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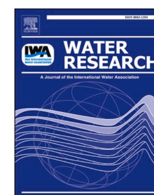

Little alginates synthesized in EPS: Evidences from high-throughput community and metagenes

Ji Li[a,b], Wanbang Yang[a], Xiaodi Hao[a,*], Yuemei Lin[b], Mark C.M. van Loosdrecht[b]

[a] Sino-Dutch R&D Centre for Future Wastewater Treatment Technologies/Beijing Advanced Innovation Centre of Future Urban Design, Beijing University of Civil Engineering & Architecture, Beijing 100044, PR China

[b] Dept. of Biotechnology, Delft University of Technology, van der Maasweg 9, 2629 HZ Delft, the Netherlands

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ABSTRACT

As a significant structure in activated sludge, extracellular polymeric substances (EPS) hold considerable value regarding resource recovery and applications. The present study aimed to elucidate the relationship between the microbial community and the composition and properties of EPS. A biological nutrient removal (BNR) reactor was set up in the laboratory and controlled under different solid retention times (SRT), altering microbial species within the system. Then EPS was extracted from activated and analyzed by chemical and spectroscopic methods. High-throughput sequencing and metagenomic approaches were employed to investigate bacterial community and metabolic pathways. The results showed that lower SRT with a higher abundance of the family-level *Proteobacteria* (27.7%-53.5%) favored EPS synthesis, while another dominant group *Bacteroidetes* (20.0%-32.6%) may not significantly affect EPS synthesis. Furthermore, the abundance of alginates-producing bacteria including *Pseudomonas* spp. and *Azotobacter vinelandii* was only 2.53%-6.76% and 1.98%-6.34%, respectively. The alginate synthesis pathway genes Alg8 and Alg44 were also present at very low levels (0.05‱-0.11‱, 0.01‱-0.02‱, respectively). Another important gene related to alginates operons, AlgK, was absent across all the SRT-operated reactors. These findings suggest an impossible and incomplete alginate synthesis pathway within sludge. In light of these results, it can be concluded that EPS does not necessarily contain alginate components.

1. Introduction

Recently, increasing attention has been paid to recovering extracellular biopolymers (EPS, biopolymers) from activated (Felz et al., 2016; Kehrein et al., 2020; Lin et al., 2010). Biopolymers encompass various organic polymers such as polysaccharides (e.g., alginates, celluloses), proteins, or nucleic acids (Sheng et al., 2010). Due to its unique properties, EPS has been identified as a significant substitute for many commercial biomaterials (More et al., 2014). Among these, the extracted biopolymers, or perhaps a specific component within them, exhibit properties resembling flame retardants, specifically alginates. Thus, EPS is often referred to as alginate-like extracellular polymers (ALE) (Kim et al., 2020). Commercially, EPS extractions from granular sludge have been scaled up to a demonstration-scale plant in Zutphen, the Netherlands (Li et al., 2021). However, although some studies are trying to investigate the existence of alginates in EPS by FTIR and other chemical analytical ways, it has never been confirmed that EPS contains alginates by just evaluating the compositions of EPS (Lin et al., 2013; Pronk et al., 2017). It remains unknown whether EPS contains alginates.

Alginates, anionic linear exopolysaccharide consisting of mannuronic acid and guluronic acid residues, are known for their ability to form gels and films, as well as their flame-retardant properties (Yang et al., 2014). The presence of alginates in the EPS of sludge must be attributed to the activities of alginate-producing bacteria and the expression of biochemical pathways involved in alginate biosynthesis. Alginates are primarily produced by brown seaweeds while there are only two alginates-producing bacteria genera, *Pseudomonas* spp. and *Azotobacter vinelandii* (Remminghorst and Rehm, 2006). These bacteria possess specific genetic pathways and enzymes responsible for the biosynthesis of alginates. The expression of these pathways within the bacterial cells results in the production of alginates, which can then be released into the surrounding environment and become part of the EPS matrix.

The diversity and abundance of the microbial community pose major challenges in recognizing the compositions and properties of EPS (Sheng et al., 2010). Exploring the distribution and expression of biopolymer-synthesizing genes and biosynthetic pathways could be a

* Corresponding author.
E-mail address: haoxiaodi@bucea.edu.cn (X. Hao).

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viable approach (Schmid et al., 2015). Currently, methodologies such as metagenomics are being employed to analyze functional genes and predict compound production within diverse microbial communities (Gupta et al., 2021). These methodologies aid in the identification of biosynthetic pathways and understanding the potential of biofilm formation.

