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De Bruin, Stefan; Riisgaard-Jensen, Marie; Hansen, Susan Hove; Van Loosdrecht, Mark C.M.; Nielsen, Per Halkjær; Lin, Yuemei

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Global insights into extracellular polymeric substances from activated sludge: Yield, composition, and microbial communities

Stefan De Bruin^{a,*}, Marie Riisgaard-Jensen^b, Susan Hove Hansen^b,
Mark C.M. Van Loosdrecht^a, Per Halkjær Nielsen^b, Yuemei Lin^a

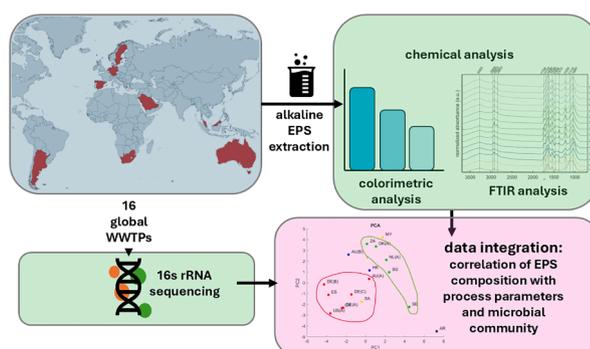
^a Department of Biotechnology, Delft University of Technology, Delft, the Netherlands

^b Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, 9220 Aalborg, Denmark

HIGHLIGHTS

- First global baseline study of alkaline extracted EPS from activated sludge.
- FTIR showed EPS composition differences in activated sludge between process types.
- Microbial community showed no clear correlation with EPS composition across samples.
- Microbial communities were diverse, with many abundant yet undescribed genera.
- Study presents high-throughput approach for analyzing EPS composition in WWTPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Activated sludge (AS) wastewater treatment generates substantial excess sludge which needs to be discarded and thereby increasing operational costs. Extracellular polymeric substances (EPS) within AS present a potential resource for recovery, reducing sludge volume and mass while adding value. Achieving this goal requires a better characterization of EPS, as the relationship between its composition and the microbial communities responsible for its production remains insufficiently understood. Here, we analysed extracted EPS from 16 wastewater treatment plants across 13 countries and 5 continents and found that alkaline extractable EPS yields varied widely (2.81–18.5 wt.% VSS). The microbial community composition of abundant species varied across plants and particularly across continents and did not correlate to the EPS yield. Only sludge retention time had a significant correlation with the EPS yield ($p < 0.005$). Traditional colorimetric assays failed to detect compositional trends of the EPS, but Fourier Transform Infrared (FTIR) analysis indicated that extracted EPS from biological phosphorus removal systems had higher lipid and polysaccharide content, while chemical phosphorus removal systems had higher relative protein content. Thus, FTIR proved effective for distinguishing extracted EPS composition, demonstrating its potential as a high-throughput characterization tool. These findings highlighted that the wastewater treatment design and operation may shape the functional groups in EPS when using the alkaline method. More investigations are needed to find possible correlations between the composition of

* Corresponding author.

E-mail address: s.debruin@tudelft.nl (S. De Bruin).

extracted EPS and the microbial community structure. Overall, the study presents a baseline for the amount and overall composition of biopolymers that can be extracted from global AS plants for recovery.

1. Introduction

Wastewater treatment is essential for preventing environmental pollution, with activated sludge (AS) being the most widely used method. This process generates substantial quantities of waste sludge. Recent findings have showcased that AS waste can be converted into biopolymers, thereby adding value to the waste stream and advancing a circular economy (Chen et al., 2022). Bacteria play a pivotal role in determining the intrinsic structure and properties of activated sludge flocs by producing extracellular polymeric substances (EPS) (Flemming et al., 2023). EPS account for approximately 10 %-20 % of the total organic solids in AS (Kim et al., 2020; Schambeck et al., 2020) and are complex polymeric structures that spans multiple classes of biomolecules, including proteins, polysaccharides, lipids and nucleic acids, as well as complex structures of these components such as glycoproteins and lipopolysaccharide (Flemming et al., 2025; Li et al., 2021; Seviour et al., 2019).

Efforts to valorise EPS are advancing, with pilot-scale recovery of Kaumera biopolymers from aerobic granular sludge (AGS) in the Netherlands moving towards commercial introduction (Bahgat et al., 2023). AS has a lower extractable EPS content (7 %-19 % of volatile suspended solids, VSS) (Kim et al., 2020; Schambeck et al., 2020) compared to AGS (35.1 ± 1.9 % of suspended solids, SS) (Li et al., 2021; Lin et al., 2010). Nonetheless, the substantial volume of excess sludge produced in AS-based wastewater treatment plants (WWTPs) globally highlights its huge unexploited potential.

Despite the significance of EPS for wastewater treatment plant operation, there is limited knowledge about its composition and the bacteria responsible for its production. Additionally, most studies on EPS extracted from AS are limited to localized contexts, and comprehensive global data on EPS variability across WWTPs and geographic regions is lacking (Li et al., 2021; Lin et al., 2013). Large-scale systematic sampling with broad geographic coverage has been undertaken before, notably in the MiDAS project, which investigated microbial communities in a global selection of full-scale AS WWTPs. It was revealed that WWTPs globally contain fewer than 1,000 distinct abundant genera, including well-studied functionally important bacteria such as nitrifiers and polyphosphate-accumulating organisms (PAOs) (Dueholm et al., 2022). However, these efforts have solely focused on microbial community composition, and little is known about whether certain bacteria are particularly important for EPS production, either in amount or composition. Incorporating EPS recovery and compositional analysis into global studies could provide insights into how geographic and operational differences may influence EPS extraction yield, the EPS composition and the factors driving these variations. The incorporation of extracted EPS in global scale AS analysis is a novel and complex challenge. EPS analysis generally involves multiple time-consuming steps, including extraction, complex functional and physicochemical characterization methods, and subsequent extensive data interpretation; all of which are further complicated by the structural complexity of EPS (Seviour et al., 2019; Zahra et al., 2024). To be able to perform the high throughput analysis needed for characterizing global samples, analytical methods should be rapid, user-friendly, and contain as much information as possible.

Colorimetric assays have been used to quantify protein and polysaccharide content in the past due to their speed and ease of use. These methods are known to have strong limitations for absolute quantification and characterisation but allow for comparing relative differences between samples (Felz et al., 2019; Le and Stuckey, 2016). Fourier Transform Infrared (FTIR) spectroscopy provides insights into relevant functional groups present in EPS. Due to the ability to measure all

functional groups in EPS it is possible to track relative differences across samples over a broad range of functional groups in a fast and straightforward manner (Zhu et al., 2012). Multivariate analysis of FTIR spectra can facilitate a more accessible interpretation of relative differences across datasets (De Bruin et al., 2024; Kassem et al., 2023).

