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Felz, Simon

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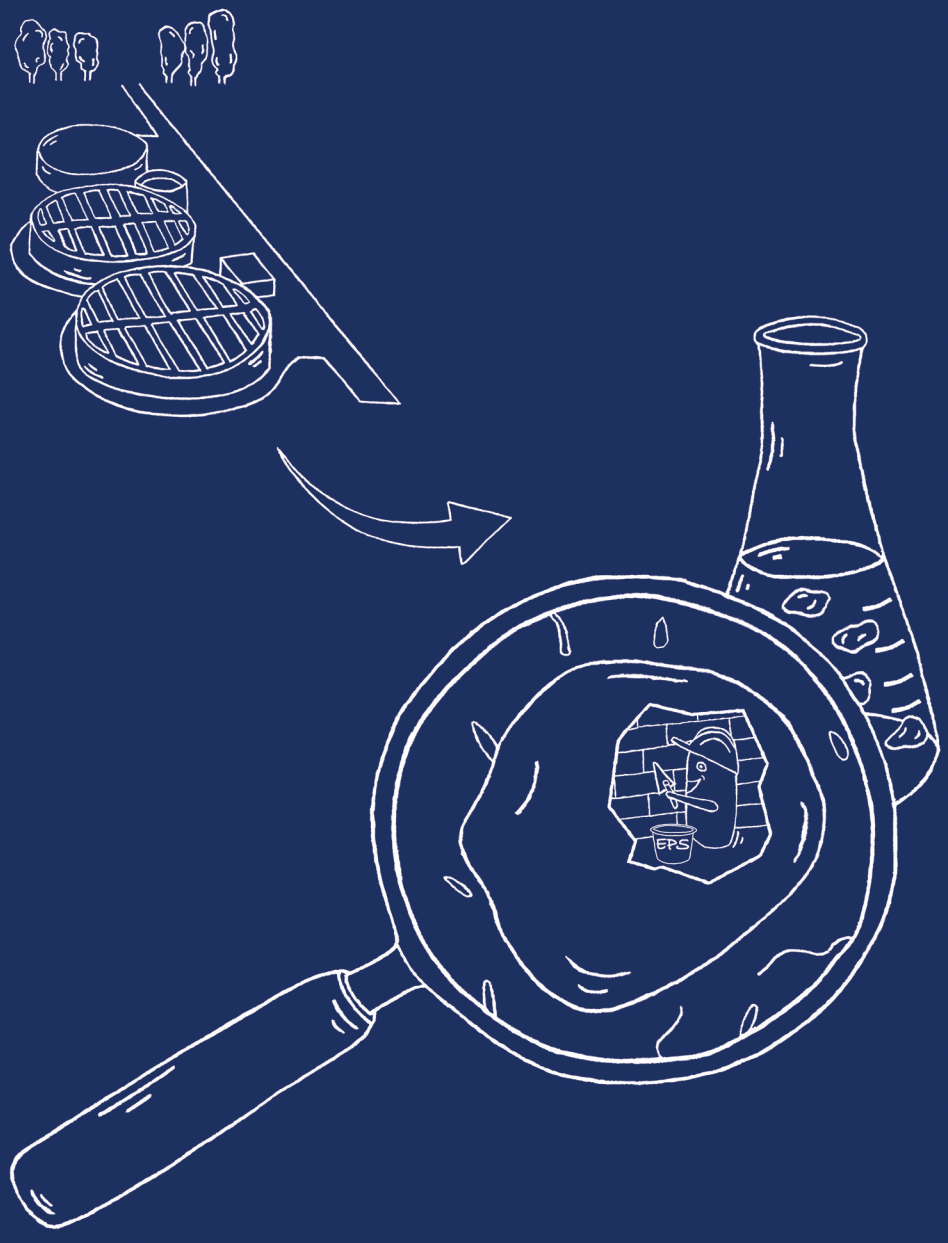

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Structural Extracellular Polymeric Substances from Aerobic Granular Sludge

Proefschrift

ter verkrijging van de graad van doctor

aan de Technische Universiteit Delft,

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voorzitter van het College voor Promoties,

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Simon FELZ

Diplom-Ingenieur Bioingenieurwesen, Karlsruher Institut für Technologie, Duitsland

geboren te Weinheim, Duitsland

Dit proefschrift is goedgekeurd door

promotor: Prof. dr. dr. h.c. ir. M.C.M. van Loosdrecht

copromotor: Dr. Y. Lin

Samenstelling promotiecommissie bestaat uit:

Rector magnificus	voorzitter
Prof. dr. dr. h.c. ir. M.C.M. van Loosdrecht	Technische Universiteit Delft, promotor
Dr. Y. Lin	Technische Universiteit Delft, copromotor

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To my parents

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Summary

Biofilms are pervasive in hydrated environments including wastewater and drinking water systems. A novel promising biological wastewater treatment process offering several advantages towards wastewater treatment with the conventional activated sludge process is the aerobic granular sludge process. Aerobic granular sludge is a special kind of biofilm of spherical shape formed by microorganisms without the addition of carrier material.

Biofilms are microbial aggregates composed of microorganisms and extracellular polymeric substances (EPS). EPS are a complex mixture of proteins, polysaccharides, uronic acids, nucleic acids lipids and humic substances. EPS have multiple important functions within a biofilm. They contribute to the initial aggregation of microbial cells and form a highly hydrated matrix being responsible for the structural integrity of a biofilm. By this EPS also provide protection, can serve as a nutrient source and bind extracellular enzymes. Being a complex mixture of multiple compounds makes EPS analysis challenging and therefore the actual composition and structure of the matrix of biofilms is still largely unknown.

Aerobic granular sludge and part of its EPS, structural EPS, has hydrogel properties. These structural EPS can be extracted from the granules and were shown to be strongly linked to the structural integrity of the sludge. Characterization of the structural EPS will help to understand the stability of granular sludge and in general of biofilms. The focus of this thesis was to analyze the composition of structural EPS from aerobic granular sludge and to analyze its hydrogel characteristics. Additionally challenges and shortcomings concerning EPS extraction and characterization are illustrated and discussed.

Chapter 1 gives a general introduction into biofilms and their EPS, as well as EPS extraction. Issues with current EPS characterization are provided and the outline of this thesis is presented.

In **Chapter 2** the impact of the extraction method on aerobic granular sludge and the obtained EPS was demonstrated with six different EPS extraction methods including mechanical and chemical treatment. Results showed that to obtain structural EPS it is necessary to dissolve the granular matrix. To dissolve the granular matrix harsh extraction methods are required, and there is no ”one fits all” method to dissolve the granular matrix for structural EPS extraction.

Chapter 3 illustrates and discusses shortcomings of current EPS analysis with colorimetric methods for the quantification of proteins, sugars, uronic acids and humic substances. Drawbacks of these colorimetric methods include: a high dependency on the standard compound selection, a lack of suitable standards which feature a similar

composition with the analyzed sample and cross-interference among EPS compounds in the measurements. Results showed that, these methods are not suitable to accurately analyze complex samples. The complexity of structural EPS was illustrated by the overall composition of granular sludge structural EPS: besides a protein fraction, the carbohydrate part itself contained a sugar alcohol, seven neutral sugars, two amino sugars and two uronic acids. Simply depending on colorimetric methods for EPS analysis is not recommended. Novel analytic methods need to be developed and implemented for in depth biofilm EPS analysis.

In **Chapter 4** structural EPS hydrogels formed with metal ions were characterized in terms of gel stiffness and structural homogeneity. Additionally the influence of the metal ion chelating reagent EDTA on the structural integrity of ionic structural EPS hydrogels was investigated. For comparison, alginate, polygalacturonic acid and κ-carrageenan were used as a reference material. The structural EPS hydrogels were less stiff than alginate hydrogels. The structure of lyophilized ionic structural EPS hydrogels was visualized with environmental SEM. Different metal ions had a different impact on the structure of the lyophilized gels. In comparison to alginate, polygalacturonic acid and κ-carrageenan, the integrity of structural EPS hydrogels was less sensitive to EDTA. After one month incubation in an EDTA solution, structural EPS gel beads were still present as a gel while the reference polysaccharide hydrogels failed to keep the gel structure. Apparently structural EPS have a different ionic hydrogel formation mechanism. Multiple functional groups are suggested to be involved in the gel formation of structural EPS.

Chapter 5 focused on the analysis of strongly anionic macromolecules in the EPS of aerobic granular sludge. The presence of glycosaminoglycans was evaluated by SDS-PAGE analysis, hyaluronic acid and sulfated glycosaminoglycan quantification kits for the mammalian extracellular matrix and glycosaminoglycan specific enzymatic digestion. The linking between sulfated glycosaminoglycans and proteins was analyzed by proteolytic enzymatic digestion. Furthermore, Heparin Red staining was used to visualize the distribution of the anionic macromolecules in the granular matrix. Macromolecules similar to Hyaluronic acid and sulfated glycosaminoglycans were discovered in the EPS, hence named Hyaluronic acid-like and sulfated glycosaminoglycans-like compounds. Sulfated glycosaminoglycans-like compounds were bound to proteins. In aerobic granular sludge the strongly anionic molecules were distributed in the microcolonies, at the outer part of the microcolonies and within the extracellular matrix between the colonies. Glycosaminoglycans-like compounds showed to be comparable to those of vertebrates. Structural EPS were therefore much more complicated than expected.

Chapter 6 represents the outlook of this thesis. Results from the previous chapters are summarized and suggestions for future EPS research are given. Suggestions include extraction of EPS, chemical analysis of EPS and general approaches.

Samenvatting

Biofilms zijn veelvoorkomend in waterige omgevingen, inclusief afvalwater- en drinkwatersystemen. Het aeroob korrelslibproces is een nieuwe en veelbelovende afvalwaterzuiveringstechnologie met veel voordelen ten opzichte van conventioneel actiefslib. Aeroob korrelslib is een speciaal soort biofilm met een ronde vorm, dat compleet gevormd wordt door micro-organismen zonder de toevoeging van een dragermateriaal.

Biofilms zijn microbiële clusters, bestaande uit micro-organismen en extracellulare polymerische substanties (EPS). EPS zijn een complex mengsel van eiwitten, suikers, uronzuren, nucleïnezuren, lipides en humuszuren. EPS hebben meerdere belangrijke functies binnen een biofilm. Ze dragen bij aan de initiële clustering van microbiële cellen door de vorming van een sterk gehydrateerde matrix. Hierdoor biedt de EPS zowel bescherming als een bron van nutriënten en binding van extracellulaire enzymen. Vanwege de complexe samenstelling van de grote hoeveelheid aan componenten is de analyse van EPS een uitdaging. Hierdoor is de exacte structuur en samenstelling van de biofilmmatrix nog grotendeels onbekend.

Aeroob korrelslib en een belangrijk deel van zijn EPS, structureel EPS, heeft eigenschappen van een hydrogel. Deze structurele EPS kunnen geëxtraheerd worden van de korrels, en hiervan is aangetoond dat ze sterk gekoppeld zijn aan de fysieke stabiliteit van het slib. Karakterisatie van het structureel EPS zal bijdragen aan de kennis over de stabiliteit van zowel korrelslib als biofilms in het algemeen. De focus van dit proefschrift ligt op de analyse van de samenstelling van structureel EPS van aeroob korrelslib en de bepaling van zijn eigenschappen als hydrogel.

Hoofdstuk 1 geeft een algemene introductie over biofilms, hun EPS en de extractie van dit EPS. Uitdagingen van de huidige EPS karakterisatie en de opzet van dit proefschrift worden beschreven.

In **Hoofdstuk 2** wordt de invloed beschreven van extractiemethode op het verkregen EPS van aeroob korrelslib. Een totaal van zes verschillende extractiemethodes zijn gebruikt, inclusief mechanische en chemische behandeling. Resultaten laten zien dat oplossing van de korrelmatrix essentieel is voor het verkrijgen van structureel EPS. Om dit te bereiken, zijn sterke extractiemethodes nodig, waar geen algemene methode voor is.

Hoofdstuk 3 beschrijft de tekortkomingen van huidige EPS-analyse met colorimetrische methodes voor kwantificatie van eiwitten, suikers, uronzuren en humuszuren. Tekortkomingen van deze methodes zijn een hoge afhankelijkheid van de referentiestof, een tekort aan geschikte referentiestoffen met een representatieve compositie als het geanalyseerde sample, en interferentie van andere EPS-componenten in metingen.

Resultaten laten zien dat deze methodes niet geschikt zijn voor het accuraat beschrijven van complexe samples. De complexiteit van structureel EPS was aangetoond door de algemene samenstelling van korrelslib EPS: naast een eiwitfractie, bevatte de koolhydraatfractie een suikeralcohol, zeven neutrale suikers, twee aminozuren en twee uronzuren. Het enkel gebruiken van colorimetrische methodes voor EPS-analyse wordt niet aangeraden. Nieuwe analytische methodes moeten ontwikkeld en geïmplementeerd worden voor diepgaande analyse van EPS biofilms.

In **Hoofdstuk 4** zijn de stijfheid en structurele homogeniteit gekarakteriseerd van hydrogels van structureel EPS met verschillende metaalionen. Ook is de invloed van de chelerende stof EDTA op de structurele stabiliteit onderzocht. Ter vergelijking zijn alginaat, polygalacturonzuur en κ-carrageenan gebruikt als referentiestoffen. De hydrogels van structureel EPS waren minder stijf dan de hydrogels van alginaat. De structuur van hydrogels van gelyofiliseerde structureel EPS waren gevisualiseerd met environmental SEM. Verschillende metaalionen hadden verschillende invloeden op de structuur van deze gelyofiliseerde hydrogels. Een hydrogel van structureel EPS was minder gevoelig voor EDTA dan hydrogels van alginaat, polygalacturonzuur en κ-carrageenan. Incubatie van een maand in een EDTA-oplossing leidde tot disintegratie van hydrogels van deze referentiestoffen, terwijl de hydrogel van structureel EPS nog steeds aanwezig was als gel. Blijkbaar hebben structureel EPS een ander ionisch vormingsmechanisme. Meerdere functionele groepen zijn gesuggereerd om een rol te spelen in de gelvorming van structureel EPS.

Hoofdstuk 5 focust op de analyse van sterk anionische macromoleculen in de EPS van aeroob korrelslib. De aanwezigheid van glycosaminoglycanen was geëvalueerd door middel van SDS-PAGE-analyse, kwantificatiekits voor hyaluronzuur en gesulfateerde glycosaminoglycanen, en enzymatische afbraak van glycosaminoglycanen. De koppeling tussen gesulfateerde glycosaminoglycanen en eiwitten was geanalyseerd door middel van proteolytische enzymatische afbraak. Verder was Heparin Red kleuring gebruikt voor de visualisatie van anionische macromoleculen in de korrelmatrix. Aan de hand van deze analyses zijn macromoleculen in de EPS ontdekt die gelijkaardig waren aan hyaluronzuur en gesulfateerde glycosaminoglycanen. Deze zijn vervolgens hyaluronzuurachtige en gesulfateerde glycosaminoglycaanachtige stoffen genoemd. Gesulfateerde glycosaminoglycaanachtige stoffen waren gebonden aan eiwitten. In aeroob korrelslib zijn de sterk anionische moleculen verdeeld in de microkolonies, aan de buitenkant van de microkolonies en in de extracellulaire matrix tussen de kolonies. Glycosaminoglycaanachtige stoffen hebben gelijkaardige eigenschappen als in gewervelde organismen. Structureel EPS waren hierom veel gecompliceerder dan verwacht.

Hoofdstuk 6 beschrijft het toekomstbeeld van onderzoek naar aanleiding van dit proefschrift. Resultaten van de voorgaande hoofdstukken zijn samengevat en suggesties voor toekomstig EPS-onderzoek zijn gegeven. Suggesties omvatten verbetering van de extractie van EPS, chemische analyse van EPS en algemene methodes van aanpak.

Chapter 1

Introduction

Biofilms: Application and composition

Biofilms are ubiquitous in natural waters and present in wastewater and drinking water systems. The first biological wastewater treatment processes were biofilm processes in the form of trickling filters. A century ago the activated sludge process based on flocculating bacteria was introduced, and has become the dominant treatment process (Henze et al., 2008). A novel biological treatment processes, the aerobic granular sludge process has gained increasing popularity in the last years (Pronk et al., 2015; Royal HaskoningDHV, 2019). This wastewater treatment process is a kind of marriage between biofilm and activated sludge processes. The aerobic granular sludge process has several advantages over conventional wastewater treatment by the activated sludge process including better effluent quality while at the same time demanding for lower investment costs and energy usage combined with an overall smaller footprint (de Bruin et al., 2004; Pronk et al., 2015). In aerobic granular sludge, different to activated sludge, spherical instead of floccular microbial aggregates are present. This is the result of a feast-famine feeding regime resulting in the selection for slow growing microorganisms which typically prefer to grow in granular form (Beun et al., 1999; de Kreuk and van Loosdrecht, 2004).

Biofilms are composed of microorganisms and surrounding extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). The first time biofilms were observed was in 1683 when Antonie van Leeuwenhoek discovered microorganisms in dental plaque (van Leeuwenhoek, 1684). Later, already in the 40's of last century microbial attachment on surfaces as "bacterial slime" (Heukelekian and Heller, 1940) or with "mucilaginous holdfast" (Zobell, 1943) was detected and shown to be advantageous for the microbial growth thereby initiating biofilm research. Despite the ubiquity of biofilm systems and the long running research into them because of their technical and medical relevance (Hall-Stoodley et al., 2004; Henze et al., 2008), still little is known about the actual composition of the polymeric matrix of the biofilms (Seviour et al., 2019). EPS form a complex matrix composed of multiple compounds such as proteins (including glycoproteins, lipoproteins, amyloids), polysaccharides (including glycolipids, lipopolysaccharides), uronic acids, nucleic acids, lipids and humic substances (Flemming and Wingender, 2010; Seviour et al., 2019). EPS are prerequisite for the formation of a biofilm and account for their slime-like appearance (Flemming, 2011; Peterson et al., 2015). Microbial cells can attach to surfaces or aggregate with each other and via produced EPS form a biofilm (Figure 1). Depending on the environmental conditions biofilms of different microbial communities and properties can occur (Flemming and Wingender, 2010).

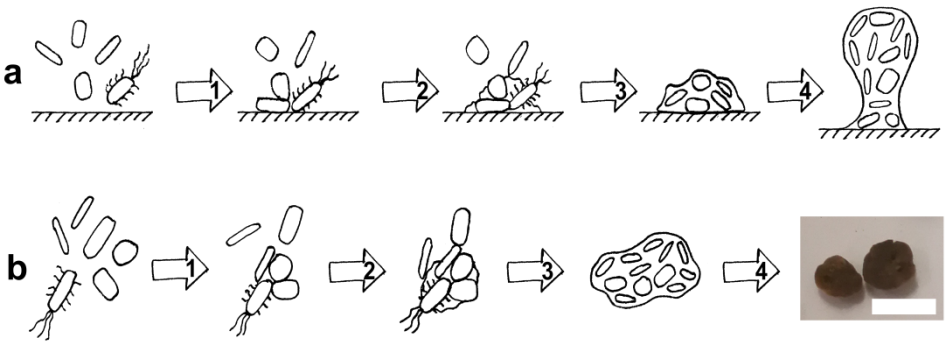

Figure 1: (a) Schematic, simplified formation of a biofilm attached to a surface. (1) Planktonic cells attach to a hydrated surface and (2) start to produce extracellular polymeric substances (EPS). (3) The aggregation and EPS formation continues and a biofilm develops. (4) The biofilm development progresses and a mature biofilm is formed. (b) Schematic, simplified formation of granular sludge (1) Planktonic cells aggregate with each other and (2) start to form extracellular polymeric substances (EPS). (3) The aggregation and EPS production continues and a floccular microbial aggregate is formed. (4) Applying conditions specifically selecting for granular sludge results in the formation of spherical microbial aggregates (scale bar: 5 mm).

EPS interact with each other to form the biofilm matrix through attractive and repulsive forces by covalent bonds, electrostatic interactions, hydrogen bonds and van der Waals interactions (Berg et al., 2002; Flemming and Wingender, 2010). EPS are not only responsible for the initial adhesion and aggregation of cells in the biofilm formation, but have multiple other functions. EPS form a highly hydrated network which provides mechanical stability and determines the structure of a biofilm. This retention of water by anionic polymers also facilitates an increased tolerance towards desiccation and results in the hydrogel characteristics of biofilms. Another protective function of EPS is an elevated resistance towards host defense mechanisms (for pathogens) and antibiotic and toxic substances. Via sorption of organic compounds EPS enable the accumulation of nutrients present in the surrounding environment of the biofilm and EPS themselves can also serve as a nutrient source. Enzymes are another part of the EPS, being bound in the matrix promoting extracellular digestion of macromolecules. In addition EPS can facilitate an exchange of genetic information within in the biofilm (Flemming and Wingender, 2010).

The before mentioned functions of EPS illustrate their importance for biofilms. Aerobic granular sludge is a novel process hence aerobic granular sludge EPS composition is so far only little analyzed. These aggregates are a special form of biofilm with spherical shape, formed by microorganism and secreted EPS without the addition of a carrier material (Beun et al., 1999). Analysis of this very stable biofilm illustrated granular sludge and also part of the extracted EPS to feature hydrogel characteristics (Lin et al., 2010; Seviour et al., 2009a). This fraction of the aerobic granular sludge EPS is regarded

as structural EPS being strongly involved in the structural integrity of the granules and having the ability to form stable hydrogels with calcium ions after extraction (Lin et al., 2010). Further characterizing structural EPS is needed to improve the understanding of aerobic granular sludge stability and that of biofilms in general. To analyze (structural) EPS, applying an extraction method dissolving the EPS matrix of the biofilm is essential.

Extraction of extracellular polymeric substances

The analysis of biofilms and their EPS is very challenging (Seviour et al., 2019). The before mentioned functions and interactions of EPS are not addressed to one type of polymer only, but to several compounds (Flemming and Wingender, 2010; Seviour et al., 2019). Furthermore biofilms which are not formed under sterile conditions by single cultures, but in natural or generally unsterile environments contain multiple microbial species and can accumulate unknown substances from their surroundings (Ali et al., 2019; Schorer and Eisele, 1997). Thus collecting single macromolecules or polymers from the EPS of a biofilm for an accurate follow up characterization is difficult or close to impossible.

The main component of biofilms is water. Aerobic granular sludge and activated sludge are composed of 80 – 90 % of water. While the water content is easy to measure, the quantification of EPS and microbial cells in a biofilm is complicated. Compositions of biofilms with an EPS content ranging from 20 – 90 % dry weight have been reported (Flemming and Wingender, 2010; Frølund et al., 1996). The accuracy of these values is often linked to the applied EPS extraction method as frequently the amount of EPS obtained after one extraction is considered the total EPS. This however can result in inaccurate measurements as it implies a complete EPS extraction. Multiple extraction methods to solubilize and collect EPS from biofilms are reported in literature (Figure 2) (Sheng et al., 2010). Extraction methods based on physical and chemical principles are applied, inducing a first differentiation criterion between reported results, defining the fraction of EPS that is going to be dissolved from the biofilm. The subsequently used method for collecting the EPS is an additional selection criterion determining the finally obtained EPS. Already the reporting of EPS that can be dissolved at acidic (Pronk et al., 2017) or basic pH (Lin et al., 2010) implies that there is no universal applicable extraction method to collect all EPS (Flemming and Wingender, 2010), but that different methods will target different compounds of the biofilm. This illustrates that besides the microbiome of the biofilm also the EPS extraction method has a big influence on the later collected and analyzed EPS.

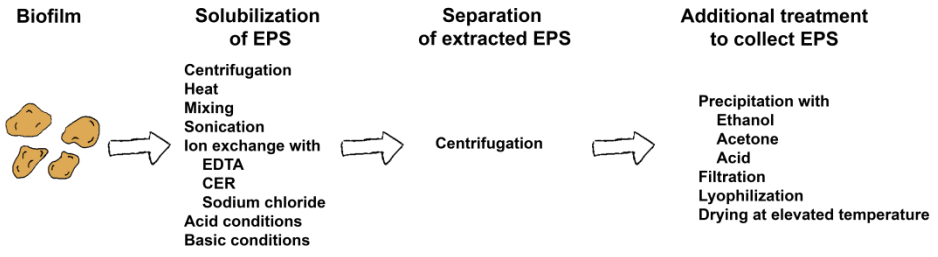

Figure 2: Schematic illustration of extraction methods for EPS from biofilms. Methods to solubilize EPS, to separate EPS from the residual biofilm and to collect solubilized EPS are shown. Abbreviations: EPS: Extracellular polymeric substances. EDTA: Ethylenediaminetetraacetic acid. CER: Cation exchange resin.

Characterization of extracellular polymeric substances

Following the extraction of EPS, EPS are frequently characterized by physical and chemical methods. Very popular methods to characterize EPS are colorimetric methods. By these methods the concentration of a group of compounds (e.g. proteins, sugars, uronic acids, humic substances) is measured indirectly via a color reaction in comparison to a known standard compound (Dubois et al., 1956; Lowry et al., 1951). Colorimetric methods are also used in other research fields than biofilm analysis and became a standard for fast and easy chemical analysis. Unfortunately, as with the extraction methods, also the colorimetric method itself has a big impact on the characterization of the EPS. If the compounds of interest in the sample react differently than the standard compound in the colorimetric assay a discrepancy of the actual value will be obtained (Dubois et al., 1956; Everette et al., 2010; Ras et al., 2008). Additionally, besides the intended compounds, other compounds present in the EPS sample can result in a color reaction, by this interfering with the measurement of the compounds of interest (Le et al., 2016; Le and Stuckey, 2016). Another disadvantage of colorimetric method is that these methods only superficially determine the EPS composition while disregarding the existence of more complex macromolecules such as glycoproteins.

