# The anammox house

## On the extracellular polymeric substances of anammox granular sludge

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## ON THE EXTRACELLULAR POLYMERIC SUBSTANCES OF ANAMMOX GRANULAR SLUDGE

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## ON THE EXTRACELLULAR POLYMERIC SUBSTANCES OF ANAMMOX GRANULAR SLUDGE

## Proefschrift

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# Contents

# SUMMARY

In biofilms, microorganisms are embedded in a hydrated matrix that provides a stable structure and protection against influences from the environment. This matrix is formed by extracellular polymeric substances (EPS) that are produced by the microorganisms of the biofilm. A major part of the microorganisms in nature lives in aggregated forms like biofilms. Yet, knowledge about biofilm formation, composition and structure is limited. A specific form of biofilm is granular sludge. A granule is a spherical biofilm that is not attached to a surface or carrier. In wastewater treatment, granular sludge systems are used for efficient wastewater treatment. Due to the high settling velocity of granules, granular sludge-based plants can be built smaller, compared to conventional plants (with flocculent sludge). Anaerobic ammonium oxidizing (anammox) bacteria are applied in granular sludge systems in wastewater treatment. Anammox bacteria are important players in the nitrogen cycle in wastewater treatment, as well as in the natural environment. Although the formation of granular sludge is not completely understood, EPS are the key components in the formation of the matrix that provides a stable structure wherein the bacteria are embedded. The aim of this thesis was to characterize the EPS composition of anammox granular sludge.

The main limitation in EPS characterization is the lack of methodologies for both EPS extraction and characterization. EPS is a complex heterogeneous mixture. In order to obtain a comprehensive understanding of the EPS components and their functions, a clearly defined target for investigation is required. Therefore, the approach was to search for candidate EPS components first, instead of characterizing the EPS as a bulk. For extraction of these candidate components, it is required to achieve disintegration of the granular sludge so that at least part of the structural components is in solution and can be recovered. In **chapter 2**, two different methods were applied for EPS extraction. The NaOH and the ionic liquid methods both dissolved the granular matrix and recovered a range of components. Proteins were found to be the dominant fraction in the extracted material. In-situ staining of granules indicated a high amount of $\beta$-sheet structures. Finding obvious extracellular candidates among the extracted proteins was a challenge due to a lack of annotated functionalities in the anammox protein database. Gel electrophoresis in combination with various other analytical methods, indicated various anionic and neutral glycoconjugates that were of interest as potential components of the extracellular matrix. Therefore, in **chapter 3 and 4**, glycosylated proteins and anionic polymers were analysed in more detail.

A highly abundant glycoprotein, carrying a heterogeneous O-glycan structure, was isolated and identified, as presented in **chapter 3**. The sequence of the protein backbone as well as the glycans and the likely glycosylation sites were determined. The protein sequence, glycan composition and glycosylation sites of this glycoprotein were highly similar with surface layer (S-layer) proteins in literature. S-layer proteins can self-assemble on the cell surface and form a crystalline layer round the cells. Although it is one of

the most commonly observed outermost cell structures of prokaryotic cells, there is no general function assigned to S-layers. The presence of the S-layer on cells in a biofilm suggest a role in linking the cells to the EPS matrix. In **chapter 4**, quantification assays and fluorescent stains indicated that sialic acids and sulfated glycosaminoglycans were present in the anammox EPS. The presence of bacterial sialic acid derivatives was confirmed with mass spectrometry. Staining specific for strongly polyanionic components indicated that the sulfated glycosaminoglycans are located around the cell envelopes of the bacteria in anammox granules. Various genes for the biosynthesis of sialic acids and sulfated glycosaminoglycans were found in the anammox draft genomes. In-situ staining's verified that the identified components in the anammox granular sludge: glycoproteins, sialic acids and sulfated glycosaminoglycans, were widely present in the granules.

The information presented in this thesis, contributes to the identification of different components of the EPS of anammox granular sludge, but also to the development of the strategy to approach EPS characterization. In **chapter 5**, the information of all chapters is combined to make a proposed image of the extracellular matrix of anammox granular sludge. The chapter also extends on the future perspectives on how to continue the research along the line of the experimental approach that was used, towards a better understanding of the EPS, the 'house' of anammox bacteria.

# SAMENVATTING

In biofilms zijn de micro-organismen omgeven door een gehydrateerde matrix. Deze matrix zorgt voor een stabiele structuur waarin de micro-organismen zijn beschermd tegen invloeden van buitenaf. De matrix bestaat uit 'extracellulaire polymerische substanties' (EPS) die worden geproduceerd door de micro-organismen in de biofilm. In de natuur leeft het merendeel van de micro-organismen in een geaggregeerde vorm zoals de biofilm. Toch is er weinig bekend over de biofilm productie, compositie en structuur. Een specifieke vorm van biofilm is korrelslib. Een korrel is een bolvormig biofilm wat niet aan een oppervlakte of dragermateriaal gehecht is. In afvalwaterzuivering worden korrelslibsystemen gebruikt voor efficiënte zuivering. Dankzij de goede bezinkeigenschappen van de korrels, kunnen systemen met korrelslib kleiner gebouwd worden in vergelijking met conventionele systemen (met vlokkig slib). Anaerobe ammonium oxidatie (anammox) bacteriën worden toegepast in afvalwaterzuivering in de vorm van korrelslib. Anammox bacterien zijn belangrijk in de stikstofcyclus van de waterzuivering, maar ook in de natuur. Hoewel de mechanismen van de vorming van korrelslib nog niet goed worden begrepen, is het wel bekend dat EPS een sleutelfactor zijn in de vorming van de matrix die voor de stabiele structuur zorgt waar de bacteriën in leven. Het doel van deze thesis was om de EPS samenstelling van anammox korrelslib te karakteriseren.