In this study the first global analysis of EPS from activated sludge is conducted. This work presents an accessible framework which aims to compare EPS yield and composition in AS collected from 16 WWTPs across 13 countries on 5 continents and search for potential correlations to WWTP design and operation and microbial community structure. Alkaline extraction was performed to ensure the solubilization of the structural matrix in the biofilms (Felz et al., 2016). This will provide the first baseline for the amount and overall composition of biopolymers that can be extracted from global AS plants for recovery, and evaluate the potential for biopolymer recovery from the numerous AS plants worldwide.

2. Materials and methods

2.1. Global sampling and sludge analysis

A global sampling campaign was conducted to collect return activated sludge (RAS) from WWTPs. WWTPs were selected to span all continents and to be typical for the given country in terms of configuration, receive primarily domestic wastewater, and preferably of a size >100,000 PE. Process types included were carbon removal (Cb), carbon removal and nitrification (Cb, N), carbon removal, nitrification, and denitrification (Cb, N, DN), and carbon removal, denitrification, nitrification, and phosphorus removal, either chemically (Cb, N, DN, P (C)) or biologically (Cb, N, DN, P (B)). National contact persons acted as sampling coordinators and were in direct contact with the WWTPs (Contributions list). A total volume of >4 L RAS were collected, and frozen immediately after collection. Samples were shipped to Aalborg University or TU Delft with cooling elements. All samples for microbial analysis never exceeded 8°C during the shipment and were stored at -20°C at Aalborg University or TU Delft until DNA extraction. Details about each WWTP and measurements of each sampling is provided in Table 1.

2.2. Community analysis by amplicon sequencing

DNA was purified using a custom plate-based extraction protocol as described in Dueholm et al. (2022). Amplicon libraries were prepared following the protocol in Dueholm et al. (2022), targeting the V1–V8 region of the 16S rRNA gene. The primers used were: 5'-TTTCGTGGTCTGATATTC-AGRGTTYGATYMTGGCTCAG-3' (NP-V1V8f) and 5'-ACTTGCCGTGCTCTATCTTC-GACGGGCGGTGWGTRCA-3' (NP-V1V8r) (Klindworth et al., 2013). PCR reactions (25 µL) were run in duplicate for each sample using 1 × PCR BIO Ultra Mix (PCR Biosystems, England), 400 nM of each primer, and 10 ng of template DNA. PCR conditions were initial denaturation at 95 °C for 2 min, followed by 25 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 60 s, with a final elongation at 72 °C for 5 min. PCR products were purified using 0.8 × CleanNGS beads (CleanNA, Netherlands) and eluted in 25 µL nuclease-free water. Sequencing libraries were prepared by barcoding the amplicons using the Oxford Nanopore Technologies (ONT) PCR Barcoding Kit (EXP-PBC096), followed by multiplexing and adaptation for Nanopore sequencing using the Ligation Sequencing Kit (SQK-LSK114) (ONT, UK). The libraries were loaded onto a MinION R10 flow cell (FLO-MIN114) in a MinION Mk1B sequencer according to the manufacturer's protocols (ONT, UK). Live base-calling was enabled using Dorado v0.9.6 (ONT, UK) with the high-accuracy model. The base-called FASTQ files were processed using the custom shell script *cmc-aau/nanopore_16Samp* workflow (version 2.0.5; https://github.com/cmc-aau/nanopore_16Samp), aligning raw reads to full-length ASVs from the

MidAS 5.3 database with a 94.5 % identity cutoff and a minimum alignment length of 1000 bp for taxonomic classification at the genus level (Yarza et al., 2014). The mappings were imported into R (R Core Team, 2025, v. 4.5.0) using RStudio (R Core Team, 2025, v. 2024.12.1+563) and processed with the *ampvis2*, *tidyverse*, and *vegan* packages (Andersen et al., 2018; Oksanen et al., 2025; Wickham et al., 2019). Sequencing data are available under BioProject PRJNA1255528, and scripts used for analysis can be accessed at https://github.com/MarieRiisgaard/2025_Global_insights_into_EPS_from_AS.

2.3. EPS extraction protocol

Extraction of EPS was performed as described previously (Felz et al., 2019). 100 g of wet activated sludge sample was poured into a 250 ml round flask and 0.5 g of sodium carbonate was added to a final concentration of 0.5 % (w/w). The mixture was stirred for 35 min at 80°C in starfish setup then centrifuged at 4000 × g and 4°C for 20 min. The supernatant was collected, and the pellet was discarded. The pH of the supernatant was adjusted to pH 2.2 with 1 M hydrochloric acid to precipitate the EPS. The acidified sample was centrifuged again at 4000 × g and 4°C for 20 min. The precipitated pellets of EPS were frozen at –80°C, lyophilized and stored at room temperature until further analysis. All activated sludges were extracted in triplicates.

2.4. Total carbohydrate and protein analysis

Lyophilized EPS fractions were dissolved in 0.01 M NaOH to 0.5 mg/mL. The total protein content was determined by the BCA protein assay following the manufacturer's instruction with bovine serum albumin as a standard (Pierce BCA protein assay Kit, Thermo Scientific). Protein absorbance was measured in duplicates at 562 nm with VIS-spectrophotometer (HACH DR3900, Ames, IA). The total carbohydrate content of the EPS solutions was determined by the phenol sulfuric acid method with glucose as a standard (DuBois et al., 1956). The carbohydrate absorbance measurements were performed in cuvettes at 490 nm in duplicates with a VIS-spectrophotometer (HACH DR3900, Ames, IA).

2.5. Functional group analysis using ATR-FTIR

Functional group analysis was performed by Fourier transform infrared (FTIR) spectroscopy a Spectrum 100 spectrometer (Perkin Elmer, Shelton, CT). The spectra of the lyophilized samples were

recorded at room temperature over a wavenumber range of 600–4000 cm⁻¹ with 10 accumulations and 2 cm⁻¹ resolution.

2.6. Multivariate analysis – PCA

For the multivariate analysis, the software Matlab R2021b, Toolboxes Stats Toolbox (Mathworks), and PLS Toolbox (Eigenvector Research Inc.) were used. Principal components analysis (PCA) was performed in the FTIR spectral window between 4000 and 600 cm⁻¹. Baseline correction was performed on 17 spectra for each sample dataset, comprising the FTIR spectra of AS and EPS from different countries. Afterward, the spectra were vector normalized. The vector normalization was carried out in the following way: spectra were first mean-centred, i.e. the average value of the absorbances was calculated for the spectral region from 4000 cm⁻¹ to 600 cm⁻¹. This value was then subtracted from the spectrum. Then, the spectra were scaled such that the sum squared deviation over the indicated wavelengths equals one. The principal components (PCs) from PC1 to PC3 were calculated and illustrated in a scores diagram (De Bruin et al., 2024; Mohsin et al., 2019).

3. Results

3.1. Characteristics of involved AS WWTPs

Samples were obtained from 16 WWTP in 13 countries on 5 continents all utilizing AS (Table 1). Countries are identified by their ISO 2-letter codes, with multiple plants from the same country labeled sequentially (A, B, C). The European WWTPs were dominated by plants with Cb, N, DN, P removal while most remaining plants had Cb, N, DN removal. The WWTP in Argentina facilitated only carbon removal (Cb). The plant size spanned from 6 × 10³–1300 × 10³ PE and all plants predominantly received municipal wastewater. The suspended solids (SS) in the aeration tanks and the sludge retention time (SRT) varied between 2.1–8.0 g/L and 5–30 days, respectively.

