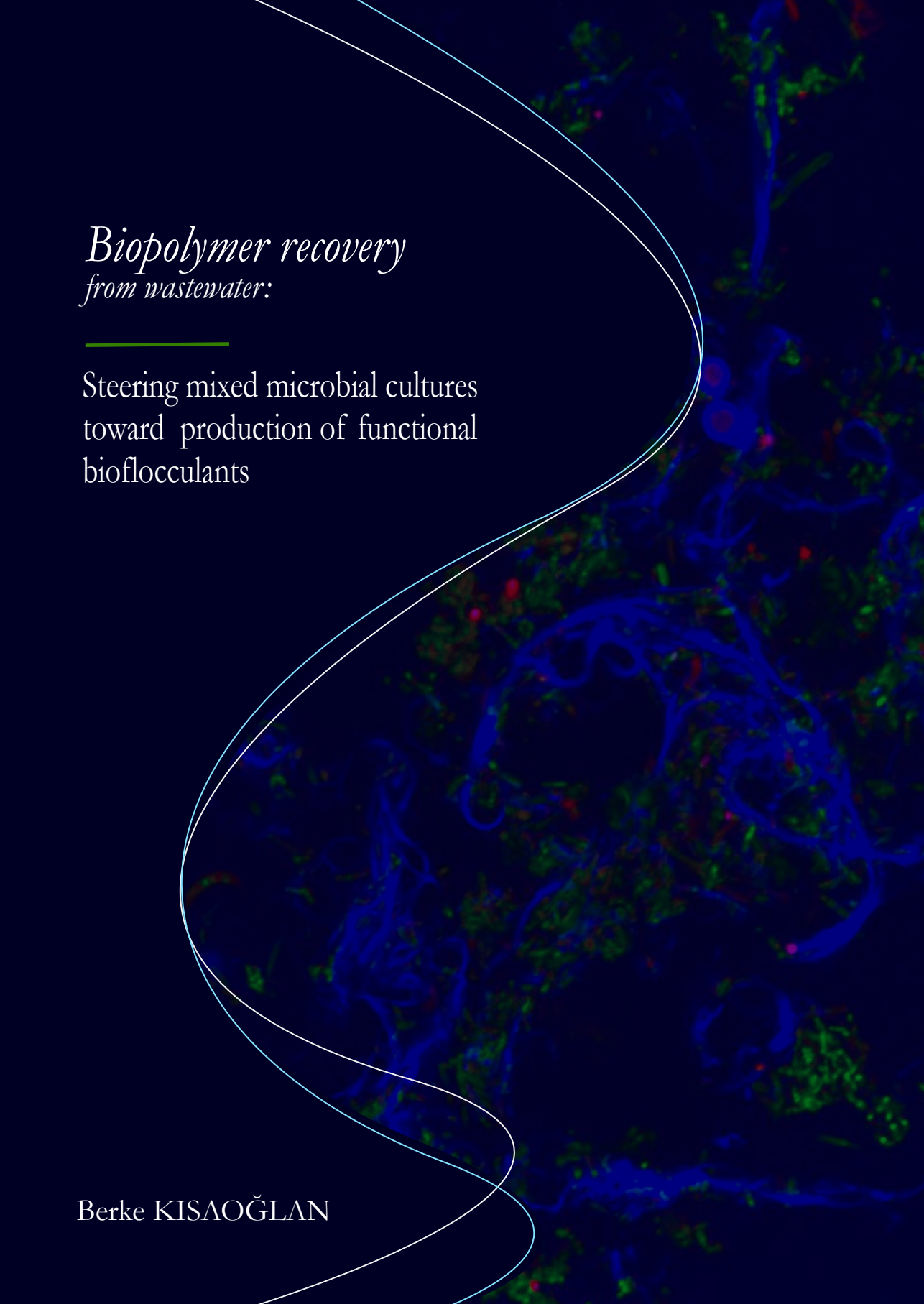


Biopolymer recovery
from wastewater:

Steering mixed microbial cultures toward production of functional bioflocculants

Berke KISAOĞLAN

Propositions

1. Wastewater organics are excellent resources for producing biopolymers.
   (this thesis)

2. The effectiveness of wastewater-derived flocculants depends on reproducible production of compounds with similar composition.
   (this thesis)

3. The duty of a scientist is to convert failure into new hypotheses.

4. The value of scientific findings depends on the researcher's ability to explain their relevance.

5. Societal acceptance of scientifically safe products depends on public perception.

6. Green innovation is held back when sustainable choices cost more.

Propositions belonging to the thesis, entitled

Biopolymer Recovery from Wastewater: Steering Mixed Microbial Cultures toward production of functional bioflocculants

Berke Kısaoğlan

Wageningen, 26 November 2026

Biopolymer recovery from wastewater: steering mixed microbial cultures toward production of functional bioflocculants

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Biopolymer recovery from wastewater: steering mixed microbial cultures toward production of functional bioflocculants

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Summary

Wastewater treatment systems need to move beyond pollution removal alone and contribute to resource recovery and circular economy goals. Wastewater contains large amounts of organic matter and part of this can potentially be recovered as valuable biobased products. One promising route is the conversion of organics to extracellular polymeric substances (EPS), which are high-molecular-weight biopolymers naturally produced by microorganisms. EPS consist mainly of polysaccharides and proteins and contain functional groups that allow them to interact with particles, metals, and other surfaces. These properties make EPS interesting as potential bioflocculants for water and wastewater treatment.

Conventional flocculation processes commonly rely on synthetic polymeric flocculants, such as polyacrylamide-based products. These polymers are effective at low dosages and are widely used for solid-liquid separation, but their fossil-based origin, persistence, and potential environmental impacts have raised concerns. Biodegradable and renewable alternatives are therefore increasingly needed. Microbial EPS may provide such an alternative, especially when produced from waste-derived organic streams by mixed microbial cultures. Compared with pure-culture processes, mixed-culture systems do not require sterile operation and can use complex, low-cost substrates. However, their practical use requires reliable process conditions that stimulate EPS overproduction and produce EPS with stable functional properties.

This thesis investigated whether mixed microbial cultures can be steered to overproduce EPS from wastewater-derived substrates and whether the recovered EPS can function as bioflocculants. The work connects three main aspects: operational steering of EPS production, microbial community development under EPS-overproducing conditions, and application of the recovered EPS for flocculation of different particles. The overall aim was to better understand how EPS production can be made reliable for future scaling up and how the resulting EPS

perform in solid-liquid separation processes.

The thesis first placed EPS recovery in the broader context of wastewater treatment and sustainable flocculant development. In conventional treatment systems, suspended and colloidal particles must be removed efficiently to ensure good effluent quality and stable operation. Coagulation and flocculation are central processes for this purpose. However, replacing fossil-based synthetic polymers with bio-based alternatives requires not only a suitable product but also a feasible production process. EPS are already present in biological wastewater treatment systems, but they are often viewed as operationally problematic because they can contribute to sludge bulking, viscosity increase, and membrane fouling. In this thesis, EPS were instead considered as a valuable recoverable product. This required identifying operational conditions that stimulate microorganisms to produce EPS beyond normal levels (EPS overproduction).

The **Chapter 2** of the thesis focused on EPS production from glycerol-rich synthetic wastewater using membrane bioreactors (MBRs). The influence of inoculum type, carbon-to-nitrogen ratio, and solids retention time (SRT) was investigated. The results showed that strong nitrogen limitation combined with short SRT was a key operational strategy to stimulate EPS overproduction by mixed microbial cultures. Under these conditions, both municipal and industrial sludge inocula were able to produce EPS, although the magnitude and stability of production differed between inocula. The recovered EPS were mainly polysaccharide-rich and had high molecular weight, which are favourable properties for flocculation. The EPS showed high flocculation efficiencies with kaolinite, demonstrating that mixed-culture-derived EPS can act as functional bioflocculants. These findings showed that EPS overproduction can be achieved in continuous reactors and that nitrogen limitation with short SRT is an important selection pressure.

The **Chapter 3** extended this strategy to volatile fatty acid (VFA)-rich wastewater, which is relevant because VFAs are common intermediates in anaerobic treatment and fermentation of organic waste streams. A membrane bioreactor and a continuous stirred-tank reactor (CSTR) were compared under nitrogen limitation and short SRT. The results showed that EPS production could also be achieved from VFA-rich substrates, indicating that the strategy developed for

glycerol-rich wastewater was transferable to another important class of waste-derived feedstocks. The CSTR showed better VFA conversion and higher EPS yields than the MBR under the tested conditions, suggesting that simpler reactor configurations may be suitable for EPS production. Importantly, although VFAs are commonly associated with Polyhydroxyalkanoates (PHA) production, the selected conditions mainly directed carbon toward EPS rather than intracellular PHA storage. This supports EPS production as an alternative route for valorising fermented waste streams.

The thesis then investigated the microbial communities associated with EPS overproduction in **Chapter 4**. Seven bioreactors operated under different conditions were compared using 16S rRNA gene amplicon sequencing. The analysis showed that EPS production in mixed microbial cultures could not be attributed to one single dominant microorganism. Instead, EPS overproduction appeared to be a community-level function supported by multiple taxa that may contribute to EPS production and degradation. Putative EPS-producing taxa such as *Brevundimonas, Pseudoxanthomonas, Microbacterium, Novosphingobium, Flavobacterium,* and *Chryseobacterium* were detected under different conditions. At the same time, potential EPS-degrading or predatory groups, including *Saprospiraceae, Bdellovibrio, Micavibrionales,* and related taxa, were also observed. These findings highlight that EPS-overproducing bioreactors are dynamic ecological systems in which production and turnover processes likely occur simultaneously. The results also showed that substrate type, reactor configuration, inoculum, and operation time shaped microbial community structure. However, 16S rRNA gene analysis alone could not confirm the active functional roles of individual microorganisms. Therefore, future studies should combine microbial community profiling with functional approaches such as metagenomics, transcriptomics, or proteomics to better identify the organisms and pathways directly involved in EPS synthesis and degradation.

The final experimental part of the thesis (**Chapter 5**) evaluated whether mixed-culture-derived EPS can flocculate particles beyond the commonly used model mineral kaolinite. EPS with different compositions were tested with kaolinite, montmorillonite, talc, and iron-rich colloids suspension. The results showed that flocculation performance depended strongly on the properties of the target

particles. EPS performed best with moderately charged and hydrophilic kaolinite, where high flocculation efficiencies were achieved under optimized calcium conditions. Performance was lower for highly charged and strongly hydrophilic montmorillonite and limited for hydrophobic talc. These differences indicate that EPS flocculation is governed not only by EPS properties but also by particle surface chemistry, hydrophilicity, charge, and morphology. Calcium was essential for robust flocculation in most systems, suggesting that calcium-mediated bridging between EPS functional groups and particle surfaces was a dominant mechanism. In the iron-rich colloidal suspension, higher EPS dosages were required than for model minerals, but high turbidity removal could still be achieved. This demonstrated that EPS can flocculate more complex particles, although application conditions must be adapted to the target water and particle type.

Overall, this thesis demonstrates that mixed microbial cultures can be steered to overproduce EPS from wastewater-derived organic streams and that the recovered EPS have potential as sustainable bioflocculants. Strong nitrogen limitation combined with short SRT was identified as a robust operational strategy for EPS overproduction. The work also showed that EPS production can be transferred from glycerol-rich to VFA-rich substrates and that reactor configuration affects process performance. Microbial community analysis indicated that EPS overproduction is likely a community-level function rather than the activity of a single dominant producer. Finally, the flocculation studies showed that mixed-culture-derived EPS can achieve high particle removal, but their effectiveness depends on matching EPS properties, calcium conditions, and particle surface characteristics. The findings contribute to the development of wastewater treatment systems that recover useful biopolymers while treating organic waste streams. By converting waste organics into functional EPS-based flocculants, this approach can support both resource recovery and reduced dependence on fossil-based synthetic polymers. Further research is still needed to test real wastewater streams for EPS overproduction, improve process control, assess long-term stability, evaluate downstream recovery and purification, and perform techno-economic and environmental assessments. Nevertheless, this thesis provides a scientific basis for developing EPS overproduction systems by mixed microbial cultures as a promising route toward sustainable flocculant production and circular water treatment.

Samenvatting

Afvalwaterzuiveringssystemen moeten verder gaan dan alleen het verwijderen van vervuiling en bijdragen aan hergebruik van grondstoffen en doelstellingen van de circulaire economie. Afvalwater bevat grote hoeveelheden organisch materiaal en een deel hiervan kan mogelijk worden teruggewonnen als waardevolle biogebaseerde producten. Een veelbelovende route is de omzetting van organische stoffen naar extracellulaire polymere stoffen (EPS), biopolymeren met hoog molecuulgewicht die van nature door micro-organismen worden geproduceerd. EPS bestaan voornamelijk uit polysachariden en eiwitten en bevatten functionele groepen waarmee ze kunnen interacteren met deeltjes, metalen en andere oppervlakken. Deze eigenschappen maken EPS interessant als potentiële bioflocculanten voor water- en afvalwaterbehandeling.

Conventionele flocculatieprocessen zijn doorgaans afhankelijk van synthetische polymere flocculanten, zoals producten op basis van polyacrylamide. Deze polymeren zijn effectief bij lage doseringen en worden veel gebruikt voor vastvloeistofscheiding, maar hun fossiele oorsprong, persistentie en mogelijke milieueffecten hebben tot zorgen geleid. Biologisch afbreekbare en hernieuwbare alternatieven zijn daarom steeds meer nodig. Microbiële EPS kunnen zo'n alternatief bieden, vooral wanneer ze door gemengde microbiële culturen worden geproduceerd uit organische afvalstromen. In vergelijking met zuivere-cultuurprocessen vereisen gemengde-cultuursystemen geen steriele bedrijfsvoering en kunnen ze complexe, goedkope substraten gebruiken. Voor praktische toepassing zijn echter betrouwbare procescondities nodig die EPS-overproductie stimuleren en EPS met stabiele functionele eigenschappen opleveren.

Dit proefschrift onderzoekt of gemengde microbiële culturen kunnen worden gestuurd om EPS over- te produceren uit substraten afkomstig van afvalwater, en of de teruggewonnen EPS kunnen functioneren als bioflocculanten. Het werk verbindt drie hoofdaspecten: operationele sturing van EPS-productie, de ontwikkeling van de microbiële gemeenschap onder EPS-overproducerende condities, en

de toepassing van de teruggewonnen EPS voor de flocculatie van verschillende deeltjes. Het overkoepelende doel was om beter te begrijpen hoe EPS-productie betrouwbaar gemaakt kan worden voor toekomstige opschaling en hoe de resulterende EPS presteren in vast-vloeistofscheidingsprocessen.

Het proefschrift plaatst eerst EPS-terugwinning in de bredere context van afvalwaterbehandeling en de ontwikkeling van duurzame flocculanten. In conventionele zuiveringssystemen moeten gesuspendeerde en colloïdale deeltjes efficiënt worden verwijderd om een goede effluentkwaliteit en een stabiele werking te waarborgen. Coagulatie en flocculatie zijn hiervoor centrale processen. Het vervangen van synthetische polymeren op fossiele basis door biogebaseerde alternatieven vereist echter niet alleen een geschikt product, maar ook een haalbaar productieproces. EPS zijn al aanwezig in biologische afvalwaterzuiveringssystemen, maar worden vaak als operationeel problematisch gezien, omdat ze kunnen bijdragen aan slibzwelling, toename van de viscositeit en membraanvervuiling. In dit proefschrift werden EPS in plaats daarvan beschouwd als een waardevol terugwinbaar product. Dit vereiste het identificeren van operationele condities die micro-organismen stimuleren om EPS te produceren boven de normale achtergrondniveaus.

Hoofdstuk 2 van het proefschrift richt zich op EPS-productie uit synthetisch afvalwater rijk aan glycerol met behulp van membraanbioreactoren. De invloed van het type inoculum, de koolstof-stikstofverhouding en de slibretentietijd werd onderzocht. De resultaten toonden aan dat sterke stikstoflimitatie gecombineerd met een korte slibretentietijd een sleutelstrategie was om EPS-overproductie door gemengde microbiële culturen te stimuleren. Onder deze condities waren zowel communale als industriële slibinocula in staat EPS te produceren, hoewel de omvang en stabiliteit van de productie verschilden tussen de inocula. De teruggewonnen EPS waren voornamelijk rijk aan polysachariden en hadden een hoog molecuulgewicht, wat gunstige eigenschappen zijn voor flocculatie. De EPS vertoonden hoge flocculatie-efficiënties met kaoliniet, waarmee werd aangetoond dat EPS afkomstig van gemengde culturen kunnen functioneren als bioflocculanten. Deze bevindingen lieten zien dat EPS-overproductie in continue reactoren kan worden bereikt en dat stikstoflimitatie met een korte SRT een belangrijke selectiedruk is.

Hoofdstuk 3 breidt deze strategie uit naar afvalwater rijk aan vluchtige vetzuren (VFA's), wat relevant is omdat VFA's veelvoorkomende tussenproducten zijn bij anaerobe behandeling en fermentatie van organische afvalstromen. Een membraanbioreactor en een continu geroerde tankreactor (CSTR) werden vergeleken onder stikstoflimitatie en een korte SRT. De resultaten toonden aan dat EPS-productie ook kon worden bereikt uit VFA-rijke substraten, wat aangeeft dat de strategie die was ontwikkeld voor glycerolrijk afvalwater overdraagbaar was naar een andere belangrijke klasse van grondstoffen afkomstig uit afval. De CSTR liet onder de geteste condities een betere VFA-omzetting en hogere EPS-opbrengsten zien dan de MBR, wat suggereert dat eenvoudigere reactorconfiguraties geschikt kunnen zijn voor EPS-productie. Belangrijk is dat, hoewel VFA's vaak worden geassocieerd met PHA-productie, de gekozen condities de koolstof voornamelijk naar EPS stuurden in plaats van naar intracellulaire PHA-opslag. Dit ondersteunt EPS-productie als een alternatieve route voor het valoriseren van gefermenteerde afvalstromen.

Het proefschrift onderzoekt vervolgens in **Hoofdstuk 4** de microbiële gemeenschappen die samenhangen met EPS-overproductie. Zeven bioreactoren, onder verschillende condities, werden vergeleken met behulp van 16S rRNA-gen-amplicon-sequencing. De analyse toonde aan dat EPS-productie in gemengde microbiële culturen niet aan één enkel dominant micro-organisme kon worden toegeschreven. In plaats daarvan leek EPS-overproductie een functie op gemeenschapsniveau te zijn, ondersteund door meerdere taxa die kunnen bijdragen aan EPS-productie en EPS-afbraak. Vermoedelijke EPS-producerende taxa zoals *Brevundimonas, Pseudoxanthomonas, Microbacterium, Novosphingobium, Flavobacterium,* en *Chryseobacterium* werden onder verschillende condities gedetecteerd. Tegelijkertijd werden ook potentiële EPS-afbrekende of predatorische groepen waargenomen, waaronder *Saprospiraceae, Bdellovibrio, Micavibrionales,* en verwante taxa. Deze bevindingen benadrukken dat EPS-overproducerende bioreactoren dynamische ecologische systemen zijn waarin productie- en omzettingsprocessen waarschijnlijk gelijktijdig plaatsvinden. De resultaten lieten ook zien dat het type substraat, de reactorconfiguratie, het inoculum en de operatietijd de structuur van de microbiële gemeenschap bepaalden. Analyse van het 16S rRNA-gen alleen kon echter de actieve functionele rollen van individuele micro-organismen niet bevestigen. Toekomstige studies moeten daarom microbiële gemeenschapsprofilering combineren met functionele benaderingen zoals

metagenomics, transcriptomics of proteomics om de organismen en routes die direct betrokken zijn bij EPS-synthese en -afbraak beter te kunnen identificeren.

Het laatste experimentele deel van het proefschrift (**Hoofdstuk 5**) evalueert of EPS afkomstig van gemengde culturen deeltjes kunnen flocculeren buiten het veelgebruikte modelmineraal kaoliniet. EPS met verschillende samenstellingen werden getest met kaoliniet, montmorilloniet, talk en een suspensie van ijzerrijke colloïden. De resultaten toonden aan dat de flocculatieprestaties sterk afhingen van de eigenschappen van de doeldeeltjes. EPS presteerden het best met het matig geladen en hydrofiele kaoliniet, waarbij hoge flocculatie-efficiënties werden bereikt onder geoptimaliseerde calciumcondities. De prestaties waren lager voor het sterk geladen en sterk hydrofiele montmorilloniet en beperkt voor het hydrofobe talk. Deze verschillen geven aan dat EPS-flocculatie niet alleen wordt bepaald door de eigenschappen van de EPS, maar ook door de oppervlaktechemie, hydrofiliciteit, lading en morfologie van de deeltjes. Calcium was essentieel voor robuuste flocculatie in de meeste systemen, wat suggereert dat calciumgemedieerde brugvorming tussen functionele groepen van EPS en deeltjesoppervlakken een dominant mechanisme is. In de ijzerrijke colloïdale suspensie waren hogere EPS-doseringen nodig dan voor de modelmineralen, maar kon nog steeds een hoge troebelheidsverwijdering worden bereikt. Dit toont aan dat EPS complexere deeltjes kunnen flocculeren, hoewel de toepassingscondities moeten worden aangepast aan het doelwater en het type deeltje.

Al met al toont dit proefschrift aan dat gemengde microbiële culturen kunnen worden gestuurd om EPS te overproduceren uit organische stromen afkomstig van afvalwater en dat de teruggewonnen EPS potentie hebben als duurzame bioflocculanten. Sterke stikstoflimitatie gecombineerd met een korte slibretentietijd werd geïdentificeerd als een robuuste operationele strategie voor EPS-overproductie. Het werk liet ook zien dat EPS-productie kan worden overgedragen van glycerolrijke naar VFA-rijke substraten en dat de reactorconfiguratie de procesprestaties beïnvloedt. Analyse van de microbiële gemeenschap gaf aan dat EPS-overproductie waarschijnlijk een functie op gemeenschapsniveau is in plaats van de activiteit van één dominante producent. Ten slotte toonden de flocculatiestudies aan dat EPS afkomstig van gemengde culturen een hoge deeltjesverwijdering kunnen bereiken, maar dat hun effectiviteit afhangt van het op elkaar afstemmen van EPS-eigenschappen, calciumcondities en oppervlakte-eigenschappen van de

deeltjes. De bevindingen dragen bij aan de ontwikkeling van afvalwaterzuiveringssystemen die nuttige biopolymeren terugwinnen terwijl organische afvalstromen worden behandeld. Door organisch afval om te zetten in functionele flocculanten op EPS-basis kan deze aanpak zowel hergebruik van grondstoffen als een verminderde afhankelijkheid van synthetische polymeren op fossiele basis ondersteunen. Verder onderzoek is nog nodig om echte afvalwaterstromen te testen op EPS-overproductie, de procesbeheersing te verbeteren, de langetermijnstabiliteit te beoordelen, de stroomafwaartse terugwinning en zuivering te evalueren, en technisch-economische en milieukundige beoordelingen uit te voeren. Desalniettemin biedt dit proefschrift een wetenschappelijke basis voor het ontwikkelen van EPS-overproductiesystemen met gemengde microbiële culturen als een veelbelovende route naar duurzame flocculantproductie en circulaire waterbehandeling.

Contents

1 General Introduction **1**
1.1 Water treatment needs sustainable flocculants . . . 3
1.2 Biopolymer-based flocculants as an alternative to synthetic flocculants . . . 10
1.3 Research gap . . . 21
1.4 Overall aim and research questions . . . 24
1.5 Thesis outline and chapter contributions . . . 25

2 Production of bioflocculants from glycerol-rich synthetic wastewater: The influence of inoculum and process parameters **49**
2.1 Introduction . . . 51
2.2 Materials and Methods . . . 54
2.3 Results . . . 60
2.4 Discussion . . . 71
2.5 Conclusions . . . 79

3 EPS overproduction from VFA-Rich wastewater by mixed microbial cultures under nitrogen limitation and short SRT: A new route for VFA valorisation **93**
3.1 Introduction . . . 95
3.2 Materials and Methods . . . 97
3.3 Results and Discussion . . . 102
3.4 Conclusions . . . 121

4 Microbial community drivers of EPS overproduction from glycerol- or VFA-rich wastewater under nitrogen limitation across different reactor configurations **129**
4.1 Introduction . . . 131
4.2 Materials and Methods . . . 133
4.3 Results and Discussion . . . 139

4.4 Conclusions 161

5 Flocculation of diverse minerals and iron-rich colloids via calcium bridging with mixed microbial culture-derived EPS 191
5.1 Introduction 193
5.2 Materials and methods 195
5.3 Results 205
5.4 Discussion 217
5.5 Conclusions 229

6 General Discussion 243
6.1 From EPS overproduction to bioflocculant application 244
6.2 Synthesis of findings across the thesis 248
6.3 The overall contribution of this thesis to advancing the application of EPS as bioflocculants 264
6.4 Limitations, uncertainties and future directions 264
6.5 Concluding Remarks 271

A Supplementary Information belonging to Chapter 2 287

B Supplementary Information belonging to Chapter 3 301

C Supplementary Information belonging to Chapter 4 309

D Supplementary Information belonging to Chapter 5 319

Acknowledgements 327

Chapter 1

General Introduction

Abstract

The availability of clean and fresh water is a critical concern for societies worldwide. In this context, the treatment of used water, commonly referred to as wastewater, plays a central role in both industrial and domestic settings. Wastewater treatment is essential not only for reducing surface water pollution, but also for enabling water reuse and recovering organic and inorganic materials that are dissolved or suspended in wastewater streams. This thesis focuses on the latter aspect, specifically the recovery of dissolved organic matter in the form of extracellular polymeric substances (EPS). In particular, it examines the formation of EPS in specific wastewater treatment configurations and the potential applications of the formed EPS. This introduction positions the research within the context of existing scientific and professional literature on recovery of organics from wastewater and the functionality of formed products, as well as previous studies conducted at the laboratories of the Wetsus European Centre of Excellence for Sustainable Water Technology and the Environmental Technology Group at Wageningen University & Research.

1.1 Water treatment needs sustainable flocculants

In the following, a brief overview of some basic principles of wastewater treatment is provided. Given the large volumes of wastewater that require treatment worldwide ($\sim 359 \times 10^9\ m^3\ yr^{-1}$)[1], a first step is efficient removal of suspended solids. This is essential to ensure stable operation and high effluent quality of treatment plants [2]. Effective removal of solids is particularly important during primary and secondary clarification (Fig. 1). Then in most treatment systems, the soluble and solubilised organic pollutants are removed by a biological process [3]. In this step, we aim to manipulate the process in so that EPS is produced from the organic waste (Fig. 1), which will be discussed further. However, such biological treatment processes often generate biosludge with variable density and settleability. As a result, the effluent from this treatment step generally requires system-specific tertiary polishing for particulate/sludge removal, followed by sludge dewatering. In this final step, insufficient particulate/sludge removal can lead to non-compliance with discharge limits and increase the chemical and energy inputs required to meet clarification targets, thereby increasing overall treatment costs. A wide range of water and wastewater streams contain fine suspended solids such as clays, metal hydroxides, and organic colloids. These particles have a small (in the order of 0.1-10 μm) size and have a surface charge, which causes electrostatic repulsion between particles that thereby remain stable in suspension [4]. As a result, these particles settle extremely slowly under gravity, taking minutes to hours or even days to settle (Table 1) [4]. This poor gravitational settleability makes physical sedimentation alone unsuitable for the reliable removal of fine and colloidal matter.

To overcome the settleability limitation, coagulation-flocculation is widely applied to destabilize colloids and convert fine particles into larger aggregates that can be removed by sedimentation, filtration, or flotation (Fig. 1) [5]. In this two-step process, stable particles are first destabilized during coagulation by dosing inorganic coagulants such as alum or ferric salts [2]. These coagulants reduce interparticle repulsion through several mechanisms; double layer compression, charge neutralization, colloid entrapment (sweep flocculation) and intra-particle bridging [6]. Once repulsive forces are sufficiently reduced, attractive forces (e.g., van der Waals) dominate and particles can collide and form microaggregates [4].

Table 1: Classification of particles sizes(reproduced from Bratby 2016 [4]).

Size (mm)	**Classification**	**Examples**	**Surface area** (m^2/cm^3)	**Time** [1]
10	Coarse dispersion (visible to naked eye)	Gravel, coarse sand, minerals, precipitated and flocculated particles	6×10^{-4}	0.1 s
10^{-2}	Fine particulate dispersion (visible under microscope)	Minerals, precipitated and flocculated particles, bacteria	0.6	11 min
10^{-4}	Fine particulate dispersion (visible under microscope)	Minerals, precipitated and flocculated particles, silt, bacteria	60	80 days
10^{-5}	Colloidal dispersion (submicroscopic)	Minerals, hydrolysis and precipitated products, macromolecules, biopolymers	600	2 years
10^{-6}	Colloidal dispersion (submicroscopic)	Minerals, hydrolysis and precipitated products, macromolecules, biopolymers	6000	20 years
$<10^{-6}$	Solution	Inorganic simple and complex ions, molecules and polymeric species.		

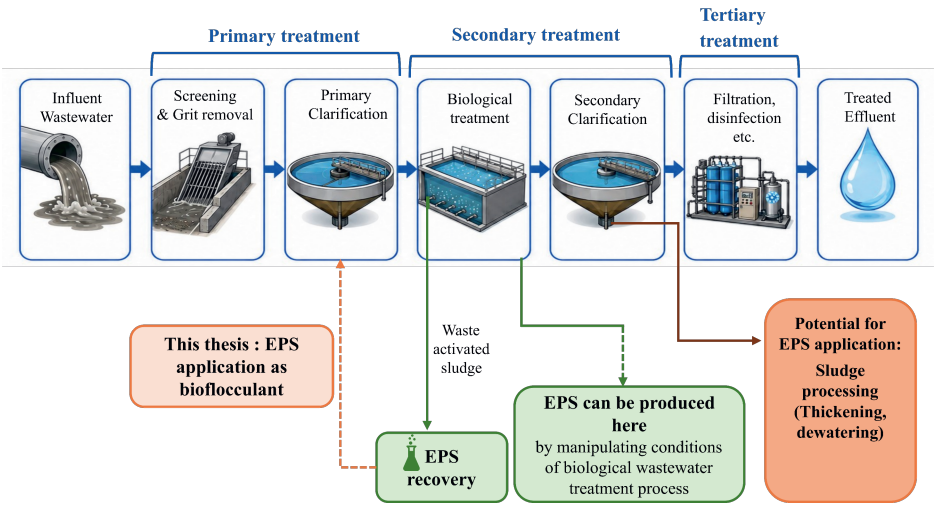


Figure 1: General scheme of wastewater treatment process - divided in four main steps as preliminary, primary, secondary, and tertiary treatment. EPS production and recovery can be performed during secondary treatment, and final EPS can be used as bioflocculants in primary treatment stage.

Following coagulation, flocculation promotes the growth of these microaggregates into larger, stronger flocs through the addition of high molecular weight, water soluble polymeric flocculants (Fig. 2) [2]. In this way settleability of flocs is increased and high removal efficiencies can be achieved. These polymers commonly enhance aggregation by several mechanisms such as charge neutralization, patching, and bridging (Fig. 3) [7]. The charge neutralization mechanism takes place when polymer carries an opposite charge to that of the suspended particles. In such cases, adsorption of the polymer onto the particle surface reduces the particle charge density, thereby decreasing electrostatic repulsion and promoting particle collisions and aggregation (Fig. 3A). This mechanism is generally associated with low molecular weight polymers ($< 10^5$ Da) that are able to adsorb directly onto particle surfaces [7].

In the patching mechanism, polymers absorb locally onto particle surfaces and create discrete patches of charge. These patches differ from the overall surface charge of the particle and can promote electrostatic attraction between neighbouring particles with complementary charged regions. In this way, particle-particle

attachment is enhanced and aggregation can occur (Fig. 3B) [4]. Lastly, the bridging mechanism (Fig. 3C) is typically associated with charged, high molecular weight polymers ($> 10^6$ Da), which may even possess the same charge as the suspended particles. A high molecular weight is essential for this mechanism, as the hydrated polymer chain must be sufficiently long to extend across at least two electrical double layers surrounding adjacent colloidal particles, thereby overcoming interparticle repulsion [9]. During bridging, some segments of the polymer adsorb onto the surface of one particle, while free loops and tails extend into the surrounding solution and attach to other particles, leading to the formation of larger aggregates (Fig. 3C) [10]. Polymer adsorption may occur through van der Waals forces, hydrogen bonding, interactions between polymer functional groups and particle surfaces, or cation-mediated electrostatic interactions [4].

In practice, flocculation frequently might proceed through a combination of these mechanisms [11], depending on polymer molecular weight, charge density, solution chemistry, and particle surface properties. Among these factors, polymer type (anionic, cationic, or non-ionic) strongly influences adsorption affinity and, in some systems, the role of multivalent cations in mediating attachment. These structure–function relationships also help explain the widespread use of polymeric flocculants in industrial applications, as their chemistry can be tailored to different particles, solution conditions, and separation requirements. The global flocculants market is currently dominated by synthetic polymeric materials, particularly polyacrylamide-based products, polyamines, and polydiallyldimethylammonium chloride (polyDADMAC) derivatives, which are generally synthesized from water-soluble monomers [12]. These synthetic flocculants vary in molecular weight (often in the range of millions of Daltons), charge (cationic, anionic, non-ionic) and conformation (branched, linear, hyperbranched) [5]. A useful compilation of commonly used and recently developed synthetic polymeric flocculants is provided by Lee et al. [13] and Vajihinejad et al. [14]. Among these, polyacrylamide (PAM)-based flocculants (available in anionic, cationic, non-ionic forms) are widely used due to their high flocculation performance at low dosages, tuneable chemistry, and flexible application [15].

Recent reports estimate the market volume of global PAM production at 2.5 million tons [16]. Water treatment represents a major application segment, accounting for approximately 35% of demand in recent market-based breakdowns

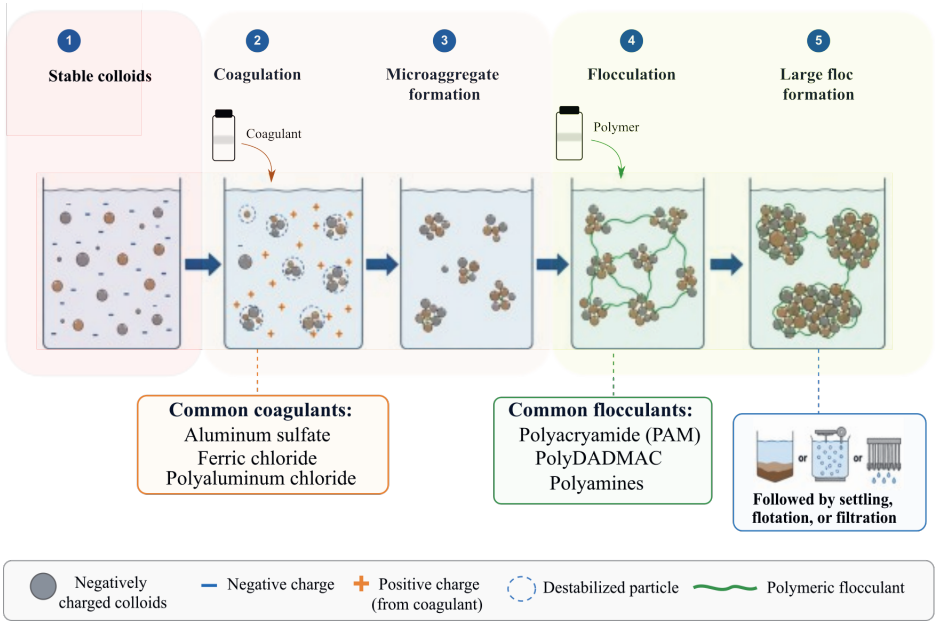


Figure 2: Schematic overview of the coagulation–flocculation process in wastewater treatment. Coagulants destabilize colloidal particles, after which polymeric flocculants, such as Polyacryamide (PAM), Polydiallyldimethylammonium chloride (PolyDADMAC), promote bridging and floc growth, resulting in larger aggregates that can be removed by settling, flotation, or filtration.

summarized in the literature [17], indicating substantial use of PAM in municipal and industrial wastewater treatment [16]. This widespread use reflects the high efficiency and versatility of PAM. However, increasing attention is being paid to the environmental and operational drawbacks associated with the production and use of these petroleum-derived polymers. Because PAM is highly water soluble, upon application in water treatment, a fraction may remain in the dissolved phase and persist as residual polymer in treated effluents, contributing to continued release into receiving environments [17], [18]. Although PAM itself is often considered less acutely toxic than many low molecular weight contaminants, its persistence, particularly when associated with solids in sludge, soils, and sediments, raises concerns regarding long-term accumulation and the management of treatment residuals [17]. Operationally, residual PAM in process streams can also interfere with downstream sludge processing [19]. For example,

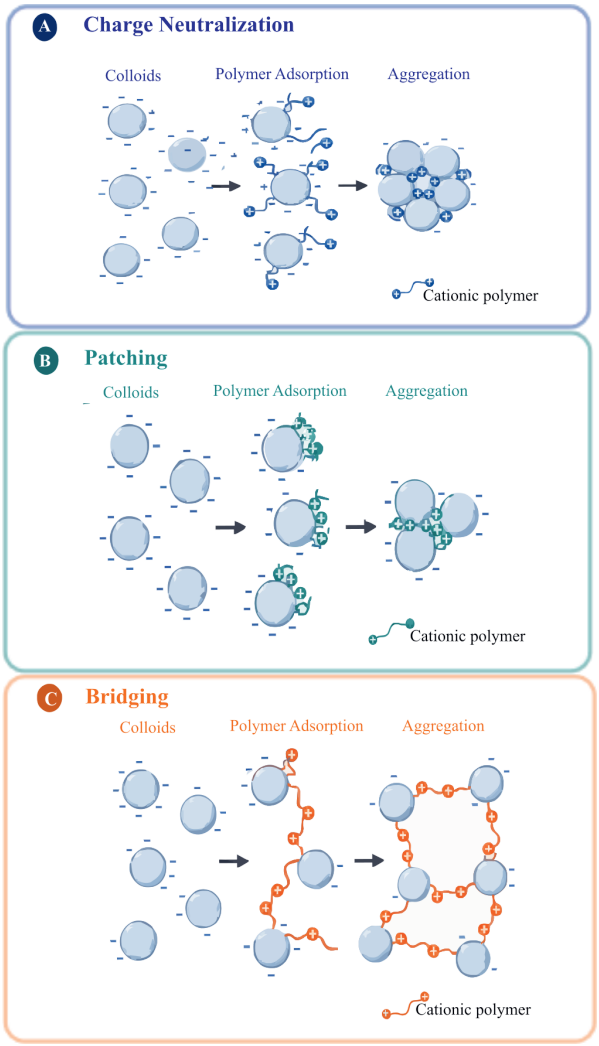


Figure 3: Schematic representation of colloidal particle flocculation through charge neutralization (A), patching (B), and bridging (C) mechanisms. Polymer properties, such as molecular weight and charge density, govern the adsorption process and the subsequent flocculation mechanism.

PAM-conditioned sludge have been reported to show reduced biodegradability and decreased methane yields during anaerobic digestion, with inhibition of microbial activity under certain conditions [20]. Importantly, potential ecological risks depend strongly on polymer ionic character: cationic PAM can interact strongly with negatively charged biological surfaces (e.g., cell membranes and extracellular coatings), and has been associated with higher acute aquatic toxicity compared with anionic or non-ionic formulations [21], [22]. While anionic and non-ionic forms are generally considered less acutely toxic, their persistence, potential transformation products, and residual monomer content still warrant careful assessment, particularly in applications involving environmental release [23], [24]. While these concerns mainly relate to the environmental fate and performance impacts of the polymer after use, additional risks arise earlier in the PAM life cycle from its monomeric precursor. Beyond polymer-related concerns, the acrylamide monomer used in PAM manufacture, which may also be released later through PAM degradation [18], represents a significant hazard [24].

Acrylamide is typically produced from acrylonitrile, a petrochemical feedstock, and is subsequently polymerized to produce PAM-based flocculants [25]. Acrylamide is substantially more hazardous than PAM itself. Due to its high solubility and lower molecular weight, it is more mobile in environmental systems [24]. It has also been classified as cytotoxic and neurotoxic, and is recognized as a carcinogenic hazard, with reported adverse outcomes including neurological effects, impaired fertility, and genotoxicity [26]. In addition to these toxicological concerns, acrylamide production relies on fossil-based feedstocks, and its use in PAM manufacturing is associated with the generation of greenhouse gases [16]. Since fossil resources are used in the production of these polymers, and their end-of-life fate is often natural or incineration-based oxidation along with flocculated sludge, polymer-derived carbon may ultimately be released into the atmosphere as carbon dioxide [27]. Consequently, the carbon footprint of PAM-based flocculants represents an important environmental concern [27]. Collectively, these issues motivate growing interest in the development of sustainable, biodegradable, and less ecotoxic flocculants capable of delivering comparable treatment performance while reducing environmental and operational risks [11].

1.2 Biopolymer-based flocculants as an alternative to synthetic flocculants

Biopolymer-based flocculants are derived from renewable natural sources, including plants, fungi, animals, and microorganisms [28]. In general, they comprise long-chain macromolecules such as polysaccharides, proteins, and glycoproteins produced by living organisms and/or extracted from renewable biomass feedstocks [29]. Representative examples include starch from plants [30], [31], chitosan from crustacean shells or fungi [32], [33], alginate from algae [34], [35], extracellular polymeric substances such as xanthan gum, gellan gum, produced by microorganisms [36], [37], [38], protein based flocculants from seeds, collogen, and gelatine [39], [40], [41], and pectin from fruit peels and plant tissues [42], [43] (Fig. 4). These polymers typically contain functional groups such as hydroxyl, amino, and carboxyl groups, which contribute to particle adsorption, charge interactions, and bridging during flocculation process. Compared with conventional petroleum-derived polymers, biopolymer flocculants offer several advantages for water and wastewater treatment; they are widely available [29], can often be chemically or physically modified to tailor performance [29], and are typically regarded as more environmentally friendly due to their low toxicity and enhanced biodegradability [29], [44]. Among biopolymer-based flocculants, microbially produced bioflocculants, also called extracellular polymeric substances (EPS) are of particular interest and form the focus of this research.

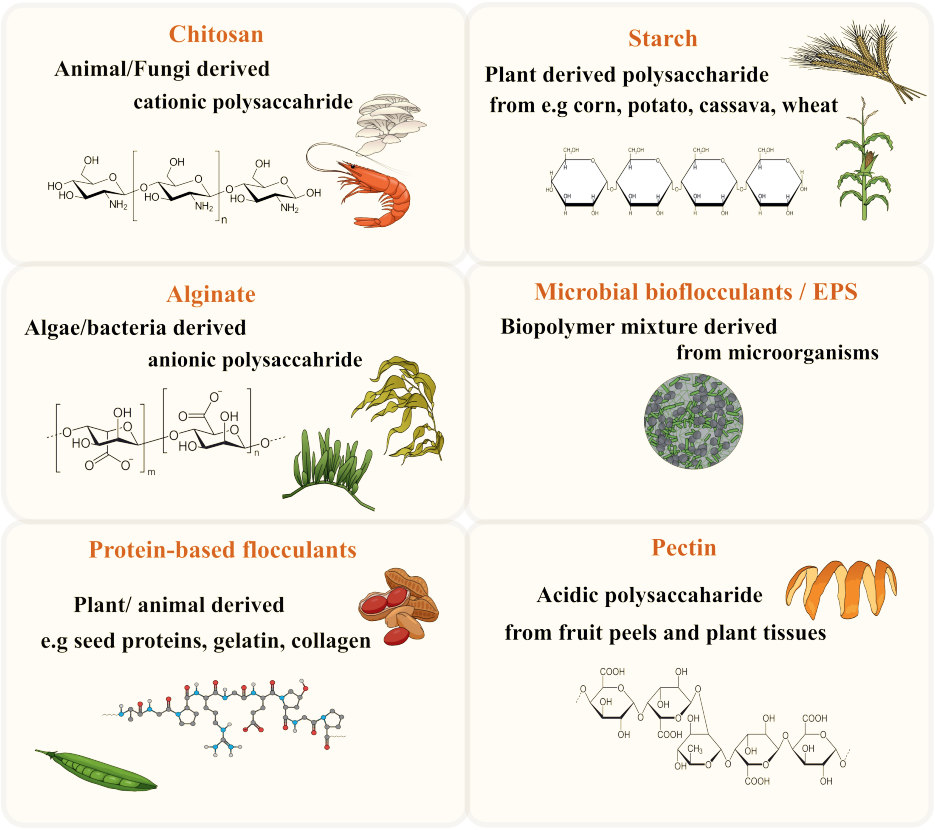


Figure 4: Biopolymer-based flocculants from various sources. Chitosan [32], [33]; Starch [30], [31]; Alginate [34], [35]; Microbial bioflocculants/Extracellular polymeric substances (EPS) [36], [37], [38]; Protein-based flocculants [39], [40], [41]; Pectin [42], [43].

1.2.1 Extracellular polymeric substances and their synthesis

As stated earlier, the soluble organic waste removal step in wastewater treatment can be manipulated so that a substantial fraction of the organic waste is converted into extracellular polymeric substances (EPS) (Fig. 1).

Extracellular polymeric substances are high molecular weight biopolymers produced and secreted by microorganisms [45]. Bacteria, fungi, and microalgae are among the most well-known EPS producers. The EPS are typically composed of proteins, polysaccharides, lipids, nucleic acids, and other minor biomolecules, and their chemical composition is strongly influenced by microbial species and their growth conditions [46]. Depending on their monomer composition and biosynthetic origin, EPS may be homopolymer or heteropolymer, ranging from repeated units of a single monomer to complex polymers containing different monomeric units and functional groups [47]. Each of the components of EPS contributes differently to the structure, stability, and functionality of the EPS matrix.

Polysaccharides, which are major components of EPS, vary in composition and may contain organic or inorganic substituents that influence their physical and biological properties. In general, polysaccharides give the EPS matrix its hydrated, viscous, adhesive, and gel-like character [48]. They support cell-cell and cell-surface interactions, enhance cohesion within the matrix, retain water, bind ions and nutrients, and protect embedded cells from environmental stress [49].

Proteins also play important roles in the EPS matrix by supporting adhesion, structural stability, enzymatic activity, and cell-cell interactions [50]. EPS-associated proteins may function as adhesins, enzymes, lectins, or structural matrix proteins, and they can interact with polysaccharides and extracellular DNA to strengthen the overall biofilm architecture [48].

Lipids are usually present in smaller amounts; however, they can strongly influence the physicochemical properties of the EPS matrix. In particular, EPS-associated lipids contribute to hydrophobicity, surface activity, membrane-associated interactions, and the formation of protective barriers [51]. Lipid-containing

compounds, such as glycolipids and lipoproteins, may also affect biofilm architecture, emulsification capacity, cell aggregation, and resistance to environmental stress [48].

Well-known EPS produced by pure cultures include defined exopolysaccharides, such as xanthan produced by *Xanthomonas campestris* [52], gellan produced by *Sphingomonas* spp.[53], dextran produced by lactic acid bacteria (e.g., *Leuconostoc* spp.)[54] and levan produced by several bacteria including *Bacillus* spp. [55]. Although EPS synthesis is a widespread trait among many microorganisms [56], the biosynthetic pathways involved in EPS production can differ among microorganisms, depending on their genome, metabolic capacity, and environmental conditions. As a result, EPS produced by different microbial species may vary in composition, structure, molecular weight, and functional groups.

Extracellular polymeric substances biosynthesis

EPS biosynthesis is a complex, enzyme- and gene-mediated process, and microorganisms can employ different biosynthetic routes depending on their taxonomic identity, cellular organization, and the specific biopolymer produced (e.g., protein or polysaccharide) [56]. For instance, in microorganisms, proteins are directly encoded by genes and synthesized through transcription and translation [57]. Therefore, whether cytoplasmic or extracellular, all proteins are first produced as polypeptide chains by ribosomes, often containing signal peptides or secretion tags that direct them to specific export pathways [58]. After translation, these proteins may undergo folding, cleavage of signal peptides, and post-translational modifications before being secreted into the extracellular environment or incorporated into the EPS matrix [57]. In contrast, extracellular polysaccharides are built from activated sugar units, such as nucleotide sugars, through glycosyltransferases and export systems. In what follows, the detailed description of extracellular polysaccharide biosynthesis is provided.

Extracellular polysaccharides biosynthesis

Extracellular polysaccharide synthesis in microorganisms is an enzyme-mediated process that can occur through different biosynthetic routes depending on the organism and the specific polymer produced. In general, four major pathways are commonly described: (i) the Wzx/Wzy-dependent pathway, (ii) the

ABC transporter-dependent pathway, (iii) the synthase-dependent pathway, and (iv) extracellular synthesis mediated by a single sucrase-type enzyme [46], [59]. The first three pathways are generally considered intracellular or membrane-associated, involving precursor assembly and polymer transport across the cell envelope, whereas the fourth represents extracellular or cell-surface-associated polymerization, in which polymer formation occurs outside the cytoplasmic membrane.

Although the site and mechanism of polymerization may differ among biosynthetic pathways, extracellular polysaccharide biosynthesis generally proceeds through several common stages. These include (i) carbon substrate uptake and metabolism; (ii) formation of activated precursor molecules, such as nucleotide sugars or sugar phosphates; (iii) enzyme-mediated chain initiation and elongation; (iv) transport or release of the polymer or its precursor units into the extracellular environment; and (v) subsequent organization, hydration, and maturation into an extracellular polymeric matrix [46]. The key distinction between intracellular/membrane-associated and extracellular biosynthetic models is the location at which polymerization occurs, corresponding to step (iii). In Wzx/Wzy-dependent, ABC transporter-dependent, and synthase-dependent systems, precursor formation and polymer assembly occur intracellularly or at the cell membrane before the polysaccharide is exported across the cell envelope. In contrast, extracellular synthesis involves the secretion of substrates or oligomeric precursors, which are then enzymatically polymerized at the cell surface or in the surrounding extracellular environment [59].

The Wzx/Wzy-dependent pathway is a common route for the biosynthesis of exopolysaccharides that are secreted into the medium and can often be harvested from culture supernatants without cell disruption. As in other extracellular polysaccharide biosynthetic pathways, synthesis begins in the cytoplasm with the formation of activated precursors, such as nucleotide sugars and sugar acids [60]. Later, repeating units of oligosaccharides are assembled on a lipid carrier, typically undecaprenyl phosphate. Once a repeat unit is complete, it is translocated to the periplasmic side of the cytoplasmic membrane by Wzx flippase [61]. Polymerization then occurs in the periplasm through the action of Wzy polymerase, forming a high molecular weight polysaccharide [61]. Finally,

the polymer is transported to the cell surface and/or secreted to the extracellular environment through additional export components, such as outer membrane polysaccharide export proteins and polysaccharide co-polymerase/regulatory proteins [62]. Exopolysaccharides produced via the Wzx/Wzy-dependent pathway are typically heteropolysaccharides with highly diverse sugar compositions, such as xanthan and sphingan, meaning that they contain multiple monosaccharide types within their repeating units.

The ABC transporter-dependent pathway is frequently associated with the biosynthesis of capsular polysaccharides that are linked to the cell surface [63]. Similar to the Wzx/Wzy-dependent route, synthesis begins at the cytoplasmic face of the inner membrane, where glycosyltransferases assemble sugar units on a lipid carrier [60]. A key distinction of this pathway is that the polysaccharide chain is assembled on the cytoplasmic side of the membrane before being exported. Depending on the number and specificity of glycosyltransferases involved, this pathway can produce either homopolymers or heteropolymers, with more complex heteropolymer structures typically requiring multiple glycosyltransferases. Once assembled, the polysaccharide is transported across the inner membrane by an ATP-binding cassette (ABC) transporter, after which additional periplasmic and outer-membrane components mediate translocation to the cell surface [64]. Exopolysaccharides produced via this pathway typically share a conserved feature: a glycolipid moiety at the reducing end that serves as the membrane-linked terminus of the polymer [65].

The synthase-dependent pathway is involved in the assembly of exopolysaccharides that are important in mucoid formation and are structural components of biofilms (e.g., alginates)[66]. Unlike other pathways, this pathway may proceed either with or without a lipid carrier, depending on the type of exopolysaccharide being synthesized [67]. Here, the cell secretes complete exopolysaccharides across the inner/outer membranes and the cell wall. Polymerization and transport across the inner membrane are carried out by an inner membrane polysaccharide synthase protein. The activity of polysaccharide synthase is sometimes post-translationally regulated by inner membrane bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) receptor [67]. Transport across the outer membrane is achieved by a periplasmic tetratricopeptide repeat (TPR) containing protein and an outer membrane β-barrel protein. This pathway is often utilized in the

synthesis of homoexopolysaccharides (e.g., curdlan or polymannuronic acid) [59].

Extracellular synthesis mediated by a single sucrase-type enzyme differs from the Wzx/Wzy-dependent, ABC transporter-dependent, and synthase-dependent pathways in that polymerization occurs extracellularly or at the cell surface. In this mechanism, secreted substrates or oligomeric precursors are enzymatically polymerized in the surrounding extracellular environment. Typical examples include the synthesis of dextran and its derivatives, as well as levan and inulin, all of which are catalysed by extracellular sucrase-type enzymes. For instance, in the case of dextran, which mainly consists of α-(1→6) linked glucose units, its polymerization occurs outside the cell through the activity of dextransucrase, the key enzyme involved in dextran synthesis. Dextransucrase belongs to the glucansucrase family and catalyses the transfer of glucose from sucrose to a growing chain of α-glycosidically linked oligo- and polysaccharides. Depending on the specific enzyme involved, different glycosidic linkages can be formed, leading to the production of structurally distinct glucans, such as α-(1→6)-linked dextran, α-(1→3)-linked mutan, or reuteran, which contains a high proportion of α-(1→4) linkages [59].

1.2.2 Stimulation of EPS production beyond basal levels: EPS-Overproduction

EPS production is a widespread and intrinsic trait of many microorganisms [56]. Under non-stress or non-stimulated environmental conditions, microorganisms typically produce EPS at basal levels as part of their normal physiology. As discussed earlier, this basal EPS production supports essential functions such as surface attachment, biofilm formation [68], and cell-cell aggregation into flocs or granular structures [69]. Therefore, EPS production can be considered a common microbial strategy that contributes to survival, spatial organization, and persistence in diverse environments.

However, EPS production is a dynamic process. At the molecular level, it is regulated by mechanisms controlling EPS biosynthetic pathways, including stress-response systems, quorum sensing, second-messenger signalling, and transcriptional regulation of EPS-related genes [70]. Research has shown that EPS biosynthesis can be upregulated beyond basal levels in response to environmental

stimuli, including nutrient limitation, osmotic stress, pH or salinity shifts, toxic compounds, and other unfavourable conditions[71]. These stimuli can alter EPS regulatory networks, leading to changes in EPS yield, composition, molecular characteristics, and functional properties. In some cases, this is manifested by the appearance of visibly mucoid or 'slimy,' EPS-rich colonies [72], [73]. In natural environments, this response is widely interpreted as an adaptive strategy that enhances survival by forming a hydrated extracellular matrix that provides protection and supports persistence [48].

This adaptive response of microorganisms can be exploited in engineered systems by cultivating pure strains or mixed microbial communities under conditions that stimulate EPS biosynthesis [71], [74]. In this thesis, this stimulated production is referred to as EPS overproduction, which describes EPS synthesis above basal physiological levels and the resulting accumulation of excess EPS under imposed environmental or operational conditions. EPS overproduction is of practical interest because it enables the production of EPS at yields suitable for downstream recovery and subsequent applications. Relevant applications include the food and fermentation industries, where EPS can serve as thickeners, stabilizers, and texture modifiers [47]; water and wastewater treatment, where they can function as bioflocculants and sludge-conditioning agents [75], [76]; and biomaterials in biotechnology [56], [77].

Several pure-culture studies have shown that EPS overproduction can be induced under specific environmental and nutritional conditions. Among these, nitrogen limitation is one of the most widely studied and industrially relevant strategies. In general, EPS production is often favoured under conditions with excess carbon and limited nitrogen availability, where growth becomes restricted while carbon flux can be redirected toward extracellular polymer formation. This principle has been reported for several well-known EPS-producing microorganisms such as *Pseudomonas* sp.[78], *Rhizobium* sp. [79] and *Xanthomonas* sp.[80] , *Lactobacillus* spp.[81] and during production of well-known EPS types, including pullulan production by *Aureobasidium pullulans* [82], xanthan production by *Xanthomonas* spp.[83], [84], and curdlan production by *Rhizobium radiobacter* [85]. Mechanistically, nitrogen limitation restricts cell growth and protein synthesis while carbon remains available, causing excess carbon to be redirected toward

extracellular polymer synthesis rather than biomass formation [86], [87]. In addition, nitrogen limitation likely triggers stress-response pathways and alters the expression of genes involved in precursor formation, polymerization, and EPS export [87], [88]. For this reason, nitrogen-limited cultivation has been widely used to promote EPS production in well-known EPS-producing microorganisms and has contributed to the development of high-yield EPS production processes.

Similar to pure cultures, mixed microbial cultures (MMCs), like in wastewater treatment configurations, engineered to produce EPS as is done in the studies reported in this thesis, can also be steered toward EPS overproduction by applying comparable strategies [74], [89], [90]. However, in mixed cultures, EPS production is regulated at the community level rather than by a single microorganism. Therefore, operational and environmental selection pressures are used to enrich EPS-producing populations or induce EPS-rich phenotypes. These pressures include environmental stressors such as nutrient limitation, particularly nitrogen limitation, as well as operational parameters that directly influence microbial growth and selection, such as sludge retention time (SRT) [91]. SRT is a key operational parameter because it controls biomass retention and washout, thereby shaping growth rates, microbial competition, and community composition [92]. Consequently, adjusting SRT can favour specific microbial groups or physiological states associated with increased EPS production. By shaping microbial community composition and metabolism, these conditions can influence both the quantity and characteristics of the EPS produced [74]. Overall, the ability to stimulate EPS production beyond basal levels has important implications for biotechnological and industrial applications, where high production yields are essential for process feasibility and commercialization. Thus, EPS overproduction represents both a microbial survival strategy and a valuable target for engineering microbial processes toward functional biopolymer production.

1.2.3 Mixed-microbial culture-derived extracellular polymeric substances as flocculants

EPS exhibit several physicochemical properties that make them attractive as bioflocculants, including high molecular weight, long and flexible polymer chains, and diverse charged and non-charged functional groups capable of interacting with suspended particles [93] (Fig. 5). These features enable EPS to interact



Figure 5: Comparison of the properties of synthetic flocculants and EPS-based flocculants.

with particle surfaces through mechanisms similar to those of synthetic polymeric flocculants, such as electrostatic attraction, polymer adsorption, bridging flocculation, and cation-mediated bridging [7] (Fig. 3). In addition, EPS are biodegradable, which reduces the risk of persistent polymer residues compared with synthetic polymeric flocculants and supports broader sustainability objectives [94]. Therefore, EPS represent promising candidates for environmentally friendly flocculation, particularly if they can be produced reliably and economically at scale. Most research has demonstrated EPS production and pronounced flocculation performance using pure microbial cultures. A comprehensive compilation of microorganisms and their reported flocculation efficiencies is provided in the review by Rebah et al., to which the reader is referred for further detail [95]. Pure-culture studies using well-studied EPS-producing microorganisms such as lactic acid bacteria, *Pseudomonas*, *Xanthomonas*, *Sphingomonas*, *Bacillus*, and

Aureobasidium have provided an important mechanistic foundation for understanding EPS biosynthesis, regulation, and stress-induced production [71], [96], [97], [98], [99], [100]. Collectively, these studies have helped identify microbial traits and cultivation conditions that promote EPS overproduction and enhance bioflocculant activity.

However, producing EPS from pure cultures specifically for flocculant applications remains economically challenging at large scale. This is mainly because pure-culture production often requires sterile conditions, controlled fermentation, and refined substrates, which increase energy demand and operating costs [74], [101]. In addition, downstream recovery and purification can further increase production costs. These constraints are particularly important for relatively low-value applications such as water and wastewater treatment, where large quantities of flocculant are required. These economic and scalability limitations have motivated increasing interest in MMCs and wastewater-based EPS production. Mixed-culture systems can operate under non-sterile conditions and can use waste-derived carbon and nutrient sources, reducing the need for refined substrates and lowering production costs [74], [102]. However, they also introduce new challenges. Because mixed cultures contain dynamic microbial communities, EPS yield, composition, molecular weight, charge density, and functional-group distribution may vary depending on the influent composition, operating conditions, and microbial community structure [103], [104]. Nevertheless, for many applications, particularly in water and wastewater treatment, performance is governed by bulk polymer characteristics such as molecular weight [105], charge density [106], and the distribution of functional groups, which can be retained in recovered mixed-culture EPS.

From a wastewater-treatment perspective, EPS produced by MMCs has long been discussed mainly as a drawback, because it is frequently associated with membrane fouling; consequently, many studies have focused on operational strategies to limit EPS formation [53], [54], [55]. At the same time, the role of EPS as a structural glue in floc formation and granulation has been extensively investigated [69], [110], [111], [112], [113], [114], since it strongly influences settleability, dewaterability, and overall process performance. More recently, this perspective has shifted toward valorising mixed-culture EPS as a renewable, biobased material with useful functional properties [76], [115], [116]. Activated sludge and other

biological wastewater treatment systems are particularly relevant in this context. In activated sludge, microorganisms naturally grow as flocs that are largely structured by EPS, meaning that EPS are inherently produced at a basal level as part of normal process operation. Recent studies have shown that organic-rich industrial wastewaters can serve as substrates for basal level EPS production in MMCs systems, including well-established treatment processes such as activated sludge [89], [117], [118], [119], [120], [121]. EPS recovered or produced in these systems has been tested as a flocculant for model suspensions, such as kaolinite, and in some cases for real wastewater matrices, indicating the potential to couple wastewater treatment with the recovery of bio-based flocculant materials [74], [89], [118], [120], [121].

