and Rhein-Knudsen et al.[20] All samples (1.0 mg of EPS, fraction I–III, and ethanol supernatant I–III) were hydrolyzed in 1 mL of 1 M HCl in an oven (105 °C) for 8 h. After centrifugation, the supernatant was neutralized with 1 M NaOH and filtered through 0.22 μm PVDF filters. Filtrates were diluted with Milli-Q water in accordance with the concentration of the standard sugar monomers. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, a Dionex ICS-5000+, a CarboPac PA20 column, and an AminoTrap precolumn) was applied to analyze the sugar monomers. The standard compounds contained seven neutral sugars (fucose, rhamnose, galactose, glucose, xylose, mannose, and ribose), two amino sugars (galactosamine and glucosamine), and two uronic acids (galacturonic and glucuronic acid). Peak identifications were done by overlaying the chromatograms of EPS with standard spiked EPS samples (EPS with the addition of sugar monomer standards). In addition, three commercial sodium alginates (Protaweld RF 6650 alginate, food grade; Protaweld S 120 and Scogin LDH alginate, industrial grade, FMC BioPolymer) were used as the control, following the same procedure as described above.

In order to get information about what kind of glycans could contribute to the carbohydrates in the extracted EPS and its fractions, the molar ratios between the sugar monomers in each sample were used to search the hits in the database of "Bacterial carbohydrate database" (http://csdb.glycoscience.ru/database/).

2.4. Determination of the Total Protein and Carbohydrate Content of the Extracted EPS and Its Fractions. The total protein and carbohydrate content of the extracted EPS and its fractions were determined by the BCA assay (with BSA as the standard)[21] and the phenol sulfuric acid method (with glucose as the standard),[22] respectively. All samples were dissolved in 0.1 M NaOH at a concentration of 1000 mg/L overnight before the analysis. Analysis was done in triplicates for each sample.

2.5. Functional Group Analysis by FTIR Spectroscopy. The Fourier transform infrared (FTIR) spectra of the extracted EPS, all fractions, and commercial sodium alginates were recorded in the 4000–600 cm^{-1} region at room temperature on an FTIR spectrometer (PerkinElmer, Shelton).

2.6. Lipopolysaccharide Isolation and Staining. Lipopolysaccharide (LPS) extraction from the EPS was conducted using the hot phenol–water protocol.[23,24] Ten milligrams of the EPS extract was dissolved in 1 mL of a 1 × PBS solution first. In order to eliminate contaminating protein, treatment with proteinase K was performed prior to the extraction step. For this purpose, 10 μL of proteinase K (10 mg/mL) was added to 200 μL of an EPS solution, and the tubes were incubated at 65 °C and 1000 rpm for 3 h. In the next step, an equal volume (200 μL) of water-saturated phenol solution (preheat at 68 °C) was added to the mixtures, followed by

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vigorous shaking at 68 °C for 15 min. Suspensions were then cooled on ice and poured in 200 μL of Milli-Q water before centrifugation at 12,000 rpm for 15 min. Gently, the tube was taken out and the top aqueous layer was transferred to 1.5 mL polypropylene tubes. The bottom layer of the phenol phase was re-extracted twice by an equal volume of Milli-Q water. All of the top layer solutions were mixed together and dialyzed against demi water in the dialysis tubing (MWCO 3.5 kDa) and lyophilized afterward.

The lyophilized samples were solubilized in sample buffer to the desired concentration (1 mg/mL) and prepared with loading buffer for the SDS gel running according to the previous study. Around 16.7 μg of each sample or 2.0 μg of the lipopolysaccharide standard (provided by the kit, *Escherichia coli* serotype 055:B5) was separated on 12% SDS gel with a 4% stacking gel under reducing conditions at 200 V for 35 min. Coomassie blue and Pro-Q Emerald 300 staining of the gels were performed according to the protocol provided by Thermo-Fisher Scientific.[25,26]

3. RESULTS

3.1. EPS Yields and Its Total Protein and Carbohydrate Content. In order to reinvestigate the composition of "alginate-like exopolymers" extracted from activated sludge and compare it with the data in the literature, the sludge was collected from one WWTP in The Netherlands and one in China, where the sludge was studied and the EPS analysis was reported.[2,18] Based on the same extraction protocol as that described in the literature, "ALE" was extracted. To avoid confusion, the extracted "ALE" will be termed as the extracted EPS in the following text.

The yields of the extracted EPS were 167.6 ± 18.4 and 179.5 ± 1.5 mg/g (VS ratio), respectively. These results are in line with the yield range reported by Li et al.[18] (90−190 mg/g VS). In addition, the total content of proteins and carbohydrates of the two extracted EPS is relatively similar (Table 1).