3.2. EPS extraction yields

To investigate the yield range and the factors influencing extraction yield, EPS extraction was performed on all samples. The resulting extraction yields exhibited significant variation across samples (Fig. 1).

Table 1

Metadata associated with each WWTP, and measurement performed at the sampling time. The background colour divides based on continent. ± denote presence or absence of a process. Climate is assigned based on GPS coordinates and using the Köppen–Geiger climate classification. PE: person equivalents, PS: Presence of a primary settling unit, Cb: Carbon removal, N: Nitrification, DN: Denitrification, P-rem.: phosphorus removal either biological (B) or chemical (C), +*: Side stream anammox, SS: Suspended solids in the aeration tank.

Country	Climate	Plant size		Plant Design							Wastewater			Measures		
		Daily Load [x10 ³ m ³]	PE [x10 ³]	Cb	N	DN	PS	P-rem.		Anammox	SRT [d]	Municipal [%]	Industrial Organic [%]	Chemical [%]	SS [g/L]	Temp. [°C]
ZA	Csb	1.5	10	+	+	+	-	-	+	-	30	100	0	0	NA	20
AR	Cfa	160	600	+	-	-	+	-	-	-	6	100	0	0	2.1	24
HK	Cwa	340	920	+	+	+	+	-	-	-	20	100	0	0	2.4	27
MY	Af	4000	440	+	+	-	-	-	-	-	11	100	0	0	3.9	29
SA	BWh	4.0	6	+	+	+	-	-	-	-	14	100	0	0	NA	30
SG	Af	2000	NA	+	+	+	+	-	+	-	5	NA	NA	NA	NA	32
CH	Cfb	180	670	+	+	+	+	+	-	+	10	75	25	0	2.9	15
DE(A)	Cfb	25	100	+	+	+	+	+	-	-	8	70	20	10	7.8	13
DE(B)	Cfb	60	340	+	+	+	+	+	-	-	10	70	30	0	3.0	12
DE(C)	Cfb	13	80	+	+	+	+	+	+	+	10	60	10	5	8.0	14
DK	Cfb	22	150	+	+	+	+	-	+	-	18.5	66	44	0	3.5	15
ES	Cfa	42	210	+	+	+	+	+	-	-	27	80	15	5	4.9	15
NL	Cfb	250	1300	+	+	+	+	-	+	-	20	100	0	0	4.0	14
SE	Cfb	52	200	+	+	+	+	-	+	-	19	70	30	0	4.5	14
AU(A)	Cfb	12000	48	+	+	+	+	-	+	+	14	99	0	1	2.9	19
AU(B)	Cfb	2500	550	+	+	+	-	-	-	-	10	57	43	0	7.1	20

RAS from ZA yielded the lowest amount of EPS, 2.81 ± 5.1 wt.% VSS, approximately seven times less than the highest yield from AR, which was 18.7 ± 2.5 wt.% VSS. Alkaline EPS extraction from the Hong Kong samples yielded too little EPS for further analysis and was therefore omitted. The average EPS yield across all samples was 12.2 wt.% VSS, with a standard deviation of 7.65 %, indicating substantial variation in extraction efficiency. This 62 % variation change underscores the broad range of yields obtained. SRT was the only operational parameter with a statistically significant effect on EPS yield ($p < 0.005$), explaining 46 % of the variability, while the remaining 54 % was attributed to unknown factors (Supp. fig. 1). Important to note is the relatively low number of WWTPs for each continent and each design and operation group, limiting the capability to obtain reliable correlations.

3.3. Protein and polysaccharide content

The analyses of the protein and carbohydrate content in the extracted EPS provided a global overview into the EPS composition (Fig. 2). In general, protein content was higher than the carbohydrate content. Protein values ranged between 13.4 ± 0.7 wt.% VSS of EPS for SA to 37.2 ± 3.4 wt.% VSS in ZA, compared to carbohydrate values ranging from 4.14 ± 0.9 wt.% in VSS of the EPS sample and 23.0 ± 10.3 wt.% VSS in ZA. The ratio of protein to polysaccharides (PN/PS) observed in the extracted EPS samples fell into three distinct categories; a majority with a low ratio between 1 and 2, some with a medium ratio ranging from 3.5 to 4.5, and a few samples exhibiting a high ratio around 6.

3.4. ATR-FTIR analysis of extracted EPS

FTIR analysis was performed to give a broad overview of EPS composition by examining the absorbance of functional groups present. The general EPS composition across all samples was highly similar, as evident from the average spectrum of all samples (Fig. 3 and supp. fig. S2). Absorbance bands associated with proteins are prominently visible in the spectra, including amide A (3283 cm^{-1}), amide I (1640 cm^{-1}), and amide II (1540 cm^{-1}) (Talari et al., 2017; Kong et al., 2007). Other distinct absorbance bands include CH_2/CH_2 functional groups found in lipids (Talari et al., 2017), as well as in proteins and polysaccharides (2853 , 2922 , 2956 , and 1456 cm^{-1}). The absorbance band at 1734 cm^{-1} is indicative of $\text{C}=\text{O}$ stretching in fatty acids but has also been associated

with the presence of sialic acids (de Graaff et al., 2019). Symmetric CH deformation in fatty acids is represented by the 1360 cm^{-1} absorbance band, in combination with asymmetric CH deformation at 1450 cm^{-1} . The band at 1220 cm^{-1} can be attributed to CH and OH stretching in carbohydrates, alongside the absorbance band at 1040 cm^{-1} , but may also result from phosphate functional groups ($\text{P}=\text{O}$ and $\text{C}-\text{O}-\text{P}$ stretching) (Ma et al., 2018; Talari et al., 2017).

All samples showcased similar absorbance bands, but differences in the relative intensity of these bands were apparent. Examining the ratio between 1640 and 1040 cm^{-1} reveals that the samples from HK, ES, CH, DE(A), DE(C), AU, and NL have a higher-than-average ratio (>1.03). Other absorbance bands, such as those in 1734 and 1220 cm^{-1} , appear relatively more intense and well-defined in samples from ZA, DK, MY, NL, SG, and HK. Additionally, the shoulder at $>3283 \text{ cm}^{-1}$ is more pronounced relative to the 3283 cm^{-1} band in samples from ES, CH, DE (A), DE(B), and SA, which may indicate the presence of OH stretching in large polysaccharides. The sample from AR was distinguishable from the others, primarily due to the absorbance at 1110 cm^{-1} . While it is possible to differentiate samples based on their spectra, assigning chemical significance to individual absorbance bands is challenging due to the overlapping contributions of multiple functional groups in EPS.