A prominent example of this valorisation approach is Kaumera Nereda® Gum [122], an EPS extracted from excess aerobic granular sludge produced in full-scale resource recovery plants in the Netherlands (e.g., Zutphen and Epe) [123]. Beyond potential use within water and wastewater treatment (e.g., bioflocculation and sludge conditioning) [124], Kaumera has been explored for a range of higher-value applications, including fire-retardant coatings [125], soil and horticultural products/biostimulant uses[126], and surface coatings for paper and related materials [127]. This example illustrates how EPS-rich residual biomass from wastewater treatment can be transformed into functional biopolymer products. Overall, MMCs and wastewater-based EPS production offer a promising route for producing bio-based flocculants from low-cost or waste-derived feedstocks. If EPS can be produced and recovered reliably at scale, this approach could provide a practical and sustainable alternative to synthetic polymeric flocculants in water and wastewater treatment, while supporting resource recovery and reducing environmental risks associated with persistent petroleum-derived polymers [128].

1.3 Research gap

Despite growing evidence that EPS can function as effective bioflocculants, several gaps still limit translation from proof-of-concept studies to robust and scalable practice, particularly for MMCs and wastewater-based production [129].

First, the operational conditions required for stable, long-term EPS overproduction in continuous MMCs systems are not yet well defined [130]. Previous studies have shown that EPS yields can be influenced by key operational parameters such as solids retention time (SRT) and nutrient composition [45], [131], [132], [133]; however, a consistent and transferable set of conditions that reliably drives EPS overproduction across reactors has not been clearly established. For instance, studies conducted by Ajao et al., [89], [90], [118] demonstrated high EPS yields from glycerol-rich wastewater when an activated sludge reactor was run under varying nutrient regimes (e.g., different COD:N ratios). Nevertheless, in these studies the SRT was largely selected arbitrarily, and a clear, generalizable EPS overproduction strategy was not formulated or validated across different reactor configurations, inoculum sources or wastewater types (beyond glycerol streams blended with substrates such as acetate or ethanol) [89], [90], [118].

More broadly, EPS production in MMCs systems remains a process control challenge, because operating conditions must be managed to maintain stable EPS yields and reproducible polymer properties [46], [74]. Mixed communities are dynamic and sensitive to operational conditions; small changes can shift community composition [103], alter EPS yield and composition [104], and affect flocculation performance [118]. Consequently, while MMC-based EPS bioflocculants are promising, their implementation requires a deeper understanding of two key aspects. First, it remains necessary to clarify how reactor operation governs EPS production and properties, and which conditions are most effective in stimulating EPS overproduction. Second, the reproducibility of EPS production outcomes across different inocula remains insufficiently understood. Because the inoculum strongly influences initial community structure and functional potential [134], [135], it is still unclear how transferable a given operational strategy is between different sludges, and how sensitive EPS yield and properties are to inoculum variability.

Secondly, the feedstock scope for wastewater-based EPS production through MMCs remains limited, particularly for VFA-rich streams. While using waste-derived substrates is central to making EPS production circular and economically realistic [117], [118], the suitability of different wastewater types for EPS overproduction is not yet well understood. This is especially relevant for widely available

VFA-rich industrial effluents, produced, for example, as by-products of anaerobic treatment or via controlled acidification, which represent attractive low-cost carbon sources.

However, EPS (over)production from VFA-rich wastewaters has only been reported to a limited extent [136] and mostly with pure microbial cultures [137], [138], [139]. Many mixed culture valorisation studies using VFAs have instead focused on conversion to intracellular storage polymers such as PHA [140], [141], [142]. Accordingly, VFA-derived EPS have so far been discussed mainly as a co-product in PHA-oriented mixed culture processes [143], [144], [145], [146].

To date, production of EPS from VFA-rich wastewaters with EPS themselves as the primary target product has rarely been proposed explicitly as the main valorisation perspective. Broadening EPS production to VFA-rich streams could diversify the range of feasible substrates and create an additional valorisation route for VFAs beyond PHA-centred platforms, while also offering opportunities to tailor EPS characteristics relevant to their application as flocculants.

In addition, there is limited integrated understanding linking process operation, microbial community assembly, and EPS properties and yields, particularly during EPS overproduction processes. Previous studies have investigated EPS-microbial community interactions in wastewater treatment systems mainly in relation to process performance and biomass structure, such as EPS production and microbial community composition in fouling membranes [147], [148], [149], in aerobic granules [150] or during the wastewater treatment process [151], [152]. However, microbial community development, particularly during EPS overproduction processes, has not been studied, or EPS overproduction is often studied without parallel ecological characterization, making it difficult to explain how operational changes drive community shifts and how these shifts translate into EPS quantity and composition. Understanding this link is crucial in terms of evaluating the effects of EPS overproduction conditions on different types of sludge inocula, in different reactor systems and from different wastewater types.

Finally, many studies have investigated the use of mixed culture-derived EPS as flocculants [74], [89], [117], [120], [121]. However, flocculation performance is often evaluated using only a single model particle system, most commonly

kaolinite [89], [102], [153], [154]. Such systems do not adequately represent EPS interactions with particles that differ in surface properties from kaolinite, which is moderately charged and hydrophilic under neutral conditions [155]. In real wastewater matrices, particles occur as complex and heterogeneous mixtures with varying surface charge, morphology, and hydrophobicity [156]. As a result, systematic insight remains limited into how different EPS types interact with heterogeneous particle surfaces and how EPS composition governs flocculation performance across realistic matrices. This knowledge gap limits the effective translation of mixed culture-derived EPS to practical applications in relevant wastewater systems. In addition, reported flocculation efficiencies do not always reflect the effect of EPS alone. In some studies, turbidity removal is calculated relative to a coagulant-containing blank, whereas in others it is compared with the untreated native suspension [157]. Consequently, reported removal efficiencies may include not only the contribution of EPS themselves, but also the combined effects of conditioning steps. This makes it difficult to isolate the true contribution of EPS to the separation process and to compare results across studies.

1.4 Overall aim and research questions

The overarching aim of this thesis is to advance the production and application of MMC-derived EPS as sustainable bioflocculants. This aim is addressed through four connected research questions, with the first research question further divided into subquestions to address its specific methodological and mechanistic aspects:

- How can EPS overproduction conditions be defined and implemented in a repeatable and reliable way? This question divides into three sub-questions: a) What are the conditions that can be defined as "EPS-overproduction conditions", b) how can the operation of continuous reactors be tuned to achieve such EPS overproduction from wastewater-based substrate in a sustained way, and c) can these conditions be applied to any type of starting inoculum?
- Can EPS overproduction conditions be used to produce EPS from VFA-rich wastewater?
- How does reactor operation shape microbial community assembly, and which community patterns are associated with EPS bioflocculant production?

- How does EPS composition govern flocculation performance across particles differing in hydrophilicity/hydrophobicity and surface charge, and how do EPS in various compositions perform in complex industrial matrices?

1.5 Thesis outline and chapter contributions

Chapter 2 establishes a continuous MMC-based production route for EPS bioflocculants using glycerol-rich synthetic wastewater. It focuses on defining operational strategies to achieve sustained "EPS-overproduction" namely applying nitrogen limitation and short SRT, and addresses core factors that determine scalability and robustness: (i) the impact of inoculum type under identical operating strategies, and (ii) the long-term stability of both EPS production and flocculation performance. EPS functionality is assessed using flocculation assays with kaolin clay as a controlled particle system. The chapter provides a process foundation for engineering MMC systems toward high-yield EPS production.

Chapter 3 expands the resource recovery landscape by demonstrating that, in addition to previously applied glycerol, volatile fatty acids (VFAs) can also be converted into EPS suitable for bioflocculant applications. The chapter broadens the range of viable waste-derived substrates for EPS-flocculant production and connects EPS recovery with emerging VFA platforms. The work described in this chapter evaluates EPS production from VFA-rich synthetic wastewater under the EPS-overproduction conditions defined earlier in Chapter 2 (nitrogen limitation and short SRT) and compares reactor configurations to reduce complexity and mitigate membrane-related limitations. A CSTR investigation examines both extracellular (EPS) and intracellular (PHA) polymer formation, clarifying how MMC distributes VFA-derived carbon into different biopolymer pathways under EPS-overproduction conditions.

While Chapters 2 and 3 demonstrate that operational strategies can drive EPS overproduction, some variability in EPS yield and flocculation efficiency remains, suggesting underlying microbial community shifts. Therefore, **Chapter 4** addresses that mechanistic gap by analysing microbial community development across seven EPS-overproducing reactors previously operated under different conditions (reactor configuration, inoculum type, substrate type, COD:N ratio, and SRT) using 16S rRNA gene amplicon sequencing. This chapter answers how

EPS-overproduction conditions shape enrichment of EPS-/biofilm-associated microbial taxa, whether different inocula converge to functionally similar microbial communities, how substrates and reactor configuration steer microbial community assembly, and how the observed shift from PHA-related to EPS-associated microbial taxa can occur under sustained conditions. Together, these insights provide an evidence-based framework to design and operate MMC reactors for consistent EPS overproduction.

Chapter 5 addresses uncertainties related to application of EPS bioflocculants. Most of the studies focus on a single model mineral system (mostly kaolinite) as the particulates to be flocculated, leaving uncertainties about flocculant performance in different types of particle systems and matrices. Therefore, by characterizing MMC-derived EPS with contrasting compositions (polysaccharide-rich vs protein-rich) and systematically testing their interaction with minerals spanning different surface properties (e.g., kaolinite, montmorillonite, talc), this work links EPS composition and physicochemical properties to particle surface charge, hydrophilicity, and resulting flocculation outcomes. Finally, EPS are tested - in a case sub-study - on an iron-rich retentate from a drinking water production plant to assess performance on iron-rich colloidal particles, gaining a first insight into applicability under conditions in practice. This chapter advances mechanistic understanding and provides practical guidance for matching EPS types to target particle systems and making a first step to real-world implementation.

Finally, **Chapter 6** brings together the findings from the experimental chapters and discusses them from a broader perspective, including the main limitations and future research directions. Taken together, the thesis advances MMC-derived EPS bioflocculants from three angles that are all required for practical application:

- Process engineering: identifying operational parameters (nitrogen limitation, short SRT, configuration) that enable sustained overproduction from wastewater-based substrates.
- Microbial ecology: linking reactor operation to community assembly to explain variability and support controllable, reproducible production.

- Application performance: moving beyond single-mineral tests to evaluate how EPS composition interacts with particle properties and how MMC-derived EPS perform in complex industrial matrices.

By integrating these elements, the thesis aims to contribute to a circular-economy approach where organic compounds present in wastewater effluents are valorised into biodegradable flocculants that can reduce reliance on environmentally detrimental petroleum-derived flocculating polymers.

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Chapter 2

Production of bioflocculants from glycerol-rich synthetic wastewater: The influence of inoculum and process parameters

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Abstract

Synthetic flocculants are applied in various fields to induce the agglomeration of dispersed particles. However, their environmental impact raises concern. Microbial extracellular polymeric substances (EPS) offer a promising green alternative to synthetic flocculants, due to their charged functional groups, high molecular weight, and biodegradability. In this study, we investigated the potential of EPS produced by mixed microbial cultures (MMCs) during wastewater treatment as bioflocculant. Two sets of experiments (Exp_1 and Exp_2) were conducted to assess the effects of inoculum sludge (municipal and industrial), changes in the COD:N ratio (from 20 to 60 to 100), and in solid retention time (SRT) (from 6 to 1.5 days) on the rapid start-up of lab-scale membrane bioreactors (MBRs) and EPS overproduction. Overproduction of EPS by the MMC was achieved by applying nitrogen limitation (COD:N 100) with short SRTs (3 to 1.5 days) from both inocula. Under these conditions, the production yield reached up to 0.2-0.25 g COD_{EPS}/g $COD_{converted}$. Quantitative analysis coupled with fluorescent staining highlighted polysaccharides as the dominant EPS component (58-75%), with an average molecular weight between 2.2-2.4 MDa. The flocculation efficiency of the EPS was evaluated against kaolinite, reaching 81-87.4% using EPS produced at 3 days SRT, and stabilising at 65.9-73.9% with EPS produced at 1.5 days SRT. This study provides a strong basis for developing a reproducible process for synthesis of polysaccharide-rich EPS-flocculants from wastewater using MMC in MBRs. We demonstrated that coupling nitrogen limitation with short SRT is crucial for achieving stable EPS overproduction with a fast start-up.

2.1 Introduction

Flocculants are widely used chemicals in water and sludge treatment to promote aggregation and removal of fine colloidal particles [1]. Currently, the flocculant market is dominated by synthetic flocculants such as polyacrylamide (PAM) and polyacrylic acid, which are highly efficient and stable across a wide pH range [1], [2]. However, their high solubility can lead to elevated residual concentrations in treated effluents [3], causing water pollution and accumulation in soils and sediments [4]. For instance, under anaerobic conditions, PAM biodegrades slowly, and may release acrylamide, a potent neurotoxin harmful to human and to aquatic biota [2], [5]. Furthermore, residual synthetic flocculants can hinder downstream processing of waste activated sludge as they alter the sludge physicochemical properties, suppress key enzymatic activities, and shift microbial community composition, ultimately impairing the efficiency of the anaerobic digestion process [6]. Recent studies on anionic PAM show that higher dosages (>10 g/ kg TS) consistently inhibit anaerobic digestion of sludge [7],[8]. Mechanistic investigations show 30–50% reductions in protease, α-glucosidase, and cellulase activities, increased sludge floc stability, and reduced release of bioavailable organics at elevated anionic PAM concentrations [8]. Moreover, as most synthetic flocculants are petroleum-derived [9], they conflict with CO_2 footprint reduction and circular economy goals. These concerns underscore the need for biodegradable and sustainable flocculants that can be applied in the coagulation–flocculation stage of primary wastewater treatment, amongst others. Microbial extracellular polymeric substances (EPS) are a promising alternative, as they are likely more compatible with downstream biological processes and have lower environmental impact [10].

Extracellular polymeric substances (EPS) are high molecular weight biopolymers secreted by microorganisms and they are primarily composed of polysaccharides, proteins, lipids, nucleic acids, and various heteropolymers [11]. Their high molecular weight and abundance of functional groups (e.g., hydroxyl, carboxyl) enable strong interactions with suspended particles [12], making EPS effective alternative to especially anionic synthetic flocculants. Moreover, their natural origin and inherent biodegradability reduce risks of secondary pollution and align with circular economy goals [10],[13]. Production of EPS is part of microbial secondary metabolism and is strongly influenced by environmental conditions [14].

Microorganisms often overproduce EPS in response to environmental stresses such as pH fluctuations [15] or increased salinity [16], which enhances their survival and adaptive capacity.

Previous research has extensively investigated EPS as flocculants, with most production relying on pure microbial cultures [17],[18] or synthetic co-cultures [19], [20]. Pure cultures provide consistent yields and produce well-characterized EPS with high flocculation capacity [12],[17], [21], and their biosynthetic pathways are better understood, allowing targeted bioengineering of EPS properties [22]. It has been demonstrated that EPS flocculants produced by pure cultures can achieve high flocculation efficiency across a wide range of polluted wastewaters. For instance, EPS produced by *Serratia ficaria* achieved turbidity removal of 84.2% from river water, 98.1% from brewery wastewater, 91.8% from meat processing wastewater, and 93.7% from soy sauce brewing wastewater. Its flocculation performance was comparable with that of conventional synthetic flocculants such as (Polyaluminium Chloride) PAC and PAM [23]. Similarly, in another recent study, EPS produced by a pure culture of *Klebsiella pneumoniae* achieved 74% of turbidity removal in polluted river water and was slightly more effective than PAM in reducing the microbial load, suggesting stronger interactions of EPS with microbial cells compared to synthetic flocculants [24]. However, producing EPS with pure cultures requires energy-intensive sterilisation to achieve axenic conditions and costly purified carbon sources (e.g., glucose, fructose, maltose), which limits scalability and sustainability of EPS production [13], [25]. This trade-off has motivated the exploration of mixed microbial cultures (MMC) as an alternative for EPS flocculant production. MMC-based processes can utilize a broad spectrum of inexpensive substrates that do not require high purity, and they can operate without sterilisation [25]. Additionally, the production of EPS-flocculants with MMC can be integrated with the treatment of wastewater, especially for those with high loads of waste organics [25], [26], offering a potentially more sustainable and scalable approach for EPS flocculant production compared to pure cultures.

Wastewater treatment plants inherently harbour MMC that naturally secrete EPS as structural components of flocs. These MMC have the genetic capacity to overproduce EPS [27] when exposed to suitable operational conditions. Key

parameters such as solids retention time (SRT) [28],[29], wastewater composition [30], organic loading rates [31], and salinity [16] strongly influence both the quantity and quality of EPS produced in these systems. Several studies have demonstrated that MMC-derived EPS can exhibit promising flocculation performance, enhancing particle aggregation and sludge settling [32],[33],[34],[35].

Despite this potential, in such MMC systems, identifying operational strategies and selective pressures required to steer microbial communities to consistently overproduce EPS with desirable flocculant properties remains a key challenge [25]. Batch experiments, such as those conducted by Ng et al. [36], have shown that nitrogen and carbon limitation can trigger significant EPS accumulation, yielding carbohydrate-rich EPS with high flocculation efficiency. Such EPS yielded near-zero turbidity (absorption at 550 nm) at an optimum stoichiometric dosage of 0.3 mg EPS per g kaolin clay and 360 mg Ca^{2+}/L [36]. The stoichiometric dose of flocculant for achieving optimum flocculation is characteristic for adsorbing flocculants thereby suggesting adsorption of EPS on kaolin clay during the coagulation-flocculation process. In the same study 70–100% coagulation-flocculation efficiency in oleochemical wastewaters was achieved, demonstrating the applicability of EPS as a flocculant in real complex matrices. However, the study did not focus on continuous EPS overproduction for practical, large-scale flocculation applications [37].

Building on earlier efforts, our group designed and patented an MMC-based membrane bioreactor (MBR) process for EPS-flocculants production [38]. Ajao et al. [39] validated this approach in controlled laboratory-scale continuous experiments using synthetic wastewater under stepwise nitrogen limitation, showing that strong nitrogen limitation (COD:N = 100) maximized EPS yields. The carbohydrate-rich EPS achieved 81–91% flocculation efficiency in both fresh and saline conditions at low doses (0.2 mg EPS/g kaolin clay). Later, they reported that EPS showed excellent flocculation efficiency (88-97%) against saline kaolinite suspension, which was comparable to anionic polyacrylamide, which showed maximum flocculation efficiency of 98.6% on the same suspension [40]. Other laboratory-scale studies have also confirmed the feasibility of directly utilizing excess activated sludge for the preparation of EPS-flocculants [41], [42], [43].

Albeit the above studies collectively demonstrate the potential of EPS produced by MMC for flocculation applications, they do not focus on investigating strategies for maximising EPS yields. The operational conditions required to stimulate EPS synthesis beyond baseline levels and to achieve sustained overproduction remain inadequately defined. Critical operational parameters, such as SRT, are often chosen arbitrarily rather than systematically investigated, and the effects of inoculum sludge type and long-term performance in continuous operation remain under-explored. These gaps limit our understanding of process stability and hinder the development of scalable strategies for consistent EPS-flocculants production.

Therefore, the aim of this study is threefold i) to investigate how SRT affects EPS overproduction with MMC under nitrogen-limited conditions; ii) to assess the effect of inoculum sludge on EPS production under identical operational strategies; iii) to evaluate whether the flocculation performance of the produced EPS remains stable over extended operation. To that end, two sets of experiments were conducted using aerobic activated sludge from two different wastewater treatment plants to inoculate MBRs operated under varying levels of nitrogen limitation. Produced EPS was applied to flocculate kaolin clay particles. Together, these insights advance the development of cost-effective and scalable MMC-based processes as an integral part of wastewater treatment, offering a practical alternative to pure cultures for producing EPS-flocculants. Moreover, they contribute to the broader effort to replace toxic synthetic flocculants with environmentally friendly and more sustainable EPS-based options.

2.2 Materials and Methods

Two sets of experiments were conducted by using membrane bioreactors (MBRs) and in each experiment, two MBRs were run in parallel. In Exp_1, the best conditions for high EPS production were defined by combining different COD:N ratios (up to N-limited conditions) and step-wise decrease in solid retention time (SRT). In Exp_2, the optimal SRT and COD:N ratio from Exp_1 were applied for investigating a rapid start-up of EPS-producing MBRs. Here, the stability of flocculating properties of the produced EPS over the operational period of the reactors was also investigated. Details of the two experiments were reported in Table S1 and outlined in the following sections.

2.2.1 Source of inocula and feed composition

Each MBR was inoculated with 4 g of volatile suspended solids (VSS) per litre with sludge sourced from two different wastewater treatment facilities. In Exp_1 and Exp_2, reactors R_{MUN-1} and R_{MUN-2} were inoculated with activated sludge from a municipal wastewater treatment plant (Leeuwarden, NL) receiving municipal and dairy wastewater. In contrast, reactors R_{IND-1} and R_{IND-2} were inoculated with activated sludge obtained from a facility processing volatile fatty acid-rich industrial wastewater (Rilland, NL). Additional details about the inocula are provided in the Supplementary Information (SI, Table S2).

The MBRs were continuously fed with synthetic wastewater containing glycerol as carbon source and NH_4Cl as source of nitrogen [44]. Detailed macro and micronutrient composition of the synthetic wastewater used in each period is reported in the Supplementary Information (SI, Table S3). The influent soluble COD (sCOD) was maintained at $1\,\mathrm{g\,COD\,L^{-1}}$, while total nitrogen content was adjusted by changing the concentration of NH_4Cl to achieve a desired COD:N ratio (SI, Table S4).

2.2.2 Bioreactor setups

The MBRs with a working volume of 3.3 L were equipped with submerged chlorinated polyvinyl chloride (CPVC) flat sheet membranes (Type 203, Kubota Membrane, JP), with a nominal pore size 0.4 μm and an area of 0.11 m^2. The setup is schematised in supplementary information (SI, Fig. S1). The MBR setting was chosen to retain biomass and EPS and to uncouple SRT and hydraulic retention time (HRT). To generate shear and reduce membrane fouling, gas spargers were placed at the bottom of the reactors. Dissolved oxygen concentration was set and maintained at 3-4 mg/L. The pH of MBRs was maintained at 7-7.3 by dosing 1M NaOH or 1M HCl. The permeate pumps (Masterflex L/S, Cole-Parmer) were operated in cycles with 15 min permeation followed by 5 min relaxation.

2.2.3 Design of Bioreactor Experiments

Exp_1- The influence of inoculum, COD:N and SRT on EPS-flocculants production

The two MBRs containing different inocula, R_{MUN-1} and R_{IND-1}, were run in parallel for 105 days under identical conditions (Table S1). Firstly, to mimic nitrogen non-limited and limited conditions, various COD:N ratios ranging from 20 to 100 were applied. This was applied together with a stepwise decrease of SRT to study whether a combination of nitrogen limitation and low SRT was required for increasing EPS yields. Furthermore, application of different inocula allowed investigation of how EPS production was influenced by the start-up biomass. During this experiment, optical microscopy was applied to monitor the EPS production by the two different inocula, and to observe changes in the sludge flocs. Extensive quantification and further characterisation of the EPS were addressed in Exp_2.

Exp_2- Overproduction of EPS-flocculants under nitrogen limitation and short SRTs

Two MBRs, R_{MUN-2} and R_{IND-2}, were run in parallel for 42 days under identical conditions (Table S1) with the aim of: i) studying whether EPS production was stable over the operational period. This was done by applying the conditions (SRT and COD:N) that yielded the most EPS during Exp_1; ii) investigating whether the flocculation performance of the produced EPS was stable over the operational period.
The reactors were fed with a sufficient amount of nitrogen (COD:N 20) after inoculation and kept in batch mode for 24 h to allow biomass adaptation and activation in the applied lab-conditions. Following that, nitrogen limitation (COD:N 100) was introduced in combination with an SRT 6 days. Later, nitrogen-limited conditions were kept constant while the SRT decreased to 3 days and 1.5 days (Table S1). During this experiment, the focus was on the quantification and characterisation of the EPS. Additionally, to gain further insight into the composition of EPS, fluorescence microscopy was utilised (see section 2.2.5).

2.2.4 Analytical techniques to study reactor performance and EPS production

COD conversion and EPS production yield

The COD removal efficiency of the reactors was evaluated based on the measurement of chemical oxygen demand (COD) concentration in effluent samples using the Dr. Lange test kits according to the manufacturer's instructions (LCK, Hach Lange, UK). Total and soluble COD (tCOD and sCOD) were determined on raw and 0.45 filtered samples, respectively.

The EPS production yields of the reactors were determined based on the EPS content of mixed liquor, the COD values of EPS (COD_{EPS}), and the converted amount of sCOD as described in supplementary information (SI-1). Chemical oxygen demand of EPS (COD_{EPS}) was determined on EPS extracts.

Total suspended solids and mixed liquor volatile suspended solids analysis

Total suspended solids (TSS) and mixed liquor volatile suspended solids (MLVSS) were analysed according to standard methods [45], with a slight modification described in supplementary information (SI-3).

EPS extraction and quantification

In Exp_1, EPS was extracted from mixed liquor only during the COD:N 100 period, at the end of SRT 6 and 3 days (day 84 and day 103, respectively) (Table S1). In Exp_2, mixed liquor was sampled at several time points (day 10, 13, 16, 23, 30, 34, 42) to cover SRT of periods 6, 3, and 1.5 days (Table S1).Samples were stored at -20 °C before EPS extraction.

Two extraction methods were applied: i) cation exchange resin (CER) [40]; and ii) heat extraction [46] considering the possible influence of extraction method on the chemical composition of EPS [47] and to assess reproducibility of EPS yield trends between methods [48]. Detailed extraction protocols are provided in the supplementary information (SI-4). EPS were quantified as the sum of polysaccharides and proteins in the extracts, determined as described in Section

2.2.5. The extraction yield was calculated by normalising the total EPS to the grams of MLVSS used in the extraction and expressed as mg EPS g^{-1} MLVSS.

2.2.5 EPS Characterization

Proteins and Polysaccharide Quantification

Proteins quantification was conducted using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific,US), according to the manufacturer's instructions. Polysaccharide concentration was determined by the colorimetric phenol-sulphuric acid method [49], measuring the absorbance at 490 nm and using glucose as standard.

Microscopy Analysis

In Exp_1, to quickly monitor biomass growth and EPS production in the reactors, fresh mixed liquor samples were periodically examined using optical microscopy (DM750, Leica, DE) equipped with a Leica ICC50 W camera. Images were analysed using the Fiji Image J software [50]. For the visualization of EPS and microorganisms, as well as acidic polysaccharides, 0.1% v/v Crystal Violet and 1% Alcian Blue stain solutions were used, respectively. For both staining procedures, mixed liquor samples were mixed with dye solutions at a ratio 1:10 (v/v) and incubated at room temperature for 2 min before observation.

For a more exhaustive visualisation of distinct components within flocs, fluorescent staining along with Confocal Laser Scanning Microscopy (CLSM, Zeiss LSM 880, DE) was employed during Exp_2. A sequential staining process allowed the visualisation of DNA, proteins, and polysaccharides. The sample was first mixed with SYTO 63 Red DNA stain (5 mM, Molecular Probes, US) at a ratio of 250:1 and incubated at room temperature in the dark for 30 min. The sample was then washed twice with Milli-Q water and centrifuged at 3750xg, 5 min, to eliminate excess dye. Afterwards, the pellet was resuspended in sterile 1x PBS and stained with SYPRO^{TM} Orange protein stain (5000x in DMSO, Molecular Probes, US) at a ratio of 100:1, for 30 min in the dark. The washing steps were repeated to remove excess dye, and the pellet was resuspended in 1x PBS buffer. 40 µl of the stained sample was placed on a microscopy slide and mixed with 20 µl of Calcofluor-white polysaccharide stain (1 g L^{-1}, Sigma-Aldrich, DE). Before

observation, an antifade mounting medium (VECTASHIELD® PLUS, Vector Laboratories, US) was applied. CLSM images were captured with a monochrome camera (Axiocam 702 mono, Zeiss, DE) and the software ZEN (Zeiss, DE). Image analysis was performed using Fiji ImageJ.

EPS Molecular Weight Determination

The determination of the average molecular weight of EPS was carried out on the dialysed EPS extracts through liquid chromatography-organic carbon detection (LC-OCD - Model 8, DOC-LABOR, DE) as described by Ajao et al [51].

2.2.6 EPS Flocculation efficiency

The flocculation ability of the EPS was evaluated using the prepared EPS extracts. To study the effect of the various SRTs on the flocculation performance of EPS, extracts from Exp_1 (R_{MUN-1} and R_{IND-1}) were selected from the end of SRT 6 days and SRT 3 days periods of the nitrogen-limited (COD:N 100) stage as EPS production was significant in this period. Later, to investigate the stability of flocculation performance of EPS produced under nitrogen-limited conditions combined with short SRT, three samples were selected from each reactor of Exp_2 (R_{MUN-2} and R_{IND-2}); from the first day and the last days of SRT 3 days period (Table S1) and from the last day of SRT 1.5 days period of the nitrogen-limited (COD:N 100) stage.

The flocculation performance of EPS samples was evaluated using $5\,\mathrm{g\,L^{-1}}$ kaolin clay (Sigma- Aldrich, DE. For details on the composition of kaolin clay see SI, Table S5), following the method described by Ajao et al. [44]. In each flocculation experiment, 0.3 mg EPS was added per g of kaolin clay, and $CaCl_2.2H_20$ (Sigma-Aldrich, DE) solution was used to provide 100 mg $Ca^{2+}L^{-1}$ as a bridging agent to the suspension. The dosage of $0.3\,\mathrm{mg\,EPS\,g^{-1}}$ kaolinite was chosen by performing dosage-flocculation efficiency tests (SI, Fig. S2).

A kaolin clay suspension without addition of any Ca^{2+} or EPS was used as a negative control. To evaluate the coagulation efficiency of Ca^{2+}, another control containing only $Ca^{2+}(100\,\mathrm{mg\,L^{-1}})$ was included. In each flocculation experiment, the controls and EPS-treated samples (EPS+ Ca^{2+}) were handled identically. After settling, supernatants were sampled from the same depth in each jar, and the

residual turbidity was measured in nephelometric turbidity units (NTU). The flocculation efficiency (FE) of EPS was calculated relative to the calcium-treated control using the following equation:

$$FE = \frac{\mathrm{NTU}_c - \mathrm{NTU}_{\mathrm{EPS}}}{\mathrm{NTU}_c} \quad \text{(Eq.1)}$$

Where NTU_c represents the residual turbidity of the control containing kaolin clay with Ca^{2+}($100\,\mathrm{mg\,L^{-1}}$) and NTU_{EPS}+Ca is the residual turbidity of the sample treated with EPS in the presence of Ca^{2+}. Total coagulation-flocculation efficiency was calculated with the same expression, but here NTU_c is the negative control of kaolin clay suspension without addition of Ca^{2+}.

2.3 Results

2.3.1 General reactors' performance

A high sCOD removal was observed for all the reactors, ranging between 90-100% in Exp_1(Fig. 1A) and 70-90% in Exp_2(Fig. 1B), indicating a high conversion of glycerol. In Exp_1, consistently stable sCOD removal was observed, and the stepwise imposition of nitrogen limitation had no noticeable impact on the MBRs' treatment performance meanwhile solids concentrations decreased and were maintained at 2–$2.5\,\mathrm{g\,VSS\,L^{-1}}$(SI, Fig, S3A). Conversely, in Exp_2, the immediate application of nitrogen limitation at the beginning of the operation led to less stable sCOD removal efficiency, albeit still mostly above 80% (Fig. 1B). The solids concentrations were measured at 1.5–$2.5\,\mathrm{g\,VSS\,L^{-1}}$ throughout the process (SI, Fig. S3B). Additional supporting data regarding steady-state operational parameters of the MBRs in Exp_1 and Exp_2 are provided in the Supplementary information (SI, Table S6).

2.3.2 Nitrogen-limited MBR operation at short SRT stimulates EPS overproduction

In R_{MUN-1} and R_{IND-1}, the EPS yields were below $0.1\,\mathrm{g\,COD_{EPS}\,g^{-1}\,COD_{converted}}$ during SRT of 6 days with and without nitrogen limitation (Fig. 2A). However, a sharp increase in EPS production yield was observed when the reactors were operated under nitrogen limitation (COD:N 100) coupled with short SRT (3 days) (Fig. 2A). Under these conditions, EPS production yield reached up

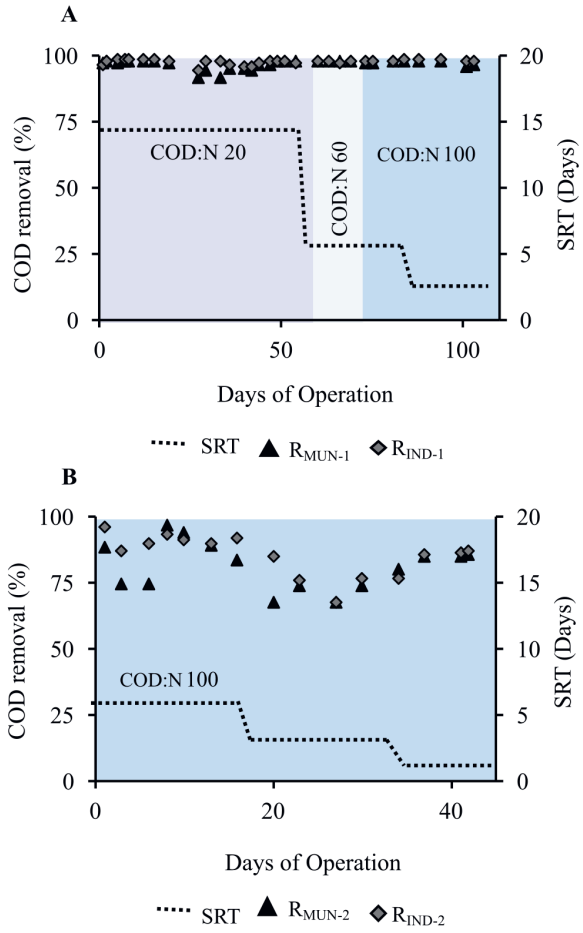


Figure 1: sCOD removal efficiency in A) R_{MUN-1} and R_{IND-1} B) R_{MUN-2} and R_{IND-2} along the process at different SRTs and COD:N ratios. COD removal efficiencies were calculated based on the soluble COD of permeate ($COD_{effluent}$).

to 0.21 g COD_{EPS} per g $COD_{converted}$, particularly in R_{MUN-1}, while under sufficient nitrogen conditions the yields were considerably lower ranging from 0.05–0.07 g COD_{EPS} g^{-1} $COD_{converted}$(Fig. 2A).

Similar observations were made in Exp_2, where COD:N ratio was kept at 100 while SRT was decreased gradually from 6 to 1.5 days. As shown in Figure 2B, EPS production yield increased in both reactors as SRT decreased. When SRT was shortened from 6 days to 3 days, in both reactors EPS yield increased from 0.05 g COD_{EPS} g^{-1} $COD_{converted}$ to 0.1–0.13 g COD_{EPS} g^{-1} $COD_{converted}$. Further decrease of SRT down to 1.5 days, resulted in even further increase of EPS yields, particularly in R_{MUN-2}, reaching up to 0.23 g COD_{EPS} g^{-1} $COD_{converted}$ (Fig. 2B).

The EPS yield results were further supported by quantifying the EPS content relative to the biomass (expressed as mg EPS per g MLVSS). When SRT was shortened to 3 days, the EPS content of R_{MUN-1} increased from 78.5 mg EPS g^{-1} MLVSS to 347.5 mg EPS g^{-1} MLVSS while in R_{IND-1} this increased from 71.5 mg EPS g^{-1} MLVSS to 115 mg EPS g^{-1} MLVSS (Fig. 3A). Similarly, in R_{MUN-2} and R_{IND-2} , EPS content increased from below 100 mg EPS g^{-1} MLVSS up to 300–400 mg EPS g^{-1} MLVSS towards the end of the process, where an even shorter SRT of 1.5 days was applied (Fig. 3B and C). Finally, while in R_{IND-2} the EPS fraction was still increasing at the end of SRT 1.5 days period (Fig. 3C), in R_{MUN-2} it reached its maximum followed by a slight decline (Fig. 3B). In summary, these results of experiments with stepwise shortened SRTs under nitrogen limitation show that at least 30-40% of the MLVSS was composed of overproduced EPS when using the industrial sludge as inoculum (Fig. 3C) and even reached 55-75% with the municipal sludge as inoculum (Fig. 3B). These outcomes show that nitrogen limited feed and short SRT are two key factors to stimulate EPS overproduction for both MMCs studied when fed with synthetic wastewater. While these findings provide a promising proof of concept, further validation with real municipal and industrial wastewaters is needed before extrapolating to full-scale applications.

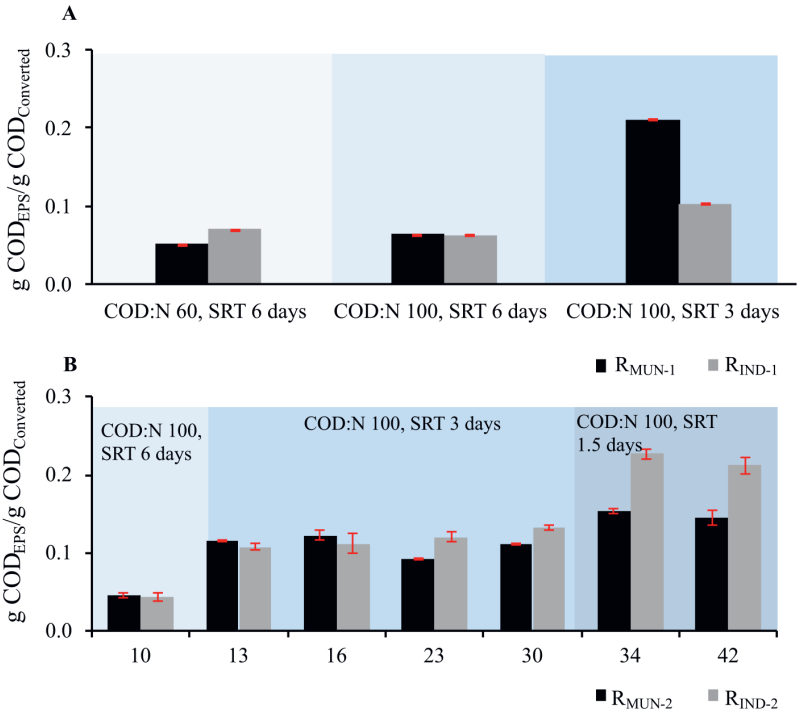


Figure 2: EPS production yields during Exp_1 (A) and Exp_2 (B). Colored boxes indicate the different experimental periods, corresponding to COD:N ratios and SRTs applied to the MBRs during the experiments.

2.3.3 Decreased SRT results in increased polysaccharide content and molecular weight of EPS

Optical microscopy and EPS staining on samples taken from R_{MUN-1} and R_{IND-1} during nitrogen limitation (COD:N 100) confirmed the quantitative results previously presented and showed increasing abundance of EPS, as SRT was shortened from 6 to 3 days (Fig. 4). During SRT 3 days, excess EPS, rich in acidic polysaccharides (identified by alcian blue staining; see Fig. 4B and D), were produced by the MMC in both reactors. This was in stark contrast to the samples obtained during the SRT 6 days, where crystal violet staining revealed only flocs composed of microbial cells and structural EPS (Fig. 4A and C). Similarly, under nitrogen-sufficient conditions (COD:N 20), no significant EPS overproduction was observed, and the reactors contained only microbial flocs (SI, Fig. S4A and C). Increasing the COD:N ratio to 60 while maintaining SRT of 6 days also did not result in a visible EPS overproduction (SI, Fig. S4B and D). Overproduction of polysaccharide-rich EPS was only achieved when both nitrogen limitation and short SRTs (3-1.5 days) were applied (Fig. 4B and D). Similar results to those observed in R_{MUN-1} and R_{IND-1} with brightfield microscopy, were found in flocs from R_{MUN-2} and R_{IND-2} via CLSM analysis. The samples from SRT 6 days did not show any polysaccharide-rich EPS production (Fig. 5A and C), while the blue signal from calcofluor white highlighted the carbohydrate portion of EPS for R_{MUN-2} and R_{IND-2}, especially during the SRT 1.5 days period (Fig. 5B and D, blue signal).

These microscopy results were followed by a quantitative carbohydrate analysis, showing that the polysaccharide content of EPS increased as the SRT decreased in all reactors (Fig. 5 and Fig. 6). In R_{IND-1} polysaccharide content increased from 9 $\mathrm{mg\,PS\,g^{-1}}$ MLVSS (0.125 $\mathrm{mg\,PS\,mg^{-1}}$ EPS) to 41.8 $\mathrm{mg\,PS\,g^{-1}}$ MLVSS (0.363 $\mathrm{mg\,PS\,mg^{-1}}$ EPS) while in R_{MUN-1} from 14.2 $\mathrm{mg\,PS\,g^{-1}}$ MLVSS (0.180 $\mathrm{mg\,PS\,mg^{-1}}$ EPS) to 202.5 $\mathrm{mg\,PS\,g^{-1}}$ MLVSS (0.582 $\mathrm{mg\,PS\,mg^{-1}}$ EPS) when measured after heat extraction (SI, Table S7). Protein content in R_{IND-1} slightly increased from 62.5 $\mathrm{mg\,PN\,g^{-1}}$ MLVSS (0.63 $\mathrm{mg\,PN\,mg^{-1}}$ EPS) to 73 $\mathrm{mg\,PN\,g^{-1}}$ MLVSS (0.87 $\mathrm{mg\,PN\,mg^{-1}}$ EPS). However, in R_{MUN-1} a sharp increase from 64.3 $\mathrm{mg\,PN\,g^{-1}}$ MLVSS (0.41 $\mathrm{mg\,PN\,mg^{-1}}$ EPS) to 145 $\mathrm{mg\,PN\,g^{-1}}$ MLVSS (0.819 $\mathrm{mg\,PN\,mg^{-1}}$ EPS) was observed.

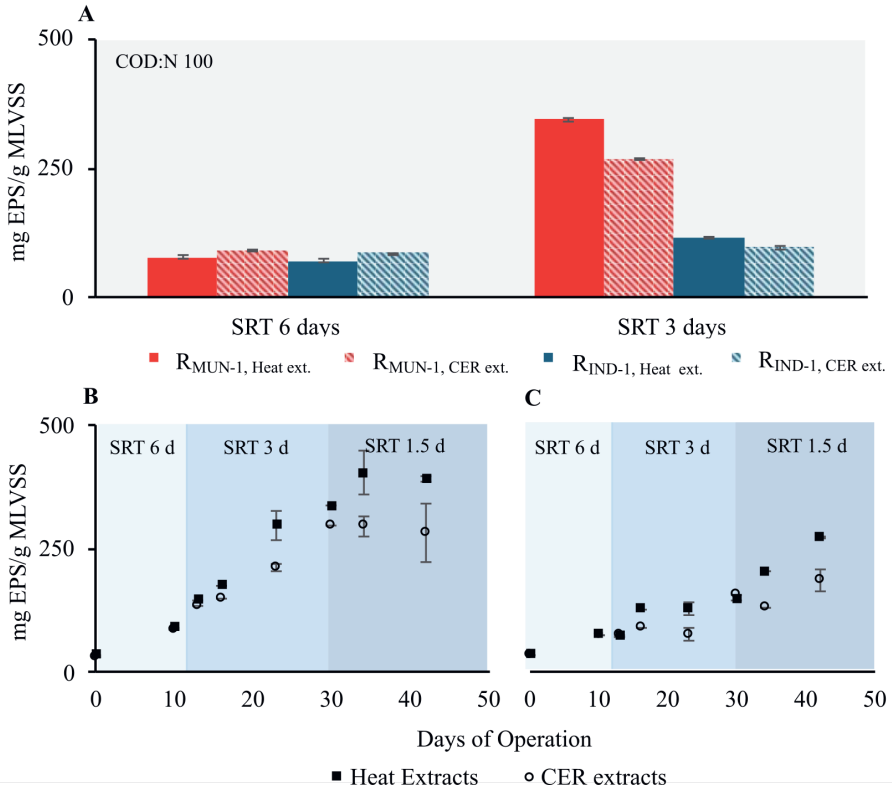


Figure 3: Fraction of EPS in mixed liquor from Exp_1 and Exp_2 quantified with various extraction methods. Colored boxes in B and C indicate different SRT periods applied during the experiments. (A) R_{MUN-1} and R_{IND-1} during COD:N 100 period, (B) R_{MUN-2} and (C) R_{IND-2} during COD:N 100 period. CER – cation exchange resin. Heat extraction performed at 50 ℃. Error bars show the absolute deviation from the average of two measurements

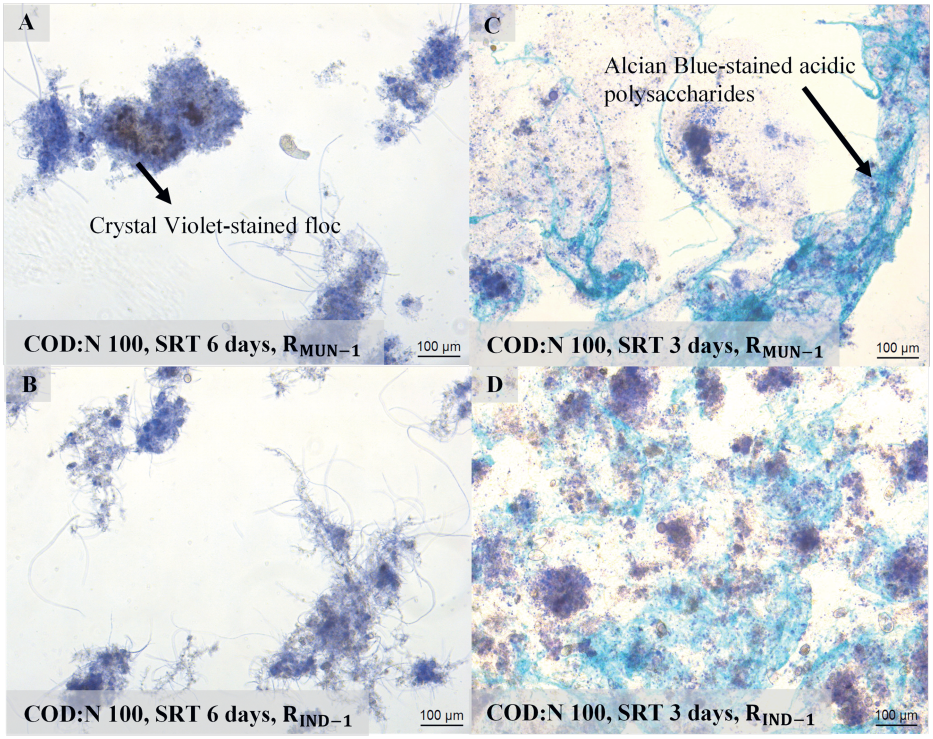


Figure 4: Brightfield microscopy images of mixed liquor (ML) samples from Exp_1- Fresh mixed liquor samples were observed to assess EPS production by applying crystal violet 0.1% (in purple) to visualize EPS structures and cell envelopes, and alcian blue 1% (in blue) to specifically target acidic polysaccharides. In A and C samples from R_{MUN-1} and R_{IND-1} under nitrogen limited conditions, SRT 6 days; in B and D samples from R_{MUN-1} and R_{IND-1} in COD:N 100, SRT 3 d.

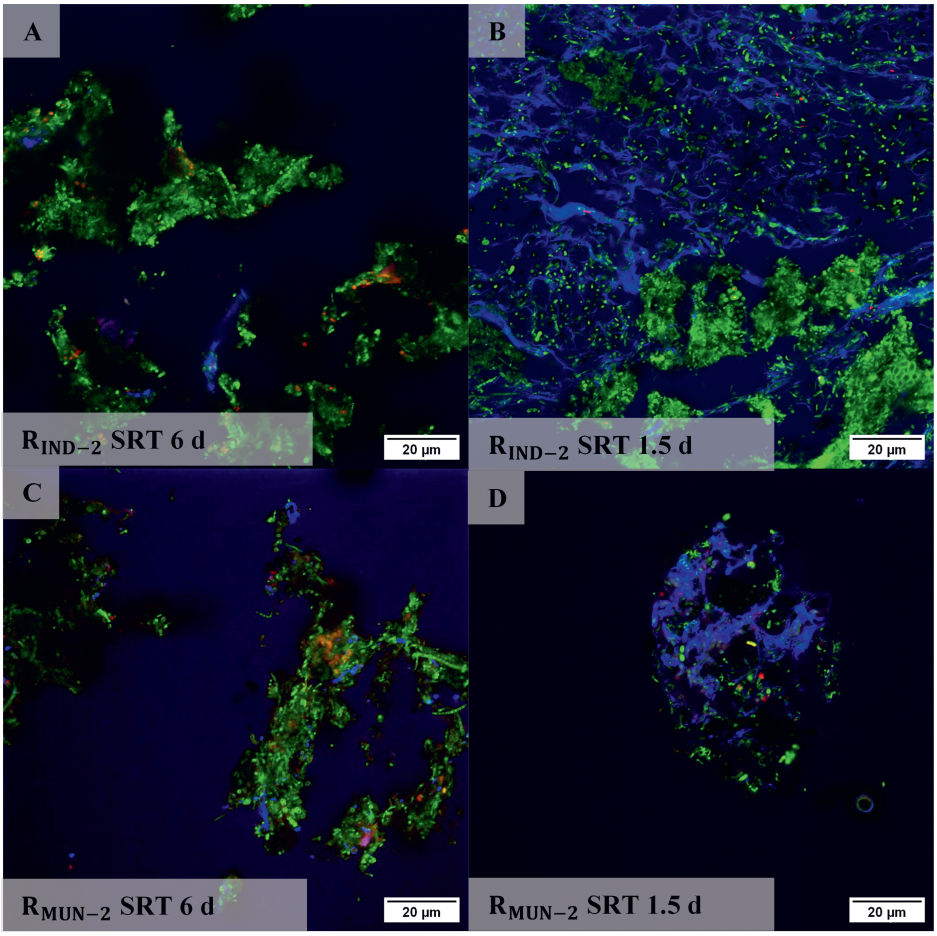


Figure 5: Confocal laser scanning microscopy (CLSM) micrographs of mixed liquor samples from Exp_2 at SRT 6 and 1.5 days. (A) R_{IND-2} SRT 6 days; (B) R_{IND-2} SRT 1.5 days; (C) R_{MUN-2} SRT 6 days; (D) R_{MUN-2} SRT 1.5 days. Both reactors had a COD:N ratio of 100 throughout the whole process. Proteins were stained with SYPRO^{TM} Orange (green), polysaccharides and DNA were stained with Calcofluor white (blue) and SYTO 63 (red). Scale bars are 10 µm.

Also, in R_{IND-2} , the polysaccharide yield increased from 5.7 mg PS g^{-1} MLVSS (0.162 mg PS mg^{-1} EPS) at SRT 6 days to a maximum of 229 mg PS g^{-1} MLVSS (0.87 mg PS mg^{-1} EPS) at SRT 1.5 days when measuring after heat extraction (Fig. 6A). The protein content of EPS in R_{IND-2} remained relatively stable at below 50 mg PN g^{-1} MLVSS (0.2-0.3 mg PN mg^{-1} EPS) throughout the experiment (Fig. 6B). Also, in R_{MUN-2}, the polysaccharide yield increased from 6.2 mg PS g^{-1} MLVSS (0.18 mg PS mg^{-1} EPS) at SRT 6 days to a maximum of 253 mg PS g^{-1} MLVSS (0.615 mg PS mg^{-1} EPS) at SRT 1.5 days (Fig. 6C). Unlike R_{IND-2}, in R_{MUN-2} , the protein yield also showed an increasing trend, ranging from a minimum of 25 mg PN g^{-1} MLVSS (0.82 mg PN mg^{-1} EPS) to a maximum of 196 mg PN g^{-1} MLVSS (0.385 mg PN mg^{-1} EPS) (Fig. 6D).

CLSM analysis further supported the quantitative results depicted in Figure 6, with a higher protein coverage for the flocs collected at SRT 6 days (red signal, Fig. 5A and C), and higher polysaccharides when operating the reactors at SRT 1.5 days (blue signal, Fig. 5B and D). Further analysis of CLSM images with merged fluorescent signals (Fig. 5 and SI, Fig. S5) showed that flocs developed at 1.5 d SRT were predominantly composed by microorganisms (positive to SYTO 63 DNA staining, in red), covered by an outer envelope rich in proteins, on top of which a polysaccharides-rich matrix deposited.

The average molecular weight of EPS significantly increased with decreasing SRT in both experiments (Fig. 7A). In R_{MUN-1} and R_{IND-1}, a sharp increase in the average molecular weight of EPS was clearly observed when SRT decreased from 6 days to 3 days during the nitrogen limited period (Fig. 7A). In R_{MUN-1} , the average molecular weight of EPS increased from 0.51 MDa to 2.2 MDa, while in R_{IND-1} it increased from 0.06 MDa to 1.87 MDa with decreasing SRT. In R_{MUN-2} and R_{IND-2}, a further decrease in SRT from 3 to 1.5 days did not affect the molecular weight of the produced EPS, which remained in the range of 1.7-2.4 MDa (Fig. 7A).

2.3.4 EPS exhibit stable flocculation performance over time

Flocculation experiments (SI, Fig. S6) conducted using EPS harvested during Exp_1 and Exp_2 showed a drastic increase in flocculation efficiency as the SRT decreased. Moreover, EPS produced at short SRTs exhibited stable flocculation

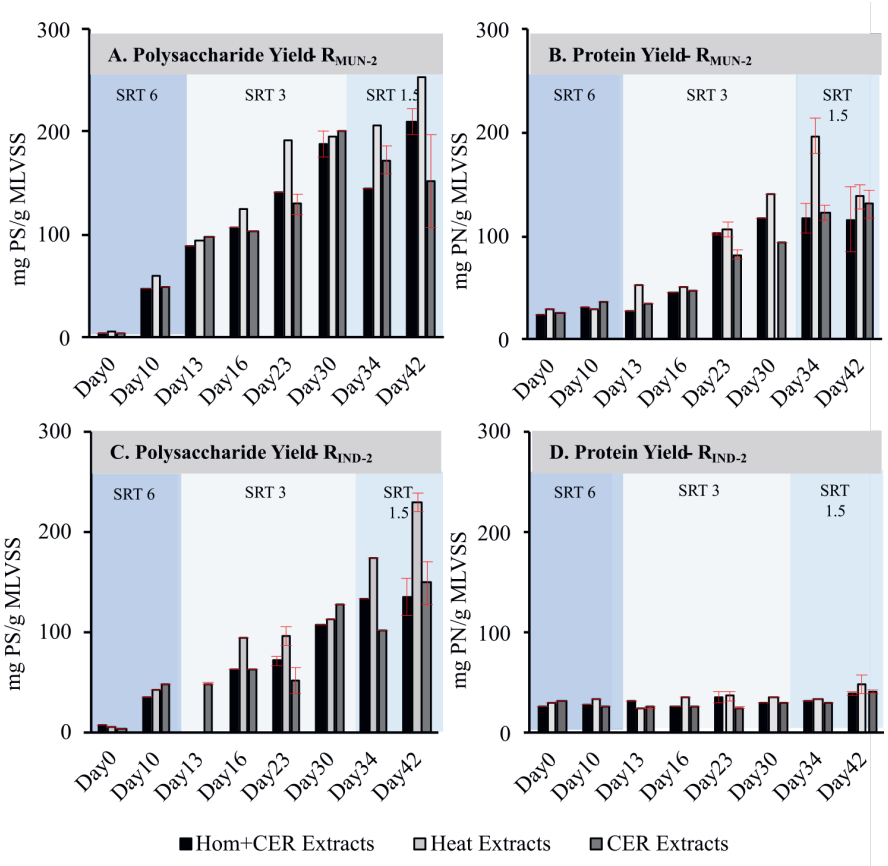


Figure 6: Polysaccharides and proteins yield of EPS extracts prepared from different time points of Exp_2 ; R_{MUN-2} and R_{IND-2} . (A) polysaccharide yield of R_{MUN-2} ; (B) protein yield of R_{MUN-2} ; (C) polysaccharide yield of R_{IND-2}; (D) protein yield of R_{IND-2}. Error bars show the standard deviation for n=2. The results are normalized per gram of MLVSS.

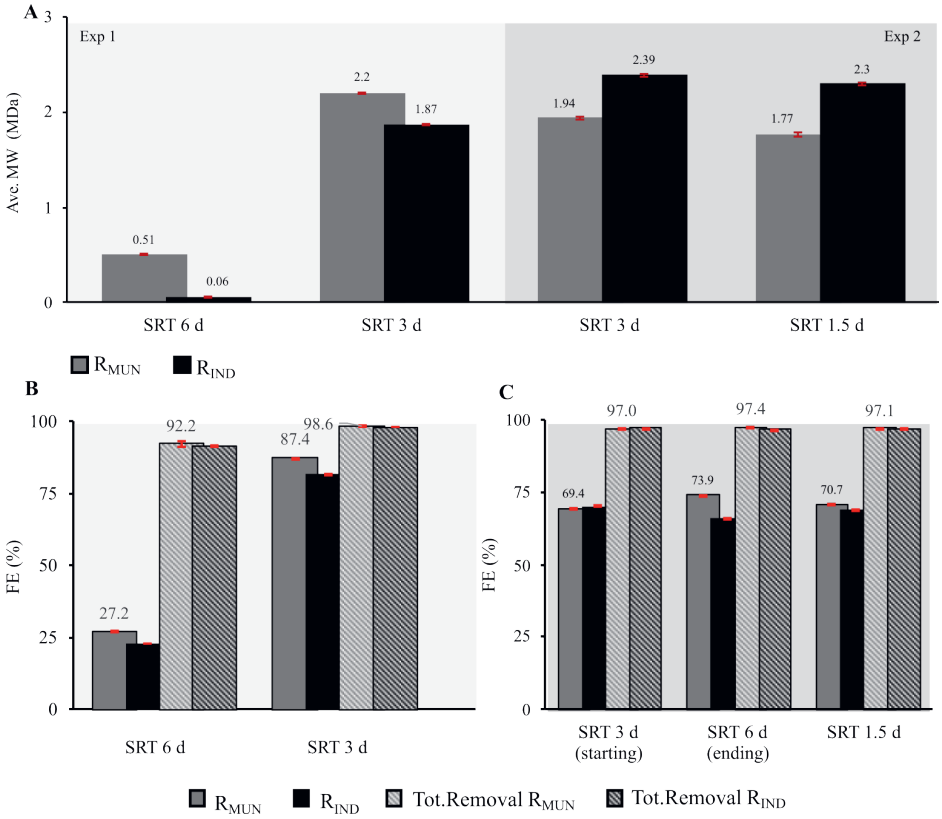


Figure 7: (A) Average molecular weight and (B) and (C) flocculation efficiencies of EPS extracts obtained through heat extraction from R_{MUN} and R_{IND} MBRs in Exp_1 and Exp_2– In (B) and (C), flocculation efficiencies were calculated for the combined EPS + Ca^{2+} treatment using two different reference controls. Values shown as solid-filled bars represent efficiencies calculated relative to the Ca^{2+} control (kaolin + Ca^{2+} only), indicating the incremental improvement provided by EPS in the presence of Ca^{2+} . Values shown as patterned bars represent total removal efficiencies calculated relative to the negative control (kaolin suspension without Ca^{2+} or EPS). Error bars indicate the standard deviation (n = 2).

efficiency over the operational period of the reactors (Fig.7 B and C).

In Exp_1, the EPS produced at COD:N 100 and SRT 6 days showed poor flocculation efficiencies of 27.2% and 22.8% for R_{MUN-1} and R_{IND-1}, respectively, when calculated relative to the Ca^{2+}-control (Fig. 7B). Decreasing the SRT to 3 days resulted in substantially improved performance, with flocculation efficiencies relative to calcium control increasing up to 87.4% (Fig. 7B). The overall coagulation-flocculation efficiencies representing the combined contribution of Ca^{2+} and EPS in flocculating negatively charged kaolin clay were also assessed. These efficiencies ranged between 90–92% at SRT 6 days and increased to a maximum of 98.6% at SRT 3 days (Fig. 7B). Such efficiencies corresponded to residual turbidity of 219 - 232 NTU and 38 - 55 NTU , respectively (SI, Fig. S7).

In Exp_2, several EPS samples from short SRT (3 and 1.5 d) were tested, with flocculation performances ranging between 69% and 74% relative to Ca^{2+}-control, with negligible differences observed between EPS samples from R_{MUN-2} and R_{IND-2}(Fig. 7C). The total coagulation-flocculation efficiencies for these samples remained consistently high at approximately 97% (Fig. 7C). Overall, only the EPS overproduced under short SRTs showed satisfactory flocculation performance relative to the Ca^{2+}-control, regardless of the origin of the inoculum. The final turbidities in Ca^{2+} treated samples ranged between 266 and 274 NTU in samples treated with EPS obtained at SRT 3 days between 80.5 and 82.5 NTU and in samples obtained at lower SRTs between 70.5 and 92 NTU (SI, Fig. S7).