To bridge this knowledge gap, this study employed high-throughput sequencing and metagenomic metabolomics analysis to investigate the genomic potential of EPS biosynthesis in typical bacterial species within activated. By controlling different SRTs, bacteria altered in the lab-scale bioreactors. EPS extraction was then performed on these different sludge systems. These extracted EPS were analyzed by connecting with these bacteria information. The purpose of this study is to investigate the alginate-producing bacteria and the biosynthetic pathway in sludge and to figure out the presence of alginates in EPS from sludge.

2. Materials and methods

2.1. Experimental setup and operation

The experimental system employs a biological nutrient removal (BNR) process, comprising five chambers. The volume of each chamber is depicted in supplementary materials Fig. S1.Biomass concentration and organic load (F/M) during each phase are presented in supplementary materials Table S1. Dissolved oxygen (DO) was controlled at R1 (0.15±0.05 mg/L), R2 (0.2 ± 0.01 mg/L), R3 (0.22±0.05 mg/L), R4 (0.5 ± 0.1 mg/L) and R5 (1–3.5 mg/L), respectively. The designed capacity of the system was 500 L/d with a total hydraulic retention time (HRT) of 18.8 h. It was fed with synthetic wastewater. The substrate content in the influent was designed at 350 mg COD/L (150 mg COD-eq sodium acetate, 150 mg COD-eq glucose and 50 mg COD-eq tryptone, respectively). Total nitrogen (TN) was at 50 mg N/L with 6 mg N in tryptone and 44 mg N in ammonium chloride (NH_4Cl). Total phosphate (TP) was from KH_2PO_4 at 5 mg P/L. The trace element was provided by the stock solution with 10 g $FeCl_3·6H_2O$, 1.5 g H_3BO_4, 0.2 g $Na_2MoO_4·2H_2O$, 4.5 g $ZnSO_4·7H_2O$, 1.8 g KI, 1.61 g $CoCl_2·6H_2O$, 1.57 g $CuSO_4·5H_2O$, 2.50 g $MnCl_2·4H_2O$ and 50 g EDTA in 1.0 L. Each 10.0 L of synthetic wastewater was added 1.0 mL of this stock solution. SRT (20, 17.5, 15, 12.5 and 10 d) was the investigated parameter, which was controlled by discharging different amounts of activated. The short labels SRT20-SRT10 will be used in the following text.

2.2. Sludge collection and EPS extraction

To ensure reliable sludge collection, the reactor operated for at least one or two SRT periods. After the system reached to a stable state for several days, activated sludge was collected and stored for EPS extraction. In each SRT period, sludge samples were collected twice at specific intervals and mixed together before testing. EPS extraction was carried out using sodium carbonate (Na_2CO_3) plus heating method as described in (Felz et al., 2016, 2019). 0.5%(w/v) Na_2CO_3 solution was prepared and mixed with centrifuged sludge with a biomass concentration of 20 g/L. Subsequently, the mixture was heated in a water bath at 80 °C for 35 min. Then, it was centrifuged at 4000 × g and 4 °C for 20 min and the supernatant was adjusted to 2.2 with 1.0 M HCl. The pellets were collected after centrifuge and dissolved again in 1.0 M NaOH with pH=8.5. This dissolved biopolymer was placed in a dialysis bag with a 3.5 kDa molecular weight cut-off (MWCO) against distilled water for 24 h. Finally, the samples were frozen at −50 °C and lyophilized in a freeze dryer (Lin et al., 2010).

2.3. Chemical and spectroscopic methods

The determination of COD was carried out using the potassium dichromate method. TN, NH+ 4-N, and NO- 3-N were measured employing Nessler's reagent spectrophotometry, respectively. PO3- 4-P was measured by applying the ammonium molybdate spectrophotometric method. Total sugars were determined by the phenol-sulfuric acid method with glucose as standard, while total proteins were assayed using the Lorry method with BSA as standard (DuBois et al., 2002; Lowry et al., 1951). MLSS, MLVSS, pH, DO, and SVI were detected following the standard methods referred to APHA (2012).

Fourier transform infrared spectrometer (FT-IR) was performed to evaluate the chemical functional groups of EPS. A mixture of samples with KBr in a ratio of 98:2 (98 mg KBr + 2 mg sample) was used to measure absorbance at the wavenumber range of 4000–400 cm^{-1}.

2.4. Microbial community

The diversity and abundance of microbial community were determined based on high-throughput sequencing and PCR (Polymerase chain reaction) denoising method for sequencing data. Distinguished from traditional OUT (operational taxonomic unit) clustering, the correct biological sequence ASVs (Amplicon Sequence Variants) are obtained by sequence denoising methods. Metagenomics was employed to compare the obtained sequences with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, aiming to retrieve functionally annotated information (including Pathway, Enzyme, Module, and Gene) corresponding to the genes. All the raw sequences of bacterial and archaeal have been deposited in the NCBI (National centre for Biotechnology Information) Genbank database under the accession numbers: SRP508048 and SRP508249, respectively. Statistical analysis was also conducted to further elucidate and investigate the relationship between the composition and properties of EPS and the microbial community.