3.5. Principal component analysis of FTIR spectra of extracted EPS

The FTIR spectra of EPS extracted from AS are a convoluted mixture of absorbance bands due to the heterogeneous nature of EPS. To systematically assess differences among samples, principal component analysis (PCA) was used (Fig. 4A). The first three principal components (PC1–3) combined accounted for 80.2 % of the total variability between samples, with PC1 explaining 39.2 %, PC2 26.7 %, and PC3 14.3 %. Using PC1–3, we were able to visually separate the two largest groups: chemical phosphate removal (Cb, N, DN, P(C)) and biological phosphate removal treatment (Cb, N, DN, P(B)). A pairwise Adonis analysis based on PC1–3 confirmed a significant difference between these two groups, with process type explaining 54.6 % of the variation ($p < 0.002$). Additionally, a significant difference was observed between Cb, N, DN, P (C) plants and Cb, N, DN plants ($R^2 = 41.8 \%$, $p < 0.05$). All other pairwise comparisons were not statistically significant (Suppl. table 1). On average, samples from systems treating Cb, N, DN, P (B) have positive scores for both PC1 and PC2 (1.43 and 1.79, respectively) and a

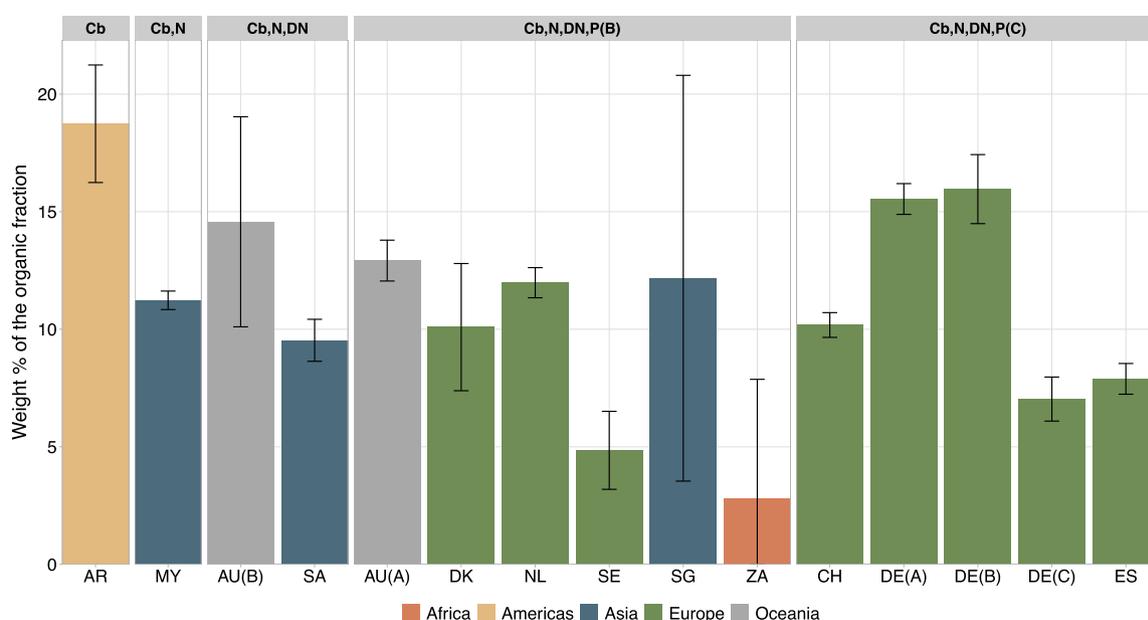


Fig. 1. EPS yield (g VSS EPS/100 g VSS AS) obtained from flocculent excess sludge samples from different countries. Bars represent the mean EPS yield, with error bars showing the standard deviation across triplicate samples.

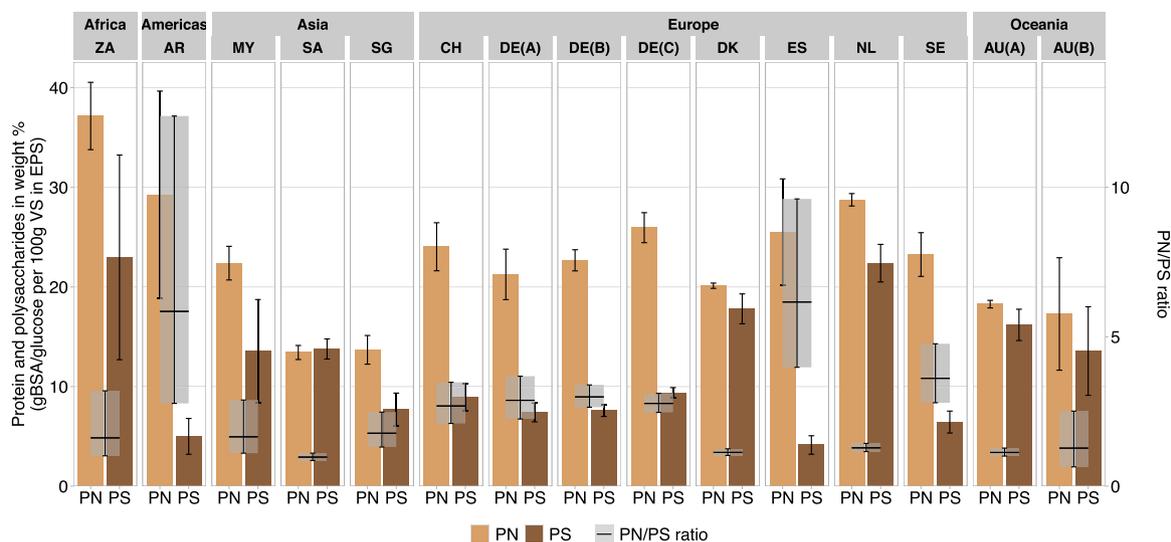


Fig. 2. Concentration of protein and polysaccharides measured as g BSA or glucose/ 100 g VSS in EPS and calculated ratio between protein and polysaccharide concentration.

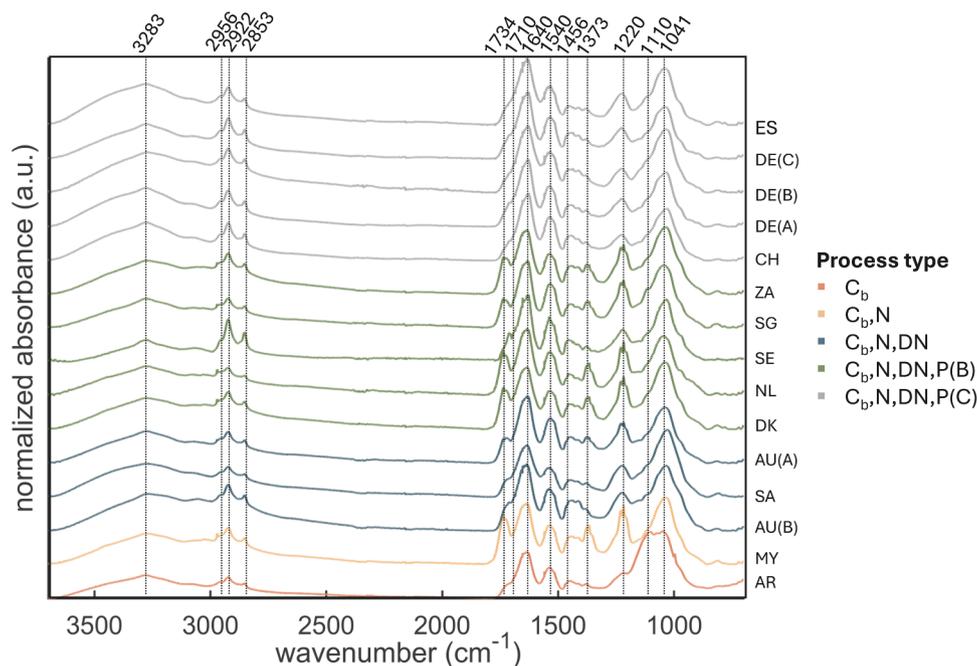


Fig. 3. Fourier transform infrared (FTIR) spectra of extracted EPS from different countries ordered by process type.