Het grootste obstakel voor het karakteriseren van EPS is het gebrek aan methodes voor de EPS extractie en karakterizatie. EPS is een complex en heterogeen mengsel. Om een samenhangend begrip te krijgen van de EPS componenten en functies is het nodig om goed gedefinieerde targets te hebben om te onderzoeken. Daarom was de aanpak om eerst potentiële componenten te zoeken die daarna meer in detail onderzocht kunnen worden, in plaats van het hele EPS als bulk te analyseren. Tijdens de extractie van deze componenten is het van belang dat de korrelstructuur uiteenvalt, zodat de kans groot is dat componenten die bijdragen aan de structuur in oplossing zijn. In **hoofdstuk 2** zijn twee verschillende methodes voor EPS extractie toegepast. Extracties gebaseerd op natronloog en ionische vloeistoffen voldeden allebei aan de eis om de korrelstructuur op te lossen en extraheerde een reeks aan componenten, waarin eiwitten de dominante fractie vormde. In-situ analyse van de korrels met behulp van fluorescent labelen wees op een grote hoeveelheid aan $\beta$-sheet structuren. Het toewijzen van duidelijke kandidaten voor eiwitten van extracellulaire origine werd belemmerd door het gebrek aan geannoteerde functionaliteiten in de database met anammox eiwitten. Gel electroforese, in combinatie met verschillende analytische methodes, wees op verschillende anionische en neutrale glycoconjugaten, welke wel als potentiele componenten voor de extracellulaire matrix werden gezien. Daarom zijn geglycosyleerde eiwitten en anionische polymeren in **hoofdstuk 3 en 4** in meer detail geanalyseerd.

In **hoofdstuk 3** is een dominant aanwezige glycoproteïne met een heterogeen O-glycaan structuur geïsoleerd en geïdentificeerd. Zowel de eiwitsequentie, de glycaan compositie en de vermoedelijke glycosylering sequentie van dit glycoproteïne kwam

sterk overeen met die van surface layer (S-layer) eiwitten (letterlijk vertaald als oppervlaktelaag eiwitten). S-layer eiwitten organiseren zich op de oppervlakte van de cel en vormen en gekristalliseerde laag om de cel. Het is een van de meest geobserveerde buitenste structuren van prokaryote cellen. Echter is er geen algemene functie bekend voor S-layer eiwitten. De aanwezigheid van de eiwitten op de cellen die in een biofilm leven, suggereert een functie bij het verbinden van de cellen en de EPS matrix. In **hoofdstuk 4** wezen quantificatie assays en fluorescent labelen op aanwezigheid van siaalzuren van gesulfateerde glycosaminoglycanen in de EPS van anammox korrels. De aanwezigheid van bacteriële siaalzuren werd bevestigd met massa spectrometrie. Het specifiek labelen van sterk polyanionische componenten wees erop dat gesulfateerde glycosaminoglycanen zich om de celenveloppen van anammox bacteriën heen bevinden. Ook werden genen die betrokken zijn bij de biosynthese van siaalzuren en gesulfateerde glycasominoglycanen gevonden in de anammox draft genomen. Met in-situ labelen werden de geïdentificeerde componenten in de anammox korrels; glycoproteinen, siaalzuren en gesulfateerde glycosaminoglycanen, wijdverspreid in de korrels teruggevonden.

De informatie zoals gepresenteerd in deze thesis, draagt bij aan het identificeren van de verschillende componenten in de EPS van anammox korrels, maar ook in het ontwikkelen van een strategie voor EPS karakterisatie. In **hoofdstuk 5** is de informatie van alle hoofdstukken gecombineerd om een beeld samen te stellen van de extracellulaire matrix van anammox korrels. In het hoofdstuk wordt ook besproken hoe het toekomstperspectief er uit ziet met vervolgonderzoek in de lijn van de experimentele aanpak die is toegepast, zodat we de EPS, het huis van de anammox bacteriën, beter kunnen begrijpen.

# 1

# General introduction

## 1.1. BIOFILMS AND GRANULAR SLUDGE

Microorganisms of all domains of life can live as suspended unicellular cells, called planktonic cells, or in aggregated forms like biofilms. In biofilms, microorganisms are embedded in a self-produced extracellular matrix [1]. Microorganisms living in a biofilm have the advantage of being protected against external influences from their environment, like pH, antimicrobial agents and salts. The biofilm is also seen as a nutrient reservoir. A mixture of different microorganisms can live in the same biofilm, as a microbial community, having the advantage of the possibility to share metabolic pathways. The matrix in which the microorganisms are embedded, consists of extracellular polymeric substances (EPS). The EPS is produced by the microorganisms and can be a wide range of different components, which will be further discussed in section 1.3. Microbial research was mostly performed on planktonic cells. In laboratory experiments, cultures are often grown in optimal conditions, stimulating growth of planktonic cells. To study the cell biology and intracellular processes of the cell it is advantageous to have planktonic cells. Also, determination of kinetic parameter of cells is preferably performed on suspended cells, since biofilms cause diffusion limitations. However, outside of the lab under environmental conditions, the majority of microorganisms live in aggregated forms [2].