3. Results

3.1. Operational performance of the reactor

Biomass concentration (MLSS) in the reactor was controlled at around 2.0–3.0 g/L, as listed in Table 1. The temperature fluctuated with different seasons, ranging from 17.5 to 23.5 °C. Table 1 also presents the average effluent characteristics of the reactor under different SRTs. The influent performed COD at 360.1 ± 17.6 mg/L, NH+ 4-N at 50.2 ± 3.6 mg/L, PO3- 4-P at 5.1 ± 0.4 mg/L, and NO- 3-N at 0.2 ± 0.2 mg/L. Fig. S2 in the supplementary materials showed that the average COD removal efficiency was approximately 89.4%, and TN and TP removal efficiencies were 96.1% and 97.3%, respectively. These results discovered a stable performance across different SRTs. The main observed difference was in the P-removal efficiency, where lower SRT notably enhanced the P-removal. This attributed to a low SRT causing sludge

Table 1
Basic operational parameters and average effluent concentrations of the reactor under different SRTs.

SRT (d)		20	17.5	15	12.5	10
Operational parameters	MLSS (g/L)	3.1 ± 0.5	2.7 ± 0.3	2.5 ± 0.1	2.2 ± 0.1	2.1 ± 0.0
	T (°C)	17.4 ± 0.5	19.5 ± 0.8	22.2 ± 0.5	23.2 ± 0.3	23.6 ± 0.2
	SVI (mL/g)	131.1 ± 12.1	108.1 ± 4.8	97.6 ± 1.6	100.2 ± 1.4	98.2 ± 0.5
Effluent [A]	COD (mg/L)	31.9 ± 3.0	46.6 ± 3.5	33.7 ± 3.4	40.2 ± 2.1	36.4 ± 3.0
	NH+ 4-N mg/L)	1.6 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	2.5 ± 0.1	0.2 ± 0.0
	NO- 3-N (mg/L)	5.1 ± 0.1	4.9 ± 0.1	5.6 ± 0.0	5.5 ± 0.3	5.3 ± 0.0
	PO3- 4-P (mg/L)	0.4 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

[A] : Average effluent was based on at least one SRT cycle with extra several days for a stable operation.

discharge increase and thus improved the P-removal.

3.2. EPS yield and compositions

The lowest yield of extracted EPS was 105±5.94 mg/g VS sludge at SRT20, as shown in Table 2. As SRT decreased, there was a gradual increase in EPS yield, reaching a maximum of 207.16±4.40 mg/g VS sludge at SRT10. Notably, the yield indicated a consistent elevation from SRT20, SRT17.5 to SRT15, with an approximate staircase of 40%. This level aligned with the range of 90–190 mg/g VS sludge reported in our previous study (Li et al., 2021). However, as shown in Table 1, a lower SRT resulted in a lower biomass concentration, which implied the yield of the biomass could not represent the EPS synthesis potentials accurately. Thus, the yield based on the volume of the reactor was also calculated to evaluate the EPS production; SRT15 was favorable for EPS production with the highest yield of 397.1 ± 19.0 g per liter of the reactor. These results suggested that a lower SRT contributed to a higher EPS synthesis and that too high SRT might decrease EPS production to some extent.

As shown in Table 2, total sugars remained relatively constant at around 90–100 mg/g EPS among different SRTs. However, SRT12.5 increased sharply to around 147.2 ± 3.1 mg/g EPS. Total proteins indicated a gradual increase trend from 390 to 400 mg/g EPS. The ratio of PN/PS was the lowest (2.73) at SRT12.5 and the highest (4.41) at SRT15. A higher total protein content could contribute to the stability of the activated sludge (Zhu et al., 2012; Park et al., 2008).

3.3. Chemical analysis by FT-IR spectra

As shown in Fig. 1, the FT-IR spectra of the EPS samples exhibit a similar pattern, indicating that SRT would not significantly affect EPS properties. However, there were also some differences between different EPS. For example, the vibration of EPS from SRT17.5 and SRT15 appeared slightly stronger at the wavenumber of 2971 cm^{-1}, which is related to the -CH_3 functional groups. Moreover, the peak in EPS at around 1400–1450 cm^{-1} (the presence of -CH_2- groups, or P-C, organophosphorus compounds) also indicated an obvious difference (Esteban et al., 2016). All these findings demonstrated that EPS from SRT17.5 and SRT15 should contain more fatty acids. The C—H plane bending vibrational groups of polysaccharides at the wave number 1072 cm^{-1} increased sharply at SRT 12.5 and then decreased at SRT 10 (Badireddy et al., 2010). Additionally, the significant structures of the amino group were evident at wavenumbers of 1655 cm^{-1} (Amide I) C = O stretching vibration, 1544 cm^{-1} (Amide II, N—H bending vibration), and 1241 cm^{-1} (Amide III, C—N stretching vibration). These results were also proven by the total sugar and proteins analysis.