2.4 Discussion

2.4.1 EPS overproduction in mixed microbial cultures: Role of nitrogen limitation and short SRT

In this study, we demonstrated the importance of combining nitrogen limitation and applying short SRT to stimulate EPS overproduction by MMCs in MBRs. The stepwise decrease of SRT allowed for more in-depth analysis of how this parameter influences EPS overproduction in time, whilst comparing two different start-up inocula.

Previously, our group reported overproduction of EPS in MBRs under nitrogen-limited conditions when reactors were operated at a short SRT of 3 days [51]. This increase in EPS production was explained using the energy spillage theory, which describes the redirection of excess energy by microorganisms through non-growth-associated processes [52]. However, in that study, EPS overproduction was attributed solely to nitrogen limitation, and the potential influence of short SRT was not considered or discussed. Based on the findings of this study, the combined influence of nitrogen limitation and short SRT appears to play a key role in EPS overproduction. This effect can be understood through several interconnected mechanisms. Nitrogen is a critical element for biomass synthesis, particularly for the formation of proteins and enzymes [53]. When nitrogen availability is low, microbial growth slows down due to restricted cell division [54]. Under these conditions, excess carbon that cannot be used for growth is redirected towards the production of EPS as an alternative carbon storage route [53]. At the same time, SRT plays a significant role in shaping microbial growth dynamics [55]. A short SRT reduces the time available for microorganisms to grow, adapt, and establish themselves in the system [56]. This environment tends to favour fast-growing microbes capable of forming aggregates or biofilms to remain in the reactors [28]. In contrast, slow-growing organisms, including those that might degrade EPS, are less competitive and may be washed out, thereby enriching the microbial community with fast-growing EPS producers [57]. In this study, the overproduction of EPS was observed in all the reactors analysed, irrespective of the inoculum type. This indicates that the operational parameters (SRT and COD:N) are relevant for different sources of inoculum, which is important considering an upscaling of the technology.

The excess sludge under nitrogen-limited conditions and low SRT consisted of a large fraction (Fig. 3) of the desired end product (up to 50% EPS), which is more than currently achievable in aerobic granular sludge systems treating municipal wastewater at full scale. Recently, Bahgat et. al reported in such systems only $\sim 30\%$ of sludge can be recovered as EPS for various applications [58]. In this study, increased production of EPS with flocculating ability can thus be seen as an alternative for sludge valorisation when the wastewater is limited in nutrients. Importantly, this flocculation ability of EPS may be further utilized

for primary treatment of said wastewaters, but this concept is yet to be demonstrated on real streams.

2.4.2 Evidence from other bioreactor systems: Effect of nitrogen limitation and short SRT on EPS production

Both nitrogen limitation and short SRT have independently been shown to promote high EPS levels in various bioreactor systems, although not always in systems specifically designed for EPS overproduction. For example, Yang et al. [59] demonstrated that operating a lab-scale sequencing batch reactor inoculated with municipal sludge, and fed with nitrogen-poor (COD:N 100:2 to COD:N 100:0) wastewater led to a gradual increase in EPS production, which in turn contributed to restoring floc structure and improving settling performance. Similarly, Chen et al. [60] showed that enhanced biomass adhesion in up-flow anaerobic filters was associated with increased EPS production when reactors were fed with nitrogen-limited feed. Ng et al. [36] reported that EPS production serves as a primary carbon storage route when activated sludge is supplied with nitrogen-deficient feedstock.

While these studies demonstrate the link between nitrogen limitation and increased EPS production, they were not designed to investigate operational parameters driving EPS overproduction and typically examined nitrogen limitation as a single factor. Likewise, a number of other studies have shown that reducing SRT can stimulate EPS production. For instance, Duan et al. [28] reported EPS production was higher at SRT 3 days compared to SRT 5 and 10 days in MBRs. They reported the biomass was fast-growing and microorganisms were more active when SRT decreased, while non-flocculating microorganisms were abundant during longer SRTs. In another study, where MBRs were operated at significantly longer SRTs than in our study, authors observed increased EPS production as SRT was shortened from 60 days to 20 days [29]. This increase was primarily attributed to a rise in protein content in the cake layer and in polysaccharide content in the gel layer, similar to results shown in our study (Fig. 6). Protein profile analysis further revealed the presence of stress, biofilm, and adhesion-related proteins at the shorter SRT [29]. Although short SRT was not intentionally applied as a strategy to enhance EPS yield in these cases, the

findings clearly demonstrate its positive impact as a sole parameter on EPS overproduction in various settings.

As a result, while studies investigating nitrogen limitation or short SRT individually have provided valuable insights into their influence on EPS production, research exploring their combined effect remains scarce. Our study directly addresses this gap, allowing us to clearly demonstrate their synergistic impact on both the quantity and composition of EPS. This approach provides new insights into how operational strategies can be optimized for targeted EPS overproduction.

2.4.3 Influence of nitrogen limitation and short SRT on EPS composition

The composition of EPS, particularly protein and polysaccharide content, can be influenced by diverse factors such as the type of carbon source, carbon and nitrogen content of the feed, operational parameters such as SRT, and the microbial community members in the reactors [29],[33],[61],[62].

In this study, a predominance of polysaccharides in the EPS composition was confirmed both through CLSM analysis of sludge flocs (Fig. 5) and by analytical methods (Fig. 6) and must be a result of operating conditions, i.e., SRT and COD:N ratios, because the carbon sources were the same in all reactors. The CLSM analysis showed that protein-rich EPS was produced as cell-envelope at higher SRT (Fig. 5A and C), while polysaccharide-rich EPS was excreted at shorter SRT (Fig. 5B and D). These distinct signals from proteins and polysaccharides may reflect a structural boundary between basal, cell-associated EPS and overproduced extracellular polymers. Similar spatial differentiation has been observed in conventional aerobic microbial aggregates, where protein-rich EPS is typically found in close proximity to microbial cells [63],[64]. In our study, feeding with nitrogen limited, excess carbon substrate might have led to the production of polysaccharide-rich EPS since the feed contains limited amount of nitrogen [65],[66]. The surplus carbon supply may have caused the activation of overflow metabolism, often observed as a result of restricted anabolic capacities [67]. This might have resulted in the production of carbonaceous compounds while prioritizing the utilization of the low nitrogen available for the basic cellular functions

and synthesis of the microbial cells themselves [68],[69]. Similarly, Liu et al. [70] also reported that the carbon content of the feed influences the composition of EPS in different type of sludge, including annamox and aerobic granular sludge and flocculent sludge. A higher protein content in EPS derived from different sources was associated with feeding on sugar-poor and undegraded organics-rich sewage water, which resulted in a low polysaccharide content in the EPS. The changes in protein and polysaccharide content of EPS is important in terms of the application of EPS as flocculant since the properties of these components have direct influence on the flocculation mechanisms of EPS [12], as discussed in section 2.4.4.

2.4.4 Flocculation efficiency of EPS-flocculant

Flocculation experiments conducted with EPS harvested during Exp_1 and Exp_2 revealed that decreasing the SRT led to improvement in flocculation efficiency, with EPS produced at short SRTs maintaining consistently high performance throughout the operational period. These findings are important for achieving reliable production of EPS-flocculants, as they demonstrate an operational strategy for obtaining EPS with stable and reproducible flocculation capacity. Such consistency is crucial for real-world applications, particularly in wastewater treatment processes seeking to replace synthetic flocculants with bio-based alternatives. The observed increase in flocculation efficiency with decreased SRT reported may be attributed to the increased polysaccharides content in the EPS (Fig. 6). Previous studies have reported similarly high flocculation performance for polysaccharide-rich EPS [1], [71]. This improvement is often linked to the abundance of anionic functional groups such as hydroxyl (-OH), carboxyl (-COOH), and sulphonate ($-SO_3^-$) present in polysaccharides, which can interact with the surrounding cations [12],[44]. Additionally, the higher flocculation efficiency observed at lower SRTs may be related to the increased molecular weight of EPS under these conditions (Fig.7A).

Molecular weight plays a critical role in flocculation, as it influences both floc formation and settling rate [72]. High molecular weight flocculants provide more adsorption sites and longer chain segments capable of connecting multiple particles, leading to the formation of larger and more stable flocs. This results in

higher flocculation ability compared to low molecular weight flocculants [71],[73].

Overall, the results demonstrate that the potential utilisation of EPS as a flocculant relies on particular properties that can be manipulated by imposing specific operational conditions such as short SRT, to different inocula. However, to achieve the best results, further optimization of the flocculation process is essential. In this study, only the ratio of EPS to kaolin clay was optimized (SI, Fig. S2). However, flocculation performance can also be influenced by several factors, including the right dose of coagulants (cations), molecular weight of EPS, charge density, pH, and particle type to be flocculated [74]. Hence, a systematic evaluation of these parameters is necessary to optimize flocculation with EPS, as well as to identify target applications where EPS-flocculants can provide the most effective alternative to conventional synthetic flocculants.

2.4.5 Proposed flocculation mechanism of EPS

Colloidal suspensions remain stable primarily due to electric repulsion between charged particles. Destabilisation of colloidal particles is the first essential step for coagulation-flocculation to occur. This is typically achieved by dosing salts of multivalent cations. Once destabilised, the repulsive forces are reduced and attractive forces (e.g., van der Waals) dominate, allowing particles to collide and aggregate [75]. This destabilisation of colloids, also referred to as coagulation, can occur through four main mechanisms: double layer compression, charge neutralisation, colloid entrapment (sweep flocculation) and intraparticle bridging [76].

Following coagulation, a flocculation step is required to promote formation of larger, better settleable aggregates from these destabilised particles. For this purpose, charged and uncharged polymers are used. In systems employing synthetic polymers, several mechanisms are known. These include bridging, polymer adsorption and charge neutralisation (including electrostatic patch effects), depletion flocculation, and displacement flocculation. With a given colloidal suspension-flocculant combination, such mechanisms occur either individually or in concert [77].

In the case of polysaccharide-based natural flocculants, the coagulation-flocculation typically proceeds via two main mechanisms: charge neutralization, and

polymer bridging (Fig.8 A, B) [1], [78]. Charge neutralization occurs when polymers carry an opposite charge to that of the suspended particles. In such cases, particle charge density is reduced due to the adsorption of polymers, which ultimately causes particle-particle collisions and aggregation. This mechanism is generally associated with low molecular weight polymers $< 10^5$ Da capable of absorbing directly on to the particle surfaces [79] (Fig. 8A). In contrast, the bridging mechanism predominates for charged, high molecular weight polymers $> 10^6$ Da which often bear the same charge as the suspended particles. The large molecular weight is required, because for this mechanism to function, the hydrated radius of the polymer should span across at least two electric double layers of the colloidal particles, preventing particle repulsion. When bridging occurs, some polymer segments adsorb onto particle surfaces, leaving free loops and tails that extend into the solution where they can attach to other particles, forming larger aggregates. Adsorption may occur via van der Waals forces, hydrogen bonding, interactions between polymer functional groups and particle surfaces or via cation-mediated electrostatic interactions [75] (Fig.8B).

In the present study, the EPS exhibited high molecular weight ($> 10^6$ Da) and an overall negative charge (SI, Fig. S8), similar to that of kaolin clay particles. Consequently, their interaction required the presence of cations. When calcium ions (Ca^{2+}) were introduced together with EPS, calcium could adsorb onto the negatively charged sites on the kaolin clay particles and the EPS, thereby reducing electrostatic repulsion and enhancing EPS-particle interaction [75]. Once the repulsion was reduced, negatively charged groups of EPS could make coordinate bonds with calcium ions bound to the kaolin clay surface [80], [81]. In other words, calcium acted as an ion bridge between negatively charged polymers (EPS) and kaolin clay particles.

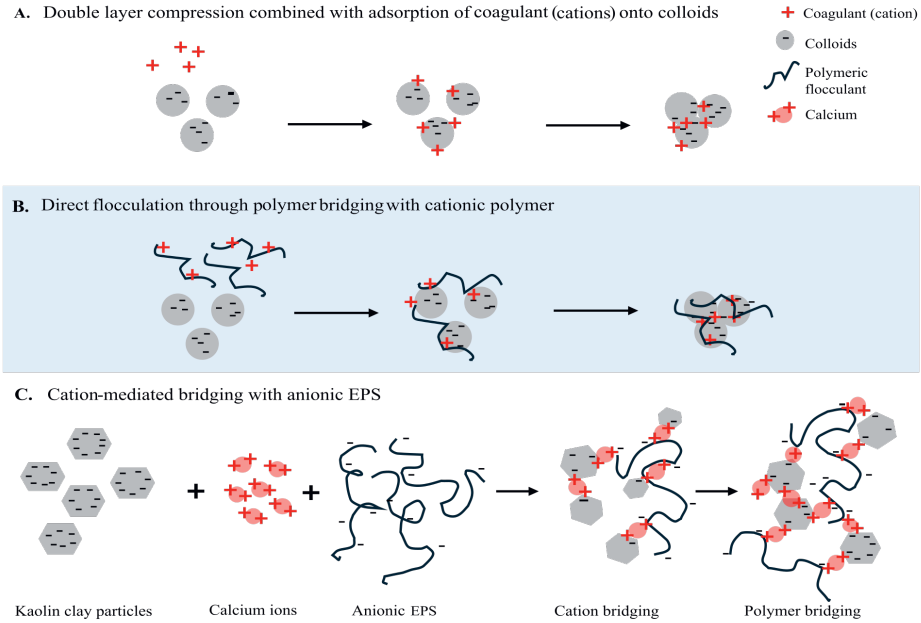


Figure 8: Coagulation-Flocculation mechanisms: (A) Charge neutralization; (B) Polymer bridging; (C) proposed coagulation-flocculation mechanism of anionic EPS showing cation-mediated bridging and polymer bridging steps individually.

Because EPS in principle are polyelectrolytes, they could interact with more particles simultaneously, forming polymer bridges resulting in formation of three dimensional large, porous, settleable floc structures (Fig. 8C) [78]. As a result, in the presence of calcium ions, EPS enhanced turbidity removal in the coagulation-flocculation tests (Fig. 7B and C), and larger, denser flocs were observed (SI, Fig. S6D), compared to the control experiments where only calcium was added (SI, Fig. S6C).

2.4.6 Valorisation of wastewater streams via EPS-flocculant production

Our results, following previous studies [51] demonstrate the potential of EPS production as a means of valorising wastewater streams rich in organics (like

glycerol and similar compounds), that are generated by various industries oriented on biodiesel, cosmetics, soap, epoxy resin [82] or fatty acid production [83]. A critical factor influencing EPS production is the type of carbon source, which affects both its yield and composition [84]. Glycerol, in particular, is a versatile substrate that can act as both an energy source and a precursor for the biosynthesis of cellular components [85],[86]. Compared to glucose, glycerol may support more efficient EPS production via secondary metabolism, potentially through a shorter and less energy-intensive pathway. This is because glycerol can enter gluconeogenesis directly, bypassing the tricarboxylic acid (TCA) cycle, and thus supply sugar nucleotide precursors for EPS biosynthesis through a more streamlined metabolic route [87],[88]. These findings highlight both the strategic advantage of utilising glycerol-rich waste streams to enhance EPS-flocculant production and support circular wastewater management using mixed microbial cultures, while also underscoring the need to explore other types of wastewater as potential substrates.

2.5 Conclusions

- Two crucial factors were identified for achieving EPS overproduction in MBRs: feeding with nitrogen-limited substrates and operating with short SRTs. A combination of these two can be used to stimulate mixed microbial communities to EPS overproduction regardless of the inoculum source.
- These conditions act as a selection pressure towards the development of microbial communities that are able to produce polysaccharide-rich EPS with high molecular weight, maintaining consistent flocculation properties over time.
- The EPS produced under these conditions ensures stable flocculation performance against kaolin clay particles, reaching up to 81-87% efficiency compared to calcium-coagulated samples.
- Our findings build upon the existing knowledge in the field, providing valuable insights for designing and operating bioreactors for EPS-flocculant production. Moreover, they reinforce the circularity of the wastewater treatment process by demonstrating effective valorization of waste sludge through EPS-flocculant preparation during wastewater treatment.

- A thorough analysis of microbial composition based on molecular techniques is part of our follow-up studies that are oriented on steering towards production of EPS as a stable source for greener flocculants.
- Although this study was performed with a glycerol-rich synthetic wastewater feed to provide controlled conditions for the experiments, further research with real industrial nitrogen-limited wastewater is necessary to confirm the robustness of EPS overproduction under more complex and variable influent compositions.

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Chapter 3

EPS overproduction from VFA-Rich wastewater by mixed microbial cultures under nitrogen limitation and short SRT: A new route for VFA valorisation

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Abstract

Volatile fatty acids (VFAs) from fermented wastewater are valuable bio polymer precursors. While microbial conversion of VFAs into polyhydroxyalkanoates (PHAs) is widely studied, their conversion into extracellular polymeric substances (EPS)-based flocculants remains largely unexplored. This study investigates EPS production from VFA-rich synthetic wastewater using mixed microbial cultures (MMC) under EPS-overproducing conditions (solid retention time (SRT) of 3 days and nitrogen limitation. A continuous stirred-tank reactor (CSTR) and a membrane bioreactor (MBR) were compared under these conditions to assess the influence of process configuration on EPS yields, composition, and flocculation performance. VFA conversion was relatively high $\sim$ 80% in the CSTR, but lower in the MBR ($<$50%). In the CSTR, up to 70% of soluble chemical oxygen demand (COD) was converted into EPS-COD at an organic loading rate (OLR) of 1 g COD L^{-1} d^{-1}. EPS yields reached 0.5–0.8 g VSS g^{-1} MLVSS, while PHA accumulation remained below 0.1 g VSS g^{-1} MLVSS. Confocal laser scanning microscopy imaging confirmed increasing EPS yields and decreasing PHA over time. EPS were predominantly polysaccharide-rich in the soluble fraction and protein-rich in the bound fraction. All EPS were anionic, with charge densities of 0.5–2.6 meq g^{-1}, and had high molecular weights of 0.17–3.7 MDa. VFA-derived EPS demonstrated high flocculation efficiency ($>$ 90%) with Ca^{2+}, comparable to commercial polyacrylamide (PAM, 92%), regardless of EPS type.These findings show EPS-flocculant production by MMC from VFA-rich wastewater, offering a sustainable route to convert waste carbon into functional biopolymers and support resource recovery and green flocculant development.

3.1 Introduction

Microorganisms can convert diverse carbon and nitrogen sources into biopolymers, which are either stored intracellularly, e.g., as polyhydroxyalkanoates (PHAs) [1], or secreted as extracellular polymeric substances (EPS) rich in polysaccharides and proteins [2]. These biopolymers serve as energy storage, adhesives, or barriers against adverse environmental conditions [2]. Hence, their synthesis is regulated in response to environmental stimuli [3]. Harnessing this regulation in pure bacterial cultures has enabled both PHA [4] and polysaccharide-rich EPS production [5]. The EPS can be applied as emulsion stabilisers, thickeners, and adhesive agents in food, pharmaceutical, textile, and paper industries [6]. PHAs, on the other hand, are biodegradable alternatives for packaging, textiles, disposable items, and carrier materials, amongst others [4]. Despite the broad range of documented uses, production of pure-culture-based biopolymers has remained costly due to the requirement for sterile conditions and refined carbon sources [7], [8].

Recently, biopolymer production using mixed (open system) microbial cultures (MMC) has gained attention [7], [8], [9]. This approach eliminates sterilisation and utilises waste feedstock, but introduces a new challenge of producing polymers with consistent physicochemical properties and yields, especially when wastewater composition varies [10]. To steer MMC towards the production of targeted biopolymers, conditions such as nutrient limitation or feast/famine regimes, together with strict control of hydraulic (HRT) and solids retention time (SRT), are required [10].

For PHA production, MMC are exposed to nitrogen limitation (COD:N $\sim$ 100), excess carbon, and repeated feast/famine whilst using pre-fermented waste streams rich in volatile fatty acids (VFAs) [11]. This enriches PHA-accumulators due to their affinity to or rapid uptake rates of VFAs [10]. Under nutrient limitation, these microorganisms do not fully oxidise activated VFAs (acetyl-CoA and propionyl-CoA) via the tricarboxylic acid (TCA) cycle, but direct them into carbon storage instead [12], making PHA recovery possible with the first full-scale application operational in the Netherlands [13]. The remaining hurdles for this

technology are the need for specified microorganisms, solvent-based PHA extraction [14] and constraint of PHA accumulation to the cell size [15].

Given the above concerns with PHA, there is a growing interest in alternative valorisation pathways for VFAs, such as EPS synthesis for specific applications (e.g bioflocculants). Synthesis of EPS offers some advantages compared to PHA: i) they are extracellular and can potentially be harvested without use of solvents [16]; ii) accumulation is not constrained by cell size iii) most bacteria possess EPS biosynthesis pathways [17]; iv) EPS synthesis is linked to central metabolic routes (e.g., glycolysis, gluconeogenesis, TCA cycle) which occur concurrently with cell growth [18]. The latter suggests that overproduction of EPS primarily requires stimulation of traits already widespread among diverse microbial taxa, translating into reduced operational complexity compared to PHA synthesis. Only a few studies exploring stimulatory conditions for EPS overproduction with MMC show that, solely or in combination, nitrogen limitation, excess carbon and short SRT are crucial [15], [19]. More recently, Kisaoglan et al. (2025) (**Chapter 2**) showed that short SRTs (3 to 1.5 days) under nitrogen limitation are critical for high-yield synthesis of polysaccharides-rich EPS.

Whilst controlling the parameters described above, EPS with high molecular weight, high surface charge, and long polymeric chains have been produced [9], [20], [21] making them attractive alternatives to synthetic flocculants for water and wastewater treatment [22]. For example, Ajao et al. (2019) demonstrated overproduction of effective EPS bioflocculants in membrane bioreactors (MBR) from glycerol/ethanol-containing wastewaters. Kisaoglan et al. (2025) further demonstrated long-term stability of EPS flocculant production in such MBRs (**Chapter 2**).

While the above-mentioned studies in MBRs provide promising proofs of concept, EPS-flocculant production requires expansion to other broadly available feedstocks with further potential to tailor EPS physicochemical properties for improved flocculation. Particularly VFA-rich streams should be considered because they are large byproducts of anaerobic wastewater treatment and many industrial wastewaters can be pre-acidified to become VFA-rich. Furthermore, reactor configurations, such as continuous stirred-tank reactors (CSTRs) with

reduced operational complexity compared to MBRs should be considered [7].

To address the above, this study investigated the feasibility of EPS production from VFA-rich wastewater using MMC in an MBR and CSTR configuration at short SRT and nitrogen limitation previously shown to promote EPS synthesis from glycerol [9] (**Chapter 2**). Furthermore, the selectivity of the process towards EPS by tracking PHA synthesis was assessed, and EPS physicochemical and flocculation properties were analysed in time. The results show that EPS flocculant synthesis can be extended to VFA-rich wastewaters and highlights the potential of these biopolymers for sustainable wastewater treatment.

3.2 Materials and Methods

3.2.1 Experimental Design

Two sets of experiments were conducted using VFA-rich synthetic wastewater. In the first experiment (Exp_1), two types of bioreactors were investigated – a CSTR with a volume of 4 L and an MBR with a volume of 3.3 L. The MBR and CSTR were chosen to study VFA conversion efficiency and EPS yields from the same start-up inoculum in different reactor configurations under nitrogen-limited conditions and a short solids retention time (SRT). The flocculation performances of the EPS recovered from the two different lab-scale settings were also evaluated. In the second experiment (Exp_2), the reactor type with superior VFA conversion and higher EPS yield, namely the CSTR, was selected to further investigate the routes for biopolymers production (PHA and EPS) under the specified conditions.

Source of inoculum and feed composition

The reactors were inoculated with 4 g L^{-1} of volatile suspended solids (VSS) of activated sludge from a municipal wastewater treatment plant located in Leeuwarden (The Netherlands). This treatment plant receives municipal wastewater mixed with an effluent from dairy production. Further information on inoculum is provided in **Chapter 2**.

The reactors were fed with synthetic wastewater, containing a mixture of volatile fatty acids (VFAs) as a carbon and energy source, and NH_4Cl as main

nitrogen source. The VFA mixture was composed of sodium acetate, sodium propionate, and sodium butyrate at a COD ratio of 60:20:20, as previously reported for VFA composition in fermented excess sludge [26]. Detailed composition of the nutrient medium is available in the supplementary information (SI-1). Nitrogen-limited conditions were applied by adjusting the influent COD to total nitrogen ratio (COD:N) to 100. This value was selected based on **Chapter 2** and previous EPS-overproduction studies, in which COD:N ratios in the range of 20–100 were evaluated and COD:N = 100, combined with short SRT, was found to promote stable EPS overproduction under nitrogen-limited conditions [20]. Influent COD concentrations applied at different time periods are reported in Table S2.

Reactor set-up and operation

Operational conditions for Exp_1 and Exp_2 are provided in supplementary information (Table S1). In both experiments, the reactors were initially operated in batch mode 24 h using nitrogen-sufficient feed (COD:N 20) and subsequently switched to continuous operation and supplied with nitrogen-limited feed (COD:N 100). The hydraulic retention time (HRT) was maintained at 72 h in CSTRs and 12 h in MBR, and SRT was 72 h in all reactors throughout the experiment (Table S1). In the MBR, HRT and SRT were decoupled by membrane retention of biomass. HRT was controlled through the influent flow rate as follows:

$$Q_{\text{in}} = \frac{V}{\text{HRT}} \quad \text{(Eq. 1)}$$

Where, Q_{in} is the influent flow rate (L h^{-1}), V is the reactor volume (L) and HRT is the hydraulic retention time (h).

The SRT was controlled independently by mixed liquor withdrawal flow rate:

$$Q_{\text{wasting}} = \frac{V}{\text{SRT}} \quad \text{(Eq. 2)}$$

Where, Q_{wasting} is the flow rate at which mixed liquor is wasted from the MBR (L h^{-1}), V is the reactor volume (L) and SRT is the solids retention time (h).

The control of SRT is possible because the membrane prevents washout of suspended solids and allows permeation of treated effluent. The membrane surface was cleaned daily to prevent biofilm formation, and the removed material was thoroughly mixed back into the mixed liquor before sludge wasting to ensure

accurate control of SRT.

The reactors were operated in two sequential phases with different organic loading rates (OLR), hereafter referred to as OLR 2 and OLR 1, corresponding to 2 and 1 g COD L^{-1} d^{-1}, respectively, while keeping the HRT and SRT constant. The lower OLR was included to evaluate the effect of reduced substrate loading on reactor performance and EPS production. These conditions have been found suitable to achieve EPS overproduction in a recent study [9]. Detailed information on flow rates, pH, F/M ratios and various flow settings is provided in the supplementary information (SI-2).

3.2.2 Analytical techniques

Reactor Performance

VFA conversion rates were regularly monitored by measuring influent and effluent VFA concentrations using ion chromatography (930 Compact IC flex, Metrohm, CH); as described in the supplementary information (SI-3). Influent samples were directly filtered through a H-PTFE 0.45 μm syringe filter (Whatman, The USA) before analysis. For effluent samples, 5 mL of mixed liquor was collected from the reactor and sequentially filtered through 0.45 μm and 0.2 μm syringe filters prior to ion chromatography analysis, ensuring the removal of any suspended solids. Chemical oxygen demand (COD) of influent and effluent samples was measured on filtered samples using the COD Cuvette Tests (LCK, Hach Lange GmbH, DE) to calculate soluble COD (sCOD) removal. Soluble COD is defined here as the fraction of COD that passed through a 0.2 μm filter. Mixed liquor volatile suspended solids (MLVSS) and volatile solids (VS) concentrations were analysed according to standard methods [27].

EPS extraction and Characterization

EPS were extracted from mixed liquor samples using the alkaline-heat method as described by Felz et al. (2016) [28] with minor modifications. A 50 mL sample was first centrifuged at 25,000 $\times$ g for 15 min at 20 °C and the supernatant was collected as the soluble EPS (sEPS) fraction. The pellet was then resuspended in 50 mL of Milli-Q® water, and 0.29 g of Na_2CO_3 was added. The mixture was heated and stirred at 80 °C, 500 rpm for 1 h. Following this, the sample

was centrifuged at 20,000 × g for 15 min at 20 °C, and the supernatant was collected as the bound EPS (bEPS) fraction. Both EPS fractions were dialyzed overnight at room temperature (RT) against deionized water using SnakeSkin^{TM} dialysis tubing (MWCO 3.5 kDa, Thermofisher Scientific, USA). EPS content of the mixed liquor was expressed as g of VS EPS per g MLVSS. Dialyzed EPS samples were freeze-dried and stored at room temperature for further analyses.

For characterization, freeze-dried EPS were resuspended in Milli-Q® at a concentration of 1 mg mL^{-1}. Protein quantification was conducted using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions, using bovine serum albumin as a standard for calibration. Polysaccharides were quantified using the phenol-sulfuric acid method, measuring the absorbance at 490 nm and using D-glucose as a standard [9]. Average molecular weight of EPS was determined from freeze-dried EPS extracts using liquid chromatography-organic carbon detection (LC-OCD-Model 8, DOC-LABOR, DE) as described by Ajao et al. (2019) [9].

PHA quantification

PHA quantification was carried out using thermogravimetric analysis (TGA) (TGA 2, Mettler Toledo, CH) on freshly collected mixed liquor samples. Before analysis, samples were acidified to pH 1.8–2.0 by adding sulphuric acid (96%) and mixed at 800 rpm for 15 min. The samples were then centrifuged at 3,750 × g for 15 min, and the resulting pellet was dried overnight at 105 °C. The dried pellet was ground into a fine powder, and 4 mg of the sample was analysed using TGA, as described by Werker et al.(2023) [29]. PHA content of the mixed liquor was expressed as g VS PHA per g MLVSS.

3.2.3 EPS staining and Microscopy observation

Optical microscopy

As a quick indicator for EPS production, fresh mixed liquor samples were regularly observed using an optical microscope (Leica DM750, Germany) equipped with a Leica ICC50 W camera. For visualisation of the protein- and polysaccharides-rich EPS portions, crystal violet 0.1% and alcian blue 1% were applied, respectively, as described in **Chapter 2**.

Confocal laser scanning microscopy

Fresh mixed liquor samples were collected at various time points from the bioreactors to monitor the temporal production of PHA and EPS. The intracellular PHA granules were identified using BODIPY® 493/503 (ThermoFisher Scientific, USA), following the protocol described by Pei et al. (2022)[30]. The protein portion of the EPS and the cell walls were visualised with SYPRO™ Red (5000× concentrate in DMSO; ThermoFisher Scientific, USA), while the β-linked polysaccharide fraction of the EPS was identified using Calcofluor White (CFW) (Merck, DE). The staining of the three portions was carried out in series.

The mixed liquor samples were centrifuged at $21000 \times g$ for 1 min. The pellet was washed 3× and resuspended in 500 μL of Milli-Q water. To 100 μL of the sample, 1 μL of SYPRO™ Red was added and incubated in the dark at RT for 30 min. Then, the sample was centrifuged again for 2 min and washed twice with Milli-Q water to remove excess dye. Next, 10 μL of the sample was placed on a glass slide, and 1 μL of BODIPY® 493/503 (2 ng μL^{-1}) was added. The slide was incubated in the dark at RT for 30 min to allow the droplet to dry. Subsequently, 10 μL of undiluted CFW stain was applied on top of the dried droplet. Finally, the slide was mounted with VECTASHIELD® Vibrance™ Antifade Mounting Medium (Vector Laboratories, USA) and sealed with nail polish before observation with a Zeiss LSM 880 confocal laser scanning microscope (CLSM) (Zeiss, Germany). Image acquisition was done as described by Pei et al. (2022) [30]. Images were captured with a monochrome camera (Axiocam 702 mono, Zeiss, DE) and the software ZEN (Zeiss, DE). Image analysis was performed using Fiji ImageJ. CLSM maximum intensity projections (MIP) were generated with ZEN and used for quantification of the biovolume occupied by proteins, polysaccharides, and PHA expressed as total pixel areas, through Fiji ImageJ, according to Pei et al. (2022)[30].

3.2.4 Flocculation Experiments

The flocculation performance of EPS samples from Exp_1 and Exp_2 was evaluated using kaolinite (Merck KGaA, DE), following the method described in **Chapter 2**. In each flocculation experiment, 0.3 mg EPS were added per g of kaolinite, and $CaCl_2 \cdot 2H_2O$ (Merck KGaA, DE) solution was used to provide 100 mg Ca^{2+} L^{-1} as a bridging agent to the suspension. Statistical analysis was performed using

Microsoft Excel to explore correlations between flocculation efficiency and the chemical composition of EPS (i.e., protein and polysaccharide content). The built-in CORREL function was used to calculate Pearson correlation coefficients, providing a measure of the linear relationship between variables. Correlation strength was interpreted based on r-values, and results are reported to support observed trends in EPS composition and functional performance. Because the sample size was relatively small, the degrees of freedom and statistical power were limited. Hence, these findings were considered exploratory.

3.3 Results and Discussion

3.3.1 Short SRT and nitrogen limitation steered EPS overproduction from VFA-rich wastewater

Results from Exp_1 showed that VFA-rich wastewater can be used as a feedstock to stimulate EPS overproduction in MMCs when they are subjected to nitrogen limitation coupled with short SRT (Fig. 1, 2 and 3). However, reactor configuration (MBR or CSTR) and OLR influenced the VFA conversion rates and EPS yields (Fig. 1). During Exp_1, VFA conversion rates above 80% were consistently observed in CSTR, whereas very low conversions ($< 50\%$) were observed in MBR (Fig. 1A) throughout the entire experiment, regardless of the applied OLR (1 or 2 g COD L^{-1} d^{-1}). This difference is primarily attributed to the shorter HRT applied in the MBR (Table S1), which also resulted in lower biomass concentration, as reflected by the MLVSS analysis (Fig. 1B).

In both reactors, MLVSS concentrations declined during the first 10 days, likely due to microbial adaptation to nitrogen-limited conditions, and stabilised thereafter. At steady state, the CSTR maintained higher biomass concentrations (2-3 g VSS per g mixed liquor) compared to the MBR (0.5-1 g VSS per g mixed liquor) (Fig. 1B). The higher biomass retention in the CSTR was likely supported by its higher VFA conversion performance, while the limited active biomass in the MBR constrained its conversion capacity regardless of OLR.

Considering overall sCOD conversion, a higher portion of COD was converted to EPS in the CSTR compared to the MBR (Fig. 2A and B). This was particularly evident during the period of OLR 2 g COD L^{-1} d^{-1}, where a clear

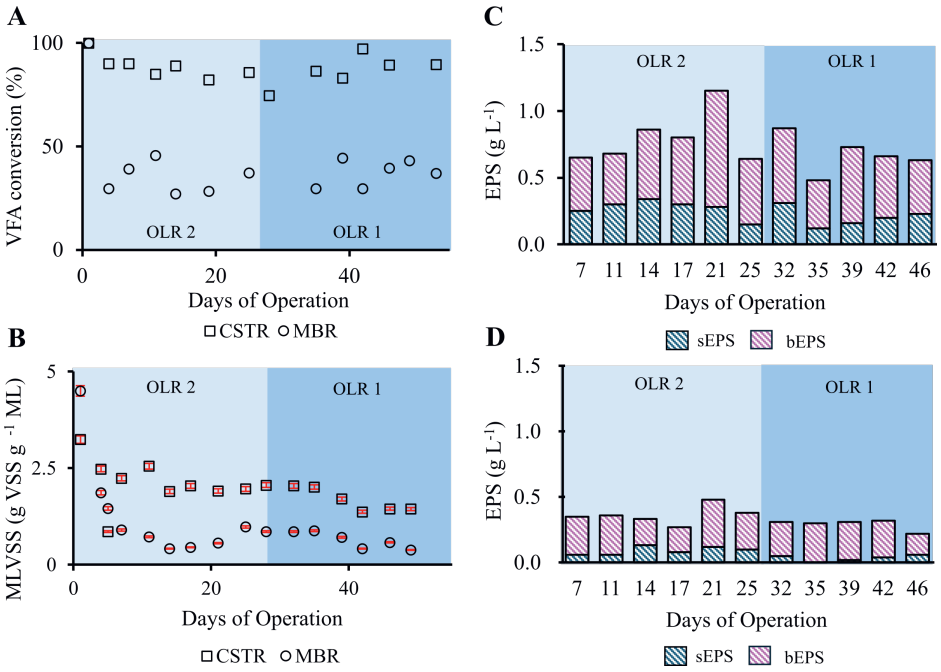


Figure 1: Volatile fatty acid (VFA) conversion efficiencies (A) and mixed liquor (ML) volatile suspended solids (VSS) concentration trends (B) in the continuous-stirred tank reactor (CSTR) and membrane bioreactor (MBR) settings during experiment 1 (Exp_1) at organic loading rate (OLR)s of 2 and 1 g COD L^{-1} d^{-1}. Error bars represent the absolute difference between duplicate measurements ($n = 2$). Concentrations of soluble extracellular polymeric substances (sEPS) and bound EPS (bEPS) during Exp_1 in CSTR (C) and MBR (D) at OLRs of 2 and 1 g COD L^{-1} d^{-1}. Due to the low variability between duplicated samples (SD $< 10\%$), error bars were omitted.

increasing trend of COD conversion to EPS was achieved in the CSTR. EPS yields showed that in the CSTR initially around 20% of the converted COD was allocated to EPS production, and it increased to as high as 70% at the end of this period (Fig. 2A). When OLR was decreased to 1 g COD L^{-1} d^{-1}, the yields were not constant and varied between 40 and 70% (Fig. 2A). In the MBR, the EPS yields fluctuated at around 20% when the applied OLR was 2 g COD L^{-1} d^{-1}(Fig. 2B). Decreasing the OLR from 2 to 1 g COD L^{-1} d^{-1} had a slight positive effect with the COD allocation to EPS increasing towards 40% but still fluctuating (Fig. 2B). Throughout the process, extracted EPS concentrations ranged from 0.55 to 1.10 g L^{-1} in CSTR (Fig. 1C), mostly composed of bound EPS (bEPS $>$ 50%) while in the MBR much lower EPS concentrations ($<$ 0.45 g L^{-1}) were measured (Fig. 1D). Similarly to CSTR, also in the MBR reactor bEPS was the dominant fraction. Thus, while both systems showed bEPS dominance, the CSTR accumulated substantially higher EPS concentrations, highlighting that reactor configuration strongly influences EPS yield and potential for recovery. This is likely attributed to the fact that HRT and SRT are coupled in a CSTR and decoupled in an MBR, thereby allowing for more efficient substrate conversion in a CSTR under nitrogen-limited conditions.

The ratio of VS EPS to MLVSS in the CSTR ranged from 0.17 to 0.7 g VS EPS g^{-1} MLVSS during operation at an OLR of 2 g COD L^{-1} d^{-1}, and narrowed to 0.42–0.48 g VS EPS g^{-1} MLVSS during OLR 1 g COD L^{-1} d^{-1} (Fig. 2C, D), indicating relatively stable EPS fraction within the mixed liquor under lower organic loading (Fig. 2C). In contrast the MBR exhibited a more variable EPS fraction under both OLRs. During operating at OLR 2 g COD L^{-1} d^{-1}, the ratio fluctuated between 0.20 and 0.80 g VS EPS g^{-1} MLVSS, showing an unstable trend, though overall higher than that observed in the CSTR. At OLR 1 g COD L^{-1} d^{-1}, the EPS fraction slightly stabilised, ranging from 0.36 to 0.78 g VS EPS g^{-1} MLVSS (Fig. 2D). These results suggest that, despite the lower VFA conversion efficiency and reduced biomass yield observed in the MBR, the system retained the capacity to produce significant quantities of EPS. In some instances, the high EPS-to-MLVSS ratios imply that a large portion of the biomass was composed predominantly of EPS rather than active microbial cells. This observation aligns with the findings of Zhang et al. (2020) [31], where stress-induced EPS accumulation was associated with reduced active biomass content and impaired substrate conversion. As such, although MLVSS levels were measurable in

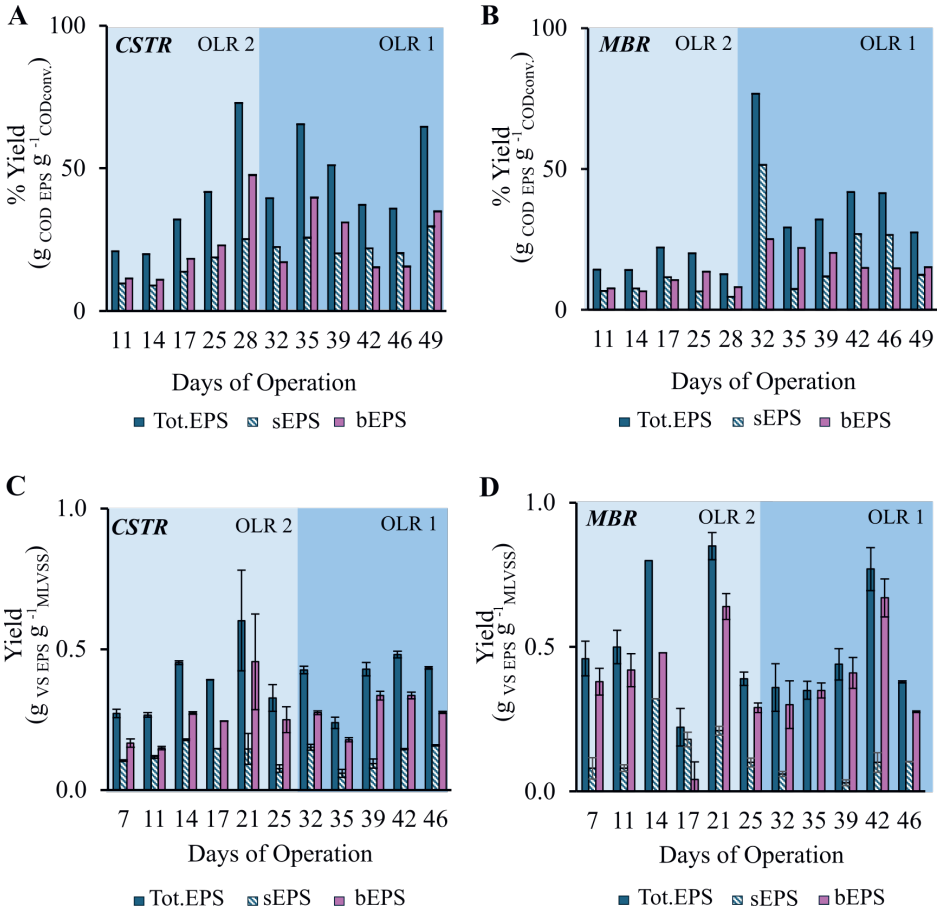


Figure 2: Extracellular polymeric substances (EPS) yields during experiment 1 (Exp_1). In (A) and (B) reported yields as a fraction of converted chemical oxygen demand (CODconv.) in a continuous-stirred tank reactor (CSTR) and a membrane bioreactor (MBR), respectively. In (C) and (D) volatile solids of EPS (VS EPS) are normalised to g MLVSS, showing the fractions of bound (bEPS) and soluble EPS (sEPS) in the mixed liquor of CSTR and MBR, respectively. Total EPS is equal to sum of sEPS and bEPS fractions. Organic loading rate (OLR) 2 and OLR 1 are the applied loading rates in g COD L^{-1} d^{-1}. Error bars represent propagated standard errors from duplicate measurements ($n = 2$). For sEPS and bEPS, errors were calculated using standard error propagation for ratios; for total EPS, using propagation of uncertainty for sums.

the MBR, a large fraction was composed of biopolymers, not contributing effectively to VFA uptake and conversion. This was observed once HRT of the MBR was reduced from 12 h to 8 h.

EPS production during the entire process in Exp_1 was also assessed by crystal violet and alcian blue staining and optical microscopy, showing that already at day 9 (OLR 2 g COD L^{-1} day^{-1}), both reactors exhibited increased EPS overproduction, as indicated by strong alcian blue signal, targeting acidic polysaccharides (Fig. S1). In the following days (day 9 to 42) in the CSTR, both staining signals were observed, indicating a mixed EPS composition, while in the MBR polysaccharides-rich flocs became less prevalent after 9 days of reactor operation. EPS overproduction based on alcian blue signal was particularly evident in the samples taken from CSTR after day 25, during the OLR 1 g COD L^{-1} day^{-1} period.

The above findings in Exp_1 align with previous studies, where application of short SRT (< 3 days) coupled with nitrogen limitation has been found crucial in directing microbial metabolism toward EPS synthesis with glycerol-rich wastewater [9], [20] and with real industrial streams from biodiesel production and potato processing [7]. Hence, in this study we extend this to VFA-rich wastewater in various reactor configurations. The obtained EPS yields here are comparable to those found with glycerol-rich wastewater as substrate. Ajao et al. (2019) reported EPS concentrations of 0.30–0.60 g L^{-1} in MBRs, while in another recent study the highest COD conversion to EPS was ranging in between 20–30% of the total converted COD under similar conditions [9] (**Chapter 2**). In this contribution, EPS concentration ranged between 0.50–1.10 g L^{-1} in CSTR and up to 70% of the total COD consumed was converted into EPS (Fig. 2). Hence, VFAs can serve as an effective carbon source for EPS overproduction with yields as high as those achieved with more commonly reported substrates such as glycerol. It should be noted that the OLR range studied here represents that characteristic to moderate-strength municipal-type streams. Higher OLRs of up to 5 g COD L^{-1} day^{-1} relevant for industrial streams have been shown to be feasible when treating biodiesel wastewater in airlift CSTRs and airlift MBRs under nitrogen-limited conditions and with efficient COD conversion. Further increases of OLR yield extremely viscous, shear-thinning mixed liquor, wherein the aeration and mixing require special attention to avoid oxygen mass transfer limitations [7]. In

this study the concentration of EPS was already relatively high, especially in the CSTR, thereby likely contributing to increased mixed liquor viscosity. Importantly complete VFA conversion was not achieved at the applied OLRs in the present study (Fig. 1A), albeit the removal was high (> 80%) in the CSTR. Therefore, further increasing OLR would likely lead to overloading, especially in the MBR, and may lead to oxygen mass transfer limitations due to increased broth viscosity. In the MBR, operation at higher OLR may require a longer HRT, whereas the CSTR appears to offer greater potential based on its better conversion performance observed here. Nevertheless, the potential of higher OLR operation with VFA-rich wastewater under optimised conditions should be explored in future work.

The differences between EPS synthesis with VFAs and glycerol may stem from the typical carbon metabolism found in microbial cells. Basic building blocks for microbial EPS synthesis are nucleotide sugars, such as guanosine diphosphate-mannuronic acid (as in the case for alginate)[32]. These are synthesised from glucose-6-phosphate and fructose-6-phosphate, the key intermediates of the central carbohydrate metabolism [32]. Glycerol can directly undergo gluconeogenesis to yield glucose-6-phosphate, whereas acetate - the main component of synthetic wastewater fed to the bioreactors in this study - is first converted in an equimolar ratio of acetyl-co-A, without any carbon loss [33]. Subsequently, acetyl-co-A can serve as a precursor of nucleotide sugars by entering the glyoxylate cycle and gluconeogenesis, leading to glucose-6-phosphate [32]. While the conversion pathway of VFAs to EPS precursors is longer, they are actively taken up by microbial cells as opposed to passive diffusion of glycerol. Hence, microbial cells with limited passive uptake may not be able to uptake this substrate as fast as acetate [34].

In Exp_1, the reactor configuration influenced the conversion efficiency of VFAs, an important factor for achieving efficient wastewater treatment alongside EPS synthesis. The same MMC achieved higher VFA conversion, EPS yields, and biomass retention in the CSTR (Fig. 1A and Fig. 1C) compared to the MBR (Fig. 1A and D). Although MBRs are generally designed to retain biomass via membrane separation, the lower biomass concentrations and reduced conversion performance observed in this study underscore the critical role of adequate HRT in enabling efficient substrate utilisation [31]. In this study, SRT was intentionally

set at 72 h and kept constant across both reactors, based on prior work identifying short SRT and nitrogen limitation as key drivers for EPS overproduction [9]. HRT, however, was different in CSTR (72 h) and MBR (12 h). The shorter HRT of the MBR has been previously applied to glycerol-rich wastewater fed MBRs with efficient glycerol conversion [9] (**Chapter 2**).

While operating an MBR with HRT = SRT is theoretically feasible, as it is done in CSTRs without additional sludge retention and recycle, such operating conditions eliminate the key advantage of membrane retention and decreased bioreactor volume, making the system behave more like a conventional CSTR, but with solids-free effluent. Therefore, the MBR was investigated under conditions that preserve its key process advantage, namely membrane-based biomass retention and decoupling of HRT (12 h) and SRT (72 h) in a compact reactor system. Clearly, this approach introduced a confounding factor when comparing CSTR with MBR, as the shorter HRT in the MBR likely limited VFA utilisation and thereby reduced biomass growth and EPS production. Hence, the observed differences between reactors reflect both, the reactor configuration and the operating strategy applied to each system and should not be interpreted as a general superiority of CSTR over MBR. Given these constraints, the MBR likely required a longer HRT to achieve comparable performance to CSTR under nitrogen-limited conditions. However, optimising HRT was beyond the scope of this study. Under the given experimental conditions, the CSTR configuration, where HRT was inherently extended to match the target SRT, proved more effective both for VFA conversion and EPS production. Additionally, differences in hydrodynamics, i.e mixing conditions, may have contributed to these outcomes. The absence of mechanical mixing in the MBR may have limited oxygen transfer efficiency, thereby limiting VFA utilisation, particularly in the highly viscous mixed liquors typical of EPS-producing systems like in this study [34]. These findings suggest that the MBR configuration and operating parameters could be further refined in future studies to achieve higher conversion of VFAs and to exploit the opportunity of producing solid-free effluents.

Nevertheless, based on the above findings, subsequent experiments (Exp_2) were performed in CSTR only, with operational parameters shown to yield effective conversion of VFAs to EPS. These investigations were focused on monitoring

both intracellular and extracellular biopolymers production (PHA and EPS) from VFA-rich wastewater as outlined in the following section.

3.3.2 Long-term CSTR experiments show preferential EPS production over PHA

In Exp_2, the CSTR was considered to have reached a steady state when both the VFA conversion and EPS production became stable, at which point the VFAs conversion rate was ranging between 70-75% (Fig. 3A). An exception is a period between day 46-53, related to technical issues with micronutrients supply. Hence, results of this period will not be further discussed. After day 53, the process performance recovered, and stable VFAs conversion between 70-80% (Fig. 3A) was reached.

Throughout the process, the fractions of PHA and EPS in mixed liquor were compared to assess the conversion of VFAs into intra and extracellular biopolymers. Only at the start-up phase of the process (on day 3) PHA fraction of biomass was nearly as high as EPS (0.23 g VS g^{-1} MLVSS and 0.35 g VS g^{-1} MLVSS, respectively) (Fig. 3B). However, PHA content of the MLVSS showed a decreasing trend over time and stabilised below 0.01 g VS PHA g^{-1} MLVSS already after day 10, irrespective of the OLR applied (Fig. 3B). In contrast, the EPS fraction increased from 0.35 to 0.54 g EPS VS g^{-1} MLVSS in the first 15 days and then fluctuated between 0.40–0.70 g EPS VS g^{-1} MLVSS when a lower OLR was applied (Fig. 3B), confirming the results of Exp_1 (Fig. 2C). Hence, these results indicate that under EPS overproducing conditions, EPS synthesis is always the favoured pathway by MMC in comparison to PHA production. It is known that in PHA-producing MMCs, such high selectivity cannot be achieved, as some part of the carbon is always diverted to EPS [35], making solely EPS-targeted MMCs more efficient.

Quantitative EPS and PHA measurements were supported by CLSM analysis of sludge samples after fluorescent staining, visually confirming that EPS were the predominant biopolymers present throughout the process (Fig. 4). Visible, big PHA granules were identified until day 3, while the majority of the cells was covered by protein-rich EPS throughout the whole process, and in some cases carbohydrate-rich spots were visible (i.e day 46 and 67, Fig. 4). Relative area

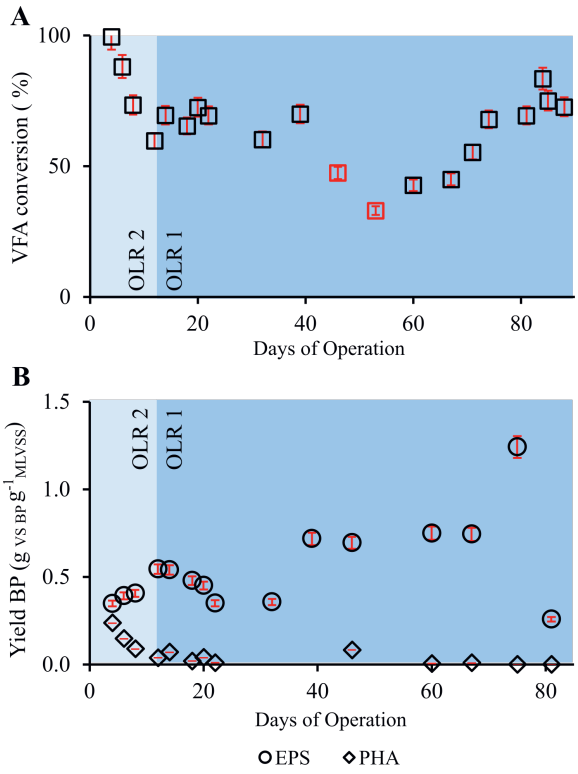


Figure 3: Performances of the continuous-stirred tank reactor (CSTR) fed with volatile fatty acid (VFA)-rich synthetic wastewater during experiment 2 (Exp_2) (A) VFA conversion rates. Red squares show the operational period with technical issues related to nutrient supply. (B) Biopolymers (EPS and polyhydroxyalkanoates (PHA)) yield normalized to mixed liquor volatile suspended solids (MLVSS). Error bars represent the absolute difference between duplicate measurements ($n = 2$).

quantification of EPS and PHA, consistent with the TGA analysis, revealed a decline in the PHA fraction over time (Fig. S2). A strong correlation ($R^2 = 0.97$) between the PHA yield obtained analytically (Fig. 3B) and the relative pixel counts of BODIPY^{TM} 493/503 (representing PHA) was found. In contrast, similar analysis for EPS proved to be more challenging, as indeed the stains applied also identify the cell's outer layer (i.e., Fig. 4, day 22 and 46), making it difficult to differentiate the portions of such complex and heterogeneous biopolymers. Hence, while useful and sufficient for qualitative and visual assessment, this limited the possibility of EPS quantification from the CLSM images.

The above results align with the initial hypothesis and previous studies, showing that nitrogen limitation coupled with short SRT stimulates EPS overproduction [9], [20]. Even if initially, both EPS and PHA-producing bacteria may have coexisted within the CSTR; because the reactors were operated under continuous feeding rather than under deliberately imposed feast/famine cycles, enrichment of PHA-accumulating microorganisms was likely not favoured. Therefore, these organisms were likely gradually outcompeted, allowing EPS producers to dominate the microbial community [36]. Since both EPS and PHA synthesis compete for carbon, it is expected that under conditions favouring EPS production, the available carbon is predominantly directed toward EPS synthesis [37]. This phenomenon was observed by Rojas-Zamora and coworkers, who studied aerobic activated sludge subjected to hydraulic and biological selective pressures to induce sludge granulation, along with a feast/famine regime aimed at selecting PHA producers [38]. Under these conditions, most of the external carbon was diverted towards EPS production rather than PHA. The authors concluded that microorganisms prioritised EPS synthesis to maintain their granular structure at the expense of reduced PHA production [38]. In another study, Rangel et al. (2024) reported the influence of ethanol on carbon diversion in a VFA conversion bioprocess. They observed that an increased EPS yield was accompanied by a decreased PHA yield when using the same carbon source, which contained lactic, butyric, acetic acid and ethanol. The diversion of carbon towards EPS production was particularly evident in the presence of a high ethanol concentration in the feed. This increased EPS yield was interpreted as a stress response to the presence of a toxic compound (ethanol) in the feed, where shielding microbial cells by EPS provided protection [37]. These studies clearly showed that diverse factors such as changing hydraulic parameters or toxicity by a chemical can regulate this

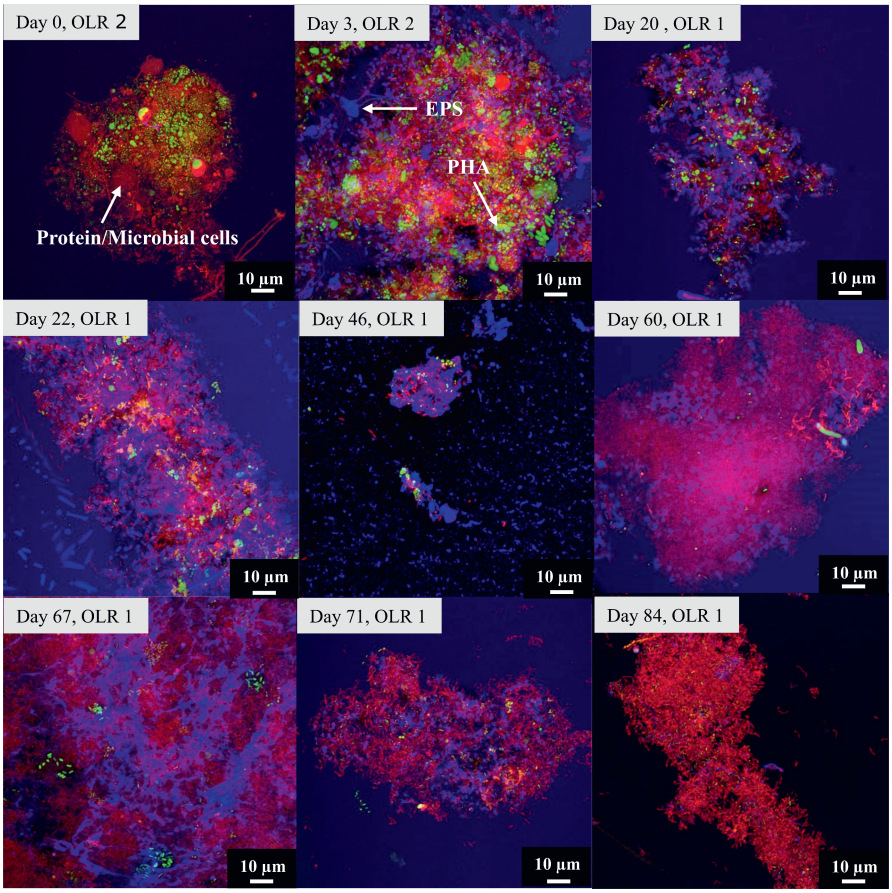


Figure 4: Maximum intensity projection micrographs of mixed liquor samples collected from the continuous-stirred tank reactor (CSTR) at various time points during experiment 2. Samples from Day 0 and Day 3 were taken during the organic loading rate (OLR) 2 period, while the others were taken during OLR 1. In red, proteins in the extracellular polymeric substances (EPS) and cell wall positive to SYPRO^{TM} Red, in blue polysaccharide components of the EPS identified with Calcofluor Wh,ite and in green PHA granules labelled by BODIPY^{TM} 493/503. Yellow spots are due to the superimposition of red and green signals, likely PHA inside protein-rich cells.

switch between EPS and PHA synthesis. However, the crucial point is that these studies collectively underscore the presence of a stress in general when the switch between EPS-PHA occurs. However, a common thread among these studies is the presence of a stress condition, which not only triggers this metabolic shift but also acts as a forcing factor, making EPS synthesis more obligatory, likely as a protective response to environmental constraints. This concept is further discussed in section 3.3.

Results from Exp_2 support this idea, showing that EPS overproduction conditions (nitrogen limitation coupled with short SRT) steered the MMC toward the production of extracellular biopolymers (EPS), rather than intracellular PHA storage (Fig. 3B). These findings suggest that channelling carbon flux toward EPS may be inherently more favourable when EPS is the target product. Most microbial communities naturally exist as flocs, biofilms, or aggregates that rely on EPS for structural integrity. As a result, EPS synthesis is an active part of normal microbial metabolism and can be overstimulated under stress, potentially explaining its persistence even under conditions otherwise favourable for PHA accumulation.

3.3.3 EPS overproduction as a microbial selection mechanism

The mechanisms underlying EPS overproduction are not yet fully understood, but several mechanistic explanations have been proposed. Nitrogen is essential for cell growth and for the synthesis of enzymes and proteins; therefore, its limitation slows cell division and biomass growth. When growth is restricted, the anabolic demand for carbon decreases, and the excess carbon is diverted toward EPS synthesis as an alternative carbon sink [39]. Meanwhile, SRT regulates microbial growth dynamics [40] and, a short SRT imposes strong selective pressure by creating dynamic, fluctuating conditions [41] . Therefore, in continuous operation, the balance between EPS synthesis and biomass proliferation is primarily governed by nitrogen limitation and short SRT. Since short SRT is shown to be required for the process, the key parameters to further control the active biomass concentration in the reactors are the organic loading rate, hydraulic residence time in the case of MBR and the extent of nitrogen limitation.

Under the combined influence of nitrogen limitation, excess carbon, and short SRT, these selective pressures favour fast-growing microorganisms with specific growth rates higher than the washout threshold of about 0.33 day^{-1} (at SRT 3 days) and traits that enhance physical retention within the reactor. In this context, EPS production functions as a trait enhancing retention of EPS producers [38] by promoting bio-aggregation and flocs formation. Microorganisms lacking this trait form weaker aggregates and are consequently more susceptible to washout. Aggregation is further promoted by increased surface hydrophobicity, often conferred by protein-rich EPS matrices, which enhances cell-to-cell and cell-to-floc adhesion. This trait was observed in CLSM analysis (Fig. 4) and is consistent with findings in the literature highlighting the role of extracellular proteins in structural integrity and microbial granule stability [42].