Biofilms are widely distributed in nature and in engineered systems. They are present in aqueous environments (Figure 1.1), in soils, and on tissues of plants, animals and humans [3]. Hence, they are an important part of the ecosystem. However, biofilms can also be detrimental. For example, in the medical field, biofilms are usually researched in the context of how to prevent the growth of biofilms. Also in membrane reactors, removal and prevention of biofilms is crucial, because biofilm growth can cause fouling of membranes, leading to clogging of the system. Other examples where biofilms are considered harmful are biocorrosion and dental plaques. On the other hand, the ability of biofilm formation can also be exploited as an advantageous characteristic of microorganisms. For cleaning of water, biofilms are applied in biofilm reactors, in which biofilms are grown on surfaces or carriers. In addition, flocculent and granular sludge are also considered as biofilms. Granular sludge is a form of biofilm in which bacteria are grown in compact spherical aggregates. For granular sludge no external carriers are needed. The EPS provide the physical structure wherein the bacteria are embedded.


Figure 1.1: Biofilms in natural environments and engineered systems. A) Biofilm formation on rocks in water stream (image credits: U.S. Geological Survey). B) Granular sludge from a full-scale anammox wastewater treatment plant.

In wastewater treatment, various granular sludge processes are applied. For example, anaerobic granular sludge is applied mainly for carbon removal [4], anaerobic ammonium oxidation (anammox) granular sludge, for the removal of nitrogen, and more recently aerobic granular sludge treatment was developed for simultaneous removal of carbon, nitrogen and phosphorus compounds from the water [5]. The advantage of granular sludge over flocculent or suspended sludge, is that granular sludge settles very fast. Therefore, the solid retention time can easily be separated from the hydraulic retention time [6]. This enables a high biomass retention in the reactor, which is especially important for the relatively slow growing bacteria. The high biomass retention increases the volumetric treatment capacity and therefore more compact treatment plants can be built. This thesis is focused on the EPS of anammox granular sludge (Figure 1.1B).

## 1.2. ANAMMOX BACTERIA

In the early nineties, the anaerobic ammonium oxidation (anammox) process was discovered in a wastewater treatment pilot plant of the company Gist-Brocades [7]. Before that time, the oxidation of ammonium in the absence of oxygen was deemed not to occur. The observed conversions in the pilot plant indicated otherwise. While ammonium and nitrate were consumed, nitrogen gas was produced. Several years later, bacteria that were able to oxidize ammonium in the absence of oxygen were enriched and identified. We refer to them as anammox bacteria. The preferred electron acceptor was shown to be nitrite. Anammox bacteria form a separate order named '*Candidatus* Brocadiales', within the phylum of *Planctomycetes*. Research showed that anammox bacteria are contributing significantly to the global nitrogen cycle. The nitrogen loss in oxygen minimum zones of oceans, which is up to 50% of the total marine environments, is attributed for a major part to anammox bacteria [8]. Another source where the bacteria are often detected is wastewater treatment plants.

Many different aspects of these bacteria have been researched. For example the kinetic properties were researched and maximum growth rates up to a doubling time of three days were achieved [9]. Concerning the structure of the cell, it was found that the anammox reaction takes place in an intracellular compartment, called the anammoxosome [10]. Anammox bacteria have a special cell biology with cell compartmentalisation. Their cell envelope does not resemble the typical gram-negative or gram-positive cell wall structure. However, for the anammox bacteria '*Ca.* Kuenenia stuttgartiensis', it was shown that they have a thin peptidoglycan layer and an outer membrane [11]. In addition, '*Ca.* Kuenenia stuttgartiensis' produces surface layer (S-layer) proteins [12]. S-layer proteins have the property to self-assemble by an entropy driven process and form surface layers (S-layers), which form the outermost envelope of the cell [13]. S-layers are two-dimensional arrays which can exhibit oblique, square or hexagonal symmetry. They are the most commonly observed cell surface structures of prokaryotes. There are several functions found for specific strains but there is no general function that can be assigned to the S-layers [13, 14]. One proposed function is a role in biofilm formation. It is essential to know if anammox bacteria that grow in biofilms also produce an S-layer, because the S-layer is the outmost layer of bacteria and in direct contact with their environment (in the case of granular sludge, the environment of most bacteria is the extracellular polymeric substances).

Anammox bacteria have a relatively slow growth rate. Therefore, they are generally cultured in an SBR (sequencing batch reactor) in the form of flocculent or granular sludge, or in a membrane bioreactor. The latter was used to achieve planktonic growth of the bacteria [15]. For research about the cell biology and determining metabolic parameters, it is beneficial to grow the bacteria in planktonic form. Since anammox have a strong tendency to grow in aggregated form, it is of interest to analyse the aggregated form (e.g. anammox biofilm) as well. The physical and chemical properties of the aggregated form depend on the composition of the EPS. Understanding the EPS composition and formation can aid in various ways. On the one hand, knowing more about EPS could help to prevent the production of EPS. For instance, presence of EPS can hamper preparation of cell extracts and purification of proteins during analysis of the cell biology [16]. On the other hand, understanding the composition may help stimulate EPS production to obtain aggregated forms, for example in granular sludge reactors. Ultimately, finding out the extracellular matrix composition aids in understanding biofilms, which is the way of bacterial life that is very common in nature [2].

In this thesis, the extracellular matrix of anammox granular sludge from wastewater treatment plants is analysed. As mentioned, anammox is applied in wastewater treatment for the conversion of ammonium and nitrite to nitrogen gas. Using the autotrophic anammox process for the nitrogen removal instead of the conventional nitrification/-denitrification process, gives the benefit of not having to use an external carbon source and no need to aerate the reactor. The anammox process is applied in wastewater treatment in two steps, where the partial nitrification and the anammox process occur in separate reactors. Or in one step, where both processes occur in the same reactor. In the latter case, the ammonium oxidizing bacteria grow at the outer layer the granule, were oxygen is present, while the anammox bacteria grow in the anoxic core of the granule.