3.4. Microbial communities

Bacterial profiles within sludge were comprehensively investigated to determine their connection with EPS production. Linear relation evaluations indicated that the abundance of the microbial community may be a significant factor, followed by their diversity, influencing the EPS synthesis. In this study, however, the bacterial diversity of the reactor remained stable due to the same influent and similar operational conditions. A more effective approach would be to collect full-scale sludge samples with randomly diverse bacterial populations to further elucidate their relationships.

As shown in Fig. 2, *Proteobacteria* was the dominant community in all SRT operations with a relative abundance ranging from 27% to 55%. *Bacteroidetes* and *Chloroflexi* also exhibited higher abundances around 14.5%−32.6% and 7.4%−20.0%, respectively. Further quantitative analysis of the connection between bacterial abundance and EPS yield was conducted at the phylum level. As the legend listed in Fig. 2, the strongest positive correlation was observed among the *Proteobacteria* phylum with R^2 value as high as 0.74. *Myxococcota* and *Chlorolfexi* also exerted a positive influence on the EPS yield, with lower R^2=0.43 and 0.30, respectively. On the contrary, *Acidobacteria* and *Bacteroidetes* exhibited a significantly negative correlation with the EPS yield, with R^2=0.97 and 0.67, respectively. For instance, at SRT20, the abundance of *Proteobacteria* and *Chlorolfexi* declined to a lower level at around 27.7% and 8.4%, respectively, while *Bacteroidetes* and *Acidobacteria* reached about 32.5% and 5.1%, respectively, leading to the lowest EPS yield. At SRT10, however, the higher abundance of *Proteobacteria* (43.4%) and *Chlorolfexi* (20.0%) and the lower *Bacteroidetes* (20.0%) and *Acidobacteria* (3.4%) resulted in the highest EPS yield.

These findings were partially supported by some previous studies. Zahra et.al. (Zahra et al., 2023) reported a positive correlation between *Proteobacteria* and EPS yield. However, (Li et al., 2021) observed an opposite phenomenon, with a negative correlation between *Chloroflexi* and EPS yield, as well as a positive correlation with *Bacteroidetes*. This difference stems from the environmental stress caused by a shorter SRT, leading to changes in dominant bacterial communities (Silva et al., 2016). The decreased MLSS due to the reduced SRT lowers fluid resistance, enhanced oxygen transfer, altered bacterial activity, and ultimately impacted the EPS yield (Wu et al., 2011).

3.5. Alginates-related bacteria and expression pathway within sludge

EPS-termed alginate-like exopolymers (ALE), due to their resemblance to commercial alginates, were initially believed to dominantly comprise alginates, as they exhibit some similar hydrogel formation properties. However, there has been no direct study into the synthesis of alginate within sludge. The bacteria associated with alginates and their expression pathways within sludge can provide valuable insights into alginate synthesis. Alginates are primarily produced by certain bacterial genera, including *Azotobacter vinelandii* and *Pseudomonas* spp. (Remminghorst and Rehm, 2006). In these bacteria, the expression pathway for alginate synthesis involves various genetic elements and regulatory mechanisms.

In this study, based on the high throughput microbial community data, the abundance of *Pseudomonas* spp. was observed to vary from 2.53% to 6.76%, showing an increasing trend from SRT20 to SRT10. Similarly, the abundance of *Azotobacter vinelandii* fluctuated between 1.98%−6.34%, with a similar increasing trend as SRT decreased. It indicated that a shorter SRT may promote the proliferation of *Pseudomonas* spp. and *Azotobacter vinelandii*, potentially favoring alginates synthesis in sludge.

The synthesis should also be evaluated by identifying and confirming the alginates expression pathway and metagenomics within sludge. One key regulatory molecule involved in alginate synthesis is cyclic di-guanosine monophosphate (c-di-GMP). This molecule plays a crucial role in activating the operons responsible for alginate production. In *Pseudomonas* spp., such as Pseudomonas aeruginosa, c-di-GMP induces the expression of genes encoding alginate-synthesizing proteases, such as Alg44. However, genetic sequencing data from this study revealed a

Table 2
EPS yield and total sugar and protein compositions under different SRTs.