Despite that recently more advanced characterization methods were introduced in EPS research including 3D-EEM, FTIR, GC-MS, HPLC, NMR, Raman spectroscopy (Sheng et al., 2010) and the drawbacks of colorimetric methods, colorimetric methods are still the method of choice. Alternative options to analyze biofilms and their composition is staining in combination with microscopy. Stains specifically targeting proteins, polysaccharides, lipids, nucleic acids or dead and living cells can be applied to visualize an overall distribution of cells and EPS compounds throughout the biofilm matrix (Chen et al., 2007; Mcswain et al., 2005; Neu et al., 2010). This however will give qualitative and structural information and less details on the actual amounts and composition of compounds present in the biofilm matrix.

These shortcomings illustrate the clear demand for new approaches in EPS analysis including a different appreciation of EPS and the implementation of novel, reasonably precise methods to more accurately characterize biofilms and EPS. A more detailed and dedicated characterization of biofilms will allow for a better understanding of biofilms and how they respond to environmental changes. This can help to optimize biofilm processes, avoid unwanted biofilms (Costerton et al., 1999) or utilize EPS as a product (Lin et al., 2015).

Outline of this thesis

This study was aiming to improve the understanding of the matrix composition of aerobic granular sludge and other multispecies biofilms to promote a better comprehension of the strong stability of biofilms. Additionally this analysis was employed to illustrate shortcomings in current biofilm EPS analysis with the examples of EPS extraction and colorimetric EPS characterization. By this encouraging the development and implementation of new analytic methods and approaches to improve future EPS analysis.

To date, there are only very few studies aiming to in depth analyze aerobic granular sludge EPS. In this thesis the focus was on one fraction of the EPS, the structural EPS, which are strongly involved in the structural integrity of aerobic granular sludge. Experiments were performed to identify the composition of structural EPS. Focusing on a smaller fraction of the total EPS will enable a more accurate analysis by limiting the present compounds. This allows for a stepwise characterization of the EPS matrix and the granules. Structural EPS were selected in particular as these polymers can help to understand the granular stability and furthermore also showed to have promising properties after the extraction (Lin et al., 2010, 2015).

The focus of **Chapter 2** is to demonstrate that to obtain EPS responsible for the structural integrity of a biofilm, the biofilm matrix needs to be dissolved. Several EPS **extraction methods** were selected to extract EPS from aerobic granular sludge and the impact of the extraction methods on the biofilm and the collected EPS is illustrated. Centrifugation, sonication, EDTA (ethylenediaminetetraacetic acid), sodium hydroxide and a combination of heat, mixing and alkalinity were investigated.

Chapter 3 gives an impression of the complexity of structural EPS from aerobic granular sludge and demonstrates the problems arising from applying **colorimetric methods** for the characterization of EPS from biofilms. Colorimetric methods frequently used in EPS analysis to quantify proteins, sugars, uronic acids and humic compounds were selected and the influence of standard selection and interfering compounds are illustrated. Structural EPS were analyzed with HPAEC-PAD (high performance anion exchange

chromatography with pulsed amperometric detection) to qualitatively determine the sugar monomer composition and by this indicate the complexity of the structural EPS composition.

In **Chapter 4** differences of **structural EPS and polysaccharide hydrogels**, with the focus on alginate, are visualized and suggestions on the gel formation of structural EPS are given. Hydrogels of structural EPS formed with metal ions were characterized in terms of gel stiffness, structure and stability towards the chelating reagent EDTA. The stiffness of hydrogels formed with alginate and structural EPS with alkaline earth metal, transition metal and zinc ions was quantified with the Young's modulus. Structural patterns of lyophilized structural EPS hydrogels were visualized with ESEM (environmental electron scanning microscopy). The stability towards EDTA of structural EPS hydrogel beads was compared to that of the polysaccharides alginate, polygalacturonic acid and κ–carrageenan.

Chapter 5 focuses on the analysis of strongly anionic macromolecules present in the extracellular matrix of aerobic granular sludge. The presence of anionic polymers such as **glycosaminoglycans** (GAG) was indicated in previous studies on EPS from wastewater sludge. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) with Commassie blue, Alcian blue and periodic acid Schiff staining were used to visualize proteins, acidic polymers and glycans. The presence of GAGs was investigated with quantitative assays targeting hyaluronic acid and sulfated GAGs as well as with glycosaminoglycan specific enzymatic digestion. The linkage of sulfated GAGs to proteins forming a proteoglycan was evaluated by enzymatic digestion of structural EPS. The spatial distribution of anionic compounds in the granular matrix was visualized by Heparin Red staining.

Chapter 6 concludes the findings from this thesis and provides an outlook for future research building up on the results obtained here.

Chapter 2

Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge

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Abstract

To evaluate and develop methodologies for the extraction of gel-forming extracellular polymeric substances (EPS), EPS from aerobic granular sludge (AGS) was extracted using six different methods (centrifugation, sonication, ethylenediaminetetraacetic acid (EDTA), formamide with sodium hydroxide (NaOH), formaldehyde with NaOH and sodium carbonate (Na_2CO_3) with heat and constant mixing). AGS was collected from a pilot wastewater treatment reactor. The ionic gel-forming property of the extracted EPS of the six different extraction methods was tested with calcium ions (Ca^{2+}). From the six extraction methods used, only the Na_2CO_3 extraction could solubilize the hydrogel matrix of AGS. The alginate-like extracellular polymers (ALE) recovered with this method formed ionic gel beads with Ca^{2+}. The Ca^{2+}-ALE beads were stable in EDTA, formamide with NaOH and formaldehyde with NaOH, indicating that ALE are one part of the structural polymers in EPS. It is recommended to use an extraction method that combines physical and chemical treatment to solubilize AGS and extract structural EPS.

1. Introduction

In recent years the aerobic granular sludge (AGS) process has become a popular biological wastewater treatment process, successfully applied at several full-scale wastewater treatment plants (Pronk et al., 2015). In contrast to the conventional activated sludge process, in the AGS process the microorganisms form granules instead of flocs (de Kreuk et al., 2007). These granules have better settleability, are able to withstand higher organic loading rates, and have higher tolerance to toxicity than activated sludge flocs (Adav et al., 2008a).

Unlike biofilms, AGS is formed spontaneously without involvement of any carrier material (Seviour et al., 2009a). In AGS, like in biofilms, microorganisms produce a significant amount of highly hydrated extracellular polymeric substances (EPS) (Flemming and Wingender, 2010) to form a hydrogel matrix in which they are self-immobilized (Flemming and Wingender, 2010; Seviour et al., 2012b, 2009a). EPS are a complex mixture, consisting of polysaccharides, proteins, nucleic acids, (phospho)lipids, humic substances and some intercellular polymers (Flemming et al., 2007; Flemming and Wingender, 2010; Wingender et al., 1999). These polymeric substances interact with each other through electrostatic forces, hydrogen bonds, attractive ionic forces and/or biochemical reactions, etc. (Flemming and Wingender, 2010), forming a dense and compact tertiary network structure. The polymers in EPS which are able to form hydrogels (Lin et al., 2013; Seviour et al., 2009a) and contribute to the formation of the tertiary network structure are in this respect considered as structural EPS, a subset of the total EPS.

EPS are responsible for the chemical structure and physical properties of granules (Flemming and Wingender, 2010). It is therefore crucial to understand the function of each EPS compound. Various approaches are applied to extract EPS (Adav and Lee, 2008; Comte et al., 2007; D'Abzac et al., 2010; Fang and Jia, 1996; Liu and Fang, 2002; Pan et al., 2010). However, due to their extreme complexity, it is almost impossible to extract all the EPS components by one single method. To date, there is no "one size fits all" method for EPS extraction. The choice of the extraction method influences not only the total amount, but also the composition of the recovered polymers (Adav et al., 2008b; Adav and Lee, 2008; Caudan et al., 2012; Comte et al., 2006; Frølund et al., 1996; Nielsen and Jahn, 1999). Depending on the type of sludge and the EPS of interest different methods are required.

Extracting gel-forming polymers, characterizing their properties and investigating their interactions with each other and with non-gel-forming EPS will help to reveal the role of EPS in aerobic granular sludge formation. Furthermore, the gel-forming polymers are also useful biopolymers in industrial applications. One possible application was already

shown by using gel-forming polymers from AGS as a coating material to increase the water resistance of paper (Lin et al., 2015).

Therefore, extraction methods, specific for gel-forming EPS are needed. The aim of this study is to develop a methodology to extract gel-forming EPS from AGS. Six extraction methods (Adav and Lee, 2008; Comte et al., 2007; D'Abzac et al., 2010; Fang and Jia, 1996; Lin et al., 2008; Liu and Fang, 2002; Pan et al., 2010), which are frequently used in literature, were selected to extract EPS from AGS. The total amount and the gel-forming property of the extracted EPS were compared for each methodology.

2. Protocol

Note: AGS was collected from the Nereda® pilot reactor at the wastewater treatment plant Utrecht, the Netherlands. The reactor was fed with municipal sewage. The granular sludge had a sludge volume index (SVI_{5min}) of 59.5 mL/g_{VSS}. The sludge was sampled in April at the end of an aerobic cycle. After sampling, the sludge was immediately transported to the laboratory, sieved and stored at -20°C until use.

2.1 EPS extraction

Note: Centrifuge granular sludge at 4,000×g and 4°C for 20 min, and decant the supernatant. Collect granules in the pellet for the extractions. The total solids (TS) and volatile solids (VS) of the granules were determined by the standard methods (APHA, 1998). The conversion factor between granule wet weight - the weight of the granules taken directly from the pellet - and the TS was determined prior to the extraction. All extractions were done in triplicates.

Note: 3 g wet granules were used for each extraction method. Their TS and VS values (0.39 g TS and 0.34 g VS), measured in triplicates, were used to calculate the extraction yield.

2.1.1 Centrifugation extraction (Liu and Fang, 2002)

2.1.1.1 Transfer 3 g (wet weight) of granules into a centrifugation tube and fill up the centrifugation tube to 50 mL with demineralized water.

2.1.1.2 Slightly shake the centrifugation tube by hand.

2.1.1.3 Centrifuge the centrifugation tube containing the mixture at 4,000×g and 4°C for 20 min.

2.1.1.4 Collect the supernatant in a glass beaker, discard the pellet and continue with the supernatant as described in section 2.1.7.

2.1.2 Sonication extraction (Fang and Jia, 1996)

2.1.2.1 Transfer 3 g (wet weight) of granules into a centrifugation tube and fill up the centrifugation tube to 50 mL with demineralized water.

2.1.2.2 Apply pulsed sonication on ice for 2.5 min at 40 W to the mixture.

2.1.2.3 Centrifuge the centrifugation tube containing the mixture at 4,000×g and 4°C for 20 min.

2.1.2.4 Collect the supernatant in a glass beaker, discard the pellet and continue with the supernatant as described in section 2.1.7.

2.1.3 Ethylenediaminetetraacetic acid (EDTA) extraction (Liu and Fang, 2002)

2.1.3.1 Transfer 3 g (wet weight) of granules into a 100 mL glass bottle and fill up the bottle to 50 mL with 2% (w/v) EDTA solution.

2.1.3.2 Slightly shake the bottle by hand and store it in the refrigerator at 4°C for 3 hours.

2.1.3.3 Transfer the mixture into a 50 mL centrifugation tube.

2.1.3.4 Centrifuge the centrifugation tube containing the mixture at 4,000×g and 4°C for 20 min.

2.1.3.5 Collect the supernatant in a glass beaker, discard the pellet and continue with the supernatant as described in section 2.1.7.

2.1.4 Formamide – sodium hydroxide extraction (NaOH) (Adav and Lee, 2008)

2.1.4.1 Transfer 3 g (wet weight) of granules into a 100 mL glass bottle and fill up the bottle to 50 mL with demineralized water.

2.1.4.2 Add 0.3 mL 99% formamide.

2.1.4.3 Slightly shake the bottle by hand and store it in the refrigerator at 4°C for 1 hour.

2.1.4.4 Add 20 mL 1 M NaOH to the granule suspension.

2.1.4.5 Slightly shake the bottle by hand and store it in the refrigerator at 4°C for 3 hours.

2.1.4.6 Transfer the mixture evenly into two 50 mL centrifugation tube.

2.1.4.7 Centrifuge the centrifugation tubes containing the mixture at 4,000×g and 4°C for 20 min.

2.1.4.8 Collect the supernatant in a glass beaker, discard the pellet and continue with the supernatant as described in section 2.1.7.

2.1.5 Formaldehyde – NaOH extraction (Liu and Fang, 2002)

2.1.5.1 Transfer 3 g (wet weight) of granules into a 100 mL glass bottle and fill up the bottle to 50 mL with demineralized water.

2.1.5.2 Add 0.3 mL 37% formaldehyde.

2.1.5.3 Slightly shake the bottle by hand and store it in the refrigerator at 4°C for 1 hour.

2.1.5.4 Add 20 mL 1 M NaOH to the granule suspension.

2.1.5.5 Slightly shake the bottle by hand and store it in the refrigerator at 4°C for 3 hours.

2.1.5.6 Transfer the mixture evenly into two 50 mL centrifugation tube.

2.1.5.7 Centrifuge the centrifugation tubes containing the mixture at 4,000×g and 4°C for 20 min.

2.1.5.8 Collect the supernatant in a glass beaker, discard the pellet and continue with the supernatant as described in section 2.1.7.

2.1.6 High temperature – sodium carbonate extraction (Na_2CO_3) (Lin et al., 2013, 2008; McHugh, 2003)

2.1.6.1 Pre-heat 150 mL tap water in a 1000 mL glass beaker on a magnetic stirrer to 80°C.

2.1.6.2 Transfer 3 g (wet weight) of granules in a 250 mL baffled flask and fill up the flask to 50 mL with demineralized water.

2.1.6.3 Add 0.25 g Na_2CO_3 anhydrous or 0.67 g $Na_2CO_3 \bullet 10H_2O$ into the flask to obtain a 0.5 % (w/v) Na_2CO_3 concentration.

2.1.6.4 Put the flask containing the mixture into the water bath. Cover the flask and the beaker glass separately with aluminum foil to prevent evaporation.

2.1.6.5 Stir the mixture for 35 min at 400 rpm and 80°C.

2.1.6.6 Transfer the mixture into a 50 mL centrifugation tube.

2.1.6.7 Centrifuge the centrifugation tube containing the mixture at 4,000×g and 4°C for 20 min.

2.1.6.8 Collect the supernatant and discard the pellet.

2.1.7 TS and VS measurement of all extracts according to the standard methods (APHA, 1998)

2.1.7.1 Take the supernatant and dialyze it for 24 hours against 1000 mL ultrapure water (dialysis bag with 3.5 kDa molecular weight cut off (MWCO)) (Comte et al., 2007; Liu and Fang, 2002). Change the dialysis water after 12 hours to enhance the effect of the dialysis.

2.1.7.2 Transfer a reasonable fraction (around 1/3) of the dialyzed supernatant to an aluminum dish for TS and VS measurement (APHA, 1998).

Note: Dry the sample overnight at 105°C. The weight difference of the empty aluminum dish and the aluminum dish containing the dried sample is the TS content. Then burn the same aluminum dish containing the sample at 550°C for 2 hours. The weight difference between the empty aluminum dish and the aluminum dish containing the burned sample is the ash content. The difference between TS and ash content is the VS content.

2.1.7.3 For each extract, transfer the residual fraction of the dialyzed supernatant to 10 mL glass beakers. Thicken the supernatant for 2 days at 60°C to a final volume of 1-2 mL to increase the polymer concentration in the supernatant.

2.2 Alginate-like extracellular polymer (ALE) extraction

2.2.1 Dialyze the extract obtained in step 2.1.6.8 according to step 2.1.7.1.

2.2.2 Transfer the dialyzed extract into a 250 mL glass beaker. Slowly stir the extraction at 100 rpm and room temperature. Constantly monitor pH changes with a pH electrode, while adding 1 M hydrochloric acid (HCl) to a final pH of 2.2 ± 0.05 to obtain ALE in the acidic form.

2.2.3 After adjusting the pH to 2.2, transfer the extract into a 50 mL centrifugation tube and centrifuge at 4,000×g and 4°C for 20 min.

2.2.4 Discard the supernatant and collect the gel-like pellet. The gel-like pellet is ALE in the acidic form.

2.2.5 To obtain the sodium (or potassium) form of ALE, slowly add 0.5 M NaOH (or 0.5 M potassium hydroxide) to the gel obtained in step 2.2.4, while mixing the gel slowly with a glass stick by hand until pH 8.5 is reached.

2.3 Ionic hydrogel formation test

Note: In order to check if the extracted EPS had ionic hydrogel formation properties, a bead formation test with Ca^{2+} ions was used (Lin et al., 2010).

2.3.1 After thickening of the extract in step 2.1.7.3 to a volume of 1-2 mL, slowly stir the mixture with a glass stick and adjust its pH to 8.5 with 0.5 M NaOH.

2.3.2 Take the extract of step 2.3.1 or the sodium ALE of step 2.2.5 and slowly drip the extract with a Pasteur pipette into a 2.5% (w/v) calcium chloride ($CaCl_2$) – solution.

Note: If the extracted EPS has ionic hydrogel gel forming properties, drop-shaped (spherical) beads will be formed. If the extracted EPS has no ionic hydrogel gel forming properties, the extract will disperse in the $CaCl_2$ – solution.

2.4. Stability test of the ionic hydrogel

Note: To further understand the role of the ionic EPS hydrogel in AGS structure formation, stability tests were performed on the ionic hydrogel beads of the Na_2CO_3 extraction, collected in step 2.3.2.

2.4.1 Keep the hydrogel beads for 30 min in the $CaCl_2$ solution.

2.4.2 Use a spoon to take out the hydrogel beads from the $CaCl_2$ solution and split the beads in four equal fractions.

2.4.3 Store fraction 1 in 10 mL demineralized water for 4 hours at 4°C.

The following stability tests were performed in the same manner as described in the extraction methods 2.1.3 – 2.1.5.

2.4.4 Store fraction 2 in 10 mL 2% (w/v) EDTA solution for 3 hours at 4°C.

2.4.5 Store fraction 3 in 7.15 mL demineralized water with 60 µL 99% formamide for 1 hour at 4°C. Then add 2.85 mL 1 M NaOH and store fraction 3 for 3 hours at 4°C.

2.4.6 Store fraction 4 in 7.15 mL demineralized water with 60 µL 37% formaldehyde for 1 hour at 4°C. Then add 2.85 mL 1 M NaOH and store fraction 4 for 3 hours at 4°C.

2.4.7 Monitor if there is visible disintegration of the beads during the storage under the conditions described in 2.4.3 – 2.4.6 to evaluate if the beads withstand the extraction conditions.

3. Results

3.1 EPS extraction

The appearance of granules after applying different EPS extraction procedures is shown in Figure 1. The shape and gel structure of granules were intact after centrifugation (Figure 1a) and EDTA extraction (Figure 1c). Granules were broken into fragments of different sizes by sonication. The turbidity in the liquid phase could be due to suspension of small fragments (Figure 1b) as the turbidity highly decreased after centrifugation. Formamide and formaldehyde alone did not have any impact on changing the shape of the granule and its gel structure (data not shown). After the addition of NaOH, the liquid phase turned yellowish. Some fluffy material was detached from the surface of the granules and formed a layer on top of the settled granules (Figure 1d and 1e). Still, the shape of the granules was not changed. The addition of NaOH apparently improved EPS solubilization, but could not damage the gel matrix structure. In comparison, granules completely disappeared after Na_2CO_3 extraction (Figure 1f). Instead a mixture of sol-like liquid and tiny jelly-like particles were formed, showing the gel matrix of granules was indeed solubilized.

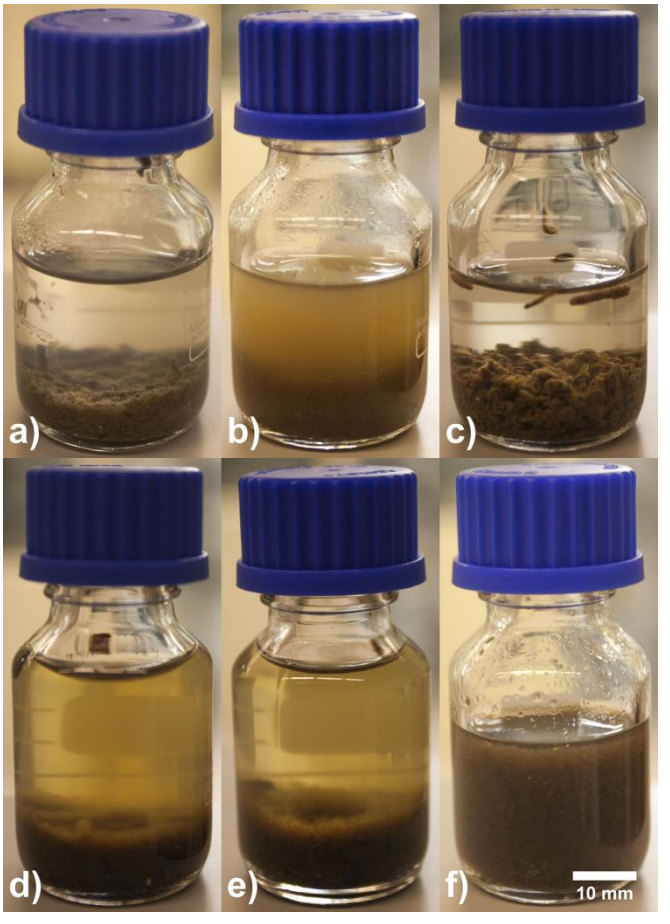


Figure 1: Aerobic granular sludge EPS extractions. For a better visualization of the impact of each extraction method on the granules, experiments were conducted in 25 mL glass bottles. After the extraction procedure, the extracts were kept for 1 hour at room temperature to allow suspended matter to settle. (a) centrifugation extraction, (b) Sonication extraction, (c) EDTA extraction, (d) Formamide – NaOH extraction, (e) Formaldehyde – NaOH extraction, (f) High temperature – Na_2CO_3 extraction.

EPS yield with respect to the VS fraction for each method is illustrated in Figure 2. The yield is presented in mg VS_{EPS} per g initial $VS_{granule}$. The amount of EPS obtained by formaldehyde + NaOH, formamide + NaOH and Na_2CO_3 + heat + mixing was higher than that of centrifugation, sonication and EDTA extraction. Similar results for these extraction techniques were also shown by previous studies (Adav and Lee, 2008; Comte et al., 2007; D'Abzac et al., 2010; Liu and Fang, 2002) indicating that alkaline conditions enhance EPS solubility (Ruiz-Hernando et al., 2015; Zorel et al., 2015). The amount of EPS recovered by Na_2CO_3 was the highest, more than 20 times of that obtained only by centrifugation. Additionally, the total EPS yield of the Na_2CO_3 extraction can be further

enhanced by multiple extractions. A second extraction using the pellet discarded in step 2.1.6.8 (protocol section) of the first extraction increased the total yield by 28 %, a quadruple extraction even increased the total yield by 46 %. The influence of the extraction parameters pre-treatment, mixing and temperature on the obtained EPS extract were investigated in terms of yield and are illustrated in Supplementary material A.

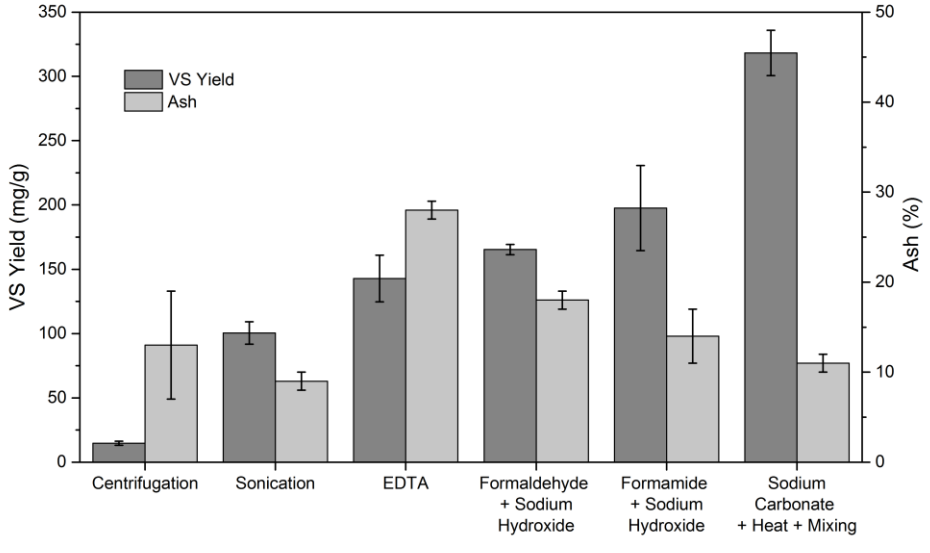


Figure 2: Results of all extraction methods with respect to VS yield and ash content. For each extraction the first bar represents the VS yield in mg VS_{EPS} per g initial $VS_{granule}$. The second bar represents the weight percentage of ash in the extracted TS. The error bars illustrate the standard deviation of the three extractions performed for each extraction technique.

3.2 Alginate-like extracellular polymer (ALE) extraction

After the pH of the EPS extracted by the Na_2CO_3 extraction was adjusted to 2.2, 63 % of the total VS was precipitated. The precipitate is acidic ALE (Lin et al., 2010). The residual fraction was likely EPS which can be solubilized under the extraction conditions, but cannot form a precipitate at pH 2.2.