Table 1. Yield and Total Protein and Carbohydrate Content of the EPS Extracted from Activated Sludge Collected in The Netherlands and China

sludge	EPS yield (mg/g VSS sludge)	total carbohydrates (mg glucose equivalent/g VS EPS)	total proteins (mg BSA equivalent/g VS EPS)
Delft, The Netherlands	167.6 ± 18.4	183.8 ± 0.3	386.5 ± 3.3
Beijing, China	179.5 ± 1.5	159.5 ± 2.0	480.0 ± 4.4

3.2. EPS Fractionation and Sugar Monomer Analysis. Alginate is the salt of alginic acid with a linear copolymer consisting only of mannuronic acid (M) and guluronic acid (G) residues, arranged in heteropolymeric blocks (i.e., MG blocks) and homopolymeric blocks (i.e., GG blocks and MM blocks). Partial acid hydrolysis has been used to fractionate alginic acid to separate those blocks with the aim of characterizing different alginates. Due to the assumption that the "alginate-like exopolymers" have similarities with alginates, this method was introduced by Lin et al.[1] to characterize the EPS extracted from sludge collected from different wastewater treatment plants.

In current research, both extracted EPS samples were fractionated according to the same protocol. The percentages of each fraction are listed in Table 2. It is interesting to note that despite the slight variation in the percentage of each fraction, the obvious similarities between the two EPS samples are that all of the collected EPS fractions contain a high amount of proteins. As alginates are carbohydrates, the higher protein content in the different fractions indicated that chemically, the EPS fractions generated by partial acid hydrolysis are different from the building blocks of alginates.

Despite the fact that EPS and its fractions are protein-dominated, there is still the possibility that the carbohydrates contain alginates. In order to investigate if the carbohydrates in the EPS fractions contain the two sugar monomers of alginates (guluronic acid and mannuronic acid), HPAEC-PAD detection and analysis were performed. The chromatograms are shown in Figure 1. Compared to the standards, within the neutral sugar and amino sugar region (Figure 1a), only one commercial alginate contained glucose and/or xylose, while all EPS fractions held eight different sugar monomers. Moreover, within the acidic sugar region (Figure 1b), all commercial alginates exhibited two sugar monomer peaks, which are the peaks of guluronic acid and mannuronic acid. However, no such peaks appeared in the chromatogram of EPS fractions. This strongly indicates that the extracted EPS chemically has no similarity with alginates. Interestingly, all of the collected EPS fractions contain fucose, galactosamine, rhamnose, glucosamine, glucose, galactose, xylose, and mannose, but the amount of these sugar monomers is different. For e.g., glucose was the dominant sugar monomer in fractions I and II, while rhamnose was dominant in ethanol supernatant I and glucosamine was dominant in ethanol supernatant III. To further explore what kind of carbohydrate polymers could be present in the extracted EPS, the molar ratios of the sugar monomers (as shown in Figure 2) were used to search in the "bacterial carbohydrate database"; most of the hits for fraction I were O-antigens of lipopolysaccharides, while for ethanol soluble parts, most were the core of lipopolysaccharides. Clearly, no alginate was found in the extracted EPS and it seemed that lipopolysaccharides might be one of the components of the extracted EPS.

3.3. FTIR Spectra. In order to study whether there were any functional groups related to lipopolysaccharides, FTIR spectroscopy was performed. The FTIR spectra of the EPS fractions extracted from the sludge of Delft (The Netherlands) are shown in Figure 3, while those from Beijing (China) are shown in Supporting Information Figure S3.

Detailed information about peak wavenumbers is listed in Supporting Information Table S4. It was observed that similar bands appeared for both EPS, i.e., the broad band between 3400 and 3100 cm^{-1} might be related to the hydroxyl groups. The peaks at around 1640 and 1540 cm^{-1} corresponded to N−H bending, which may be ascribed to amide I and amide II of nitrogen compounds, respectively.[27] The peaks at around 1230 cm^{-1} could be attributed to phosphate and/or C−N stretching vibrations of amide III. The stretching vibrations of both C−H (2820 to 2940 cm^{-1}; 1460 to 1470 cm^{-1}) and phosphate (1200 to 1265 cm^{-1}, 1106 cm^{-1}, and 960 to 983 cm^{-1}) might indicate the presence of lipid A in the lipopolysaccharides. The band at 1050−1085 cm^{-1} could be assigned to the C−O−C stretching of polysaccharide bonds.