The first anammox full scale plant was built as a two-step system, and is located in Rotterdam (Sluisjesdijk)[17]. This plant was used for a lot of research on the anammox process. In this thesis, the anammox granular sludge collected from this plant was the main used sludge for the research to extracellular matrix polymers. Using granular sludge of full-scale plants brings the access to a high amount of biomass, which gives the opportunity to explore many different extraction and analysis methods. In addition, it is representative for the real and 'matured' biofilm.

## 1.3. EPS CHARACTERIZATION METHODS

To understand the formation and the stability of the granular sludge matrix, it is necessary to characterize the composition of the extracellular matrix. The constituents of this matrix are referred to as extracellular polymeric substances, or EPS in short. EPS are macromolecules, produced by the microorganisms in the biofilm. Due to adsorption, macromolecules from the environment possibly get incorporated in the matrix as well. However, the major part of the matrix is deemed to be produced by the microorganisms themselves. The polymers of the microorganisms can become part of the extracellular matrix through different processes: active secretion, shedding of cell surface material and cell lysis [3]. The EPS composition is reported to be a range of different type of proteins, polysaccharides, lipids to DNA and other components. Although particular components have been identified in different biofilm types, there is no consensus on general

composition and functions of EPS. The chemical composition and physical properties determine the functions of EPS. In the case of anammox granular sludge or other aggregated forms of anammox bacteria, there is little information on the EPS matrix composition, structure and function. Improved understanding of the matrix composition could aid in directing the morphology of the anammox bacterial growth into suspended or aggregated growth. In addition, bacteria have the potential to produce polymers with unique properties. Exploring the EPS with different (mechanical and physical) properties can pave the way for biopolymer production from granular sludge.

Two components that are mostly focussed on during EPS analyses are proteins and polysaccharides. There is a major amount of variations of both components. Proteins are formed by chains of amino acids. Since the protein sequences can be translated from the DNA, genomes from databases can be of help for unravelling protein sequences. However, post-translational modifications of the proteins (e.g glycosylation, methylation, sulfation) cannot so easily be predicted. That is also the case for polysaccharides. Polysaccharides contain many (sometimes hundreds or even thousands) monosaccharides, that are connected by glycosidic bonds [18]. Because of the variety in monomers, and different orientation of the glycosidic bonds, there is a high variability in possible polysaccharide structures. Different configurations impart different properties and functionalities. Moreover, more complex components with other macromolecules, like glycoproteins and glycolipids, can also be formed by bacteria. It is worth pointing out that the current EPS analyses are mostly limited to general quantification of polysaccharides and proteins by colorimetric methods. Information provided by these analyses do not reveal the real composition of EPS, a more dedicated characterization with clear targets is required to understand the structural functions of the EPS.

Another hurdle in EPS research is that the methods for EPS extraction are not always effective. Ideally, in-situ EPS characterization (e.g. through confocal laser scanning microscopy (CLSM)) can visualize EPS location while inducing little change to the EPS [19]. However, with little information on EPS composition, the in-situ analysis lack specific targets. To define a specific target, EPS needs to be extracted and analysed. There are a range of physical and chemical extraction methods, or a combination thereof, for EPS extraction [20]. Physical methods applied in literature include centrifugation, sonication and heat treatments. Chemical methods include extracting agents like bases, acids, EDTA, cation exchange resins and organic solvents. Besides physical and chemical methods, enzyme digestion is used. Due the fact that there is no clear target for EPS extraction and the extraction conditions are variable in different studies in literature, it is difficult to compare results from different studies. The diversity of EPS among different organisms complicates the extraction more, since it requires for each biofilm to test which extraction works. On top of that, it is important to keep in mind that different extraction methods may extract different parts of the EPS. Thus, a clear target for EPS characterization is already critical at the extraction step.

In granular sludge, EPS directly involved in the formation of the physical structure are considered as structural EPS [21]. When EPS is extracted with the purpose to characterize the structural EPS, the prerequisite step for extraction is the solubilization of the polymers [21]. The solubilization is seen as a crucial step: when the granules stay intact during extraction, it indicates that the structural polymers are not recovered. However,

structural EPS has not been a common focus yet. As EPS extraction may cause cell lysis it is important to verify that the extracted EPS is originating from the extracellular space in the biofilm. Therefore, when components are identified in the extracted EPS, also an in-situ analysis that targets the identified components is required. This information cannot be obtained with the commonly used EPS analysis methods. Therefore, to better characterize EPS, a roadmap which includes extraction, characterization and in-situ location visualization has to be established.

## 1.4. APPROACH FOR CHARACTERIZATION OF EPS OF ANAMMOX GRANULAR SLUDGE

Along the way of establishing the roadmap for EPS analysis, this thesis describes the exploration and characterization of the EPS of anammox granular sludge. The main aim was to find target components and analyse them regarding structural functionality in the extracellular matrix. In chapter 2, sludge from different reactors were analysed by applying two different extraction methods. The alkaline extraction and ionic liquid extraction were compared in order to evaluate their suitability for extraction of structural EPS from anammox granules and secondly to improve the understanding of the underlying extraction mechanism. This led to various interesting candidates with potential extracellular origin. The in-detail analyses of these candidates are described in chapter 3 and 4. In chapter 3, identification and characterization of a candidate glycoprotein is presented. Using mass spectrometry, both the protein sequence and the glycan composition were determined. The chapter described the potential role of glycoproteins and more specifically, surface layer proteins, in biofilm formation. In chapter 4, polyanionic groups were characterized, focussing on sialic acids and sulfated polysaccharides. In the last chapter, the information of chapter 2-4 is used to make an integrated picture, as hypothesis of the composition and arrangement of the extracellular matrix of anammox granular sludge.