As a result, under these conditions EPS producing microorganisms become enriched [19], while slow-growing, possibly EPS-degrading microorganisms, are outcompeted, and washed out [43]. As a result, the combination of redirected carbon flux under nitrogen limitation and selective pressures imposed by short SRT promotes the enrichment of EPS-producing microorganisms within MMCs. As this study shows, under these conditions, PHA accumulation is not the dominant metabolic route for excess carbon utilisation. While intracellular PHA storage provides an energy reserve at the individual cell level, it does not contribute to biomass retention and thus offers limited advantage under high washout pressure [44] .

3.3.4 Flocculation performances of VFA-derived EPS

In the present study, EPS derived from VFA-rich wastewater exhibited high flocculation efficiency (FE), comparable to that of a commercial synthetic flocculant (anionic polyacrylamide, 92% at the same dosage as EPS) in the presence of calcium. Total turbidity removal reached up to $\sim$ 99% (Fig. 5). These results demonstrate the strong flocculation capacity of the EPS, as the negative control showed very high turbidity (2760 NTU) without addition of any calcium or EPS (Fig. S3). When only calcium was added, acting as a coagulant, turbidity decreased to 285–330 NTU. Importantly, further turbidity reduction below 100 NTU was only achieved when EPS was applied as a flocculant. Similar results were observed with glycerol-rich wastewater-derived EPS in former studies [9],

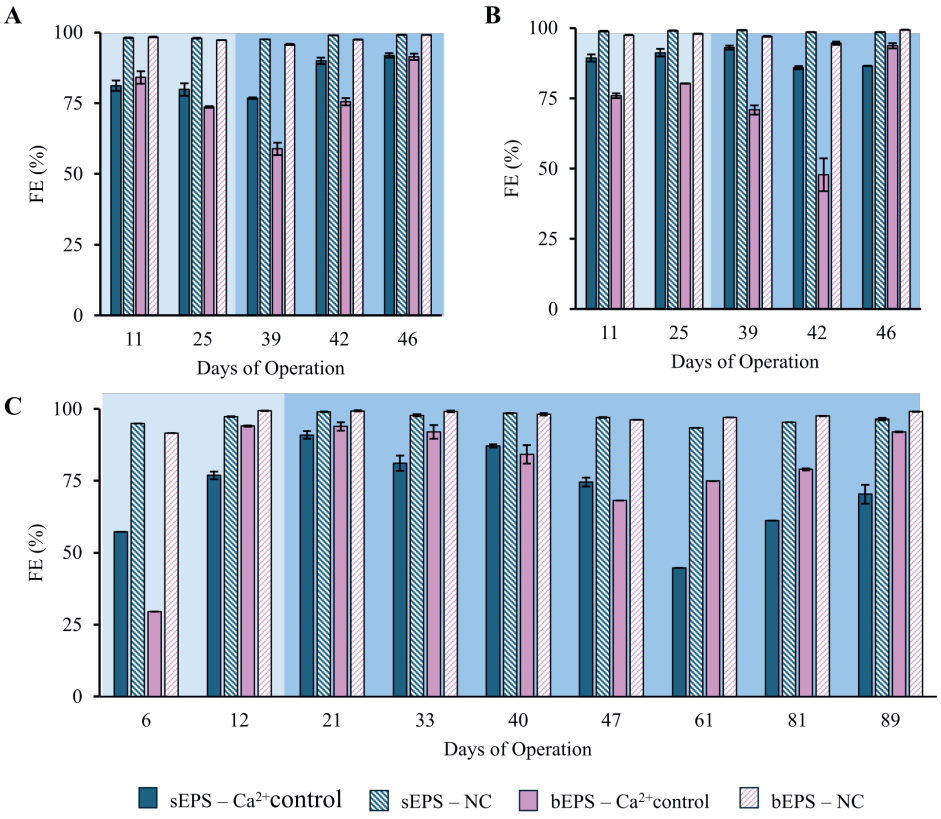


Figure 5: Flocculation efficiency of soluble and bound extracellular polymeric substances (sEPS and bEPS) extracts in in continuous-stirred tank reactor (CSTR) (A) and a membrane bioreactor (MBR) in experiment 1 and CSTR (C) in experiment 2. Light blue boxes represent the organic loading rate (OLR) 2 and dark blue boxes OLR 1 period of the bioreactors. NC is flocculation efficiency relative to a negative control, containing only kaolinite suspension without addition of calcium (Ca^{2+}). The Ca^{2+} control indicates flocculation efficiency relative to kaolinite suspension destabilized with Ca^{2+} but without EPS. Error bars represent the absolute difference between duplicate measurements ($n = 2$).

[45], and recently also with EPS synthesised from real wastewaters [7], [35]. This indicates the potential of VFA-derived EPS as an effective flocculant in various industrial applications.

In *Exp*$_1$, particularly during OLR 2 g COD L^{-1} d^{-1}, EPS extracts from both the CSTR and MBR exhibited high FE of over 90% (Fig. 5A and B) relative to Ca^{2+}-Control, while higher FEs ranging from 94% to 99% were obtained compared to negative control (NC) which represents the total NTU removal efficiency of the EPS samples. The highest FE relative to Ca^{2+}-Control observed during this phase was 94%, exhibited by the bEPS fraction from the MBR. Interestingly, the EPS samples that showed high FE (> 90%) relative to Ca^{2+}-Control were both from sEPS and bEPS fractions. This suggests that both fractions of EPS can be utilised as effective bio-flocculants, offering potential advantages in downstream processing, particularly as sEPS can be more readily recovered from the mixed liquor.

During OLR 1 g COD L^{-1} d^{-1}, EPS extracts from the MBR exhibited superior FE compared to those from the CSTR. The highest FE relative to Ca^{2+}-Control in the MBR reactor was 91%, achieved by the sEPS fraction (Fig. 5B), while the highest FE in the CSTR reactor was 84%, achieved by the bEPS fraction at day 39 (Fig. 5A). Hence, these results highlight the need to balance EPS yield and flocculation efficiency and indicate potential for further optimisation of MBR performance.

In *Exp*$_1$, some EPS samples exhibiting high FE in the presence of Ca^{2+} cations were protein-rich, while others were more polysaccharide-rich (Fig. 6A and B). Notably, certain samples, particularly from the MBR, showed exceptionally high polysaccharide content. This may be attributed to potential interference between EPS-associated compounds and the colorimetric assays used for EPS quantification, leading to overestimation [28]. The average molecular weight (MW) of the EPS varied widely, from 0.17 MDa to 3.70 MDa (Fig. 6D), and EPS with both high and low MW exhibited high FE. For instance, some EPS with MW of 2.70 MDa displayed higher than 90% FE, while others with a low MW of 0.34 MDa showed similar excellent flocculation performance (Fig. 5A and B and Fig. 6D). Future studies should clarify whether the flocculation mechanisms of these EPS are similar, i.e., whether the smaller MW EPS rely on electrostatic patching and the larger MW EPS on bridging.

In *Exp*$_2$, bEPS fractions from the CSTR exhibited consistently high FE (> 90%) from day 12 to day 33 relative to Ca^{2+}-Control (Fig. 5C), while sEPS

fractions mostly exhibited moderate FE (70–90%). FEs compared to NC were higher ($\sim$ 99%) for all samples. Decreasing OLR from 2 to 1 g COD L^{-1} d^{-1} did not influence FE of bEPS fraction as it showed only negligible changes until day 40. In contrast, sEPS fractions demonstrated higher FE during OLR 1 g COD L^{-1} d^{-1}, particularly between days 12 and 40. After day 40, FE for both sEPS and bEPS fractions dropped below 80%. This reduced performance could be a result of the technical issues encountered with nutrient dosing (See section 3.1). In Exp_2, EPS exhibiting FE higher than 90% contained slightly more polysaccharides than proteins (Fig. 6C). The average molecular weight of EPS samples mostly varied between 1–1.5 MDa showing a more stable trend compared to Exp_1 (Fig. 6E).

A notable finding across both experiments was the higher protein content in VFA-derived EPS, as indicated by a PS:PN ratio ranging from 1 to 2.9 depending on the reactor and EPS type (Fig. S3), compared to glycerol-derived EPS, where PS:PN ratio ranged between 1.2 to 6.8 [9], which may have contributed to the improved FE observed. However, no strong correlation was found between FE and the physicochemical properties of the EPS. The polysaccharide content showed the strongest (though still weak) positive correlation with FE (r = 0.22, n=36), while protein content (r = 0.14, n=36) had a minor positive effect.

Polysaccharides have been reported as key drivers of flocculation by several researchers in previous studies, likely due to the presence of anionic functional groups (hydroxyl (-OH), carboxyl (-COOH), and sulphonate ($-SO_3^-$) [46]. By interacting with surrounding cations, these functional groups can aid better flocculation [47]. Proteins, on the other hand, may contribute to flocculation through several mechanisms. For instance, some proteins carry positively charged amino acids (arginine, lysine) that can neutralise the surface charge of the colloidal particles and promote aggregation [48]. Others may have hydrophobic regions that interact with hydrophobic particles, enhancing their flocculation. Several studies have reported variations in FEs and the physicochemical composition of EPS. In some cases, high FEs were observed in EPS rich in polysaccharides [35], [48], while in others, EPS comprised a mixture of polysaccharides, proteins, and other components [49]. These studies highlight the significant roles of both polysaccharides and proteins in the flocculation process. In this study, many EPS samples with varying physicochemical compositions were tested for flocculation. Although no strong relationship was found between FE and polysaccharide/protein content,

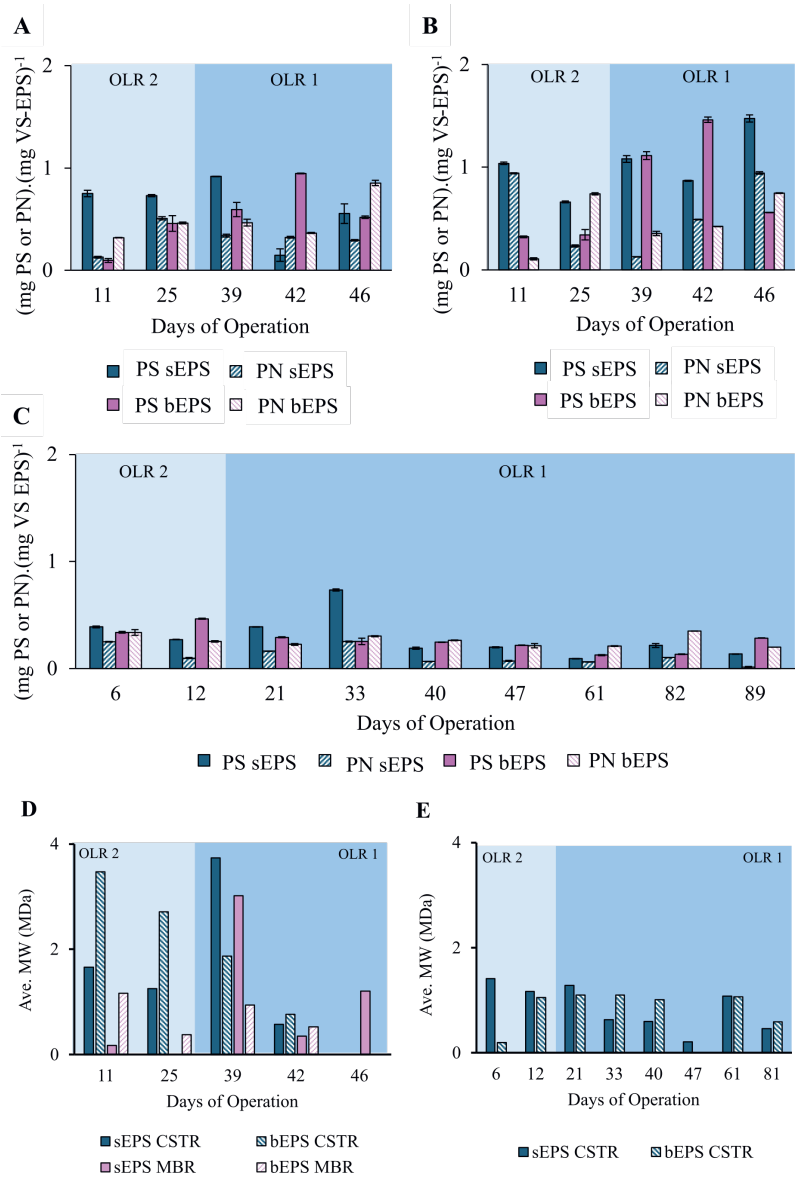


Figure 6: Polysaccharides (PS) and proteins (PN) content of soluble and bound extracellular polymeric substances (sEPS and bEPS) extracts presented as mg PS or PN in mg volatile solids (VS-EPS) in a continuous-stirred tank reactor (CSTR) (A) and a membrane bioreactor (MBR) (B) from Exp_1 and in CSTR(C) from Exp_2. Error bars represent the absolute difference between duplicate measurements ($n = 2$). Average molecular weight (MW) of EPS samples extracted from either CSTR and MBR and used in flocculation experiments from experiment 1 (D) and experiment 2 (E).

the presence of several functional groups and net negative charge (Fig. S3) provided by polysaccharides and proteins likely contributed to the observed results.

In addition to chemical composition, the origin of EPS (soluble or bound) was also investigated for its influence on FE. No consistent differences were observed between the sEPS and bEPS fractions, as both fractions showed the potential for high and moderate FE at different time points. This variability occurred despite the reactors operating under constant conditions during the OLR 2 g COD L^{-1} d^{-1} and OLR 1 g COD L^{-1} d^{-1} periods. These findings suggest that temporal changes in EPS characteristics occurred throughout the experimental period. This most likely happens due to the microbial metabolism shift overtime depending on the environmental conditions prevailing in the bioreactors [50]. Because both fractions could flocculate, either can be used as a flocculant without significant differences in performance. This offers potential advantages in downstream processing, particularly as sEPS can be more readily recovered from the mixed liquor.

A similar trend was observed with respect to the average molecular weight of EPS. Both high and low MW EPS fractions could achieve high FE (Fig. 5 and Fig. 6D, E), complicating understanding of the direct influence of MW on FE. In Exp_2, certain EPS samples with an average MW below 0.60 MDa exhibited poor FE. However, in Exp_1 EPS fractions with comparable average MW were still able to achieve high FE. These findings are partially consistent with those of Ajao et al., who reported poor FE ($< 25\%$) for low MW EPS on kaolinite and montmorillonite. They suggested that short polymer chains may be trapped within the electrostatic repulsion layer, limiting their ability to form bridging interactions between particles. In that study, molecular weight was identified as a key factor influencing flocculation, particularly when bridging was the dominant mechanism [21]. This also agrees with recent findings by Contreras-Davila et al.,(2025) [7].

This limited correlation may be attributed to the generally high flocculation efficiencies observed across all EPS samples, which may have masked subtle compositional effects. To better distinguish the influence of specific physicochemical properties on flocculation performance, future experiments could be conducted at lower particle concentrations, where reduced particle–particle interactions would

make coagulation–flocculation more challenging. Under such conditions, the contribution of EPS composition to flocculation efficiency may become more apparent. Therefore, while these findings highlight the potential of diverse EPS types derived from VFA-rich wastewater as effective bioflocculants, further targeted research is needed to explain the flocculation mechanisms and relationships between EPS characteristics and flocculation properties.

3.3.5 The future prospects of EPS flocculants from VFA-rich wastewaters

Flocculants are broadly used in e.g., water and wastewater treatment, dredging, and the food industry to effectively remove suspended colloidal particles. Currently, mostly synthetic flocculants, such as PAM are applied due to their efficacy and lower price compared to, e.g., biobased alternatives. Hence, the market value of PAM alone was estimated at 4.7–6.3 billion in 2022 and with a projected annual increase of 5% between 2023–2030 and with applications in water treatment contributing to about 35% of the demand [51]. However, such chemicals are fossil-based and are receiving increased scientific scrutiny regarding their environmental fate, persistence, and toxicity of breakdown products [52].

Therefore, non-toxic EPS flocculants as obtained from VFA-rich wastewaters in this study offer obvious advantages and opportunities. As a wastewater-derived product generated in parallel with the wastewater treatment process itself, EPS have the potential to provide a more sustainable alternative to conventional synthetic flocculants, while increasing the value recovered from carbon-rich waste streams and enhancing the overall sustainability of the treatment process.

An argument could also be made that EPS must be more cost-effective compared to synthetic flocculants when considering wastewater treatment as a must. Furthermore, the reduced mineralization of the substrate could even reduce the aeration costs related to the treatment. Hence, further work should look for concrete applications, where wastewater-derived flocculants fit from a legislative and safety perspective e.g., by producing the flocculants by the same industry which will apply them.

From a technical perspective the maximum productivity of 0.4 g L^{-1} d^{-1} reported in this study could be further increased, and consistent flocculation on real wastewater streams should be demonstrated. Furthermore, the high EPS yields in this study were obtained with synthetic fermented wastewater, but these could be different when the complexity of real wastewaters is introduced. For example, it has recently been shown that changing from pure glycerol to glycerol-rich biodiesel wastewater reduced the EPS yield from 40% to 30%, probably due to presence of long-chain fatty acids [7]. To that end real fermented wastewater streams should be used to compare the obtained EPS yields with the results reported here.

3.4 Conclusions

This study shows that conditions previously optimised for glycerol-based extracellular polymeric substances (EPS) overproduction (**Chapter 2**) also drive EPS synthesis from volatile fatty acid (VFA)-rich wastewater. Under these conditions, carbon was mainly channelled to extracellular polymers rather than intracellular polyhydroxyalkanoates (PHA) storage. Although EPS and PHA were initially detected, the lack of selective enrichment for PHA-storing microorganisms likely favoured EPS accumulation over time. This shift supports targeted valorisation of VFA-rich waste streams with mixed microbial cultures, converting waste carbon into functional biopolymers and shaping EPS properties relevant to flocculation. VFA-derived EPS were high-molecular weight, anionic, and relatively more protein-rich achieved $>$ 90% flocculation, comparable to polyacrylamide (92%). As this study was conducted with synthetic VFA-rich wastewater, validation with real wastewater is needed to confirm process robustness under more complex and variable conditions. Furthermore, links between EPS overproduction and microbial community dynamics should be made, and flocculation across particles with varied surface properties investigated.

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Chapter 4

Microbial community drivers of EPS overproduction from glycerol- or VFA-rich wastewater under nitrogen limitation across different reactor configurations

A modified version of this chapter is in preparation for publication: B. Kisaoglan, H.P.J. van Veelen, H.H.M. Rijnaarts, D. Sudmalis, M.C. Gagliano, "Microbial community drivers of EPS overproduction from glycerol- or VFA-rich wastewater under nitrogen limitation across different reactor configurations".

Abstract

Mixed microbial cultures (MMCs) offer a promising route for producing extracellular polymeric substances (EPS) from wastewater-derived substrates, but little is known about the microbial communities that develop during EPS-overproduction processes. This study investigated MMC development in seven bioreactors operated under EPS-overproduction conditions using either glycerol- or volatile fatty acid (VFA)-rich synthetic wastewater. The reactors differed in inoculum, configuration, substrate type, organic loading rate, and operational duration. Across the glycerol-fed membrane bioreactors, EPS-overproduction conditions consistently stimulated EPS production for all different configurations and enriched communities containing both putative EPS producers and EPS degraders. Recurrent EPS-producing taxa included *Brevundimonas*, *Microbacterium/ Microbacteriaceae*, *Pseudoxanthomonas*, *Novosphingobium*, *Asticcacaulis*, and *Derxia*, while taxa such as *Saprospiraceae, Pseudoclavibacter, Bdellovibrio,* and *Terrimonas* were associated with EPS turnover. Direct start-up under nitrogen limitation resulted in comparable EPS yields, indicating that acclimation was not required. In VFA-fed systems, configuration strongly shaped community assembly and process performance. The continuous stirred-tank reactor achieved higher COD removal and EPS yields than the membrane bioreactor, despite lower bacterial diversity. VFA-fed reactors enriched taxa potentially involved in both EPS and polyhydroxyalkanoates (PHA) metabolism, including *Chryseobacterium, Flavobacterium, Brevundimonas, Pseudoxanthomonas,* and *Acinetobacter,* together with polymer-degrading and predatory groups such as *Microscillaceae, Kapabacteriales, Micavibrionales*, and *Bdellovibrio*. Generally, EPS production in MMCs was not linked to one dominant taxon but appeared to be a community-level function supported by multiple EPS-producing taxa at moderate abundance. These findings highlight the ecological complexity of EPS-overproducing MMCs. Further delineating the active roles of EPS producers, EPS degraders, and polymer-turnover microorganisms, as well as their contributions to the overall functionality of strong EPS-producing reactor communities, requires approaches beyond 16S rRNA gene profiling.

4.1 Introduction

Extracellular polymeric substances (EPS) are high-molecular-weight biopolymers secreted by microorganisms that facilitate adhesion, aggregation, energy storage, and protection against environmental stresses [1]. Microorganisms may overproduce EPS in response to stressors such as salinity [2], nutrient limitation, and pH fluctuations [3]. This stress-induced EPS secretion creates opportunities to deliberately steer microbial communities towards EPS overproduction and to use the resulting polymers as bio-based products in various applications [4]. Microbial EPS produced under sterile conditions by single-strain bacteria already find application as thickeners, gelling agents, and stabilisers in several industrial sectors [5], [6], [7]. In water and wastewater treatment, EPS have recently gained attention for their application as bioflocculants [8], [9], [10]. Their high molecular weight, abundance of charged functional groups, and biodegradability make them promising and potentially more sustainable alternatives to synthetic flocculants [9], [11], which have increasingly been associated with environmental and health concerns [12].

However, for EPS-bioflocculants to become viable at scale, both production and flocculation performance must be reliable and controllable. To date, most EPS-bioflocculants studies have focused on EPS produced by pure cultures [13], [14], [15], [16]. These EPS can vary widely in chemical composition and molecular weight [17],[18], and although pure cultures can deliver consistent products, their reliance on sterile operation and costly substrates makes large-scale implementation economically and technically challenging [19].

To overcome the limitations of pure cultures and enable more sustainable, cost-effective production of EPS bioflocculants, the use of natural mixed microbial cultures (MMCs) has recently been proposed [19], [20], [21]. MMCs are typically sourced from man-made biotechnological systems (municipal/industrial wastewater treatment bioreactors) where naturally occurring microorganisms find suitable habitats and, over time, develop into stable microbial consortia aggregating in biofilms or flocs/granules harbouring diverse EPS- and aggregation-forming populations [22]. MMCs do not require sterilisation, allowing the use of variable organic-rich waste streams as substrates while inherently producing EPS for their aggregation [23]. Consequently, direct recovery of EPS from MMC-based systems

has been explored, including from excess activated sludge [24], [25], [26] as well as through the integration of EPS production within existing wastewater treatment processes [27], [28], [29]. While existing MMCs are effective EPS-producing consortia, their variability may negatively affect EPS yields and chemical composition. Therefore, in practice, a dedicated process utilizing these MMCs must be developed to produce high quantities of EPS with consistent quality. In this context, specific bioprocesses that apply stress factors to MMCs to induce EPS overproduction have been proposed as a feasible strategy to increase yields and support more consistent bioflocculant production [29], [30], [31]. For instance, nitrogen limitation stimulates EPS production by MMCs in many natural and man-made settings, such as soil [32], rhizosphere [33], aerobic granules [34] or biofilm reactors [35]. Such limitation activates the secretion of polysaccharide-rich EPS, as excess COD is channelled toward carbohydrate storage rather than growth [36]. In many cases, EPS derived from MMCs have shown flocculation efficiencies comparable to those of EPS from pure cultures and even commercial synthetic flocculants [27], [29]. For example, Ajao et al. (2018) demonstrated that EPS-bioflocculants produced during the treatment of both fresh and saline biodiesel wastewater flocculated kaolinite suspensions under non-saline and saline conditions with efficiencies up to 89% and 97%, respectively, comparable to a commercial anionic synthetic flocculant, polyacrylamide (PAM), at lower dosages [27].

Despite promising results reported in previous studies [27], [29], [30], achieving consistent EPS yields and quality with MMCs remains challenging because reactor operation governs microbial selection. Parameters such as reactor configuration [37], solids retention time (SRT) [38], hydraulic regime [39], carbon-to-nitrogen (COD:N) ratio, and carbon-source type shape the community and thereby control which biopolymers are produced, in what amounts, and with which functional properties. The carbon source can be a particularly strong selector towards microorganisms with different metabolic guilds and can shift EPS yields and physicochemical properties relevant to flocculation (e.g., charge density and molecular-weight distribution) [40], [41], [42]. At the same time, this responsiveness provides an opportunity to steer MMCs towards producing the desired products. In terms of EPS-bioflocculants, the production of high-molecular-weight, long-chain, and sufficiently charged (often anionic) polymers is desired [43], [44]. A variety of studies on pure-culture bioflocculant-producing microorganisms were analysed by Ben Rebah et al. (2018) [45], showing that many

high-performing EPS-bioflocculants (>80% flocculating activity) contained both polysaccharides and proteins, but were often polysaccharide-rich. These studies can serve as a benchmark for adjusting process conditions to obtain an optimal MMC community composition for EPS synthesis.

In the previous chapters (**Chapters 2 and 3**), we demonstrated that nitrogen limitation (COD:N 100) coupled with short SRT (3 to 1.5 days) can be used to enrich MMCs that overproduce EPS from glycerol substrate in continuously operated membrane bioreactors(MBRs) (**Chapter 2**). These so-called EPS-overproducing conditions were then applied in continuous stirred tank reactor (CSTR) and MBR configurations fed with VFAs to cultivate MMCs with similar functionality (**Chapter 3**). In all cases, the recovered EPS exhibited relatively high flocculation efficiencies (80-95%), while varying in their polysaccharide-to-protein composition [29].

This study investigates the microbial communities associated with seven EPS-overproducing bioreactors operated under different conditions across four experiments. The reactors differed in configuration (MBR vs CSTR), inoculum type (municipal vs industrial aerobic sludge), substrate (glycerol-rich vs VFA-rich synthetic wastewater), COD:N ratio, and SRT applied. Using 16S rRNA gene amplicon sequencing at selected time points, we combined microbial community data with process performance to assess which microbial groups from different inocula were enriched under EPS-overproducing conditions with different substrates and process duration. While extensive scientific literature is available on MMCs involved in the production of other biopolymers, such as polyhydroxyalkanoates (PHA), to the best of our knowledge, no previous study has investigated microbial community assembly under EPS-overproducing conditions. Such insight may support the rational design and optimization of scalable and reliable EPS-bioflocculant production processes.

4.2 Materials and Methods

4.2.1 Bioreactor setup and experimental design

Seven bioreactors were utilized to investigate EPS overproduction under nitrogen-limited conditions during four separate experiments (Exp_1,Exp_2, Exp_3 and Exp_4),

and the main operational parameters of these reactors are summarized in Table 1. The reactors used had two different configurations, either a membrane bioreactor (MBR) or a continuous stirred tank reactor (CSTR), and they were inoculated with two different inoculum types, one originating from a wastewater treatment plant (RWZI, Leeuwarden, NL) treating municipal and dairy wastewater, and another one from an installation treating industrial wastewater (RWZI-Bath, Rilland, NL) [29]. During the experiment, the reactors were fed with synthetic wastewater containing either glycerol or a mixture of VFAs (acetate/butyrate/propionate) as substrate [29], [31]. Details of these experiments are described in **Chapter 2** and **3**.

Experiment 1- Microbial community development under nitrogen limitation and short SRT in MBRs

In experiment 1 (Exp_1), two MBRs were operated in parallel to determine optimal EPS-overproduction conditions in terms of applied carbon to nitrogen (COD:N) ratio and solid retention time (SRT). Reactor R1 was inoculated with municipal sludge and R2 with industrial sludge, and both were fed with glycerol-rich synthetic wastewater (SI-1). The reactors were operated for 105 days with decreasing nitrogen supply (COD:N 20, 60 and 100) and a decreasing SRT of 15, 6 and 3 days at an organic loading rate (OLR) of 2 g COD L^{-1} d^{-1}. Detailed operational parameters are reported in Table S1 and in **Chapter 2**. To monitor the evolution of the microbial community from the two different start-up inocula, microbial community analysis was carried out by 16S rRNA gene amplicon sequencing (as described in section 2.3) on multiple samples; the initial inoculum, and sludge sampled at day 75 (end of COD:N 60/SRT 6 period) and day 105 (end of process, COD:N 100/SRT 3).

Experiment 2 - Development of microbial community from different inocula under direct application of EPS-overproduction conditions

In experiment 2 (Exp_2), two MBRs, R3 and R4, were inoculated with the same source of municipal and industrial sludge inocula as in Exp_1, respectively. However, the inoculum samples were collected in different seasons. The MBRs were fed with the same glycerol-rich synthetic wastewater and operated for 42 days. Based on the results collected in Exp_1, the reactors were directly started applying nitrogen limitation (COD:N 100) while SRT was decreased from 6 (until day 12)

to 3 (until day 34) and 1.5 days until the end of the process (Table S1, **Chapter 2**). The microbial community development was monitored by comparing the inoculum and the sample taken at day 34 (end of SRT 3) and day 42 (end of SRT 1.5 and of the process).

Experiment 3 - Microbial community development from VFA-rich feed under EPS-overproduction conditions

To test whether the same EPS-overproduction conditions applied in Exp_1 and Exp_2 would also be effective using VFAs instead of glycerol, in Experiment 3 (Exp_3), an MBR (R5) and a CSTR (R6) were operated using the same municipal sludge inoculum (Table 1). The reactors were operated under identical conditions using the same VFA-rich synthetic wastewater (containing a mixture of acetate, propionate, and butyrate at a COD ratio of 60:20:20) (SI-2) for 46 days. The initial OLR was 2 g COD L^{-1} d^{-1} which was decreased to 1 g COD L^{-1} d^{-1} at day 25. Operational details are provided in Table S2 and in **Chapter 3**. Microbial community development in both reactors was analysed to assess how reactor configuration influences both microbial community structure and VFA conversion under EPS-overproduction conditions from the same inoculum. To this end, the inoculum and the sludge taken at the end of the reactors' operations (day 46) were collected and analysed.

Experiment 4- Microbial community development in a CSTR during EPS-overproduction

Since the CSTR (R6) in Exp_3 achieved the best performance in terms of VFA conversion and EPS yields (Table 1), a second CSTR (R7) was operated in Experiment 4 (Exp_4) under the same conditions but for a longer period (89 days) (Table 1). This reactor was inoculated with the same municipal sludge, but sampled during a different period of the year, and fed with the same VFA-rich synthetic wastewater as in Exp_3. The OLR was decreased from 2 to 1 g COD L^{-1} d^{-1} at day 12. During the operation, the accumulation of intracellular polyhydroxyalkanoates (PHA) was also monitored besides EPS, as nitrogen limitation and VFAs could also favour production of such biopolymers [19] (**Chapter 3**). For this purpose, sludge samples from R7 were collected at multiple time points (inoculum, day 12, 22, 39, 75 and 84) for 16S rRNA gene amplicon sequencing, allowing the monitoring of putative EPS- and PHA-producing populations.

4.2.2 Analytical methods

Soluble chemical oxygen demand (sCOD) was measured in filtered influent and effluent samples from the bioreactors across the four experiments(0.45 μm, H-PTFE, Whatman, The USA) using the COD Cuvette Tests (LCK, Hach Lange GmbH, DE) to calculate sCOD removal.

For EPS characterization and quantification, EPS were extracted from raw sludge samples freshly collected from each reactor during the experimental period, as described in **Chapters 2** and **3**. Solutions of extracted EPS (1 g L^{-1}) were prepared by resuspending the freeze-dried EPS in Milli-Q water and used to quantify polysaccharides and proteins. Polysaccharide concentration was determined using the colorimetric phenol–sulphuric acid method [46] by measuring absorbance at 490 nm with glucose as the calibration standard. Protein concentration was quantified using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

In Exp_4, polyhydroxyalkanoates (PHA) were quantified using thermogravimetric analysis (TGA) as described in **Chapter 3**. For comparability, all yields (EPS and PHA) were normalized and reported as mg VS biopolymer per g VS sludge (**Chapter 3**). The values are reported in Figure S1.

4.2.3 Sampling and DNA extraction

Fresh sludge samples (25 mL) were collected at defined time points and immediately stored at -20 °C until further processing. Before DNA extraction, thawed samples were concentrated by centrifugation (5 min, 3750 rpm). The supernatant was discarded, and 500 mg of the resulting pellet was transferred into a clean Eppendorf tube. The pellet was washed five times with 1x PBS, resuspended in 1 mL PBS and sonicated (40 kHz, 50 W, 30 s) to break the EPS matrix and make the cells more accessible to the extraction procedure. Genomic DNA was extracted using a FastDNA® SPIN kit for soil (MPBio, USA) according to the manufacturer's instructions. The quantity and purity of the extracted DNA were assessed with Quantus fluorometer (Promega, USA) and NanoDrop ND-1000 spectrophotometer (NanoDrop, USA), respectively.

Table 1: Operational conditions, process performances, and EPS yield and PS/PN ratio in the four experiments. Abbreviations: COD, chemical oxygen demand; N, total nitrogen; SRT, solids retention time; OLR, organic loading rate; PS, polysaccharides; PN, proteins; MBR, membrane bioreactor; CSTR, continuous stirred-tank reactor; VFA, volatile fatty acids.

	Exp_1		Exp_2		Exp_3		Exp_4
Reactor ID	R1	R2	R3	R4	R5	R6	R7
Reactor type	MBR	MBR	MBR	MBR	MBR	CSTR	CSTR
Inoculum sludge	Municipal	Industrial	Municipal	Industrial	Municipal	Municipal	Municipal
Feed type	Glycerol	Glycerol	Glycerol	Glycerol	VFA	VFA	VFA
COD/N ratio	20, 60, 100	20, 60, 100	100	100	100	100	100
SRT (days)	15, 6, 3	15, 6, 3	6, 3, 1.5	6, 3, 1.5	3	3	3
Length (days)	105	105	42	42	46	46	89
OLR ($g\ COD\ L^{-1}\ d^{-1}$)	2	2	2	2	2 (day 1–25); 1 (day 25–46)	2 (day 1–25); 1 (day 25–46)	2 (day 1–12); 1 (day 13–89)
COD removal (%)	90–100%	90–100%	70–90%	70–90%	40–50%	80–97%	70–80%
EPS yields ($g\,VS_{EPS}\,g^{-1}\,VSS$)	0.08 (COD:N 60/SRT 6); 0.35 (COD:N 100/SRT 3)	0.071 (COD:N 60/SRT 6); 0.114 (COD:N 100/SRT 3)	0.13–0.3 (SRT 3); 0.3–0.4 (SRT 1.5)	0.15 (SRT 3); 0.27 (SRT 1.5)	0.39 (OLR 2, day 25); 0.38 (OLR 1, day 53)	0.33 (OLR 2, day 25); 0.43 (OLR 1, day 53)	0.64 (OLR 2, day 12); 0.71 (OLR 1, day 39); 1.2 (OLR 1, day 75); 0.25 (OLR 1, day 84)

4.2.4 16S rRNA gene amplicon sequencing and bioinformatic analysis

Prior to microbial community analysis, PCR efficiency was assessed by sample dilution series and qPCR-based PCR efficiency estimation for n=5 top samples ranked by DNA concentration. PCR efficiency (E) was calculated as follows;

$$E = 10^{-1/\text{slope}} - 1 \qquad \text{(Eq.1)}$$

where the slope is the regression estimate of $\log_{10}$ dilution factor against the quantification cycle (C_q) value [47], and $0.9 < E < 1.1$ is accepted as efficient PCR amplification. To ensure unbiased analysis, DNA was accordingly diluted to 5 ng μL^{-1} before PCR, and library preparation and amplicon sequencing were conducted at Macrogen Europe (Amsterdam, The Netherlands). Briefly, the V4-V5 region of the 16S rRNA gene was amplified in a PCR using prokaryotic universal primers 515F-Y and 926R [48], [49]. The Nextera XT (Illumina, USA) libraries were sequenced on an Illumina MiSeq i100 system (Illumina, USA) producing 2x300bp paired-end (PE) reads. Sequence data were processed with QIIME2 (v. 2022.2) with error correction by DADA2 [50] and taxonomic classification using a naive Bayesian classifier trained on SILVA v.138.1. Sequencing data are deposited in the ENA database under the accession number PRJEB115358.

Microbial community responses to sludge type, reactor configuration, and operational conditions were analysed in R (R Core Team) using *phyloseq* [51]. Since bacteria represented 99% of all sequences, non-bacterial kingdoms were excluded, together with mitochondrial and chloroplast amplicon sequence variants (ASV) and ASVs with a mean abundance of less than 0.1%. The minimal read depth after filtering was 34448 sequences per sample, and a total of 2396 ASVs represented by 96.6% of reads from the unfiltered data set. All samples showed saturated rarefaction curves (Fig. S2). Subsets for each experiment were created and rarefied to each respective minimal sequencing depth. Beta diversity was calculated using the Bray-Curtis dissimilarity index on rarefied relative abundance tables and visualized using principal coordinates analysis (PCoA). This analysis can be reproduced from `https://github.com/pietervanveelen/BKIS_EPS_sludge_feed.Bray-Curtis`. These PCoAs were used to compare MMCs between inocula, reactor types, substrates and operating conditions (e.g. COD:N 60 vs. COD:N 100, MBR vs. CSTR, glycerol vs. VFAs) and to identify the most similar and most divergent communities over time.

4.3 Results and Discussion

4.3.1 Nitrogen limitation and short SRT enrich putative EPS-producers and degraders in MBRs fed with glycerol

Over the 105 days of operation, both glycerol-fed MBRs, R1 and R2, maintained high COD removal (90–100%) (Table 1). Stronger nitrogen limitation and shorter SRT increased EPS production in both reactors; in R1, EPS rose from 0.08 mg VS_{EPS} g^{-1} VSS (COD:N 60/SRT 6d) to 0.35 mg VS_{EPS} g^{-1} VSS (COD:N 100/SRT 3), while in R2 they increased from 0.071 to 0.114 mg VS_{EPS} g^{-1} VSS over the same shift. Overall, R1 achieved substantially higher EPS yields under the tested conditions, suggesting that the municipal sludge inoculum may have supported EPS production more effectively during start-up.

Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity of the microbial community profiles (Fig. S3A) showed that the municipal and industrial inocula initially formed distinct microbial assemblages. During EPS production, both communities followed a similar developmental trajectory, resulting in end-stage communities that became more similar while still retaining inoculum-specific differences.

The microbial community composition in both bioreactors consistently developed EPS producers alongside EPS degraders (Fig. 1). This is expected, as the EPS matrix is not static; several components of it (particularly polysaccharides and proteins) can serve as carbon and energy sources under nutrient limitation or starvation. This creates a natural ecological niche for degraders, leading to a dynamic turnover of EPS [52], [53].

In R1, the municipal inoculum community was primarily dominated by members of the families *Saprospiraceae,* and *Comamonadaceae*, together with other important genera such as *Competibacter, Terrimonas, Caldilineaceae, Anaerolinaceae, Ferruginibacter, Tetrasphaera* (Fig. 1A). These taxa are commonly detected in activated sludge systems, and they contribute to the degradation of diverse organic matter-related substrates [54], [55], [56].

During moderate nitrogen limitation (COD:N 60/SRT 6) in R1, the community shifted remarkably, with the genus *Hydrogenophaga* becoming dominant (11.5% relative abundance) (Fig. 1A). While in wastewater treatment plants (WWTPs) *Hydrogenophaga* are heterotrophic carbon degraders [57], in nutrient-limited conditions they have also been identified as putative PHA and EPS producers [58], [59]. Other groups whose relative abundance increased included *Pseudoxanthomonas* (4.4%), *Flavobacterium* (4.1%), *Arenimonas* (3.6%) and *Sphaerotilus* (3%), while *Comamonadaceae* remained consistently abundant in comparison to the inoculum ($\sim$ 5.5%) (Fig. 1A). Among these, *Pseudoxanthomonas* has been reported as EPS producer in several habitats [60], [61], [62], while *Sphaerotilus* is a well-known mucus-forming activated sludge organism which was reported to produce EPS [63]. Similarly, *Flavobacterium*, frequently associated with surface-attached lifestyles and EPS-rich matrices, has also been reported to produce EPS [64], [65]. *Arenimonas* is a biofilm-producing denitrifier [66], [67].

When nitrogen limitation was intensified in combination with shorter SRT (COD:N 100/SRT 3), the community shifted again and was dominated by *Saprospiraceae* and *Chthoniobacteraceae* LD29 (Fig. 1A), commonly found in wastewater treatment systems and linked to organic matter degradation [68], [69]. These groups were likely involved in EPS turnover/degradation, together with *Ferruginibacter*, which can degrade complex organic polymers, including EPS components [70], *Prosthecobacter* [71], and *Bdellovibrio* [72], whose presence may reflect predator-prey dynamics within the sludge community (Fig. 1A). The presence of the genus *Berkiella* (2.1%), belonging to the order *Legionellales*, whose members can form parasitic relationships with various eukaryotic hosts [73], may indicate the presence of protozoa in the bioreactor. Among the other representative bacteria, *Ancylobacter* (3.1%) was likely the dominant EPS producer (Fig. 1A), as strong abilities in biofilm formation and adhesion have been shown for strains like *Ancylobacter* sp. TS-1 [74]. EPS production could also be related with members of the family *Comamonadaceae*, which can produce biofilm-associated EPS [75] and EPS linked to aggregating phenotypes [76] and *Ferrovibrio*, which is often found to be dominant in oligotrophic drinking water biofilms [77].

The industrial sludge inoculum, which was used to seed R2, was dominated by *Microthrix, Dokdonella, Comamonadaceae, Terrimonas, Hyphomicrobium,*

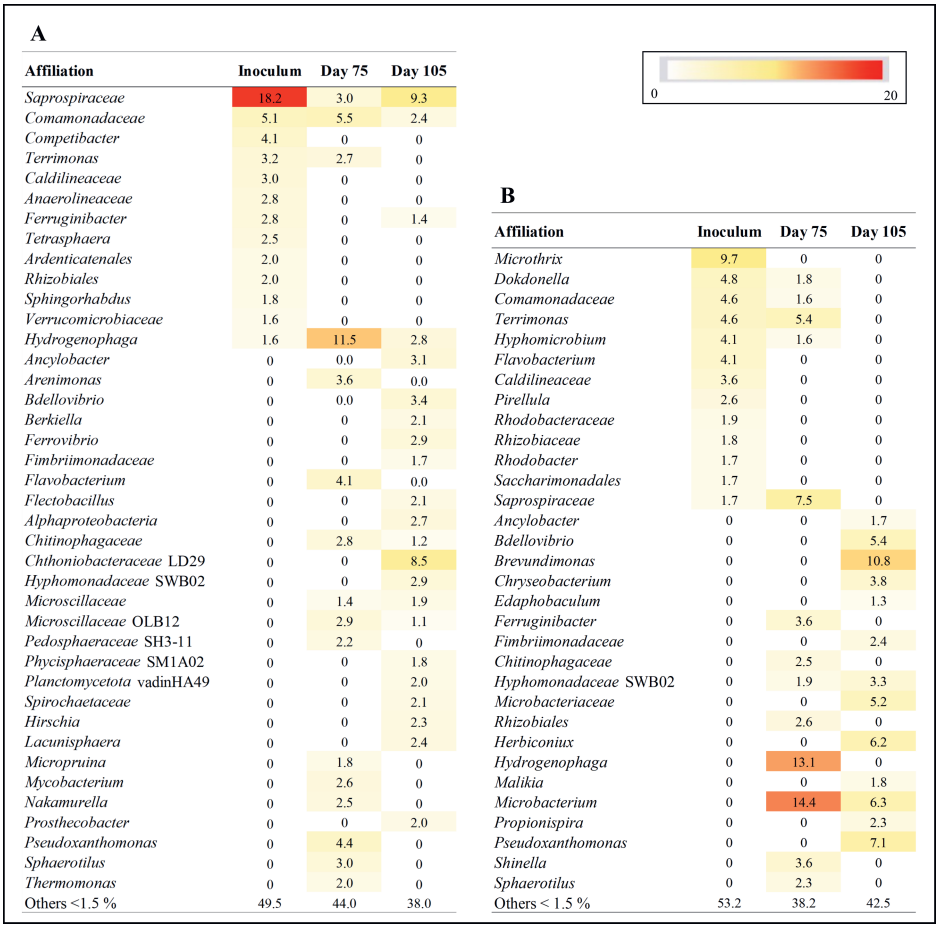


Figure 1: Microbial community analysis for Experiment 1 – Relative abundances (between 0% and 20%) of dominant microbial ASVs (> 1.5% relative abundance in at least one of the samples reported) for reactor R1 (A) and reactor R2 (B) in the start-up inocula and at the end of each operational period (day 75 for COD:N 60/SRT 6 and day 105 for COD:N 100/SRT 3). Microbial groups were identified using 16S rRNA gene amplicon sequencing, and their taxonomy classification is reported at the identified level. All ASVs unidentified or with relative abundances below 1.5% are grouped as "Others" and not included in the heatmap colour format.

Flavobacterium and *Caldilineaceae* (Fig. 1B). Most of the taxa overlap with the community composition of the municipal sludge inoculum of R1 (Fig. 1A), reflecting typical activated-sludge microorganisms involved in carbon and nitrogen cycling [78], [79], [80], [81]. Similar to R1, operation under moderate nitrogen limitation (COD:N 60/SRT 6) caused remarkable changes in the community of R2, with a clear differentiation between EPS producers and EPS degraders. As in R1, in R2 *Hydrogenophaga* became the dominant microorganism (13.1%) and may have contributed to EPS production under these conditions [58]. *Microbacterium* (14.4%) was also abundant and has been reported as an EPS producer frequently detected in WWTPs [82], [83], [84] (Fig. 1B). Additional putative EPS-producing taxa included *Shinella*, which has been reported to produce cellulose-like EPS with potential bioflocculant applications [85], [86], and and which may contribute to floc stability in WWTPs through EPS secretion [63]. The group of potential EPS-degraders comprised *Saprospiraceae, Terrimonas*, *Ferruginibacter* [69], [78], [87], [88] (Fig. 1B). *Chitinophagaceae*, which are heterotrophic ammonia-oxidizing bacteria (AOB) [89], were also identified as EPS producers in aerobic MBRs [90].

After shifting to stronger nitrogen limitation (COD:N 100/SRT 3d), the putative EPS-producing community in R2 was dominated by *Brevundimonas*, members of which are known EPS producers in various environments [91], [92], [93]. The community also included the previously discussed *Pseudoxanthomonas* and *Microbacterium*, species of which have been reported to produce anionic EPS [82], [83], [84]. Other putative EPS producers detected in the reactor were *Microbacteriaceae*, which have been identified as key players in aerobic granulation [94], and *Chryseobacterium*, for which several strains have been optimised for high-yield EPS production [95] and linked to EPS production in aerobic systems [96]. Among the EPS degraders, in addition to the previously mentioned predatory *Bdellovibrio* [72] and the polysaccharide-degrading *Herbiconiux* [97], *Hyphomonadaceae* SWB02 may also have been involved. Members of *Hyphomonadaceae* SWB02 have been described as oligotrophic aerobic microorganisms associated with manganese oxidation in sand filters [98] and have previously been linked to extracellular matrix degradation in marine sponges [99]. In addition, although *Microbacterium* was considered here as a putative EPS producer, some strains are known to produce hydrolytic enzymes [100], [101], suggesting that this genus

may also have contributed to EPS degradation or matrix turnover.

Together, these results suggest that the EPS-overproducing conditions favour microbial consortia in which EPS formation and degradation likely occur simultaneously. In conventional wastewater systems, a dynamic balance between EPS producers and degraders is expected [102]. This continuous turnover contributes to floc/granule resilience, nutrient cycling, and overall process stability. Under stress conditions, such as nutrient limitation, transient EPS degradation may also represent an adaptive strategy that enables microorganisms to sustain metabolic activity [96].

Notably, although R1 achieved higher EPS yields than R2 under EPS-overproducing conditions, the dominant taxa in R1 were mainly putative EPS degraders, including *Saprospiraceae* and *Chthoniobacteraceae* LD29. In contrast, reactor R2 was dominated by putative EPS producers, including *Brevundimonas, Microbacterium, Pseudoxanthomonas, Microbacteriaceae*, and *Chryseobacterium*. In addition, other EPS-associated taxa, such as *Shinella* and *Sphaerotilus*, were already prominent in R2 at earlier stages (COD:N 60/SRT 6). Overall, these findings indicate that 16S rRNA gene amplicon sequencing alone may be insufficient to resolve the quantitative contribution of the diverse microbial groups to the functional balance between EPS production and degradation in EPS-overproducing bioreactors. For example, in R1, the activity of less abundant but potentially important EPS producers, such as *Ancylobacter* with 3.1% relative abundance, may still have contributed substantially to EPS formation toward the end of the operation. This is because relative abundance does not necessarily reflect absolute abundance, metabolic activity, or cell-specific EPS production. Therefore, relative abundance should not be directly interpreted as functional activity, and additional methods would be needed to confirm the active roles of producer and degrader populations.

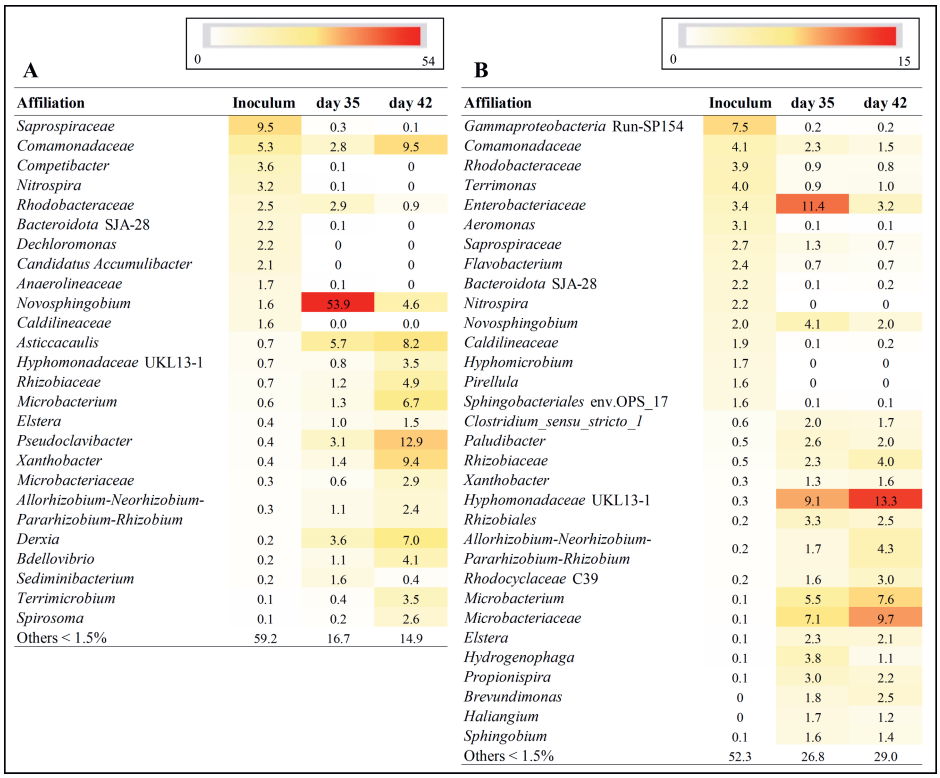
4.3.2 Immediate start-up with nitrogen limitation and short SRT enriched EPS-producing bacteria in MBRs fed with glycerol

In Exp_2, both glycerol-fed MBRs were directly started under nitrogen-limited conditions (COD:N 100). Both reactors maintained moderate to high COD removal throughout operation, ranging between 70 and 90% (Table 1). Similar to observations in Exp_1, R3, which was inoculated with municipal sludge, reached higher EPS yields at the end of the process than R4, which was inoculated with industrial sludge (Table 1). Further decreasing the SRT to 1.5 days resulted in an additional increase of EPS yields, particularly in R3, where the yield reached up to 0.4 $g\,VS_{EPS}\,g^{-1}\,VSS$. Notably, a comparison of reactors with similar inocula, such as R3 and R1 from Exp_1, showed that direct start-up under nitrogen limitation resulted in comparable EPS yields under the same operational conditions (COD:N 100/SRT 3) (Table 1). This indicates that nitrogen-limited start-up can immediately stimulate MMCs toward EPS overproduction without requiring a separate acclimation phase.

Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity of the microbial community profiles (Fig. S3B) showed that the communities in R3 and R4 started to diverge between municipal and industrial activated sludge inocula. This suggests that, under direct nitrogen-limited start-up, the municipal sludge inoculum supported a community development pattern more favourable for EPS accumulation than the industrial sludge inoculum, which followed a different trajectory without an acclimation step.

At the microbial community level, operation under SRTs of 3 and 1.5 days during nitrogen limitation caused substantial shifts from the original inocula in both R3 and R4 (Fig. 2). The resulting communities contained both putative EPS producers and EPS degraders, consistent with the Exp_1. Thus, direct start-up under strict nitrogen limitation not only stimulated EPS production but also favoured the development of communities with comparable functional roles related to EPS formation and turnover.

The municipal inoculum used for R3 was similar to that used for R1 (Fig. 1A) and was dominated by taxa typically associated with organic matter degradation



A

Affiliation	Inoculum	day 35	day 42
Saprospiraceae	9.5	0.3	0.1
Comamonadaceae	5.3	2.8	9.5
Competibacter	3.6	0.1	0
Nitrospira	3.2	0.1	0
Rhodobacteraceae	2.5	2.9	0.9
Bacteroidota SJA-28	2.2	0.1	0
Dechloromonas	2.2	0	0
Candidatus Accumulibacter	2.1	0	0
Anaerolineaceae	1.7	0.1	0
Novosphingobium	1.6	53.9	4.6
Caldilineaceae	1.6	0.0	0.0
Asticcacaulis	0.7	5.7	8.2
Hyphomonadaceae UKL13-1	0.7	0.8	3.5
Rhizobiaceae	0.7	1.2	4.9
Microbacterium	0.6	1.3	6.7
Elstera	0.4	1.0	1.5
Pseudoclavibacter	0.4	3.1	12.9
Xanthobacter	0.4	1.4	9.4
Microbacteriaceae	0.3	0.6	2.9
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0.3	1.1	2.4
Derxia	0.2	3.6	7.0
Bdellovibrio	0.2	1.1	4.1
Sediminibacterium	0.2	1.6	0.4
Terrimicrobium	0.1	0.4	3.5
Spirosoma	0.1	0.2	2.6
Others < 1.5%	59.2	16.7	14.9

B

Affiliation	Inoculum	day 35	day 42
Gammaproteobacteria Run-SP154	7.5	0.2	0.2
Comamonadaceae	4.1	2.3	1.5
Rhodobacteraceae	3.9	0.9	0.8
Terrimonas	4.0	0.9	1.0
Enterobacteriaceae	3.4	11.4	3.2
Aeromonas	3.1	0.1	0.1
Saprospiraceae	2.7	1.3	0.7
Flavobacterium	2.4	0.7	0.7
Bacteroidota SJA-28	2.2	0.1	0.2
Nitrospira	2.2	0	0
Novosphingobium	2.0	4.1	2.0
Caldilineaceae	1.9	0.1	0.2
Hyphomicrobium	1.7	0	0
Pirellula	1.6	0	0
Sphingobacteriales env.OPS_17	1.6	0.1	0.1
Clostridium_sensu_stricto_1	0.6	2.0	1.7
Paludibacter	0.5	2.6	2.0
Rhizobiaceae	0.5	2.3	4.0
Xanthobacter	0.3	1.3	1.6
Hyphomonadaceae UKL13-1	0.3	9.1	13.3
Rhizobiales	0.2	3.3	2.5
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0.2	1.7	4.3
Rhodocyclaceae C39	0.2	1.6	3.0
Microbacterium	0.1	5.5	7.6
Microbacteriaceae	0.1	7.1	9.7
Elstera	0.1	2.3	2.1
Hydrogenophaga	0.1	3.8	1.1
Propionispira	0.1	3.0	2.2
Brevundimonas	0	1.8	2.5
Haliangium	0	1.7	1.2
Sphingobium	0.1	1.6	1.4
Others < 1.5%	52.3	26.8	29.0

Figure 2: Microbial community analysis for Experiment 2 – Relative abundances (between 0% and 54% in A and 0% and 15% in B) of dominant microbial ASVs (> 1.5% relative abundance in at least one of the samples reported) for reactor R3 (A) and reactor R4 (B) in the start-up inocula and at the end of the application of SRT 3 (day 35) and SRT 1.5 (day 42). Microbial groups were identified using 16S rRNA gene amplicon sequencing, and their taxonomy classification is reported at the identified level. All ASVs unidentified or with relative abundances below 1.5% are grouped as "Others" and not included in the heatmap colour format.

and heterotrophic carbon and nitrogen removal and nitrogen cycling, including *Saprospiraceae, Comamonadaceae,* and *Competibacter,* and *Nitrospira* (Fig. 2A) [54], [55], [56]. After applying an SRT of 3 days, *Novosphingobium* strongly dominated the community, reaching 53.9% relative abundance. This high dominance suggests that the imposed conditions exerted a strong selective pressure favouring this taxon. Although relative abundance alone does not confirm its actual activity or quantitative contribution to EPS production, members of this genus are known to produce polysaccharide-rich EPS [103] and have also been identified as gel-forming bacteria in sewage-treating MBRs [104]. Other potential EPS producers present at lower relative abundance included *Asticcacaulis* and *Derxia* (Fig. 2A). The genus *Asticcacaulis* harbours conserved genes involved in holdfast (a polysaccharide-rich adhesive matrix) synthesis [105], and has also been detected in membrane-fouling layers [106]. *Derxia* are nitrogen-fixing bacteria, and the species *Derxia gummosa* has been reported to secrete a polysaccharide-rich slime that adheres to plant roots [107]. In addition, *Derxia* can produce heavy, sticky biomass growth under nitrogen limitation [108], further indicating the strong ability to produce EPS-like substances. Potential abundant EPS degraders included, *Pseudoclavibacter*, which has been associated with organic matter and carbohydrate degradation [109], [110], and the predatory *Bdellovibrio*. At the end of the process (SRT 1.5, day 42), in R3 the relative abundance of *Novosphingobium* drastically decreased (Fig. 2A), while *Asticcacaulis* and *Derxia* increased and became the dominant putative EPS producers, together with earlier detected *Microbacterium* and *Xanthobacter* whose subspecies have been reported to produce polysaccharide-rich EPS [111], [112]. Additionally, members of *Rhizobiaceae* were part of the community at this stage; similar to *Derxia*, they adhere to plant roots by secreting different types of EPS [113], [114]. *Hyphomonadaceae* UKL13-1, which has been identified as a key microorganism of aerobic granulation [115], was also part of the community that potentially contributed to EPS production. Similar to the earlier results, the community also included microorganisms that have been reported to be involved in both EPS production and degradation. These included the previously discussed *Microbacterium*, as well as *Rhodobacter*, which has been associated with EPS and biofilm formation [116], [117] but also has been identified as a carbohydrate degrader [118]. Among the more clearly putative EPS degraders, the dominant group was the previously detected *Pseudoclavibacter* with 13% relative abundance [109], [110], together with

Bdellovibrio and *Terrimicrobium*.

The inoculum of R4 shared several taxa with that of R3 (Fig. 2), including *Comamonadaceae*, *Rhodobacteraceae* and *Novosphingobium*, and additionally contained genera such as *Terrimonas* and *Aeromonas*, which are typically found in activated sludge systems and contribute to organic matter degradation [119]. At the end of the SRT 3 period (day 35), the dominant genus was an unclassified *Enterobacteriaceae* (Fig. 2B), several members of which are known to produce polysaccharide-rich EPS [120], [121] with potential application as bioflocculants [122]. The community was also dominated by *Hyphomonadaceae* UKL13-1, *Microbacteriaceae* and *Microbacterium* (Fig. 2B), the putative EPS producers previously reported. Similarly, *Rhizobiaceae, Xanthobacter*, and *Novosphingobium* were also present but at lower relative abundances than in R3 (Fig. 2). The higher microbial diversity in R4 compared with R3 was reflected by the presence of additional putative EPS producers during this phase, including *Brevundimonas, Comamonadaceae*, and *Sphingobium* (Fig. 2B) [123]. Among the putative EPS degraders, abundant groups were *Hydrogenophaga*, which was already identified in Exp_1 and assigned a potential dual role in both EPS production and degradation, as well as *Propionispira* and *Paludibacter*, both of which are able to secrete extracellular enzymes involved in polysaccharide degradation [124], [125].

At the end of SRT 1.5 (day 42), the community composition of R4 remained relatively stable, except for a significant decrease in *Enterobacteriaceae*, which was accompanied by an increase in *Hyphomonadaceae* UKL13-1, *Microbacteriaceae*, and *Microbacterium* (Fig. 2B). Most other putative EPS producers and degraders observed during operation at SRT 3 remained abundant under SRT 1.5 (Fig. 2B). Overall, more putative EPS producers were observed in R4 than in R3, despite the higher EPS yields measured in R3 (Table 1). Considering both reactors, decreasing the SRT from 3 to 1.5 days changed the relative abundance of some groups, but the dominant functional roles remained similar, irrespective of inoculum source.