3.3 Ionic hydrogel formation test

Aerobic granules have been described as being similar to a hydrogel. The granulation process has been regarded as a gel-forming phenomenon with glycosides as the gelling agent (Lin et al., 2010, 2013, Seviour et al., 2009a, 2009b). Normally, Ca^{2+} is one of the most common cations in wastewater. In addition, it easily binds with acidic polysaccharides (e.g. alginates and poly-galacturonic acid), presumably as a counter-ion

to mediate gelation (de Kerchove and Elimelech, 2007). Thus resulting in an ionically cross-linked hydrogel. It was observed that the addition of Ca^{2+} ions can accelerate aerobic sludge granulation (Jiang et al., 2003). Therefore, Ca^{2+}-EPS (ionic hydrogel) could play an important role in building up the gel matrix structure in aerobic granular sludge. In this respect, whether the extracted EPS forms an ionic hydrogel with Ca^{2+}-ions could be used as a test to check if the extracted EPS is a structural polymer contributing to the formation of the gel matrix in aerobic granular sludge (Lin et al., 2013).

In this research, for the EPS extracted from AGS (Figure 3a) by various methods, only the EPS extracted by Na_2CO_3 held the shape of a droplet in 2.5 % (w/v) $CaCl_2$ solution and formed stable ionic hydrogel beads. Moreover, the sodium ALE obtained from this EPS by additional steps (ALE polymer extraction, Figure 3b) displayed the same property as well. The color and morphology of the Ca^{2+}-ALE gel beads (Figure 3c) are similar to aerobic granular sludge (Figure 3a). Apparently, the EPS extracted by the Na_2CO_3 method contributes to the formation of the gel matrix in aerobic granular sludge. ALE, which is a main component of this EPS are structural polymers, able to form an ionic hydrogel.

3.4 Stability test of the ionic hydrogel

It was observed that during EPS extraction, aerobic granules kept their spherical shape in EDTA, formaldehyde + NaOH and formamide + NaOH (Figure 1). In order to understand if the extracted structural polymers play a role in the stability of the granules, Ca^{2+}-ALE beads were treated exactly the same way as aerobic granules during the extraction. Interestingly, Ca^{2+}-ALE beads displayed the similar stabilities as that of AGS (Figure 3d - 3f), i.e. Ca^{2+}-ALE beads were extremely stable in EDTA. There was little amount of ALE detached from the surface of Ca^{2+}-ALE beads (tiny brownish floc in Figure 3e and 3f), when the Ca^{2+}-ALE beads had been soaked in formaldehyde + NaOH and formamide + NaOH for three hours, respectively. This similarity in terms of stability between Ca^{2+}-ALE beads and aerobic granules indicates that ALE are one part of the important structural polymers forming the AGS gel matrix.

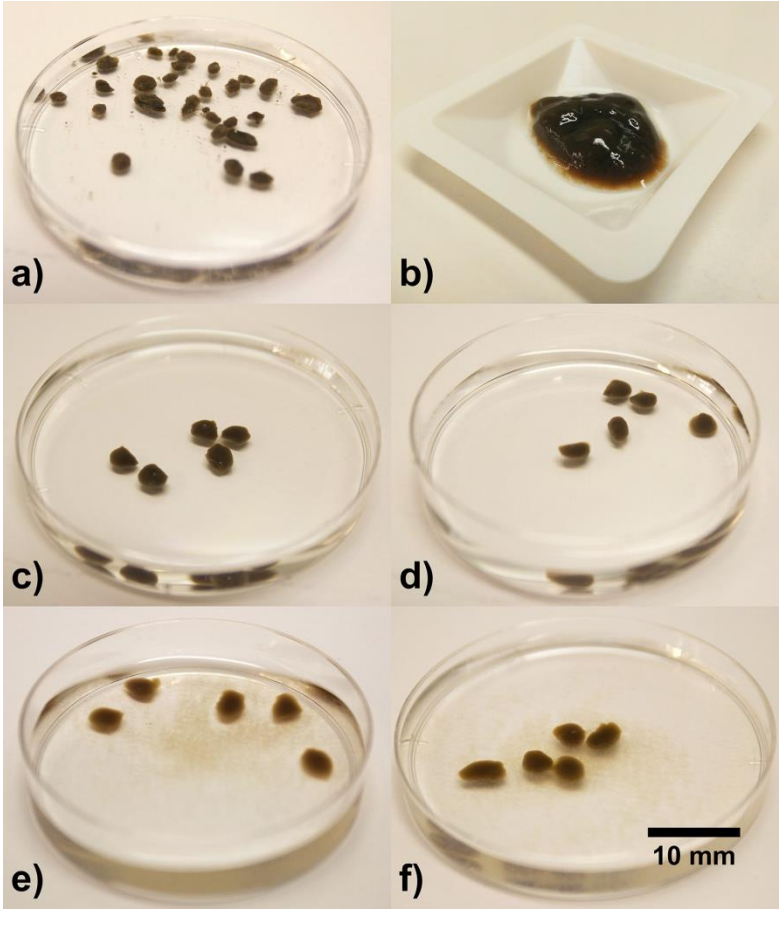


Figure 3: Aerobic granules and extracted ALE. (a) Granules in demineralized water prior extraction, (b) acidic ALE (extracted according to the paragraphs 2.1.6 and 2.2) after centrifugation at 4,000×g and 4°C for 20 min. Results of the stability test of the ionic hydrogel. (c) Ca^{2+}-ALE-beads stored in demineralized water for 4 hours at 4°C, (d) Ca^{2+}-ALE-beads stored in 2 % EDTA, for 3 hours at 4°C (e) Ca^{2+}-ALE-beads stored in formamide + NaOH for 4 hours at 4°C, (f) Ca^{2+}-ALE-beads stored in formaldehyde + NaOH for 4 hours at 4°C.

4. Discussion

4.1 Remarks for the protocol section

The extraction of EPS/ALE is described for a volume of 50 mL and 3 g of granules. These values are intended as guidelines. Extractions with higher granule concentrations can decrease the yield of the extracted EPS. During the extraction of ALE the temperature should be kept constant at 80°C for 30 min. The time required for the mixture to heat up (around 5 min) is included in the protocol. Furthermore, the extraction efficacy is

enhanced by using a magnetic stir bar of the same size as the diameter of the flask bottom. This will result in good mixing properties and milling effects, promoting the extraction of EPS.

Later in the protocol section, TS and VS yields of all extractions (supernatant collected in steps 2.1.1-2.1.6) are determined. Dialysis needs to be performed prior to TS and VS measurement to decrease possible errors owing to the presence of chemicals used for extractions. A MWCO of 3.5 kDa is recommended to remove these chemicals while retaining the EPS macromolecules within the dialysis bag. The dialysis bag should have a larger volume than the volume of the extract. This is necessary, because the volume of the extract will increase during the dialysis (e.g. for EDTA extraction up to 40% volume increase). The extent of chemical removal by dialysis can be determined by measuring the pH in the sample prior and after dialysis. Alternatively, conductivity measurements of the dialysis water show the extent of ion removal.

To obtain ALE from the total extracted EPS (steps 2.1.6 and 2.2) the dialysis step is optional. Nevertheless, dialysis has three benefits: it reduces the amount of HCl needed for the precipitation, it improves the acid mass transfer in the extract and decreases the ash content of the obtained ALE. For the precipitation of ALE it is recommended to use a glass beaker with a much larger volume than the extract. Na_2CO_3 is normally overdosed in the extraction. The added HCl will first react with the Na_2CO_3 left in the extract, resulting in carbon dioxide formation and, if the sample was not dialyzed before, in foaming. During the addition of HCl, the extract should be stirred slowly with a magnetic stir bar of the same size as the bottom of the beaker. A stir bar of this size and slow stirring will result in even mixing without breaking the structure of the precipitate. If acidic gel clumps are formed in the extract, the beaker should be swirled slightly by hand. The precipitation is conducted with an acid concentration of 1 M to avoid a large volume increase of the extract while still obtaining a homogeneous distribution of the acid in the sample. Higher acid concentrations can result in a regional pH decrease and acidic gel clumps formation. A pH lower than 2.0 decreases the amount of ALE that can be recovered, probably due to structural changes of the polymers at lower pH. It is therefore important to keep the final pH at 2.20 ± 0.05.

4.2 Limitations

The ALE extraction method aims to extract structural extracellular polymers of the EPS from AGS or biofilms in general and is not intended to extract all present EPS. To extract all EPS, a combination of more than one extraction method is necessary. Moreover, as shown with the increase of the VS_{EPS} yield by applying a double and quadruple extraction, one single extraction will not extract all structural EPS. ALE extraction is a harsh EPS extraction method, combining constant mixing with heat and alkaline

conditions. For this reason it is possible that some intracellular material is extracted together with the EPS. Although cell lysis can be caused by physical and chemical extraction techniques (sonication (Guo et al., 2014; Liang et al., 2010), NaOH (Guo et al., 2014; Liang et al., 2010), EDTA (Guo et al., 2014; Liu and Fang, 2002), CER (Guo et al., 2014), heat (Guo et al., 2014) and high shear rates by mixing (Frølund et al., 1996)), the presence of intracellular material in recovered EPS still needs to be verified. The ionic gel-forming property of the extracted EPS is the main focus of this research, whether the recovered EPS contains intracellular material was not analyzed. Future research will focus on identifying intracellular material in the extracted EPS.

4.3 Solubilizing the hydrogel matrix of AGS is crucial to extract structural EPS

EPS forms a dense and compact hydrogel matrix in AGS. Although EPS contains various classes of organic macromolecules such as polysaccharides, proteins, nucleic acids, (phospho)lipids, humic substances and some intercellular polymers (Flemming et al., 2007; Flemming and Wingender, 2010; Wingender et al., 1999), not all of them form a gel. Only those gel-forming polymers are here considered as structural polymers in EPS.

The aim of EPS extractions is to first solubilize EPS and then to collect the solubilized EPS. If the structural EPS (i.e. the EPS forming a hydrogel) is the target of the extraction, the gel matrix of AGS has to be solubilized first. Only methods that can solubilize the gel matrix are capable of extracting structural EPS. In this research, some frequently used EPS extraction methods such as centrifugation (Adav and Lee, 2008; Comte et al., 2007; D'Abzac et al., 2010; Fang and Jia, 1996; Liu and Fang, 2002; Pan et al., 2010), sonication (D'Abzac et al., 2010; Fang and Jia, 1996; Pan et al., 2010), EDTA (Comte et al., 2007; D'Abzac et al., 2010; Fang and Jia, 1996; Liu and Fang, 2002; Pan et al., 2010), formaldehyde + NaOH (Adav and Lee, 2008; Comte et al., 2007; D'Abzac et al., 2010; Fang and Jia, 1996; Liu and Fang, 2002; Pan et al., 2010) and formamide + NaOH (Adav and Lee, 2008) could not efficiently isolate the structural EPS. This is due to the fact that the hydrogel matrix of the aerobic granules was not solubilized by these methods. For this reason, stability tests in section 2.4 were only performed with conditions present in EDTA, formamide + NaOH and formaldehyde + NaOH extraction. These three extractions were not capable of isolating structural EPS, but still obtained the highest VS_{EPS} yield besides the Na_2CO_3 extraction. Conditions of the Na_2CO_3 extraction were not applied as this extraction method clearly solubilized the AGS matrix. Hence the applied conditions during the stability test were considered representative.

Extraction with cation exchange resin (CER), another frequently used EPS extraction method, was not considered for this comparison, as previous studies on EPS extraction with CER did not yield better results than the chemical extractions used here.

4.4 Gel-forming EPS in AGS

Gel-forming EPS are considered as the structural EPS in the hydrogel matrix of AGS. It is worth pointing out that there are various kinds of hydrogels such as ionic gels, temperature-induced gels and pH induced gels. This study only focuses on EPS that form ionic gels. Regarding the large fraction of structural gel material extracted, this is likely to be the dominant structural EPS. There are certainly possibilities that other kinds of EPS that form different kinds of hydrogels (e.g. pH induced gel (Seviour et al., 2009b)) exist in the same or other type of aerobic granules. Nevertheless, no matter what kind of hydrogel is targeted, solubilizing the EPS gel matrix is the most important step to extract gel-forming EPS.

Currently, little research has been done on structural EPS of granular sludge. The ALE extraction described in this protocol is capable of extracting gel-forming EPS from AGS and will be used in future studies to characterize structural EPS. More research needs to be done on AGS, structural EPS and non-structural EPS to better understand the process and function of granulation and EPS. Especially the following three points need to be investigated: why microorganisms produce such a large amount of EPS, what is the exact composition of EPS and how is the composition of EPS modified depending on environmental changes. Detecting and analyzing all involved compounds and their interactions will help to understand biofilms and how to use them to our advantage.

Supplementary Material

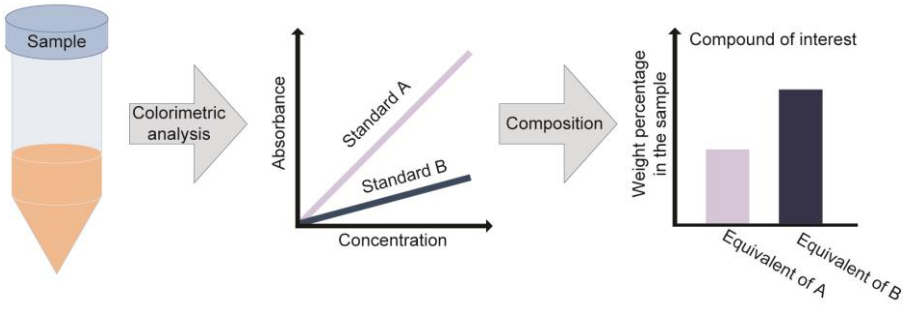
Supplementary material A: Influence of extraction parameters on the EPS yield

Table A: Influence of pre-treatment, mixing and temperature on the EPS yield. Extractions were performed with aerobic granular sludge from the pilot wastewater treatment plant Utrecht, the Netherlands. Conditions used to obtain structural EPS in Chapter 2 are highlighted with a grey background. The VS yield represents the amount of EPS obtained after acid precipitation with hydrochloric acid at pH 2.2. Hydrogel beads formation was tested after re-dissolving EPS at pH 8.5.

Pre-treatment	Extraction conditions					VS yield [mg/g]	Beads formed with $CaCl_2$
	Temperature [°C]	Mixing [rpm]	Duration	Chemical	pH		
-	80	400	35 min	0.5 % (w/v) Na_2CO_3	10	282	Yes
Pulsed sonication (40 W, 2.5 min)	80	400	35 min	0.5 % (w/v) Na_2CO_3	10	288	Yes
-	20	400	60 min	0.5 % (w/v) Na_2CO_3	10	20	Not tested
Blending (13.500 rpm, 30 sec)	20	400	60 min	0.5 % (w/v) Na_2CO_3	10	58	Not tested
Blending (13.500 rpm, 30 sec)	20	100	4 days	0.5 % (w/v) Na_2CO_3	10	122	Yes
Blending (13.500 rpm, 30 sec)	20	-	9 days	0.5 % (w/v) Na_2CO_3	10	64	Yes
-	20	-	9 days	0.5 % (w/v) Na_2CO_3	10	38	Yes
Blending (13.500 rpm, 30 sec)	20	-	9 days	1.2 % (w/v) NaOH	13	75	No
-	20	-	9 days	1.2 % (w/v) NaOH	13	67	No

Chapter 3

Chemical Characterization Methods for the Analysis of Structural Extracellular Polymeric Substances (EPS)



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Abstract

Biofilm structure and functionality depend on extracellular polymeric substances (EPS), but analytical methods for EPS often lack specificity which limits progress of biofilm research. EPS were extracted from aerobic granular sludge and analyzed with frequently applied colorimetric methods. The colorimetric methods were evaluated based on their applicability for EPS analysis. EPS fractions of interest were proteins, sugars, uronic acids and phenolic compounds. The applied methods (Lowry method, bicinchoninic acid assay, phenol sulfuric acid method, carbazole sulfuric acid method) were investigated in terms of their sensitivity towards the selected standard compound. Interference of compounds present in EPS with the colorimetric methods was further evaluated. All methods showed to be highly depending on the choice of standard compound and susceptible towards interference by compounds present in EPS. This study shows that currently used colorimetric methods are not capable of accurately characterizing EPS. More advanced methods are needed to be able to draw conclusions about biofilm composition, structure and functionality.

1. Introduction

Biofilms are present in natural systems as well as in wastewater and drinking water systems. Biofilms are composed of microorganisms and extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). A thorough analysis of EPS is prerequisite to study the structure and stability of biofilms.

The analysis of EPS is challenging and highly depending on analytical methods that are using standard compounds for quantification. Fractions of EPS (proteins, saccharides, uronic acids, humic substances) are currently characterized with colorimetric methods. Colorimetric methods are fast, easy to use and cheap. These methods were however developed to analyze known substances of unknown concentration (Dubois et al., 1956; Lowry et al., 1951). In the initial publications introducing these methods the authors mention the susceptibility of the methods to interfering compounds (Dische, 1946; Dubois et al., 1956; Lowry et al., 1951). The current use of methods was evaluated based on a search in the Scopus database targeting recent publications focusing on EPS analysis. With this evaluation colorimetric methods were selected for this study. It is clear that no common analytical approaches are used, complicating the evaluation of data in literature.

Analyzing EPS is performed to understand the biofilm composition and biofilm adjustment to environmental changes. Interpretations based on the composition however can only be done correctly if the results are reasonably accurate and carefully used. Previous studies already showed the unreliable results obtained for protein analysis in EPS with Lowry, Bradford or bicinchoninic acid (BCA) assay (Avella et al., 2010; Le et al., 2016; Ras et al., 2008). In these studies the interference of humic acids with the Lowry method (Avella et al., 2010; Le et al., 2016), the variance of the protein content in the same sample when analyzed with Lowry method and BCA assay (Ras et al., 2008) and the general inconsistency and unreliability of the methods (Le et al., 2016) were emphasized.

Not only the protein quantification in EPS samples is unreliable, but also the humic substance and saccharide quantification has certain inaccuracies (Everette et al., 2010; Le and Stuckey, 2016). The Folin-Ciocalteu reagent is used to quantify humic substances in EPS. The reactivity of the Folin-Ciocalteu reagent with multiple compound classes was analyzed suggesting it to be used to measure the total antioxidant capacity of a sample, but not to quantify the phenolic content (Everette et al., 2010). The anthrone sulfuric acid method and the phenol sulfuric acid method are widely applied in EPS research. Similar to the protein analysis (Le et al., 2016) also here interfering substances and the choice of the method can easily result in an under or overestimation of the actual sugar content (Le and Stuckey, 2016).

The purpose of this study is to evaluate EPS analysis with colorimetric methods. This study is building up on previous evaluations of EPS analysis with colorimetric methods (Avella et al., 2010; Le et al., 2016; Le and Stuckey, 2016; Ras et al., 2008). Structural EPS (Felz et al., 2016), further denoted here simply as EPS, from aerobic granular sludge was used. This sample was used as an exemplary sample for wastewater sludge or biofilm to illustrate drawbacks of colorimetric analytic methods in EPS research. Additionally to protein and saccharide detection, uronic acids and humic substances were included. The cross-interference of compounds present in EPS in the colorimetric measurements was evaluated. The significance of the standard compound used in colorimetric methods on the final result was illustrated. Standard compounds evaluated were proteins (bovine serum albumin, cytochrome C), neutral saccharides (xylose, glucose, mixture of neutral saccharides), uronic acids (galacturonic acid, glucuronic acid) and phenolic compounds (humic acid, gallic acid). Suggestions are given to improve the standard selection by a detailed analysis of the monosaccharide composition in EPS. Overall, this study aimed to provide a critical discussion about drawbacks of colorimetric methods in particular for biofilm EPS analysis as well as to display a way forward in this research field.

2. Materials and Methods

2.1 Sample collection

Aerobic granular sludge was collected from the municipal wastewater treatment plant Dinxperlo, the Netherlands in July 2017 (Royal HaskoningDHV, 2018). To separate granular sludge from flocs and other unwanted matter, the sludge was sieved with a stainless steel woven wire mesh sieve with a mesh size of 2 mm. The fraction > 2mm was collected and washed thoroughly on the mesh with demineralized water. Subsequently the washed granules were centrifuged at 4,000×g and 10°C for 20 min. The supernatant was discarded and the pellet of granules was stored at -20°C until being further used.

2.2 Extraction of structural EPS from aerobic granular sludge

Extraction of structural EPS was performed as described previously (Felz et al., 2016). 6 g of the sieved, washed and centrifuged aerobic granular sludge were transferred into a baffled flask containing 100 mL of demineralized water. Sodium carbonate was added to the granules in the demineralized water to a final concentration of 0.5 % (w/v). The mixture was stirred for 35 min at 80°C in a water bath and then centrifuged at 4,000×g and 4°C for 20 min. The supernatant was collected and the pellet was discarded. The pH of the supernatant was adjusted to 2.2 with 1 M hydrochloric acid. The acidified supernatant was centrifuged at 4,000×g and 4°C for 20 min. The pellet containing the structural EPS was collected and re-dissolved with 1 M sodium hydroxide at pH 8.5. The

dissolved structural EPS was dialyzed for 24 hours against demineralized water in dialysis tubing with a molecular weight cut-off of 3.5 kDa, frozen at -80°C and freeze-dried.

To avoid confusion with other publications on EPS and for the ease of this study structural EPS will only be called EPS throughout the following text.

The organic and the ash fraction of the EPS were determined by drying the sample at 105°C and combusting it at 550°C (APHA, 1998). The obtained values were used for the calculations of the weight percentages of the compounds measured with colorimetric assays.

2.3 Colorimetric assays

EPS was characterized with colorimetric methods. Furthermore standards for proteins, sugars, uronic acids, phenolic substances, amino sugars and sugar alcohols were analyzed in terms of their interference in all applied colorimetric methods. EPS were dissolved in 0.02 M sodium hydroxide and analyzed at concentrations of 200 mg L^{-1} and 100 mg L^{-1}, respectively. Standard lines were prepared in a concentration range from 5 - 100 mg L^{-1}. Cross-interference of standard compounds was tested at concentrations of 50 mg L^{-1}, 100 mg L^{-1} and 1000 mg L^{-1}. Analysis of cross-interference was performed with bovine serum albumin (BSA), glucose, a mixture of neutral sugars, galacturonic acid, humic acid, gallic acid, glucosamine and glycerol. Demineralized water together with the corresponding reagents of the assays was used as a blank in all colorimetric assays. Measurements were performed in triplicates for all colorimetric assays.

2.3.1 Protein determination

The presence of proteins was analyzed with two different assays, the Lowry method and the BCA assay.

2.3.1.1 Lowry method

Proteins were determined according to the modified Lowry method (Frølund et al., 1996, 1995). The Lowry method was performed with BSA and cytochrome C from equine heart as standards. BSA is the most commonly used standard for this assay. Cytochrome C was chosen to compare the method for two commercially available proteins of different size and composition. Measurements were performed in a 96-well plate, the absorbance was measured at 750 nm.

Not only proteins, but also humic acids will result in an increased absorbance intensity in the original Lowry method (Box, 1983; Frølund et al., 1995). In the modified Lowry

method (Frølund et al., 1995) a correction factor is included to decrease the impact of humic acids on the protein measurement. A detailed explanation of the correction factor is shown in the original publication (Frølund et al., 1995). The correction factor was calculated for BSA and cytochrome C. We applied the Lowry method with and without correction factor as both methods are applied in EPS research and we wanted to illustrate the effect of standard selection and cross-interference for both methods.

2.3.1.2 BCA assay

Additionally to the Lowry method the BCA assay (Smith et al., 1985) is frequently applied in EPS research and available as commercial kit for protein quantification. This assay is using a similar principle to that of the Lowry assay, but shows less variability for different proteins and was therefore also applied in this study (Ras et al., 2008; Smith et al., 1985). Analysis was performed with a commercially available kit (BC assay protein quantification kit, Interchim). Standard lines were prepared with BSA and cytochrome C. Measurements were performed in a 96-well plate, the absorbance was measured at 562 nm.

2.3.2 Saccharide determination with the phenol sulfuric acid method

Saccharides were determined using the phenol sulfuric acid method (Dubois et al., 1956). Used standards were glucose, xylose and a sugar mixture (equal amounts of fucose, rhamnose, galactose, glucose, xylose, mannose, ribose). Glucose is the most commonly applied standard in EPS research. Xylose was selected to illustrate the impact of the measurement wavelength on the final result. Sugars used in the sugar mixture were all detected in EPS and are therefore used as a standard. Measurements were performed in cuvettes at absorbance maxima of the sugar standards. The corresponding wavelengths were 480 nm (xylose), 482 nm (sugar mixture), 487 nm (glucose). In the original publication (Dubois et al., 1956) xylose was measured at 480 nm and glucose at 490 nm. In our experiments glucose resulted in a slightly higher absorbance at 487 nm than at 490 nm. Therefore 487 nm was used.

2.3.3. Uronic acid determination with the carbazole sulfuric acid method

Uronic acids were determined using the carbazole sulfuric acid method (Dische, 1946; Filisetti-Cozzi and Carpita, 1991; Galambos, 1967; Li et al., 2007). A modified version of this method was applied (Li et al., 2007). Standard lines were prepared with galacturonic acid and glucuronic acid. Both compounds are commonly used in EPS research. Measurements were performed in cuvettes, the absorbance was measured at 525 nm.

2.3.4 Phenolic compound determination with the modified Lowry method

Phenolic compounds were determined using the modified Lowry method which was also used for the protein determination (Frølund et al., 1996, 1995). Standard lines were prepared with humic acid and gallic acid. Humic acid is the commonly used standard in EPS research. Gallic acid is an unconventional standard in EPS research, but is commonly used in the food and plant research to measure the total phenolic content in a sample. Measurements were performed in a 96-well plate, the absorbance was measured at 750 nm.