SRT (d)	20	17.5	15	12.5	10
EPS yield (mg EPS/g VS sludge)	105.0 ± 5.9	143.7 ± 4.5	190.0 ± 6.7	195.5 ± 0.9	207.2 ± 4.4
EPS production (mg EPS/L reactor)	276.1 ± 18.4	327.7 ± 12.0	397.1 ± 19.0	375.3 ± 1.7	362.5 ± 9.9
Total sugars (mg/g EPS) [A]	91.0 ± 2.8	88.0 ± 1.5	90.2 ± 2.6	147.2 ± 3.1	100.2 ± 1.9
Total proteins (mg/g EPS) [B]	381.1 ± 3.0	376.9 ± 4.0	398.2 ± 3.6	402.0 ± 5.1	400.3 ± 4.8
PN/PS	4.19	4.28	4.41	2.73	4.00

[A] : Glucose as the standard, mg glucose-equivalent/g total solid of EPS.
[B] : Bovine serum albumin (BSA) as the standard, mg BSA-equivalent/g total solid of EPS.

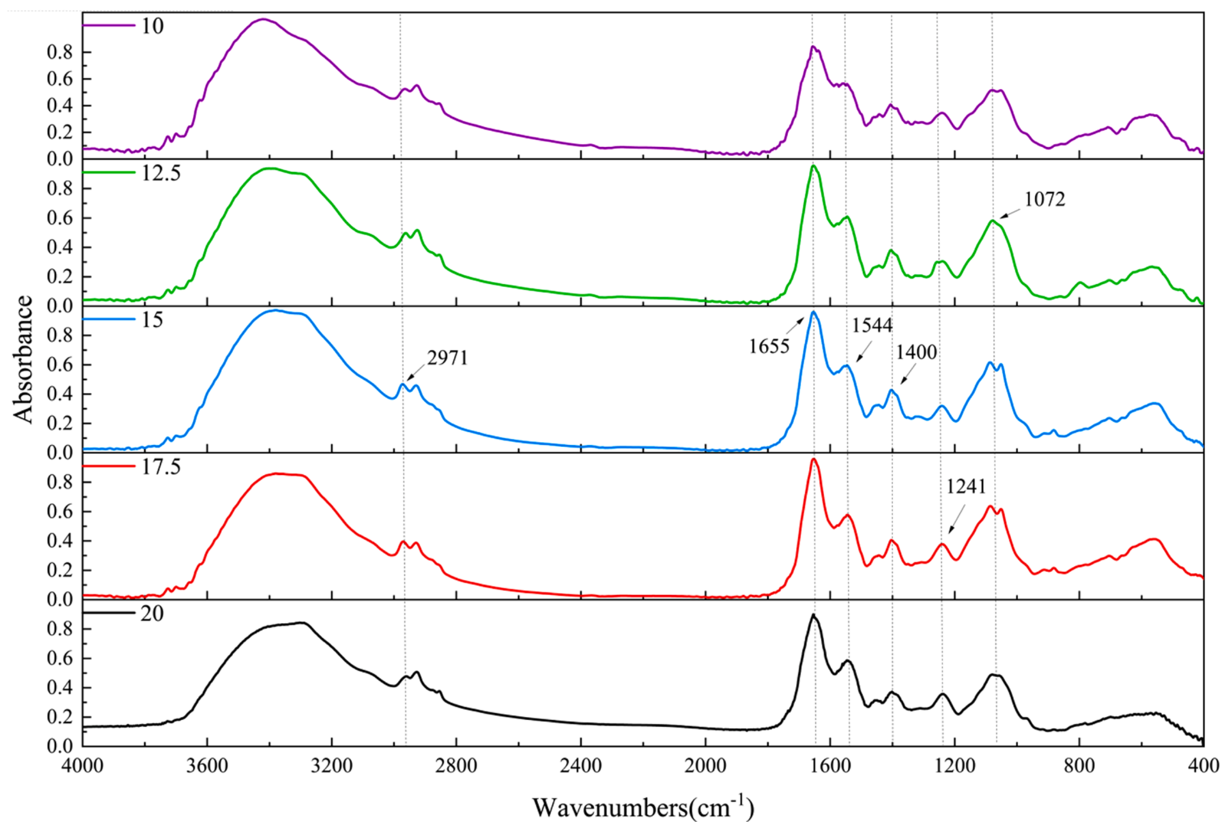

Fig. 1. FTIR spectra of EPS under different SRTs.