It was noted that there was almost no lipid-related peak in fraction I, while in ethanol supernatant III, the peaks at 2920 and 2850 cm^{-1} were much stronger compared to other fractions. These two peaks were attributed to the stretching vibration of the $-CH_2$ group, where a significant contribution

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Table 2. Percentage of Each Fraction and Chemical Characterization of the Total Proteins and Carbohydrates

		fractions (precipitated in an ethanol solution)[c]			ethanol supernatants (not precipitated in an ethanol solution)		
sludge	contents	fraction I	fraction II	fraction III	ethanol supernatant I	ethanol supernatant II	ethanol supernatant III
Delft, The Netherlands	fractions[a]	121.3 ± 6.3	27.5 ± 2.7	273.2 ± 14.0	324.5 ± 2.2	31.6 ± 6.9	107.7 ± 9.3
	total carbohydrates[b]	255.9 ± 7.7	245.5 ± 14.5	82.1 ± ± 1.1	97.8 ± 34.8	50.0 ± 5.6	120.9 ± 10.2
	total proteins[b]	377.2 ± 13.8	290.6 ± 4.8	638.6 ± 4.2	422.5 ± 9.3	258.2 ± 9.5	444.9 ± 3.1
Beijing, China	fractions[a]	82.1 ± 0.4	48.1 ± 8.3	359.5 ± 1.5	189.6 ± 1.1	67.4 ± 16.0	116.2 ± 4.0
	total carbohydrates[b]	257.2 ± 3.5	320.1 ± 26.8	124.7 ± 3.6	119.5 ± 13.0	152.2 ± 5.4	122.0 ± 1.5
	total proteins[b]	557.1 ± 4.1	336.1 ± 7.9	693.3 ± 5.8	596.2 ± 0.6	626.6 ± 1.0	594.4 ± 3.8

[a]Fraction content was presented based on the volatile solid of fractions to the volatile solid of EPS (mg/g VS ratio). [b]Total proteins and carbohydrates were calculated based on the volatile solids of each fraction (total carbohydrates unit: mg glucose equivalent/g VS of the EPS fraction; total proteins unit: mg BSA equivalent/g VS of the EPS fraction). [c]Final concentration of the ethanol solution was 80% vol/vol.