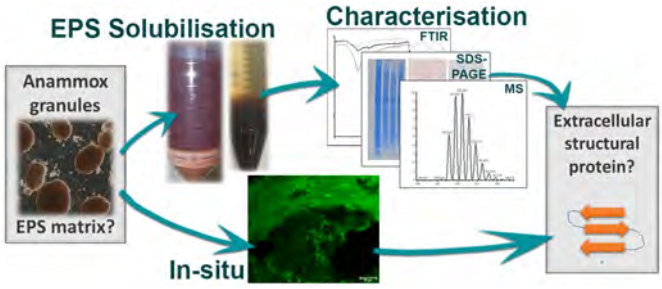
# 2

# SOLUBILIZATION AND CHARACTERIZATION OF EXTRACELLULAR PROTEINS IN ANAMMOX GRANULAR SLUDGE

This chapter has been published in *Water Research* as: 'Solubilisation and Characterisation of Extracellular Proteins from Anammox Granular Sludge', by Marissa Boleij, Thomas Seviour, Lan Li Wong, Mark C. M. Van Loosdrecht, and Yuemei Lin (2019).

## ABSTRACT

ELUCIDATING extracellular polymeric substances (EPS) of anammox granular sludge is important for stable nitrogen removal processes in wastewater treatment. However, due to a lack of standardized methods for extraction and characterization, the composition of anammox granule EPS remains mostly unknown. In this study, alkaline (NaOH) and ionic liquid (IL) extractions were compared in terms of the proteins they extracted from different "*Candidatus* Brocadia" cultures. We aimed to identify structural proteins and evaluated to which extend these extraction methods bias the outcome of EPS characterization. Extraction was focussed on solubilization of the EPS matrix, and the NaOH and IL extraction recovered on average 20% and 26% of the VSS, respectively. Using two extraction methods targeting different intermolecular interactions increased the possibility of identifying structural extracellular proteins. Of the extracted proteins, 40% were common between the extraction methods. The high number of common abundant proteins between the extraction methods, illustrated how extraction biases can be reduced when solubility of the granular sludge is enhanced. Physicochemical analyses of the granules indicated that extracellular structural matrix proteins likely have $\beta$-sheet dominated secondary structures. These $\beta$-sheet structures were measured in EPS extracted with both methods. The high number of uncharacterised and possible moonlighting proteins confounded identifying structural (i.e. $\beta$-sheet dominant) proteins. Nonetheless, new candidates for structural matrix proteins are described. Further current bottlenecks in assigning specific proteins to key extracellular functions in anammox granular sludge are discussed.



## 2.1. INTRODUCTION

Anaerobic ammonium oxidation (anammox), a process involving the oxidation of ammonium to dinitrogen gas with nitrite as electron acceptor, is significant across many biogeochemical landscapes [22–24]. The means to transform ammonium to nitrogen gas without oxygen also makes it a very attractive nitrogen removal process in wastewater treatment, where aeration is a major operating cost for complete nitrification. Since its discovery almost thirty years ago [7], more than 100 full-scale applications have been commissioned in which anammox is coupled with partial nitritation [25]. This includes a wide spectrum of wastewater that can be treated by anammox, from digester effluents [17], black water from source separated sanitation [26], to various industrial wastewaters with low C/N ratio [25]. The configuration of the process ranges from two-stage to single-stage applying to either mainstream or side stream wastewater treatment plants. Currently there is a strong emphasis on developing anammox technology for mainstream municipal wastewater treatment [27–29].



Anammox processes rely on the immobilisation of anammox bacteria as granules or biofilms, in order to reach sufficient biomass retention [30]. Biofilm formation is facilitated by the production of extracellular polymeric substances (EPS) [31]. EPS are reported to consist of polysaccharides, proteins, DNA, and other polymers. The tendency of their EPS to mediate the formation of dense anammox biofilms is an important aspect of stable anammox reactor operation. When the EPS are not sufficiently stabilised, this can impair the anammox process stability [32]. On the other hand, EPS production can hamper the characterization of anammox cell biology [16]. Hence, there are both cases in which EPS production is desirable and in which it is not. In any case it is a process with many unknowns, and therefore difficult to measure or control. To improve on that, it is important to first understand the composition of the EPS matrix.

In recent years, much information has been obtained regarding population, cell structure and compartmentalization (e.g. anammoxosomes) [33], and kinetics [34]. For example, five different genera have been identified that are capable of performing anammox [8]. Nonetheless, despite their importance and ubiquity, the EPS are perhaps the least understood aspect of anammox communities. This is largely because EPS are difficult to analyse due to the fact that they are poorly soluble and compositionally heterogeneous [35].

One major challenge, or objective in EPS characterization, is how to link EPS composition with function. Thus, instead of focussing on the amount of EPS (e.g. proteins and polysaccharides) that can be recovered, the composition should be identified to resolve the exact nature[35–37]. In previous work it was shown that proteins form the dominant fraction in the EPS [36, 38], and anammox EPS contains a relatively high amount of $\beta$-sheets [39]. $\beta$-sheet have the potential to self-assemble into various conformations that can have structural functions in the matrix [40] (e.g. as fibers, like silk [41] or amyloid-like [42]). Hence, $\beta$-sheet rich proteins potentially have a structural function in the EPS matrix.

The prerequisite step for recovering and identification of the structural polymers, is to solubilize the EPS. Because different biofilms have different EPS, various biofilms need a different treatment to be solubilized. For example, some aerobic granules were effectively solubilized with alkaline conditions [21], while this treatment did not work for

aerobic granules enriched with *Defluviicoccus* [43] and aerobic granules enriched with ammonia-oxidizing bacteria [44]. Instead, they were solubilized with acidic conditions and SDS treatment, respectively.