negative score for PC3 (-0.81). In contrast, samples treating C_b, N, DN, P (C) exhibit lower scores across all three principal components, with values of -2.14 for PC1, -1.85 for PC2, and -0.64 for PC3. The AR sample stands out as a notable outlier, possibly because it is the sole carbon removal WWTP. Samples from other removal types fall between these two major groups in terms of principal component scores.

Analysis of the loading spectra of the principal components provides insight into the EPS composition of the samples (Fig. 4B). The loading spectrum for PC1 shows positive correlations with absorbance bands at 1739 cm⁻¹ (C=O vibrations of ester bonds in lipids), 1366 cm⁻¹ (CH₂ vibrations), and the region from 1218 to 1104 cm⁻¹ (P=O, P-O-C, and polysaccharide C-O-C vibrations, respectively). In contrast, negative bands at 3283 cm⁻¹ and 1600 cm⁻¹ suggest a relatively lower presence of protein-related functional groups. PC2 exhibits strong positive absorbance at 1740, 1366, and 1218 cm⁻¹, corresponding to C=O stretching,

CH₂ stretching, and C-O stretching vibrations, respectively. The similarity of these bands to those in the acetone spectrum, commonly used for cleaning the ATR crystal, suggests possible contamination. For PC3, negative bands are observed at 1640 cm⁻¹ and 1540 cm⁻¹, corresponding to amide I and amide II bands of proteins. Conversely, a positive absorbance is observed for functional groups associated with polysaccharides, particularly C-O-C and C-O stretching at 1100 cm⁻¹ and 998 cm⁻¹, respectively.

3.6. Microbial community analysis

The samples were analysed using 16S rRNA gene sequencing to investigate the community structure across the plants and to assess whether variations in extracted EPS were reflected in the microbial community of the raw AS. A total of 64,512 to 172,131 16S rRNA gene

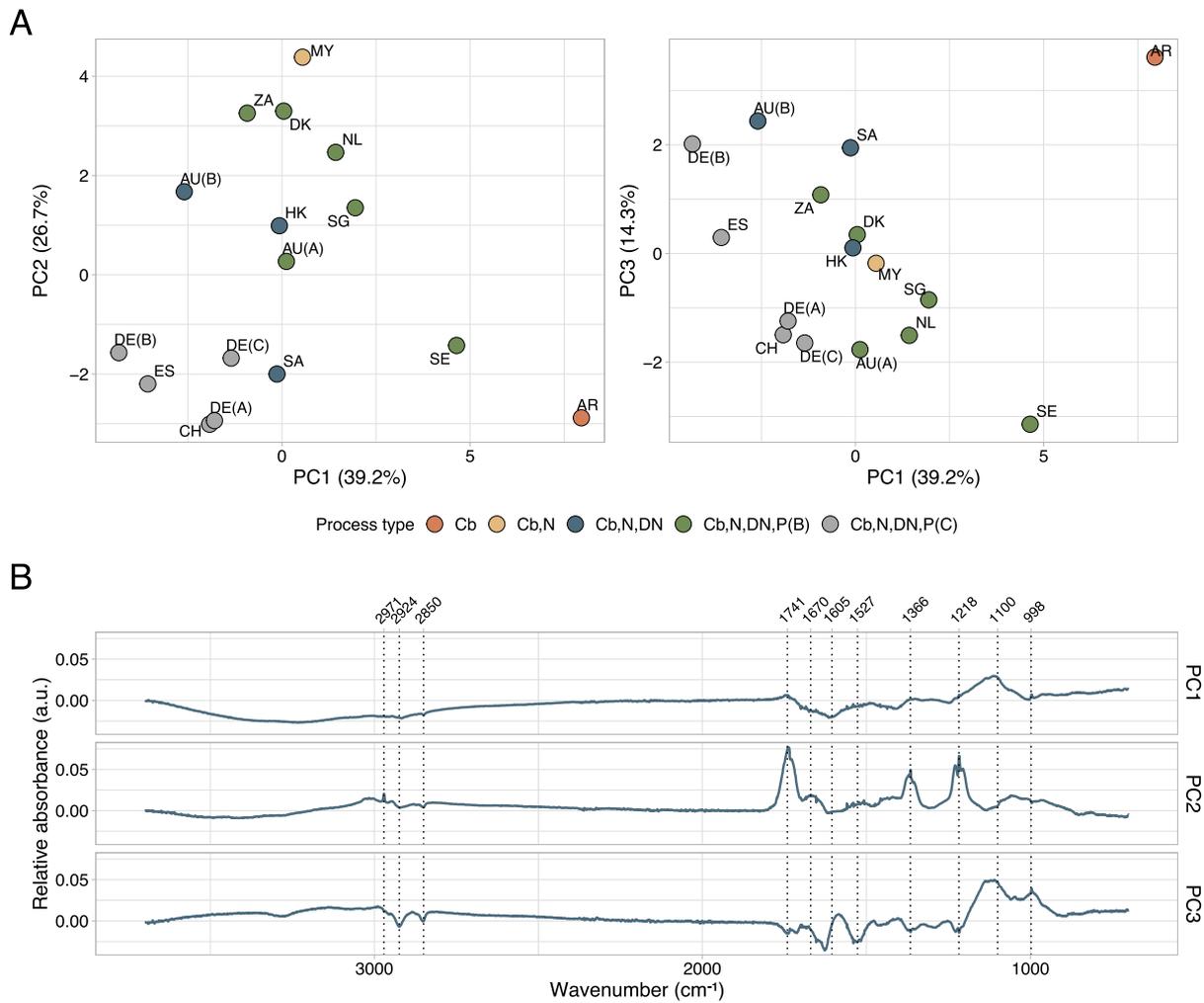


Fig. 4. Principal component analysis (PCA) scores and loading spectra of extracted EPS FTIR spectra. A) Score plots: PC1 vs. PC2 (left) and PC1 vs. PC3 (right), colored by process type. B) Loading spectra for PC1–PC3.