Taken together, Exp_1 and Exp_2 indicate that EPS overproduction from glycerol in the MBR was governed mainly by operational regime rather than inoculum origin. Under nitrogen limitation and short SRT, both municipal and industrial sludge developed communities with putative EPS-producing and EPS-degrading

lineages and produced polysaccharide-rich EPS, although yields remained reactor-specific. Similar functional performance was observed across reactors despite differences in dominant taxa, indicating that different sludge sources formed distinct microecosystems with comparable EPS production and turnover. Moreover, Exp_2 showed that applying strict nitrogen limitation from start-up resulted in comparable reactor performance and EPS production, suggesting that an extended acclimation period is not required.

4.3.3 Microbial community development in VFA-fed bioreactors at decreasing OLR and in two different reactor configurations

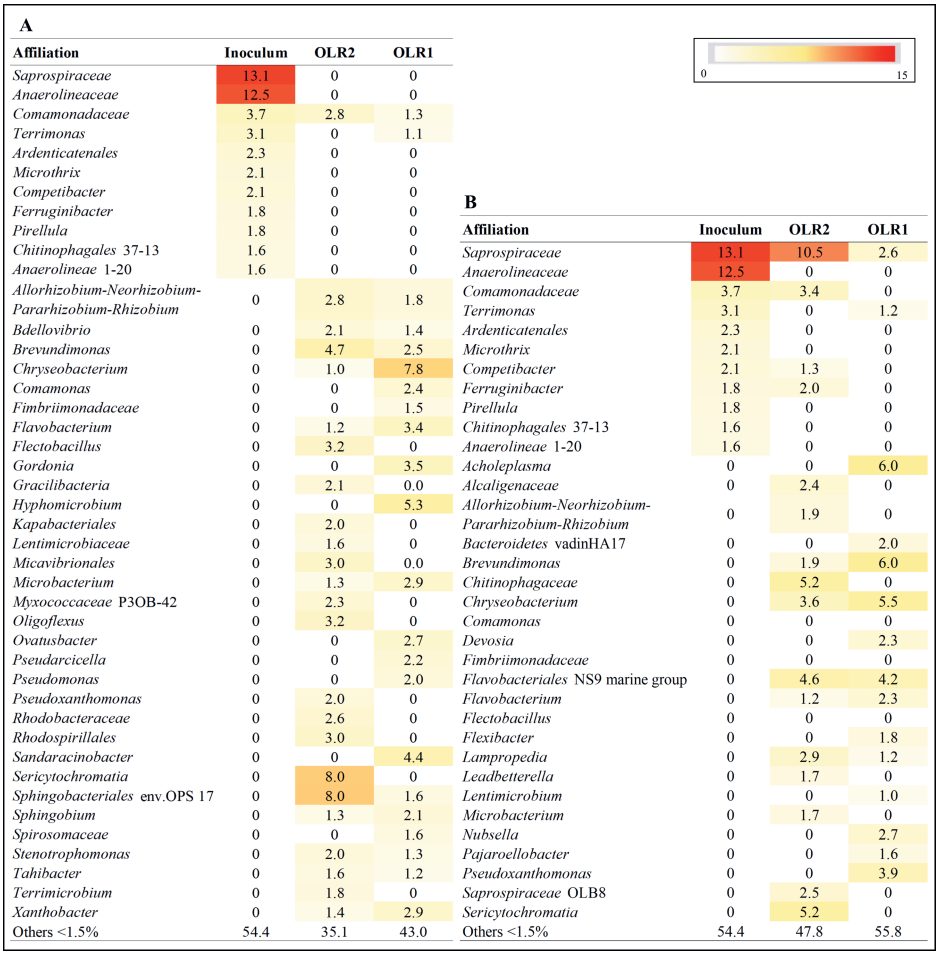
In Exp_3, the VFA-fed reactors, R5 (MBR) and R6 (CSTR), showed clearly different COD removal performances over 46 days of operation, with the CSTR achieving higher removal efficiencies (80–97%) than the MBR (40–50%). Under EPS-overproduction conditions (COD:N 100/SRT 3 days), both reactors showed similar EPS yields at an OLR of 2 g COD L^{-1} d^{-1} (Table 1). When the OLR was decreased to 1 g COD L^{-1} d^{-1} on day 25, EPS yield increased only in the CSTR (R6), rising from 0.33 to 0.43 mg VS_{EPS} g^{-1} VSS by the end of the process (Table 1). These results indicate that both reactors supported EPS secretion under VFA-fed, nitrogen-limited conditions, but differed in substrate-removal efficiency and in their response to changes in organic loading. Under nitrogen-limited conditions, VFAs could potentially be converted into polyhydroxyalkanoates (PHA) rather than EPS [31]. However, PHA were measured only in Exp_4 (see Section 3.4), whereas Exp_3 was designed to identify the most suitable bioreactor configuration and operating conditions for EPS production from VFAs [31].

Although both reactors were inoculated with the same start-up inoculum, their microbial communities developed along distinct trajectories during operation (Fig. 3). PCoA based on Bray-Curtis dissimilarity of the microbial community profiles (Fig. S3C) showed that the MBR and CSTR communities followed a predictable and broadly similar shift during operation, resembling the trajectory observed in Exp_1. However, the communities remained distinct at the end of operation (Fig. S2C), indicating that reactor configuration influenced the final microbial community structure.

The inoculum comprised lineages similar to those observed in Exp_1 and Exp_2 and was dominated by *Saprospiraceae* (13%) and *Anaerolineaceae* (12%). Additional common activated-sludge heterotrophs found in the inoculum were *Comamonadaceae, Terrimonas, Ardenticatenales* and *Ferruginibacter* [119].

At OLR 2, most inoculum-derived groups with relative abundances above 1.5% were outcompeted in R5. *Sphingobacteriales* env.OPS 17 became the likely dominant putative EPS producer, reaching 8% relative abundance, as diverse members of this order have been associated with EPS production and biofilm formation [126]. Among the previously identified putative EPS producers, *Xanthobacter, Microbacterium, Flavobacterium, Brevundimonas, Sphingobium*, and *Pseudoxanthomonas* were detected in R5 (Fig. 3A). Interestingly, some members of these genera have also been reported as PHA/PHB producers in mixed or pure cultures, indicating that part of the community may have had the genetic potential to divert carbon toward intracellular polyester synthesis under suitable conditions [127], [128], [129], [130], [131]. In R5, the class *Sericytochromatia* also reached relatively high abundance (8%) (Fig. 3A). This class has recently been described among non-photosynthetic cyanobacteria [132]. Although its functional role in wastewater bioreactors is still poorly understood, *Sericytochromatia* has been detected in systems associated with biofilm or aggregate formation, including a bioreactor treating polyol-rich wastewater [133], stable aerobic granules [134], and biofilms on polyurethane carriers in a synthetic-wastewater biofilm reactor [135]. This suggests that it may be adapted to attached-growth or EPS-rich environments, although its specific contribution in R5 remains unclear.

Another relevant group was *Micavibrionales*, which reached 3% relative abundance. Similar to *Bdellovibrio* detected in the same reactor, *Micavibrionales* belongs to the *Bdellovibrio*-and-like organisms (BALOs), predatory bacteria that can invade Gram-negative hosts and degrade host-derived biopolymers, including PHA [136]. Its previous association with alginate-like exopolymer [137] and PHA-producing systems [136] suggests that it may be linked to both polymer turnover and polymer-rich microbial systems. Around the same relative abundance, *Oligoflexus, Rhodospirillales*, and *Flectobacillus* were also detected (Fig. 3A). *Oligoflexus* and *Rhodospirillales* have been associated with nitrogen metabolism [138], [139], while *Rhodospirillales*, together with *Sphingomonadales*, have been identified as major contributors to biofouling in MBRs, likely through



A

Affiliation	Inoculum	OLR2	OLR1
Saprospiraceae	13.1	0	0
Anaerolineaceae	12.5	0	0
Comamonadaceae	3.7	2.8	1.3
Terrimonas	3.1	0	1.1
Ardenticatenales	2.3	0	0
Microthrix	2.1	0	0
Competibacter	2.1	0	0
Ferruginibacter	1.8	0	0
Pirellula	1.8	0	0
Chitinophagales 37-13	1.6	0	0
Anaerolineae 1-20	1.6	0	0
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0	2.8	1.8
Bdellovibrio	0	2.1	1.4
Brevundimonas	0	4.7	2.5
Chryseobacterium	0	1.0	7.8
Comamonas	0	0	2.4
Fimbriimonadaceae	0	0	1.5
Flavobacterium	0	1.2	3.4
Flectobacillus	0	3.2	0
Gordonia	0	0	3.5
Gracilibacteria	0	2.1	0.0
Hyphomicrobium	0	0	5.3
Kapabacteriales	0	2.0	0
Lentimicrobiaceae	0	1.6	0
Micavibrionales	0	3.0	0.0
Microbacterium	0	1.3	2.9
Myxococcaceae P3OB-42	0	2.3	0
Oligoflexus	0	3.2	0
Ovatusbacter	0	0	2.7
Pseudarcicella	0	0	2.2
Pseudomonas	0	0	2.0
Pseudoxanthomonas	0	2.0	0
Rhodobacteraceae	0	2.6	0
Rhodospirillales	0	3.0	0
Sandaracinobacter	0	0	4.4
Sericytochromatia	0	8.0	0
Sphingobacteriales env.OPS 17	0	8.0	1.6
Sphingobium	0	1.3	2.1
Spirosomaceae	0	0	1.6
Stenotrophomonas	0	2.0	1.3
Tahibacter	0	1.6	1.2
Terrimicrobium	0	1.8	0
Xanthobacter	0	1.4	2.9
Others <1.5%	54.4	35.1	43.0

B

Affiliation	Inoculum	OLR2	OLR1
Saprospiraceae	13.1	10.5	2.6
Anaerolineaceae	12.5	0	0
Comamonadaceae	3.7	3.4	0
Terrimonas	3.1	0	1.2
Ardenticatenales	2.3	0	0
Microthrix	2.1	0	0
Competibacter	2.1	1.3	0
Ferruginibacter	1.8	2.0	0
Pirellula	1.8	0	0
Chitinophagales 37-13	1.6	0	0
Anaerolineae 1-20	1.6	0	0
Acholeplasma	0	0	6.0
Alcaligenaceae	0	2.4	0
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0	1.9	0
Bacteroidetes vadinHA17	0	0	2.0
Brevundimonas	0	1.9	6.0
Chitinophagaceae	0	5.2	0
Chryseobacterium	0	3.6	5.5
Comamonas	0	0	0
Devosia	0	0	2.3
Fimbriimonadaceae	0	0	0
Flavobacteriales NS9 marine group	0	4.6	4.2
Flavobacterium	0	1.2	2.3
Flectobacillus	0	0	0
Flexibacter	0	0	1.8
Lampropedia	0	2.9	1.2
Leadbetterella	0	1.7	0
Lentimicrobium	0	0	1.0
Microbacterium	0	1.7	0
Nubsella	0	0	2.7
Pajaroellobacter	0	0	1.6
Pseudoxanthomonas	0	0	3.9
Saprospiraceae OLB8	0	2.5	0
Sericytochromatia	0	5.2	0
Others <1.5%	54.4	47.8	55.8

Figure 3: Microbial community analysis for Experiment 3 – Relative abundances (between 0% and 15%) of dominant microbial ASVs (> 1.5% relative abundance in at least one of the samples reported) for reactors R5 (A) and R6 (B) in the start-up inoculum and at the end of the operational periods with applied organic loading rate (OLR) of 2 and 1 g COD L^{-1} d^{-1}. Microbial groups were identified using 16S rRNA gene amplicon sequencing, and their taxonomy classification is reported at the identified level. All ASVs unidentified or with relative abundances below 1.5% are grouped as "Others" and not included in the heatmap colour format.

EPS production [139].

At the same time, at OLR 2, R6 developed a different community than R5, although *Sericytochromatia* was present in both reactors (Fig. 3B). In R6, *Sericytochromatia* co-dominated with *Saprospiraceae, Chitinophagaceae*, and the *Flavobacteriales* NS9 marine group (Fig. 3B). As noted above, the first two groups are mainly associated with detrital organic matter degradation, whereas the NS9 marine group has often been linked to EPS production and granule formation [140]. Putative EPS producers previously identified in other experiments were also detected in R6, including *Chryseobacterium*, *Microbacterium, Flavobacterium*, and *Brevundimonas*. Novel genera developing in R6 at this stage included *Lampropedia* (2.9%) and *Leadbetterella* (1.7%). *Lampropedia* has been described as a phosphate-accumulating bacterium capable of producing PHA and PHB from acetate [141] . *Leadbetterella* has also been identified among PHA-producing mixed microbial communities, in association with genera such as *Brevundimonas, Ferruginibacter,* and *Pseudoxanthomonas* [142]. Together, these results indicate that reactor configuration shaped the microbial response under high organic loading, with R6 showing stronger enrichment of degradation-associated taxa while still maintaining putative EPS- and PHA-producing populations.

When the OLR was decreased to 1 g COD L^{-1} d^{-1} , the community composition shifted again in both reactors, in some cases leading to the emergence of microbial groups that were not detected at the higher OLR (Fig. 3). In R5, the putative EPS-producers *Flavobacterium* [143], also known for its PHA production capacity [144], and *Brevundimonas* remained present from the OLR 2 period. They co-occurred with other previously mentioned putative EPS producers, including *Xanthobacter, Sphingobium, Microbacterium, Sphingobacteriales* env.OPS 17, *Stenotrophomonas* [145] and *Pseudomonas*. The latter is particularly notable because of its well-documented genetic capacity to produce extracellular polysaccharides such as alginate, Psl, and Pel [146], [147]. New groups including *Hyphomicrobium, Sandaracinobacter*, and *Chryseobacterium*, emerged only after the OLR decrease and exceed 1.5% relative abundance threshold. While *Chryseobacterium* was identified as a potential EPS or PHA producer, *Hyphomicrobium* and *Sandaracinobacter* have previously been associated with nitrogen removal and degradation of toxic hydrocarbons [148], [149], [150]. Interestingly, other abundant taxa linked to hydrocarbons degradation were also detected, including

Gordonia [151], *Comamonas* [152], *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* [153] and *Stenotrophomonas* [145]. Their presence in R5 may therefore be more related to the degradation of complex biopolymers produced by other community members than to direct biopolymer production. This interpretation is supported by reports of some *Stenotrophomonas* species being able to degrade polyesters [154]. Although VFAs may have promoted some intracellular polymer storage, the observed response was likely limited compared with the enrichment levels typically achieved under feast–famine PHA-producing conditions. One possible explanation is the longer retention time applied in this bioreactor compared with typical PHA-producing systems, which are often operated at SRTs of 1.7 to 0.8 days [155] may have favoured subsequent polymer release and biodegradation. These results suggest that, in R5, polymer turnover may have been high, with degradation potentially counterbalancing polymer production.

After the OLR was reduced to 1 g COD L^{-1} d^{-1}, the community in R6 also shifted, while remaining consistent with continued EPS formation and turnover. Among the putative EPS producers, *Flavobacterium* and *Brevundimonas*, already present during the OLR 2 period, remained detectable. In addition, *Pseudoxanthomonas* increased above the 1.5% relative-abundance threshold, while the previously mentioned NS9 marine group and *Chryseobacterium* were also likely among the EPS-producing taxa in this reactor. Organic matter degraders such as *Saprospiraceae* were no longer dominant, whereas new genera emerged, including *Acholeplasma*, which reached a substantial relative abundance of 6% (Fig. 3B). Interestingly, *Acholeplasma* has been identified in bioreactors as an anaerobic fermenter producing VFAs [156] and has also been linked to biofilm formation [157]. This suggests that, despite complete mixing in R6, anoxic microzones may have developed within EPS-rich flocs. The putative PHA producers *Lampropedia* and *Leadbetterella* decreased substantially at the lower OLR (Fig. 3B), whereas genera such as *Devosia* and *Nubsella* became abundant and may be associated with the degradation of complex hydrocarbons [158], [159], [160], similar to the pattern observed in R5 for other microbial groups. *Nubsella* has previously been identified together with *Brevundimonas, Chryseobacterium*, and *Xanthobacteraceae* in a biofilm reactor treating phenanthrene through biodegradation [160]. This observation highlights that, irrespective of reactor configuration, many microorganisms developing in Exp_3 may have flexible roles in bioreactors, and that microbial community composition alone is insufficient to reconstruct metabolic

networks.

Finally, the presence of *Pajaroellobacter* (1.6%), a genus of predatory myxobacteria similar to *Bdellovibrio* and known to produce a wide range of exoenzymes [157], [161], further supports the possibility of intracellular or extracellular polymer turnover in R6. Overall, these results indicate that reactor configuration played a central role in shaping microbial community assembly and process performance from the same inoculum. Although both reactors contained diverse putative polymer producers and degraders, the presence of putative EPS producing taxa alone was not sufficient to explain EPS accumulation. The MBR (R5) harboured a broader and more complex community, including several taxa potentially associated with EPS, PHA, and polymer turnover, but showed lower EPS yields than the CSTR (R6). In contrast, the CSTR achieved higher substrate removal and EPS yield despite its lower bacterial diversity. This suggests that the stirred CSTR configuration, particularly at low OLR, may have favoured VFA conversion toward EPS production, while keeping PHA accumulation low either because PHA formation was limited or because intracellular polymers were rapidly released, degraded, or converted into soluble substrates.

4.3.4 Hydrocarbons degraders and predatory bacteria developed with putative EPS-producers within the microbial community of a CSTR fed with VFAs

In Exp_4, a VFA-fed CSTR was operated with a shortened high-OLR phase. Specifically, an OLR of 2 g COD L^{-1} d^{-1} was applied for the first 12 days, after which it was decreased to 1 g COD L^{-1} d^{-1} until the end of the process. Over the 89 days of operation, R7 showed moderate COD removal of 70–75% under EPS-overproduction conditions (COD:N 100/SRT 3) (Table 1).

Throughout the process, PHA and EPS fractions were monitored to estimate how VFAs were partitioned between intracellular and extracellular biopolymer synthesis (Fig. S1). Only on day 3, during start-up, was the PHA fraction comparable to the EPS fraction, reaching 0.23 g VS_{PHA} g^{-1} MLVSS and 0.35 g VS_{EPS} g^{-1} MLVSS, respectively (Fig. S1) [31]. Thereafter, PHA decreased rapidly and remained below 0.01 g VS_{PHA} g^{-1} MLVSS after day 10, regardless of the applied OLR. In contrast, the EPS increased after the shift from OLR 2 to

OLR 1, rising from 0.64 mg VS_{EPS} g^{-1} VSS at day 12 to 0.71 mg VS_{EPS} g^{-1} VSS at day 39, before declining to 0.25 mg VS_{EPS} g^{-1} VSS by day 84 [31]. A reactor failure between days 46 and 53 caused partial biomass washout and affected the observed yields. Nevertheless, the trends indicate that, at SRT of 3 days, carbon flux rapidly shifted from PHA accumulation toward EPS production as the process progressed.

The municipal sludge inoculum composition for Exp_4 was in line with the previous experiments (Fig. 1 to 4), depicted as a gradually shifting trajectory in PCoA (Fig. S3D). Consistent with the significant PHA fraction measured at the beginning of the process, some of the inoculum taxa have been reported as PHA-producing or PHA-gene-carrying organisms (Fig. 4). Members of the family *Saprospiraceae*, and in particular the OLB8 lineage, have been shown to store intracellular polymers such as glycogen and PHA [69]. *Competibacter* is a well-known glycogen-accumulating organism capable of PHA storage [162]. Genera within *Comamonadaceae* have frequently been identified as key members of PHB-producing consortia [163], while *Anaerolineaceae* have occasionally been linked to PHA-enriched communities [164].

At day 12, corresponding to the end of the OLR 2 phase, several of these PHA-related taxa were still present, and additional known PHA and/or EPS producing taxa appeared (Fig. 4), These included *Brevundimonas* (4.6%), which can accumulate PHA under suitable conditions [165][166] and was also previously identified as a putative EPS producer; *Ferruginibacter* (3%), a potential PHA producer [167]; and *Flavobacterium* (1.6%), previously associated with a potential dual role in PHA and EPS production.

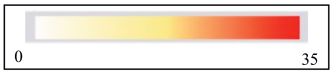
At this point, organic matter degraders and predatory bacteria were also part of the community, including *Prosthecobacter* [168], *Phycisphaeraceae* CL500-3 [169], *Terrimonas* and *Bdellovibrio*. The *Bacteroidota* SJ-28 lineage, which was highly enriched at day 12 (21.2%), has been described as a fermentative heterotroph, facultative H_2-oxidizing autotroph, denitrifier, and N_2O-reducing bacterium [170]. However, its progressive decrease suggests that this lineage was probably not directly associated with sustained EPS production (Fig. 4). A similar interpretation may apply to the highly abundant *Bacteroidetes* VC2.1 Bac22 (Fig. 4), an uncultured clade widely distributed in anoxic, sulfide-rich marine

environments [171]. Nevertheless, their involvement in PHA accumulation or utilization cannot be excluded, as previously hypothesized for other members of the class *Bacteroidia* [172].

At day 22, ten days after the OLR decrease, the microbial community was dominated mainly by potential organic matter or hydrocarbon degraders, despite the high EPS yields and low PHA levels observed at this stage (Table 1). These included *Saprospiraceae, Dysgonomonas* [173], *Alcaligenes* and *Parapusillimonas* [174], *Prosthecobacter* and *Phycisphaeraceae* CL500-3. Moreover, two eukaryotic obligate endosymbionts, *Berkiella* (5.6%, already seen in reactor R1) and *Rickettsiales* SM2D12 (3.6%) [175], were among the abundant taxa (Fig. 4). Their presence suggests the occurrence of protozoa, which may represent active members of PHA-producing MMCs, as previously reported [176]. As hypothesized above for the predatory role of *Micavibrionales* and *Bdellovibrio* (Section 4.3.3), some bacteria under these conditions may have survived in association with, or inside, other organisms while degrading accumulated intracellular polymers. Interestingly, the dominant group on this day was *Arenimonas* (Fig. 4), a biofilm-producing denitrifier already identified in R1 and associated with environments characterized by high PHA utilization [177]. The previously reported *Chryseobacterium* was also present in the community at this time point and, may have been linked to EPS production, as its relative abundance increased further with increasing EPS yields, from 5.4% to 13.4% by day 39 (Fig. 4). Other putative EPS producers included *Brevundimonas, Spirosomaceae* [177], and *Xanthomonadales* [178] (Fig. 4).

During the stable operational phase between days 22 and 39, several putative EPS producers increased in relative abundance. In addition to *Chryseobacterium*, which was already detected earlier [95], *Spirosomaceae* also increased and may have contributed to EPS production, as members of this group have been associated with granule formation in aerobic reactors [179]. *Xanthobacter* and *Pseudoxanthomonas* also increased substantially during this period (Fig. 4) while relative abundance of *Arenimonas* was stable. Another relevant polymer-associated genus was *Agaricicola*, which has been linked to biofilm formation in drinking water environments [180] and PHB accumulation [181].

Alongside these putative EPS producers, several potential organic matter degraders also increased in abundance. These included facultative anaerobes such



Affiliation	Inoculum	day 12	day 22	day 39	day 75	day 84
Saprospiraceae	17.2	14.2	7.1	0.8	0.2	0.2
Anaerolineaceae	6.4	0.6	0.3	0	0	0
Ardenticatenales	4.8	0.8	0.4	0.2	0	0
Competibacter	4.3	0.3	0.3	0	0	0
Comamonadaceae	3.3	0.5	0.7	0	0.2	0.3
Terrimonas	2.4	1.8	0	0	1.5	0.4
Nitrospira	2.1	0	0	0	0	0
Caldilineaceae	2.0	0.3	0.2	0.1	0	0
Anaerolineae SBR1031	2.0	0.2	0	0	0	0
Anaerolineae 1-20	1.9	0.2	0.3	0	0	0
Ferruginibacter	1.8	3.0	0.6	0	0	0
Bacteroidota SJA-28	1.4	7.6	5.3	1.7	0	0
Flavobacteriales NS9 marine group	0.8	0.2	0.1	0.0	5.0	0.8
Microscillaceae	0.5	0	0	0.1	11.4	13.9
Chitinophagaceae	0.5	0.9	0	0	3.2	1.9
Phycisphaeraceae CL500-3	0.4	3.1	1.0	0.3	0.0	0.0
Kapabacteriales	0.3	0.7	0.8	3.1	5.1	8.1
Micavibrionales	0.1	0.1	1.5	2.2	2.7	34.2
Bdellovibrio	0.1	2.6	0.6	1.1	0.4	0.6
Flavobacterium	0.1	1.6	0.3	0.3	5.2	0.6
Prosthecobacter	0.1	3.1	1.8	1.3	0	0
Rickettsiales SM2D12	0.1	0.2	3.6	0.9	0	1.5
Pseudoxanthomonas	0	0.2	0.7	5.0	0.7	0.1
Babeliales	0	0	1.1	4.5	0	0
Chryseobacterium	0	0.4	5.4	13.4	2.4	0.7
Brevundimonas	0	4.6	1.7	1.1	0.5	0.6
Spirosomaceae	0	0.2	2.2	9.3	2.2	2.4
Fluviicola	0	2.1	0.3	0.1	0	0
Acinetobacter	0	0	0.1	0.3	8.2	0.3
Arenimonas	0	0.7	10.3	9.9	0.7	0.4
Dysgonomonas	0	0.1	2.7	5.9	0.7	0.4
Taibaiella	0	0.9	0.2	1.3	5.3	1.9
Bacteroidetes VC2.1 Bac22	0	21.2	1.2	0.2	0	0
Xanthobacter	0	0.4	0.4	3.2	0.1	0.2
Pseudochelatococcus	0	0	0.2	1.6	6.5	3.5
Tannerellaceae	0	0	0.5	0.4	3.7	1.9
Berkiella	0	0.1	5.6	1.3	0.2	0
Saprospiraceae OLB8	0	5.5	3.1	0.3	0	0
Sphingobacteriaceae	0	0	0	0	5.8	1.3
Alcaligenaceae	0	0.5	2.6	1.1	0.3	0.1
Xanthomonadales	0	0.1	1.0	0.5	3.2	2.4
Parapusillimonas	0	0	2.6	4.0	0	0
Agaricicola	0	0	0.3	4.8	0	0
Others <1.5%	47.4	21.2	33.3	20.2	24.8	21.6

Figure 4: Microbial community analysis for Experiment 4 – Relative abundances (between 0% and 35%) of dominant microbial ASVs (> 1.5% relative abundance in at least one of the samples reported) for reactor R7 in the start-up inoculum and during the reactor operation with applied organic loading rate (OLR) of 2 (days 0–12) and 1 g COD L^{-1} d^{-1} (days 12–84). Microbial groups were identified using 16S rRNA gene amplicon sequencing, and their taxonomy classification is reported at the identified level. All ASVs unidentified or with relative abundances below 1.5% are grouped as "Others" and not included in the heatmap colour format.

as *Dysgonomonas*, known for the degradation of complex organics and polysaccharides [182], *Kapabacteriales,* described as hydrolytic heterotrophs [179], and *Parapusillimonas*, a nitrifying bacterium associated with the degradation of complex organic matter [183]. In addition, eukaryotic endosymbionts belonging to the genus *Babeliales* were detected at 4.5% relative abundance. Members of this group have been reported to possess strong cell-decomposition abilities [184], [185] and co-occurred with *Berkiella, Micavibrionales,* and *Bdellovibrio* (Fig. 4). Together, these results suggest that the stable phase of operation was characterized by the coexistence of putative EPS-producing taxa and organisms potentially involved in polymer or biomass turnover.

During the temporary nutrient feeding failure (between day 46 and 53) and subsequent recovery phase (by day 75), the microbial community structure changed significantly (Fig. 4). At this time point, the previously abundant putative EPS producers *Chryseobacterium* and *Spirosomaceae* had decreased substantially. At day 75, they were replaced by a broader set of putative EPS- producing taxa, including *Acinetobacter, Sphingobacteriaceae, Flavobacterium, Chitinophagaceae*, the *Flavobacteriales* NS9 marine group, and *Xanthomonadales* (Fig. 4). Most of these groups had already been identified in previous experiments, except for *Acinetobacter*, which has been reported to produce EPS on carbohydrate substrates [186], including EPS with flocculating properties [187].

Potential polymer degraders were also strongly represented at day 75 by several taxa. The dominant group was *Microscillaceae* (11.4%), previously identified as carbohydrate- and aromatic-compound degraders in biofilm reactors treating organic pollutants [188]. Other abundant taxa with similar potential included *Pseudochelatococcus* (6.5%), associated with VFA and hydrocarbon utilization [189], [190], *Taibaiella* (5.3%), described as a lignocellulose degrader in bioreactors treating petroleum refinery wastewater [191], and *Tannerellaceae* (3.7%), reported as dominant in industrial-scale bioreactors treating oil-refinery wastewater [192] (Fig. 4). *Kapabacteriales* (5%) were also abundant and have been found in granular sludge reactors degrading phenol-based lignin model compounds [193], with metagenomic analyses confirming this degradation potential [194].

The presence of many taxa with enzymatic potential to degrade complex organic compounds and hydrocarbons suggests, as also proposed for Exp_3, that

intracellular polymers such as PHA may still have been produced but rapidly degraded by community members [195]. This interpretation is supported by reports showing that microorganisms capable of hydrocarbon degradation may also degrade synthetic polyesters [196], [197], [198]. Importantly, the EPS yield at day 75 was considerably higher than at day 84 (Table 1). This distinction supports the interpretation that EPS production was temporarily restored after the reactor failure, but that the accumulated EPS may have been degraded toward the end of the process. The strong increase of predatory *Micavibrionales* from 2.7% to 34.2% between days 75 and 84, together with the increase of *Microscillaceae* and *Kapabacteriales,* supports this hypothesis, as these groups are associated with predation and polymer/biomass turnover (Fig. 4).

By day 84, the low EPS yields observed (Table 1) corresponded with a lower abundance of putative EPS producers, which were mainly represented by *Sphingobacteriaceae, Xanthomonadales*, and *Spirosomaceae* (Fig. 4). These results suggest that the reactor disturbance shifted the community toward stronger polymer and biomass turnover, while reducing the dominance of taxa associated with sustained EPS production.

Overall, within the optimal operational window up to day 39, R7 showed good EPS yields and a balanced community in which putative EPS producers were well represented. The results from Exp3 and Exp_4 also indicate the development of microorganisms potentially involved in intracellular polymer storage and turnover. The lower COD removal efficiencies observed in the VFA-fed reactors compared with the glycerol-fed reactors from Exp_1 and Exp_2 (Table 1) were more likely influenced by operational factors and/or differences in community adaptation to the VFA feed. Intracellular polymer storage may therefore have affected the partitioning of converted carbon between storage polymers, EPS, biomass, and residual soluble COD.

4.3.5 The black box of complex MMC function for EPS overproduction: conclusions and perspectives

The experiments carried out in this study showed that a range of EPS-producing MMCs can develop from two different aerobic sludge inocula in seven reactors operated for EPS overproduction under nitrogen-limited conditions, using either

glycerol or VFAs as substrate. Recent research on mixed-culture-derived EPS production remains limited (e.g., [29], [31], [199]), and many aspects still require deeper understanding. To the best of our knowledge, only a limited number of studies have explored microbial composition and diversity in mixed-culture systems aimed at EPS-flocculant production, although these studies used a different strategy from the EPS-overproduction approach applied in this thesis. In the study by Ng et al. [200], an MMC was first enriched from activated sludge using glycerin-pitch under an aerobic dynamic feeding strategy, primarily intended to select for PHA-accumulating microorganisms. The enriched culture was then tested in a separate batch reactor with glycerin-pitch wastewater, where EPS were recovered as a possible coproduct rather than as the main target of a dedicated continuous EPS-overproduction process. This strategy enriched several putative EPS-producing taxa, including *Thauera, Dokdonella* , and *Phaselicystis*.

In our study, once the EPS-overproduction strategy was applied (COD:N 100/SRT 3 days) to the reactors, *Brevundimonas* was found to be one of the most consistently enriched microorganisms, being detected in all bioreactors except R1, irrespective of inoculum source or substrate type. A similar pattern was observed for taxa affiliated with *Microbacterium*/*Microbacteriaceae* and *Pseudoxanthomonas* (Table 2). In contrast, *Chryseobacterium* and *Flavobacterium* were specifically enriched in all VFA-fed reactors (R5, R6, and R7). All these groups were discussed as putative EPS-producing microorganisms and may represent key players in maintaining community functions related to biopolymer production.

Notably, clear dominance by a single putative EPS producer was observed only in R3, where *Novosphingobium* reached 53.9% relative abundance (Fig. 2A). This likely represents a reactor-specific selection event, in which the imposed conditions strongly favoured this EPS-producing taxon. In all other reactors, EPS production appeared to be supported by a broader consortium of taxa present at moderate to low relative abundances, approximately between 2 and 11% (Figs. 1, 2, 3, and 4). This suggests that EPS production in these MMC systems was generally a community-level process rather than being dominated by a single taxon.

Several genera and bacterial groups discussed in this chapter are well adapted to nutrient-limited (oligotrophic) conditions. Such microorganisms can persist under macronutrient scarcity by using alternative metabolic pathways, including

nitrification, metal or sulphur oxidation, inorganic carbon fixation, or polymer storage [201], [202]. With excess carbon, they often employ alternative metabolic pathways or storage strategies to survive and grow when key nutrients (e.g., nitrogen, phosphorus) are scarce [203]. Activated sludge from aerobic bioreactors and WWTPs often contains these functionally versatile organisms, because feast/famine cycles, nutrient limitation, and engineered nutrient removal create strong selective pressures. When such sludge is used as inoculum, MMCs can therefore be steered toward the accumulation of intracellular or extracellular polymers.

However, which polymer is favoured depends on the substrate and operating strategy. Both glycerol and VFAs can support PHA production, with VFAs being particularly suitable because they are readily obtained by waste acidogenic fermentation and are preferentially stored as PHA by many microorganisms [204]. However, in the absence of an aerobic dynamic feeding strategy, based on alternating carbon-rich and carbon-depleted periods, PHA accumulation in MMCs is generally reduced. In contrast, the operational conditions applied in the glycerol-fed MBRs of Exp_1 and Exp_2 directed the system toward EPS production [29], with communities composed of both putative EPS producers and degraders.

This producer-degrader balance is relevant because EPS are not only storage-related polymers, but also the main structural components of flocs and granules in bioreactors. EPS producers build and stabilise the floc/biofilm matrix, while EPS degraders recycle matrix material during endogenous or low-substrate phases. In this way, the system may reach a quasi-equilibrium that supports aggregate stability and long-term reactor function. However, if this balance shifts too strongly toward degradation, matrix destabilisation may occur, potentially leading to poorer settling, bulking, or biomass loss.

In the case of Exp_3 and Exp_4, where VFAs were used as substrate, many of the identified bacterial groups have been reported to produce both EPS and PHA, including *Brevundimonas, Flavobacterium, Pseudoxanthomonas, Microbacterium, Sphingobium*, and *Acinetobacter* [127], [128], [129], [131]. This makes it difficult to discriminate their specific role based on phylogenetic analysis alone. Therefore, based on the available literature, the producer-degrader equilibrium in Exp_3 and Exp_4 appeared to be linked not only to EPS, but also to possible PHA turnover. Although analytical analysis performed in Exp4 largely

excluded substantial PHA accumulation (Table 2) [31], the presence of several taxa with potential to degrade hydrocarbons and polyesters, including *Sandaracinobacter, Stenotrophomonas, Gordonia, Microscillaceae, Pseudochelatococcus, Taibaiella, Tannerellaceae, Kapabacteriales*, together with predatory or parasitic bacteria such as *Micavibrionales, Bdellovibrio, Babeliales, Berkiella*, and *Rickettiales* (Figs. 3B and 4), suggests that part of the COD may have been transiently channelled into intracellular polymers that were rapidly degraded. This interpretation is plausible because PHA synthesis and degradation can occur simultaneously within cells [195]. PHA-producing microorganisms can depolymerize intracellular PHA into monomeric R-hydroxycarboxylic acids, which may subsequently be released extracellularly [205]. Thus, low measured PHA levels do not necessarily exclude intracellular PHA turnover but may instead reflect the absence of a targeted PHA-accumulation strategy. In our case, the presence of such bacteria may reduce the potential of some community members to accumulate EPS due to nutrient availability. Importantly, this does not compromise the main goal of identifying operational conditions that promote EPS production while minimizing carbon loss to PHA accumulation [31]: the analytical data indicate that this was achieved.

To resolve these open questions, future studies should move beyond 16S rRNA gene-based community profiling and apply metagenomics and transcriptomic or other functional approaches. Only by applying such methods can it be determined whether genes related to EPS biosynthesis, PHA synthesis, PHA degradation, hydrolysis, and alternative energy metabolism are actively expressed during different operational phases. Existing transcriptomic studies on pure-culture EPS overproduction could provide useful benchmarks for developing similar approaches in MMC systems. In parallel, future research should further optimize operational parameters to improve EPS production from different substrates and to steer the balance between polymer producers and degraders toward higher recoverable EPS yields.

4.4 Conclusions

- EPS-overproduction conditions consistently steered glycerol-fed MMCs toward EPS overproduction and enriched communities containing both putative EPS producers and EPS degraders.

- Both municipal and industrial sludge inocula developed functionally similar communities under EPS-overproduction conditions.
- EPS production was generally not driven by one dominant microorganism; it appeared to be a community-level function supported by multiple taxa at moderate abundance.
- In VFA-fed reactors, reactor configuration strongly shaped microbial community assembly and process performance. The CSTR supported higher COD removal and EPS yield than the MBR, despite lower bacterial diversity.
- VFA-fed systems enriched microorganisms potentially involved in both EPS and PHA metabolism, together with polymer-degrading and predatory taxa, indicating active polymer turnover.
- The presence of putative EPS producers alone was insufficient to explain EPS accumulation, showing that EPS yield depends on the broader producer-degrader balance and reactor conditions.

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Chapter 5

Flocculation of diverse minerals and iron-rich colloids via calcium bridging with mixed microbial culture-derived EPS

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Abstract

Efficient solid-liquid separation is fundamental for stable water treatment. This study evaluates mixed culture-derived extracellular polymeric substances (EPS) as bioflocculants and investigates how EPS composition and particle surface properties jointly govern flocculation performance. Distinct EPS types were studied for their flocculation performance using mineral suspensions (kaolinite, montmorillonite, talc) with varying hydrophilicity, and with an iron-rich colloidal suspension. Calcium dosing at a minimum concentration of 0.16–0.24 g Ca^{2+} g^{-1} TSS was essential to achieve robust performance with EPS, acting as a mediator in cation-bridged polymer attachment. Under optimized Ca^{2+} conditions 0.24 g Ca^{2+} g^{-1} TSS, EPS achieved high turbidity removal (>90%) in moderately charged, hydrophilic kaolinite suspension, with flocculation efficiencies exceeding 90%. In contrast, for highly charged and strongly hydrophilic montmorillonite, turbidity removal and flocculation efficiency were lower ($\sim$ 70% and 45–52%, respectively). EPS had a limited effect on flocculation of hydrophobic talc, with maximum flocculation efficiency reaching $\sim$ 30% and turbidity removal of $\sim$ 50%. The different EPS types performed similarly across the mineral suspensions, indicating that flocculation was governed primarily by mineral type rather than by EPS characteristics. In a more complex matrix, namely an iron-rich colloidal suspension, the required EPS dose increased substantially compared with that for the model minerals. To achieve high turbidity removal $\sim$ 90% and a flocculation efficiency of 69%, the required dose increased from 0.3 to 0.5 mg EPS g^{-1} TSS. Notably, comparable flocculation efficiency ($\sim$ 75%) could be achieved without Ca^{2+} addition only when the dosage of polysaccharide-rich EPS was increased to 5 mg EPS g^{-1} TSS. Correlation analysis showed that molecular weight was the most consistent predictor of flocculation efficiency across particle types, whereas the influence of EPS composition was secondary and depended on particle type. Overall, this work demonstrates that mixed-culture derived EPS have the potential to replace conventional flocculants, thereby providing a mechanistic basis for advancing EPS flocculants from model systems toward practical treatment applications. Their success, however, depends on matching EPS physicochemical characteristics with the surface chemistry and hydration state of the target solids.

5.1 Introduction

Flocculants play a crucial role in water and wastewater treatment, where rapid and efficient suspended solid removal is essential for process stability and effluent quality [1]. In conventional coagulation- flocculation systems, metal-based coagulants such as alum or ferric salts are first applied to neutralize particle charges and destabilize colloids [2]. Subsequently, high molecular weight flocculants are added to facilitate the aggregation of destabilized particles into larger, denser flocs that settle or filter more readily [1], [2].

Synthetic polymeric flocculants, especially polyacrylamide (PAM) and its derivatives, are widely used in the flocculation process due to their high efficiency at low dosages [3]. However, PAM is produced from petroleum-derived acrylamide monomers, meaning its manufacture relies on fossil feedstocks and is therefore associated with greenhouse gas emissions [4]. In addition, PAM may degrade slowly under anaerobic conditions [5], potentially releasing acrylamide monomers that are toxic and persistent in the environment [6]. Residual PAM can impair downstream sludge treatment, including anaerobic digestion, where it has been linked to reduced biodegradation, methane production, and inhibition of microbial activity [7], [8]. These limitations have increased interest in sustainable, biodegradable alternatives to synthetic flocculants [9].

Extracellular polymeric substances (EPS) produced by microorganisms are promising alternatives to synthetic flocculants [9] as they inherently possess desirable features in flocculants such as high molecular weight, flexible polymer chains, and diverse functional groups [10]. EPS are biopolymer mixtures (mainly polysaccharides and proteins) [11] that can promote particle aggregation through the same key mechanisms as synthetic polymers, such as charge neutralization and polymer bridging [1], [10]. Because EPS are biodegradable, bio-(non-fossil) based, and generally non-toxic, they offer a sustainable alternative to synthetic flocculants [12].

Recent research has demonstrated that EPS flocculants can be generated from low-cost or waste-based feedstocks such as biodiesel wastewater [13], glycerol-pitch residues [14], and volatile fatty acid (VFA)-rich effluents [15] with open mixed microbial cultures (MMCs). Compared with pure culture processes, this

approach lowers production costs and supports resource recovery. Importantly, these MMC systems have yielded EPS with flocculation efficiencies comparable to those of synthetic flocculants. For example, Ng et al. reported polysaccharide-rich EPS production with 70 to 100% turbidity removal from oleochemical wastewater through batch culturing [14]. In another study, MMC-derived EPS were produced in continuous reactors treating synthetic biodiesel wastewater. These EPS displayed 81–97% flocculation efficiency on kaolinite [13]. These pioneering studies clearly indicate the potential of EPS as a viable, environmentally friendly flocculant.

Despite such promising findings, large-scale application of EPS as flocculants remains limited. This is because, besides process-related barriers, such as variability in EPS composition or unpredictable yields [16], [17], there is uncertainty about the flocculation performance of EPS across a diversity of particles encountered in real water systems. The latter concern is because EPS flocculation studies generally relied on simplified model suspensions, often involving a single mineral, such as kaolinite [13], [18], [19], [20], [21], [22], [23].

However, flocculation is a surface-phenomenon-dominated process where the different particle properties greatly affect the outcome. For instance, while investigating the interaction between model EPS (such as dextran) and silica colloids, Kwon et al. showed that H-bonds and electrostatic interactions were dominant, with phosphate groups in EPS forming stronger hydrogen bonds with neutral silanol groups than hydroxyl groups [24]. Similarly, Fang et al. reported that bacterial exopolymers interact strongly with goethite primarily through ligand exchange between phosphate groups in EPS and surface hydroxyl groups, resulting in inner-sphere complex formation [25].

From a morphological perspective, biopolymers may preferentially adsorb to rough or irregular surfaces, while smoother, plate-like particles can exhibit weaker interactions [26]. Industrial wastewaters typically contain heterogeneous mixtures of minerals, metals, organic particulates, and colloids with widely varying surface charge, hydrophobicity, functional groups, and morphology [27], [28]. Consequently, the dominant flocculation mechanisms, i.e., electrostatic interactions, polymer bridging, hydrophobic association, and ligand-polymer complexation, can differ substantially among particle types. Hence, there is a critical knowledge

gap in understanding how EPS composition governs flocculation across particles with diverse surface chemistries [24], [25], [26].

In our recently published contributions, we demonstrated that EPS production with MMCs can be significantly enhanced under nitrogen-limited, short-SRT conditions in continuously operated reactors fed with glycerol-rich [19] (**Chapter 2**) or VFA-rich synthetic wastewater [15] (**Chapter 3**). These conditions yielded EPS with distinct physicochemical profiles: glycerol-fed reactors produced high molecular weight (2.2-2.4 MDa) polysaccharide-dominant EPS [19] (PS:PN ratio >1), whereas VFA-fed reactors produced lower molecular weight EPS (1-2 MDa) with an increased protein fraction (PS:PN ratio < 1) relative to glycerol-fed reactors [15]. These EPS exhibited flocculation efficiencies between 70 and 96% with kaolinite. Yet, similarly to the commonly used flocculation-testing approach in the field, their performance on other mineral surfaces and real industrial streams has not yet been investigated.

In the present study, we investigate the flocculation efficiency of these EPS derived from glycerol and VFA-rich synthetic wastewaters across mineral particles with different surface properties (kaolinite, montmorillonite, and talc). Furthermore, the EPS were applied to an iron-rich colloidal suspension that was prepared by diluting sludge originating from a drinking water production plant to examine their performance in a complex matrix. Hence, to the best of our knowledge, this is the first study systematically investigating MMC-derived EPS of various compositions and linking flocculation performance with a wide range of particle properties, with bulk liquids with defined composition and with more complex water matrices.

5.2 Materials and methods

5.2.1 Particle suspensions and iron-rich colloidal suspension

Single particle suspensions and their physicochemical characteristics

Montmorillonite (Sigma-Aldrich, USA), kaolinite (Sigma-Aldrich, USA), and talc (VWR, USA) were selected as model minerals to simulate suspensions with contrasting surface properties. Their properties are well-documented in the literature [29], [30] and are summarised in Table 1. Montmorillonite was chosen to

represent particles with highly hydrophilic and strongly charged surfaces [31]. Kaolinite represented moderately hydrophilic and moderately charged surfaces [30]. Finally, talc was selected as a relatively hydrophobic mineral with a weakly charged surface [32]. Each suspension was prepared at 1 g L^{-1} TSS, and pH was adjusted to $\sim$ 7 before flocculation experiments.

Preparation of iron-rich colloidal suspension

Iron-rich sludge from drinking water production (provided by Pidpa, Belgium), was used to prepare an iron-rich colloidal suspension to perform flocculation experiments with colloids encountered in real streams. Total suspended solids (TSS) content of the sludge stock was 52 g L^{-1}, measured with standard methods [46]. Cation and anion profile of the liquid phase of the sludge was analysed by ion chromatography (SI-1). The iron-rich sludge contained on average 33 wt% Fe, 10 wt% Ca, and 0.12 wt% Mn (all on dry solid basis), with a dry matter content of 35% (w/w)(Table S1). The high Fe and Ca contents correspond to ferric hydroxide and calcium carbonate phases, consistent with the orange colour and alkaline pH (8-8.5). The specific surface area of the iron-rich sludge was 292.04 m^2 g^{-1}, determined by BET analysis as described in Section 2.4.2. Before conducting flocculation experiments, the sludge was diluted to 1 g L^{-1} TSS using Milli-Q water. Before dilution, the sludge was vigorously shaken and then stirred for 1 h to ensure homogeneity. The required volume was withdrawn using a wide-bore pipette while the stock was stirred continuously. The pH was not further adjusted and was measured as $\sim$ 8.2.

Table 1: Physicochemical characteristics of the mineral particles used in the flocculation experiments

Property	Description	References
Montmorillonite		
Structure	2:1 dioctahedral aluminosilicate; interlayer with exchangeable hydrated cations	[31], [29], [30]
Typical morphology / size	Thin nanoplates; highly disperse	
Main surface functional groups	Basal siloxane Si–O–Si, octahedral M–OH; exchange sites in interlayer	[33], [29], [30]
Specific surface area (SSA)	217.2874 m^2 g^{-1}	*
Cation-exchange capacity (CEC)	$\sim$ 70–120 cmol(+) kg^{-1} (high)	[34]
Zeta potential (water)	Typically, negative over a wide pH range (changes little with pH; electrolyte valence matters)	[35], [36]
Hydrophobicity	Hydrophilic surfaces; strong water uptake in interlayer (High hydrophilicity)	[37], [38]
Mean diameter [D4, 3]	23.5 μm	*
Kaolinite		
Structure	1:1 dioctahedral ($Al_2Si_2O_5(OH)_4$); non-swelling	[30], [29], [30]
Typical morphology / size	Platy/"booklet" hexagonal plates; μm-scale agglomerates	[32]
Main surface functional groups	Edge Al–OH (aluminol), basal Si–OH (silanol)	[29], [30]
Specific surface area (SSA)	6.9941 m^2 g^{-1}	*
Cation-exchange capacity (CEC)	$\sim$ 3–15 cmol(+) kg^{-1} (variable charge from edges; increases with pH)	[32], [39]
Zeta potential (water)	Usually negative from $\sim$pH 4–11; IEP often absent or near $\sim$2–3 depending on electrolyte	[32]
Hydrophobicity	Generally hydrophilic (edge –OH dominated; basal siloxane is neutral) (Moderate hydrophilicity)	[29], [40]
Mean diameter [D4, 3]	24.9 μm	*
Talc		
Structure	2:1 trioctahedral $Mg_3Si_4O_{10}(OH)_2$; non-swelling	[32], [29], [30]
Typical morphology / size	Very platy, lamellar; easily cleaves into fine flakes	
Main surface functional groups	Basal Si–O–Si (non-polar), minor edge Si–OH/Mg–OH	[32]
Specific surface area (SSA)	3.6173 m^2 g^{-1}	*
Cation-exchange capacity (CEC)	$\sim$ 0–2 cmol(+) kg^{-1} (practically negligible)	[30], [41], [42]
Zeta potential (water)	Usually negative across pH 2–12; reported IEP $\approx$ pH 2–3	[43], [44]
Hydrophobicity	Hydrophobic basal faces; edges slightly hydrophilic (Lower hydrophilicity)	[32], [44], [45]
Mean diameter [D4, 3]	23.8 μm	*

Note: *Properties measured experimentally in this study as described in Section 5.2.4. IEP: Isoelectric point.

5.2.2 Source and extraction of EPS

Mixed culture-derived EPS were produced in continuously operating bioreactors inoculated with municipal activated sludge as described in (**Chapter 2** and **3**). In short, reactors (either membrane or continuously stirred configuration) were fed with glycerol-rich and VFA-rich synthetic wastewaters and subjected to EPS overproduction conditions, namely nitrogen limitation (COD:N 100) and SRT/HRT of 3 days.

EPS extraction from the EPS-rich mixed liquor was performed using an alkaline heat extraction method. Briefly, 50 mL of fresh mixed liquor sample was centrifuged (25,000 $\times$ g, 15 min, 20 °C) and the supernatant was collected as soluble EPS (sEPS). The pellet was resuspended in 50 mL Milli-Q water, amended with Na_2CO_3 (0.29 g), heated to 80 °C and stirred at 500 rpm for 1 h. After centrifugation (20,000 $\times$ g, 15 min, 20 °C), the supernatant was collected as bound EPS (bEPS). Both sEPS and bEPS fractions were dialysed overnight at $\sim$ 20 °C. The purified sEPS and bEPS samples were freeze-dried and stored at room temperature in airtight tubes. For the characterisation and flocculation experiments, 1 g L^{-1} EPS solutions were prepared by dissolving freeze-dried EPS in Mili-Q water. The two fractions of EPS obtained, sEPS and bEPS, often differ in composition (e.g., polysaccharide/protein distribution), molecular weight, and functional groups [47], [48], which can influence flocculation mechanisms and performance. Therefore, both fractions were evaluated during flocculation experiments.

5.2.3 Physicochemical characteristics of EPS

The extracted EPSs were characterised for polysaccharide and protein content, molecular weight, charge density, functional groups, and cation content, as described in the following sections (Table 2). Based on the polysaccharide-to-protein ratios (PS/PN) of the EPS, the following categories were defined: PS-rich sEPS and bEPS, PN-enriched sEPS and bEPS, and PS–PN balanced sEPS and bEPS.

Table 2: Physicochemical properties of EPS samples used in flocculation experiments

Sample	EPS	Feed	PS*	PN*	PS/PN	MW*	CD*	Na+	M*. ion
PS-PN balanced	sEPS	Gly	0,14±0,002	0,116±0,004	1,16	90,2	0,72	0,13	0,03
	bEPS		0,22±0,013	0,23±0,011	0,96	561,7	2,48	0,12	0,02
PS-rich	sEPS	Gly	0,39±0,007	0,12±0,053	3,22	637,8	3,99	0,05	0,05
	bEPS		0,17±0,002	0,13±0,007	1,33	463,3	1,55	0,13	0,04
PN-enriched	sEPS	VFA	0,05±0,006	0,08±0,002	0,63	200,6	0,76	0,14	0,03
	bEPS		0,01±0,001	0,08±0,001	0,19	168,0	0,84	0,18	0,06

Note: *M: miscellaneous ion content in mg mg^{-1}, CD: charge density in meq g^{-1} EPS, MW: average molecular weight in kDa, PS: polysaccharide in mg mg^{-1}, PN: protein in mg mg^{-1}, Gly: glycerol,sEPS: soluble EPS, bEPS: bound EPS.

Composition, charge density and molecular weight of EPS

Polysaccharides were measured using the phenol–sulphuric acid method (absorbance at 490 nm) with glucose as the calibration standard [49]. Proteins were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (ThermoFisher Scientific, USA) following the manufacturer's protocol. Polysaccharide-to-protein (PS/PN) ratios were calculated from the measured contents. Charge density was measured following the method described by Ajao et al. [50] using a Mütek PCD 03 particle charge detector (230 V, 50 Hz. BTG, Sweden). Briefly, samples were titrated with a cationic standard solution (Poly(diallyldimethylammonium chloride, BTC, DE)), added drop-wise using a pre-weighed 10 mL syringe, until the streaming potential reached zero (charge neutralisation point). The syringe was reweighed to determine the mass (and volume) of titrant added. Sample charge density was then calculated from the titrant charge concentration, the amount of titrant required to reach the endpoint, and the sample EPS concentration [50]. The determination of the average molecular weight of EPS was carried out on the resuspended EPS samples through liquid chromatography-organic carbon detection (LC-OCD - Model 8, DOC-LABOR, DE) as described by Ajao et al. [13]. Sodium and other inorganic ions in the EPS samples were quantified by ion chromatography (IC), as described in Supplementary Information (SI-1). Prior to analysis, all samples were filtered through a 0.45 µm PTFE membrane.

Functional groups analysis via Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was performed on freeze-dried EPS samples using an ALPHA II FTIR spectrometer (Bruker Optics, Germany). Spectra were collected over 4000–450 cm^{-1} with 40 scans per sample, at a spectral resolution of 4 cm^{-1}. Spectral processing and analysis were conducted using the instrument software (OPUS) over 4000–400 cm^{-1}. Band assignments were made according to the peak list provided in the Supplementary Information (Table S2).

5.2.4 Colloidal particle characterisation

Particle size distribution

Particle size distributions of kaolinite, montmorillonite, and talc suspensions were measured using a laser diffraction analyser (Mastersizer 3000, Malvern Instruments, UK). Particle suspensions were circulated through the optical unit using a peristaltic pump (6 mm ID tubing) at 2.5 L min^{-1}. Water was used as dispersant (refractive index 1.33) and particles were treated as non-spherical. Particle optical parameters (refractive and absorption indices) were set in the software (e.g., kaolinite: RI 1.56, absorption 0.01; density 2.56 g cm^{-3}; montmorillonite: RI 1.52, absorption 0.01, density 2.2–2.6 g cm^{-3}; talc: RI 1.57, absorption 0.01, density 2.75–2.80 g cm^{-3}). Obscuration limits were 0.1–20%. Background and sample acquisition times were 30 s for both red and blue measurements with a 120 s stability criterion. Each sample was measured five times, and results were averaged. Particle size was reported as the volume-based median diameter, $D_{[4,3]}$.

5.2.5 Brunauer–Emmett–Teller (BET) specific surface area measurement

Specific surface area of kaolinite, montmorillonite, talc, and iron-rich sludge were determined by N_2 gas adsorption using a TriStar 3000 BET surface area and porosity analyser (Micromeritics, NL). Samples were loaded into glass sample tubes ($\frac{1}{2}$-inch stem diameter) and sealed with butyl rubber stoppers. Before adsorption measurements, samples were degassed using a SmartPrep^{TM} 065 degassing unit (Micromeritics, NL) to remove adsorbed moisture and volatile contaminants. After degassing, the tubes were transferred to the analyser and adsorption-desorption isotherms were collected at 77 K using liquid nitrogen. Helium was used for free-space determination. The BET specific surface area was calculated from the adsorption data using the BET model, applying the linear region of the BET plot within the selected P/P_0 range and using the instrument software (TriStar analysis program, v. 6.04). The results are reported as m^2 g^{-1}.

5.2.6 Flocculation experiments

Multiple flocculation experiments were performed to investigate i) the influence of coagulant and EPS flocculant dose (Experiment 1), ii) differences in EPS

flocculation behaviour of various particles (Experiment 2), iii) the influence of EPS composition on flocculation of various particles (Experiment 3) and iv) the feasibility of flocculation of particles in a complex matrix with EPS of various compositions with or without a coagulant (Experiment 4). The core parameters, namely Ca^{2+} dosage, EPS dosage, EPS type, and particle type, are shown in Table 3. The total available surface area for each particle in the flocculation experiments is presented in Table 4. Details of the calculations are provided in the Supplementary Information (SI-3).

In Experiment 1, a wide dosage range of both Ca^{2+} and EPS was screened using kaolinite and PS-PN balanced EPS to study the dose effects of EPS and Ca^{2+}. Albeit not directly translatable to other particles, these results were used to screen a narrower range of doses of EPS and Ca^{2+} for flocculation of montmorillonite and talc in subsequent experiments (Experiments 2 and 3). Finally, in Experiment 4, the optimal Ca^{2+} and EPS dosages were re-evaluated because a more complex matrix was used. Under these conditions, the applied Ca^{2+} and EPS dosages differed slightly from those used for the single-particle suspensions. In particular, for direct flocculation with EPS in the absence of Ca^{2+}, a higher EPS dosage (5 mg EPS g^{-1} TSS) was applied to ensure sufficient interaction between EPS and the particles.

Flocculation experiments were conducted following the method described in **Chapter 2**. In short, for each experiment, Ca_{2+} and EPS were used at varying dosages, and a particle suspension without addition of any Ca_{2+} or EPS was used as a negative control. To evaluate the flocculation efficiency of EPS relative to samples coagulated with Ca_{2+}, another control containing only Ca_{2+} was included. In each flocculation experiment, the controls and EPS-treated samples (EPS+Ca_{2+}) were handled identically. After settling, supernatants were sampled from the same depth (1 cm) in each jar, and the residual turbidity was measured in nephelometric turbidity units (NTU).

The flocculation efficiency (FE) of EPS was calculated relative to the calcium-treated control using the following equation (Eq.1):

$$FE = \frac{\mathrm{NTU}_c - \mathrm{NTU}_{\mathrm{EPS+Ca}}}{\mathrm{NTU}_c} \qquad \text{(Eq.1)}$$

where NTU_c represents the residual turbidity of the control containing particles with Ca^{2+}, and $\mathrm{NTU}_{\mathrm{EPS+Ca}}$ is the residual turbidity of the sample treated with EPS in the presence of Ca^{2+}. Total coagulation-flocculation efficiency was calculated with the same expression, but here NTU_c is the negative control of particle suspension without addition of Ca^{2+}.

Table 3: Experimental design

Objective	Experiment 1	Experiment 2	Experiment 3	Experiment 4	
				With coagulant	Without coagulant
EPS flocculant type	PS-PN balanced bEPS	PS-PN balanced sEPS and bEPS	PS-rich sEPS and bEPS, PN-enriched sEPS and bEPS	PS-rich sEPS and bEPS, PN-enriched sEPS and bEPS	PS-rich sEPS and bEPS, PN-enriched sEPS and bEPS
Particle type	Kaolinite	Kaolinite, montmorillonite, talc	Kaolinite, montmorillonite, talc	Iron-rich colloidal suspension from drinking water production	Iron-rich colloidal suspension from drinking water production
Coagulant	$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$	No coagulant
Coagulant dosage (g Ca^{2+} g^{-1} TSS)	0.02, 0.04, 0.08, 0.16, 0.24, 0.32, 0.40	0.16 and 0.24	0.24	0.16	–
Flocculant dosage (mg EPS g^{-1} TSS)	0, 0.1, 0.3, 0.5	0.3, 0.5	0.3	0.5, 1.0	0.5, 5.0

5.2.7 Correlation analysis

Correlation analysis was performed to explore relationships between flocculation efficiency and EPS characteristics (molecular weight, charge density, polysaccharide content, protein content, and PS/PN ratio) for each mineral-EPS combination. Pearson correlation coefficients (r) were calculated in Microsoft Excel using the built-in CORREL function based on six paired observations (n = 6). The correlations were descriptively interpreted based on the magnitude and direction of r, and no formal statistical significance testing was applied due to the limited sample size.