2.4 HPAEC-PAD analysis of monosaccharides in EPS

EPS was hydrolyzed in 1 M hydrochloric acid with a sample concentration of 10 g L^{-1}. The sample suspension was prepared in caped bottles and shortly vortexed. Hydrolysis was performed in an oven at 105°C for 8 hours without mixing. After hydrolysis the sample was centrifuged at 13,300 × g for 5 min. The supernatant was collected and neutralized with 1 M sodium hydroxide. The neutralized sample was diluted 1:5 with ultrapure water and filtered through a 0.22 µm PVDF filter.

For the qualitative analysis of monosaccharides in the EPS, the hydrolyzed and filtered EPS was analyzed with and without the addition of monosaccharides. Monosaccharides were added with a concentration of 0.01 g L^{-1}. Selected compounds were glycerol, galactosamine, fucose, glucosamine, rhamnose, galactose, glucose, xylose, mannose, ribose, galacturonic acid and glucuronic acid. Multiple other sugar monomers were also analyzed. However as these sugars were not detected in the here analyzed EPS they are not mentioned.

Analysis was performed with a Dionex ICS-5000^{+}, a CarboPac PA20 column and an AminoTrap pre-column. Eluents used were ultrapure water, 200 mM sodium hydroxide, and 50 mM sodium acetate with 200 mM sodium hydroxide. Samples were analyzed with a quadruple waveform. Before injection of the first sample the columns were thoroughly washed and re-equilibrated. Samples were analyzed with the elution pattern shown in Table 1. Chromatograms of EPS and spiked EPS were overlaid to detect present monosaccharides.

Table 1: Sample elution for monosaccharide analysis with HPAEC-PAD

Time point	Eluents	Purpose
0 – 30 min	2 mM sodium hydroxide	Elution of sugar alcohols, small neutral sugars and amino sugars
30 – 45 min	50 mM sodium acetate with 200 mM sodium hydroxide	Elution of large neutral sugars and uronic acids
45 – 60 min	200 mM sodium hydroxide	Washing of the columns
56 – 68 min	2 mM sodium hydroxide	Re-equilibration of the columns

3. Results

3.1 Chemical characterization of EPS with colorimetric methods

EPS extracted from aerobic granular sludge was analyzed with colorimetric methods that are currently frequently applied in EPS research and described before. The results are visualized in Figure 1 below. The commonly used standard compounds (Figure 1a) and a set of alternative standards (Figure 1b) were evaluated to see the impact of the standard selection on the EPS composition.

The protein measurement was affected by the selection of the standard and the method. The change from BSA to cytochrome C resulted in a difference of 5.2 wt% for the equivalents in EPS. Using the modified Lowry method also the correction factor has to be taken into account when changing the standard. This is not visible in Figure 1. The obtained correction factors were 0.29 for BSA and 0.50 for cytochrome C. Measuring the equivalent without a correction factor resulted in 61.7 wt% BSA equivalents and 49.3 wt% cytochrome C equivalents in the EPS. In contrast to the Lowry method, the BCA assay resulted in the same equivalents for BSA and cytochrome C with 45.7 wt%. Overall using these methods as applied in literature would give a variation in protein standard equivalents of 38 to 62 wt% in total organic mass.

The quantification of saccharides was sensitive towards the selection of the standard and the used wavelength. Different sugars will result in different absorbance maxima in the phenol sulfuric acid method (Dubois et al., 1956). Measuring the sugar equivalent of EPS with xylose at 480 nm and 487 nm resulted in 10.7 wt% and 12.9 wt%, respectively. Measuring the equivalent in EPS with glucose at 480 nm and 487 nm resulted in 14.6 wt% and 13.8 wt%, respectively. With these methods the saccharide standard equivalent mass would vary from 11 – 15 wt% of the organic mass and that of uronic acids from 7 – 13 wt%.

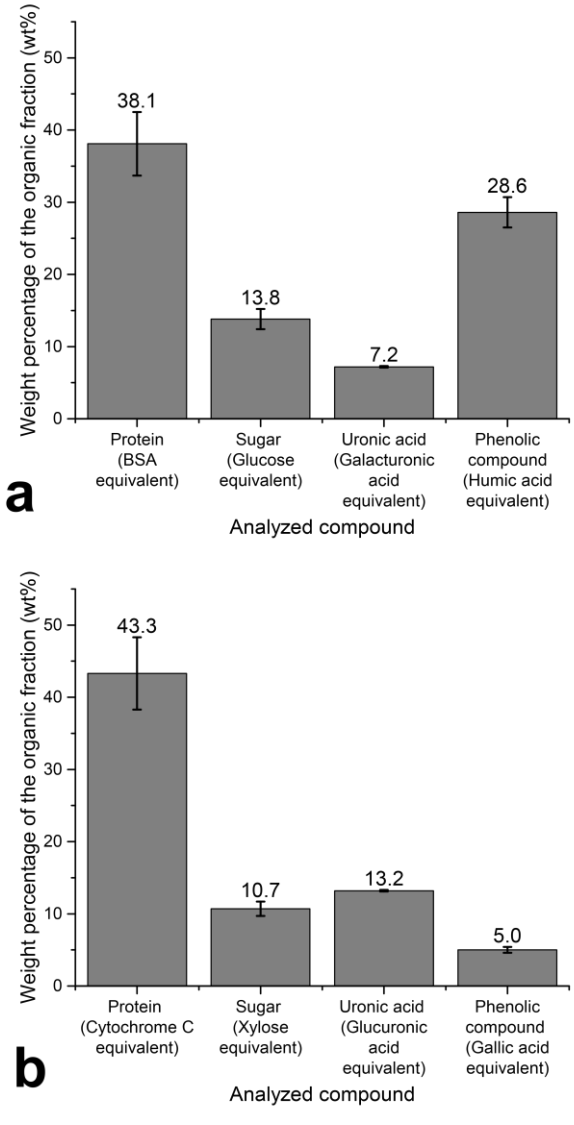


Figure 1: Overall composition of EPS presented in weight percentage of the organic fraction of EPS. Compounds were measured with colorimetric methods and are presented in equivalents of the standards used for the corresponding measurement. Proteins were analyzed with the modified Lowry method, sugars with the phenol sulfuric acid method, uronic acids with the carbazole sulfuric acid method and phenolic compounds with the modified Lowry method. Figure (a) shows the results using BSA (at 750 nm), glucose (at 487 nm), galacturonic acid (at 525 nm) and humic acid (at 750 nm) as standards. Figure (b) illustrates the results using cytochrome C (at 750 nm), xylose (at 480 nm), glucuronic acid (at 525 nm) and gallic acid (at 750 nm) as standards.

An alternative standard for the quantification of saccharides was introduced based on the monomer composition of EPS. As follows later, HPAEC-PAD analysis of EPS showed the presence of several sugars in EPS. Using this result a standard mixture composed of various neutral sugars was additionally applied in the phenol sulfuric acid method. Glycerol, galactosamine and glucosamine were excluded from this sugar mixture as these compounds did not result in an increased absorbance intensity in the range of 480 nm – 490 nm. Using the sugar mixture in the phenol sulfuric acid method resulted in an absorbance maximum at 482 nm which is close to that of EPS with 485 nm. The sugar mixture showed a good linearity in the here used concentration range of 5 mg L^{-1} – 100 mg L^{-1} with an R^2 of 0.9981. Applying the sugar mixture as standard compound for EPS resulted in a equivalent of 14.6 wt% of the organic mass.

The measurement of phenolic compounds was influenced by the selection of the standards for phenolic compound and protein quantification. Commercial humic acid standard yielded a much higher phenolic compound equivalent than gallic acid. The absorbance of gallic acid in the assay was on the average 5.7 times higher than that of humic acid. The correction factor applied in the protein analysis also affects the phenolic compound measurement. The results illustrated in Figure 1a and 1b are obtained using the correction factor of BSA. Using the correction factor of cytochrome C resulted in 6.4 wt% humic acid equivalents and 1.1 wt% gallic acid equivalents. Depending on the applied standards for phenolic compounds and proteins the measured content of phenolic compounds would vary from 1 to 29 wt% of the organic mass.

3.2 Possible cross-interferences of compounds present in EPS with the here applied colorimetric methods

The colorimetric analysis above indicated the impact of the standard selection on the obtained EPS composition. Another problem is formed by the cross-interference among EPS components. EPS contains proteins, neutral sugars, uronic acids and phenolic compounds. The colorimetric quantification of one component may be interfered by the presence of other components. Compounds used to evaluate possible cross-interferences are listed in the material and methods section. The cross-interferences are displayed in Table 2 below.

Table 2: Visualization of cross-interferences of substances present in EPS with the compound of interest analyzed with colorimetric assays. The interference is represented in equivalents of the standard used in the corresponding assay. White color indicates compound of interest. Grey color indicates the degree of interference of the analyzed compound. The darker the grey color the higher was the interference of the analyzed compound with the compound of interest. The interference is presented in five increments with negligible (≤ 1 wt% standard equivalents), small (1 – 10 wt%), considerable (10 – 20 wt%), large (20 – 50 wt%) very large (> 50 wt%). Selected standards for the compounds of interest were BSA (proteins), glucose (sugars), galacturonic acid (uronic acids) and humic acids (phenolic compounds).

Compound of interest to be measured	Used colorimetric method	Compound analyzed for its interference with the compound of interest						
		BSA	Glucose	Galacturonic acid	Humic acid	Gallic acid	Glucosamine	Glycerol
Proteins	Modified Lowry assay							
	Lowry assay							
	BCA assay							
Sugars	Phenol sulfuric acid assay							
Uronic acid	Carbazole sulfuric acid assay							
Phenolic compounds	Modified Lowry assay							
	Lowry assay							

All of the analyzed methods were prone to interference of the selected standard compounds. The mixture of sugars showed the same behavior as glucose and is therefore not included into the table. Glucosamine and glycerol were detected in the EPS with HPAEC-PAD and were also included in the cross-interference analysis. Glycerol did not increase the absorbance intensity of any of the here analyzed colorimetric methods, which is similar to previously reported results for the sugar alcohols sorbitol and mannitol (Le et al., 2016; Le and Stuckey, 2016).

Protein measurements were influenced differently depending on the applied method. Both Lowry method and BCA assay were susceptible to humic acid, gallic acid and glucosamine, with the BCA assay exhibiting a much higher interference by glucosamine. Additionally there was small interference by glucose and galacturonic acid in the BCA assay. Interference of humic acid and gallic acid was removed in the modified Lowry assay by the correction factor as proposed by Frølund et al. (1995). Protein quantification can be overestimated in the presence of phenolic compounds, glucose, galacturonic acid and glucosamine.

The saccharide measurement was sensitive towards the presence of galacturonic acid and humic acid. The phenol sulfuric acid method showed negligible interference to BSA. As illustrated here and in the initial publication (Dubois et al., 1956) uronic acids result in an increased absorbance intensity in this method. Humic acid and galacturonic acid can result in an overestimation of the measured saccharide content.

The measured value of uronic acids was affected by BSA, glucose and humic acid. Uronic acid measurement exhibited small interference with glucose and humic acid. Not visible in this chart is the interference of proteins. Proteins were reported to influence the uronic acid measurement by decreasing the measured value (Balazs et al., 1965; Dische, 1946). The carbazole sulfuric acid assay was performed with galacturonic acid by itself (50 mg L^{-1}), EPS by itself (200 mg L^{-1}) and a mixture of galacturonic acid (50 mg L^{-1}) and EPS (200 mg L^{-1}). The measured value of the mixture resulted in an 8 % lower value than the sum of the single compound values. For BSA similar results were obtained. Quantification of uronic acids can be overestimated by glucose and humic acid and underestimated in the presence of BSA.

The quantification of phenolic compounds was only sensitive towards proteins. The interference of BSA was removed by the correction factor of BSA. The correction factor of BSA was only able to completely remove interference of proteins if the protein standard behaved in the same way as BSA. Using the correction factor of BSA and cytochrome c as protein standard gave small interference of 2.3 wt%. Without the correction factor the quantification of phenolic compounds had a large interference by BSA. The measurement of phenolic compounds can be overestimated in the presence of proteins.

3.3 Monosaccharide analysis of EPS with HPAEC-PAD

Qualitative analysis of monosaccharides present in EPS was performed using HPAEC-PAD. Detected monosaccharides can be seen in Figure 2a and 2b. In the initial elution with diluted sodium hydroxide (Figure 2a) the presence of one sugar alcohol (glycerol (1)), two amino sugars (galactosamine (3), glucosamine (5)) and seven neutral sugars (fucose (2), rhamnose (4), galactose (6), glucose (7), xylose (8), mannose (9) and ribose (10)) was confirmed. Further elution with sodium acetate/sodium hydroxide (Figure 2b) revealed galacturonic acid (11) and glucuronic acid (12) in EPS.

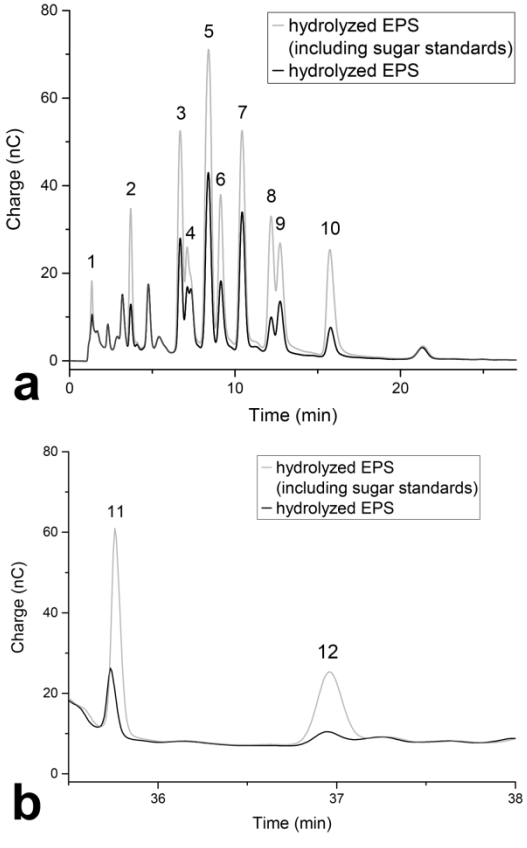


Figure 2: Qualitative analysis of monosaccharides in acid hydrolyzed EPS with HPAEC-PAD. (a) Elution with 2 mM sodium hydroxide revealed the presence of sugar alcohols, neutral sugars and amino sugars. Detected compounds were (1) glycerol, (2) fucose, (3) galactosamine, (4) rhamnose, (5) glucosamine, (6) galactose, (7) glucose, (8) xylose, (9) mannose, (10) ribose. (b) Further elution with 50 mM sodium acetate and 200 mM sodium hydroxide showed the presence of galacturonic acid (11) and glucuronic acid (12).

4. Discussion

The colorimetric methods used are all based on the same principle: A sample containing a compound of interest is directly or indirectly reacting with a selected color reagent resulting in an increased absorbance intensity at a known wavelength. The absorbance intensity is then compared to that of a standard compound of known concentration. Based on this comparison the concentration of the standard in the sample is calculated. A detailed explanation of the mechanisms of the used colorimetric methods is given in previous studies (Le et al., 2016; Le and Stuckey, 2016; Prior et al., 2005; Smith et al., 1985).

4.1 Selection of the standard

Colorimetric methods were used to measure the concentration of standards representing proteins, saccharides, uronic acids and humic substances in EPS. For all of the analyzed compounds changing the standard compound resulted in a change of the corresponding equivalent in EPS. This shows the sensitivity of colorimetric methods to the choice of standard and that results of colorimetric methods should only be presented in equivalents of the standard. Furthermore not only the standard, but also the applied method had an influence on the obtained equivalent in EPS. Using the same EPS sample and the same standard yielded different equivalents when applying Lowry method and BCA assay.

Results of colorimetric methods need to be interpreted very carefully. Inaccuracies of available colorimetric methods were shown for protein and sugar quantification in general and for wastewater and EPS samples in particular (Le et al., 2016; Le and Stuckey, 2016; Ras et al., 2008). Similar findings were shown on the variance of the result when changing the standard compound in the applied methods (Le et al., 2016; Le and Stuckey, 2016; Ras et al., 2008). This study is building up on these previous findings about protein and sugar analysis. Additionally illustrated are problems of colorimetric measurements in EPS analysis with uronic acids and phenolic compounds, which are frequently investigated in wastewater and EPS samples.

The variance of the results obtained for different standards in colorimetric protein analysis can be explained by the mechanism of Lowry method and BCA assay. Peptide bonds, the amino acids cysteine, tryptophan, tyrosine and side groups similar to that of the three amino acids are responsible for the color reaction in these assays (Folin and Ciocalteau, 1927; Lowry et al., 1951; Smith et al., 1985; Wiechelman et al., 1988). Proteins with a different composition to that of the applied standard can result in an over- or underestimation of the protein content in the sample. In the BCA assay cytochrome C and BSA showed a very similar behavior resulting in the same equivalents for the EPS. This can be a coincidence for those two proteins as previous studies showed varying results when using the BCA assay with different proteins (Avella et al., 2010; Le et al., 2016). Acceptable results in EPS analysis can only be obtained with these assays, if a standard of similar composition to that of the EPS is used.

Already when introducing sugar quantification with the phenol sulfuric acid method a variance in maximum absorbance wavelength and absorbance intensity for monosaccharides was illustrated with xylose, mannose, glucose, galactose and fucose (Dubois et al., 1956). Looking at the amount of different sugars detected in EPS with HPAEC-PAD, a standard composed of one saccharide will not be an adequate standard for EPS analysis. A possible optimization for a more representative standard was shown

in this study. Using a mixture of sugars can better compensate for the different maximum absorbance wavelengths and intensities of the single sugars and therefore more accurately represent EPS.

The high variance of absorbance intensity for different compounds was also visible in the uronic acid measurement. The glucuronic acid equivalent in EPS was almost double the amount to that of galacturonic acid. Both uronic acids were shown to be present in EPS. Similar to the standard applied for the saccharide measurement, a mixture of glucuronic and galacturonic can be a more representative standard for EPS analysis.

The two standards used in the analysis of phenolic compounds especially showed the impact of the standard selection in colorimetric methods. Based on this result it cannot be stated which standard is more representative for the analysis of phenolic compounds in EPS. Humic acid resulted in this assay in a very low absorbance. If an inaccurate standard with a very low absorbance is used the measured equivalent in the EPS can easily be overestimated. The types of phenolic compounds present in EPS need to be analyzed to be able to select a suitable standard.

It is very important to keep in mind that colorimetric methods are highly standard dependent and that inadequate standards can easily result in a misleading result. Representative standards are needed, but can still not ensure an accurate result. Thus interpretation of results obtained with the above described methods should be done with great care.

4.2 Cross-interference in EPS analysis

It was demonstrated that all used methods suffer from interference towards compounds present in EPS. This analysis illustrated that the measured values obtained with colorimetric methods do not only represent the amount of the targeted compound, but also of other compounds present in the sample. It is crucial to take interfering compounds into account when performing colorimetric methods on EPS.

Interfering substances for the quantification of proteins and saccharides were reported (Frølund et al., 1995; Le et al., 2016; Le and Stuckey, 2016). In this study standards of compounds found to be present in EPS were included in the analysis of interfering compound in the protein and saccharide measurement. Furthermore the analysis of interfering compounds was extended to the measurement of phenolic compounds and uronic acids.

Results show different interference for the Lowry method and the BCA assay. Both assays are very prone to the presence of phenolic compounds. The very large interference with phenolic compounds in the Lowry method is due to the ability of

phenolic compounds to directly interact with the color reagent (Folin-Ciocalteu reagent) (Box, 1983; Everette et al., 2010). The BCA assay in addition suffered from a large interference of glucosamine. Possibly BCA is interacting stronger with amino sugars and other sugars than the Folin-Ciocalteu reagent. Interference in this assay can originate from compounds with similar side groups to those of the amino acids interacting with the assay (Wiechelman et al., 1988).

To overcome the interference of phenolic compounds in the protein measurement, a correction factor was proposed (Frølund et al., 1995). The correction factor is based on the absorbance values of a protein standard. In the interference analysis this correction factor completely removed the interference of phenolic compounds with the protein measurement. However despite the positive impact on the decreased interference of humic and gallic acid, the correction factor was shown to be depending on the standard. As the correction factor is affecting the measurement of both, proteins and phenolic compounds, an inaccurate protein standard will falsify both results. If a sample behaves differently than the standard, the correction factor can also cause detection of phenolic compounds in a sample in which no phenolic compounds are present (Avella et al., 2010).

Not only do the protein assays suffer from interfering compounds, but also from other interfering parameters such as the structure (Wiechelman et al., 1988) or dissolution of the proteins. Lowry method and BCA assay are performed under highly alkaline conditions. EPS that are only soluble under acidic conditions (Pronk et al., 2017) can therefore not be analyzed properly with these assays.

At the current state neither of the here used protein assays will give a reliable result for protein quantification in EPS. Other authors already named the measured substances “proteinaceous” material (Le et al., 2016) instead of proteins. Proteomic studies of biofilms will help to analyze present proteins. By this it can be evaluated if there is a dominant type of protein present which can be used as a more representative standard. Also, amino acid analysis of EPS can help to compare the amount of cysteine, tyrosine and tryptophan in relation to a standard.

The measurement of saccharides was interfered by the presence of galacturonic acid and humic acid, both leading to an overestimation of the measured equivalent in EPS. This is similar to previous studies (Dubois et al., 1956; Le and Stuckey, 2016). The interference of the saccharide measurement was also shown for a very similar method, the anthrone sulfuric acid method (Dreywood, 1946). A suggestion to overcome this interference was introduced with measuring both, uronic acids and saccharides in the same method at different wavelengths (Rondel et al., 2013). This can be an improvement to reduce the interference, but can still lead to a falsification of the result

if the selected standard is not accurate as shown before. Because of the drawbacks of this method, recent studies question the applicability of sugar quantification with colorimetric method for wastewater samples (Le and Stuckey, 2016).

In comparison to protein and saccharide analysis, the measurement of uronic acids showed small interference by compounds present in EPS. Glucose and humic acid resulted in a small overestimation of the measured equivalent. Neutral sugars react with the reagents of this method. To decrease the impact of neutral sugars sulfamate was added to the assay (Balazs et al., 1965), thus decreasing the interference. Important for the uronic acid measurement is to be aware of the interference of proteins (Balazs et al., 1965; Dische, 1950, 1946) which can lead to an underestimation of the measured equivalent.

The phenolic compound measurement was only susceptible towards the interference of proteins. This interference can be explained by the reactivity of the Folin-Ciocalteu reagent towards proteins (Everette et al., 2010; Frølund et al., 1995; Lowry et al., 1951). A correction factor was introduced as described before. The correction factor calculated based on the absorbance of proteins highly affected the phenolic compound measurement. Thus having an inaccurate standard for the protein measurement will have a severe impact on the measurement of phenolic compounds. Measurement of phenolic compounds in EPS with and without correction factor will be affected by proteins.

Aside of the shown interference, the phenolic compound measurement suffered from the vast variety of compounds that can interact additionally with the Folin-Ciocalteu reagent, such as thiol derivatives, vitamins and nucleotide bases (Box, 1983; Everette et al., 2010; Ikawa et al., 2003; Sharma and Krishnan, 1966). The reactivity of the assay is that broad that it is recommended to use it as a measure of the total antioxidant capacity rather than to measure the phenolic compounds in a sample (Everette et al., 2010).

Interference of compounds present in EPS was visible in all the tested assays, partly at severe levels. The analysis of interfering compounds and of the standard dependency show that colorimetric methods should only be used as an orientation to analyze EPS. Optimization of colorimetric methods with more adequate standards or correction factors can improve these methods for EPS analysis, but will not solve all the drawbacks. When using colorimetric methods it is important to be aware of other compounds that will be measured besides the intended compounds and obtained values should not be used as absolute values. Thus colorimetric methods should only be used to obtain an overview of the EPS composition and also this overview has to be interpreted very carefully.

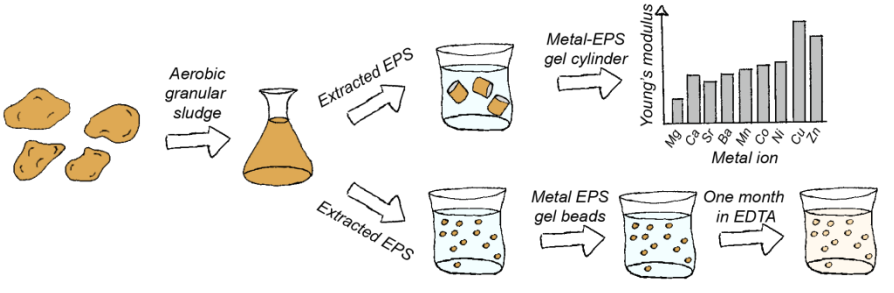
From the analysis above it becomes clear that colorimetric EPS analysis is at best indicative of the different fractions of polymers in EPS. The use of different methods and the presence of varying interfering compounds make a comparison of results between different studies almost impossible. The EPS research field needs to shift the focus from analyzing the present polymeric material with colorimetric methods to analyzing the different compounds more in depth. The high complexity of the present sugars in the polymers was indicated by the result of HPAEC-PAD analysis. A next step is to further understand the function and origin of these sugars (e.g. polysaccharides, glycosylated groups, etc.) as well as to analyze the exact bond between the sugars. There are good options to do so by using mass spectrometric analysis (Dell and Morris, 2001; Kumirska et al., 2010; Mariño et al., 2010). The same can be stated for proteins. Analysis with SDS-PAGE can reveal the presence of certain sizes and types of proteins. Further analyzing such proteins with mass spectrometry can reveal the composition and function of the present proteins (Kaltashov et al., 2012; Svensäter et al., 2001). Hereto more elaborate meta-proteomics techniques will have to be developed (Garza and Dutilh, 2015; Myrold et al., 2014). Seen to the importance of EPS in the functioning of biofilms and biofouling, only a strongly improved analysis of the EPS constituents might bring biofilm research to the next level.