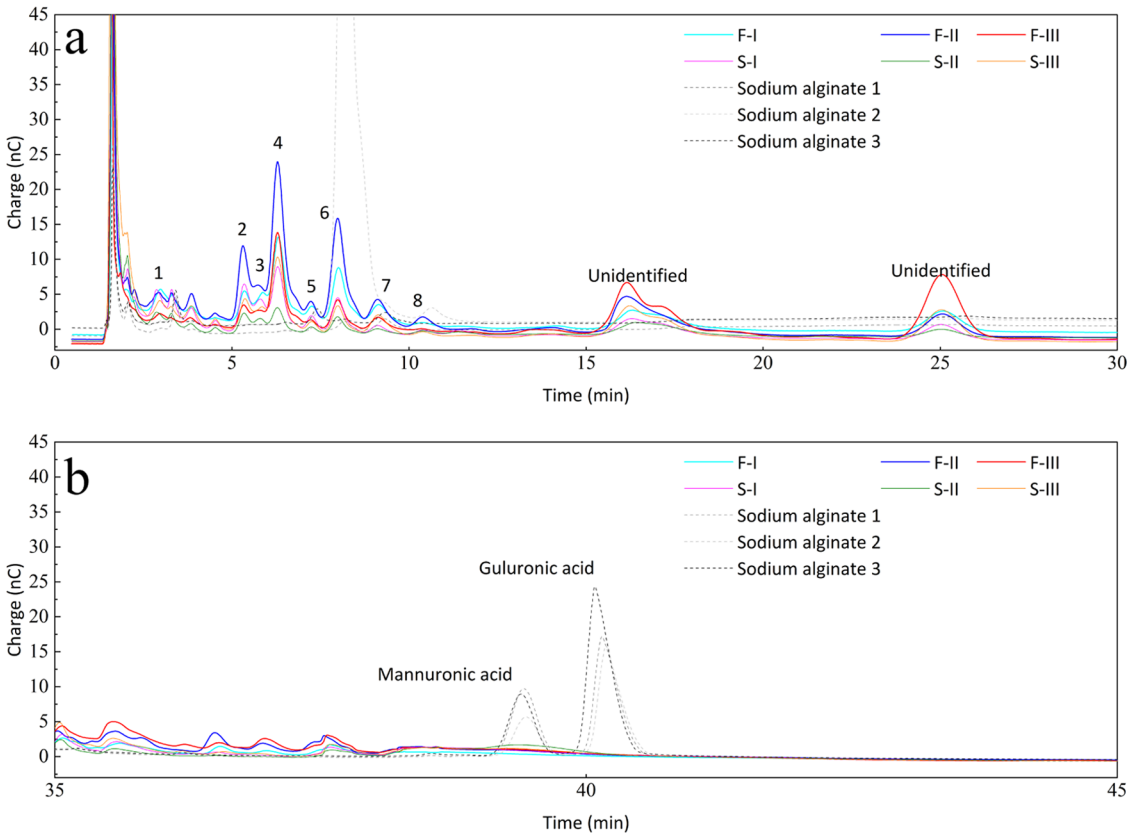

Figure 1. HPAEC-PAD chromatograms of standards, different EPS fractions, and three commercial alginates after complete acid hydrolysis. Sugar monomer speaks: 1-fucose, 2-galactosamine, 3-rhamnose, 4-glucosamine, 5-galactose, 6-glucose, 7-xylose, and 8-mannose. Sodium alginate concentration was at 50 mg/L. (a) Retention time at 0−30 min with the eluent of 2 mM sodium hydroxide and (b) retention time at 35−45 min with the eluent of 50 mM sodium acetate plus 200 mM sodium hydroxide. The baseline drifted at 30−35 min periods as the eluent was changed. F I−III and S I−III represent fractions I−III and ethanol supernatants I−III, respectively.

may come from the lipid.[28,29] In addition, as there were bands at 1460 and 1230 cm^{-1}, which indicated the presence of lipid A, and bands at 1050 cm^{-1}, which implied the presence of polysaccharides, there was a high possibility for the existence of lipopolysaccharides.

3.4. LPS Extraction and Validation. As there was an indication from the FTIR spectra on the presence of lipopolysaccharides (LPSs), the hot phenol−water extraction method, specific for LPS extraction, was performed on the EPS.[23] The yields of LPS extraction from the EPS of The Netherlands (Delft) and China (Beijing) were about 20.5 ± 0.8 and 23.5% ± 1.0% VS of the extracted EPS, respectively. FTIR spectra were obtained to compare the difference between the EPS and the LPS (Figure 4a). The extracted LPS samples exhibited a broad and smooth band between 3400 and 3100 cm^{-1}, which was in accordance with the hydroxyl group of the carbohydrates, and a strong band at 1050 cm^{-1}, which can be attributed to the C−O−C stretching of polysaccharides. The stretching vibrations of C−H (2820 to 2940 cm^{-1}; 1460−1470 cm^{-1}) and phosphate (1200 to 1265 cm^{-1}) are indicative of lipid A presence. Additionally, the amide I peak at around 1640 cm^{-1} was maintained, while amide II at 1540 cm^{-1} disappeared. This was probably due to the proteinase K digestion followed by phenol isolation of proteins from EPS,

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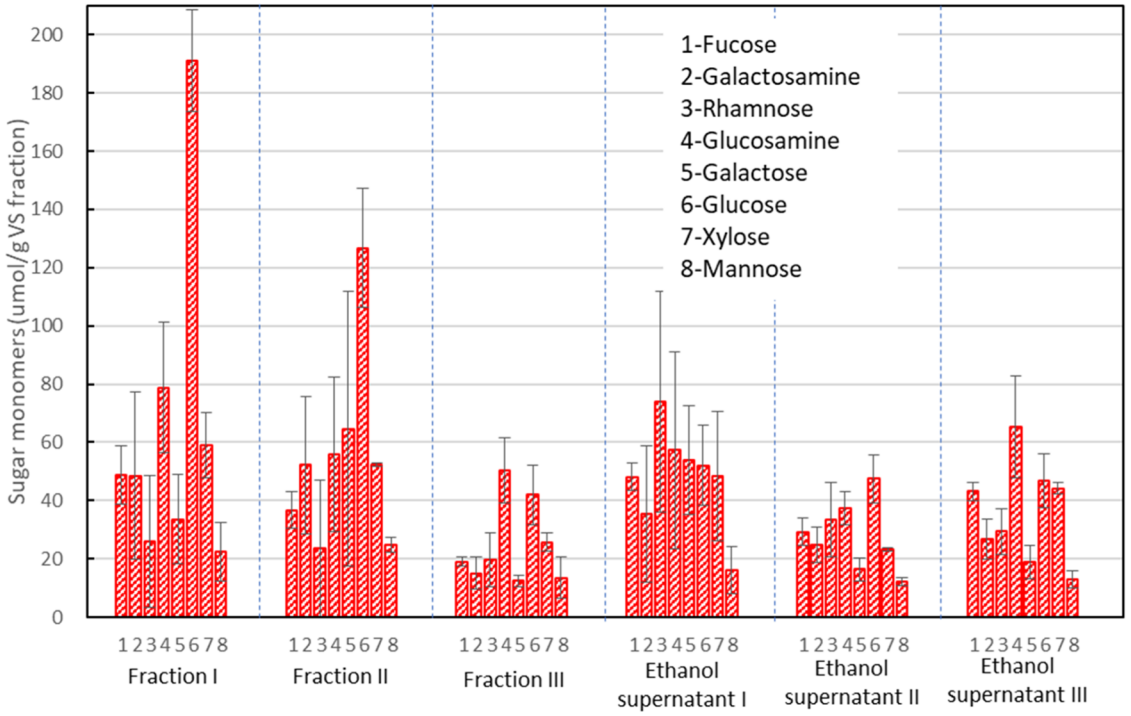