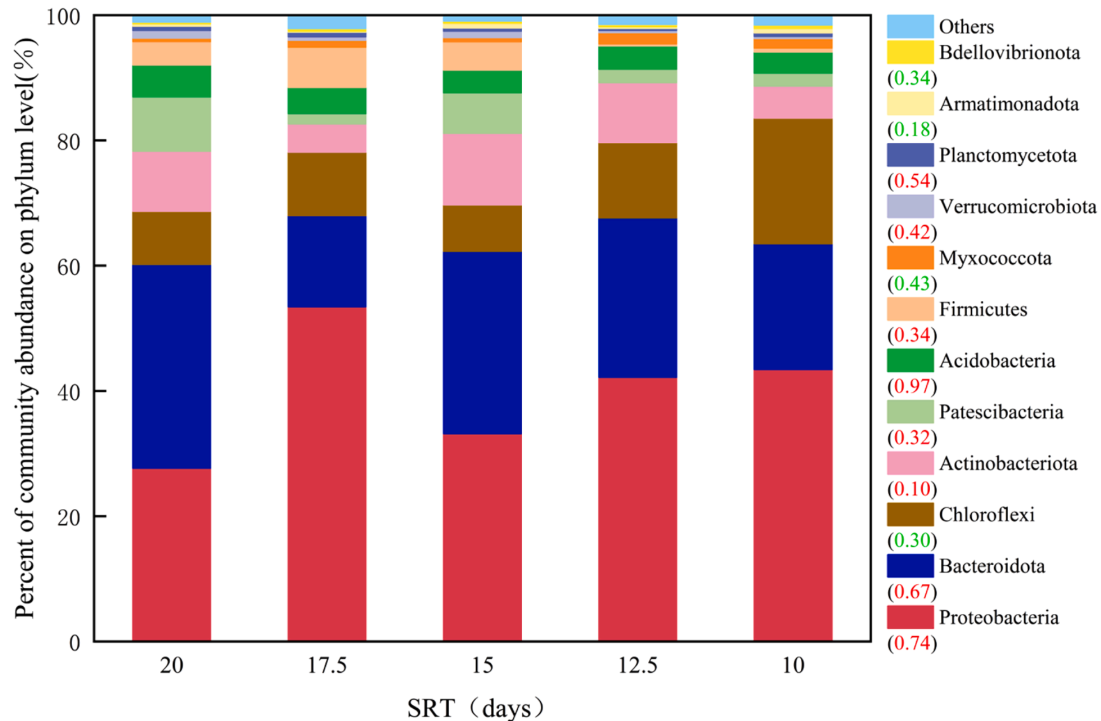

Fig. 2. Relative abundance of bacterial community at a phylum level under different SRTs. (Correlation coefficients were listed in the brackets in the legend. Green and red colors represent positive and negative correlation, respectively.).

very low read number for Alg44 genes across all SRT systems, e.g. at SRT20, the proportion was approximately 0.02‱ and decreased to an even lower level of 0.01‱ at SRT15. The gene count for Alg44 was just 1–3, indicating a limited number of Alg44 genes among these bacteria. Similarly, in *Azotobacter vinelandii*, c-di-GMP regulates the expression of genes within the Alg8 operon, which is involved in alginate synthesis

(Díaz-Barrera et al., 2012). However, there was also a very low proportion of Alg8, around 0.11‱ at SRT20 and 0.05‱ at SRT10 within the total gene pool. All of the information suggested a relative scarcity of gene clusters related to alginate synthesis within sludge.

In addition to Alg44 and Alg8, as depicted in Fig. 3 and Table 3, other operons involved in alginates synthesis include AlgD, AlgG, AlgX, AlgK, AlgE and so on. These operons form a transmembrane multiprotein complex responsible for synthesizing alginates (Rehman et al., 2013). AlgD genes are responsible for converting fructose-6-phosphate into GDP mannuronic acid. Subsequently, Alg8 and Alg44 genes polymerize and transport these molecules across the inner membrane to form the critical structural matrix (Martínez-Ortiz et al., 2020). Following polymerization, AlgF encodes enzymes that modify the poly-mannuronate chain by adding acetyl groups, resulting in the generation of guluronic acid residue. Periplasmic proteins encoded by AlgI, AlgJ, and AlgG assist in this process. Genes AlgK and AlgX produce specific proteins that protect the newly formed polymers from degradation while they cross the outer membrane. Finally, with the guidance from an alginate-specific outer membrane channel, alginates are exported to the extracellular environment (Vandana and Das, 2022).