5. Conclusion

Current EPS research largely depends on analytical colorimetric methods that have a significant bias. These biases are due to the unavoidable choice of a standard compound, a lack of suitable standard compounds and the cross-interference of the many EPS compounds in the individual assays. Results obtained with colorimetric methods have to be interpreted very carefully. Current EPS research for natural and wastewater biofilms should focus first on improving and developing more advanced analytical methods (e.g. based on FTIR, GC, HPLC, EEM, NMR, MS) before it progresses with studying the functionalities of the material that comprises biofilm EPS.

Chapter 4

Impact of Metal Ions on Structural EPS Hydrogels from Aerobic Granular Sludge



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Abstract

Structural extracellular polymeric substances (structural EPS) can form stable hydrogels and are considered to be responsible for the stability of biofilms and aerobic granular sludge. Structural EPS were extracted from aerobic granular sludge and characterized for their gel-forming capacity with different alkaline earth and transition metal ions. The structural EPS hydrogels were compared to alginate hydrogels. Alginate is a well characterized polymer which is able to form stiff hydrogels with multivalent ions. The stiffness of the obtained hydrogels was measured with dynamic mechanical analysis and quantified by the Young's modulus. Furthermore the stability of structural EPS hydrogels towards disintegration in the presence of ethylenediaminetetraacetic acid (EDTA) was evaluated at pH 4.5 – 10.5 and compared to that of alginate, polygalacturonic acid and κ-carrageenan. The stiffness of alginate hydrogels was multiple times higher than that of structural EPS. Alkaline earth metals resulted in stiffer alginate hydrogels than transition metals. For structural EPS this trend was opposite to alginate. Independent of the pH, polysaccharide hydrogels were quickly disintegrated when being exposed to EDTA. Structural EPS hydrogels demonstrated greater stability towards EDTA and were still intact after one month at pH 4.5 – 8.5. It is suggested that the gelling mechanism of structural EPS is not only related to metal ion complexation of the polymers, but to a combination of interactions of multiple functional groups present in structural EPS. This study helps to further understand and characterize structural EPS from aerobic granular sludge, and therewith understand its stability and that of biofilms in general.

1. Introduction

The aerobic granular sludge process is an emerging new wastewater technology (Pronk et al., 2015). Granular sludge is a spherical biofilm with hydrogel properties (Seviour et al., 2009a). Structural extracellular polymeric substances (structural EPS) are hydrogel-forming polymers and considered to be strongly involved in the mechanical strength of aerobic granular sludge (Felz et al., 2016). Understanding the gel forming properties and stability of structural EPS will help in better understanding of the aerobic granular sludge stability.

Structural EPS from aerobic granular sludge were previously denoted as alginate-like EPS (Lin et al., 2008). Their extraction method is similar to that of alginate (McHugh, 2003). Initial studies on structural EPS and the reported presence of alginates in microbial EPS (Davies and Geesey, 1995; Remminghorst and Rehm, 2006) suggested that alginate-like compounds were present in structural EPS. Both, structural EPS and alginate form hydrogels with calcium ions, precipitate as a gel at acidic pH and in both compounds carboxyl groups were detected with FTIR (Draget et al., 1994; Lin et al., 2010). The FTIR spectrum of structural EPS was more complex than that of pure alginate. Possible reasons for the appearance of the FTIR spectrum were the higher complexity of structural EPS compared to alginate or impurities included in the extract together with the alginate-like compounds. Besides the before mentioned similarities, follow up research demonstrated structural EPS to be more complex than alginate. Structural EPS were composed of proteins, neutral sugars, amino sugars, uronic acids and polyphenolic compounds (Felz et al., 2019).

Hydrophilic polymers containing acidic groups such as alginate have the ability to form ionic cross-linked hydrogels with metal ions. The structure and stability of the ionic cross-linked hydrogel is not only affected by the structure and available functional groups of the polymer, but also by the type of metal ion (Haug and Smidsrød, 1970; Lee and Mooney, 2012; Ouwerx et al., 1998). To understand and assess the gelling behavior of structural EPS, the ionic gel-forming property, the structure of lyophilized gels and the gel stability of structural EPS were compared to that of known, well characterized polymers. Hydrogels of structural EPS were prepared with alkaline earth, transition metal and zinc ions to evaluate the impact of the ion on the gel. The stiffness of structural EPS hydrogels was measured by dynamic mechanical analysis (DMA) and quantified by the Young's modulus. The obtained Young's moduli were compared to those of alginate. Structural elements of lyophilized structural EPS hydrogels were analyzed by environmental scanning electron microscopy (ESEM). Furthermore the stability towards disintegration of structural EPS hydrogels in the presence of the strong chelating agent ethylenediaminetetraacetic acid (EDTA) was studied in comparison to hydrogels of the pure polymers alginate, polygalacturonic acid and κ-carrageenan.

Little is known about the exact composition of EPS from aerobic granular sludge and biofilms in general. The comparison with well characterized polymers can be used as a starting point to enable a positioning of physical properties of structural EPS hydrogels into context with literature. Thus, polymers and functional groups of the EPS involved in the gelling can be evaluated. In this way, this manuscript aims to better understand the gelling and gel stability of structural EPS and tries to improve the understanding of the formation and stability of aerobic granular sludge and biofilms in general.

2. Materials and Methods

2.1 Collection of the sludge sample and extraction of structural EPS

Aerobic granular sludge was collected from the Nereda® pilot reactor at the wastewater treatment plant Utrecht, the Netherlands in March 2016. The reactor was fed with municipal sewage to reduce the COD, phosphorus and nitrogen content. The reactor was operated with 1 hour anaerobic feeding and 8 hours aeration. The sludge had an average solid retention time (SRT) of 20 – 30 days and a sludge volume index (SVI_{30min}) of 33 – 43 mL/g_{VSS}. The sludge was sampled at the end of an aerobic cycle. Granular sludge and flocs were separated by sieving the sludge sample with a stainless steel mesh sieve with a mesh size of 1 mm. The retained fraction containing only the granular sludge was collected and washed with demineralized water. The washed sample was centrifuged at 4,000×g, the supernatant was discarded and the pellet was frozen at -20°C until being further used. The extraction of structural EPS was performed as described previously (Felz et al., 2019). Extracted structural EPS were frozen at -80°C and freeze-dried.

2.2 Preparation of gel cylinders

Extracted structural EPS from section 2.1 were dissolved in 0.01 M sodium hydroxide with a concentration of 10 % (w/v), guluronic acid rich alginate from the stipe of *Laminaria hyperborea* was dissolved with a concentration of 2.5 % (w/v). The composition of the alginate is illustrated in Supplementary material A. Different concentrations were used for structural EPS and alginate to ensure obtaining complete dissolution of the polymers and stable hydrogel cylinders. A hollow plastic cylinder was covered on one side with a dialysis bag with a molecular weight cut off (MWCO) of 3.5 kDa. Dissolved structural EPS and alginate solution, respectively, were transferred into the plastic cylinder and the plastic cylinder was closed with a dialysis bag (Skjåk-Bræk et al., 1989). The cylinders containing the structural EPS and alginate solutions were dialyzed in a glass beaker containing 150 mL of 100 mM metal solution. Metal solutions used for the dialysis were magnesium chloride, calcium chloride, strontium chloride, barium chloride, manganese sulfate, cobalt sulfate, nickel sulfate, copper chloride and zinc acetate. Dialysis was performed for 24 hours with changing of the

dialysis solution. After dialysis, gel cylinders of 8 mm height and diameter were obtained and used for the mechanical analysis with DMA.

2.3 Dynamic mechanical analysis (DMA) of hydrogel cylinders

The deformation of hydrogel cylinders prepared as described in section 2.2 was measured by DMA. DMA measurements were performed on a Perkin Elmer 7E with parallel plate disc geometry. Hydrogel cylinders were placed on the bottom plate and compressed with the top plate by a force increase of 25 mN per minute. From the stress-strain curve we obtained the Young's modulus. The measurements were performed in triplicate.

2.4 Environmental scanning electron microscopy (ESEM) of structural EPS hydrogels

Structural EPS gel cylinders were prepared with magnesium chloride, calcium chloride and zinc acetate as described in section 2.2 and dialyzed against demineralized water to remove unbound metal ions. A follow up dialysis of the gel cylinders against ethanol was performed to obtain gel cylinders of higher structural integrity after freeze-drying. The gel cylinders were frozen at -80°C and subsequently freeze-dried. Freeze-dried gel cylinders were cut into half to analyze the cross-section of the former hydrogel. ESEM analysis was performed under vacuum with a Philips XL30 ESEM Tungsten filament electron microscope.

2.5 Hydrogel stability test towards EDTA

Extracted structural EPS, alginate and polygalacturonic acid were dissolved in 0.01 M sodium hydroxide with a concentration of 2.5 % (w/v). K-carrageenan was dissolved in 0.01 M sodium hydroxide with a concentration of 2.5 % (w/v) and heated to 60°C. Using a 1 mL Pasteur pipette small drops of the structural EPS and polysaccharide solutions were dripped into 100 mM metal solutions of calcium chloride, copper chloride and zinc acetate. The resulting hydrogel beads were allowed to rest for 3 hours in the metal ion solutions. To remove unbound metal ions the hydrogel beads were transferred into demineralized water and kept in the demineralized water for 1 hour.

EDTA solutions with a concentration of 2 % (w/v) EDTA and a pH of 4.5, 6.5, 8.5 and 10.5 were prepared. Hydrogel beads of each polymer were transferred into glass beakers containing 45 mL of the EDTA solutions. Of all polymers four hydrogel beads with each metal ion were analyzed at the four different pH levels. Once a day the glass beakers were slightly swiveled to evaluate the disintegration of the hydrogel beads. The disintegration of hydrogel beads was monitored for one month. Gels were considered stable if the gel bead was present as one spherical gel and visually intact after swiveling the glass beakers.

3. Results

3.1 Dynamic mechanical analysis of structural EPS hydrogels

To draw conclusions about the overall stiffness and the gel formation of structural EPS hydrogels, structural EPS hydrogel stiffness was compared to that of the well characterized polysaccharide alginate, which is frequently reported to be a constituent of biofilm matrices (Davies and Geesey, 1995; Remminghorst and Rehm, 2006). The gel stiffness was evaluated with different metal ions. Alkaline earth and transition metals were selected as members of both groups of metals were present in aerobic granular sludge (Supplementary material B), and are able to form hydrogels with anionic polymers. In addition their interactions with alginate showed a distinct behavior in terms of affinity and gel stiffness (Chan et al., 2011; Haug, 1961; Haug and Smidsrød, 1970; Ouwerx et al., 1998). Alkaline earth metals other than Mg^{2+} resulted in stiffer hydrogels with increasing ionic radius and were with the exception of Cu^{2+} stiffer than those formed with transition metals (Mn^{2+}, Co^{2+}, Ni^{2+}) and Zn^{2+} (Chan et al., 2011; Ouwerx et al., 1998; Straccia et al., 2015; Topuz et al., 2012).

Alginate interacts with metal ions via its carboxyl groups. The composition of structural EPS is more complex than that of alginate, thus multiple functional groups can be involved in the gel formation. To conceive which functional groups and types of polymers are involved in the formation of structural EPS hydrogels and to compare the metal preference of structural EPS to that of alginate, different alkaline earth (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}), transition metals (Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}) and Zn^{2+} were assessed in terms of the obtained gel stiffness. The pH of the metal solutions (3.82 – 6.58) was higher than that applied for the precipitation of structural EPS to avoid gel formation by acid precipitation. The exact pH values are illustrated in Supplementary material C. All metal ions successfully resulted in the formation of stable structural EPS hydrogels (Figure 1a). The stiffness of the gels increased in the order $Mg^{2+}<Sr^{2+}<Ba^{2+}\approx Ca^{2+}<Mn^{2+}<Co^{2+}<Ni^{2+}<Zn^{2+}<Cu^{2+}$. Overall hydrogels of alkaline earth metals were less stiff than those of transition metal ions.

The stiffness of alginate hydrogels with alkaline earth and transition metal ions is summarized in Figure 1b. The alginate gel formed with Mg^{2+} was weak and not strong enough to be determined by DMA, which is in accordance to literature (Topuz et al., 2012). The copper alginate hydrogel was inhomogeneous and did not allow for an accurate and representative stiffness measurement. This was likely due to the high affinity for complex formation of copper with alginate or the pH of the copper solution (Lee and Mooney, 2012; Ouwerx et al., 1998). The alginate gel stiffness increased in the order $Mn^{2+}<Ni^{2+}<Co^{2+}<Zn^{2+}<Ca^{2+}<Sr^{2+}<Ba^{2+}$. Other studies showed similar results with Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} resulting in weaker alginate gels than alkaline earth metals

(Ouwerx et al., 1998; Straccia et al., 2015). For copper and barium hydrogels there were inconsistent results. It was reported that copper alginate hydrogels were stiffer than barium hydrogels (Chan et al., 2011) and also that barium hydrogels were stiffer than copper hydrogels (Ouwerx et al., 1998).

Overall it can be seen that even with only ¼ of the polymer concentration alginate hydrogels were significantly stronger than structural EPS hydrogels, especially for alkaline earth metals. With the exception of copper, alginate favored alkaline earth metals to form stiff hydrogels. For structural EPS instead transition metals and Zn^{2+} resulted consistently in stiffer hydrogels than alkaline earth metals. Structural EPS hydrogels did not exhibit the same preference for alkaline earth metals in terms of ionic radius as alginate and in contrast to alginate were able to form stable hydrogels with magnesium. Structural EPS hydrogel stiffness increased for transition metal ions with increasing atomic number with the exception of copper. These results indicated a quite different chemistry for structural EPS versus alginate. It should be noted that reconstituted structural EPS beads had a weaker strength than original aerobic granules. This could not be quantified with the current used methods.

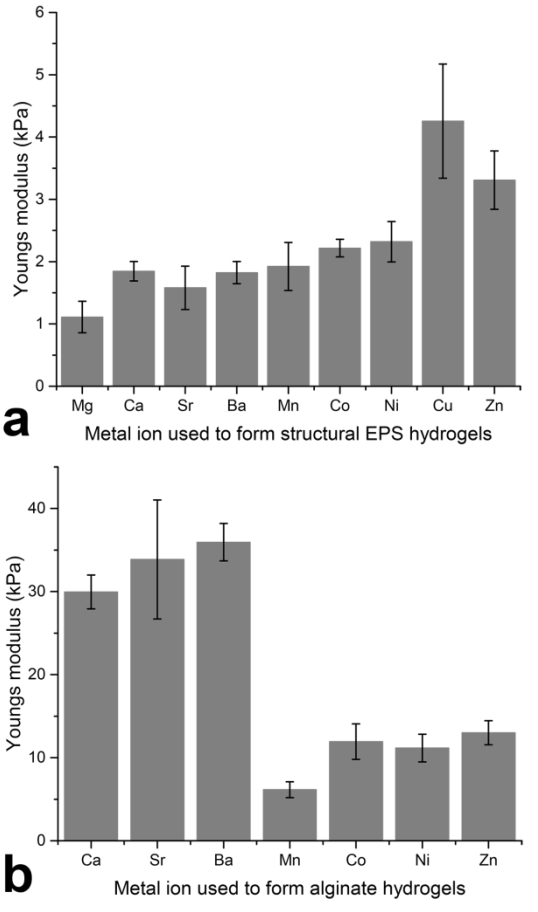


Figure 1: Dynamic mechanical analysis of the gel stiffness quantified in Young's modulus of hydrogels with divalent metal ions of (a) 10 % (w/v) structural EPS with Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} and (b) 2.5 % (w/v) alginate with Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}.

3.2 Environmental scanning electron microscopy (ESEM) of structural EPS hydrogels

ESEM analysis of freeze-dried structural EPS hydrogels was performed to visualize structural homogeneity and structural differences of gels prepared with different metal ions. For this evaluation the ions Mg^{2+}, Ca^{2+} and Zn^{2+} were selected as these ions showed different trends for alginate and structural EPS in terms of gel stiffness (section 3.1). When discussing (E)SEM pictures it is important to take into account that ESEM pictures do only show a small section of the freeze-dried hydrogel and do not resemble the actual structure of the original hydrogel. The gel structure can be disrupted by released liquids or collapse during the freeze-drying procedure. Despite these drawbacks, ESEM pictures can still be used to illustrate overall structural variations of different hydrogels.

Freeze-dried structural EPS hydrogels formed with Mg^{2+}, Ca^{2+} and Zn^{2+} featured different structural patterns indicating that the metal ion had an impact on the overall structure (Figure 2a – 2c). Independent of the metal ion inhomogeneous structures were present. The visible cavities in the three freeze-dried gels can be structural elements from the original gel or a result of released liquids. Alginate hydrogel structure analysis with SEM was also affected by the drying process and showed structural variations depending on the metal ion (Ouwerx et al., 1998; Topuz et al., 2012; Ye et al., 2017).

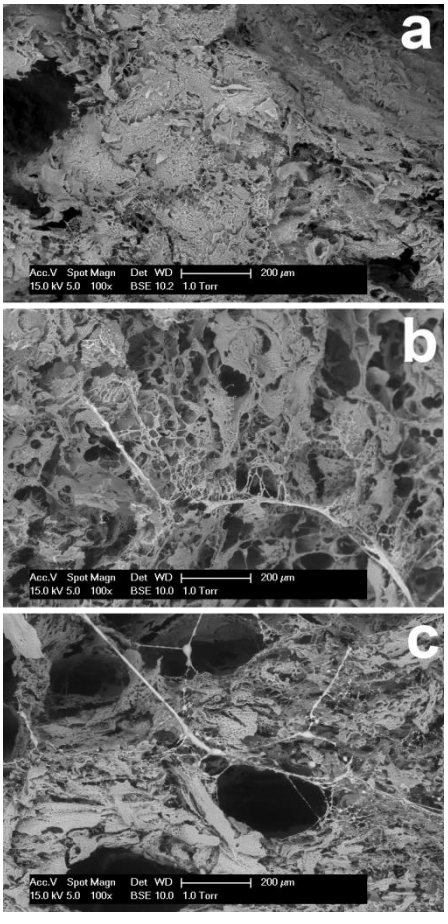


Figure 2: ESEM analysis of freeze-dried structural EPS hydrogels with different metal ions at 100 times magnification. Freeze-dried gels were prepared with (a) magnesium, (b) calcium and (c) zinc.

3.3 Gel stability of hydrogel beads towards the chelating reagent EDTA

EDTA is a common chelating agent used to complex metal ions (Harris, 2010). It can also be applied to extract EPS from biofilms (Liu and Fang, 2002) and was demonstrated to dissolve alginate gels (Schweiger, 1964). EDTA solutions of different pH were prepared to evaluate the impact of the available carboxyl groups of EDTA (Harris, 2010) on the disintegration of the hydrogel beads. For this experiment the concentration of EDTA used was the same as that reported to extract EPS from biofilms (Liu and Fang, 2002). Hydrogel beads were prepared with Ca^{2+}, Cu^{2+} and Zn^{2+}. These metal ions were selected as representative ions as Ca^{2+} and Cu^{2+} gave stiff hydrogels with alginate and Cu^{2+} and Zn^{2+} with structural EPS. Furthermore Cu^{2+} and Zn^{2+} were reported to bind with proteins (Grenács and Sóvágó, 2014; Morgan, 1981).

To further understand the gel properties of structural EPS, the gel stability towards the strong chelating agent EDTA was tested in comparison to the acidic polysaccharides alginate, polygalacturonic acid and κ-carrageenan. Polygalacturonic acid was chosen because galacturonic acid was shown to be present in structural EPS (Felz et al., 2019) and it was selected instead of pectin as de-esterification likely occurred during the alkaline extraction of structural EPS (Castro et al., 2017). K-carrageenan was included to evaluate polysaccharides with different gelling mechanism and other acidic groups, e.g. sulfate half-esters. Polysaccharides containing acidic groups other than carboxyl groups were of interest as the elemental analysis (Supplementary material D) illustrated significant amounts of sulfur and phosphorous in structural EPS. Regarding previous research on EPS this can indicate the presence of sulfate and phosphate groups (Amjres et al., 2015; Boleij et al., 2018; Bourven et al., 2015; Guibaud et al., 2005; Mata et al., 2006; Toner et al., 2005).

Independent of the metal ion and the pH of the EDTA solution all polysaccharide hydrogel beads were disintegrated and dissolved after 2 hours. Structural EPS hydrogel beads were disintegrated at pH 10.5 after 24 hours and completely dissolved after 48 hours. At pH 4.5 – 8.5 structural EPS hydrogel beads were still present after one month. While there was no visible change at pH 4.5 after 24 hours, leaching out of some components of the structural EPS hydrogel took place at pH 6.5 and 8.5 (Figure 3a). This leaching out continued throughout the measurement at all pH levels, resulting in a turbidity increase of the solution while the hydrogel beads got a lighter color (Figure 3b). The visual stability of structural EPS hydrogel beads towards EDTA was the same irrespective of the metal ion. Pictures shown were taken after one day incubation since at pH 4.5 – 8.5 there was only a small difference in the appearance of hydrogel beads after 2 hours. The different stability towards EDTA of hydrogel beads of structural EPS and the here analyzed polysaccharides indicated a different gelling or complexing mechanism of structural EPS and the polysaccharides.

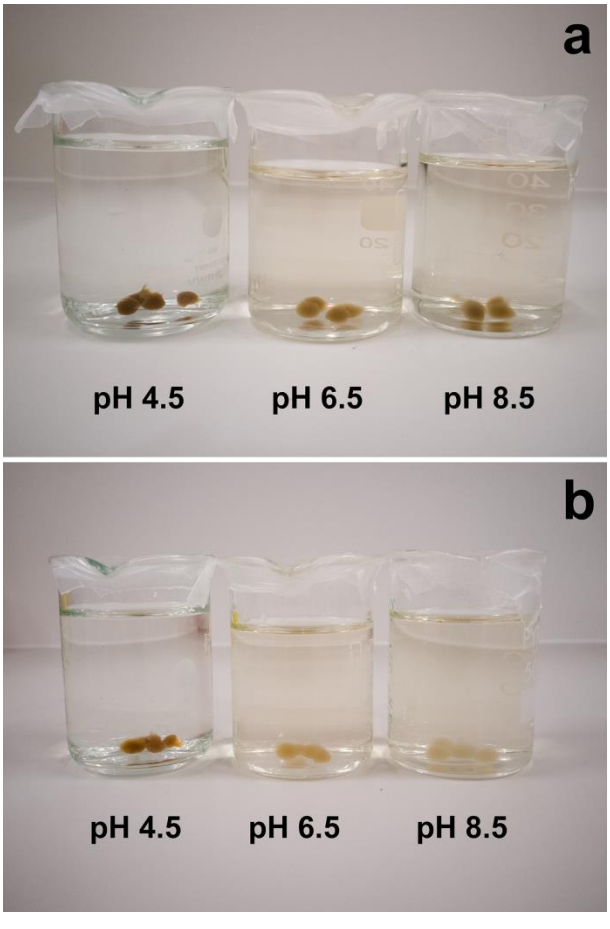


Figure 3: Ca-structural EPS hydrogel beads in 2 % (w/v) EDTA solutions at different pH levels after storage at room temperature for (a) one day and (b) for one month.

4. Discussion

4.1 Comparison of structural EPS and alginate hydrogels

Previous studies showed some similarities of alginate and structural EPS (Lin et al., 2010, 2008). Both are alkaline extracted, form stable hydrogels with Ca^{2+} and contain uronic acids (Lin et al., 2010; McHugh, 2003). Recent analysis on structural EPS illustrated structural EPS to be much more complex than alginate (Felz et al., 2019). Alginate is a linear polysaccharide composed of alternating guluronic and mannuronic acid units (McHugh, 2003). Structural EPS is a mixture of proteins, neutral sugars, amino sugars, uronic acids and humic compounds (Felz et al., 2019). Alginate is a very well characterized polymer and reported as constituent of biofilm EPS (Davies and Geesey,

1995; Remminghorst and Rehm, 2006). For this reason, despite the compositional differences of alginate and structural EPS, alginate is an adequate reference as a starting point for investigating and better understanding the properties of structural EPS hydrogels.

The results from this study clearly illustrated differences of alginate and structural EPS hydrogels. Independent of the metal ion, the absolute stiffness values of alginate hydrogels were multiple times higher than those of structural EPS. Alginate formed stiffer hydrogels with copper and alkaline earth metals than with transition metals (Chan et al., 2011; Ouwerx et al., 1998; Straccia et al., 2015). In contrast to alginate structural EPS formed stiffer hydrogels with Zn^{2+} and transition metal ions, especially with copper, than with alkaline earth metals. Interestingly structural EPS were able to form a stable hydrogel with Mg^{2+} which was not possible for alginate. Alginate was reported to from only weak gels with Mg^{2+} (Topuz et al., 2012). The structure of both, dried alginate gels and freeze-dried structural EPS gels was affected by the metal ion used to form the hydrogel and showed different structural patterns (Ouwerx et al., 1998; Topuz et al., 2012). The inhomogeneous structures of dried structural EPS gels can result from the high complexity of their composition and the various molecular interactions. Compared to alginate, structural EPS hydrogels demonstrated a significantly stronger resistance towards disintegration in the presence of the complexing agent EDTA.