Figure 2. Quantitative analysis of sugar monomers in different EPS fractions determined by HPAEC-PAD. The molar mass of different standard sugar monomers is listed in Supporting Information Table S2.

while other compounds containing a −NH group (such as the N-acetyl glucosamine from lipid A) still conferred the amide I peak at 1640 cm^{-1}. Apparently, the FTIR spectra indicate that there were lipopolysaccharides in the extracted EPS.

Separation over SDS-PAGE gel followed by Pro-Q Emerald 300 staining was used to validate the presence of LPS structures in these extracts.[25,26] LPS is a large glycolipid composed of three structural domains: lipid A, the core oligosaccharide, and the O-antigen (Figure 5). Lipid A is the hydrophobic portion of the LPS. The core oligosaccharide is a nonrepeating oligosaccharide that is linked to the N-acetyl glucosamines of lipid A. The O-antigen is an extended polysaccharide that is attached to the core oligosaccharide. It is composed of a repeating oligosaccharide made of two to eight sugars (as shown in Figure 5). As shown in Figure 4b, LPS from both the two EPS gave a characteristic staircase (ladder-like) pattern of bands, indicating the presence of O-antigens composed of repeating units (as shown in Figure 4b within the blue rectangle). On the other hand, Coomassie blue staining of the gels showed no band, indicating the absence of contaminating proteins (Figure 4b). Therefore, with the specific LPS extraction and staining, it was verified that a significant amount of the extracted EPS was LPS.

4. DISCUSSION

4.1. Alginate Monomers Are Not Present in the EPS Extracts from Activated Sludge. In the field of activated sludge research, over the past decade, a significant increasing amount of EPS research has focused on "alginate-like exopolysaccharides" (ALE, also called "alginate-like exopolymers" in some works of the literature), which is heavily associated with alginate. Although various chemical analyses have been performed to characterize the extracted ALE and it was assumed that ALE contained the building blocks (GG, MG, MM blocks) made from the two typical sugar monomers mannuronic acid (M) and guluronic acid (G) of alginates, there was no direct proof that these two sugar monomers have ever been detected in the ALE. In order to search for a direct proof, in the current research, activated sludge EPS was extracted by following the same protocol for alginate extraction from algae (also the same as ALE) and fractionated to obtain the different "building blocks". The sugar monomers of all of the EPS fractions were identified based on the standards and compared with the commercial alginates. Interestingly, no mannuronic and guluronic acids were found in the extracted EPS at all, which clearly suggested that there is no alginate in the extracted EPS from the activated sludge collected.

When looking back at the literature on ALE, there are a few reports describing the dissimilarity of ALE with alginates. Some studies focused on identifying the presence of mannuronic acid and guluronic acid by FTIR, but only mannuronic acid was claimed to be detected,[9,15] while others concluded that their EPS samples did not contain guluronic or mannuronic acid.[32] In addition, despite the fact that the ALE extracted from aerobic granular sludge could form a hydrogel with metal ions, which was considered one of the similarities between ALE and alginates, the stiffness of EPS hydrogels formed was significantly weaker than that of alginate hydrogels.[33] Especially, the integrity of the EPS hydrogel was still maintained after the addition of EDTA, while the alginate ionic gels could be completely solubilized by the EDTA.

Besides the comparison between ALE and alginate, recent investigations related to the genetic potential of alginate biosynthesis in sludge raised a similar doubt. Based on metagenome-assembled genomes (MAGs) of 23 Danish activated sludge samples, it was observed that the genetic potential for alginate production was restricted to a few low-abundant bacteria, leading to the conclusion that it is unlikely for alginates to be a dominant EPS in the sludge of WWTPs.[34] Doloman et al.[35] examined the MAGs that revolved around the