5.2.8 Electric double layer thickness (Debye Length) calculation

To interpret the coagulation and flocculation behaviour, the electric double-layer (EDL) thickness was estimated for the tested suspensions. The EDL controls the range of electrostatic repulsion between colloidal particles: thick double layers at low ionic strength promote colloidal stability, whereas increasing ionic strength (e.g., by Ca^{2+} addition) compresses the EDL, reduces repulsive forces, and increases the probability of particle-particle collisions and aggregation [51]. Electric double-layer thickness, also called the Debye length κ^{-1}, was calculated from the ionic strength (I) of the suspension [51]. Ionic strength was determined from measured ion concentrations using Eq.2:

$$I = \frac{1}{2} \sum c_i z_i^2 \qquad \text{(Eq.2)}$$

where c_i is the molar concentration of ion i and z_i is its charge. The Debye length was then calculated (Eq.3) assuming aqueous solution at 25 °C:

$$\kappa^{-1}\ (\text{nm}) \approx \frac{0.304}{\sqrt{I\ (\text{M})}} \qquad \text{(Eq.3)}$$

The resulting κ^{-1} values indicate the EDL thickness under the tested conditions.

Table 4: The total available surface area for each particle in the flocculation experiments, calculated considering a TSS concentration of 1 g L^{-1} and a volume of 0.3 L per experiment (SI-3)

Particle type	BET SSA (m^2 g^{-1})	Total surface area in the experiments (m^2)
Kaolinite	6.99	2.1
Montmorillonite	217.28	65.2
Talc	3.61	1.1
Iron-rich sludge	291.04	87.3

Note: BET SSA: Brunauer–Emmett–Teller specific surface area.

5.3 Results

5.3.1 Investigating the influence of coagulant and flocculant dose

Most studies in the field of natural flocculants do not report the rationale behind choosing a given dose of a coagulant, whereas it is known to be of paramount importance and is typically determined experimentally [51]. Hence, the effect of coagulant (Ca^{2+}) and EPS dosages on flocculation was investigated in Experiment 1 (Table 3). The PS-PN balanced bEPS were applied in this experiment.

As shown in Fig. 1A, the initial EDL of kaolinite particles was $\sim$ 7.8 nm at the lowest Ca^{2+} concentration of 0.02 g Ca^{2+} g TSS^{-1} (0.5 mM Ca^{2+}), corresponding to an initial turbidity of about 900 NTU. Increasing Ca^{2+} concentration without EPS addition reduced turbidity considerably, with an apparent maximum of 80% removal at 0.08 g Ca^{2+} g^{-1} TSS (2 mM Ca^{2+}) and very similar turbidity removals at increasing Ca^{2+} dosages. This particle destabilization is mainly attributed to double layer compression and may also be aided by specific adsorption (association) of Ca^{2+} at kaolinite surface sites (e.g., $Si - O^-$) that promotes charge neutralization [2].

Upon addition of EPS, a distinct increase in residual turbidity removal was observed, reaching 91–96% across different Ca^{2+} - EPS combinations (Fig. 1B). Even at an EPS dosage of only 0.1 mg g^{-1} TSS, turbidity removal was consistently high (>90%) across a wide range of Ca^{2+} dosages (Fig. 1B). This improvement in flocculation by EPS addition most likely reflects the transition from simple

coagulation to combined effect of coagulation-flocculation, in which the negatively charged EPS act as bridging polymers linking particles into larger, settle-able flocs [9]. Flocculation efficiency (FE) increased progressively with both Ca^{2+} and EPS dosages, showing that a minimum of 0.16 g Ca^{2+} g^{-1} TSS (4 mM Ca^{2+}) and 0.3 mg EPS g^{-1} TSS were required to achieve high FE (> 74%) (Fig. 1C). Further increment of EPS dosage to 0.5 mg EPS g^{-1} TSS only lead to a minor improvement of FE, reaching up to 75% at the same Ca^{2+} dosage. The EDL decreased from 7.8 nm to 2.8 nm as Ca^{2+} increased to 0.16 g Ca^{2+} g^{-1} TSS (4 mM Ca^{2+}), and further to 2.3 nm at 0.24 g Ca^{2+} g^{-1} TSS (6 mM Ca^{2+}), beyond which no significant effects on EDL compression occurred (Fig. 1A). Hence, 0.24 g Ca^{2+} g^{-1} TSS (6 mM Ca^{2+}) with 0.3 mg EPS g^{-1} TSS was selected as the optimum condition for subsequent experiments.

5.3.2 Flocculation of kaolinite, montmorillonite and talc with polysaccharide and protein balanced EPS-flocculants

In single particle suspensions (Experiment 2), both the sEPS and bEPS fractions of PS-PN balanced EPS exhibited particle-dependent flocculation performance (Fig. 2). With kaolinite, flocculation efficiency (FE) was high (>90%) at both previously identified optimal Ca^{2+} concentration (0.24 g Ca^{2+} g^{-1} TSS) and the slightly lower concentration (0.16 g Ca^{2+} g^{-1} TSS), as well as at both EPS doses tested (0.3 and 0.5 mg EPS g^{-1} TSS) (Fig. 2A). Furthermore, differences between bEPS and sEPS were small (Fig. 2A), despite the general tendency of sEPS fractions to exhibit higher PS/PN ratios (Table 3). This consistency in residual turbidity and FE suggests that once destabilization and bridging conditions were met, EPS-specific differences such as PS/ PN ratio, molecular weight, and charge density (Table 3) played a secondary role. Such plateau behaviour is consistent with Ca^{2+}- mediated bridging-dominated flocculation, where EDL compression facilitates EPS adsorption and interparticle linkage in kaolinite systems [50], [52], [53].

In contrast to kaolinite, montmorillonite showed only moderate turbidity removal ($\sim$ 70%) and comparatively low flocculation efficiency (45–55%). However, similarly to kaolinite, only minor differences between bEPS and sEPS were observed (Fig. 2B). The native montmorillonite suspension (without addition of Ca^{2+} or EPS) exhibited lower turbidity ($\sim$ 78 NTU) compared to kaolinite ($\sim$ 840 NTU). Addition of Ca^{2+} alone reduced residual turbidity to $\sim$ 35

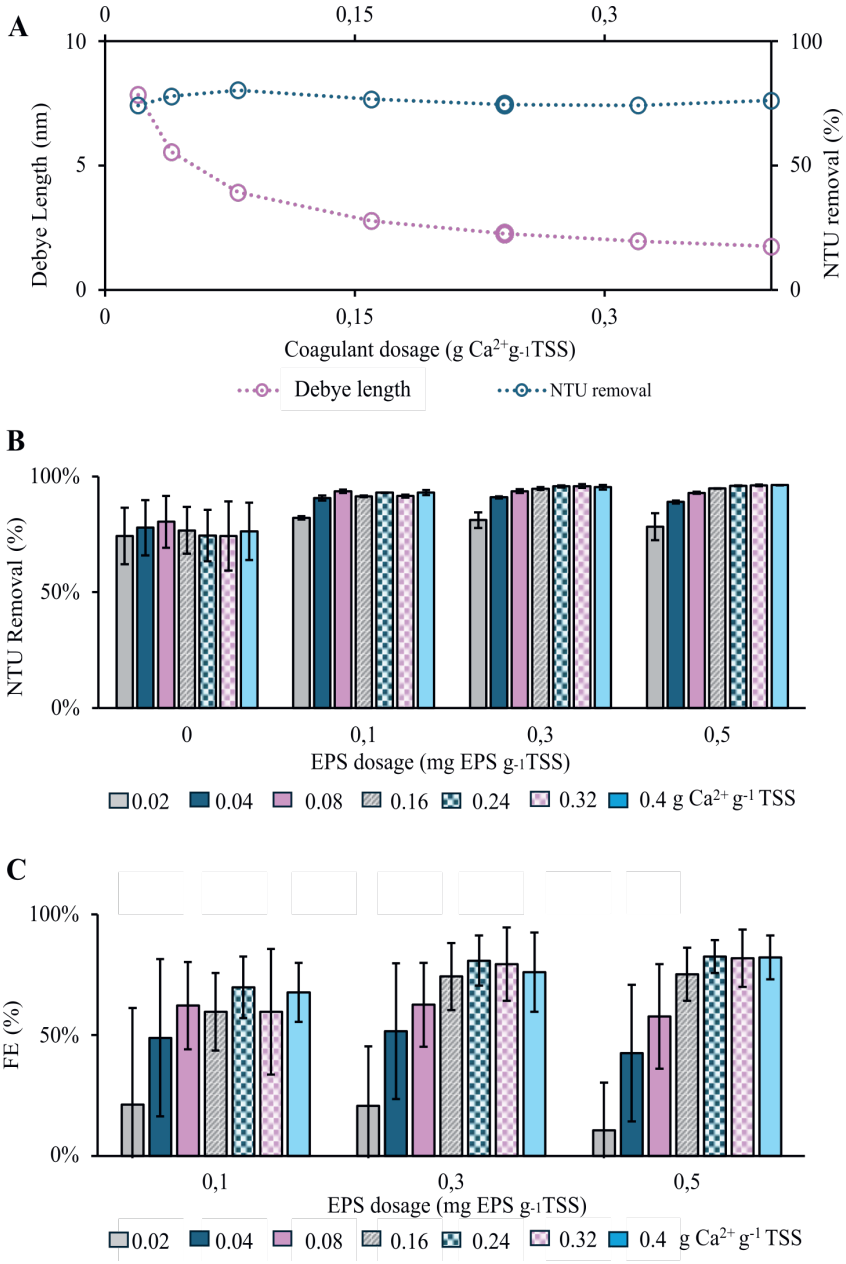


Figure 1: Coagulant-flocculant dosage experiment with kaolinite (Experiment 1). Ca^{2+} was used as coagulant. A) Debye length-turbidity removal relation of kaolinite when coagulated with Ca^{2+} at different dosages (g Ca^{2+} g TSS^{-1}) without flocculation with EPS. B) Turbidity removal percentage of kaolinite at different PS-PN balanced bEPS and Ca^{2+} dosages (indicated by coloured boxes in g Ca^{2+} g TSS^{-1}). C) Flocculation efficiency of EPS relative to coagulation under the same conditions as in (B). Each value is the average of two samples (absolute error, $n = 2$).

NTU (50% removal) at both 0.16 and 0.24 g Ca^{2+} g^{-1} TSS dosages, indicating substantial cation-induced destabilization. Such decreased turbidity generally complicates flocculation due to reduced probability of successful collisions [51]. Nevertheless, combined Ca^{2+}-EPS treatment further decreased residual turbidity to $\sim$ 20 NTU (73–75% removal) (Fig. 2B). The corresponding FE ranged from 38 to 44% at 0.3 mg EPS g^{-1} TSS and decreased slightly at 0.5 mg EPS g^{-1} TSS, particularly for sEPS while bEPS generally maintained higher FE under comparable conditions (Fig. 2B). The latter is consistent with the higher surface charge of montmorillonite compared to kaolinite (Table 1) making bEPS with higher charge (2.5 meq g^{-1} EPS) more effective than sEPS (0.72 meq g^{-1} EPS). Since the applied Ca^{2+} concentration (4 and 6 mM), exceeded the theoretically required amount to screen all charges on the surface of montmorillonite ($\sim$ 0.425 mM Ca^{2+}), bEPS likely adsorbed more effectively and thereby enhanced polymer bridging.

Talc exhibited the lowest turbidity removal and FE (<30%), indicating weak EPS-particle interactions (Fig. 2C). Native talc showed an initial turbidity of $\sim$ 169 NTU. At 0.16 g Ca^{2+} g^{-1} TSS (4 mM Ca^{2+}) the turbidity was reduced to $\sim$ 78 NTU. Increasing Ca^{2+} to 0.24 g Ca^{2+} g^{-1} TSS (6 mM Ca^{2+}) lowered turbidity to $\sim$ 59 NTU. This trend is consistent with salt-induced destabilization of relatively hydrophobic talc suspensions (Table 1), where increased ionic strength compresses the EDL, reduces already modest electrostatic repulsion, and promotes aggregation [51]. EPS addition only slightly improved turbidity removal, reflecting weak EPS-talc interaction. The maximum FE (29%) occurred with bEPS at 0.3 mg EPS g^{-1} TSS and 0.16 g Ca^{2+} g^{-1} TSS (4 mM Ca^{2+}),suggesting limited adsorption. Hence, compared to typically studied kaolinite, the flocculation efficiency decreased with more hydrophobic particles (talc) or hydrophilic particles carrying more charge (montmorillonite).

5.3.3 Flocculation of kaolinite, montmorillonite and talc with polysaccharide and protein enriched EPS

In Experiment 3, which investigated the effect of EPS composition on the flocculation of various particle types, both PS-rich and PN-enriched EPS enhanced turbidity removal from mineral suspensions compared with Ca^{2+}-destabilized suspensions (Fig. 3). Hence, the results were similar to PS-PN balanced EPS

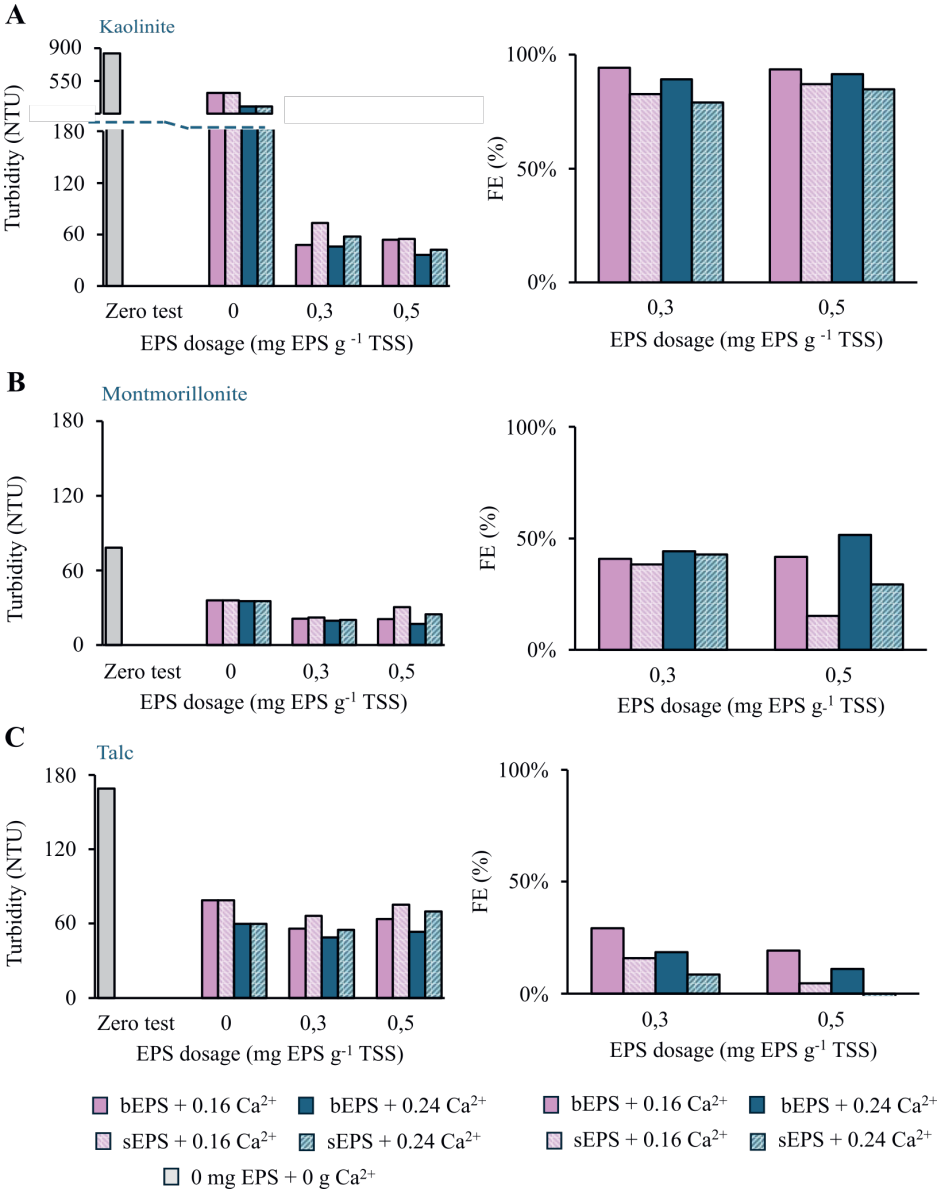


Figure 2: Residual turbidity measurements (NTU) and flocculation efficiencies (FE) reported for (A) kaolinite, (B) montmorillonite and (C) talc with two different EPS dosages (0.3 and 0.5 mg EPS g^{-1} TSS) in the presence of 0.16 and 0.24 g Ca^{2+} g^{-1} TSS (Experiment 2). The bound EPS (bEPS) and soluble EPS (sEPS) applied are from a PS-PN balanced EPS type (see Materials and Methods).

(Fig. 2). Variations in turbidity removal and FE were observed mainly among mineral types, while differences between PS-rich and PN-enriched EPS were most evident for kaolinite (Fig. 3). As noted above, despite the identical TSS concentration (1 g L^{-1}) in all suspensions, initial turbidity varied among particle types. This difference likely affected the probability of successful collisions between destabilized particles during orthokinetic flocculation and, therefore, may partially explain the higher turbidity removal observed for kaolinite compared to talc and montmorillonite.

The strongest flocculation effect was observed for kaolinite, where turbidity removal exceeded 90% for all EPS samples, compared with 74% removal achieved by Ca^{2+} alone (Fig. 3B). The native kaolinite suspension (without Ca^{2+} or EPS addition) exhibited a turbidity of approximately 834 NTU. Addition of Ca^{2+} alone reduced the residual turbidity to 220 NTU, while subsequent EPS addition further lowered it to 30–70 NTU, depending on the EPS type (Fig. 3A). Flocculation efficiency was higher for PS-rich EPS (85% and 33 NTU for sEPS; 83% and 37 NTU for bEPS) than for PN-enriched EPS (64% and 75 NTU), suggesting that PS-rich EPS provided a stronger bridging effect with kaolinite (Fig. 3C).

For montmorillonite, the improvement in turbidity removal was modest, increasing from 25% with Ca^{2+} coagulation alone to 46–58% following EPS addition (Fig. 3B). The native montmorillonite suspension exhibited a turbidity of approximately 65 NTU, which decreased slightly to 50 NTU after Ca^{2+} addition and further to around 30 NTU in the presence of EPS (Fig. 3A). Notably, differences in turbidity removal and FE between PS-rich EPS and PN-enriched were limited for montmorillonite (Fig. 3A, B, C), except for PN-enriched bEPS, which yielded slightly lower turbidity removal and FE (Fig. 3B, C). These results suggest a lower probability of successful particle collisions in montmorillonite suspensions and therefore weaker EPS–particle interactions than in kaolinite suspensions. This is consistent with the high charge density and strong hydration of montmorillonite surfaces, which result in strong electrostatic and hydration repulsion, respectively. Together, these repulsive interactions can hinder EPS adsorption and polymer bridging in the montmorillonite system [54].

Similar to earlier observations (section 3.2), Ca^{2+} coagulation reduced the turbidity of the native talc suspension from approximately 163 NTU to about 75 NTU, corresponding to ∼ 50% turbidity removal (Fig. 3B). The additional effect of EPS was limited: the lowest residual turbidity (approximately 65 NTU) was achieved with PS-rich bEPS, corresponding to 61% turbidity removal. Flocculation efficiencies ranged from 7 to 17%, whereas PN-enriched bEPS showed a negative effect (Fig. 3C). These results suggest that EPS adsorption to talc remained limited despite differences in EPS composition (Table 3), and that turbidity removal was driven mainly by reduced electrostatic repulsion at higher ionic strength [51].

5.3.4 Flocculation of iron-rich colloids from drinking water production facility with different types of EPS flocculants

Similarly to single particle suspensions (Section 5.3.1), in experiment 4, first the effective Ca^{2+} dose required to destabilise the iron-rich colloids was determined. At a colloidal particle concentration of 1 g TSS L^{-1} (ionic strength of 0.00014 mM, pH ∼ 8.2), the initial EDL thickness was approximately 26 nm (Fig. 4A). This corresponded to an initial turbidity of ∼ 760 NTU, indicating that the colloidal suspension was highly stable. Upon coagulation with Ca^{2+}, the residual turbidity decreased to ∼ 160 NTU, while the EDL thickness decreased sharply to 3.92 nm at 0.08 g Ca^{2+} g^{-1} TSS (2 mM Ca^{2+}) and to 2.78 nm at 0.16 g Ca^{2+} g^{-1} TSS (4 mM Ca^{2+}) (Fig. 4A). This compression of the EDL was consistent with the observed turbidity removal trends: Ca^{2+} alone achieved 73% turbidity removal at 0.08 g Ca^{2+} g^{-1} TSS (2 mM Ca^{2+}), which increased to 78% at 0.16 g Ca^{2+} g^{-1} TSS, after which removal reached a plateau (Fig. 4A). Accordingly, 0.16 g Ca^{2+} g^{-1} TSS (4 mM Ca^{2+}) was selected as the effective dose to promote destabilization of the colloidal particles in the iron-rich colloidal suspension.

Dosage screening using PS-PN balanced EPS showed that, at EPS doses of 0.1–0.3 mg EPS g^{-1} TSS, turbidity removal remained comparable to that achieved by Ca^{2+} coagulation alone (∼ 67%, 250 NTU) (Fig. 4B). Increasing the EPS dose to 0.5 mg EPS g^{-1} TSS improved turbidity removal to 85% (66 NTU), suggesting that a minimum dose of ∼ 0.5 mg EPS g^{-1} TSS was required to achieve satisfactory flocculation efficiency under these conditions. Evidently,

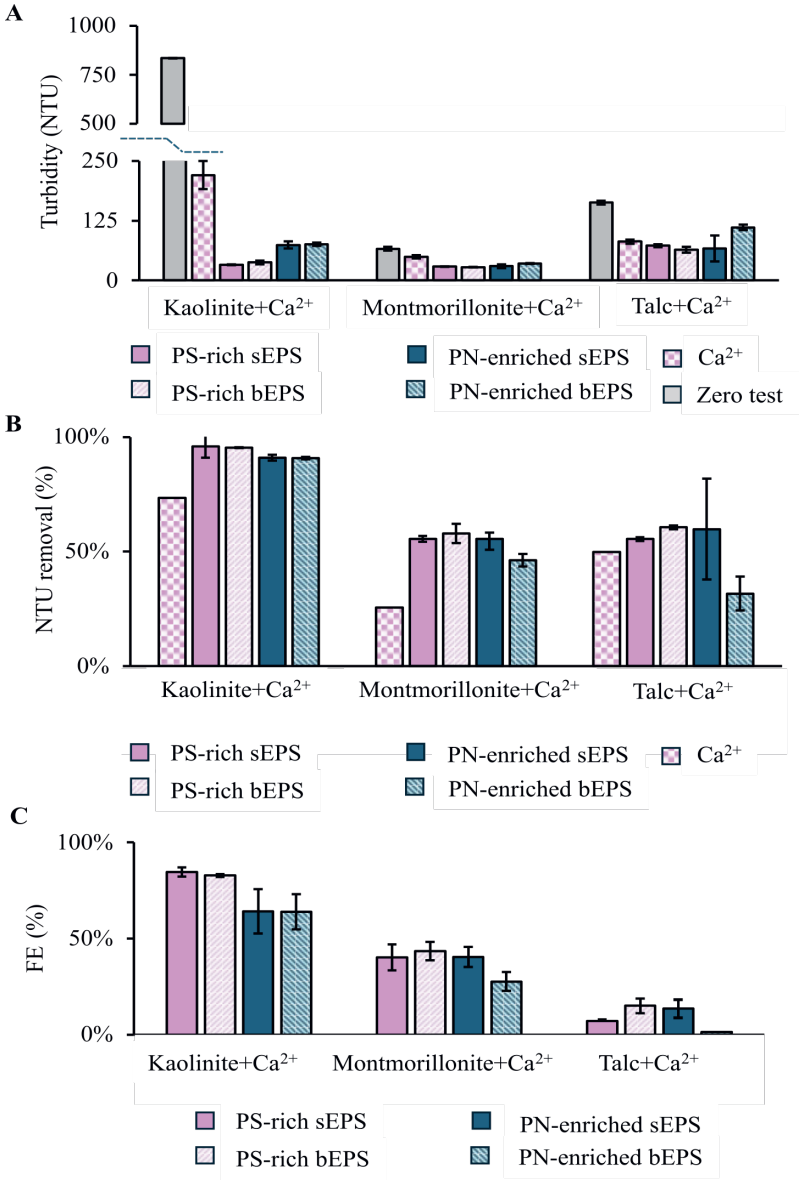


Figure 3: Influence of EPS composition on flocculation of various particles at the optimal dosages of Ca^{2+} of 0.24 g Ca^{2+} g^{-1} TSS and EPS of 0.3 mg EPS g^{-1} TSS (Experiment 3). In (A) the residual turbidity measurements (NTU), in (B) the average turbidity removal, and in (C) the average flocculation efficiency (FE). Values are reported for two samples (absolute error, $n = 2$).

the iron-rich colloidal suspension required a higher flocculant dose than the model colloidal particles (kaolinite, montmorillonite and talc) examined in this study. Therefore, subsequent experiments comparing PS-rich and PN-enriched EPS were conducted at two EPS doses, namely 0.5 and 1.0 mg EPS g^{-1} TSS (Fig. 4C, D).

During combined coagulation-flocculation with 0.16 g Ca^{2+} g^{-1} TSS and PS-rich EPS, a substantial improvement over coagulation alone (65–67% turbidity removal) was achieved (Fig. 4C, D). This effect was particularly pronounced for sEPS, for which turbidity removal efficiencies of 90–94% were obtained. Accordingly, the turbidity of the suspension decreased from 250–280 NTU to 50–77 NTU when PS-rich sEPS was applied as the flocculant (Fig. 4C, D). The PS-rich bEPS also further reduced turbidity relative to coagulation alone, although it was less effective than sEPS (Fig. 4C, D). Its turbidity removal efficiency was approximately 82% (140 NTU), corresponding to flocculation efficiencies of 44–51%, with no significant difference between the 0.5 and 1.0 mg EPS g^{-1} TSS doses. The PN-enriched EPS showed performance similar to that of PS-rich bEPS, with turbidity removal efficiencies ranging from 79% to 83% (130–160 NTU) (Fig. 4C, E), regardless of dose (0.5 or 1.0 mg EPS g^{-1} TSS), and with no measurable difference between sEPS and bEPS.

To assess the potential for direct flocculation by EPS, experiments were performed in the absence of Ca^{2+}, using the same PS-rich and PN-enriched EPS (Fig. 5) and the same iron-rich colloidal suspension. Without divalent cations, the iron-rich colloids remain highly stable in suspension, as explained earlier, with a native turbidity of $\sim$ 760 NTU. Under these conditions, flocculation depends entirely on the ability of the added polymers to overcome colloidal stability. Accordingly, at the lower EPS dose (0.5 mg g^{-1} TSS), residual turbidity remained high, ranging from 630 to 740 NTU, which indicated very poor flocculation efficiency ($<$ 17%) (Fig. 5). In contrast under Ca^{2+} coagulated conditions, at the same EPS dose reduced residual turbidity to as low as 77 NTU (FE 69%), indicating a clear synergistic effect between Ca^{2+} and EPS (Fig. 4 C and D).

High turbidity removal comparable to that observed under Ca^{2+}-coagulated conditions (Fig. 4C) was achieved only at substantially higher EPS dose of 5 mg EPS g^{-1} TSS (Fig. 5B). The lowest residual turbidity achieved was 179 NTU, corresponding to a FE of 76%. Notably, this behaviour was observed only for the PS-rich EPS fractions, whereas increasing the dose of PN-enriched EPS did not significantly improve turbidity removal in the absence of Ca^{2+}. This suggests that PS-rich EPS can interact more effectively with the surfaces of iron-rich colloids under these conditions, possibly through multiple hydrophilic functional groups and extended chain conformations that favour bridging.

In contrast, the poor flocculation performance of PN-enriched EPS in the absence of Ca^{2+} (Fig. 5B), together with its improved performance under Ca^{2+}-coagulated conditions (Fig. 4C and D), suggests that this fraction depended more strongly on Ca^{2+} for effective flocculation. One possible explanation is that protein-associated functional groups interacted with Ca^{2+}, thereby promoting bridging or cross-linking between EPS and colloidal particles. This interpretation is consistent with previous studies indicating that proteins within EPS can contribute to the binding of multivalent cations, including Ca^{2+} [55], [56].

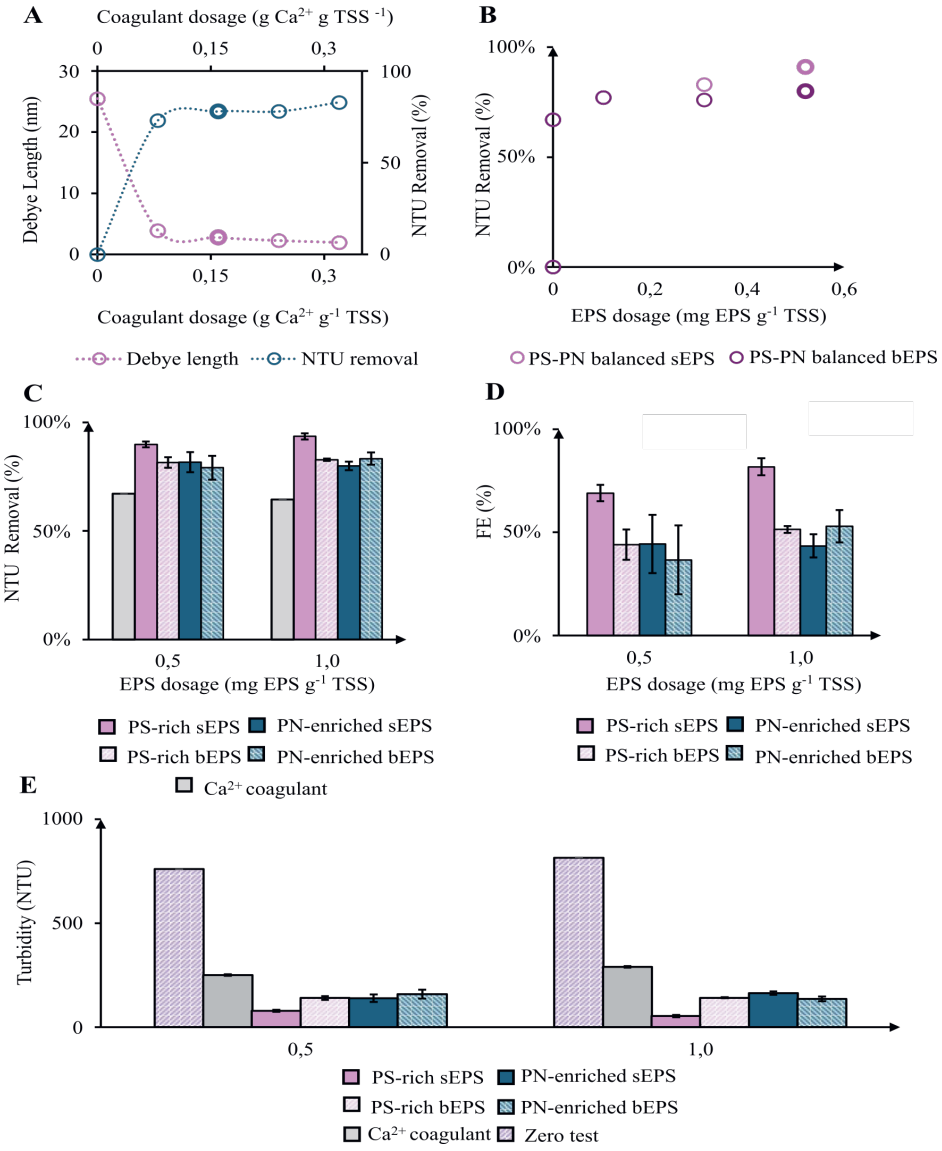


Figure 4: Flocculation of particles in a complex matrix with EPS (Experiment 4). In (A), Debye length and coagulation efficiency of iron-rich colloidal suspension at increasing concentrations of Ca^{2+} (no EPS added). In (B), turbidity removal efficiency with PS-PN balanced sEPS and bEPS at various dosages with 0.16 g Ca^{2+} g^{-1} TSS as coagulant. Turbidity removal efficiency in (C) and flocculation efficiency in (D) with PN-enrichen and PS-enriched sEPS and bound bEPS with 0.16 g Ca^{2+} g^{-1} TSS as coagulant. In (E) residual turbidity values measured during the experiment. Each value is the average of two samples (absolute error, $n = 2$).

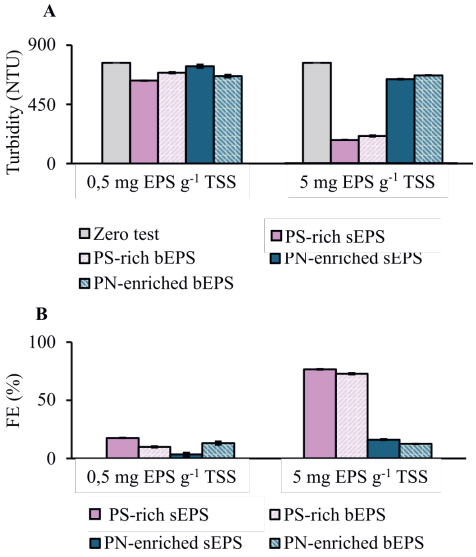


Figure 5: Residual turbidity values (A) and flocculation efficiencies (B) of flocculation test carried out on iron-rich colloidal suspension with PS-rich EPS and PN-enriched EPS at dosages of 0.5 and 5 mg EPS g^{-1} TSS without addition of calcium as coagulant. Each value is the average of two samples (absolute error, $n = 2$).

5.4 Discussion

5.4.1 What makes mixed culture-derived EPS flocculate particles with calcium as a coagulant?

In previous studies on EPS-based bioflocculants using kaolinite as a model particle, the optimal Ca^{2+} concentration has been shown to depend on several factors, including EPS composition (e.g., protein/polysaccharide ratio), pH (typically 6-8), kaolinite concentration (usually 4–7 g L^{-1}), EPS dose (0.5-40 mg L^{-1}), and the microbial source of the EPS. Overall, the reported optimum generally falls within the range of 0.5-10 mM Ca^{2+} to achieve FE above 90% [57], [58]. Although higher Ca^{2+} concentrations have sometimes been applied, they may lead to overdosing and subsequent particle re-stabilization [55].

In this study, under the applied experimental conditions, the addition of divalent cations was essential for mediating mineral-EPS interactions, as all tested minerals carried an overall negative surface charge ranging from strongly to mildly charged particles, while the mixed culture-derived EPS were also anionic in nature [52]. Cations play a key role in promoting the destabilization of suspended particles by EPS flocculants by reducing electrostatic repulsion and decreasing the separation distance between particles [59]. In particular, divalent Ca^{2+} ions can simultaneously compress the EDL, screen surface charges of colloidal particles, and form coordination complexes with EPS functional groups, thereby promoting cation-mediated bridging between particles and polymer chains [50], [52], [53], [60].

For the EPS used in this study, FTIR analysis indicated the presence of Ca^{2+}-binding functional groups, including carboxylate groups (COO^- symmetric stretch, $\sim$ 1406–1426 cm^{-1}) and abundant hydroxyl groups (broad O–H stretch, $\sim$ 3270–3305 cm^{-1}) and protein-related amide bands (amide I/amide II) (Fig. 6). Together, these findings suggest that both polysaccharide- and protein-associated functional groups can coordinate Ca^{2+} and thereby facilitate interparticle and particle–polymer bridging [61]. Therefore, identifying optimal Ca^{2+} and EPS dosages for efficient bridging flocculation is crucial. For this reason, in this study a broad range of increasing Ca^{2+} and EPS doses were screened using kaolinite and PS-PN balanced EPS to determine appropriate operating window (section

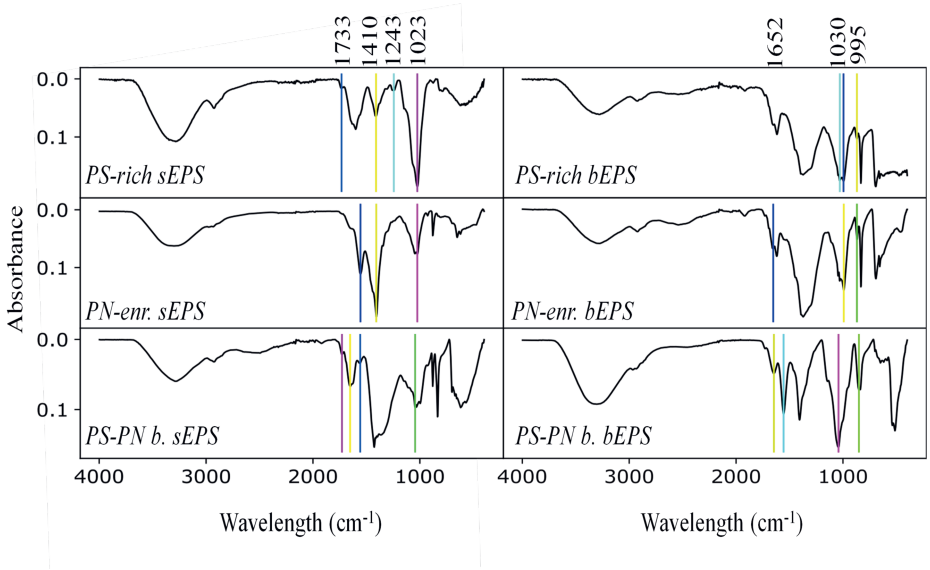


Figure 6: FTIR spectra of the different EPS samples investigated in this study, namely soluble EPS (sEPS) and bound EPS (bEPS) fractions of PS-rich, PN-enriched and PS-PN balanced EPS. Functional-group regions are indicated with colored lines: Signature carbohydrate region (C–O–C/C–O), (1200–950 cm^{-1}), C=O of carbonyl (1733 cm^{-1}), Amide ($\sim$ 1650 cm^{-1}), Amide II ($\sim$ 1550 cm^{-1}), carboxylate (1410 cm^{-1}), β-linkage zone (850 cm^{-1}).

3.1; Fig. 1). Kaolinite was selected as the model particle for flocculation assessment because it is widely used as a benchmark mineral in flocculation studies and provides a well-characterized and reproducible system for dose optimization [13], [18], [19], [20].

Based on the charge stoichiometry (SI-4, Table S4), the lowest Ca^{2+} dose applied in the kaolinite suspension (1 g L^{-1}), namely 0.02 g Ca^{2+} g^{-1} TSS (0.5 mM Ca^{2+}), was theoretically sufficient to completely neutralize the estimated surface charge, assuming complete sorption of Ca^{2+} onto the surface. However, FE remained low at this dosage (Fig. 1C), likely because the ionic strength at 0.5 mM Ca^{2+} was still insufficient to achieve adequate EDL compression (7,8 nm) (Fig. 1A), thereby limiting close particle approach [1],[7]. As ionic strength increased further with increasing Ca^{2+} and Cl-1 concentrations, the EDL became

more compressed (< 2 nm) (Fig. 1A). This enabled closer particle approach and enhanced Ca^{2+} bridging between EPS and kaolinite particles [51]. Consistent with this, EPS addition at various doses (0.1, 0.3, 0.5 mg EPS g^{-1} TSS) had a positive effect on FE and turbidity removal only when combined with at least 0.04 g Ca^{2+} g^{-1} TSS (1 mM Ca^{2+}; EDL 5.5 nm), indicating the onset of Ca^{2+} -mediated polymer bridging (Fig. 1B, C). This effect became even more pronounced at higher Ca^{2+} concentrations across all applied EPS doses (Fig. 1B, C).

Increasing ionic strength may, however, also affect the conformation of EPS in the solution through charge screening [63]. In the presence of Ca^{2+}, this effect is particularly important, as Ca^{2+} plays a central role in neutralizing negatively charged EPS groups and promoting the formation of three-dimensional matrices. Such Ca^{2+} -mediated structuring can enhance bridging flocculation when polymer chains remain sufficiently extended to form loops and tails between particles [64]. However, at excessively high ionic strength, EPS chains may adopt a more compact conformation, thereby reducing the number of available bridging sites [64]. In this study, increasing the Ca^{2+} concentration up to 0.4 g Ca^{2+} g^{-1} TSS resulted in a plateau in FE near the maximum value for all tested EPS doses (Fig. 1C), suggesting that the EPS retained a conformation favourable for bridging under these conditions [63].

Overall, these initial results confirm that the flocculation of hydrophilic particles such as kaolinite with mixed culture-derived EPS is governed primarily by cation-mediated bridging mechanisms. This finding is consistent with previous studies on bioflocculants, which generally report optimal kaolin flocculation only in the presence of added cations such as Ca^{2+}, typically within the low-to-mid millimolar range[58], [65]. However, most of these studies have focused on EPS bioflocculants derived from pure cultures [58], [66], [67]. The present results extend this body of literature by demonstrating that a similar cation-dependent flocculation behaviour also applies to EPS produced by mixed microbial cultures at comparable Ca^{2+} concentrations.

5.4.2 The anionic EPS flocculants interact better with moderately hydrophilic particles than with highly hydrophilic and hydrophobic particles

In this study, the flocculation behaviour of mixed culture-derived EPS was evaluated across minerals with contrasting surface properties (kaolinite, montmorillonite, and talc, Table 1) and EPS types differing in physicochemical composition (sEPS and bEPS fractions of PS-PN balanced, PS-rich, PN-enriched, Table 3) (Fig. 2 and 3). Overall, flocculation performance was governed mainly by mineral type rather than by EPS type. Across all tested EPS types, EPS achieved consistently high flocculation efficiencies with kaolinite, moderate improvement with montmorillonite, and poor performance with talc (Fig. 2 and 3).

In contrast to these findings, other studies have reported greater EPS absorption on montmorillonite than kaolinite [68], and reported higher flocculation efficiencies, showing the incremental effect of EPS after Ca^{2+} coagulation [50]. Such discrepancies between studies likely reflect differences in experimental conditions (e.g., TSS concentration and the dosages of EPS and cations), as well as differences in EPS characteristics, since particle–polymer interactions are strongly influenced by molecular weight and functional group composition [69]. Under the conditions applied here, the differences among EPS types for a given mineral were less pronounced than differences between minerals. This suggests that, although mixed culture-derived EPS were produced under different experimental conditions (**Chapter 2 and 3**), they showed broadly similar flocculation potential, i.e., mineral surface properties were the dominant factor controlling flocculation performance.

Several factors likely contributed to the observed mineral-specific behaviour. First, differences in surface charge density and hydrophilicity influence the strength of repulsive interactions and thus the extent to which Ca^{2+} can promote close particle approach and Ca^{2+} -mediated polymer bridging. Second, the initial turbidity of suspensions differed among minerals despite identical initial TSS concentrations, implying differences in dispersion state and potential effective collisions, which can affect aggregation once destabilisation is initiated [70]. Third, for strongly hydrophilic minerals, surface hydration can hinder close approach and polymer attachment, making adsorption/attachment mechanisms important [71],

[72].

Finally, FTIR confirmed that all EPS types share major functional groups relevant for Ca^{2+} association, partially explaining why differences in between EPS types in terms of flocculating the same type of mineral were small. As depicted in Fig. 6, hydroxyl/amine ($\sim$ 3300 cm^{-1}), proteins associated amide I–II (1650–1550 cm^{-1}), carboxylate groups (COO^- symmetric stretch, $\sim$ 1406–1426 cm^{-1}) and abundant hydroxyl groups (broad O–H stretch, $\sim$ 3270–3305 cm^{-1}) (SI-2, Fig. 6 and Table S2) were the main functionalities present in all EPS samples. Despite these shared features, the spectra still indicate subtle compositional differences among EPS types (Fig. 6). This suggests that the limited differences observed in flocculation performance were not due to the EPS being chemically identical, but rather because these compositional variations were apparently less influential than mineral surface properties under the conditions tested. The PS-rich sEPS and bEPS fractions appeared to show somewhat more evident carbohydrate-associated C–O/C–O–C bands, while the PN-enriched sEPS and bEPS fractions displayed relatively more noticeable amide I–II features. These differences are consistent with variations in polysaccharide and protein contributions, although such interpretations from FTIR alone should be made with caution. A contribution near $\sim$ 1410 cm^{-1} may also arise from protein-associated carboxyl groups. The PS–PN-balanced EPS exhibited a spectral pattern without a clearly dominant carbohydrate- or protein-associated signal. In addition, the band near $\sim$ 1652 cm^{-1} (amide I) appeared visually more distinct in the bEPS fractions than in the corresponding sEPS samples (Fig. 6), though this remains a qualitative observation. Taken together, the spectra suggest that oxygen- and nitrogen-containing functional groups are present across all EPS types, which may be relevant to adsorption and Ca^{2+}-mediated bridging once electrostatic repulsion is reduced.

EPS showed the strongest and most consistent flocculation performance with kaolinite. In both the PS-PN balanced EPS experiments (Fig. 2) and the broader comparison across PS-rich and PN-enriched EPS types (Fig. 3), highest turbidity removal and FE were observed with kaolinite suspension. EPS treated samples typically achieved turbidity removal >90% (Fig. 3), indicating efficient Ca^{2+}-mediated polymer bridging under the applied conditions. EPS-type effects were observable but secondary compared to the mineral effect; PS-rich EPS generally

produced higher FE and turbidity removal than PN-enriched EPS (Fig. 3). This suggests that PS-rich EPS promoted more effective polymer bridging on kaolinite, likely due to higher molecular weight and charge density (Table 3) together with the presence of Ca^{2+}-binding functionalities (such as carboxylate and hydroxyl groups) (Fig. 6) on the EPS.

For montmorillonite, addition of EPS increased turbidity removal compared to coagulation with Ca^{2+} alone. However, differences between EPS types were relatively small; the effect of EPS type was not clear (Fig. 2 and 3). Although the EDL thickness theoretically is identical for kaolinite and montmorillonite at the same Ca^{2+} concentration, the higher negative charge of montmorillonite results in a more negative surface potential and a greater electrostatic repulsion between particles [73] and its strong hydration shell can further hinder close particle approach and polymer attachment [71], [72]. Consequently, kaolinite particles can approach each other and EPS more readily compared to montmorillonite, and anionic EPS can efficiently bridge with Ca^{2+} and kaolinite under the same ionic conditions [31]. This might explain the lower efficiency of the same EPS sample under similar conditions on more hydrophilic particles. To achieve higher efficiencies on montmorillonite, further optimization of the Ca^{2+}/EPS dosage or the use of EPS with different physicochemical properties may be required. This is consistent with Fig. 2B, where increasing EPS dosage affected the two EPS fractions differently: sEPS showed a sharp FE decrease at higher dosages, whereas bEPS remained stable or slightly increased, suggesting increased potential for effective bridging at higher concentrations with bEPS. Differences in molecular weight and charge/functional group composition between sEPS and bEPS (Table 3; Fig. 6) likely contribute to these trends.

In the case of talc, the very low initial turbidity of the suspensions indicates that talc particles were already poorly dispersed in water, inherently limiting particle–polymer contact and reducing the likelihood of effective EPS bridging. Accordingly, the poor flocculation efficiencies observed with EPS (Figs. 2 and 3) can be explained, at least in part, by the limited opportunity for bridging between talc particles and EPS. Talc basal planes are hydrophobic and nearly uncharged, whereas the particle edges may carry a slight negative charge at neutral pH [32]. Owing to these surface characteristics, talc does not readily remain dispersed

in water, as also reflected by its low initial turbidity ($\sim$ 160 NTU), and therefore provides only limited opportunity for interaction with Ca^{2+}. Under these conditions, any flocculation observed is likely to arise mainly from hydrophobic interactions between non-polar regions of EPS and the talc basal planes [74], [75], while the slight negative charge at the particle edges may still permit some Ca^{2+}-mediated EPS bridging. Consistent with this interpretation, similar weak interactions between anionic polysaccharides and talc have been reported for carboxylated starch, where starch adsorption on talc was limited by electrostatic repulsion, and only the presence of hydrophobic functional groups enhanced the absorption [76]. Likewise, another study reported poor adsorption of both anionic and cationic modified guar on talc, whereas non-ionic guar exhibited strong adsorption, attributed primarily to hydrophobic interactions between the polymer backbone and talc basal surfaces [77]. The residual turbidity removal ($\sim$ 50–60%; Figs. 2 and 3) is therefore attributed largely to ionic-strength effects rather than EPS bridging. Although talc carries minimal surface charge, the addition of Ca^{2+} ions still promotes particle aggregation. This likely occurs through salt-induced hydrophobic attraction and compression of the EDL, which together reduce repulsive forces and strengthen the tendency of hydrophobic particles to aggregate [78].

Normalizing EPS dose to the total particle surface area highlights another important point when comparing flocculation efficiencies across studies. At the same EPS dose based on mg EPS g^{-1} TSS, the surface area normalized dose (mg EPS m^{-2}) differed by orders of magnitude among the minerals due to differences in SSA (Table 4). For example, at 0.3 mg EPS g^{-1} TSS the applied dose corresponded to 8.31×10^{-2} mg EPS m^{-2} for talc, 4.2×10^{-2} mg EPS m^{-2} for kaolinite, and only 1.38×10^{-3} mg EPS m^{-2} for montmorillonite. Thus, at identical EPS dosing, montmorillonite received $\sim$ 30–60$\times$ less EPS per unit surface area than talc and kaolinite. Despite these large differences, FE was to a limited extent controlled by mg EPS m^{-2} upon screening with PN-PS balanced EPS. This indicates that particle surface-EPS affinity and sorption mechanisms govern flocculation with EPS. In kaolinite suspensions, increasing the EPS dose from 0.3 to 0.5 mg g^{-1} TSS increased the surface area normalized dose from 4.2×10^{-2} to 7.15×10^{-2} mg EPS m^{-2}, yet FE remained at 94%, suggesting particle surfaces may already be sufficiently covered to allow for bridging and additional EPS no longer further increases flocculation since bridging is at its maximum. In montmorillonite suspensions, the same dosage increase raised the applied dose from

1.38×10^{-3} to 2.3×10^{-3} mg EPS m^{-2}, but FE remained similar, consistent with under-dosing on a surface-area basis and/or limited attachment caused by strong hydration. In the case of talc, talc received the highest mg EPS m^{-2} across all conditions (Table 5), yet FE remained comparatively low and even decreased at higher EPS dosage. This was consistent with limited adsorption/anchoring sites of talc due to its surface chemistry; therefore, bridging capacity was very limited.

Overall, these results indicate that Ca^{2+}-mediated EPS flocculation was primarily controlled by mineral surface properties (charge density, hydrophilicity/hydration, and dispersion state), while EPS-type effects were generally modest for the clay minerals. These results indicate that effective Ca^{2+}-mediated EPS bridging occurs primarily on moderately charged, hydrophilic surfaces (kaolinite and montmorillonite), whereas nearly neutral, more hydrophobic particles like talc are effectively coagulated with Ca^{2+}. Therefore, for these particles, affinity to EPS and low particle concentration can both contribute to poor flocculation efficiency.

Table 5: Surface area normalized EPS dosages for each particle type at different EPS dosages (mg EPS g^{-1} TSS) and constant Ca^{2+} dosage

Particle	A(m^2)	mg EPS m^{-2}		
		0.1	0.3	0.5
Kaolinite	2.097	1.43×10^{-2}	4.2×10^{-2}	7.15×10^{-2}
Montmorillonite	65.184	4.6×10^{-4}	1.38×10^{-3}	2.3×10^{-3}
Talc	1.083	2.77×10^{-2}	8.31×10^{-2}	1.39×10^{-1}

Note: A: Total surface area per jar, 0.1, 0.3 and 0.5 are EPS dosages in mg EPS g^{-1} TSS

5.4.3 Correlations of EPS properties with flocculation performance

Overall, our results suggested EPS-particle interactions and resulting flocculation efficiencies are influenced by multiple factors, such as molecular weight, charge density, PS/PN ratio of EPS, and particle characteristics such as hydrophilicity and surface charge. Therefore, to interpret the influence of physicochemical properties of EPS on interactions between various EPS types with kaolinite, montmorillonite, and talc, flocculation efficiencies were correlated with different physicochemical properties of EPS, such as molecular weight, charge density,

polysaccharide and protein content, and PS/PN ratio.

For kaolinite, flocculation efficiencies showed strong positive correlations with molecular weight ($r = 0.75$, $n=6$), polysaccharide content ($r = 0.81$, $n=6$), protein content ($r = 0.81$, $n=6$), PS/PN ratio ($r =0.61$, $n=6$), and EPS charge density ($r = 0.68$, $n=6$) (Table S5). These results are consistent with previous findings showing that molecular weight and charge density are dominant parameters governing biopolymer flocculation, particularly when bridging mechanisms contribute to the flocculation process [79]. This was explained by the higher capacity of adsorption and bridging of high-molecular-weight polymers with the particles compared to low-molecular-weight polymers, which tend to adsorb densely on the particles, forming thin layers that inhibit bridging [50], [80]. Similar results were reported by Ajao et. al. [50], who demonstrated that high-molecular-weight EPS achieved flocculation efficiencies of 75–95% at low dosages (0.1—0.2 mg g^{-1}), while low-molecular-weight EPS performed poorly at the same concentrations [50].

For talc and montmorillonite, correlations between flocculation efficiency and these parameters were weaker but still evident (Table S5). Protein content showed the strongest positive association with talc FE ($r = 0.65$, $n = 6$), suggesting that proteinaceous components may support interactions with more hydrophobic particles, potentially due to hydrophobic amino acid residues [81]. The weaker correlations observed for both protein and polysaccharide contents in relation to flocculation efficiency for montmorillonite and talc indicate that the relative proportions of these components alone are not sufficient to predict flocculation performance.

Rather than behaving as two independent fractions, EPS function as a structurally organized matrix, potentially containing glycoprotein-like components, in which macromolecular conformation governs the exposure and accessibility of functional groups involved in interfacial interactions [82]. Accordingly, compositional and structural characteristics such as the type and abundance of functional groups, chain conformation, and the hydrophilic or hydrophobic nature of constituent monomers may be more important in determining how EPS interacts with different particle surfaces.

EPS contain ionizable functional groups provided both by polysaccharides and proteins, enabling interactions with a wide range of particle types depending on surface chemistry. Proteins are particularly versatile because variation in amino acid composition and sequence gives rise to diverse physicochemical properties, influencing side chain pKa values, molecular conformation, and the accessibility of surface-exposed functional groups [83], [84]. By contrast, polysaccharides tend to provide more consistently negative charge, mainly through carboxyl-containing sugar residues, while their long chains facilitate adsorption onto multiple particles simultaneously [1]. Thus, in this context, the presence of both types (PN and PS) in EPS seems to be important to allow for robustness in EPS flocculation of diverse particles encountered in real-world systems. Given the fact of the heterogeneous nature of mixed culture-derived EPS, separating the two would require quite an effort. Similar conclusions were drawn by Subramanian et al., who reported that differences in flocculation performance among slime, capsular, and broth EPS were likely related to variations in polymer chain length and the number of active sites associated with their biochemical composition [18].

Overall, these results indicate that (i) molecular weight exerts the strongest and most consistent influence on flocculation across all particles (ii) charge density plays a secondary role, being most relevant for moderately hydrophilic particles such as kaolinite; and (iii) Stronger PS–PN correlations for kaolinite suggest that hydrophilic, low-charge surfaces respond more directly to differences in EPS composition, whereas strong hydration/charge effects (montmorillonite) or limited surface affinity (talc) reduce this sensitivity.

5.4.4 Flocculation efficiency of mixed culture-derived EPS on iron-rich colloidal suspension

In this study, experiments with the iron-rich colloidal suspensions started in a slightly alkaline (pH $\sim$ 8.2), low ionic strength (I = 0.00014 mM) medium with 1 g L^{-1} TSS. The suspension most probably was dominated by hydrated Fe(III) oxyhydroxide colloids with hydroxylated, hydrated surfaces and near-neutral to slightly negative surface charge [85]. The low ionic strength implies a thick EDL (Fig. 4A), which can promote colloidal stability. In addition, Fe(III) (hydr)oxide particles may exhibit a highly hydrophilic character, which could promote strong surface hydration and introduce short-range repulsive hydration forces [86]. Such

effects may further hinder close particle approach and spontaneous aggregation. In the iron-rich colloidal suspension, dissolved Fe(III) remained minimal (0.051 mg L^{-1}, Table 2), consistent with the low aqueous solubility of Fe(III) phases at circumneutral to mildly alkaline pH [87]. Any dissolved Fe(III) would mainly occur as hydrolysed complexes, primarily $Fe(OH)_3(aq)$, with minor $Fe(OH)_4^-$ [88]. However, due to dilution for the flocculation experiments, concentrations of such species were expected to be negligible in the experimental medium. Under these conditions, EPS likely promoted particle aggregation and separation, particularly when combined with Ca^{2+} coagulation, which may have favoured the formation of larger, denser, and more settle-able flocs.

Mechanistically, Ca^{2+} dosing is expected to act primarily on compressing EDL and enabling cation-aided polymer bridging between negatively charged functional groups of EPS and colloidal particles in the iron-rich colloidal suspension [89]. As summarized in Table 7, at the same EPS dosage (0.5 mg EPS g^{-1} TSS), the surface area normalized EPS availability was identical with or without Ca^{2+} ($\sim 1.72 \times 10^{-3}$ mg EPS m^{-2}). Nevertheless, turbidity removal differed substantially, indicating that the presence of Ca^{2+} affects the effectiveness of EPS adsorption and bridging. In particular, Ca^{2+} can increase the fraction of surface-associated EPS that adopt bridging conformations between EPS coated colloidal particles, whereas in Ca^{2+}-free conditions a larger fraction of EPS may remain dissolved or weakly adsorb without the capacity to form bridging configurations.

In the absence of Ca^{2+}, EPS adsorption and interparticle bridging were less effective at low dosage (0.5 mg EPS g^{-1} TSS, Fig. 5) because electrostatic repulsion remained high and cation bridging sites were unavailable. Consequently, much higher EPS dosages ($\sim 10\times$) were required to provide sufficient polymer-particle interactions to achieve comparable removal (Fig. 5) under slightly alkaline conditions of the experiment (pH $\sim$ 8.2). Without Ca^{2+}, EPS-particle absorption likely relies on weaker surface interactions (hydrogen bonding and specific surface complexation, potentially including ligand-exchange-type interactions) [90]. Increasing the EPS concentration can partially compensate by increasing the probability of productive polymer-particle contacts and by enabling sufficient partial surface coverage for bridging [50] [91]. Consistent with this, Table 7 shows that the surface area normalized EPS dose increased by $\sim 10\times$ at 5 mg EPS g^{-1} TSS compared with 0.5 mg EPS g^{-1} TSS. This indicates that Ca^{2+} plays a key role

in destabilizing the colloidal particles and enabling efficient bridging, as reported earlier with single particle systems (section 4.1) and decreases the effective dosage of EPS needed to achieve high turbidity removal.

As shown in Fig. 4C and D, the PS-rich and PN-enriched sEPS and bEPS used in the single-particle experiments were applied here, and flocculation performance differed significantly between EPS types. PS-rich sEPS achieved the highest turbidity removal and flocculation efficiency in the iron-rich colloidal suspension, both with and without Ca^{2+} addition. PS-rich sEPS exhibited higher molecular weight and charge density than the other EPS samples, while also having the lowest sodium content (Table 2). FTIR analysis further indicated a pronounced contribution of carboxylate groups (1410 cm^{-1}), together with notable aliphatic content (2925 cm^{-1}) and a strong carbonyl band at 1733 cm^{-1} (esters/COOH), suggesting a moderately amphiphilic character (Table 2; Table S2; Fig. 6). These properties likely enhanced interactions with suspended particles in the iron-rich colloidal suspension via H-bonding to surface Fe–OH groups and, where Ca^{2+} was present, cation bridging between EPS–COO^-/O-donor sites and deprotonated surface sites (≡Fe–O^-), which are expected to be present to some extent at pH ∼ 8.2 [92]. PN-rich EPS, on the other hand, showed similar but slightly lower or more variable performance, possibly due to different conformations or fewer binding sites, much lower molecular weight and charge density compared to PS-rich EPS samples.

Correlating EPS physicochemical properties with flocculation efficiencies showed that when Ca^{2+} and EPS are used together, flocculation efficiencies show very high positive correlation ($r > 0.8$) with molecular weight, charge density, and polysaccharide content at low and high EPS dosages. These results agree with what was observed with single particle systems, especially with moderately hydrophilic particles. At pH ∼ 8.2, the suspended solids in iron-rich colloidal suspension are hydrophilic due to surface hydroxylation [93]. Additionally, there might be some Fe-organic complexes that are also hydrophilic. Thus, as observed earlier, mixed culture-derived EPS can readily interact with these particles and show similar flocculation mechanisms.

Table 6: Surface area normalized EPS dosages for the iron-rich colloidal suspension at each EPS dosage applied with and without Ca^{2+} coagulation

Particle	Condition	A(m^2)	mg EPS m^{-2}		
			0.5	1.0	5.0
Iron-rich colloids	With Ca^{2+}	87.612	1.72×10^{-3}	3.4×10^{-3}	–
Iron-rich colloids	Without Ca^{2+}	87.612	1.72×10^{-3}	–	17.2×10^{-3}

Note: A: Total surface area per jar, 0.5, 1 and 5 are EPS dosages in mg EPS g^{-1} TSS

5.5 Conclusions

- Mixed culture-derived EPS flocculate particles with varying surface chemistries in the presence of Ca^{2+} as a bridging cation. Therefore, the dominant flocculation mechanism in EPS–clay systems is most probably cation-mediated bridging.
- Particles with moderately hydrophilic characteristics are flocculated more effectively by both polysaccharide- and protein-rich EPS. Turbidity removal and flocculation efficiency were ranked accordingly: kaolinite > montmorillonite > talc.
- Molecular weight and charge density of EPS were found to be important physicochemical properties, indicating flocculation by polymer–cation–mineral bridging mechanisms.
- Polysaccharide- and protein-enriched EPS can flocculate particles in complex matrices, such as iron-rich colloidal suspensions, with or without Ca^{2+}, but require higher EPS dosages compared to model clay suspensions.