Studies involving EPS extraction from anammox granular sludge, in general don't report the solubilisation of the EPS matrix. Two previously proposed extraction methods that were able to solubilize and recover EPS of anammox granular sludge, are the alkaline (NaOH) extraction [36] and ionic liquid (IL) extraction [45]. However, they were never directly compared. Since there are no standardized extraction and characterization methods, it is currently unknown if these two extraction methods lead to comparable extracted EPS or not.

Here we evaluated two different extraction methods (NaOH and IL) and analysed the extracted EPS from anammox granular sludge from various reactors, with a focus on the proteins. The molecular weight distribution and the functional groups of the recovered EPS were investigated, as well as the secondary structure of the extracted proteins. Mass spectrometry (MS) was applied for identification of extracted proteins. The recovered polymers were characterized in order to evaluate the influence of different extraction methods, and better understand the underlying extraction mechanisms. In addition, by combining information of the characterisation of EPS extracted with both methods we aimed for identification of the structural components.

## 2.2. MATERIALS AND METHODS

### ANAMMOX GRANULAR SLUDGE

Anammox granular sludge samples were collected from three full-scale plants from the Netherlands (NlAmx1, NlAmx2 and NlAmx3) as well as from a lab-scale reactor in Singapore (SgAmx). The characteristics of all reactors are shown in Table 2.1. A clone library analysis was performed to identify the dominant anammox species in the granules This was complemented by Fluorescent In Situ Hybridization (FISH), which was performed as described by Johnson *et al.* [46]. (see supplemental materials for details about clone library analysis and FISH). Anammox granules were visualized with an optical microscope, scanning electron microscope and transmission electron microscope. The granular sludge sample with the highest inorganic content (NlAmx1) was analysed by micro-computed tomography (Micro-CT). Micro-CT was performed as described by Lin *et al.* [47], using a MCT-12505MF (Hitachi Medical, Kashiwa, Japan). Thioflavin T staining was performed to indicate $\beta$-sheet rich structures in the granule. 0.5% (w/v) Thioflavin T (THT) (Sigma Aldrich) was prepared in 0.1N HCl and filtered. Cryosectioned anammox granules (Leica Cyrostats CM1950) on glass slide (5 $\mu$m) was stained with THT working solution for 15 min and the slide was rinsed two times with PBS and imaged on Leica SPP8WLL confocal microscope with a 40x objective.

### EPS EXTRACTION BY IONIC LIQUID AND ALKALINE TREATMENT TREATMENT

Prior to extraction, granules were washed with MilliQ water and lyophilized. The EPS extraction process was performed as follows: Ionic liquid (IL) 1-ethyl-3-methyl imidazolium acetate (EMIA) was mixed with dimethylacetamide (DMAc) to a volumetric ratio of 40:60 as described in Seviour *et al.* [48]. Freeze-dried anammox granules were directly

Table 2.1: Characteristics of the different reactors and anammox granular sludge.

| **Sample** | **NlAmx1** | **NlAmx2** | **NlAmx3** | **SgAmx** |
|---|---|---|---|---|
| **Reactor** | full-scale | full-scale | full-scale | lab-scale |
| **Wastewater** | municipal | industrial[†]/ municipal | industrial[‡] | synthetic |
| **System** | anammox | nitritation / anammox | nitritation / anammox | anammox |
| **Feed $NH_4^+$ (mg N $L^{-1}$)** | 500 - 700 | 300 - 400 | 1000 - 2000 | $300 \pm 20$ |
| **Feed $NO_3^-$ (mg N $L^{-1}$)** | 500 - 700 | none | none | $360 \pm 20$ |
| **Volume ($m^3$)** | 70 | 600 | 3000 | 0.004 |
| **DO (mg $O_2$ $L^{-1}$)** | 0 | 0.5 - 2.0 | 0.5 - 1.5 | < 0.01 |
| **pH** | 7.0 - 7.5 | 7.5 - 8.0 | 7.0 - 7.5 | 7.0 - 7.5 |
| **VSS in granules (%)** | 71 | 88 | 87 | 88 |

[†] Potato plant
[‡] Rendering plant



added to 15 mL 40% v/v EMIA mixture to a concentration of 30 mg/mL in a Falcon tube. The tube was incubated in a 55°C water bath for 16 hours. Soluble and semi-soluble fractions were captured by precipitation with ethanol (70% v/v), separated by centrifugation, cleaned by dialysis and lyophilized for further analysis. The alkaline (NaOH) extraction was performed as described in [36]. Freeze-dried anammox granules were added to 0.1 M NaOH to a concentration of 50 mg/ml. The mixture was incubated for 5 hours while being stirred with a magnetic stirrer at 400 rpm. After centrifugation at 4000 g for 20 minutes at 4°C, the pellet was discarded. Polymers in the supernatant were precipitated out by decreasing the pH to 5. For 40 ml solution, approx. 2.5 ml HCl (1M) was used to obtain pH 5. The precipitated polymers were collected by centrifugation at 4000 g for 20 minutes at 4°C. Subsequently the extracted EPS were dialyzed and lyophilized.