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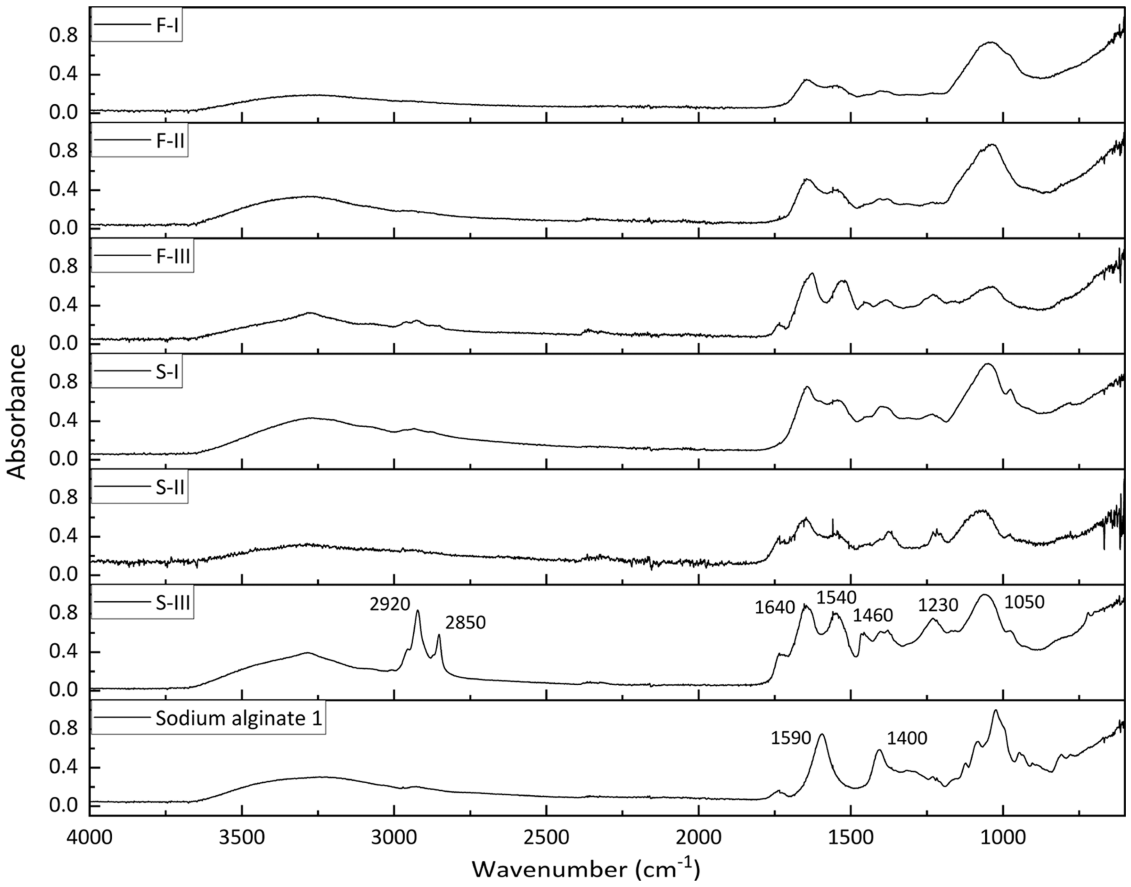

Figure 3. Comparisons of the FTIR spectra between different EPS fractions (from Delft, The Netherlands) and commercial sodium alginates. F I–III and S I–III represent fractions I–III and ethanol supernatants I–III, respectively. Three commercial sodium alginates (another two are depicted in Supporting Information Figure S5) exhibited strong-sharp asymmetric stretching bands near 1590 and 1400 cm^{-1}, which were the typical bands of carboxylate functional groups of both guluronic and mannuronic acid units in alginates.[30,31] On the contrary, all EPS fractions did not show these peaks, supporting the conclusion that the EPS has no chemical connection with alginates.

alginate metabolism cluster in anaerobic granular sludge but only found one sludge sample having the full operons for the synthesis of alginates, while the other two sludge samples were lacking over five important operons out of a total of 12 operons.

Based on current research and the literature, it can be concluded that there is a low possibility for the existence of alginates not only in the activated sludge but also in other types of sludge such as aerobic granular sludge and anaerobic granular sludge. Regarding the term "alginate-like exopolysaccharides" (ALE, also as "alginate-like exopolymers"), it is recommended to keep in mind that ALE is definitely not identical to alginates. It mostly reflects that the polymers form a gel with calcium ions.

4.2. There Are Lipopolysaccharides in the EPS. It is well known that bacteria can synthesize a diverse array of glycans, often attached to proteins (glycoproteins) and lipids (glycolipids) or as loosely associated polysaccharides.[36] If alginate is not the component of the glycans in the EPS, what could be the possible components is an interesting topic to be investigated. Intrigued by the strong indication of lipopolysaccharides from the FTIR spectrum of ethanol supernatant III and phenomena that half of the extracted EPS did not precipitate out from the 80% ethanol–water solution after partial acid hydrolysis, a specific LPS extraction was performed. With the characteristic staircase (ladder-like) pattern of bands shown in the SDS-PAGE gel after staining, it was verified that there was LPS in the extracted EPS.