4.2. Hydrogel stiffness

Gel formation of alginate and pectin, the partly methylated form of polygalacturonic acid, are considered to be similar (Morris et al., 1982). Both polysaccharides form stiffer hydrogels with calcium ions than structural EPS (Chan et al., 2011; Markov et al., 2017; Ouwerx et al., 1998). The stiffness of alginate and pectin hydrogels is closely related to their composition. A high guluronic acid content in alginate results in the formation of stiff hydrogels due to crosslinking of guluronic acid blocks (Chan et al., 2011; Mancini et al., 1999). Mannuronic acid rich alginates are weaker and more flexible than guluronic acid rich alginates (Mancini et al., 1999; Smidsrød and Haug, 1972). The stiffness and calcium ion uptake of pectin hydrogels is influenced by the degree and distribution of methylation as well as by the occurrence of rhamnose gaps in the galacturonic acid chain (Axelos and Thibault, 1991; Powell et al., 1982; Ström et al., 2007; Thibault and Rinaudo, 1985). This shows that not only the amount of available carboxyl groups, but also the conformation and distribution of carboxyl groups are of importance for the hydrogel stiffness. Alginates and pectins have a very large amount of available carboxyl groups which are distributed on a linear polysaccharide chain. Previous analysis of structural EPS has indicated that structural EPS consist of proteins and a much higher diversity of sugar monomers than alginates (Felz et al., 2019). It is therefore not

surprisingly that gelling with ions that complex with carboxyl groups gave weaker gels for structural EPS than for alginate.

4.3 Metal ion sequence of hydrogel stiffness

A correlation of metal ion affinity of alginate and its hydrogel stiffness was reported (Chan et al., 2011; Ouwerx et al., 1998; Smidsrød and Haug, 1968). The ion selectivity is influenced by the available functional groups and the conformation of a polymer (Haug and Smidsrød, 1970; Smidsrød and Haug, 1968). Proteins, sugars, uronic acids and polyphenolic compounds in structural EPS can interact with metal ions and have different metal ion affinities.

Proteins were shown to bind with copper and zinc (Grenács and Sóvágó, 2014; Morgan, 1981) and to gel with ions (Munialo et al., 2017). Multiple amino acids have a higher stability constant with transition metal ions than with alkaline earth metals (Berthon, 1995; Furia, 1972). The binding of proteins with zinc was indicated in aerobic granular sludge (Wei et al., 2016) and copper was illustrated to bind with proteins and humic substances in activated sludge EPS (Sheng et al., 2013). Humic compounds were demonstrated to have a good binding to transition and alkaline earth metals (Mantoura et al., 1978; Pandey et al., 2000). The phosphate group is another functional group that can be involved in the gelling process of structural EPS. The metal binding of phosphate groups in EPS was previously suggested (Guibaud et al., 2005; Toner et al., 2005) and the presence of phosphorous in structural EPS is an indication for phosphate groups. Phosphate groups of biological molecules were demonstrated to bind with alkaline earth metals (Burton, 1959) and polyphosphates were shown to favor transition over alkaline earth metal ions (Wazer and Campanella, 1950).

The ion preference of compounds containing functional groups also present in structural EPS can be the reason for structural EPS to form stiffer hydrogels with transition than with alkaline earth metal ions. The ability of amino acids and phosphates to bind with magnesium (Berthon, 1995; Burton, 1959; Furia, 1972; Wazer and Campanella, 1950) can explain stronger Mg^{2+} hydrogels of structural EPS than of alginate. An experimental analysis of metal selectivity towards structural EPS and additional analysis of functional groups present in structural EPS will further strengthen this assumption.

4.4 Stability towards dissolution by EDTA

Exposing the hydrogel beads of the polysaccharides alginate, polygalacturonic acid and κ-carrageenan to EDTA quickly resulted in the dissolution of these hydrogels. Structural EPS hydrogels showed a stronger integrity in the presence EDTA and were only quickly dissolved with EDTA at pH 10.5. The dissolution at this pH is in accordance with structural EPS also being extracted at pH 10.5 thus being well soluble under these

conditions. The partial leaching out of structural EPS hydrogel compounds at lower pH levels was closely related to the pH increase which was the result of two different effects. Increasing the pH approaches the extraction pH for structural EPS and increases the binding capacity of EDTA for divalent cations (Harris, 2010).

The stability of structural EPS hydrogels towards EDTA is difficult to explain. Independent of the metal ion EDTA has very high stability constants with metal ions which are generally higher than those of compounds present in structural EPS (Berthon, 1995; Furia, 1972). There are different possibilities for the stability of structural EPS hydrogels towards EDTA. Structural EPS contain proteins, carboxyl and possibly sulfate and phosphate groups. Proteins can interact with phosphate and sulfate (Chakrabarti, 1993; Copley and Barton, 1994) and can gel through covalent and non-covalent interactions (Munialo et al., 2017). Thus compounds of structural EPS may have not only interacted with the metal ions, but also with each other during the gel formation. These interactions could be the reason for the hydrogel to remain intact, even when metal ions were removed from the gel by EDTA. Furthermore the tertiary structure formed by structural EPS could hinder or retard the complexing of metal ions by EDTA. This indicates a strong metal binding that strengthens the assumption of EPS being a protective layer for microbial cells against increased metal concentrations (Guibaud et al., 2005). The structure and properties of gel-forming bacterial polysaccharides were reported to be stabilized by hydrogen bonding (gellan, granulan) (Chandrasekaran and Radha, 1995; Seviour et al., 2012a) and hydrophobic interactions (curdlan) (Zhang and Edgar, 2014). It is possible that such intermolecular interactions are also present in structural EPS hydrogels and that hydrophobic interactions are involved in shielding metal ions from chelation by EDTA. These possibilities however are speculative and need to be further investigated. More in-depths analysis of structural EPS hydrogels can also reveal if distinct gelling mechanisms such as the for alginate described egg-box model (Braccini and Pérez, 2001; Sikorski et al., 2007) are involved in the gel formation.

The gel beads were still visibly intact, but there was a slow and partial leaching out of structural EPS at pH 4.5 – 8.5. This can indicate a slow disintegration of the overall gel structure or a dissolution of structural EPS compounds which were not or only little involved in the hydrogel structure. It is possible that the gel was still present, but with lower stiffness than before the treatment with EDTA. Analyzing the amount of EDTA complexed metals, the EDTA released structural EPS compounds and comparing the stiffness of the gel beads before and after treatment will give more information on the gelling and gel stability of structural EPS. Additionally more insights into the gelling mechanisms of structural EPS can be obtained by isothermal titration calorimetry (ITC). This technique was already used to investigate the gelling mechanism of alginate (Fang et al., 2007). ITC is a very sensitive analysis which allows to measure ion-polymer and

polymer-polymer interactions. Once the composition of structural EPS is better known, this technique will be valuable to further analyze the interactions of structural EPS with itself and metal ions.

4.5 Hydrogel analysis of structural EPS and aerobic granular sludge

The here reported findings gave new insights into the metal hydrogel properties of extracted structural EPS from aerobic granular sludge in terms of gel stiffness and gel stability. Structural EPS are considered to be responsible for the structure of the granular matrix (Felz et al., 2016). In this study structural EPS were shown to form stable hydrogels with multiple metal ions, and several molecular interactions were suggested to be involved in the gel formation and stability. Not only extracted structural EPS interact with metal ions and behave like hydrogels, but also the granule itself (Seviour et al., 2009a; Yu et al., 2001). Considering the previously demonstrated effects of calcium ions on biofilms with stimulating faster granulation (Yu et al., 2001) and stiffer biofilms (Körstgens et al., 2001) and the here obtained results of different metals resulting in different stiffness and structural features of EPS hydrogels, the composition of present metal ions in the medium around a biofilm will likely also affect the stiffness and structure of intact biofilms.

The here used structural EPS were extracted from the granular sludge and were not present in their native form. Based on the here obtained results it cannot be stated to which extent structural EPS contribute to the overall stiffness of intact granules. For instance the alkaline extraction procedure will lead to (partial) deacetylation of the sugars (Castro et al., 2017) thus changing the composition of the extracted structural EPS. Stiffness values quantified as Young's modulus of intact biofilms of different origin were already reported (Blauert et al., 2015; Körstgens et al., 2001). The stiffness measurement of intact granules will also be beneficial here. To compare the stiffness of granules and structural EPS hydrogel beads can illustrate to what extent extracted structural EPS resemble the granular stability. This would also indicate to which extent the high granular stability is related to the combination of cells linked to EPS or to EPS only. Expanding this analysis on other types of biofilm will help to understand the stability of biofilms and the impact of EPS on biofilm stability and strength in general.

5. Conclusion

- Structural EPS favor transition metals over alkaline earth metals to form stiff hydrogels
- Structural EPS are highly complex and have a different gelling mechanism than the acidic polysaccharides alginate, polygalacturonic acid, κ-carrageenan
- Structural EPS hydrogels show strong integrity towards the chelating reagent EDTA

Supplementary Material

Supplementary material A: Alginate composition

Table A: Composition of alginate isolated from the stipe of *Laminaria hyperborea*. The composition is presented in the fractions of guluronic acid F(G), mannuronic acid F(M), guluronic acid dimers F(GG), mannuronic acid dimers F(MM) and alternating guluronic and mannuronic acid dimers F(GM+MG). The size of the used alginate was 220 kDa. The composition was provided by Olav Andreas Aarstad (Aarstad et al., 2012).

F(G)	F(M)	F(GG)	F(MM)	F(GM+MG)
0.68	0.32	0.57	0.21	0.22

Supplementary material B: X-ray fluorescence analysis of aerobic granular sludge

2 g freeze-dried granules were grinded to a fine powder and mixed with 0.5 g Boreox® binder (Fluxana). The mixture was further grinded and added evenly in a steel mold. On top of the mixture another 0.5 g Boreox® binder was evenly added. The sample-binder mixture was compressed at 200 kp/m². The obtained pellet was analyzed with a Panalytical Axios-Max WD-XRF with Super-Q software. The content of carbon, nitrogen and oxygen in the granular sludge was approximated with the elemental composition of structural EPS. The obtained results are illustrated in Figure B.

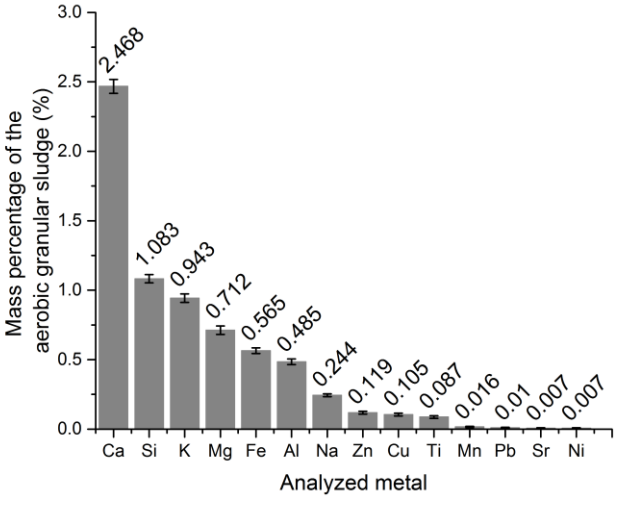


Figure B: X-ray fluorescence measurement of metals in aerobic granular sludge. Results are presented as mass percentage of the freeze-dried sludge sample.

Supplementary material C: pH values of metal solutions utilized to form structural EPS and alginate hydrogels

Table C: pH values of the solutions containing the metal ions used to form hydrogel cylinders with structural EPS and alginate.

Metal ion	Mg^{2+}	Ca^{2+}	Sr^{2+}	Ba^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}
pH of the metal solution (-)	5.94	5.45	5.84	6.42	4.24	5.11	5.64	3.82	6.58

Supplementary material D: Elemental analysis of C, H, N, P and S in structural EPS from aerobic granular sludge

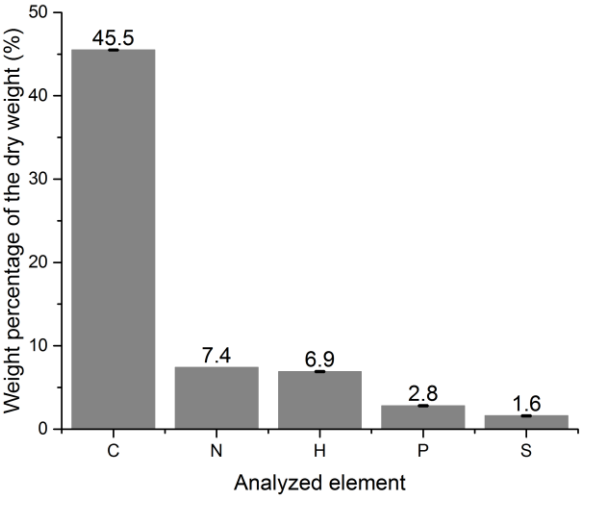
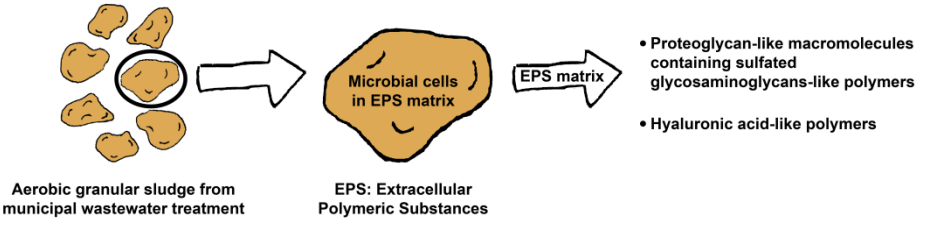


Figure D: Elemental composition of structural EPS from aerobic granular sludge in terms of carbon, nitrogen, hydrogen, phosphorous and sulfur content in weight percentages of the dry weight. Analysis was performed by Mikroanalytisches Laboratorium Kolbe, Oberhausen, Germany.

Chapter 5

Aerobic Granular Sludge Contains Hyaluronic acid-like and Sulfated Glycosaminoglycans-like Polymers



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Abstract

Glycosaminoglycans (GAGs) are linear heteropolysaccharides containing a derivative of an amino sugar. The possibility of the presence of GAGs in aerobic granular sludge was studied by combining SDS-PAGE with Alcian Blue staining (at pH 2.5 and 1), FTIR, mammalian Hyaluronic acid and sulfated GAG analysis kits, enzymatic digestion and specific in situ visualization by Heparin Red and lectin staining. GAGs, including Hyaluronic acid-like and sulfated GAGs-like polymers were found in aerobic granular sludge. The sulfated GAGs-like polymers contained Chondroitin sulfate and Heparan sulfate/Heparin based on their sensitivity to the digestion by Chondroitinase ABC and Heparinase I & III. Heparin Red and lectin staining demonstrated that, the sulfated GAGs-like polymers were not only present in the extracellular matrix, but also filled in the space between the cells inside the microcolonies. The GAGs-like polymers in aerobic granules were different from those produced by pathogenic bacteria but resemble those produced by vertebrates. Findings reported here and in previous studies on granular sludge described in literature indicate that GAGs-like polymers might be widespread in granular sludge/biofilm and contribute to the stability of these systems. The extracellular polymeric substances (EPS) in granular sludge/ biofilm are far more complicated than they are currently appreciated. Integrated and multidisciplinary analyses are significantly required to study the EPS.

1. Introduction

The aerobic granular sludge process is a wastewater treatment process which gained increasing popularity as an alternative to the conventional flocculent sludge processes (Pronk et al., 2015). The sludge granules consist of bacteria embedded in a matrix of extracellular polymeric substances (EPS) (Lin et al., 2010; Seviour et al., 2009b). The EPS not only provide a structural matrix in which cells can be embedded, but also have various compositions and properties (Flemming and Wingender, 2010).

Recent studies on EPS composition revealed that not only proteins and polysaccharides are the components of EPS, but complex glycoconjugates are present as well. E.g. a highly abundant glycoprotein, carrying a heterogeneous O-glycan structure, was identified in the EPS of anaerobic ammonium oxidation (anammox) granular sludge enriched with *Ca. Brocadia sapporoensis* (Boleij et al., 2018; Varki, 2017). Glycoproteins were hypothesized to be strongly involved in the structure of anammox granules, having a similar role as glycans in the extracellular matrix (ECM) of multicellular organisms like vertebrates (Boleij et al., 2018; Varki, 2017). In seawater-adapted aerobic granular sludge, sialic acids present as sialoglycoproteins were widely distributed in the EPS. Sialic acids were covering the penultimate galactose unit by this providing stability of the sugar chain (de Graaff et al., 2019). Glycoconjugates have also been found in saline anaerobic granular sludge. The major sugar monomers reported were mannose and N-acetyl galactosamine (Gagliano et al., 2018). The amino sugars galactosamine and glucosamine, the uronic acids glucuronic acid and galacturonic acid and several neutral sugars were identified as monomers in the structural EPS of aerobic granular sludge (Felz et al., 2019). The presence of amino sugars and glucuronic acid, as well as the highly negative charge of structural EPS and their gel-forming ability (Felz et al., 2016) indicate that glycosaminoglycans (GAGs) might be another type of glycoconjugates in the EPS of granular sludge. GAGs are heteropolysaccharides that contain a hexosamine (either glucosamine or galactosamine, generally N-acetylated) and a uronic acid (glucuronic/iduronic acid) or galactose as part of their repeating unit (Esko and Lindahl, 2001; Silbert and Sugumaran, 2002).

GAGs are building blocks of the ECM in mammalian cells and can be covalently bound to proteins, forming a large and negatively charged proteoglycan (Lindahl et al., 2017; Williams, 1998). By attracting cations and water molecules, these macromolecules form a hydrated gel. Proteoglycans are an important constituent of the connective tissue forming the ground substance which facilitates structural support and enables the diffusion of soluble compounds, e.g. nutrients or signaling molecules, and cell migration within the ECM (Williams, 1998). It is interesting to investigate if there are similar macromolecules present in the EPS of granular sludge. Due to the fact that amino sugars (e.g hexosamines) do not react with phenol-sulfuric acid assay (Felz et al., 2019; Manzi

and Esko, 1995), the presence of GAGs might be overlooked by the commonly used colorimetric methods. Thus, it is significantly necessary to establish appropriate methodologies to investigate the possible existence of GAGs in granular sludge or biofilms.

In the current research, it is assumed that there are various GAGs (e.g. Hyaluronic acid, Chondroitin sulfate and Heparan sulfate/Heparin) in the EPS of aerobic granular sludge, which are similar to the polymers forming the ECM in higher organisms. Their possible presence was evaluated by commercial extracellular matrix assays (for use with mammalian cells, tissues and fluids), specific enzymatic digestion and FTIR. The location of highly negatively charged macromolecules (e.g. sulfated GAGs) in granular sludge was visualized by Heparin Red staining.

2. Materials and Methods

The analysis of GAGs-like polymers (including Hyaluronic acid-like and sulfated GAGs-like polymers) in aerobic granular sludge was summarized in Figure 1.

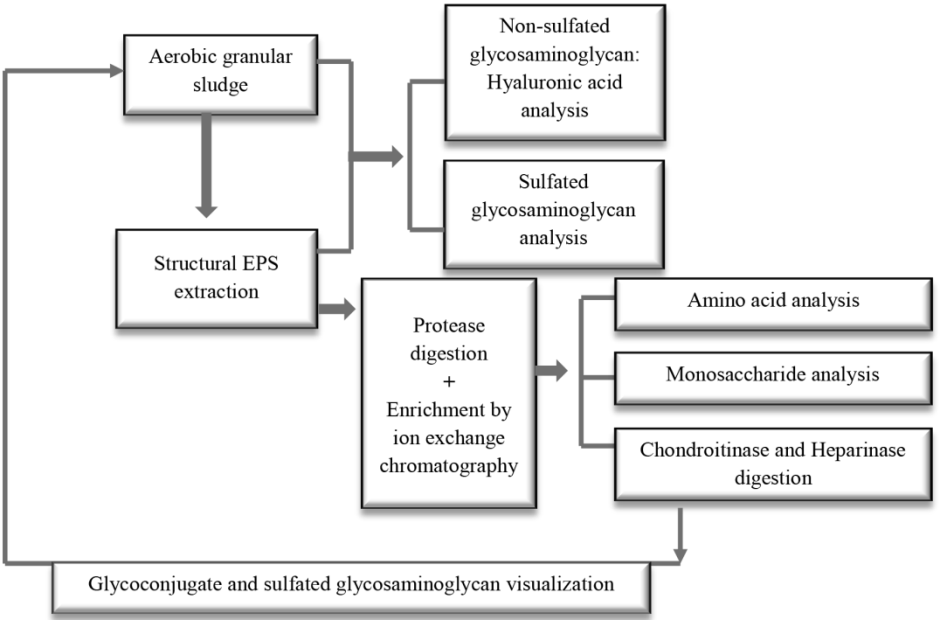

Figure 1: Schema for analyzing glycosaminoglycans-like polymers in aerobic granular sludge.

2.1 Aerobic granular sludge collection

Aerobic granular sludge was collected from the municipal wastewater treatment plant Dinxperlo, the Netherlands in July 2017 (Royal HaskoningDHV, 2018). The granular sludge was used to decrease COD, total phosphorous and total nitrogen in the wastewater. The reactor was operated with 1 hour anaerobic feeding and 4 hours aeration. The sludge volume index ($SVI_{30\ min}$) was 50 mL/g_{VSS} and the average solid retention time (SRT) was > 30 days. The sludge was sampled at the end of an aerobic cycle. Collected granular sludge was sieved, washed with demi-water, frozen at -80°C and lyophilized.

2.2 Extraction of structural EPS from aerobic granular sludge and SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) analysis

Structural EPS were extracted as described previously (Felz et al., 2019), frozen at -80°C and lyophilized. The organic and ash fractions were quantified according to the standard methods (APHA, 1998).

Structural EPS were analyzed by SDS-PAGE, as described in Boleij et al. (2018) using NuPage® Novex 4-12% Bis-Tris gels (Invitrogen). The gels were stained by following three different staining protocols. Proteins were visualized with Coomassie Blue staining (Colloidal Blue staining kit, Invitrogen) according to manufacturer's instructions. Glycoprotein staining was performed based on the periodic acid-Schiff (PAS) method using the Thermo Scientific Piercie Glycoproteins Staining Kit, which is specific for glycans containing vicinal hydroxyl groups. For staining of carboxyl and sulfate groups (Shori et al., 2001), Alcian blue staining at pH 2.5 (carboxyl-rich glycoconjugates and/or sulfated glycoconjugates) and pH 1.0 (sulfated glycoconjugates) were performed separately.

2.3 Analysis of hyaluronic acid content in aerobic granular sludge and the structural EPS

The Hyaluronic acid content in both lyophilized aerobic granular sludge and isolated structural EPS was measured by following the protocol of the Purple-Jelley™ Hyaluronan Assay provided by biocolor (UK). In brief, the sample (15 mg, dry weight) was digested by proteinase K (0.5 mg/mL) overnight at 55°C. After centrifugation at 12,000×g for 10 min, the supernatant was collected. Hyaluronic acid in the supernatant was recovered by a sequence of precipitation steps using ethanol saturated with sodium acetate and cetylpyridinium chloride containing sodium chloride. Afterwards, Hyaluronic acid was precipitated by ethanol (98%) and fully hydrated with 100 µL of water. The extracted Hyaluronic acid gel was quantified by using 3,3'-Diethyl-9-methyl-4,5,4',5'-dibenzothia carbocyanine bromide supplied in 55% solution of dimethyl sulfoxide.

The Fourier transform infra-red (FTIR) spectrum of the recovered Hyaluronic acid and the Hyaluronic acid standard (extracted from rooster comb) was recorded on a FTIR Spectrometer (Perkin Elmer, Shelton, USA) at room temperature, with a wavenumber range from 600 cm^{-1} to 4000 cm^{-1}.

2.4 Analysis of sulfated GAGs in aerobic granular sludge and structural EPS

The sulfated GAGs content in both lyophilized aerobic granular sludge and structural EPS was measured by following the protocol of the Blyscan™ glycosaminoglycan assay provided by biocolor (UK). In brief, the sample (1.5 mg, dry weight) was digested by papain extraction reagent overnight at 65°C. After centrifugation at 10,000×g for 10 min, the supernatant was collected. Total sulfated GAGs were quantified by using the Blyscan™ dye reagent containing 1,9-dimethyl-methylene blue (DMMB) with bovine tracheal Chondroitin-4-sulfate as the standard. In addition, the ratio of O-and N-sulfated GAGs within the test samples was determined by following the nitrous acid cleavage method. Nitrous acid reacts with the N-sulfated-D-glucosamine and cleaves the N-sulfated site (Bienkowski and Conrad, 1985). After this reaction, the amount of O-sulfated GAGs was measured again using the Blyscan™ protocol. The difference between the total sulfated GAGs and the amount of O-sulfated GAGs gave the amount of N-sulfated GAGs.

In animal tissue, Hyaluronic acid is a non-sulfated GAG and is not covalently bound to proteins. In contrast, the other GAGs are sulfated and are covalently bound to a protein backbone to form proteoglycans (Lindahl et al., 2017). In order to study if the sulfated GAGs in granular sludge are also covalently bound to proteins, proteinase digestion was performed on structural EPS to remove proteins which are not covalently bound to GAGs.

2.4.1 Proteinase digestion of structural EPS

Structural EPS were digested with papain and proteinase K in sequence. Papain digestion was performed as described in the Blyscan™ protocol (biocolor, UK). Lyophilized structural EPS (200 mg) was digested in 100 mL papain extraction reagent containing 5 mg papain at 65°C for 24 hours. 5 mg papain was added afterwards and the mixture was incubated at 65°C for another 24 hours. The mixture was heated to 100°C for 5 min to deactivate the enzyme, and dialyzed overnight against demineralized water in a dialysis bag (molecular weight cut-off (MWCO) 3.5 kDa).