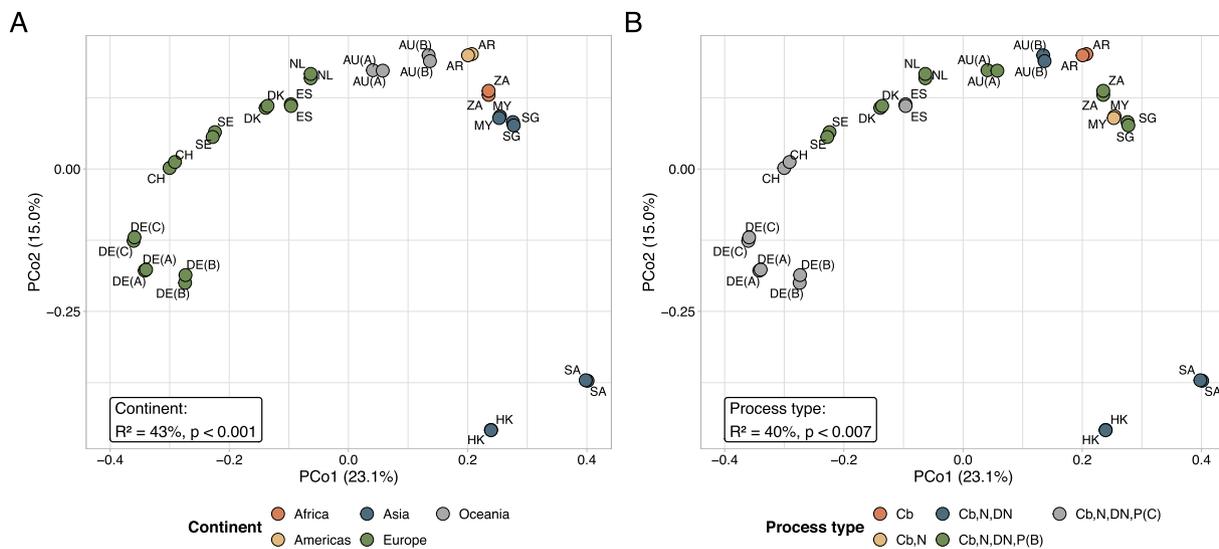


Fig. 5. Effect of geographical location (continent) (A) and process type (B) on the beta diversity of microbial community structure across all included WWTPs. Beta diversity was assessed using a Bray-Curtis distance matrix at the genus level, excluding genera with a relative abundance $< 0.1\%$. The variation explained by continent and process type was quantified using PERMANOVA (Adonis R^2 -values).

sequences were obtained from 32 AS samples (16 WWTPs, two replicates each). These sequences were mapped to the MiDAS reference database and clustered into over 5,700 distinct genera at 94.5 % identity, classifying 62–93 % of the reads (Supp. fig 3). Among these, only 678 genera were observed to be abundant in the WWTPs (>0.1 % relative abundance). The number of distinct genera exceeding 0.1 % relative abundance per WWTP ranged from just 58 in SA to 186 in AU(A) (Supp. fig 4). Notably, more than 300 of the 678 genera were found to be abundant (>0.1 % relative abundance) in only a single WWTP, indicating a high number of unique taxa per plant. This highlights the strong site-specificity of dominant bacteria across the investigated WWTPs. The beta diversity analysis revealed that samples clustered primarily based on geographic factors, with continent, climate, and process tank temperature explaining 21–54 % of the variation. Process type accounted for 40 % of the variation ($p < 0.007$) (Fig. 5 and supp. fig. 5).

Since the most abundant bacteria are likely to contribute the most to the biomass (and EPS) fraction, we focused on the 15 most abundant genera for each plant, resulting in 112 genera. This analysis revealed that these genera accounted for 28 % in MY to 60 % in DE(C) of the total read abundance (Fig. 6). The highest numbers of cumulative relative

abundance were observed in regions with denser sampling, particularly in Europe, which also showed most overlap in genera. In contrast, plants located in distinct climate zones (Table 1) exhibited unique microbial profiles. For instance, the most abundant genera in MY, SA and HK were either absent or present at low abundance in other regions.

Notably, the phylum Planctomycetota was unique to SA, while genera such as *Marinicella*, *Ca. Thiobios*, and *Thiohalocapsa*, typically associated with marine environments, were exclusive to HK with its seawater-based wastewater. Both SA and HK exhibited significantly lower genus diversity within Bacteroidota compared to other plants. Distinct Chloroflexota genera were identified, though most were restricted to a few treatment plants. Across all plants, only a few genera had an abundance >5 %. In Europe, *Ca. Microthrix* was frequently among these dominant genera. Some genera, such as *Ottowia* and *Novosphingobium*, were found abundant globally, indicating a broader geographical distribution. Among nitrifiers, the genera *Nitrosomonas*, *Nitrospira* and *Nitrotoga* were often among the 15 most abundant, among PAOs it was *Azonexus*, *Ca. Phosphoribacter* and *Tetrasphaera* while *Ca. Accumilibacter* was not, perhaps because amplicon sequencing tends to underestimate its abundance (Kleikamp et al., 2023). Among GAOs, only *Ca. Competibacter* was found abundant. Interestingly, almost half of the