LPS are glycolipids. It is an amphiphilic molecule with a hydrophobic lipid A region embedded in the outer membrane of Gram-negative bacteria.[37] Beyond lipid A, there is a core sugar region and the O-antigen. The O-antigen is composed of 20–70 repeating units of 3–5 sugar monomers.[37] As cell turnover and lysis result in the presence of both "cell-bound" and "free" LPS in natural aquatic systems,[38] it can be assumed that there are both "cell-bound" and "free" LPS in the EPS of activated sludge. Since LPS is anionic due to phosphate and acid groups in lipid A, it can be solubilized with alkaline extraction and end up in the extracted EPS. During partial acid hydrolysis, the O-antigens were cleaved from the LPS.[39] As the O-antigens are polysaccharides and hydrophilic, they form a precipitate in ethanol and thus can be collected in fractions I and II, whereas the leftover (lipid A + core region of LPS) is hydrophobic, it may form micelles in the 80% ethanol solution without precipitating out and enriching ethanol supernatant III. Therefore, the FTIR spectrum of ethanol supernatant III showed the strongest bands for lipids.

As shown in Figure 5, LPS is an anionic polymer. Each LPS molecule has multiple negative charges from phosphate and carboxylic acid groups in lipid A and the core region. It was suggested by Adams et al.[40] that there are at least six negatively charged groups per LPS. Probably, this anionic property could result in gelling with calcium and has led to a misinterpretation

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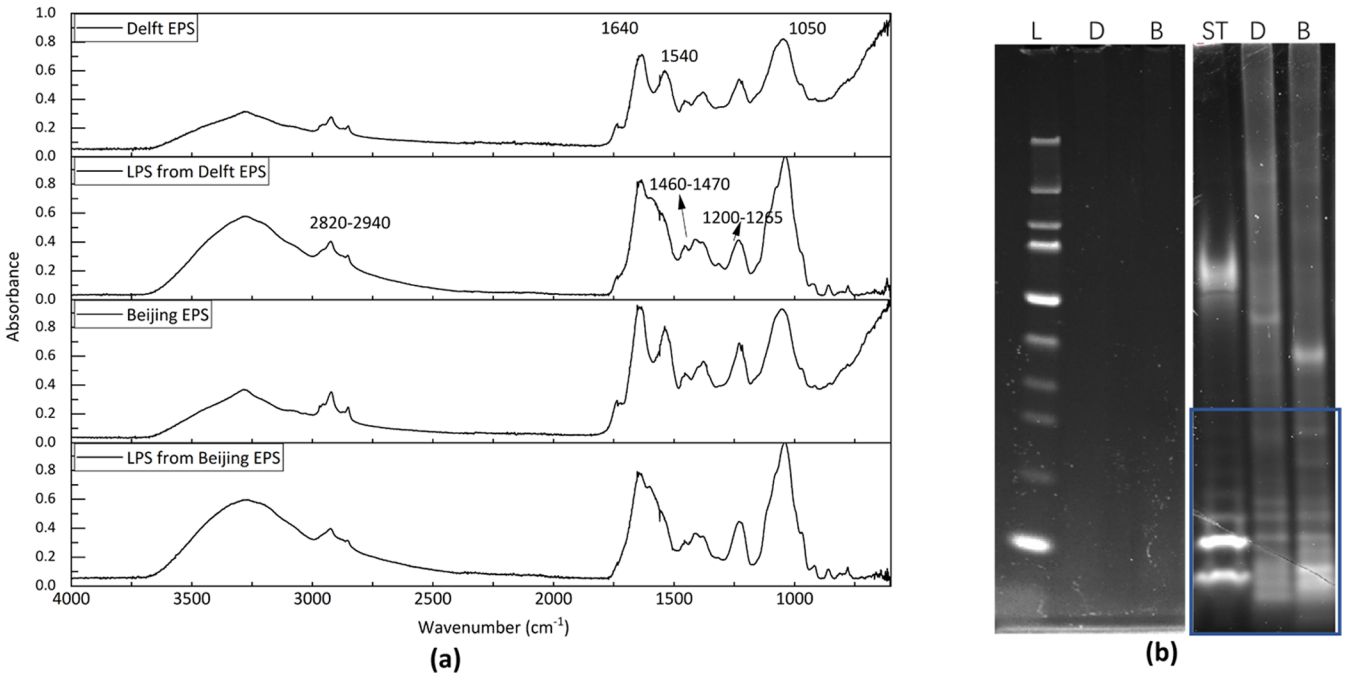

Figure 4. Characteristics and analysis of lipopolysaccharide (LPS) extraction. (a) FITR spectra of EPS from Delft (The Netherlands) and Beijing (China), and LPS extraction from these EPS. (b) Images of LPS polyacrylamide gel after staining (4−12% acrylamide gel). Left: Coomassie blue staining was used for proteins. Right: Pro-Q Emerald 300 staining was used for the LPS. L: protein ladder; D: LPS was extracted from Delft EPS; and B: LPS extracted from Beijing EPS. ST, lipopolysaccharides standard from *E. coli* serotype 055:B5.