Subsequently, the dialyzed mixture was further digested by proteinase K according to the manufacturer's instructions with modifications (5 mg proteinase K in 50 mM Tris (pH 8), 4 mM calcium chloride and 10 mM sodium chloride) at 37°C for 24 hours with gentle mixing. After 24 hours another 5 mg of proteinase K were added and the

digestion continued for 24 hours. When the digestion was finished, the mixture was heated to 100°C for 5 min to deactivate the enzyme, and dialyzed overnight against demineralized water in a dialysis bag (MWCO 3.5 kDa). Consequently, the content inside the dialysis bag was centrifuged at 4,000×g for 20 min and the supernatant was collected and lyophilized.

2.4.2 Enrichment of sulfated GAGs containing polypeptides by ion exchange chromatography

Lyophilized protease digested structural EPS were dissolved in 7 M urea containing 30 mM Tris (pH 8.5) and filtered through a 0.45 µm PVDF filter (Millex®HV Millipore®). The filtrate was separated with DEAE Sepharose® fast flow resin (GE healthcare) in a XK 16 column (GE healthcare) with a column volume of 30 mL using a NGC medium pressure chromatography system (Biorad). The column was washed with three column volumes of 7 M urea containing 30 mM Tris (pH 8.5) prior to introducing the sample. The sample was applied at a flow rate of 3 mL/min. After sample application on the column, the column was washed with 1.5 column volumes of 7 M urea containing 30 mM Tris (pH 8.5). Sample elution was performed with ten column volumes of a sodium chloride gradient from 0 – 2 M. Fractions of 5 mL were collected. Absorbance intensity was measured at 215 nm, 280 nm and 350 nm. After the elution, 50 µL of each fraction was mixed with 200 µL DMMB reagent to determine the sGAG-like containing fractions (Zheng and Levenston, 2015). Fractions showing precipitation and lower absorbance intensity at both 215 nm and 280 nm which were eluted at sodium chloride concentrations of 0.8 – 1 M were combined (Supplementary material A), dialyzed against demineralized water in a dialysis bag with 3.5 kDa MWCO, frozen at -80°C and lyophilized. The reason of collecting these specific fractions is as follows: 1) there is still a signal of the peptide bond which indicates the presence of polypeptides; 2) at the same time the sodium chloride concentration (mainly Cl^-) is high enough to exchange compounds that contain highly negatively charged groups, e.g. sulfated GAGs containing O-sulfate groups and N-sulfate groups. Sulfated GAGs content of the combined fraction was quantified by the Blyscan™ kit (biocolor, UK).

2.4.3 Monosaccharide and amino acid analysis of the enriched sulfated GAGs containing polypeptides fraction

Monosaccharide analysis of the combined fraction was performed as described previously (Felz et al., 2019). In short, samples were hydrolyzed at a concentration of 10 mg/mL in 1 M hydrochloric acid at 105°C for 8 hours. The hydrolyzed sample was centrifuged at 10,000×g for 5 min and the supernatant was collected. The supernatant was neutralized with 1 M sodium hydroxide, diluted 1:5 with ultrapure water and

filtered through a 0.45 µm PVDF filter. The sample was analyzed by a Dionex ICS 5000$^+$ HPAEC-PAD with an AminoTrap pre-column (Dionex) and a PA20 column (Dionex).

The amino acids were analyzed by gas chromatography – mass spectrometry (GC-MS) after acid hydrolysis. Hydrolysis was performed with sample concentrations of 2 mg/mL in 6 M hydrochloric acid at 105°C for 24 hours. After hydrolysis samples were neutralized with 6 M sodium hydroxide and centrifuged at 10,000×g for 5 min. The supernatant was filtered with a 0.45 µm PVDF filter and diluted 1:5 with ultrapure water. Then 100 µL of each diluted sample were mixed with 20 µL of an internal amino acid standard (Wahl et al., 2014) in a GC-vial and lyophilized. Each lyophilized sample was mixed with 75 µL acetonitrile and 75 µL N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), heated for 60 min at 70°C in a heating block. After cooling down to room temperature, samples were centrifuged at 10,000×g for 2 min. Finally 80 µL of each sample were subsequently analyzed with GC-MS (7890A GC (Agilent) together with a 5975C single quadrupole mass spectrometer (Agilent)) as described previously (de Jonge et al., 2011). A sample of 1 µL was injected on a Zebron ZB-50 column (30 m × 250 µm internal diameter, 0.25 µm film thickness; Phenomenex, Torrance, Ca, USA) for injection in splitless mode by a programmed temperature vaporizer (PTV; Gerstel, Mühlheim, Germany). Straight glass liners with glass wool were utilized (Agilent). MS was operated in selected ion monitoring mode and the quantification of the amino acids was performed by isotope dilution mass spectrometry.

2.4.4 Enzymatic digestion of the enriched sulfated GAGs containing polypeptides with Chondroitinase ABC and Heparinase I&III

In order to understand if there were Chondroitin sulfate and/or Heparan sulfate (including Heparin) in the enriched sulfate GAGs, Chondroitinase ABC from *Proteus vulgaris* (Sigma-Aldrich) and a blend of Heparinase I&III from *Flavobacterium heparinum* (Sigma-Aldrich) were used, respectively for digestion. The specificity of the enzymes is described in Supplementary material B. Samples (1 mg/mL) were digested according to manufacturer's instructions, with Chondroitinase ABC (0.1 mg/mL) at 37°C and pH 8 in 0.01 % (w/v) bovine serum albumin (BSA), 50 mM Tris and 60 mM sodium acetate for 24 hours. In parallel, samples (1 mg/mL) were digested with Heparinase I&III (0.1 mg/mL) at 25°C and pH 7.5 in 0.01 % (w/v) BSA, 20 mM Tris, 50 mM sodium chloride and 4 mM calcium chloride for 24 hours. The amount of sulfated GAGs in the samples after digestion was quantified by using the Blyscan™ glycosaminoglycan assay (biocolor, UK).

2.5 Visualization of highly negatively charged macromolecules (e.g. Heparan Sulfate/Heparin) and other glycoconjugates in aerobic granular sludge

Highly negatively charged macromolecules (e.g. Heparan sulfate/Heparin) were localized in the granular matrix by staining with the fluorescent probe Heparin Red (RedProbes, Münster, Germany). Staining using Heparin Red was done according to the supplier's data sheet. Briefly, 8.8 µL Heparin Red and 1 mL enhancer solution were mixed and added to the granules for 1 hour. Other glycoconjugates were examined on the basis of fluorescence lectin bar-coding with subsequent fluorescence lectin-binding analysis (Neu and Kuhlicke, 2017). For this purpose all commercially available lectins (FITC, Fluorescein, Alexa488) were screened and individually tested for binding. Granules were stained with lectins (0.1 mg/mL) for 20 min at room temperature in the dark and washed 3 times in order to remove the unbound probe. For both stainings the granules were washed with tap water and mounted in coverwell chambers (Thermofisher) with various spacers (1, 1.7, 2 mm) to keep the original shape of the granule. The samples were examined at a Leica SP5X instrument (Leica Germany) equipped with an upright microscope and a super continuum light source. The microscope was controlled by the LAS AF software version 2.4.1. Confocal images were recorded as single scan or serial scan (step size 1 or 0.5 µm) using a 25x NA 0.95 or a 63x NA 1.2 water immersion lens. Laser excitation was at 480 nm and 567 nm, emission was from 470-490 nm (reflection) and 590-650 nm (Heparin Red). For lectins, laser excitation was at 490 nm, emission was from 485-495 nm (reflection) and 505-600 nm (lectins). Images were collected with different zoom factors to either get an overview or to match optical and pixel-resolution. In order to optimize image resolution and contrast, the lectin image datasets were subjected to blind deconvolution with Huygens version 18.10.0 (SVI, The Netherlands). Data were finally projected using Imaris version 9.2.1. Heparin Red data sets were loaded in Fiji (https://fiji.sc/) and color coded with the lookup table called "rainbow". For improved color separation of pixel intensities the contrast was set to auto. All image data sets were printed from Photoshop (Adobe).

3 . Results

3.1 Extraction and analysis of structural EPS from aerobic granular sludge

The structural EPS extracted from aerobic granular sludge represented 253 ± 14 mg/g volatile content of the sludge. The glycoconjugates in the extracted EPS were further characterized using SDS-PAGE in combination with different staining protocols. Following Coomassie Blue staining, a smear was observed with the molecular weight range from 50 kDa to 10 kDa (Figure 2, lane 1), no clear bands could be seen. Alcian Blue staining was applied with pH 2.5 and pH 1.0 (Figure 2, lane 2 and 3). At pH 2.5, both carboxyl-rich glycoconjugates ($-COO^-$) and sulfated glycoconjugates ($-SO_3^-$) were stained,

while with pH 1.0 only the sulfated glycoconjugates were stained (Shori et al., 2001). A smear appeared at the high molecular weight range (above 235 kDa) with decreasing intensity until 70 kDa at both pH 2.5 and pH 1, with the intensity of the smear at pH 2.5 higher than that at pH 1, implying there are both carboxyl-rich glycoconjugates and sulfated glycoconjugates in the structural EPS. In addition, part of the structural EPS was retained in the wells. It was strongly stained by Coomassie Blue, PAS and Alcian blue (both at pH 2.5 and 1), indicating that it contained proteins/polypeptides, carboxyl-rich glycoconjugates and sulfated glycoconjugates as well.

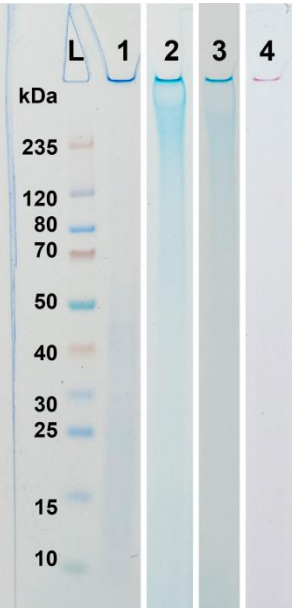

Figure 2: SDS-PAGE analysis of structural EPS. Visualization of proteins and glycoconjugates. Structural EPS was stained with Coomassie blue (1), Alcian blue at pH 2.5 (2), Alcian blue at pH 1 (3) and PAS (4). The size distribution is illustrated by a broad band ladder (L) of proteins ranging from 10 – 235 kDa.

3.2 Hyaluronic acid content in aerobic granular sludge and extracted structural EPS

The lyophilized granular sludge as well as the extracted structural EPS was treated according to the protocol provided in the commercial kit for Hyaluronic acid (HA) extraction. The extracted polymers turned purple when reacted with 3,3'-Diethyl-9-methyl-4,5,4',5'-dibenzothia carbocyanine bromide, which is the standard stain to indicate the presence of HA. Based on the calibration curve with the commercial HA standard provided in the kit, the HA content in aerobic granular sludge was determined as 294 ± 22 µg/g. In structural EPS the HA content was much lower with 15 ± 6 µg/g.

In addition, the FTIR spectra of the extracted HA from granular sludge (HA extracted from EPS was not analyzed by FTIR due to its low amount) and the commercial standard were compared. As shown in Figure 3, they were similar in: The peak at 3447 cm^{-1} and 3150 cm^{-1} are attributed to -NH and -OH stretching region. The peak at 2925 cm^{-1} can be attributed to stretching vibration of -CH. The peak at about 1580 cm^{-1} corresponds to the amide carbonyl of amino sugar and the peak at 1420 cm^{-1} is the stretching of COO^-, which refers to the acid group of HA. The peak at 1028 cm^{-1} is attributed to the linkage stretching of -C-OH (de Oliveira et al., 2017). The peaks at around 3800 cm^{-1} and 2750 cm^{-1} which only present in the spectrum of the polymer extracted from granular sludge are probably due to impurities. Therefore, based on the chemical reaction and the FTIR spectrum, it is confirmed that there is Hyaluronic acid-like polymer in aerobic granular sludge.

Hyaluronic acid contains glucuronic acid and N-acetyl-glucosamine as repeating units (de Oliveira et al., 2017). It is reported to be synthesized as an extracellular capsule by a few pathogenic bacteria (e.g. Lancefield group A and C *streptococci*) (Chong et al., 2005). The yield of Hyaluronic acid through fermentation of group C *streptococci* can reach to 0.3 g/g dry weight (Chong et al., 2005). In comparison, the amount of Hyaluronic acid in human extracellular matrix is variable, approximately 15–150 µg/g in lung tissue, 500–2500 µg/g in articular cartilage and 300-500 µg/g in skin (Cowman et al., 2015; Kuo, 2006; Piehl-Aulin et al., 2017). The amount of Hyaluronic acid-like polymer in aerobic granular sludge is comparable with that in the human skin.

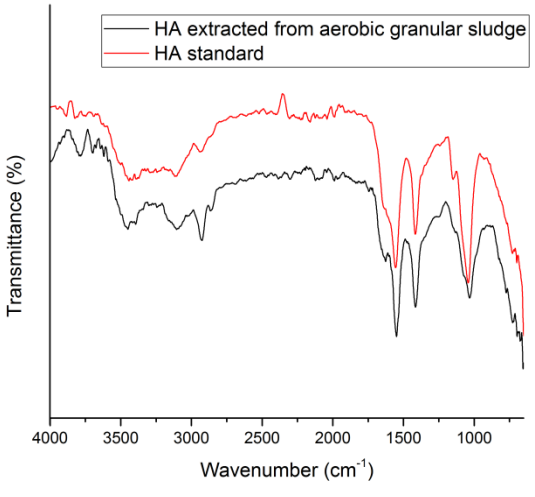

Figure 3: FTIR spectrum of the extracted Hyaluronic acid (HA) from aerobic granular sludge in comparison to the commercial standard. The two spectra were similar, especially in the wavenumber region 1750 cm^{-1} to 750 cm^{-1}.

3.3 Sulfated GAGs content in aerobic granular sludge and extracted structural EPS

Based on the reaction with the dye 1,9-dimethylmethyene blue (DMMB), the total sulfated GAGs in aerobic granular sludge was determined as 31 ± 5 mg/g in aerobic granular sludge and 87 ± 6 mg/g in structural EPS (Chondroitin-4-sulfate as the standard). If the yield of structural EPS (253 ± 14 mg/g) is considered, the extracted structural EPS contains the major part (approximately 71%) of the total sulfated GAGs in aerobic granular sludge. It is known that, there are five different kinds of sulfated GAGs in mammals: Chondroitin sulfate, Keratan sulfate and Dermatan sulfate which contain O-sulfated hexosamines; Heparin and Heparan sulfate which contain N-sulfated hexosamines. Thus, by determining the ratio between O- and N-sulfated GAGs within test samples, it is possible to detect the presence of Heparin and Heparan sulfate. The ratio between O- and N-sulfated GAGs in granular sludge and in the extracted structural EPS is listed in Table 1. It seems that the major fraction is O-sulfated GAGs, with roughly 10% N-sulfated GAGs in both aerobic granular sludge and the extracted structural EPS.

Similar with Hyaluronic acid, Chondroitin and Heparosan (also called unsulfated Heparin) can be produced by a few pathogenic bacteria (*E. coli* K4, *E. coli* K5, *Pasteurella multoccida* Type F and Type D, *Avibacterium paragallinarum*) (DeAngelis, 2012). However, those microbial Chondroitin and Heparosan are not sulfated. Moreover, neither of them are covalently bound to proteins. In comparison, the Chondroitin and Heparin in mammals are sulfated and are covalently bound to proteins (DeAngelis, 2012).

Aerobic granular sludge and the structural EPS contain sulfated GAGs, which are different from the GAGs produced by pathogenic bacteria reported in literatures. To further understand if these sulfated GAGs are covalently bound to proteins, two different proteases were applied to digest the proteins in the extracted structural EPS in sequence. Nevertheless, after such intense protease digestion, there was still absorbance at both 215 nm and 280 nm in the ion exchange chromatogram (Supplementary material A). Absorbance at both 215 nm and 280 nm are considered as the absorbance of carboxylate groups and aromatic amino acids, respectively. This indicates that there are still polypeptides in the sample, even in those fractions that were eluted out with high concentration of sodium chloride (0.8 – 1 M). In fact, these fractions were supposed to be highly negatively charged compounds such as sulfated GAGs. To investigate if the sulfated GAGs are bound to polypeptides, the fractions which were eluted out at sodium chloride concentration of 0.8 – 1 M were collected and combined as one fraction. Its total sulfated GAGs content was determined as 264 ± 5 mg/g (with 77% of O-sulfated GAGs and 24% of N-sulfated GAGs, as shown in Table 1). Therefore, this fraction was considered as enriched sulfated GAGs-containing polypeptides.

Further analysis of the sugar monomers and amino acids provided details of the monosaccharides and amino acids of the enriched sulfated GAGs-containing polypeptides. The detected monosaccharides were: fucose, galactose, glucose, mannose, rhamnose, xylose, galactosamine, glucosamine, galacturonic acid and glucuronic acid. The detected amino acids were: alanine, glycine, isoleucine, leucine, phenylalanine, proline, valine, serine, threonine, tyrosine, aspartate, glutamate, lysine and histidine. Interestingly, the weight percentage of the total amino acids decreased from 22.7 % (w/w) in structural EPS to 1.5 % (w/w) in the enriched polypeptides fraction, but amino acids could not be entirely removed by the intense protease digestion. Apparently, with both complex sugar monomers and amino acids existing together, the enriched sulfated GAGs-containing polypeptides are glycopolypeptides.

Therefore, different from the GAGs produced by pathogenic bacteria, the GAGs-like polymers (except for Hyaluronic acid-like polymers) in aerobic granular sludge were both sulfated and covalently bound to polypeptides.

Table 1: The amount of total sulfated GAGs and the ratio between O-sulfated GAGs/N-sulfated GAGs in aerobic granular sludge, structural EPS and the enriched sulfated-GAGs-containing polypeptide.

	Total sulfated GAGs (mg/g) with Chondroitin-4-sulfate as the standard	O-sulfated GAGs (% of the total sulfated GAGs)	N-sulfated GAGs (% of the total sulfated GAGs)	Total amino acids (% w/w)
Aerobic granular sludge	31 ± 5	90 ± 4	10 ± 3	N.A.
Structural EPS	87 ± 6	89 ± 3	11 ± 3	22.7
Enriched sulfated-GAGs- containing polypeptides fraction	264 ± 5	77 ± 2	24 ± 2	1.5

3.4 Enzymatic digestion of the enriched sulfated GAGs-containing polypeptides fraction

In order to study if there was Chondroitin sulfate and Heparan sulfate/Heparin in the enriched sulfated GAGs-containing polypeptide fraction, the sample was digested with Heparinase I & III and Chondroitinase ABC. After enzymatic digestion, the amount of total sulfated GAGs was decreased by 54 % and 43 %, respectively, indicating the enriched fraction was sensitive to the digestion with Heparinase I & III and Chondroitinase ABC. Unfortunately, disaccharides were not detected by the follow-up HILIC-MS analysis (data not shown) after the enzymatic digestion, which was different from the property of the Chondroitin sulfate and Heparan sulfate/Heparin from mammals. The reason could be: there are hydrolysis sites which can be recognized by those enzymes (recognition sites of these two enzymes are provided in Supplementary

material B), but disaccharides may not be the final product of the enzymatic digestion of the enriched glycopolypeptides fraction. Therefore, there were sulfated GAGs-like polymers, such as Chondroitin sulfate-like and Heparan sulfate/Heparin-like polymers in aerobic granular sludge.

3.5 Visualization of the location of sulfated GAGs-like polymers and other glycoconjugates in aerobic granular sludge

The location of the sulfated GAGs-like polymers in the granules was of interest in order to illustrate their spatial distribution and to understand their function. The visualization was conducted by Heparin Red staining of the whole granule (Figure 4).

Heparin Red is a fluorescent molecular probe. It is used for direct detection of Heparins in blood plasma. Heparin Red is a polycationic probe. Once there are polyanionic macromolecules (e.g. Heparin), the Heparin Red probe forms supramolecular complex aggregation at the polyanionic template (Figure 4). In this way, it is possible to visualize the location of the polyanionic macromolecules based on the fluorescence of the probe. Moreover, it is important to note that, the fluorescence intensity of Heparin Red probe is quenched once it forms stable aggregates on the polyanionic chains. Thus, opposite to the normal fluorescence stains, using Heparin Red, a low signal intensity demonstrates a strong binding of the probe (Warttinger et al., 2016a).

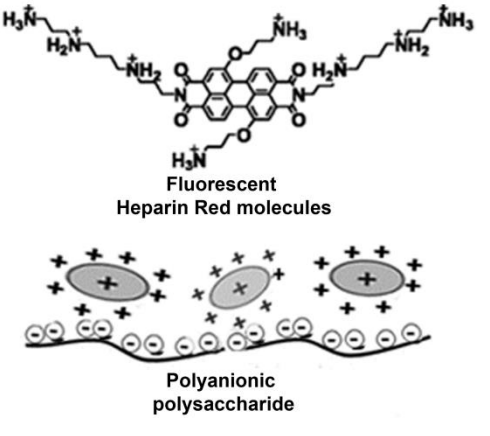

Figure 4: Molecular structure of the polycationic fluorescent probe Heparin Red (Warttinger et al., 2016a) and Schematic representation of forming aggregation on polyanionic polysaccharides chains.

As shown in Figure 5, the distribution of negatively charged polymers in aerobic granular sludge can be mapped by Heparin Red staining. The Heparin Red (HR) images are presented using a look up table in which the pixel intensities are color coded as: very low pixel intensities in blue – no binding of HR, high pixel intensities in red – binding of HR and intermediate pixel intensities in green – strong binding of HR due to quenching. According to literature, the fluorescent signal of Heparin Red is quenched when the negative charge density of the polyanionic macromolecules is higher than -0.81 per monosaccharide (Warttinger et al., 2016a, 2016b). Above this threshold, the higher the negtive charge intensity, the lower the fluorescent intensity. For example, the charge density of Chondroitin sulfate in bloodplasma is -0.70 per monosaccharide, while the charge density of Heparin is -1.7 per monosaccharide and Heparan sulfate -1 per monosaccharide (da Costa et al., 2017; Warttinger et al., 2016a). Heparin Red does not bind Chondroitin sulfate in bloodplasma but Heparin and Heparan sulfate, with Heparin resulting in lower fluorescent intensity (Warttinger et al., 2016a, 2016b). Thererfore, the green colour region in Figure 5 is where the the charge density of the polyanionic macromolucles is higher than -0.81 per monosaccharide; the lower the fluorescent intensity, the higher the charge density.

It was observed that the negatively charged macromolecules were not only present in the microcolonies, where the typical globular structures with the size around 10-20 µm can be identified (red), but at the outer boundary of the microcolonies and also in the extracellular matrix in between the colonies (green) (Figure 5A, B and C). In the microcolonies, the bacterial cells are not visible, indicating Heparin Red rather stained the capsular region or the space in between capsules (Figure 5C and D). On top of that, since the lower the intensity of the signal is, the higher the negative charge density is, the differences in brightness indicated various negative charge levels of the macromolecules. Figure 5D was representative for areas showing microcolonies with a high degree of quenching (green). As a result, signal intensity and resolution appeared to be rather low, but indicated a strong binding of the Heparin Red probe and a high level of negative charge density.

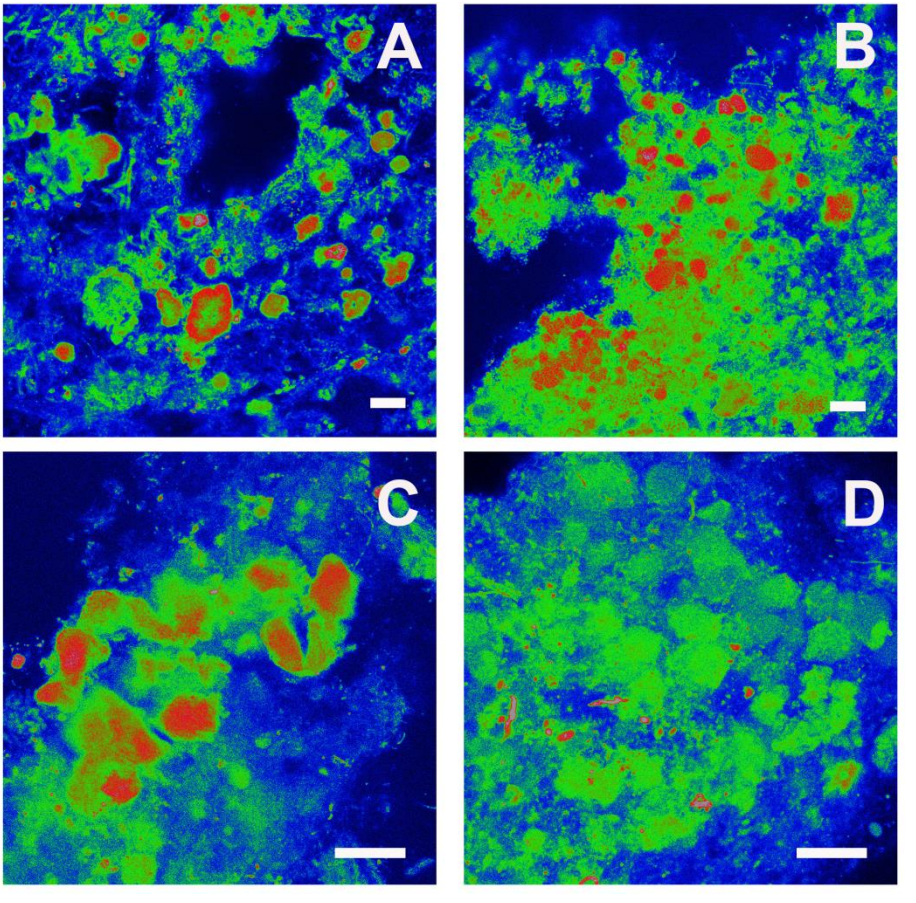

Figure 5: Distribution of negatively charged polymers in aerobic granular sludge based on Heparin Red staining. Blue: no binding of Heparin Red. Red: Binding of Heparin Red. Green: Strong binding of Heparin Red due to quenching, implying high negative charge density. Scale bars = 10 µm. A and B show the typical globular sub-structures of the granules with areas of low and high binding of Heparin Red, respectively. C and D show that the polymers with higher negative charge density are at the outer boundary of the microcolonies and also in the extracellular matrix in between the colonies.