### LIVE/DEAD STAINING

IL and NaOH treated anammox granules were examined by live/dead staining. Briefly, anammox granules (treated and untreated), were washed two times with double distilled water and freeze-dried. Subsequently they were stained with BacLight Live/Dead viability stain (Thermo Fisher Scientific). Live/dead stain was prepared by adding 6 $\mu$L of 1:1 SYTO 9 (3.34 mM in DMSO) and propidium iodide (20 mM in DMSO) mixture to 1 mL of double distilled water. After staining for 15 min, the granules were washed two times with double distilled water for 5 min, smeared on glass slides and imaged on confocal microscope (Leica SP8WLL and Zeiss LSM 780 with a 63x objective).

### FOURIER-TRANSFORM INFRARED (FT-IR) SPECTROSCOPY AND EXCITATION-EMISSION MATRIX (EEM) FLUORESCENCE SPECTROSCOPY

The Fourier-transform infrared (FT-IR) spectra of the extracted EPS were recorded on a FT-IR Spectrometer (Perkin Elmer, Shelton, USA) at room temperature, with the wavenum-

ber range from 550 $cm^{-1}$ to 4000 $cm^{-1}$. 3D fluorescent spectroscopy of extracted EPS solutions (25 mg/L, pH 11) was performed using a FluoroMax-3 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ, U.S.A.). EEM spectra were scanned with excitation wavelengths from 220 to 450 nm (2 nm increment) and emission wavelengths from 270 to 500 nm (4 nm increment). Graphs were generated using MATLAB.

### SODIUM DODECYL SULFATE – POLYACRYLAMIDE GEL ELECTROPHORESIS

The extracted EPS were analysed by SDS-PAGE, as described in Boleij *et al.* [36]. SDS-PAGE was performed using NuPage® Novex 4-12% Bis-Tris gels (Invitrogen). EPS samples were prepared in NuPAGE LDS-buffer and DTT (dithiothreitol) was added to a final concentration of 10 mM. The proteins were denatured by incubation at 70°C for 10 minutes. Subsequently, 10 $\mu$l sample was loaded per well. The Thermo Scientific Spectra Multicolor Broad Range Protein Ladder was used as molecular weight marker. The gel electrophoresis was performed at 200 V for 35 minutes. The gels were stained by three different stains afterwards.

For visualization of proteins, the Colloidal Blue staining kit (Invitrogen) was used according to manufacturer's instructions. For visualization of glycoproteins, the Thermo Scientific Pierce Glycoprotein Staining Kit was used, which is based on the periodic acid-Schiff (PAS) method and is specific for glycans bearing vicinal hydroxyl groups. For staining of acidic glycoconjugates, Alcian Blue 8GX (Fluka, Sigma Aldrich) was used. Alcian Blue is a cationic dye. It was used with pH 2.5 to stain dissociated (ionic) acidic groups. An adapted protocol of Møller and Poulsen [49] was used. After electrophoresis, the gels were extensively washed in solution I (25% (v/v) ethanol and 10% (v/v) acetic acid) for 2.5 hours while refreshing the solution 4 times. Subsequently, the gel was stained in 0.125% (w/v) Alcian Blue in solution I for 30 minutes and washed in solution I overnight.

### MASS SPECTROMETRY (MS) ANALYSIS

The samples, containing 100 $\mu$g of proteins, were polymerized in a 4% SDS gel and fixed with 50% methanol and 12% acetic acid, for 30 min in room temperature. The gel was cut into small pieces (1 $mm^3$), the pieces were washed three times with 50 mM TEAB/50% (v/v) acetonitrile (ACN) and dehydrated using 100% ACN. Samples were reduced with 5 mM TCEP at 57°C for 60 min, followed by alkylation with 10 mM MMTS for 60 min at room temperature with occasional vortexing. Following reduction and alkylation, the gel pieces were washed with 500 $\mu$l of 50 mM TEAB. They were dehydrated with 500 $\mu$l of ACN and 500 $\mu$l of 50 mM TEAB added for re-swelling. A final dehydration step was performed using 100 $\mu$l of ACN. 1 $\mu$g of trypsin per 20 $\mu$g of proteins was added and trypsinization performed at 37 °C for 16 h. The digested peptides were extracted sequentially with 200 $\mu$l each of 50 mM TEAB, 5% formic acid (FA) in 50% ACN and then 100% ACN. The solutions were added, allowed to stand for 5-10 min and centrifuged at 6000 rpm. The supernatant with the digested peptides were collected. Elutes were then desalted in a Sep-Pak C18 cartridge (Waters, Milford, MA), dried and then reconstituted in 20 $\mu$l of 2% ACN and 0.05% FA in water. An Eksigent nanoLC Ultra and ChiPLC-nanoflex (Eksigent, Dublin, CA, USA) column was used in Trap Elute configuration to separate the peptides. Desalting was with a Sep-Pak tC 18 $\mu$ Elution Plate (Waters, Miltford, MA, USA), followed by reconstitution in 20 $\mu$l of 2% ACN and 0.05% FA in water. 5 $\mu$l of the samples

were loaded on a 200 $\mu$m x 0.5 mm trap column and eluted through an analytical 75 $\mu$m × 150 mm column made of ChromXP C18-CL, 3 $\mu$m (Eksigent, Germany). Peptides were separated by a gradient formed by 2% ACN, 0.1% FA and 98% ACN, 0.1% FA. A TripleTOF 5600 system (AB SCIEX, Foster City, CA, USA) in Information Dependent Mode was used for MS analysis. MS spectra were acquired across the mass range of 400–1250 m/z in high resolution mode (>30000) using 250 ms accumulation time per spectrum. Tandem mass spectra were recorded in high sensitivity mode (resolution >15000) with rolling collision energy on adjustment. Survey- IDA Experiment, with charge state 2 to 4 was selected. Peptide identification was carried on the ProteinPilot 5.0 software Revision 4769 (AB SCIEX) using the Paragon database search algorithm (5.0.0.0.4767) for peptide identification and the integrated false discovery rate (FDR) analysis function. The data were searched against a "*Ca.* Brocadia" database (total 33264 sequences). These protein data were searched against a protein reference database obtained from analysis of translated predicted genes from metagenome assemblies of the sampled reactor communities combined with protein sequence from five extant draft AnAOB genomes [50].