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Chapter 6

General Discussion

6.1 From EPS overproduction to bioflocculant application

Coagulation-Flocculation is a core operation in water and wastewater treatment, enabling rapid separation of suspended solids and improving downstream settling, filtration, and overall process stability [1]. In practice, high molecular weight synthetic polymeric flocculants (especially polyacrylamide (PAM)-based products) are widely used in this process due to their effectiveness at low doses and broad applicability [2], [3]. However, the use of these polymers has increasingly been questioned in recent years because of sustainability and environmental concerns, including their persistence in the environment [4], reliance on petroleum-derived monomers [5] , and potential downstream impacts on sludge handling and biological treatment processes[6], [7], [8]. These concerns highlight the need for biodegradable, sustainable, and more environmentally friendly alternatives that can deliver comparable performance under the conditions of practice.

Microbial extracellular polymeric substances (EPS) present a promising alternative to synthetic polymeric flocculants [3]. Naturally produced by microorganisms, EPS consist mainly of polysaccharides and proteins and exhibit key flocculant-like characteristics, including high molecular weight, flexible polymer chains, and diverse charged functional groups [9], [10]. These features enable strong interactions with suspended, charged particles through mechanisms such as polymer bridging [3], [11], while their biodegradability makes EPS attractive for more sustainable flocculation [12]. In addition, EPS can potentially be produced from low-cost, waste-derived feedstocks, using open mixed microbial cultures (MMCs)[13], [14], [15], [16]. Such renewable feedstocks replace fossil resources used for traditional flocculant production that have a significant carbon footprint. Moreover, by shifting from sterile pure-culture based production systems to open and organic wastewater-based feedstock systems, EPS production becomes aligned with circular resource recovery in wastewater treatment, an ongoing development to make that sector sustainable [14].

While EPS contribute to biofilm formation, floc structure, and settling behaviour of aggregates, they are also associated with operational challenges such as sludge bulking [17], increased viscosity [18], and membrane fouling [19], [20]. In that framing, EPS are often treated as an unavoidable by-product that must be controlled or minimized to maintain stable operation. In contrast, more recent research has demonstrated that EPS are not merely a passive waste fraction; instead, they are valuable, steerable products, creating the possibility of reducing dependency on conventional synthetic flocculants in the future [13], [21], [22], [23], [24].

Despite this promise, two fundamental barriers have limited the transition of EPS bioflocculants from laboratory demonstrations to scalable implementation (Fig.1). **The first barrier is production reliability, i.e., reproducible EPS yields, composition, and flocculation performance.** Although MMC-derived EPS can achieve high flocculation efficiencies, many studies focus on short-term or batch-based production rather than sustained continuous production compatible with large-scale application [15], [21], [25], [26], [27]. However, the operational conditions required to reliably stimulate EPS synthesis above baseline levels (EPS-overproduction) and to maintain stable production over time have remained thus far insufficiently defined. Even when the effect of nutrient limitation and critical operational parameters such as solids retention time (SRT) on EPS production are discussed in the literature, these factors are generally selected ad hoc, and not as a result of a systematic investigation. Furthermore, inoculum effects and long-term stability of the flocculation performance in continuous systems have remained underexplored. Resolving these gaps is necessary to design robust processes that consistently deliver high yields of highly functional EPS, which is essential for enabling MMC-derived EPS to compete with conventional fossil-based commercial polymers.

The second barrier is application uncertainty. Most EPS flocculation studies assess performance using simplified model suspensions, typically kaolinite, and implicitly assume that strong performance in these systems will translate to other particle types and real water matrices. However, flocculation is governed by complex interfacial interactions, and particle properties such as charge, hydrophobicity, functional groups, and morphology can strongly affect which interaction mechanisms dominate and how effectively a given polymer performs. In real water

matrices, this complexity is further increased by dissolved interfering substances, such as humic and fulvic acids, natural organic matter, salts, and competing ions [28], which can modify particle surfaces, compete for polymer binding sites, or screen electrostatic interactions [29], [30]. As a result, EPS that perform exceptionally well in one particle system may perform differently in another. This creates an important knowledge gap: not only whether MMC-derived EPS can function as bioflocculants, but under which particle and matrix conditions they are reliable, and which EPS properties are most important for broad applicability.

Against this background, this thesis was structured to connect the full chain from operational steering to microbial selection, EPS properties, and functional performance. Specifically, **Chapter 2** defines conditions that stimulate EPS overproduction and investigates (i) how SRT under nitrogen limitation shapes EPS overproduction, (ii) whether inoculum type affects EPS production under identical strategies, and (iii) whether flocculation performance remains stable over extended continuous operation. The outcomes of **Chapter 2** establish short SRT combined with nitrogen limitation as a robust EPS-overproduction strategy that can be applied across different sludge inocula to enrich EPS-overproducing microbial consortia.

Building on this foundation, **Chapter 3** advances the work toward practical implementation by applying the EPS-overproduction strategy to widely available substrates, volatile fatty acids (VFAs), and by assessing the influence of reactor configuration. The results demonstrate that the defined EPS-overproduction conditions are transferable across substrates and can be implemented in different reactor types. In particular, EPS overproduction was achieved in a continuous stirred-tank reactor (CSTR), which exhibited fewer process-control challenges than membrane bioreactors (MBRs) in maintaining stable EPS overproduction. In addition, the findings indicate that the selected operating conditions direct the embedded carbon in the waste-organics preferentially toward EPS production, with only a very limited diversion to intracellular storage polymers such as polyhydroxyalkanoates (PHA), which is not the desired product in EPS recovery units.

Chapter 4 adds a microbial ecology perspective by systematically examining how operational conditions, substrate type, and reactor configuration shape

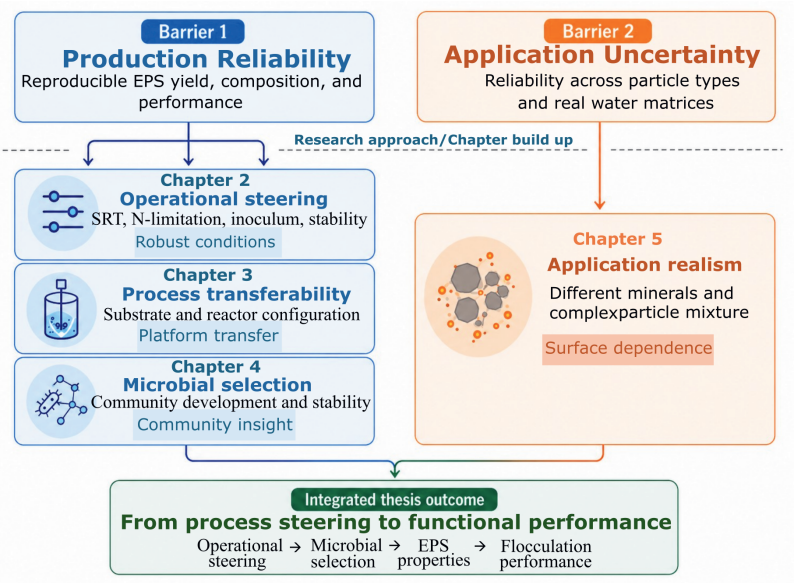


Figure 1: Thesis roadmap showing how Chapters 2-5 address the two main barriers for implementation of MMC-derived EPS as bioflocculants. Production reliability is addressed through robust conditions (Chapter 2), platform transfer (Chapter 3), and community insight (Chapter 4), while application uncertainty is addressed through surface dependence/application realism (Chapter 5). Together, these chapters establish the link between operational steering, microbial selection, EPS properties, and flocculation performance.

microbial community development in EPS-(over)producing bioreactors, providing mechanistic insight into the stability and reproducibility of EPS production. This chapter tracks microbial community dynamics across seven bioreactors operated with the EPS overproduction strategy and shows that these conditions consistently enrich taxa that are putative EPS producers and/or EPS degraders. However, shifts in relative abundance do not always correlate directly with EPS yields, and individual reactor conditions can promote distinct, reactor-specific communities that are likely to differ in their contributions to EPS production and turnover as well as organic matter degradation.

Finally, **Chapter 5** addresses application realism by evaluating the performance of MMC-derived EPS across minerals with diverse surface properties and

within a complex particle mixture, thereby clarifying the robustness and limitations of MMC-derived EPS bioflocculants under conditions closer to practice. The results show that flocculation performance was governed primarily by particle surface properties rather than by EPS physicochemical properties alone. Overall, EPS performed best with moderately charged, moderately hydrophilic mineral surfaces, whereas high surface hydrophilicity reduced flocculation efficiency. The dominant flocculation mechanism was inferred to be calcium-mediated polymer bridging, consistent with the observed importance of divalent cations. Across the tested minerals, EPS molecular weight and charge characteristics showed the strongest correlations with flocculation performance.

6.2 Synthesis of findings across the thesis

6.2.1 Nitrogen limitation and short SRT act as consistent selection pressures for EPS overproduction

Across the experimental chapters, a consistent pattern emerged: the combination of strong nitrogen limitation (COD:N 100) and short solids retention time (SRT) steered the metabolism and community selection of mixed microbial cultures (MMCs) toward enhanced EPS synthesis.

This concept was first established in **Chapter 2**, where the research aimed to define broadly applicable "EPS overproduction conditions" across different inocula and later expanded to different substrates, and reactor configurations in **Chapter 3**. Addressing a long-standing gap in the development of MMC-derived EPS bioflocculants, the study combined two key selective factors: (i) nitrogen limitation, a well-known trigger for secondary metabolite production that has been studied extensively in pure microbial cultures [31], [32], [33] ,[34] , and (ii) SRT, an important operational parameter in bioreactors that enable control over microbial growth and community composition [35], [36]. Together, these conditions enabled the enrichment of EPS-producing microorganisms from diverse inocula in different reactor settings and from different substrate types. Under these conditions, visible changes in the mixed liquor were observed, including increased viscosity and localized biofilm formation, providing early evidence of EPS overproduction [37], [38].

These observations were later confirmed by EPS quantification, (fluorescence)dye-based microscopic staining, and microbial community analysis. EPS quantification and microscopic staining confirmed the high EPS content, while microbial community analysis showed the presence of microorganisms associated with EPS production and turnover (**Chapter 4**).

Importantly, direct start-up under EPS overproduction conditions also proved feasible. When bioreactors were directly started at COD:N 100 and SRT 3 days, microorganisms were still able to produce EPS with high yield (**Chapter 2**&**3**). This finding is practically significant, as it indicates that stable EPS production can be achieved without a prolonged adaptation phase, thereby reducing start-up time and accelerating process implementation.

The combined effect of nitrogen limitation and short SRT can be explained by complementary metabolic and ecological mechanisms. Under nitrogen limitation, biomass growth is restricted because nitrogen is essential for protein synthesis, enzyme production, and cell division [39]. As growth slows, the carbon demand for biomass formation decreases, and excess carbon can be redirected toward EPS synthesis as a carbon overflow mechanism [40]. At the same time, short SRT imposes strong selective pressure by favouring microorganisms that can both grow rapidly enough to avoid washout and maintain effective retention within the reactor [41], [42]. EPS production provides such an advantage because it promotes bioaggregation and floc formation. In contrast, microorganisms lacking this trait form weaker aggregates and are therefore more susceptible to washout. Under these conditions, EPS-producing populations become enriched, while slower-growing and potentially more organic matter-degrading microorganisms are progressively removed from the system [43]. Together, these findings indicate that nitrogen limitation redirects carbon flux toward EPS production, while short SRT selectively enriches EPS-producing microorganisms within MMCs.

This proposed EPS overproduction strategy paves the way for sustainable bioflocculant production from waste streams using MMCs. This is especially relevant because EPS intended for bioflocculant applications do not require high-purity recovery. Unlike applications in food or medicine, where purity, safety, and compositional consistency are critical, bioflocculant use is mainly governed by functional performance, such as the ability to promote particle aggregation and

solid-liquid separation. Hence, crude or partially purified EPS extracts may be suitable if they retain sufficient molecular weight, charge density, and functional groups for flocculation. Therefore, achieving continuous and reliable production with MMCs is a crucial step towards transferring this technology to larger-scale production. From this perspective, defining operational conditions that can be generalised across different systems to stimulate EPS production above basal levels was essential, yet this aspect has not been sufficiently addressed in previous research.

Previous studies, although not specifically focused on maximising EPS yields, have reported individual stimulatory effects of nitrogen limitation and solid retention time on EPS synthesis. In the context of microbial granulation and floc formation, nitrogen limitation has been shown to enhance EPS production and support aggregate formation [44], [45], [46]. Similarly, membrane fouling studies have demonstrated that operation at shorter SRTs often leads to increased EPS biosynthesis. However, these studies generally treated EPS as an operational factor affecting fouling or treatment performance, rather than as a target product for downstream recovery [47], [48], [49]. Therefore, the potential of SRT control as a deliberate strategy for EPS overproduction remains insufficiently explored.

A particularly relevant study by Ajao et al. demonstrated that decreasing nitrogen concentration in the feed under a fixed SRT of 3 days increased EPS yield, with the highest production observed at a COD:N ratio of 100. In that work, enhanced EPS formation under nitrogen-limited conditions was attributed to the conversion of excess carbon into EPS under nutrient-imbalanced conditions. However, the role of SRT as a selective pressure was not discussed in detail, and the observed increase in EPS production was mainly linked to nitrogen limitation and the presence of secondary substrates such as ethanol or methanol in the feed [23].

Overall, this work offers a consistent selective framework that can be applied across systems to enrich EPS-overproducing MMCs. In this way, the study moves beyond earlier observations of isolated stimulatory effects and provides a more generalisable process concept for EPS overproduction with MMCs. Importantly, this framework builds on knowledge derived from pure culture EPS studies, where growth-limiting conditions such as nitrogen limitation are known

to shift microbial metabolism away from biomass formation and toward EPS synthesis. By translating this principle to MMCs, this work demonstrates that a strategy originally understood mainly at the level of individual microorganisms can also be applied at the community level in non-sterile, wastewater-based systems. This contributes both to the fundamental understanding of how operational parameters shape microbial functionality and to the practical development of MMC-derived bioflocculant production as a more sustainable alternative to conventional pure-culture approaches.

6.2.2 Substrate type as a route to tune EPS composition and application potential

For the application of EPS as a bioflocculant, not only the production yield but also physicochemical properties are highly important. In particular, bulk characteristics such as molecular weight, branching, charge density, charge distribution and the nature of functional groups largely determine how EPS interact with suspended solids, and consequently, how effectively they perform during flocculation.

This study showed that, when MMCs were used for EPS bioflocculant production from wastewater-based substrates, it is possible to tune not only the yield of EPS produced but also, to some extent, their composition (Chapter 2&3). Specifically, the relative contribution of key EPS fractions, including proteins and polysaccharides, could be influenced by operational conditions, indicating that reactor operation can shape both the quantity and biochemical characteristics of the recovered EPS.

This was consistent with previous literature, which has shown that EPS biochemical composition is highly sensitive to factors such as carbon type, nutrient availability (particularly nitrogen and phosphorus), and the identity of EPS-producing microorganisms [50], [51], [52], [53]. However, achieving comparable control in MMCs is more challenging because EPS composition reflects the combined activity of multiple populations, whose abundance and metabolic state can shift with reactor operation. Thus, while operational parameters can steer the relative contributions of proteins, polysaccharides, and lipids, precise control over EPS composition remains more difficult in MMC systems than in pure cultures.

If EPS biosynthesis is divided into three main stages, these include precursor synthesis, polymerisation, and transfer across the cytoplasmic membrane followed by export through the outer membrane [25]. These steps can vary depending on the carbon source and from one microorganism to another. The first stage is particularly relevant to the final EPS composition, because during EPS biosynthesis, assimilated carbon is first converted into intermediate sugar metabolites, which are subsequently transformed into activated sugar precursors, such as nucleotide sugars (for example, GDP-sugars) [54]. The nature of these precursors depends on the substrate being metabolized and determines the building blocks available for EPS formation [55]. For each type of polymer (such as dextran, alginate, xanthan) specific precursors and enzymes are involved in the biosynthesis. Supporting this, Ye et al. examined activated sludge grown in laboratory-scale completely mixed reactors fed with starch, glucose, or sodium acetate as the main carbon source; in particular, the polysaccharide content of the loosely bound EPS fraction was lower under acetate feeding than under glucose or starch feeding [56].

In this thesis, similar results were obtained when glycerol-rich and VFA-rich wastewaters were used as carbon sources. In **Chapter 2**, when glycerol-rich synthetic wastewater was used, despite differences in inoculum origin, the overall outcome was consistent: all reactors produced EPS that was predominantly rich in polysaccharides. Chemical characterization showed high polysaccharide-to-protein (PS/PN) ratios, while fluorescence-based microscopic staining confirmed the abundance of carbohydrates within the EPS matrix (Fig. 2). Together, these results indicated that glycerol-fed systems favoured the production of EPS with a clearly polysaccharide-dominated composition. However, a different pattern emerged when VFA-rich synthetic wastewater was used in **Chapter 3**. In these systems, irrespective of inoculum type or reactor configuration, the produced EPS showed lower PS/PN ratios than the glycerol-derived EPS, indicating a relatively greater contribution of proteins to the overall matrix (Fig. 2). Depending on the specific sample, the resulting EPS ranged from clearly protein-enriched to compositions that remained polysaccharide-dominant but still contained more protein than those obtained under glycerol-fed conditions.

The underlying cause of this difference may be related to the distinct metabolic pathways involved in the assimilation of glycerol and VFAs such as acetate, butyrate, and propionate. The citric acid cycle plays a major role in the metabolism

of organic compounds and the biosynthesis of microbial materials [57]. VFAs such as acetate can be readily converted to acetyl-CoA [58], which then enters the citric acid cycle, whereas glycerol must first pass through intermediate metabolic steps before contributing to central carbon metabolism, eventually forming pyruvate and acetyl-CoA [59]. Compared with acetate utilization, glycerol metabolism is therefore more complex and likely involves a different set of enzymes and metabolic intermediates. For instance, in many bacteria, such as *Escherichia coli*, *Pseudomonas* spp., and *Klebsiella pneumoniae*, glycerol can be metabolized through phosphorylative pathways involving glycerol kinase and glycerol-3-phosphate dehydrogenase, or through oxidative pathways involving glycerol dehydrogenase and dihydroxyacetone kinase, producing intermediates such as glycerol-3-phosphate, dihydroxyacetone, and dihydroxyacetone phosphate before conversion toward pyruvate and acetyl-CoA [60], [61], [62].

This metabolic difference may also influence the formation of EPS precursors, thereby contributing to variations in final EPS composition. Glycerol can enter sugar-phosphate metabolism through dihydroxyacetone phosphate (DHAP) [63]. These sugar-phosphate intermediates can then be converted into activated nucleotide sugars, which serve as precursors for extracellular polysaccharide biosynthesis. In contrast, acetate must first be assimilated through acetyl-CoA, the glyoxylate/TCA-cycle network, and gluconeogenesis before contributing to nucleotide-sugar precursor formation [59]. Since nucleotide sugars are key precursors for extracellular polysaccharide biosynthesis, these different assimilation routes may affect the relative balance between polysaccharide and protein fractions in the recovered EPS [55], [64]. Therefore, the higher relative protein content in VFA-derived EPS may partly reflect substrate-dependent differences in EPS precursor formation. However, this explanation should be interpreted cautiously, as EPS composition is also influenced by microbial community structure, physiological state, and operational conditions.

Nevertheless, this shift in EPS composition is significant because it suggests that wastewater type can serve as an important starting point for steering the biochemical profile of MMC-derived EPS. This is similar to pure cultures, where the choice of carbon source, nutrient availability, and cultivation conditions can influence EPS yield and composition [64], [65], [66]. This could open the possibility of producing a diversity of EPS types from different wastewater streams,

which may be advantageous for tailoring EPS toward different applications such as, bioflocculation, sludge conditioning, soil amendment, biomaterials, or other biotechnology-related uses. However, such diversity may also complicate standardization and increase application uncertainty, because differences in EPS composition may translate into differences in flocculation behaviour across particle types and water matrices. Thus, wastewater-driven tunability should be considered both an opportunity for product diversification and a challenge for reproducible bioflocculant performance.

The practical importance of these compositional differences was investigated in **Chapter 5**, which focused specifically on the application of EPS as a bioflocculant. In that chapter, EPS samples that were produced in **Chapter 2** and **3** were grouped according to their PS/PN ratios. Polysaccharide-rich EPS obtained from glycerol-fed reactors were compared with more protein-enriched EPS from VFA-fed systems, as well as with samples containing a more balanced proportion of polysaccharides and proteins. These EPS types were then evaluated across different suspension matrices to assess whether composition alone could explain differences in flocculation performance. No consistent advantage was observed for either polysaccharide-rich or protein-rich EPS. Instead, flocculation performance was context-dependent and varied across the particle suspensions tested. EPS that performed efficiently in one system did not necessarily perform equally well in another, indicating that performance was strongly influenced by particle-specific properties such as surface charge, hydrophobicity, morphology, and functional-group chemistry. Therefore, the effectiveness of a given EPS type was governed not only by its biochemical composition, but also by its compatibility with the target particle matrix.

The PS/PN ratio of EPS has often been considered an important factor in wastewater treatment studies, as lower PS/PN ratios are generally associated with better sludge-settling characteristics [67], [68]. In some studies, this has been explained by the more hydrophobic nature of proteins compared with polysaccharides [69]. A lower PS/PN ratio is thought to favour the hydrophobic character of EPS, helping to promote the accumulation and flocculation of microbial cells [70].

Although such studies suggest a strong relationship between protein content and the flocculation or aggregation behaviour of sludge, this may mainly apply to EPS in their native state. In the present study, extracted EPS were used.

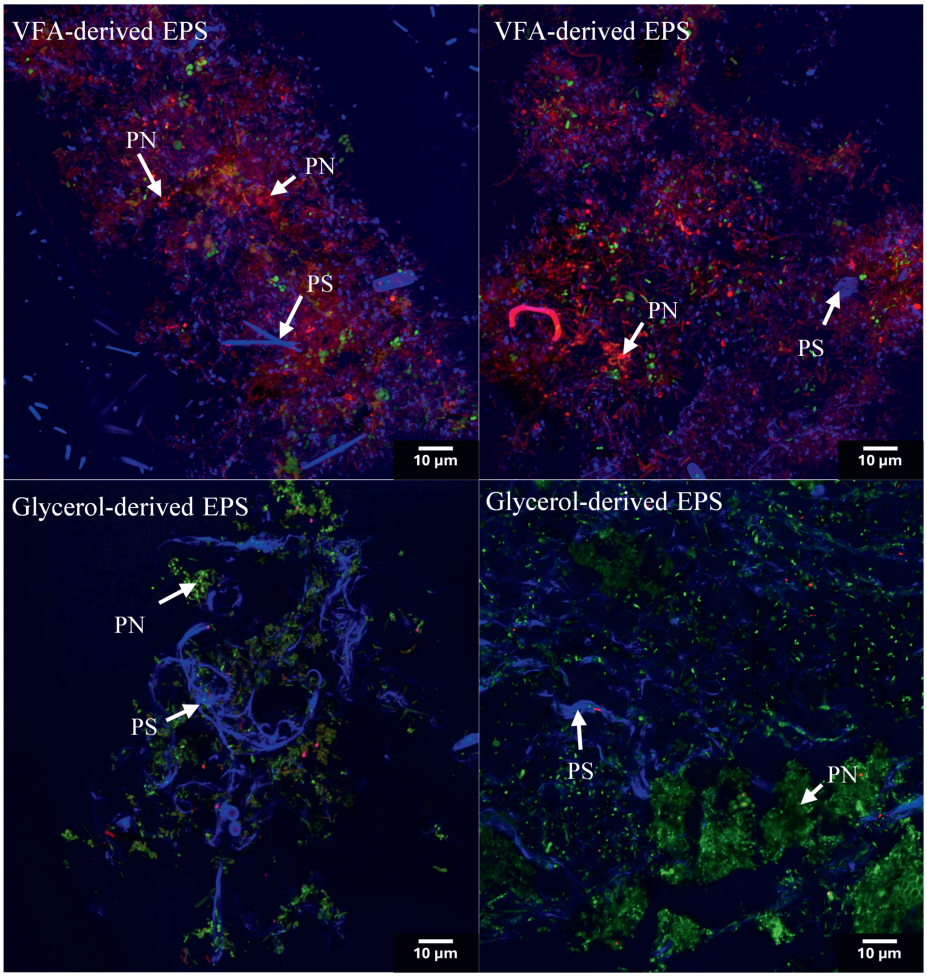


Figure 2: Micrographs showing differences in EPS composition, based on polysaccharide and protein content, in glycerol-derived and VFA-derived extracellular polymeric substances (EPS). Samples were collected from bioreactors operated under EPS-overproduction conditions (COD:N 100, SRT 3 days) in **Chapter 2** and **3**. Polysaccharide components of EPS are shown in blue and were identified using Calcofluor staining. In VFA-derived EPS samples, proteins and SYPRO^{TM} Red-positive cell wall-associated components are shown in red, whereas in glycerol-derived EPS samples, the same components were labelled with SYPRO^{TM} Orange and are shown in green. PN: protein, PS: polysaccharide.

The extraction process itself may influence functionality, particularly in the case of proteins, which are more sensitive to physicochemical disruption such as heat and alkalinity [71], [72], [73]. As a result, proteins may no longer retain the same functionality after extraction as they do in the native matrix. This is important because EPS-associated proteins can contribute to flocculation through charged amino acid residues and functional groups (i.e., amine, sulfhydryl, hydroxyl, carboxyl) [74], which may participate in electrostatic interactions, hydrogen bonding, and metal-ion complexation [74]. Aromatic amino acid residues may also support π-related interactions with metal ions or particle surfaces [75]. Therefore, extraction-induced changes in protein conformation or binding-site accessibility could alter the flocculation behaviour of protein-rich EPS and may partly explain why trends reported for native sludge EPS were not directly reflected in the extracted EPS tested here.

However, flocculation performance of EPS samples correlated well with molecular weight and charge density of EPS. These are indirectly related to qualitative and quantitative properties of polysaccharide and protein components of EPS and are directly linked to the main mechanisms involved in flocculation, such as charge neutralisation, interparticle bridging, and the formation of stable aggregates [76], [77]. This interpretation is in line with previous studies on bioflocculants. Polysaccharides are often considered particularly effective because they typically possess high molecular weights and relatively high charge density, making them well suited for bridging between particles [76], [77]. Proteins may also contribute strongly to flocculation through different mechanisms, including the presence of charged amino acid residues, hydrophobic domains, and adsorption sites that facilitate particle attachment and aggregate stabilisation [25], [78]. Therefore, flocculation performance is likely governed less by the simple proportion of polysaccharides and proteins, but by the specific molecular features of these fractions, including chain length, charge density, functional-group accessibility, and conformational integrity.

From this perspective, changing the substrate in MMC-based EPS production may not provide precise control over all EPS properties, but it can still be considered a practical strategy to direct EPS composition toward a desired range. Substrate selection may therefore function as an operational lever to enrich for certain classes of biopolymers, even if it does not allow full control over

polymer architecture or functionality. In the present work, the analysis focused primarily on polysaccharides and proteins because these are generally the dominant EPS fractions and are most directly relevant for flocculation applications. However, EPS is a complex matrix that may also contain lipids, humic-like substances, nucleic acids, and other minor components, all of which could influence its functional behaviour [74]. For example, lipids may affect EPS hydrophobicity and interactions with hydrophobic particle surfaces [79], while humic-like substances and nucleic acids can introduce additional charged functional groups [9] that may contribute to electrostatic interactions, metal-ion binding, or bridging between particles [9], [80]. Therefore, differences in these minor fractions could also influence flocculation behaviour, even if they were not the focus of this study.

This creates an important perspective for future research. Different wastewater streams could be explored not only to maximise EPS yield, but also to promote the formation of EPS with alternative compositional profiles, including matrices enriched in components other than polysaccharides and proteins. Such an approach could help broaden the application potential of mixed-culture-derived EPS beyond bioflocculation alone. Overall, the results indicate that substrate type should not be viewed merely as a carbon source for EPS production, but also as a relevant process variable that shapes EPS composition and, consequently, its functional properties.

6.2.3 Functional robustness of EPS-producing microbial communities

Another important outcome of this study was that the effect of the EPS-overproduction conditions was not only reflected in EPS yield and composition, but also clearly manifested at the microbial community level as discussed in **Chapter 4**.

In this research, seven bioreactors were operated under EPS-overproduction conditions, while differing in substrate type, inoculum source, and reactor configuration. Despite these differences, the microbial community analysis revealed a consistent overall pattern, especially in glycerol-fed reactors. As summarized in Table 1, in all reactors, the final communities became enriched in microorganisms associated with both EPS production and EPS degradation; in VFA-fed reactors, in addition to these groups, the presence of PHA-related groups was

also detected. These results indicate that these operational conditions selected for a functionally similar microbial consortium. Thus, although the taxonomic composition varied between reactors, the dominant ecological roles within the communities remained comparable. This suggests that the microbial communities were able to reorganize taxonomically while maintaining a similar functional capacity for EPS production. Thereby, EPS overproduction appeared to be a robust community-level function rather than a trait dependent on the persistence of a single microbial strain.

Among all reactors, only one reactor showed dominance of a single microbial taxon at the end of the SRT 3 period. However, when the SRT was further decreased, this dominance was replaced by a more diverse community composed of several taxa. This pattern is estimated to be reactor-specific, as it was never observed again. In all other reactors, the final communities consisted of broad consortia of putative EPS-producing taxa with moderate relative abundances. This represents an important advantage of MMC-based systems compared with sterile, pure culture-based production processes, namely that EPS overproduction can be maintained by a flexible microbial consortium rather than depending on the persistence of a single selected strain.

This functional robustness is directly relevant to the first barrier discussed above (Fig. 1). With respect to production reliability, it suggests that EPS-overproduction conditions can select for a similar functional outcome across different substrates, inocula, and reactor configurations, thereby supporting the development of more robust continuous production processes. With respect to composition, the enrichment of functionally similar EPS-related communities may help reduce variability in broad EPS characteristics, such as protein and polysaccharide content, while not necessarily implying conservation of the exact EPS chemical structure. Therefore, the observed community-level convergence can be viewed as a promising feature of MMC-derived EPS production, while still requiring further validation in relation to long-term stability and application-specific performance.

This was particularly evident when comparing reactors exposed to the same EPS-overproduction conditions but differing in other features, such as inoculum source, feed type, or reactor configuration. In **Chapter 2**, reactors inoculated with different sludges developed distinct putative EPS-producing communities, although some overlap between taxa was still observed. This reflects the influence of the initial inoculum on community assembly, while at the same time highlighting the broader applicability of the EPS-overproduction conditions across different inoculum sources.

Chapter 3 further showed that this functional convergence was maintained even when substrate type and reactor configuration were changed. In the VFA-fed systems, R5 and R6 were operated as an MBR and a CSTR, respectively, and developed distinct microbial communities from the same start-up inoculum. Despite these differences, both reactors contained microorganisms potentially involved in EPS formation, PHA/PHB storage, and polymer turnover. Putative EPS-producing taxa shared between the two reactors included *Microbacterium, Flavobacterium, Brevundimonas*, *Pseudoxanthomonas*, and *Chryseobacterium*, while additional EPS-associated groups such as *Xanthobacter, Sphingobium*, *Sphingobacteriales* env.OPS 17, *Pseudomonas*, and the *Flavobacteriales* NS9 marine group were enriched depending on the reactor and operational period. Several taxa were also linked to PHA/PHB production or intracellular polymer storage, suggesting that VFA conversion under nitrogen-limited conditions could potentially be directed toward different polymeric products. This possibility became particularly relevant during the microbial community analysis of the VFA-fed reactors, as VFAs are commonly used substrates for PHA/PHB production [81]. In R5 and R6, PHA content was not analytically monitored during operation; therefore, a dedicated experiment with R7 was later performed to partially address this question. In R7, similar patterns were observed, as potentially PHA-associated taxa appeared at different time points together with putative EPS producers. However, the main trend was that PHA-associated taxa were more relevant during start-up and early operation, whereas the community later shifted toward EPS-associated taxa, especially up to day 39. After the reactor disturbance, the community shifted again toward polymer and biomass turnover, which likely contributed to the lower EPS yield observed by the end of the process. This pattern was consistent with the analytical data, as PHA was

mainly detected during the early stage of operation and rapidly decreased thereafter. It was also consistent with microscopy observations showing a decreasing presence of PHA in the sludge toward the end of the process [82].

Together, these results indicate that EPS-overproduction conditions favoured the enrichment of EPS-associated taxa and did not support enrichment of PHA-related taxa or significant PHA accumulation. This is important and promising for the establishment of a process specifically dedicated to EPS overproduction. In parallel, all VFA-fed reactors contained taxa associated with organic matter degradation, hydrocarbon degradation, predation, and polymer turnover. This indicates that the VFA-fed EPS-overproducing communities were functionally complex and included microorganisms potentially involved in both polymer formation and polymer degradation.

However, the presence of putative EPS-producing taxa was not sufficient to explain EPS accumulation. The MBR harboured a broader community, including several taxa associated with EPS, PHA, and polymer turnover, but showed lower COD removal and EPS yield than the CSTR. In contrast, the CSTR achieved higher substrate removal and EPS yield, particularly at the lower OLR, despite having lower bacterial diversity. Thus, **Chapter 3** demonstrated that reactor configuration influenced both microbial community assembly and process performance. EPS-producing communities could develop in different reactor configurations, but production efficiency depended on how the reactor environment shaped the balance between EPS production, possible PHA storage, and polymer turnover.

From the mechanistic perspective, such a dynamic balance between EPS production and degradation is also consistent with the role of EPS in microbial aggregates (Fig. 3). EPS are part of a continuously renewed matrix that supports floc/biofilm stability, microbial retention, nutrient exchange, and stress protection [9]. Under nutrient limitation, EPS degradation may additionally provide a local source of carbon, nitrogen, and energy, allowing the community to maintain metabolic activity while other members continue matrix production. From this perspective, the coexistence of producer and degrader populations should not be viewed as contradictory to EPS overproduction, but rather as a characteristic

feature of a stable EPS-rich consortium.

Another important observation from the microbial community analysis was that some taxa appeared to be associated with both EPS or PHA production and EPS turnover. Genera such as *Hydrogenophaga, Brevundimonas, Flavobacterium*, and *Chryseobacterium* could not be assigned unambiguously to a single functional role, as literature reports link them to EPS and/or PHA synthesis as well as degradation-related processes. This overlap is biologically plausible, since microorganisms in sludge aggregates may contribute to matrix formation or internal polymer storage under some conditions, while under other conditions they may produce hydrolytic enzymes or utilize released polymers [54], [83]. Also from a microbial ecology perspective, the ability to perform both functions can provide an adaptive advantage under fluctuating environmental conditions [84], [85], [86]. A similar interpretation also applies to several microorganisms discussed in **Chapter 4**, where potential roles in EPS/PHA production or degradation were assigned based on available literature and interpreted in the context of the nitrogen-limited reactor conditions. However, some microorganisms may behave differently under nutrient-rich conditions, and their activity may also differ between mixed microbial communities and pure cultures. Therefore, assigning a specific function to individual taxa remains challenging in mixed-culture studies and requires more targeted approaches, as discussed later in Section 6.4.

Taken together, these findings suggest that EPS-overproduction conditions do not select for a single specific microbial composition, but rather for a functional community structure capable of sustaining high EPS production together with continuous matrix turnover. Different microorganisms may occupy these roles depending on the initial inoculum, reactor configuration, and substrate type, but the enriched communities consistently converged toward the same broad functional organization. This indicates a degree of ecological flexibility in the establishment and maintenance of EPS-overproducing systems. From an application perspective, this may be advantageous for wastewater-based EPS production, as it suggests that the process may not depend strictly on one specific inoculum or wastewater source. Although this was not directly investigated in the present study, it also opens an interesting perspective for future research to assess whether sequential feeding with different wastewater types could still maintain communities with similar functional characteristics.

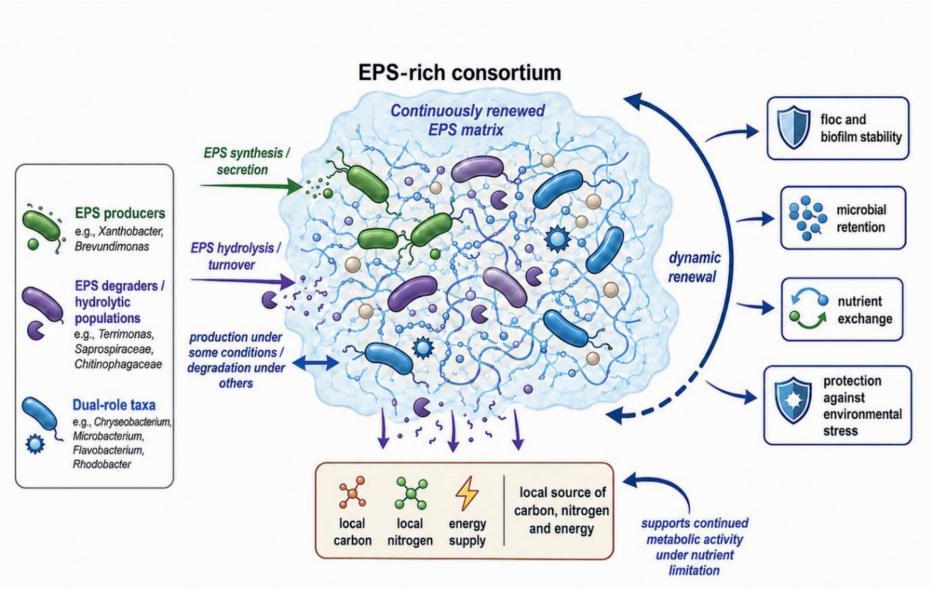


Figure 3: Conceptual representation of dynamic EPS production and turnover in an EPS-rich microbial consortium. EPS-producing, EPS-degrading, and dual-role taxa coexist within a continuously renewed EPS matrix. Partial EPS hydrolysis may release local carbon, nitrogen, and energy sources that support microbial activity under nutrient-limited conditions, while the matrix contributes to floc and biofilm stability, microbial retention, nutrient exchange, and protection against environmental stress.

Table 1: Putative EPS producers and degraders in bioreactors operated under EPS-overproduction conditions. Samples were collected at the end of specific operational periods from each reactor, as detailed in Chapter 4. Although the taxa differed depending on the conditions, their putative functions were similar.

Reactor ID	Reactor specifications	Putative EPS producers	Putative EPS degraders / EPS turnover-associated taxa
R1	Glycerol-fed MBR (municipal inoculum) (Ch. 2)	*Hydrogenophaga*, *Pseudoxanthomonas*, *Flavobacterium*, *Sphaerotilus*, *Arenimonas*, *Ancylobacter*, Comamonadaceae, *Ferrovibrio*	Saprospiraceae, Chthoniobacteraceae LD29, *Ferruginibacter*, *Prosthecobacter*, *Bdellovibrio*
R2	Glycerol-fed MBR (industrial inoculum) (Ch. 2)	*Hydrogenophaga*, *Microbacterium*, *Shinella*, Chitinophagaceae, *Brevundimonas*, *Pseudoxanthomonas*, Microbacteriaceae, *Chryseobacterium*	Saprospiraceae, *Terrimonas*, *Ferruginibacter*, *Bdellovibrio*, *Herbiconiux*, Hyphomonadaceae SWB02
R3	Glycerol-fed MBR (municipal inoculum) (Ch. 2)	*Novosphingobium*, *Asticcacaulis*, *Derxia*, *Microbacterium*, *Xanthobacter*, Rhizobiaceae, Hyphomonadaceae UKL13-1, *Rhodobacter*	*Pseudoclavibacter*, *Bdellovibrio*, *Terrimicrobium*, *Rhodobacter*
R4	Glycerol-fed MBR (industrial inoculum) (Ch. 2)	Enterobacteriaceae, Hyphomonadaceae UKL13-1, Microbacteriaceae, *Microbacterium*, Rhizobiaceae, *Xanthobacter*, *Novosphingobium*, *Brevundimonas*, Comamonadaceae, *Sphingobium*	*Hydrogenophaga*, *Propionispira*, *Paludibacter*
R5	VFA-fed MBR (municipal inoculum) (Ch. 3)	Sphingobacteriales env.OPS 17, *Xanthobacter*, *Microbacterium*, *Flavobacterium*, *Brevundimonas*, *Sphingobium*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Pseudomonas*, *Chryseobacterium*	Micavibrionales, *Bdellovibrio*, *Hyphomicrobium*, *Sandaracinobacter*, *Gordonia*, *Comamonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Stenotrophomonas*
R6	VFA-fed CSTR (municipal inoculum) (Ch. 3)	Flavobacteriales NS9 marine group, *Chryseobacterium*, *Microbacterium*, *Flavobacterium*, *Brevundimonas*, *Pseudoxanthomonas*	Saprospiraceae, Chitinophagaceae, *Devosia*, *Nubsella*, *Pajaroellobacter*
R7	VFA-fed CSTR (municipal inoculum) (Ch. 3)	*Brevundimonas*, *Flavobacterium*, *Chryseobacterium*, Spirosomaceae, Xanthomonadales, *Xanthobacter*, *Pseudoxanthomonas*, *Agaricicola*, *Acinetobacter*, Sphingobacteriaceae, Chitinophagaceae, Flavobacteriales NS9 marine group	*Prosthecobacter*, Phycisphaeraceae CL500-3, *Terrimonas*, *Bdellovibrio*, Saprospiraceae, *Dysgonomonas*, *Alcaligenes*, *Parapusillimonas*, Babeliales, Micavibrionales, Microscillaceae, *Pseudochelatococcus*, *Taibaiella*, Tannerellaceae, Kapabacteriales

6.3 The overall contribution of this thesis to advancing the application of EPS as bioflocculants

The contributions of this thesis to advancing the application of MMC-derived EPS as bioflocculants are summarized in Figure 4.

6.4 Limitations, uncertainties and future directions

The results of this thesis demonstrate that EPS overproduction conditions can stimulate EPS synthesis in MMCs beyond the basal production level. This provides an important proof of concept for the development of wastewater-based EPS production processes. However, the work was carried out under controlled laboratory conditions using synthetic wastewater and a relatively narrow range of operational settings. This approach was necessary to isolate the effects of the selected EPS overproduction conditions and to enable consistent comparison between experiments. At the same time, it limits direct transfer of the findings to full-scale or real wastewater systems. Real wastewater is inherently more complex and may introduce additional constraints related to substrate heterogeneity, competing microbial processes, and fluctuations in loading [13]. Therefore, future research should test the EPS overproduction strategy under real wastewater conditions and across a broader range of organic loading rates in order to evaluate process robustness and applicability under realistic treatment scenarios. Such studies will be critical for determining whether the process remains stable under fluctuating influent composition and operational disturbances.

A second important limitation concerns the range of substrates investigated. In this thesis, glycerol and VFAs were used as model substrates to represent distinct wastewater carbon compositions under nitrogen-limited conditions. Within this range, substrate type clearly influenced EPS composition, particularly by increasing the protein contribution in VFA-derived EPS. This indicates that substrate selection may be used as a first operational tool to steer EPS composition. However, only a small part of the possible substrate spectrum relevant to wastewater treatment was explored. A broader investigation of wastewater types and carbon compositions is therefore needed to determine the extent to which EPS properties can be tuned through feed selection alone. This concept is already well

established in the PHA field, where the composition of volatile fatty acid feedstocks is known to influence polymer yield, but also polymer composition and properties [84]. For example, acetate-rich feeds mainly favour 3-hydroxybutyrate formation, whereas increasing the contribution of propionate promotes incorporation of 3-hydroxyvalerate into PHBV, thereby altering crystallinity, melting behaviour, flexibility, and other material properties [87], [88], [89]. This demonstrates that feed composition can be used as a steering parameter for biopolymer functionality and suggests that a similar concept may be relevant for MMC-derived EPS, although this remains less systematically established.

The characterization of EPS in this study was also intentionally focused on the parameters most directly relevant to flocculation, especially polysaccharides, proteins, molecular weight, charge density, and functional groups. This level of characterization was sufficient for the objectives of the work. However, the complex and heterogeneous nature of MMC-derived EPS leaves substantial room for more in-depth investigation in future research. In addition to polysaccharides and proteins, such EPS may contain lipids, extracellular DNA, humic-like substances, and other minor components that could also influence functionality. Moreover, the analytical methods applied here, including colorimetric assays for polysaccharide and protein quantification and CLSM-based staining, provide only a partial view of EPS composition and structure. Future studies should therefore incorporate more comprehensive compositional and structural analyses, particularly when different wastewater streams are used as feedstocks. In particular, sugar and amino acid composition, and the distribution of functional groups should be examined in greater detail to better understand how EPS composition relates to application performance. For example, in terms of flocculation applications, determining sugar composition may be important because the relative abundance of neutral sugars, amino sugars, and uronic acids can influence EPS properties, charge, hydrophilicity, calcium-binding capacity, and polymer conformation [26], [90], thereby affecting adsorption and bridging during flocculation. Such analyses would provide a more mechanistic basis for linking EPS composition to application performance and further clarify structure–function relationships that cannot be resolved when EPS is described only by bulk PS/PN ratios.

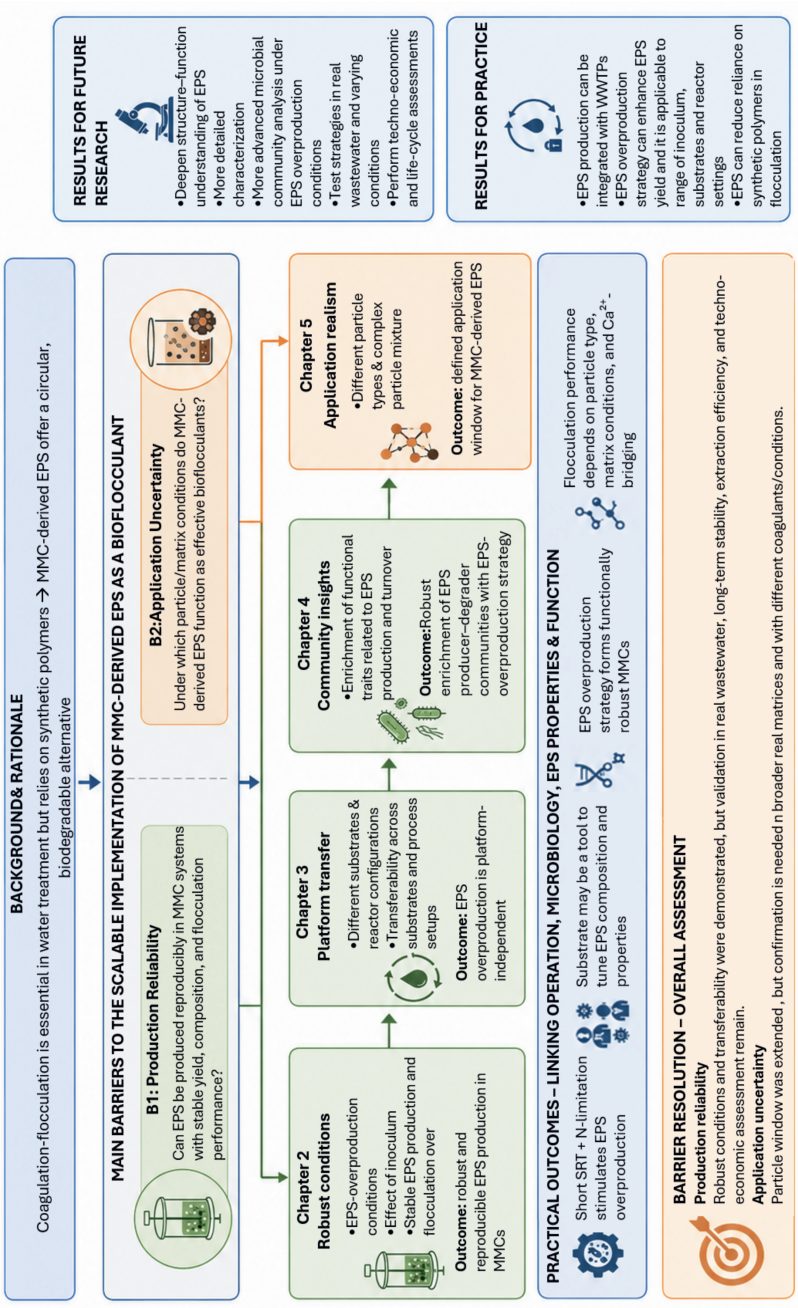


Figure 4: Overview of the overall contribution of this thesis to advancing MMC-derived EPS as bioflocculants.

Another limitation of the present work is that only nitrogen limitation was explored as the main trigger for EPS overproduction. While nitrogen limitation was chosen for a clear mechanistic reason, other nutrient limitations (e.g. phosphorus limitation) may induce different cellular responses and potentially lead to different EPS yields or compositions; they therefore represent promising additional strategies for stimulating EPS production. Exploring these alternatives could broaden the range of wastewater suitable for the process and provide further opportunities to direct EPS properties toward specific applications. A more systematic comparison of nutrient limitation strategies would also help distinguish which responses are specific to nitrogen limitation and which reflect a broader stress-induced EPS production mechanism.

Uncertainty also remains regarding the microbial community assessment underlying EPS/PHA production and turnover. One of the key findings of this thesis was that EPS overproduction appeared to be a community-level function. However, the 16S rRNA gene amplicon sequencing approach used in this thesis provides mainly taxonomic information, mostly at genus-level resolution, and cannot directly confirm the metabolic activity of individual microorganisms under the studied conditions. Nor can this method determine whether a given microorganism was producing EPS, degrading it, storing PHA, or performing more than one of these roles depending on the operational conditions. Therefore, the functional assignments made in this thesis should be considered informed indications rather than definitive proof. Future work should combine community profiling with metagenomics, metatranscriptomics, metaproteomics, enzyme activity analysis, or isotope-based approaches in order to link taxonomic identity more directly to EPS-related function. This will be especially important for resolving whether EPS-rich systems are maintained by distinct producers and degrader guilds or by metabolically versatile populations that can switch roles depending on environmental conditions.

The application experiments performed in this thesis were designed primarily to assess the potential of extracted EPS as a bioflocculant. For this reason, EPS performance was tested against different particle types rather than being evaluated only with standard kaolinite suspensions. This provided a broader view of how particle properties influence flocculation outcomes. Nevertheless, the application experiments were still limited compared with the complexity of

real wastewater systems, where particles vary in hydrophobicity, size distribution, surface chemistry, and organic content. Although a complex particle matrix, which was prepared by diluting iron-rich sludge, was included in the experiments, this was not sufficient to fully evaluate the practical application window of the produced EPS. Future research should therefore investigate EPS performance in a wider range of real process streams and under more application-oriented conditions.

Additionally, in this thesis, effective flocculation was achieved by combining EPS with Ca^{2+}, and cation-mediated bridging was inferred to be the dominant mechanism driving flocculation. In this context, Ca^{2+} essentially acted as a coagulation agent. The choice to use Ca^{2+} was based not only on previous research [21], but also on the known role of Ca^{2+}, in three-dimensional biofilm matrix formation [9], [91]. Calcium can stabilise EPS networks by promoting interactions between negatively charged functional groups, such as carboxyl groups, thereby contributing to matrix cohesion, aggregate stability, and particle bridging [88]. However, in full-scale water treatment practice, other metal-based coagulants, particularly Fe- and Al-based salts, are more commonly used [92]. Therefore, future studies should evaluate EPS performance in combination with different coagulants, including ferric, ferrous, and aluminium-based coagulants. Such tests would help clarify whether EPS are most effective as independent bioflocculants, co-flocculants, or polymer aids following coagulation.

In this thesis, MMC-derived EPS were only considered for flocculation applications; however, other separation processes such as flotation may also be promising application routes for MMC-derived EPS. Flotation efficiency depends on particle-bubble collision, attachment, and detachment, which are typically controlled using reagents such as collectors, activators, depressants, and frothers [93]. Collectors increase the hydrophobicity of target particles and promote their attachment to air bubbles, while activators enhance collector adsorption on specific mineral surfaces. In contrast, depressants selectively reduce the floatability of unwanted particles by making their surfaces more hydrophilic or by preventing collector adsorption. Frothers stabilise air bubbles and the froth phase, improving the separation of floated particles from the suspension [93]. In this context, MMC-derived EPS may be relevant as bio-based flotation modifiers. A recent review [94] summarized studies on the usage of microbial polysaccharides, such

as xanthan gum, gellan gum, and pullulan, in mineral flotation. These microbial polysaccharides can interact with metal sites on mineral surfaces through abundant hydroxyl and carboxyl groups, allowing them to act as flotation inhibitors, or depressants, for specific minerals [94]. For example, gellan gum has been used as a depressant for barite, where chemical interactions between gellan gum and Ba^{2+} ions on the barite surface were proposed to drive selective adsorption [94]. Xanthan gum has shown a strong depression effect for talc; adsorption of xanthan gum on talc increased the surface roughness and hydrophilicity of the particles, thereby reducing their floatability [94]. Similarly, pullulan has been shown to function as a depressant for talc during the flotation separation of chalcopyrite and talc. Pullulan adsorbed more strongly onto talc than onto chalcopyrite, forming a polymer layer that increased talc hydrophilicity and inhibited talc flotation [94]. Therefore, MMC-derived EPS could potentially be explored as bio-based depressants, flotation modifiers, or conditioning agents. However, while EPS used as depressants in flotation may need strong surface adsorption and hydrophilicity-enhancing properties, EPS intended to promote particle-bubble attachment may require more hydrophobic or amphiphilic characteristics [93]. Future research should therefore examine whether MMC-derived EPS can be tailored toward specific flotation functions, including selective depression or hydrophobicity-enhanced particle-bubble attachment.

In addition, future studies should evaluate EPS not only as a stand-alone replacement for synthetic polymers, but also as components of hybrid flocculation strategies. For example, combining EPS with reduced doses of synthetic polymers such as PAM could provide a transitional route toward lowering synthetic polymer consumption while maintaining reliable separation performance. This approach has already been demonstrated in a dual PAM-starch system, where the PAM dosage required for surface water flocculation was reduced by 50–70% [95]. Similarly, another study used a different hybrid strategy in which a pure-culture-derived bioflocculant was grafted with polyacrylamide. The resulting hybrid flocculant achieved high removal efficiencies for COD, BOD, N, and P in domestic wastewater, reaching 98%, 54%, 53%, and 57%, respectively [96].

Economic uncertainty is another important limitation of the present work. No detailed techno-economic or life-cycle assessment was carried out, and therefore the sustainability and competitiveness of the process remain only conceptually

supported. The main advantage proposed in this thesis is that EPS production is integrated into a biological wastewater treatment process rather than relying on pure-culture fermentation using refined substrates. This concept has clear potential benefits from a circularity perspective, since it seeks to convert wastewater carbon into a functional biopolymer while simultaneously supporting wastewater treatment.

A useful benchmark is provided by Kumar et al. (2020), who performed a techno-economic analysis of pure-culture-based EPS bioflocculant production using activated sludge fortified with crude glycerol [97]. In that study, the estimated price of soluble EPS was approximately 14 USD kg^{-1}, whereas the reported price of PAM-based flocculants was approximately 4.0–4.2 USD kg^{-1}. Their cost breakdown showed that fermentation dominated the production cost, contributing 86.6%, whereas the extraction step by centrifugation accounted for 13.4%. Importantly, their sensitivity analysis showed that an approximately 30% increase in EPS concentration in the fermenter can reduce the unit production cost by about 25%.

Considering that, in the process evaluated by Kumar et al. (2020) [97], EPS was produced without a specific operational strategy to stimulate EPS overproduction, and that sterilisation steps contributed substantially to the production cost, these findings indicate clear room for process improvement. An MMC-based process integrated into wastewater treatment together with EPS-overproduction strategies could potentially reduce these requirements by avoiding sterile pure-culture operation and selectively stimulating EPS production. However, the unit price and the broader economic and environmental benefits of this approach will still depend on measurable reactor-performance indicators, such as EPS yield and productivity, carbon conversion to EPS, COD and nutrient removal, effluent quality, process stability, energy demand for aeration and mixing, sludge production, and chemical requirements, as well as EPS extraction efficiency and downstream processing needs. Therefore, a full process assessment is needed before its industrial feasibility can be determined.

Among the downstream steps, EPS extraction represents a particularly important source of uncertainty and cost. In this thesis, a standard literature-based extraction method was applied under predefined conditions, but no systematic

optimization of this step was performed. As a result, it remains unclear whether all available EPS was recovered or whether certain EPS fractions were preferentially recovered over others. This has direct implications for the reported yield values as well as for the measured composition and functionality of the extracted product [98], [99], [100]. Future work should address extraction more explicitly by comparing methods, optimising recovery conditions, and evaluating how extraction influences polymer composition and application performance.

Overall, this thesis provides a strong proof of concept that EPS overproduction can be stimulated in mixed microbial cultures and that substrate type, microbial community structure, and polymer properties are closely interconnected in this process. At the same time, several important uncertainties remain regarding performance under real wastewater conditions, the full compositional complexity of the produced EPS, the exact microbial mechanisms involved, the role of extraction in shaping the recovered product, and the economic feasibility of large-scale implementation. Future research should therefore move toward more integrated studies in which reactor operation, microbial ecology, EPS characterization, downstream recovery, and end-use performance are evaluated together. Only through such a combined approach will it be possible to determine whether wastewater-based EPS production can be developed into a robust, scalable, and practically relevant platform for circular biopolymer production.

6.5 Concluding Remarks

Mixed microbial culture (MMC)-based EPS production offers a promising route to integrate biopolymer production with wastewater treatment practice. Until now, two main barriers have limited further implementation: production reliability and application uncertainty. This thesis addressed the first barrier by showing that EPS overproduction can be reproducibly stimulated in open MMC systems through operational steering, particularly under short SRT and nitrogen-limited conditions, and that this response can be maintained across different inocula, substrates, and reactor configurations. The second barrier was addressed by demonstrating that MMC-derived EPS can function as bioflocculants across different particle systems, while also showing that performance depends strongly on particle surface properties and matrix conditions.

From a scientific perspective, this thesis shows that EPS production by MMCs is a community-level function shaped by operational conditions, substrate composition, and reactor configuration. Importantly, EPS overproduction does not appear to depend on the selection of specific microbial taxa, but rather on the enrichment of functional traits associated with EPS production and turnover. It also highlights that EPS functionality cannot be predicted from bulk composition alone, but requires understanding the link between operational conditions, microbial ecology, polymer properties, and interfacial performance. The way forward scientifically is therefore to deepen structure–function analysis of MMC-derived EPS, including more detailed compositional characterization, extraction effects, and application-specific testing.

For practice, this thesis demonstrates that MMC-derived EPS bioflocculant production has clear potential, but also that implementation will require further validation under realistic operating conditions. Future steps should focus on long-term process stability, optimized EPS recovery, flocculation performance in real water matrices, and full techno-economic and life-cycle assessment. Overall, this thesis provides a foundation for developing MMC-derived EPS from a promising laboratory concept into a scalable platform for circular and more sustainable bioflocculant production.

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Appendix A

Supplementary Information belonging to Chapter 2

SI-1 Calculation of EPS Production Yield

To determine EPS production yield, the following steps were followed; i) EPS extraction was performed as described in section 2.4.3 (Materials and Methods). Later, the chemical oxygen demand (COD) of the extracted EPS was measured to quantify the organic content associated with EPS. ii) The daily amount of COD corresponding to produced EPS was calculated using the sludge wasting rate (Q_{wasting}) of the MBR, as this represents the EPS production rate of the reactor. iii)The soluble COD (sCOD) converted (i.e removed during the process) by the MBRs was calculated based on the influent and effluent COD concentrations and corresponding flow rates (Q_{in} and Q_{effluent}).