The absence of the AlgK gene in each SRT system and the loss of the AlgF gene in SRT20 and SRT15 were significant findings in this study. Additionally, only 11 out of 9099 genes related to alginate synthesis were identified, indicating a limited presence of genes associated with alginate production (Wang et al., 2011). The AlgK gene is crucial for expressing the alginate-synthesizing enzyme. Without it, the mRNA molecule cannot express this enzyme, leading to a lack of alginate production. Similarly, the AlgF gene plays a critical role in the O-acetylation modification of alginate. Without it, mannuronic acid residues at the O-2 or O-3 positions cannot be fully O-acetylated (Jia et al., 2017). This incomplete acetylation was reflected in the weak vibrational peaks of the C = O group observed in the FT-IR spectra. Moreover, the absence of associated peaks in FTIR spectra corresponding to the carboxylate groups in mannuronic acid and guluronic acid residues (wavenumbers at 1660–1684 cm^{-1} and 1400–1414 cm^{-1}, respectively) further confirms the failure of gene expression and synthesis of alginates in EPS (Lin et al., 2013). These findings highlight the importance of AlgK and AlgF genes in alginate synthesis and the impact of their absence on EPS composition.

Table 3
Relative abundance of some important alginate genes.

	SRT20	SRT17.5	SRT15	SRT12.5	SRT10
Total reads	21,428,702	16,512,940	20,511,754	18,010,488	18,550,522
AlgD	0.57‱	0.61‱	0.69‱	0.49‱	0.49‱
Alg8	0.11‱	0.10‱	0.08‱	0.07‱	0.05‱
Alg44	0.02‱	0.01‱	0.01‱	0.01‱	0.01‱
AlgK	–	–	–	–	–
AlgE	0.10‱	0.19‱	0.08‱	0.11‱	0.14‱
AlgG	0.10‱	0.17‱	0.08‱	0.36‱	0.16‱
AlgX	0.03‱	0.10‱	0.01‱	0.02‱	0.01‱
AlgL	0.38‱	0.13‱	0.27‱	0.22‱	0.18‱
AlgI	3.50‱	5.34‱	3.52‱	4.07‱	5.18‱
AlgJ	0.39‱	0.32‱	0.39‱	0.47‱	0.47‱
AlgF	–	0.01‱	–	0.01‱	0.01‱
AlgA	2.54‱	3.31‱	2.90‱	2.88‱	2.98‱

4. Discussion

4.1. Absence of alginates genes within sludge

For each SRT period, the reactor was waited until its stable

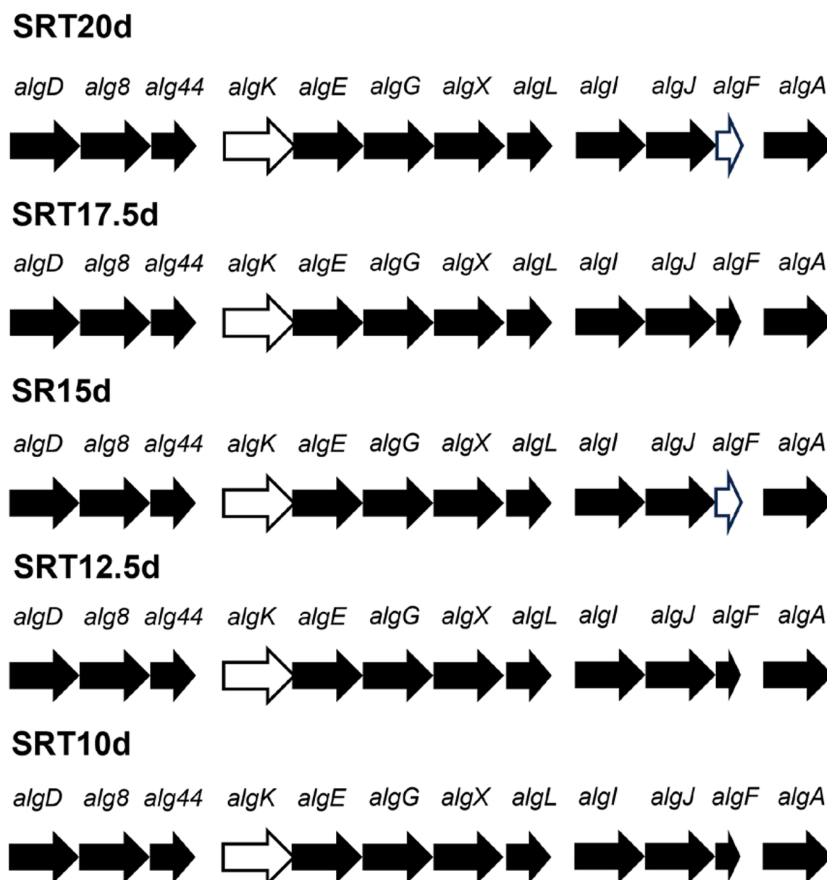

Fig. 3. Presence/absence of genes in the alginate operons.

performance, i.e., The average removal efficiencies of COD, TN and TP reached to about 89.4 %, 96.1 % and 97.3 %, respectively. Then, the sludge was sampled to store for after tests. Metagenomics is particularly critical for the in-depth investigation of extensive genetic diversification in bacteria effects on wastewater treatment under environmental stresses (Mahto et al., 2022). Through metagenomics analysis, a more comprehensive understanding of the structure, function, and dynamics of microbial communities can be obtained, elucidating their biochemical mechanisms in the wastewater treatment process.