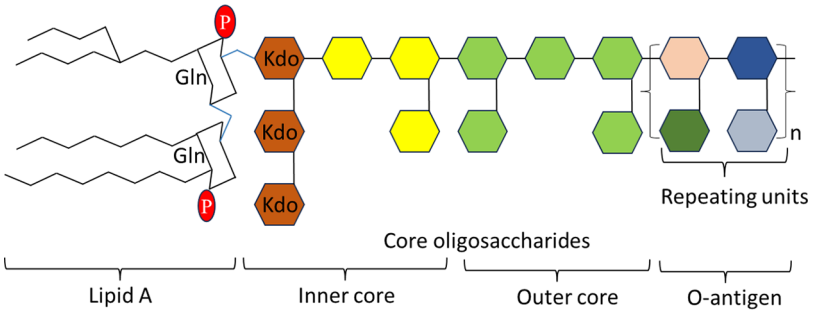

Figure 5. Schematic of the basic structure of lipopolysaccharides (LPS). Gln: N-Acetyl glucosamine, Kdo: keto-deoxyoctulosonate, and P: phosphate.

of certain resemblances between the extracted EPS (ALE) and alginates.

LPS is the major component of the surface of Gram-negative bacteria and its glycan portion is situated at the outermost region.[41] If there are both "cell-bound" and "free" LPS in the EPS of activated sludge, its anionic and amphiphilic properties certainly influence the property of the EPS. Regarding carbohydrate research in the EPS, most of the studies were focused on free polysaccharides. However, besides free polysaccharides, glycoproteins and glycolipids contain carbohydrates as well. Both of them were found in the EPS of the sludge.[42,43] Therefore, to obtain a complete overview of the glycans in EPS, it is necessary to include the structure and function of glycoproteins and glycolipids (e.g., LPS) in addition to free polysaccharides.

5. CONCLUSIONS

By reinvestigating the sugar monomer composition of EPS extracted as alginate-like exopolymers (ALE) from activated sludge samples, it was revealed that no mannuronic acid and guluronic acid were found in the extracted EPS. If present alginates would likely be a rare feature in extracellular polymers from wastewater sludges, further characterization of the EPS suggested that lipopolysaccharides (LPS) could be a significant fraction of the carbohydrates in the EPS. Therefore, direct proof of the composition by chemical analysis is significantly important for EPS research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.4c00217.

Figure S1 Diagram of EPS fractionation according to partial acid hydrolysis.[1] **Table S2** molar mass of different sugar monomers. **Figure S3** comparisons of FTIR spectra between different EPS fractions (from Beijing, China). **Table S4** summary of the peak wavenumbers in the FTIR spectra. and **Figure S5** FTIR spectra of three commercial sodium alginates (PDF)

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AUTHOR INFORMATION

Corresponding Author

Ji Li − *Department of Biotechnology, Delft University of Technology, 2629 HZ Delft, The Netherlands;* orcid.org/0000-0001-6477-2892; Email: J.Li-24@tudelft.nl

Authors

Xiaodi Hao − *Sino-Dutch R&D Centre for Future Wastewater Treatment Technologies, Beijing University of Civil Engineering & Architecture, Beijing 100044, P. R. China*

Rozalia Persiani − *Department of Biotechnology, Delft University of Technology, 2629 HZ Delft, The Netherlands*

Mark C. M. van Loosdrecht − *Department of Biotechnology, Delft University of Technology, 2629 HZ Delft, The Netherlands;* orcid.org/0000-0003-0658-4775

Yuemei Lin − *Department of Biotechnology, Delft University of Technology, 2629 HZ Delft, The Netherlands;* orcid.org/0000-0002-8952-1253

Complete contact information is available at: https://pubs.acs.org/10.1021/acsestwater.4c00217

Author Contributions

CRediT: **Ji Li** data curation, methodology, writing-original draft; **Xiaodi Hao** supervision, writing-review & editing; **Rozalia Persiani** methodology; **Mark C.M. Van Loosdrecht** funding acquisition, supervision, writing-review & editing; **Yuemei Lin** formal analysis, investigation, supervision, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

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