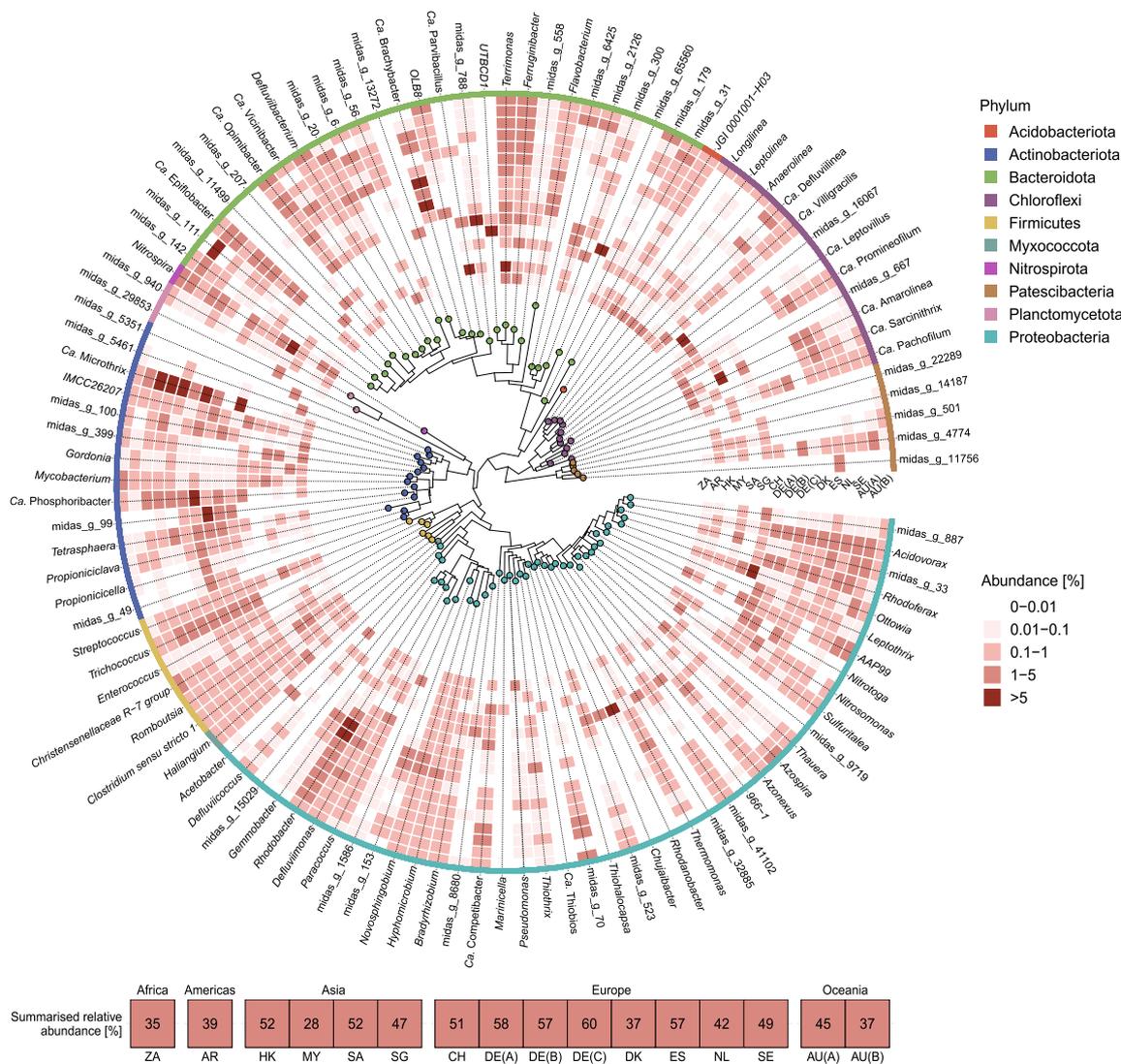


Fig. 6. The 15 most abundant genera in each WWTP and their relative read abundance across all WWTPs (112 genera in total). The phylogenetic tree was constructed using full-length 16S rRNA gene sequences from a representative species for each genus, based on the MiDAS 5.3 database and IQ-TREE. Tree tips and the outer ring are colored by phylum. The summarized relative abundance of all 112 genera is shown for each WWTP below the phylogenetic tree.

15 most abundant genera across all plants had only genus placeholder names, indicating they are novel with nothing known about their physiology including EPS production.

3.7. Comparison of microbial beta diversity, PN, PS, Yield, and FTIR profiles

We assessed whether microbial beta-diversity correlated with the extracted EPS measurements. However, no consistent patterns emerged. While FTIR variation was primarily associated with process type, it only partially explained differences in microbial community composition. Conversely, FTIR profiles did not correlate with geographic factors such as continent, climate, or temperature despite these factors being important for microbial diversity patterns. Consequently, no clear associations were observed between FTIR groupings and microbial beta-diversity at any taxonomic level (data not shown). Similarly, microbial beta-diversity showed no consistent relationship with EPS yield, protein, or polysaccharide content.

4. Discussion

4.1. EPS recovery from global activated sludge plants

The EPS yield from AS ranged from 2.81 to 18.5 wt.% VSS. The average yield aligns with the range reported by Li et al. (2021) for AS extracted using a similar method. However, this study exhibited greater variability than previously reported, likely due to differences in process conditions and geographical locations. Li et al. (2024) demonstrated that SRT influenced the extraction yield in biofilms, and explained that the increased environmental stress can cause a change in the dominant microbial community. In this study, SRT was the only operational parameter with a statistically significant effect on EPS yield. The model explained 46 % of the variability, suggesting that while SRT has a measurable influence, other factors such as organic loading, nutrient balance, hydrodynamic shear, or microbial community shifts may also play important roles. With the current dataset, possible collinearity between SRT and other parameters could not be tested. The linear fitting should therefore be regarded as exploratory, and future studies should apply targeted experimental designs to better disentangle the relative importance of SRT and other operational variables. Additionally, the diversity of microbial communities across samples was so high that it could not explain variations in EPS yield. Since EPS production is influenced by multiple interdependent variables, pinpointing contributing factors is challenging. In addition, Chen et al. (2023) demonstrated that EPS composition is not static but responds dynamically to environmental changes. They showed that the amount of negatively charged polymers in the biofilm changed as salt levels in the substrate varied but eventually stabilized after the biofilm adapted to the new conditions. This highlights how external stress can affect EPS composition within similar microbial populations.

Alkaline extraction generally favours negatively charged biopolymers. Therefore, differences in yield could be due to variations in both the total amount and composition of these polymers in the biofilm. Interestingly, FTIR spectra and PCA analysis indicated relatively consistent EPS composition despite yield variations, suggesting that similar types of biopolymers were extracted across samples. The precipice of this study was to evaluate EPS as a potential resource recovered from activated sludge. Alkaline extraction was shown to be able to solubilize the structural component of biofilms with a high extraction yield (Felz et al., 2016). Another comparative study by Liu et al. (2024) demonstrated that alkaline extraction resulted in the highest polysaccharide recovery compared to the five other tested extraction methods. Alkaline extraction is generally effective for biofilm EPS recovery, although Pronk et al. (2017) reported instances where it was less efficient, implying that biofilm composition influences extraction efficiency. Specifically, differences in negatively charged polymers may

play a role. The amount of intracellular content, well known to be released when using the alkaline extraction may also affect yield and composition (García Becerra et al., 2010). Ultimately, EPS production is affected by multiple factors, making it challenging to attribute yield variations to any single parameter, particularly when comparing globally sourced samples with substantial differences.

4.2. Influence of assay bias and microbial variability on EPS composition analysis

On average, the protein content for the global samples was higher than the carbohydrate content. This aligns with previous studies that reported that proteins are the dominant macromolecules in AS EPS (Comte et al., 2006; Frølund et al., 1996). The PN/PS ratios of EPS in activated sludges typically range between 1.0 and 2.5, with higher ratios being attributed to differences in e.g. the SRT, substrate composition or microbial stress. In our data set it was not possible to correlate the PN/PS ratio to SRT or other operational conditions. Even though the changes in the operational conditions did seem to change the microbial composition, these changes could not directly be linked to the PN/PS ratio. An important factor to consider when interpreting the PN/PS ratio is that both the protein and polysaccharide content are an approximation and depend on the reactivity of sugar monomers and amino acids to the assays (Felz et al., 2019). Different microbial communities can synthesize slightly different EPS, with similar functional characteristics but variations in the exact monomer compositions (Chen et al., 2023). Colorimetric assays like the BCA assay, are sensitive to specific amino acids such as cysteine, tyrosine, and tryptophan (Smith et al., 1985). As a result, the amino acid composition, driven by the microbial community, can artificially inflate or deflate the PN/PS ratio, creating a methodological bias that complicates comparisons across studies. To minimize potential biases in the PN/PS ratio, direct quantification of the constituent monomers would be recommendable. High-performance anion exchange chromatography with pulsed amperometric detection can be used for sugar monomers, while liquid chromatography–mass spectrometry can be applied for amino acids. To measure the influence of microbial composition on PN/PS ratios, these detailed analyses could be combined with a less heterogeneous sample set, which allows for a better attribution of compositional differences to specific microbial or operational factors.