Calculation of the amount of COD that was converted to EPS (g COD/d):

For MBR:

$$\text{g COD}_{\text{EPS,produced}} = Q_{\text{wasting}} \cdot \text{COD}_{\text{EPS}} \tag{Eq.1}$$

Calculation of the amount of converted COD (g COD/d):

$$\text{g COD}_{\text{converted}} = Q_{\text{in}} \cdot \text{COD}_{\text{in}} - Q_{\text{effluent}} \cdot \text{COD}_{\text{effluent}} \tag{Eq.2}$$

Calculation of the production yield:

$$Y = \frac{\text{g COD}_{\text{EPS produced}}}{\text{g COD}_{\text{converted}}} \tag{Eq.3}$$

Q_{in}: Influent flow rate (L d^{-1})
Q_{effluent}: Effluent flowrate (L d^{-1})
Q_{wasting}: sludge wasting flowrate (L d^{-1})
COD_{in}: Influent soluble COD concentration (g L^{-1})
$\text{COD}_{\text{effluent}}$: Effluent soluble COD concentration (g L^{-1})
COD_{EPS}: EPS COD concentration (g L^{-1})

Table S1: Detailed operational parameters of Exp_1 and Exp_2. Total nitrogen concentration was calculated for each COD:N ratio separately while the supplied amount of COD was kept constant. SRTs were controlled by changing sludge wasting flow rate (Q_{waste})

Exp.	R. ID	Inoculum	SRT	Time	COD:N	HRT	DO	pH	COD_{in}	Total N	Q_{in}	Q_{eff}	Q_{waste}	F/M	ML	V
			(day)	(day)		(h)										(L)
Exp_1	R1	Municipal sludge	>15	0–60	20	12	3–4	7–7.3	1000	50	6.6	6.6	–	0.5	4	3.3
			6	60–75	60					16.6	6.6	6.05	0.55			
			6	75–84	100					10	6.6	6.05	0.55			
			3	84–105	100					10	6.6	5.5	1.1			
Exp_1	R2	Industrial sludge	>15	0–60	20	12	3–4	7–7.3	1000	50	6.6	6.6	–	0.5	4	3.3
			6	60–75	60					16.6	6.6	6.05	0.55			
			6	75–84	100					10	6.6	6.05	0.55			
			3	84–105	100					10	6.6	5.5	1.1			
Exp_2	R3	Municipal sludge	6	0–12	100	12	3–4	7–7.3	1000	10	6.6	5.5	1.1	0.5	4	3.3
			3	12–34	100						6.6	5.5	1.1			
			1.5	34–42	100						6.6	5.5	1.1			
Exp_2	R4	Industrial sludge	6	0–12	100	12	3–4	7–7.3	1000	10	6.6	5.5	1.1	0.5	4	3.3
			3	12–34	100						6.6	5.5	1.1			
			1.5	34–42	100						6.6	5.5	1.1			

Note: DO: Dissolved oxygen concentration in mg L^{-1}, COD: Chemical oxygen demand in mg COD L^{-1}, Total N: Total Nitrogen in mg L^{-1}, Q : flow rate in L d^{-1}, F:M ratio: Food to microorganism ratio in g COD g $MLVSS^{-1}$ d^{-1}, ML: Mixed liquor volatile suspended solids in g L^{-1}

SI- 2 Source of inocula and synthetic wastewater composition and preparation

Table S2: Information about inocula – municipal sludge and industrial sludge

	Municipal Sludge	Industrial Sludge (Concentrated)
MLVSS (g/L)	3.5	40
pH	6.5–8	6.5–7
DO (mg/L)	0–2	NA
Influent Type	Municipal wastewater + Industrial wastewater from milk factory	VFA (acetic, propionic and butyric acid)
Process	Biological C and N removal, chemical P precipitation	Biological C and N removal
COD:N	15–20	NA
F/M ratio (kg COD/kg MLSS.d)	0.125	NA
FM ratio (kg BOD/kg MLSS.d)	0.068	NA
Nitrogen load (kg N/kg MLSS.d)	0.011	NA
SRT (d)	15	NA
HRT (h)	24	NA
COD:N	11.3	NA

Table S3: Composition of synthetic wastewater and stock trace solution used in experiments 1 and 2 – Each solution was prepared according to the recipe presented below. Based on the desired COD:N ratios, the total nitrogen content was adjusted, while the concentration of glycerol, which was the main carbon source, was kept constant. The synthetic wastewater was prepared using distilled water and used without sterilization. To prevent bacterial growth, the carbon source (glycerol) and other components of the synthetic wastewater were supplied from separate tanks.

Concentration for each period	COD:N 20	COD:N 60	COD:N 100
Glycerol	819.6	819.6	819.6
NH_4Cl	150.7	43.5	18.07
Yeast Extract	100	50	50
K_2HPO_4	15	15	15
KH_2PO_4	25	25	25
$MgCl_2 \cdot 6H_2O$	200	200	200
$CaCl_2 \cdot 2H_2O$	150	150	150
Trace Solution (mL/L)	2	2	2

Trace solution recipe	Concentration (mg/L)
$FeCl_3 \cdot 6H_2O$	1500
H_3BO_3	150
$CoCl_2 \cdot 6H_2O$	150
$ZnSO_4 \cdot 7H_2O$	120
$MnCl_2 \cdot 4H_2O$	145
$Na_2MoO_4 \cdot 2H_2O$	60
$CuSO_4 \cdot 5H_2O$	60
KI	30

Table S4: Concentration of COD and Total nitrogen in the synthetic wastewater at different periods of the experiment 1 and 2.

Concentrations (mg/L)	COD:N 20	COD:N 60	COD:N 100
Total COD	1000	1000	1000
Total Nitrogen	50	16	10

SI- 3 Modified TSS/VSS analysis

TSS and VSS were measured with a modified method as it was stated in section 2.4.2 of the manuscript. The modification included the centrifugation of mixed liquor samples at 3750xg for 25 min to separate the liquid from the solid phase. This step was required due to the very poor filterability of the viscous liquid in the EPS-rich reactors.

SI-4 EPS extraction methods

For each method listed in section 2.4.3 of the manuscript, 40 ml of mixed liquor was first diluted with 1x PBS buffer (1:1 v:v) to increase solubility of EPS in the mixed liquor whilst keeping pH of EPS solutions constant during extraction steps. Subsequently, samples were divided into 4 aliquots of 20 ml to perform the two EPS extraction methods in duplicates. In what follows we briefly outline each of the extraction methods.

- **Cation exchange resin (CER) extraction**: the samples were mixed with CER (DOWEX Marathon C, sodium form, 20 - 50 mesh size, Sigma-Aldrich, DE) at a ratio 700 mg of CER per gram VSS. This mixture was stirred for 1 h at 600 rpm at room temperature.

- **Heat extraction:** the samples were stirred on a magnetic hotplate stirrer for 1 h, at 600 rpm and 50 °C

At the end of each extraction, the samples were centrifuged at 25000xg for 25 min, and the resulting EPS-containing supernatants were collected and dialysed against demineralized water for 24 h using a cellulose membrane with a molecular weight cut-off of 3.5 kDa (SnakeSkin dialysis membrane, Thermo Fisher Scientific, US). EPS extracts were used for further characterization and flocculation experiments.

Supplementary Figures

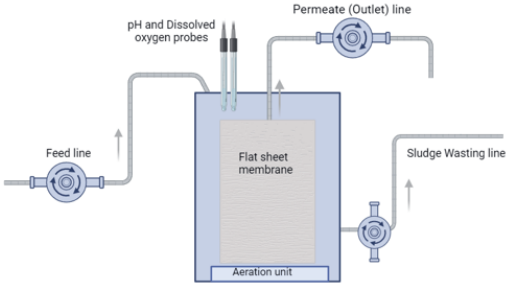


Figure S1: Schematic representation of the MBR setup used to produce EPS – MBRs were equipped with chlorinated polyvinyl chloride (CPVC) flat sheet membranes with 0.4 μm nominal pore size and 0.11 m^2 surface area. The aeration unit was placed at the bottom of the reactor to prevent membrane fouling. Glycerol-rich synthetic wastewater was fed to the MBRs. The outlet contained biologically treated and filtered wastewater; the solid retention time (SRT) was controlled via sludge wasting pumps.

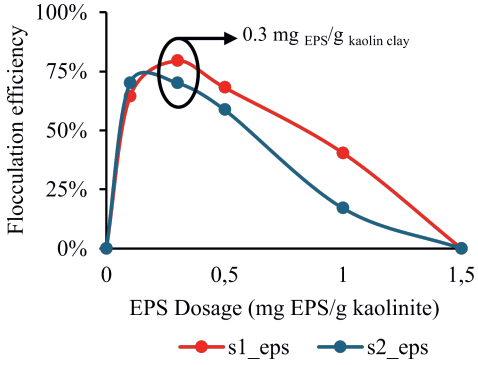


Figure S2: EPS dosage - flocculation efficiency test – The flocculation efficiency of two types of EPS (S1 and S2) was tested using a kaolin clay suspension across a range of dosages from 0 to 1.5 mg EPS per gram of kaolinite.

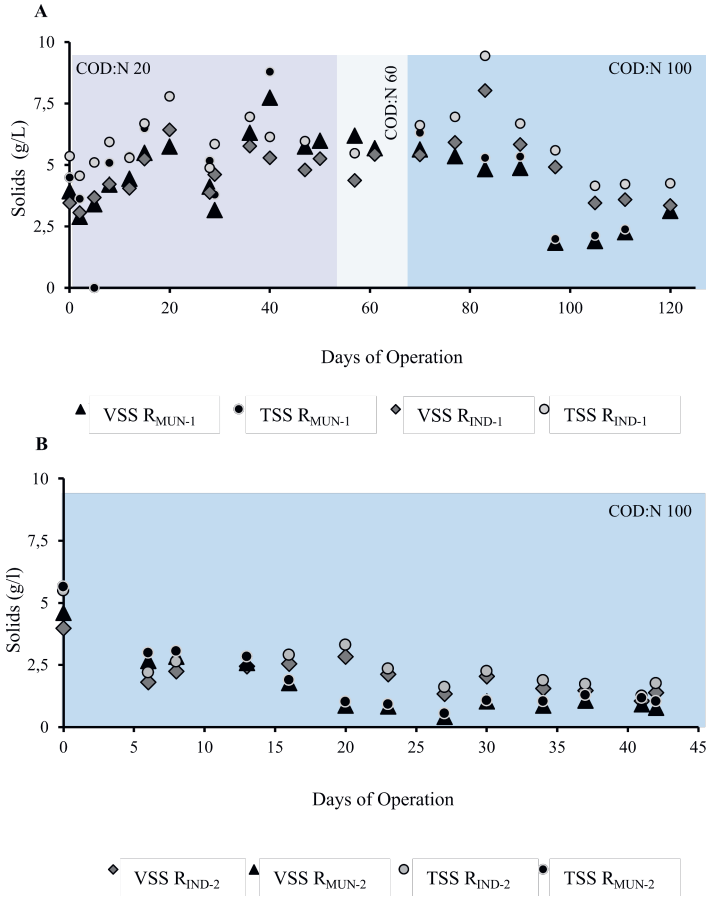


Figure S3: Solids concentration during (A) experiment 1 and (B) experiment 2 in the MBRs

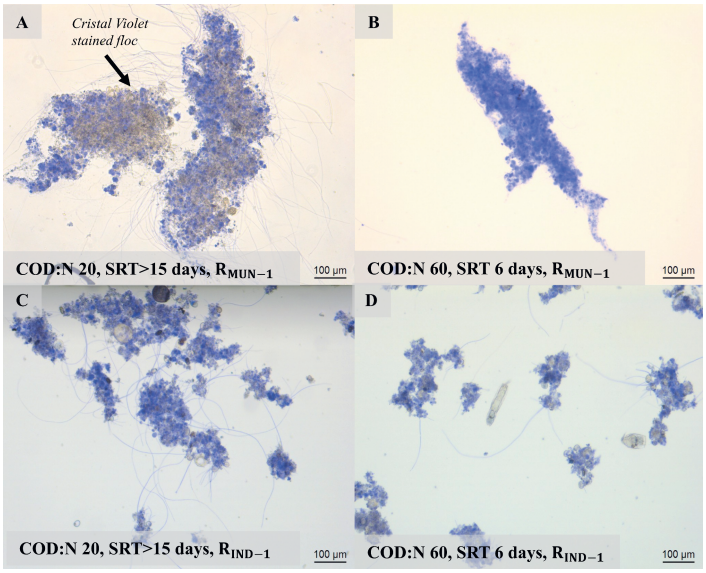


Figure S4: Brightfield microscopy of mixed liquor (ML) samples from Exp_1 – Fresh mixed liquor samples were observed to assess EPS production by applying crystal violet (in purple) to visualize EPS structures and cell envelopes, and alcian blue (in blue) to specifically target acidic polysaccharides. In A and C samples from R_{MUN-1} and R_{IND-1} under nitrogen-sufficient (COD:N 20) conditions; In B and D samples from R_{MUN-1} and R_{IND-1} in COD:N 60, SRT 6 days.

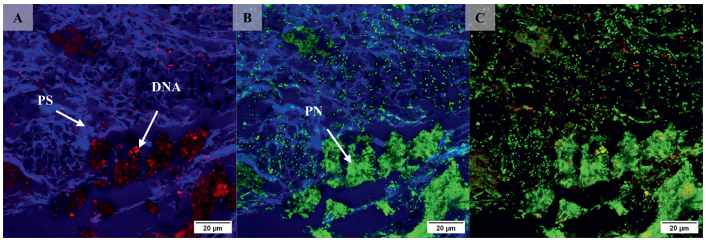


Figure S5: Confocal laser scanning microscopy (CLSM) micrographs of mixed liquor samples of RIND-2 at SRT 1.5 days – Merged images of fluorescently stained proteins (Sypro orange, in green), polysaccharides (Calcofluor white, in blue), and DNA (SYTO 63, in red). A) Visualization defined cell clusters (DNA) and a cloudy polysaccharide-rich EPS (PS); B) Compartmentalization of protein (PN) and polysaccharide components within the EPS; C) Visualization of proteins and DNA components, showing that most cells are covered with protein-rich EPS. D) PS, DNA and microbial cells

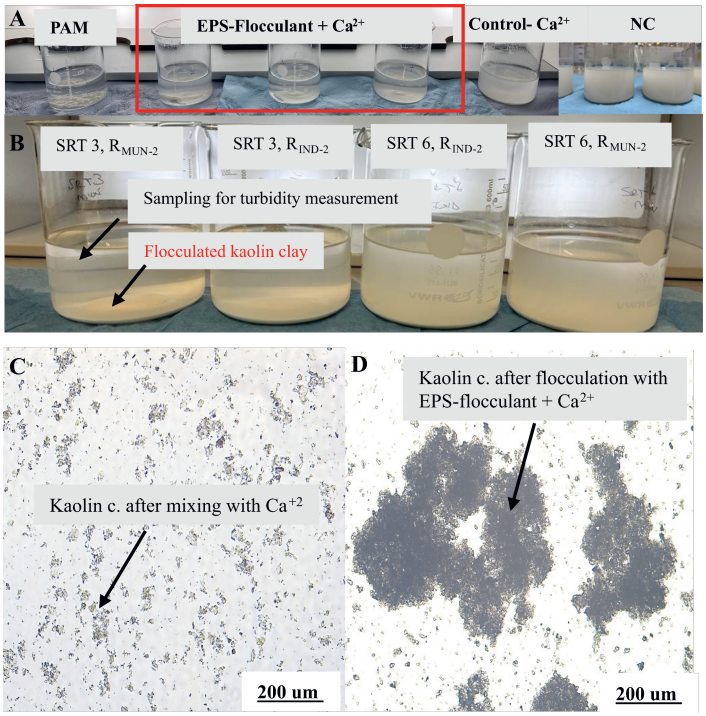


Figure S6: Flocculation of kaolinite particles A) by polyacrylamide (PAM) synthetic flocculant and EPS-flocculant (produced under nitrogen limitation COD:N 100, short SRT (3 days)), control contains only clay solution and Ca^{2+} and Negative control (NC) containing only kaolin clay. B) Qualitative comparison of flocculation efficiencies of EPS from SRT 6 days and SRT 3 days periods. On the bottom, brightfield microscopy of sediments after the flocculation process C) Control – Kaolinite particles after addition of Ca^{2+}. D) Clay particles flocculated with EPS-flocculant. To make the observations presented in C and D, settled samples were carefully collected from the bottom of the beakers using a Pasteur pipette at the end of the jar test settling period. The samples were then placed on microscopy slides and examined under an optical microscope.

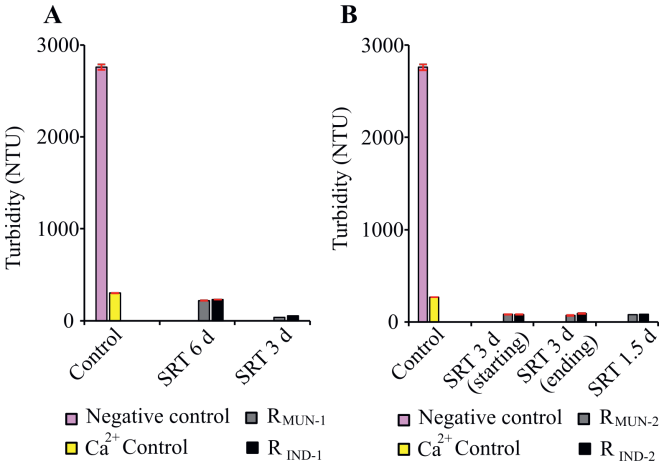


Figure S7: Nephelometric turbidity units measured in flocculation experiments in (A) experiment 1 and (B) experiment 2 - Negative control contains only kaolin clay suspension without addition of calcium or EPS, while Ca-control contains only calcium in kaolin clay suspension. Other samples presented correspond to the samples shown in Figure 7. B and C in the manuscript.

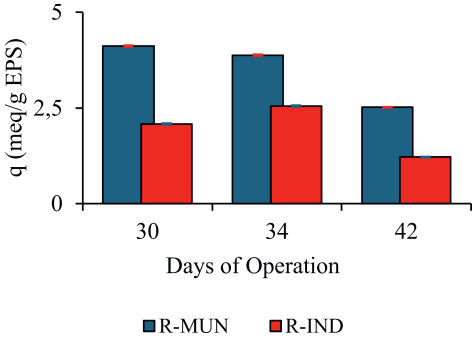


Figure S8: Charge density of EPS from R_{MUN-2} and $R_{IND-2}Exp_2$ – Charge density was measured by potentiometric titration using poly(diallyldimethylammonium chloride) (PolyDADMAC) as the titrant for EPS samples.

Supplementary Tables

Table S5: Chemical analysis of kaolin clay, as provided by Sigma-Aldrich

Compound	wt%
SiO_2	50.4
Al_2O_3	34.3
K_2O	1.9
Fe_2O_3	0.5
TiO_2	0.4
Na_2O	0.2
Loss on ignition	11.5%

Table S6: Average steady-state operational parameters of R_{MUN-1} and R_{IND-1} from experiment 1 and R_{MUN-2} and R_{IND-2} from experiment 2 . The reactors were fed from the same feed tank during the experiments. All parameters were kept identical for both reactors. Effluent samples were collected separately and $COD_{effluent}$ was measured for each reactor.

RMUN-1			
Parameter	COD:N 60, SRT 6 days	COD:N 100, SRT 6 days	COD:N 100, SRT 3 days
$COD_{influent}$ (mg/L)	1088.25 ± 34	942.7 ± 19	939.95 ± 8.4
$COD_{effluent}$ (mg/L)	21.4 ± 0.57	15.1 ± 0.47	26 ± 0.65
COD_{Waste} (mg/L)	7672 ± 110	8376 ± 249	3664 ± 97
COD_{EPS-1} (mg/L)	604	654	1056
COD_{EPS-2} (mg/L)	602	649	1045
$Q_{influent}$ (L/day)	6.6	6.6	6.6
$Q_{effluent}$ (L/day)	6.05	6.05	5.5
Q_{Waste} (L/d)	0.55	0.55	1.1
Total Kjeldahl Nitrogen (mg/L)	17.2 ± 0.93	8.8 ± 0.8	9.5 ± 0.17
SRT (d)	6	6	3
HRT (h)	12	12	12
COD load (mg/L.d)	2176.5 ± 128	1885.4 ± 38	1879.8 ± 17
MLVSS (g/L)	5.84 ± 0.146	5.02 ± 0.14	2.28 ± 0.25
Volume (L)	3.3	3.3	3.3
FM ratio (g COD/g MLVSS.d)	0.37 ± 0.01	0.37 ± 0	0.82 ± 0.07
RIND-1			
Parameter	COD:N 60, SRT 6 days	COD:N 100, SRT 6 days	COD:N 100, SRT 3 days
$COD_{influent}$ (mg/L)	1088.25 ± 34	942.7 ± 19	939.95 ± 8.4
$COD_{effluent}$ (mg/L)	20.2 ± 0.38	12.53 ± 0.86	73 ± 7.4
COD_{Waste} (mg/L)	6568 ± 111	7992 ± 85	4348 ± 92
COD_{EPS-1} (mg/L)	828	640	492
COD_{EPS-2} (mg/L)	821	641	496
$Q_{influent}$ (L/day)	6.6	6.6	6.6
$Q_{effluent}$ (L/day)	6.05	6.05	5.5
Q_{Waste} (L/d)	0.55	0.55	1.1
Total Kjeldahl Nitrogen (mg/L)	17.2 ± 0.93	8.8 ± 0.8	9.5 ± 0.17
SRT (d)	6	6	3
HRT (h)	12	12	12
COD load (mg/L.d)	2176.5 ± 128	1885.4 ± 38	1879.8 ± 17
MLVSS (g/L)	5.07 ± 0.28	6.6 ± 0.58	3.8 ± 0.31
Volume (L)	3.3	3.3	3.3
FM ratio (g COD/g MLVSS.d)	0.42 ± 0.01	0.28 ± 0	0.49 ± 0.0

Table S6: Average steady-state operational parameters of R_{MUN-1} and R_{IND-1} from experiment 1 and R_{MUN-2} and R_{IND-2} from experiment 2 – continued.

RMUN-2			
Parameter	COD:N 100, SRT 6	COD:N 100, SRT 3	COD:N 100, SRT 1.5
$COD_{influent}$ (mg/L)	1081 ± 14	1081 ± 14	1081 ± 14
$COD_{effluent}$ (mg/L)	279.5 ± 0	307.5 ± 17	162.3 ± 2
COD_{Waste} (mg/L)	4241 ± 255	1516 ± 196	1253 ± 135
$Q_{influent}$ (L/day)	6.91	6.91	6.91
$Q_{effluent}$ (L/day)	6.32	5.73	4.55
Q_{Waste} (L/d)	0.59	1.18	2.36
Total Kjeldahl Nitrogen (mg/L)	10.2 ± 0.8	10.2 ± 0.8	10.2 ± 0.8
SRT (d)	5.59	2.8	1.4
HRT (h)	11.4	11.4	11.4
COD load (mg/L.d)	2263.54 ± 30	2263.54 ± 30	2263.54 ± 30
MLVSS (g/L)	2.17 ± 0.08	1.4 ± 0.26	0.9 ± 0.05
Volume (L)	3.3	3.3	3.3
FM ratio (g COD/g MLVSS.d)	1.04 ± 0.1	1.62 ± 0.02	2.52 ± 0.03
RIND-2			
Parameter	COD:N 100, SRT 6	COD:N 100, SRT 3	COD:N 100, SRT 1.5
$COD_{influent}$ (mg/L)	1081 ± 14	1081 ± 14	1081 ± 14
$COD_{effluent}$ (mg/L)	92.6 ± 9	289.6 ± 25	151 ± 2.62
COD_{Waste} (mg/L)	2550 ± 290	2513 ± 92	2038 ± 38
$Q_{influent}$ (L/day)	6.91	6.91	6.91
$Q_{effluent}$ (L/day)	6.32	5.73	4.55
Q_{Waste} (L/d)	0.59	1.18	2.36
Total Kjeldahl Nitrogen (mg/L)	10.2 ± 0.8	10.2 ± 0.8	10.2 ± 0.8
SRT (d)	5.59	2.8	1.4
HRT (h)	11.4	11.4	11.4
COD load (mg/L.d)	2263.54 ± 30	2263.54 ± 30	2263.54 ± 30
MLVSS (g/L)	2.35 ± 0.07	2.09 ± 0.02	1.46 ± 0.06
Volume (L)	3.3	3.3	3.3
FM ratio (g COD/g MLVSS.d)	0.96 ± 0.1	1.08 ± 0.01	1.55 ± 0.02

Table S7: Polysaccharide and protein yields of EPS extracted in experiment 1

Extraction Method	SRT (days)	COD:N	Reactor	PS Yield (mg PS/g VSS)	PN (mg PN/g VSS)
Heat Extracts	6	100	RMUN-1	14.2 ± 0.61	64.3 ± 10.71
	6	100	RIND-1	9.0 ± 0.49	62.5 ± 7.47
	3	100	RMUN-1	202.5 ± 4.12	145.0 ± 7.46
	3	100	RIND-1	41.8 ± 1.53	73.2 ± 2.47
CER Extracts	6	100	RMUN-1	13.75 ± 1.12	78.3 ± 0.05
	6	100	RIND-1	6.2 ± 0.008	80.6 ± 0.64
	3	100	RMUN-1	149.0 ± 3.5	120 ± 1.20
	3	100	RIND-1	27.1 ± 2.18	70.0 ± 10.67

Appendix B

Supplementary Information belonging to Chapter 3

SI-1 Synthetic wastewater composition and preparation

Macro and micronutrients used for the synthetic wastewater preparation are listed below. The chemical oxygen demand to nitrogen (COD:N) ratio was maintained at 100. The organic loading rate (OLR) was set at 2 g L^{-1} d^{-1}. When the OLR was reduced to 1 g L^{-1} d^{-1}, the concentrations of volatile fatty acids (VFAs) in the synthetic wastewater were proportionally decreased by half and needed nitrogen amount (provided by mainly NH_4Cl and 10% w/w from yeast extract) was calculated again considering COD:N ratio was 100.

Synthetic wastewater composition: Synthetic wastewater was prepared using sodium acetate ($C_2H_3O_2Na$), sodium butyrate ($C_4H_7O_2Na$), sodium propionate ($C_3H_5O_2Na$), yeast extract, ammonium chloride (NH_4Cl), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), and calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$). Two feed formulations were used, one for the MBR and one for the CSTR, as detailed below.

- Membrane bioreactor (MBR) feed (mg L^{-1}): $C_2H_3O_2Na$ 770; $C_4H_7O_2Na$ 137; $C_3H_5O_2Na$ 173; yeast extract 10; NH_4Cl 34.1; K_2HPO_4 15; KH_2PO_4 25; $MgCl_2 \cdot 6H_2O$ 200; $CaCl_2 \cdot 2H_2O$ 150. In addition, 2 mL L^{-1} of trace solution was added.
- Continuous-stirred tank reactor (CSTR) feed (mg L^{-1}): $C_2H_3O_2Na$ 4620; $C_4H_7O_2Na$ 830; $C_3H_5O_2Na$ 1040; yeast extract 60; NH_4Cl 206.2; K_2HPO_4 90; KH_2PO_4 150; $MgCl_2 \cdot 6H_2O$ 200; $CaCl_2 \cdot 2H_2O$ 150. In addition, 2 mL L^{-1} of trace solution was added.

Trace solution (stock) composition (mg L^{-1}): The trace solution stock contained $FeCl_3 \cdot 6H_2O$ (1500), H_3BO_3 (150), $CoCl_2 \cdot 6H_2O$ (150), $ZnSO_4 \cdot 7H_2O$ (120), $MnCl_2 \cdot 4H_2O$ (145), $Na_2MoO_4 \cdot 2H_2O$ (60), $CuSO_4 \cdot 5H_2O$ (60), and KI (30).

Table S1: Operational conditions applied to the bioreactors used in experiment 1 (Exp_1) and experiment 2 (Exp_2).

Exp.	Reactor	Inoculum	COD:N	SRT (h)	HRT (h)	V(L)	OLR	Influent COD	
								OLR 1	OLR 2
Exp_1	CSTR	Aerobic activated sludge from WWTP	100	72	72	4	Day 0–25: OLR = 2; day 25–46: OLR = 1	3	6
Exp_1	MBR	Aerobic activated sludge from WWTP	100	72	12	3.3	Day 0–25: OLR = 2; day 25–46: OLR = 1	3	6
Exp_2	CSTR	Aerobic activated sludge from WWTP	100	72	72	4	Day 0–12: OLR = 2; day 12–89: OLR = 1	3	6

Note: CSTR: continuous-stirred tank reactor, MBR: membrane bioreactor, COD: chemical oxygen demand in (g COD L^{-1}), N: total nitrogen, SRT: solid retention time, HRT: hydraulic retention time, OLR: organic loading rate in (g COD L^{-1} d^{-1})

SI-2 Experimental design of MBR and CSTR

In both experiments, the reactors were initially operated in batch mode for 24 h using nitrogen-sufficient feed (carbon-to-nitrogen ratio (COD:N) =20) and subsequently switched to continuous operation and supplied with nitrogen-limited feed (COD:N =100). Parameters were set as presented below.

Table S2: Experimental design parameters for membrane bioreactor (MBR) and continuous-stirred tank reactor (CSTR) operation at organic loading rates (OLRs) of 2 and 1 g COD L^{-1} d^{-1}

	MBR		CSTR	
Parameter	OLR 2	OLR 1	OLR 2	OLR 1
SRT (days)	3	3	3	3
Volume (L)	3.3	3.3	4	4
COD:N ratio	100	100	100	100
HRT (hours)	12	12	72	72
DO (mg L^{-1})	3–4	3–4	3–4	3–4
pH	7.2–7.5	7.2–7.5	7.2–7.5	7.2–7.5
COD inlet (mg L^{-1})	1000	500	6000	3000
Total nitrogen (mg L^{-1})	60	30	60	30
Q influent (L d^{-1})	6.6	6.6	1.33	1.33
Q effluent (L d^{-1})	5.5	5.5	1.33	1.33
Q waste (L d^{-1})	1.1	1.1	–	–
F/M ratio (g COD g $MLVSS^{-1}$ d^{-1})	0.5	0.25	0.5	0.25
MLVSS (g L^{-1})	4	4	4	4
COD load (g COD d^{-1})	6.6	3.3	8	4
OLR (g COD L^{-1} d^{-1})	2	1	2	1

Note: SRT: Solid retention time, HRT: Hydraulic retention time, COD: Chemical oxygen demand N: Total nitrogen, DO: Dissolved oxygen, Q: Flowrate, F/M : Food to Microorganism ratio, MLVSS: Mixed liquor volatile suspended solids, OLR: Organic loading rate

SI-3 VFA concentration detection with ion chromatography

Volatile fatty acids (VFAs) were measured with ion chromatograph (Metrohm 930 Compact IC Flex) equipped with a conductivity detector, a Phenomenex Synergi 4u hydro-RP 80A column and a Metrohm Metrosep Organic Acids Guard/4.6 precolumn. The mobile phase was 0.5 mM sulphuric acid and 1.5% diethyl ether and the suppressor solution was 0.1 M sodium chloride. The flowrate was 1.5 mL min^{-1} for the mobile phase and 0.3 mL min^{-1} for the suppressor solution with an injection volume of 100 μL. Limit of detection (LOD) ranged between 0.75–1.00 mg L^{-1}.

Figures

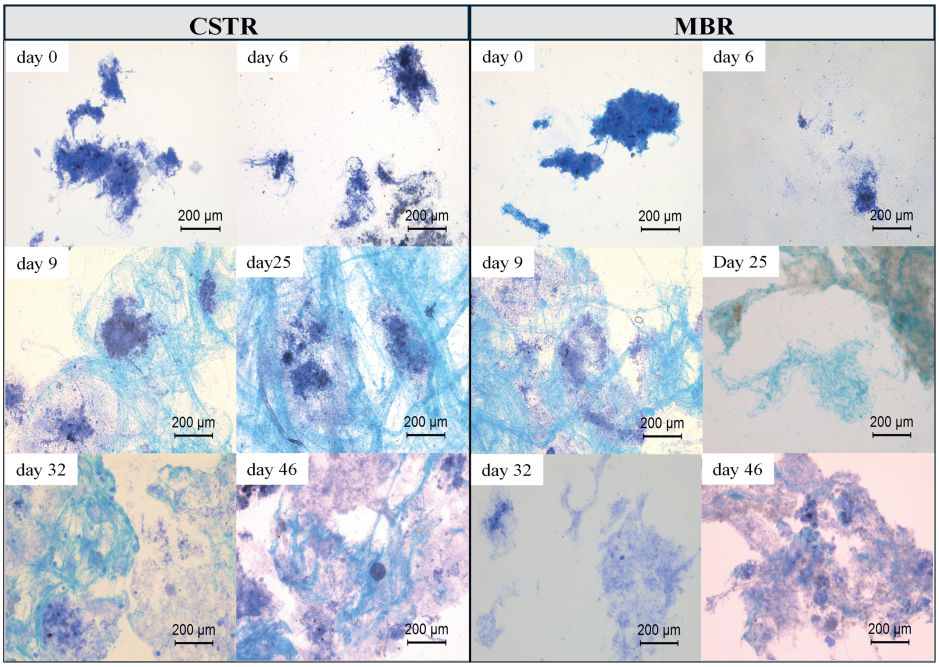
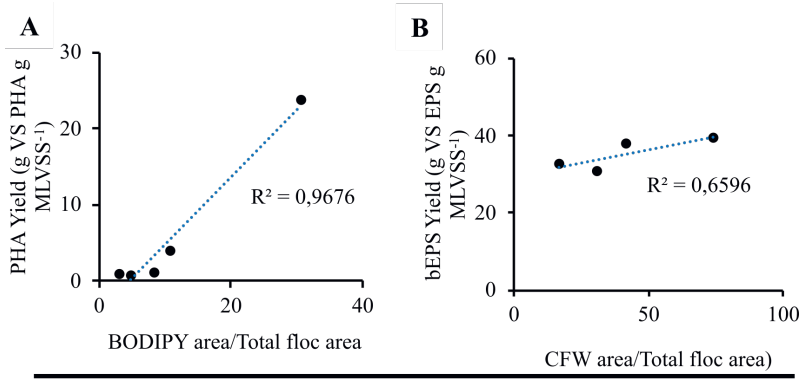


Figure S1: Brightfield microscopy pictures of mixed liquor samples taken during experiment 1 (Exp_1 – from the continuous-stirred tank reactor (CSTR) and membrane bioreactor (MBR)) after combined staining with crystal violet and alcian blue. Alcian blue indicates overproduction of extracellular polymeric substances (EPS).



	BODIPY- Lipids (PHA)		CFW- Carbohydrates (EPS)	
Sample ID	Mean area (%)	Coefficient of variation (%)	Mean area (%)	Coefficient of variation (%)
Inoculum	5.0	11.8	6.3	56.8
OLR 2 D3	30.6	20.2	16.7	15.3
OLR1 D9	10.6	50.2	41.9	24.4
OLR1 D11	8.3	21.1	30.8	9.6
OLR1 D35	2.9	47.3	74.2	32.7
OLR1 D49	4.8	59.0	17.6	15.9
OLR1 D56	2.8	14.2	50.4	52.9

Figure S2: (A) Correlation of polyhydroxyalkanoates (PHA) yield presented in Figure 4B with quantification of area in confocal laser scanning microscopy (CLSM) images presented as BODIPY/Total floc area (B) Correlation of bound extracellular polymeric substances (bEPS) yield with quantification of area in CLSM images presented as Calcofluorwhite (CFW)/Total floc area. BODIPY was used to stain PHAs and CFW was used to stain polysaccharides. (C) Mean area and coefficient of variation were calculated from image analysis of three CLSM images per sample (n=3). Correlation and statistical analyses were conducted using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

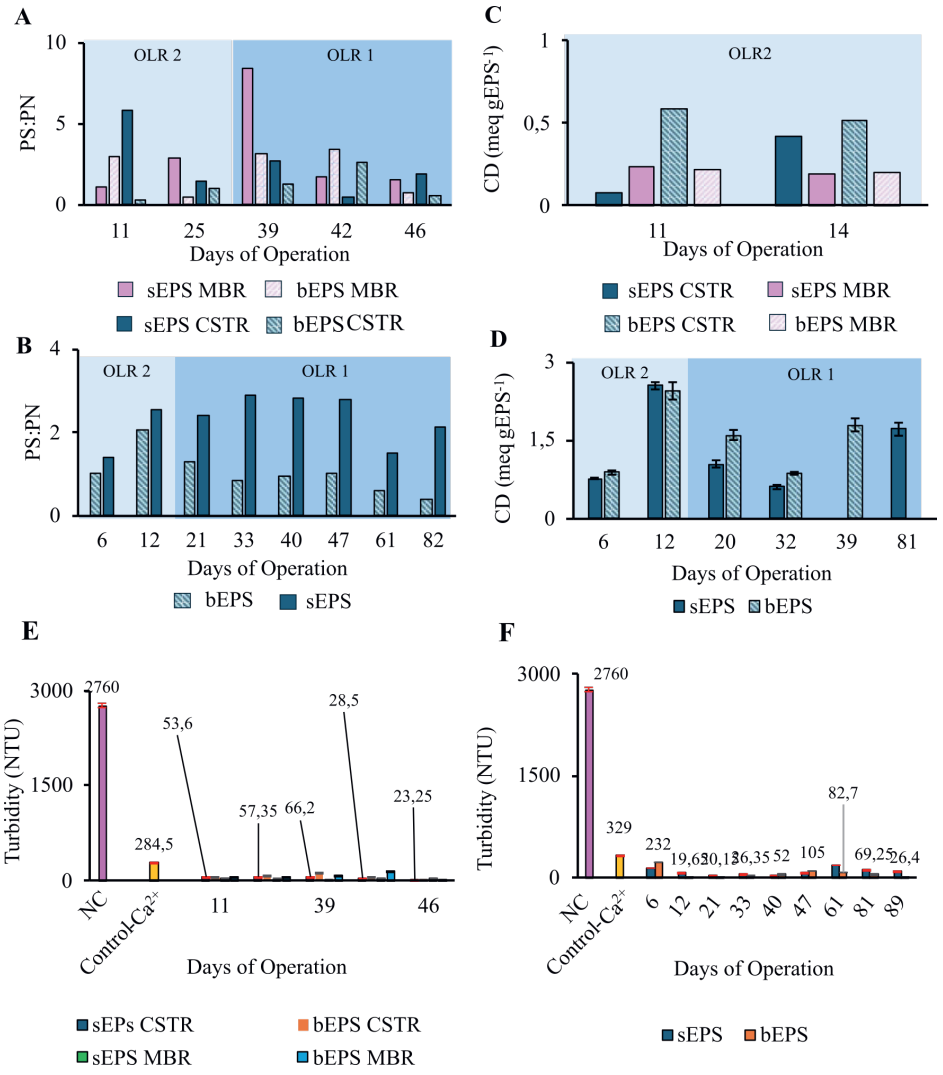


Figure S3: Polysaccharide (PS) to protein (PN) ratio of soluble and bound extracellular polymeric substances (sEPS and bEPS) fractions of EPS samples in (A) experiment 1 (Exp_1) and (B) experiment 2 (Exp_2). Charge density (CD) sEPS and bEPS samples in (C) Exp_1 and (D) Exp_2. Error bars represent the absolute difference between duplicate measurements ($n = 2$). Nephelometric turbidity units measured in flocculation experiments in (E) Exp_1 and (F) Exp_2. Negative control contains only kaolin clay suspension without addition of calcium or EPS while Ca-control contains only calcium in kaolin clay suspension. Other samples presented correspond to the samples shown in Figure 7B and C in the manuscript. Error bars represent the absolute difference between duplicate measurements ($n = 2$, excluding NC; $n = 8$).

Appendix C

Supplementary Information belonging to Chapter 4

SI-1 Glycerol-rich synthetic wastewater composition for Exp_1 and Exp_2

The synthetic wastewater used in Exp_1 and Exp_2 was prepared with glycerol as the main carbon source. The glycerol concentration was kept constant at 820 mg L^{-1} for all experimental periods, while the ammonium chloride concentration was adjusted to obtain the desired COD:N ratios of 20, 60, and 100. Specifically, NH_4Cl was added at 150.7, 43.5, and 18.07 mg L^{-1} for COD:N ratios of 20, 60, and 100, respectively. Yeast extract was added at 100 mg L^{-1} for COD:N 20 and 50 mg L^{-1} for COD:N 60 and 100. The basal medium further contained K_2HPO_4, KH_2PO_4, $MgCl_2 \cdot 6H_2O$, and $CaCl_2 \cdot 2H_2O$ at concentrations of 15, 25, 200, and 150 mg L^{-1}, respectively. A trace-element solution was added at 2 mL L^{-1}.

The trace-element solution contained $FeCl_3 \cdot 6H_2O$, H_3BO_3, $CoCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, and KI at concentrations of 1500, 150, 150, 120, 145, 60, 60, and 30 mg L^{-1}, respectively.

All synthetic wastewater solutions were prepared using distilled water and were used without sterilization. To minimize bacterial growth in the feed tanks, glycerol and the remaining medium components were supplied from separate tanks.

Table S1: Detailed operational parameters of Exp_1 and Exp_2. Total nitrogen concentration was calculated for each COD:N ratio separately while the supplied amount of COD was kept constant. SRTs were controlled by changing sludge wasting flow rate (Q_{waste})

Exp.	R. ID	Inoculum	SRT	Time	COD:N	HRT	DO	pH	COD_{in}	Total N	Q_{in}	Q_{eff}	Q_{waste}	F/M	ML	V
			(day)	(day)		(h)										(L)
Exp_1	R1	Municipal sludge	> 15	0–60	20	12	3–4	7–7.3	1000	50	6.6	6.6	–	0.5	4	3.3
			6	60–75	60					16.6	6.6	6.05	0.55			
			6	75–84	100					10	6.6	6.05	0.55			
			3	84–105	100					10	6.6	5.5	1.1			
Exp_1	R2	Industrial sludge	> 15	0–60	20	12	3–4	7–7.3	1000	50	6.6	6.6	–	0.5	4	3.3
			6	60–75	60					16.6	6.6	6.05	0.55			
			6	75–84	100					10	6.6	6.05	0.55			
			3	84–105	100					10	6.6	5.5	1.1			
Exp_2	R3	Municipal sludge	6	0–12	100	12	3–4	7–7.3	1000	10	6.6	5.5	1.1	0.5	4	3.3
			3	12–34	100						6.6	5.5	1.1			
			1.5	34–42	100						6.6	5.5	1.1			
Exp_2	R4	Industrial sludge	6	0–12	100	12	3–4	7–7.3	1000	10	6.6	5.5	1.1	0.5	4	3.3
			3	12–34	100						6.6	5.5	1.1			
			1.5	34–42	100						6.6	5.5	1.1			

Note: DO: Dissolved oxygen concentration in mg L^{-1}, COD: Chemical oxygen demand in mg COD L^{-1}, Total N: Total Nitrogen in mg L^{-1}, Q : flow rate in L d^{-1}, F:M ratio: Food to microorganism ratio in g COD g $MLVSS^{-1}$ d^{-1}, ML: Mixed liquor volatile suspended solids in g L^{-1}

SI-2 VFA-rich synthetic wastewater composition for Exp_3 and Exp_4

Macro and micronutrients used for the synthetic wastewater preparation are listed below. The chemical oxygen demand to nitrogen (COD:N) ratio was maintained at 100. The organic loading rate (OLR) was set at 2 g L^{-1} d^{-1}. When the OLR was reduced to 1 g L^{-1} d^{-1}, the concentrations of volatile fatty acids (VFAs) in the synthetic wastewater were proportionally decreased by half and needed nitrogen amount (provided by mainly NH_4Cl and 10% w/w from yeast extract) was calculated again considering COD:N ratio was 100.

Synthetic wastewater composition: Synthetic wastewater was prepared using sodium acetate ($C_2H_3O_2Na$), sodium butyrate ($C_4H_7O_2Na$), sodium propionate ($C_3H_5O_2Na$), yeast extract, ammonium chloride (NH_4Cl), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), and calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$). Two feed formulations were used, one for the MBR and one for the CSTR, as detailed below.

- Membrane bioreactor (MBR) feed (mg L^{-1}): $C_2H_3O_2Na$ 770; $C_4H_7O_2Na$ 137; $C_3H_5O_2Na$ 173; yeast extract 10; NH_4Cl 34.1; K_2HPO_4 15; KH_2PO_4 25; $MgCl_2 \cdot 6H_2O$ 200; $CaCl_2 \cdot 2H_2O$ 150. In addition, 2 mL L^{-1} of trace solution was added.
- Continuous-stirred tank reactor (CSTR) feed (mg L^{-1}): $C_2H_3O_2Na$ 4620; $C_4H_7O_2Na$ 830; $C_3H_5O_2Na$ 1040; yeast extract 60; NH_4Cl 206.2; K_2HPO_4 90; KH_2PO_4 150; $MgCl_2 \cdot 6H_2O$ 200; $CaCl_2 \cdot 2H_2O$ 150. In addition, 2 mL L^{-1} of trace solution was added.

Trace solution (stock) composition (mg L^{-1}): The trace solution stock contained $FeCl_3 \cdot 6H_2O$ (1500), H_3BO_3 (150), $CoCl_2 \cdot 6H_2O$ (150), $ZnSO_4 \cdot 7H_2O$ (120), $MnCl_2 \cdot 4H_2O$ (145), $Na_2MoO_4 \cdot 2H_2O$ (60), $CuSO_4 \cdot 5H_2O$ (60), and KI (30).

SI-3 EPS Extraction and PHA quantification

EPS Extraction

For Exp_1 and Exp_2: 40 ml of mixed liquor was diluted with 1x PBS buffer (1:1 v:v) to increase the solubility of EPS while keeping pH of EPS solutions constant during extraction. Subsequently, samples were divided in two aliquots of 20 ml to perform the EPS extraction in duplicate. Using the heat extraction method, the samples were stirred on a magnetic hotplate stirrer for 1 h, at 600 rpm and 50 °C. Then they were centrifuged at 25000xg for 25 min, and the resulting EPS-containing supernatants were collected and dialysed against demineralised water for 24 h using a cellulose membrane with a molecular weight cut-off of 3.5 kDa (SnakeSkin dialysis membrane, Thermo Fisher Scientific, US).

For Exp_3 and Exp_4: EPS were extracted from mixed liquor samples using the alkaline-heat method as described by Felz et al. [1] with minor modifications. A 50 mL sample was centrifuged at 25,000xg for 15 min at 20 °C and the supernatant was collected as the soluble EPS (sEPS). The pellet was then resuspended in 50 mL of Milli-Q water, and 0.29 g of Na_2CO_3 was added. The mixture was heated and stirred at 80 °C, 500 rpm for 1 h. Following this, the sample was centrifuged at 20,000xg for 15 min at 20 °C, and the supernatant was collected as the bound EPS (bEPS). Both EPS fractions were dialysed overnight at room temperature (RT) against deionised water using the same SnakeSkin dialysis tubing. EPS content of the mixed liquor was expressed as g of VS EPS per g MLVSS. Dialysed EPS were freeze-dried and stored at RT for further analyses.

PHA quantification

PHA quantification was carried out using thermogravimetric analysis (TGA) (TGA 2, Mettler Toledo, CH) on freshly collected mixed liquor samples. Prior the analysis, samples were acidified to pH 1.8–2.0 by adding sulfuric acid (96%) and mixed at 800 rpm for 15 min. The samples were then centrifuged at 3,750xg for 15 min, and the resulting pellet was dried overnight at 105 °C. The dried pellet was ground into a fine powder, and 4 mg of the sample was analyzed via TGA a as described by Werker at al. [2] PHA content of the mixed liquor was expressed as g VS PHA per g MLVSS.

Table S2: Detailed operational parameters of Exp_3 and Exp_4

Exp.	R. ID	Type	Inoculum	COD:N	SRT	HRT	Volume	Period	COD_{in} (g COD L-1)	
					(h)	(h)	(L)		OLR 1	OLR 2
Exp_3	R5	MBR	Municipal sludge	100	72	12	3.3	Day 0–25: OLR = 2 Day 25–46: OLR = 1	3	6
	R6	CSTR	Municipal sludge	100	72	72	4	Day 0–25: OLR = 2 Day 25–46: OLR = 1	3	6
Exp_4	R7	CSTR	Municipal sludge	100	72	72	4	Day 0–12: OLR = 2 Day 12–89: OLR = 1	3	6

Figures

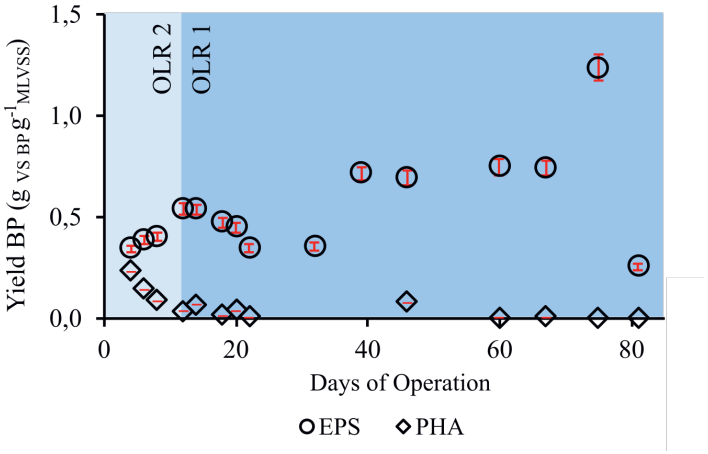


Figure S1: Comparison of EPS and PHA yields in reactor R7 during Exp_4.

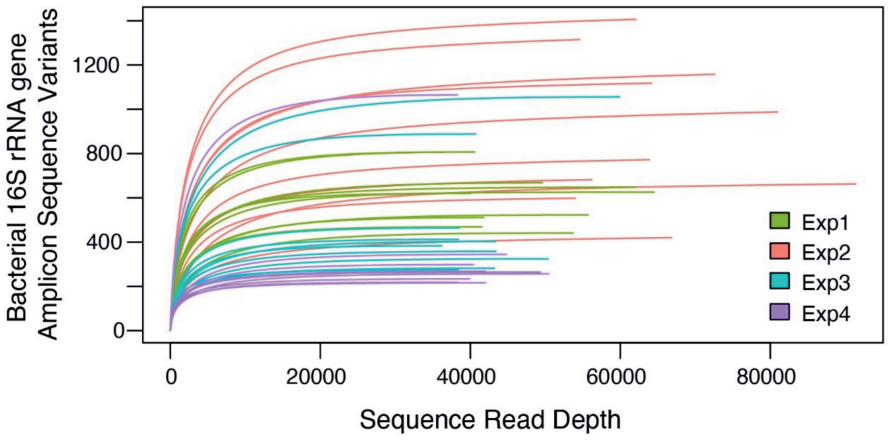


Figure S2: Rarefaction curves

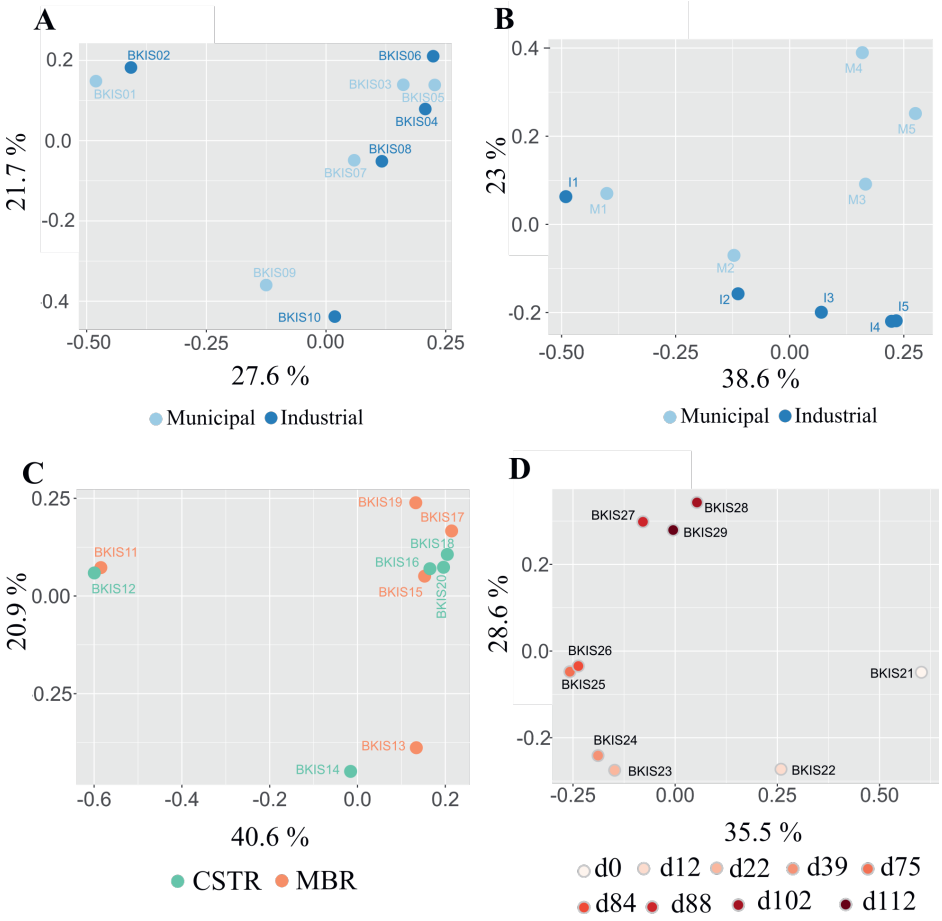


Figure S3: Mixed Microbial Community (MMC) composition dynamics. Principal Coordinates Analysis plots visualise (Bray-Curtis) dissimilarity among microbial communities, as captured by the first two PCoA axis after ordination as dimensionality reduction. More distant samples (symbols) have more dissimilar MMCs, and differences among reactors, conditions and during operation are interpreted as changes in abundance of the dominant bacteria. A) In Exp_1 inocula (SRT 0) of municipal and industrial activated sludge developed into communities following a similar trajectory throughout operation under nitrogen limiting conditions, while maintaining compositional difference. B) In Exp_2, when directly nitrogen limited without acclimation, both EPS producing biomasses developed into divergent communities. C) In Exp_3, MMC comparison between MBR and CSTR reactors showed that the community change during operation of both reactors was predictable and approximate to the observed trajectory of Exp_1 (A), although distinct communities remained by the end of operation.D) Exp_4

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Appendix D

Supplementary Information belonging to Chapter 5

SI-1 Ion Chromatography details

IC-Anions: Anions were quantified using an ion chromatograph (Dionex Aquion IC) equipped with a conductivity detector, an electrolytically regenerated suppressor (Dionex AERS 500 Carbonate, 2 mm, 085028), an online degasser, and an autosampler (Dionex AS-AP). Separation was performed using a Dionex IonPac AS22 RFIC analytical column (2 × 250 mm) with a Dionex IonPac AG22 RFIC guard column (2 × 50 mm). Data acquisition and processing were carried out using Chromeleon 7 software.

The eluent consisted of 4.5 mM sodium carbonate and 5.7 mM sodium bicarbonate prepared by dissolving 954 mg Na_2CO_3 and 235 mg $NaHCO_3$ in 2 L Milli-Q water. Milli-Q water was used as the rinsing solution.

Analyses were conducted at a flow rate of 0.30 mL min^{-1} with a suppressor current of 11 mA, an injection volume of 5 μL, and a total run time of 18 min. The method detection limit was 0.1—0.5 mgL^{-1} . The upper quantification limit was 80 mgL^{-1} for chloride, and 20 mgL^{-1} for the remaining anions.

IC-Cations: Cations were quantified using ion chromatography with Metrohm Compact IC Flex systems (881 and 930) equipped with a conductivity detector, an online degasser, and an autosampler (Spark Optimas). Separation was performed using a Metrohm Metrosep C 4 column (150/4.0 mm) with a Metrohm Metrosep RP 2 Guard/3.6 guard column. Data acquisition and processing were carried out using Metrohm MagIC Net software (version 2.3 and 3.2). The mobile phase consisted of 3 mM nitric acid prepared by adding 470 μL nitric acid and 25 mL acetone to 2.5 L Milli-Q water. A 20% (v/v) isopropanol solution was used as the autosampler rinse solution, prepared by mixing 40 mL isopropanol with 200 mL Milli-Q water. Analyses were conducted at a flow rate of 0.9 $mLmin^{-1}$ with an injection volume of 100 µL and a run time of 18 min. The method detection limit was 0.10 mgL^{-1}. The upper quantification limit was 40 mgL^{-1} for sodium and 20 mgL^{-1} for the remaining cations.

Table S1: Chemical composition of liquid phase of iron-rich sludge

Compound	Liquid phase (mg L-1)
Chloride	49.2
Nitrate	2.2
Phosphate	6
Sulphate	70.2
Calcium	79.1
Magnesium	11
Potassium	6.2
Iron	0.051

SI-2 FTIR band assignments

Table S2: Annotations for detected peaks in FTIR spectrum of EPS samples

Wavenumber (cm^{-1})	Assignments	Reference
3000–3678	O–H stretching (polysaccharides) and N–H stretching (proteins; amide A)	[1], [2]
2880–3000	C–H stretching (CH_2/CH_3) from aliphatic/lipid-like moieties or aliphatic side chains	[3]
2450–2555	Broad O–H of carboxylic acids (–COOH)	
1720–1760	Carbonyl C=O stretching and/or carboxylic acids (–COOH)	[4]
1600–1700	Amide I (C=O stretch) of proteins (secondary structure); may overlap with H_2O bending / aromatic C=C shoulders	[5]
1500–1600	Amide II (N–H bending + C–N stretching) → proteinaceous EPS	[5]
1360–1460	COO^- symmetric stretch (carboxylates in EPS) + (CH_2/CH_3) deformations (alkyl contribution)	[2], [4]
1180–1275)	Amide III (proteins) and/or S=O (sulfate); can include polysaccharide C–O–C/C–O contributions	[6]
950–1150	Polysaccharide region: C–O–C / C–O stretching (glycosidic bonds + ring vibrations)	[4]
800–930	Anomeric / β-glycosidic / ring modes (β-linkage zone; carbohydrate structural bands)	[7]

SI-3 Total surface area and normalisation of EPS amount to surface area

Table S3: Input parameters used to calculate surface area-normalized EPS dosages

Particle	BET SSA $m^2\ g^{-1}$	TSS $g\ L^{-1}$	Jar volume L	EPS dosage mg EPS g^{-1} TSS
Kaolinite	6.99	1	0.3	0.1
Montmorillonite	217.28	1	0.3	0.3
Talc	3.61	1	0.3	0.5
Iron-rich sludge	291.04	1	0.3	0.5, 1, 5

Calculations for a flocculation jar with volume of 0.3 L mineral suspension:

Particle mass per jar:

$$m_{\text{particle}} = \text{TSS} \times \text{Volume} \quad \text{(g)} \tag{Eq.1}$$

Total surface area per jar:

$$A = \text{SSA}_{\text{particle}} \times m_{\text{particle}} \quad (\text{m}^2) \tag{Eq.2}$$

EPS amount per jar:

$$\text{EPS} = \text{EPS dosage (mg g}^{-1}\text{ TSS)} \times \text{TSS} \quad \text{(mg)} \tag{Eq.3}$$

$$\text{Surface area normalised EPS dosage} = \frac{\text{EPS}}{A} \quad (\text{mgEPS m}^{-2}) \tag{Eq.4}$$

SI-4 Charge stoichiometry

Charge stoichiometry was used to estimate the Ca^{2+} dosage required to neutralize the net negative surface charge of the suspended solids. The particle charge demand was determined from the charge density of the mineral suspension (expressed as equivalents of negative charge per mass of TSS) and converted to a theoretical Ca^{2+} requirement assuming electrostatic charge balance (2 equivalents of negative charge neutralized per mole of Ca^{2+}). The calculated charge-neutralization dosage provides a reference point for interpreting coagulation-flocculation behaviour: below this dosage, electrostatic repulsion is expected to dominate and inhibit aggregation, whereas at or above it, charge neutralization and double-layer compression should reduce repulsion and promote polymer adsorption and bridging. Importantly, exceeding the charge-neutralization dosage does not guarantee high flocculation efficiency, because aggregation can remain limited by insufficient ionic screening, particle collision frequency, and polymer conformation/availability.

Table S4: Estimated charge stoichiometry of kaolinite-EPS system assuming 100% sorption efficiency of Ca^{2+} and charge density of kaolinite

Ca^{2+} **g g^{-1} TSS**	**EPS mg g^{-1} TSS**	Ca^{2+} **meq L^{-1}**	**Kaolin^{-} meq L^{-1}**	**EPS^{-} meq L^{-1}**	**Net meq L^{-1}**	**FE %**	**Removal %**
0.02	0.0	0.998	0.142	0.000000	0.856	21	74
0.02	0.1	0.998	0.142	0.000072	0.856	21	82
0.02	0.3	0.998	0.142	0.000216	0.856	21	81
0.02	0.5	0.998	0.142	0.000360	0.856	11	78
0.04	0.0	1.996	0.142	0.000000	1.854	49	78
0.04	0.1	1.996	0.142	0.000072	1.854	52	91
0.04	0.3	1.996	0.142	0.000216	1.854	43	91
0.04	0.5	1.996	0.142	0.000360	1.854	43	89
0.08	0.0	3.992	0.142	0.000000	3.850	62	80
0.08	0.1	3.992	0.142	0.000072	3.850	63	94
0.08	0.3	3.992	0.142	0.000216	3.850	58	94
0.08	0.5	3.992	0.142	0.000360	3.850	58	93
0.16	0.0	7.984	0.142	0.000000	7.842	60	77
0.16	0.1	7.984	0.142	0.000072	7.842	74	91
0.16	0.3	7.984	0.142	0.000216	7.842	75	95
0.16	0.5	7.984	0.142	0.000360	7.842	75	95
0.24	0.0	11.976	0.142	0.000000	11.834	70	75
0.24	0.1	11.976	0.142	0.000072	11.834	81	93
0.24	0.3	11.976	0.142	0.000216	11.834	83	96
0.24	0.5	11.976	0.142	0.000360	11.834	83	96
0.32	0.0	15.968	0.142	0.000000	15.826	60	74
0.32	0.1	15.968	0.142	0.000072	15.826	79	92
0.32	0.3	15.968	0.142	0.000216	15.826	82	96
0.32	0.5	15.968	0.142	0.000360	15.826	82	96
0.40	0.0	19.960	0.142	0.000000	19.818	68	76
0.40	0.1	19.960	0.142	0.000072	19.818	76	93
0.40	0.3	19.960	0.142	0.000216	19.818	82	95
0.40	0.5	19.960	0.142	0.000360	19.818	82	96

Note: Removal refers to turbidity removal. FE: flocculation efficiency.

SI-5 Correlations between EPS properties and flocculation efficiencies

Table S5: Correlations in between flocculation efficiency on kaolinite, montmorillonite, and talc and physicochemical features of EPS (molecular weight(MW) , charge , polysaccharide(PS) content, protein (PN) content and Polysaccharide to protein ratio (PS/PN)). (n=6)

Correlations	**MW-FE**	**Charge -FE**	**PS-FE**	**PN-FE**	**PS/PN -FE**
Kaolinite	0,75	0,68	0,81	0,81	0,61
Montmorillonite	0,38	0,26	0,49	0,59	0,37
Talc	0,41	0,10	0,19	0,65	0,35

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