Furthermore, as GAGs contain a hexosamine (either N-acetyl-glucosamine or N-acetyl-galactosamine) as part of their repeating structure, the locations of glycoconjugates with N-acetyl glucosamine or N-acetyl galactosamine were visualized by staining with different lectins (Figure 6). It was observed that the hexosamine-containing glycoconjugates were located in the microcolonies (Figure 6A and 6E) as well as in the extracellular matrix (Figure 6D and 6F). In the microcolonies, the bacterial cells were not visible, indicating those hexosamine-containing glycoconjugates rather stay in the capsular region or the space in between capsules, which is in line with the result from Heparin Red staining (Figure 5D).

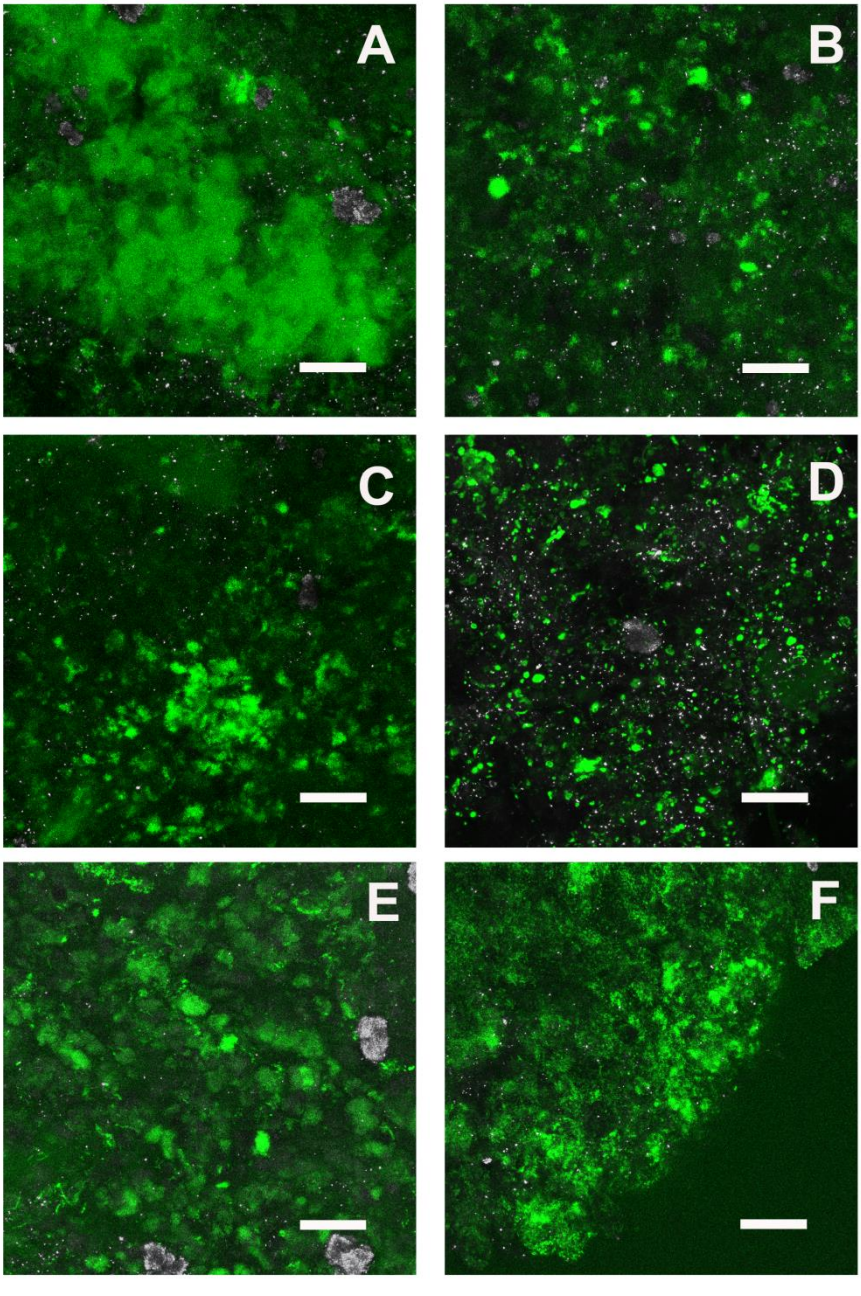

Figure 6: Confocal laser scanning microscopy showing maximum intensity projections of aerobic granular sludge data sets. Glycoconjugates were stained with N-acetyl hexosamine specific lectins. The different lectins are, A: STA, B: SJA, C: PHA-L, D: LEA, E: ABA, F: SSA. Scale bars = 10 µm. The binding specificity of lectins are listed in Supplementary material C. Color allocation: lectin signal – green, reflection signal – grey.

4. Discussion

4.1 GAGs-like polymers in aerobic granular sludge

In the current research, it was confirmed that there were GAGs-like polymers in aerobic granular sludge, such as Hyaluronic acid-like, Chondroitin sulfate-like and Heparan sulfate/Heparin-like polymers. Different from those produced by pathogenic bacteria, the Chondroitin- and Heparan/Heparin-like polymers in aerobic granules were sulfated and covalently bound to polypeptides. Sulfated glycoconjugates were reported in a few EPS related studies in different granular sludge: In anaerobic granular sludge, high molecular weight proteoglycan-like and sulfated proteoglycan-like substance were found in the Bound-EPS (Bourven et al., 2015). In the extracted structural EPS of anammox granular sludge, a glycoprotein with the molecular weight at around 12 kDa contained neutral sugar, carboxyl and sulfated glycoconjugates (Boleij et al., 2018). In aerobic granular sludge enriched with ammonium-oxidizing bacteria, there were glycosylated amyloid-like proteins which have glycocconjugates of neutral sugar, carboxyl groups and sulfate groups in the structural EPS (Lin et al., 2018). The presence of both carboxyl and sulfate groups in those EPSs implies the possible existence of sulfated GAGs-like polymers. Moreover, all those reported glycoconjugates were linked with proteins. Thus, one could reasonably assume that “the existence of sulfated GAGs-like polymers (e.g. Chondroitin sulfate and Heparan sulfate/Heparin) covalently bound to proteins” might be a common phenomenon in granular sludge/biofilms.

4.2 Potential role of GAGs-like polymers in granular sludge

It is interesting to look at the distribution of glycosaminoglycans-like polymers in nature:

The microbial production of GAGs has only been studied in certain pathogenic bacteria. The GAGs produced by *Streptococcus, Escherichia* and *Pasteurella* are similar or identical to the backbone of vertebrate polymers, thus the immune system of higher organisms fails to recognize the capsules from those pathogens as a foreign entity, and consequently no antibodies are formed to kill the microbes (Whitfield and Robert, 1999). Basically, the GAGs containing capsule is a camouflage. The GAGs produced by those pathogenic bacteria are neither sulfated nor covalently bound to proteins (DeAngelis, 2002).

Fresh water and land plants do not contain any sulfated glycoconjugates extracellularly. Marine macroalgae produce GAG mimetics (sulfated polysaccharides consist of only neutral sugars instead of hexosamine and uronic acids). These GAG mimetics are not covalently bound to proteins (Vasconcelos and Pomin, 2017).

Invertebrates generally produce the same types of GAGs as vertebrates, except that Hyaluronic acid is not present and the Chondroitin chains tend to be non-sulfated (Lindahl et al., 2017). In comparison, vertebrate cells produce GAGs and secrete them into the ECM or incorporate them into the plasma membrane (Alberts and Johnson, 2002). GAGs are crucial for the pericellular space in which they can define physical properties of tissues or adjust biological functions of cells (da Costa et al., 2017). Among the properties of GAGs, negative charge, which is intrinsic to all GAGs, is paramount. In general, the negative charge of GAGs is linked to the sulfate groups. The only exception is Hyaluronic acid. Here the negative charge is only originating from glucuronic acid (da Costa et al., 2017).

The GAGs in the ECM of animals are able to attract cations and to bind water molecules (Prydz, 2015). Hydrated GAG gels have long been known to play an important role in the absorption of pressure changes in joints and tissues. Proteoglycans with its sulfated GAGs in the extracellular matrix were discovered to influence cellular signaling, largely through electrostatic interactions with charged proteins such as growth factor, morphogens, and other chemokines, acting as a molecular sponge to embed those proteins (Gandhi and Mancera, 2008; Lim and Temenoff, 2013; Prydz, 2015).

Looking at the evolution of GAGs, it seems that the GAGs-like polymers in aerobic granular sludge resemble vertebrates' GAGs due to the presence of Hyaluronic acid-like polymers, the sulfation and the covalent bond with proteins. At this point, it is logical to speculate that, the function of GAGs-like polymers in granules is similar as that in vertebrates, such as attracting cations, forming a hydrogel, binding proteins and signaling in biological processes. Further research should focus on finding out the important functions of GAGs-like polymers in granular sludge/biofilm.

4.3 Development of proper methodologies to analyze GAGs-like polymers in granular sludge/ biofilm

GAGs are one of the major components in mammalian extracellular matrix. GAGs-like polymers were found in aerobic granular sludge by combining various analytical approaches: SDS-PAGE with Alcian Blue staining (at pH 2.5 and 1), FTIR, commercial extracellular matrix assays (for use with mammalian cells), monosaccharide and amino acid analysis, enzymatic digestion and specific in situ visualization by Heparin Red fluorescent probe and lectin staining (Figure 1). The resemblance to vertebrate GAGs implies that, the EPS in granular sludge/biofilm is far more complicated than it is currently appreciated. Thus, appropriate methodologies are needed, aiming at studying EPS components based on their important functions, rather than superficially measuring the relative amount of proteins and polysaccharides. On top of that, as it is gradually clear that protein glycosylation is a widespread phenomenon in EPS, the complexity of

glycoconjugates imparts EPS not only various functions but also complicates the analysis. For example, sulfated GAGs can have variable sulfation patterns. Possible structural variations of sulfated GAGs result in GAGs being one of the most complex groups of macromolecules discovered in nature (Zamfir et al., 2011). It is reported that GAGs' activity is dictated by sulfation patterns (Gama et al., 2006; Tully et al., 2004). Thus, obtaining the detailed molecular structure and the sulfation pattern of the GAGs-like polymers in granular sludge/biofilm will facilitate the understanding of EPS functionality and stability. At this aspect, Integrated and multi-disciplinary analyses such as MS, NMR and metagenomic analysis (Seviour et al., 2019) are significantly required.

5. Conclusion

- Besides pathogenic bacteria, microorganisms in aerobic granular sludge produce Hyaluronic acid-like and sulfated GAGs-like polymers in the extracellular matrix.
- The GAGs-like polymers in aerobic granules are different from those produced by pathogenic bacteria but resemble those produced by vertebrates: there are both Hyaluronic acid-like polymers and sulfated GAGs-like polymers; further the sulfated GAGs-like polymers are bound to proteins.
- The EPS in granular sludge/ biofilm is far more complicated than it is currently appreciated. Integrated and multidisciplinary analyses are significantly required.

Supplementary Material

Supplementary material A: Separation of the papain – proteinase K digest with DEAE resin from section 2.4.2

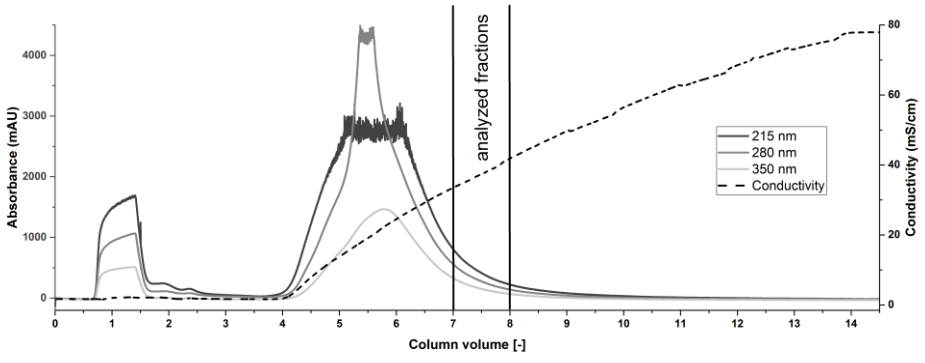

Figure A: Absorbance at 215 nm, 280 nm, 350 nm and conductivity measurement during the separation of the structural EPS – papain – proteinase K digest with DEAE Sepharose® resin. The collected fractions analyzed were eluted between column volume 7 and 8.

Supplementary material B: Enzymatic specificity of Chondroitinase and Heparinase

Heparinase I and II cleave linkages of Heparin and Heparan sulfate. Heparinase III is only active on Heparan sulfate. The (1→4) glycosidic linkages are cleaved by Heparinase I between α-D-glucosamine and O-sulfated α-L-iduronic acid, by Heparinase II between (N-Acetyl-)α-D-glucosamine and both uronic acids (β-D-glucuronic acid, α-L-iduronic acid) and by Heparinase III between N-Acetyl-α-D-glucosamine and β-D-glucuronic acid (Sigma-Aldrich, 2007).

Chondroitinase ABC is active on chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate and slowly active on hyaluronic acid. It cleaves the (1→3) glycosidic linkage between β-D-glucuronic acid and N-Acetyl-β-D-galactosamine-4-sulfate and β-D-glucuronic acid and N-Acetyl-β-D-galactosamine-6-sulfate. Chondroitinase ABC can also cleave (1→3) and (1→4) glycosidic linkages between β-D-glucuronic acid and N-Acetyl-β-D-glucosamine (Sigma-Aldrich, 2007).

Supplementary material C: Lectin binding specificity (Section 2.5)

Table C: Names, abbreviations and specificities of lectins used for N-acetyl hexosamine staining.

Lectins	Abbreviation	Glycoconjugates binding specificity
Solanum tuberosum lectin/agglutinin	STA	GlcNAc (2-4)
Sophora japonica	SJA	GalNAc (β 1-6)Gal
Phaseolus vulgaris Leucoagglutinin	PHA-L	Gal (β 1-4)GlcNAc(β 1-2)Man(α1-6)
Lycopersicon esculentum agglutinin	LEA	GlcNAc (β 1-4)GlcNAc oligomers
Agaricus bisporus agglutinin	ABA	Gal (β 1-3)GalNAc
Sambucus sieboldiana	SSA	α GalNAc

Chapter 6

Outlook

Biofilms are an important factor in wastewater treatment and clinical applications having both positive and negative effects. The complex composition, especially that of multispecies biofilms in natural environments, complicates chemical characterization of their extracellular matrix. This analysis is important to improve the understanding of biofilm stability and adjustments to environmental changes. Being a novel process little is known about the EPS composition of aerobic granular sludge. The goal of this thesis was to enable a better understanding of aerobic granular sludge by characterizing its structural EPS in chemical and physical terms. The matrix of aerobic granular sludge is still mostly unidentified, but the presented results help to pursue a better understanding of biofilms and their extracellular matrix.

Extraction of (structural) extracellular polymeric substances

Structural EPS are EPS which are involved in the formation of the structure of the granular matrix and are able to form hydrogels after being extracted. To obtain structural EPS, the biofilm matrix needs to be dissolved. In **Chapter 2** the outcome of different extraction methods applied on one sludge sample was visualized. Before selecting and applying an extraction method to investigate the extracellular material of a biofilm, it is important to be aware of the mechanism of the method and the substances aimed to be extracted. If the targeted compounds are e.g. structural EPS an extraction method capable of solubilizing the biofilm matrix is crucial. A multitude of extraction methods is reported in literature, ranging from very gentle (sole centrifugation) to harsh (alkaline with heat and mixing) (Lin et al., 2010; Liu and Fang, 2002; Sheng et al., 2010). All of these methods target different compounds in the EPS inducing biases when being used individually to characterize the total EPS. With the current knowledge about multispecies biofilm EPS it is recommended to focus on a single fraction of the EPS, such as here structural EPS, and to thoroughly characterize this fraction together with linking its function to the original biofilm.

Analytical methods for the characterization of extracellular polymeric substances

EPS characterization with currently used colorimetric methods has significant shortcomings. The colorimetric methods used in **Chapter 3** are popular analytic methods for a reason. These methods are cheap, only using standard chemicals and standard equipment, do not demand much time and are simple to execute. Being frequently applied in research covering multiple disciplines these methods seem as being a gold standard thus perceived to offer reliable results. This can also explain why these methods are applied in highly complex samples such as EPS from wastewater sludge despite their known shortcomings which were already described in one of the initial publications (Dubois et al., 1956). Measurements of in **Chapter 3** applied methods suffer from a high standard dependency on the selected reference standard compound and a

lack of suitable standards resulting in a reduced accuracy. Additionally there is cross-interference among EPS compounds in the measurements while at the same time other compounds (e.g. amino sugars) are overlooked entirely accounting for a further decreased precision of these methods.

Primarily these methods are only helpful to get a first impression of the composition, e.g. protein or sugar dominated, but not to draw conclusions on the actual composition. Until more accurate methods are implemented these colorimetric methods should not be entirely neglected, but also not be used without being aware of their drawbacks. For analyzing complex samples such as multispecies biofilms, especially those grown outside from laboratory reactors, applying two or more different types of analytic methods targeting the same compound/group of compounds can decrease the possible misrepresentation of the actual composition enabling a more accurate characterization of the biomass.

Structural extracellular polymeric substances from aerobic granular sludge

Structural EPS from aerobic granular sludge were shown to be much more complicated than anticipated. **Chapter 4** and **5** displayed chemical and physical characteristics of structural EPS. Analysis of ionic structural EPS hydrogels clearly differentiated structural EPS from the known polysaccharides alginate, polygalacturonic acid and κ–carrageenan indicating them to have a different gelling mechanisms involving multiple functional groups. Sulfated glycosaminoglycans-like compounds linked to proteins and hyaluronic acid-like compounds similar to those in the extracellular matrix of vertebrates were discovered in the EPS of aerobic granular sludge demonstrating the high complexity of the aerobic granular sludge matrix. Such complex macromolecules show why chemical methods applied in **Chapter 3** will not allow to comprehend the complexity of biofilm EPS and should not be used exclusively to draw conclusions on EPS. Neither amino sugars, nor sulfation, or that polysaccharides are bound to proteins will be detected. Multidisciplinary approaches are needed to use the full potential of analytic methods to untangle the EPS composition.

Next steps for the analysis of extracellular polymeric substances

Overall approaches in sludge EPS research have to target in depth EPS analysis. Having a large variety of types of compounds present in the EPS only a detailed analysis, including their three dimensional structure, will allow for a better understanding of biofilms and their behavior. To achieve this small and careful steps have to be taken and considered as a success to avoid rash conclusions. Possible future directions facilitating an improved compositional EPS analysis with advanced analytical methods were already pointed out, including a combination of compositional analysis of extracted EPS by e.g. NMR or LC-

ESI-MS coupled with microscopy analysis of the biofilm by CLSM and FTIR imaging or Raman microscopy (Boleij et al., 2018; Lin et al., 2018; Seviour et al., 2019).

Besides using advanced analytical methods, simplifying the sample aimed to be characterized can promote more accurate EPS characterization (Seviour et al., 2019). This can be achieved by pre-treatments to enrich for the particular compound(s) of interest. Selecting an extraction method to solubilize the targeted compound(s) following purification steps will decrease the influence of interfering compounds. A purification can be based on e.g. solubility using different solvents or the addition of salts, charge using chromatography as shown in Chapter 5, or size using size exclusion chromatography. All these steps will confine the variety of present compounds. After such pre-treatments the purified compound(s) can be analyzed with more accuracy by techniques such as FTIR, Raman, NMR, MS, SDS-PAGE, HPAEC-PAD coupled with enzymatic or acid hydrolysis to determine the composition.

More simplified samples can also be obtained by focusing on analysis of e.g. granular sludge grown in laboratory reactors. This decreases the influence of unknown substances accumulated in the EPS matrix compared to that in natural environments (Schorer and Eisele, 1997). Lab-scale reactor analysis also enables the characterization of direct responses of the biofilm to changes in environmental conditions. The knowledge obtained from these reactors can then be transferred to analyze full-scale reactor granules.

In addition to focusing on secreted EPS, including (meta)genomic analysis can be supportive for the EPS characterization. Also here, starting with lab-scale granular sludge can simplify the analysis. 16S rRNA or FISH analysis (fluorescence in situ hybridization) give an overview of the microbial community in granular sludge (Ali et al., 2019; Winkler et al., 2012) and FISH analysis together with lectin staining can reveal present microorganisms and their surrounding EPS (Böckelmann et al., 2002; Weissbrodt et al., 2013). Detecting dominant strains and their genome may reveal the presence of dominant EPS producing strains. A very detailed genomic and biofilm analysis on *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* revealed the presence and activity of genes involved in biofilm and EPS formation (Beenken et al., 2004; Boles et al., 2010; Chai et al., 2008; Fux et al., 2005; Matsukawa and Greenberg, 2004; Resch et al., 2005). Such a detailed analysis may not be possible or very difficult with mixed microbial biofilms such as granular sludge, but aiming in the direction of identifying dominant species and their genome can be a first step in detecting genes involved in the EPS production. If successful this genomic analysis can then give new insight into e.g. secreted proteins, sugar production pathways or enzymes involved in sulfation or acetylation.

Ongoing EPS analysis will not only advance understanding of biofilms and by this helping to optimize biofilm based processes, but can also help to change the perception of what is considered as waste and as a resource. Structural EPS have promising properties such as increasing the hydrophobicity of the surface of paper when being applied as a coating material (Lin et al., 2015). Structural EPS from aerobic granular sludge are already branded with the trade name Kaumera (Waterschap Rijn en IJssel, 2019) and targeted for commercial applications. An extraction plant combined with an aerobic granular sludge (Nereda®) wastewater treatment plant treating dairy wastewater has been gone in operation in 2019 in Zutphen, the Netherlands. This project involving the water authorities Rijn en Ijsel, Vallei en Veluwe, the Energie- en Grondstoffenfabriek, Royal HaskoningDHV, Stowa, Chaincraft and TU Delft demonstrates the future potential of the aerobic granular sludge process and aerobic granular sludge EPS. Considering the huge demand for wastewater treatment a successful implementation of structural EPS as a commercial product can be a prosperous contribution to a more circular, bio-based society.

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Curriculum Vitae

Simon Felz was born on May 13th 1988 in Weinheim, Germany. Following his interests he begin with his studies in bioengineering in 2008 at the Karlsruhe Institute of Technology in Karlsruhe, Germany.

During his studies he became passionate in industrial biotechnology and water treatment and also enjoyed working in multicultural environments. For his six months student research project he went in 2012 to the Nanjing Tech University, Nanjing, China to work on succinic acid production with genetically modified *E. coli*. Further continuing with his study, he completed a five months internship at the hyglos GmbH in Bernried, Germany with the focus on using bacteriophages to detect different *E. coli* strains. In order to gain more industrial working experience in wastewater treatment, he worked during an internship for three months at Maymuse Environmental Technology Co., Ltd. in Changzhou, China on treating dyeing industry wastewater. He finished his studies with the diploma thesis at the Karlsruhe Institute of Technology characterizing biofilms grown on moving bed biofilm reactor carriers with 2D and 3D imaging techniques. He received his diploma in bioengineering from the Karlsruhe Institute of Technology in 2014.

Being very interested in continuing research in biotechnology, especially with an environmental aspect, he started his PhD in 2015 in the Environmental Biotechnology Section at the Delft University of Technology under the supervision of Yuemei Lin and Mark van Loosdrecht. The aim of the research project was to better understand the extracellular matrix of aerobic granular sludge with the focus on structural extracellular polymeric substances.

Currently he is working on the optimization of the extraction of Kaumera Nereda® gum from aerobic granular sludge at the Delft University of Technology.

List of Publications

List of publications

Felz, S., Vermeulen, P., van Loosdrecht, M.C.M., Lin, Y.M., 2019. Chemical Characterization Methods for the Analysis of Structural Extracellular Polymeric Substances (EPS). Water Res. 157, 201–208. doi:10.1016/j.watres.2019.03.068

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Conference contributions

Oral presentations

IWA Biofilms: Granular Sludge Conference, Delft, The Netherlands, 2018. **Felz, S.**, van Loosdrecht M.C.M., Lin Y. Characterization of Alginate-like Extracellular Polymers Will Enable their Full Potential.

9th European Symposium on Biopolymers, Toulouse, France, 2017. **Felz, S.**, van Loosdrecht M.C.M., Lin Y. Gel-forming Polymers as an Opportunity for Resource Recovery from Wastewater (Treatment).

Vakantiecursus Delft, The Netherlands. 2016. **Felz, S.**, van Loosdrecht M.C.M., Lin Y. Using Wastewater Treatment Plants to Produce Valuable Polymers.

Poster presentations

Biofilm 7, Porto, Portugal, 2016. **Felz, S.**, van Loosdrecht M.C.M., Lin Y. Solubilizing the Hydrogel Matrix of Aerobic Granular Sludge is Crucial to Extract Structural Extracellular Polymeric Substances.