4.3. Process driven variations in EPS composition: Insights from FTIR and PCA analysis

FTIR and PCA analysis revealed that EPS composition is primarily influenced by wastewater treatment process type rather than geographical location. Despite significant differences in microbial communities across sites, the extracted EPS clustered into two distinct groups based on their spectral profiles, corresponding to biological (Cb, N, DN, P(B)) or chemical (Cb, N, DN, P(C)) phosphorus removal systems. The largest variation in FTIR data was driven by differences in lipid-related absorbance bands (1739 and 1366 cm^{-1}) and phosphate- and polysaccharide-related absorbance bands (1218 and 1104 cm^{-1}). Specifically, biological phosphorus removal systems (Cb, N, DN, P(B)) exhibited relatively higher absorbance in bands related to lipid, phosphate and polysaccharide functional groups, while chemical phosphorus removal systems (Cb, N, DN, P(C)) had a higher absorbance in bands related to protein functional groups. In biological phosphorus removal systems, PAOs are known to contribute to EPS production (Paez-Watson et al., 2024), which may explain the elevated phosphate- and polysaccharide-related signals. The stronger lipid, phosphate, and polysaccharide signals might also reflect higher lipopolysaccharide (LPS) content. These results suggest that process conditions help shape the EPS composition. Given that differences in polymer composition cause distinct functional properties, future research should investigate how physical-chemical properties change in EPS from different

operational processes. This could determine functional performance in applications such as flocculation, contaminant adsorption, and material engineering. The ability to differentiate EPS samples based on FTIR spectra, which provide a broad overview of functional groups present, highlights the potential of FTIR as a high-throughput alternative to traditional analytical methods for EPS characterization.

4.4. Correlation of microbial community with extracted EPS measurements

It is well established that different AS bacteria can produce distinct polysaccharides, many of which remain uncharacterized (Dueholm et al., 2023). While the role of microorganisms in EPS production has been described, current literature remains inconclusive and often system-specific, providing only a partial understanding of how microbial community composition influences EPS yield and properties in complex, real-world environments (Seviour et al., 2019). In this study no direct correlation was observed between microbial community composition and EPS composition. This suggests that EPS composition is not solely determined by which microbes are present, but seems to be influenced by different processes. Functional redundancy among microbial taxa, and the adaptive regulation of EPS production may explain why shifts in community composition did not necessarily translate into predictable changes in EPS composition. The chemical analysis performed in this study did not allow for a further identification of the specific polymers inside of the extracted EPS mixture. To analyse the samples based on the chemical composition but still maintain a high-throughput approach, broad analytical methods were used that could still distinguish important components in the EPS. The number of WWTPs per continent and within each operation process was relatively low, which reduced statistical power. Whether the lack of correlation reflects a true absence of association or is influenced by chemical limitations or limited sample size remains uncertain.

Our understanding of EPS synthesis pathways in microorganisms is still developing, yet evidence indicates that different microorganisms can produce polymers with similar functions. For example, Chen et al. (2023) found similar EPS compositional patterns in anaerobic and aerobic granular sludge, two fundamentally different systems. This raises the intriguing possibility that such functional similarity could also occur in our study, where distinct microbial communities might still produce broadly comparable EPS compositions. To advance the understanding of microbial community contributions to EPS, future studies could apply metagenomic analyses to assess the genetic potential for EPS production, enabling the identification of key EPS-producing taxa and dominant biosynthetic pathways. This approach may help clarify which EPS components are prevalent and how their expression is influenced by operational conditions. Additionally, future research could focus on time-series analyses of individual WWTPs, where operational settings remain constant and only community composition varies, allowing for more controlled investigation of these relationships.

5. Conclusion

- In this study, we aimed to develop a high-throughput EPS characterization method that could be directly compared with yield and microbial community data. FTIR spectroscopy, combined with multivariate analysis, effectively identified trends in EPS composition.
- The EPS extracted by the alkaline method correlated only with SRT, not with microbial community structure or other investigated factors. Despite significant microbial diversity across treatment plants, the EPS composition remained relatively stable across the global samples.
- Microbial communities were highly diverse and revealed many abundant but hitherto undescribed genera. As both process type, operation and geography varied, the limited number of WWTPs

made it difficult to establish reliable correlations to the community structure and impossible to link specific taxa to EPS type or properties. However, future timeseries studies focusing on a more similar sample population could help establish such links.

- Exploring the genetic potential of microbial communities may help uncover key EPS-producing taxa and pathways, offering insights into how EPS composition is shaped by microbial communities and operational conditions
- Overall, the study presents a baseline for the amount and overall composition of EPS that can be extracted from global AS plants for recovery.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve clarity and grammar. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Contributors

Australia

Kris Coventry, Melbourne Water, Melbourne, Australia
Steve Petrovski and Robert Seviour, Environmental Microbial Genetics Lab, La Trobe University, Melbourne, VIC, Australia

Sweden

Magnus Christensson, Veolia Water Technologies, AnoxKaldnes, Lund, Sweden

The Netherlands

Rogier van Kempen, Delfluent Services, Den Hoorn, The Netherlands

Spain

Maite Pijuan, Technologies and Evaluation Area, Catalan Institute for Water Research, ICRA, Girona, Spain

Germany

Harald Horn, Water Chemistry and Water Technology and DVGW Research Laboratories, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

Switzerland

Eberhard Morgenroth, Swiss Federal Institute of Aquatic Science and Technology (Eawag), Dübendorf, Switzerland

Singapore

Wong Lan Li, Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, Singapore

Saudi Arabia

Pascal Saikaly, King Abdullah University of Science and Technology (KAUST), Rabigh, Saudi Arabia

Malaysia

Adeline Chua Seak May, Department of Chemical Engineering, Faculty of Engineering, University of Malaya, Kuala Lumpur, Malaysia

Hong Kong

Dou Wang, The University of Hong Kong, Room LG209, Composite Building, Hong Kong SAR, China

Argentina

Leonardo Erijman, INGEBI-CONICET, University of Buenos Aires, Buenos Aires, Argentina

South Africa

Jac Wilsenach, Department of Forest and Wood Science, Stellenbosch University, Stellenbosch, South Africa

CRediT authorship contribution statement

Stefan De Bruin: Writing – original draft, Methodology, Investigation, Formal analysis. **Marie Riisgaard-Jensen:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Susan Hove Hansen:** Methodology, Investigation, Formal analysis, Data

curation. **Mark C.M. Van Loosdrecht:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization. **Per Halkjær Nielsen:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization. **Yuemei Lin:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization.

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.

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Supplementary materials

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Data availability

Data will be made available on request.

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