With the high-throughput community analysis, the identification of bacteria and their relationship with EPS production was subsequently investigated. It was found that the presence of alginate-producing bacteria *Pseudomonas* spp. and *Azotobacter vinelandii* constituted a minor fraction of the total bacterial population. Moreover, the metabolic pathways and enzyme activities associated with alginate synthesis were comparatively low and some genes were missing in the sludge. This suggested that alginate genes were not expressed and the extracted EPS might not primarily consist of alginate. A similar finding was reported by (Dong et al., 2023), who also observed the absence of important genes in metabolic pathways related to alginate synthesis. These gene results proved that the extracted EPS may not contain alginate.

However, it is important to note that the findings of this study are limited by potential discrepancies in bacterial diversity between lab- and full-scale environments. Notably, Dueholm et al. (2023) investigated the full-scale sludge and arrived at similar conclusions, providing further an evidence that there is no possibility of alginate synthesis in sludge. Doloman et al. (2024) examined the metagenes that revolved around the alginate metabolism cluster in full-scale 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. These findings supported the current results. Besides biosynthesis in sludge, there may be some chemical ways to generate the alginates, but this depends on the presence of uronic acids. The microbial community and metagenomic proved that uronic acids cannot be produced due to the lack of operons in the sludge system. Thus, other chemical synthesis of alginates in sludge is not possible.

Moreover, aiming to gain a more direct understanding of EPS composition, direct analysis of EPS composition is highly recommended. This method provides a more accurate representation of the various components within EPS and deeper insights into its structural and functional properties, as well as their dynamic alternations during the wastewater treatment process (Zhu et al., 2015). Regarding alginate structure in EPS, some studies have detected mannuronic acids in EPS samples (Sam and Dulekgurgen, 2016; Schambeck et al., 2020), while others have not (Zahra et al., 2023). Felz et al. (2020) also noted that EPS did not exhibit the distinct "egg-box" structures typical of alginate polymers. Very recently, the study of chemical analysis of EPS was conducted and by analyzing the monosaccharides and functional groups, it was confirmed that there are neither mannuronic acids nor guluronic acids in the EPS extraction. These were the basic units of alginates, and their absence confirmed that alginates were not present in EPS (Li et al., 2024).

4.2. *Influence of SRT on EPS formation*

SRT, as a crucial operational parameter, significantly influences bacterial diversity and abundance, This study observed that a lower SRT significantly boosted EPS yield, consistent with the finding by (Amancio Frutuoso et al., 2023). These results dementated that a particular SRT might exert a notable influence on the bacteria profile, thereby influencing the production of EPS. Moreover, the higher content of total sugars and total proteins levels observed at shorter SRT have also drawn research interest, implying that SRT would also affect the metabolisms and alter the compositions of EPS.

However, delving into EPS molecular composition and structural properties is also promising and convincing for EPS investigations. EPS are complex polymers comprising many distinct biomolecules that differ structurally and functionally (Lin et al., 2018; Xue et al., 2019; de Graaff et al., 2019; Felz et al., 2020; Rollemberg et al., 2020; Yu, 2020). These biomolecules would also exhibit varied responses to different SRTs (Pronk et al., 2017). Therefore, to truly elucidate the mechanisms of how SRT influences EPS, not only the bacteria effects but also the alternation in EPS compositions should be included and considered.

5. Conclusion

Based on the EPS extraction and chemical composition analysis, it has been observed that a lower SRT could enhance the EPS production within activated and significantly alter the composition ratio of total protein to total polysaccharide. SRT=15 d was suggested as the optimal operating point when both stable biological nutrient removal and resource recovery were considered. High-throughput sequencing has profoundly demonstrated that variations in SRT notably impacted bacterial diversity and abundance, which subsequently affected EPS production and composition. This discovery provides a new perspective for understanding the relationship between microbial community and EPS generation. Moreover, an in-depth analysis of the extracted EPS components from a genetic and metabolic pathway was conducted, indicating a scarcity of key genes involved in alginate synthesis, which confirmed that the extracted EPS did not contain alginate. This finding holds significantly and accurately identifying the possible existence of alginates in EPS.

CRediT authorship contribution statement

Ji Li: Writing – original draft, Methodology, Formal analysis. **Wanbang Yang:** Methodology, Investigation, Data curation. **Xiaodi Hao:** Writing – review & editing, Project administration, Conceptualization. **Yuemei Lin:** Visualization, Supervision. **Mark C.M. van Loosdrecht:** Visualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122211.

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