### ANALYSIS OF ABUNDANT PROTEIN SEQUENCES

To estimate the protein abundance in the samples, Exponentially Modified Protein Abundance Index (emPAI) analysis was performed according to Li *et al.* [51]. The sequences of the abundant proteins, obtained with the emPAI analysis, were subjected to the PredictProtein tool to predict structural and functional features [52], and the ProtParam tool from ExPaSy to predict physical and chemical parameters [53].

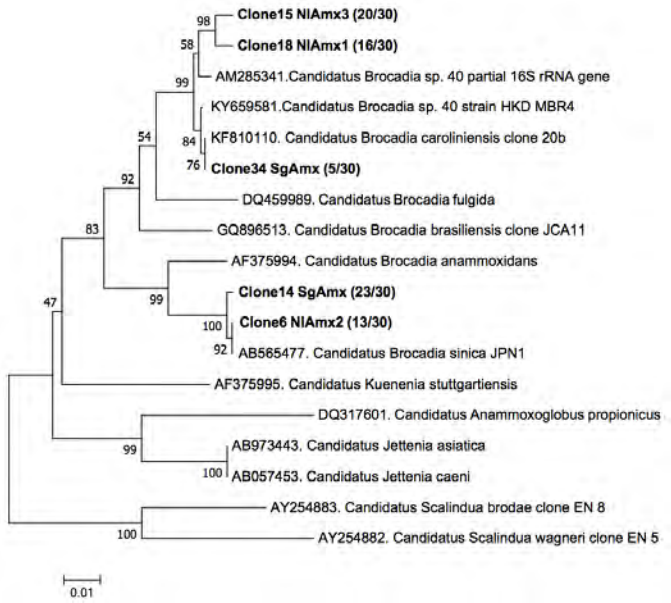



Figure 2.1: Phylogenetic tree with the anammox bacteria of the different reactors, determined by a clone library analysis.

## 2.3. RESULTS

### CHARACTERIZATION OF THE ANAMMOX GRANULAR SLUDGE

Granules from three full-scale (municipal and industrial) and one laboratory-scale anammox reactor (see Table 2.1) were collected in order to extract their EPS and resolve the composition. The phylogenetic tree in Figure 2.1 shows that the dominant anammox species in NlAmx1 and NlAmx3 cluster together, and in both instances are closely related to "*Candidatus* (*Ca.*) Brocadia sapporoensis" (previously "*Ca.* Brocadia sp. 40"). NlAmx2 and SgAmx cluster together and are closely related to "*Ca.* Brocadia sinica". A smaller part of SgAmx is closely related to "*Ca.* Brocadia caroliniensis". FISH indicated a high abundance of the anammox bacteria in the granules (see supplemental Figures S1 and S2).

The volatile suspended solid (VSS) content of the granules ranged from 71-88% (Table 2.1). In Figure 2.3B, the inorganic part appears bright and is not only present in the core of the granules, but also forms layers around the core. The inorganic fraction of NlAmx1 was previously determined to be hydroxyapatite [47]. In between these hydroxyapatite layers the space is filled with the organic matrix, which appears as the grey part. The matrix of the granules has a heterogeneous structure, as was observed using electron microscopy: both regions with low and high cell densities were observed. In regions with low cell densities, both a compact matrix and a relatively open, fibrous structure can be seen (Figure 2.3C and D). At the regions with high cell densities, the EPS appear in between the cells, which glue the cells tightly to each other (Figure 2.3E and F). Thus, the challenge for the EPS characterization lies not only in the fact that the EPS matrix is difficult to solubilize, but also in the heterogenous structure of the granules.

Thioflavin T (ThT) staining was applied to indicate $\beta$-sheets in the anammox granules. ThT becomes strongly fluorescent when it binds to $\beta$-sheet rich structures. The images in Figure 2.2 show that ThT binds to the anammox granules enriched with "*Ca.* Brocadia sinica", which indicates that $\beta$-sheets are indeed a dominant secondary structure in the EPS. Since $\beta$-sheets are abundant, and also found before in anammox EPS [42], for the EPS analysis in this study, $\beta$-sheet rich proteins will be taken into account as a candidate for structural extracellular proteins.

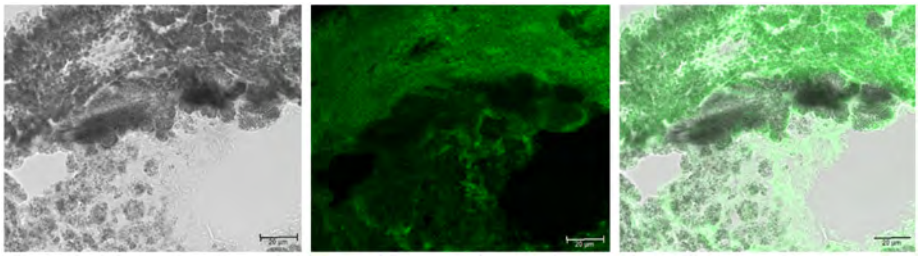


Figure 2.2: Staining of the anammox granule enriched with "*Ca.* Brocadia sinica" with Thioflavin T (ThT). Microscopy image (left), fluorescent microscopy image after staining with ThT (middle) and overlay of both images (right). (Scale bar is 20 $\mu$m.)

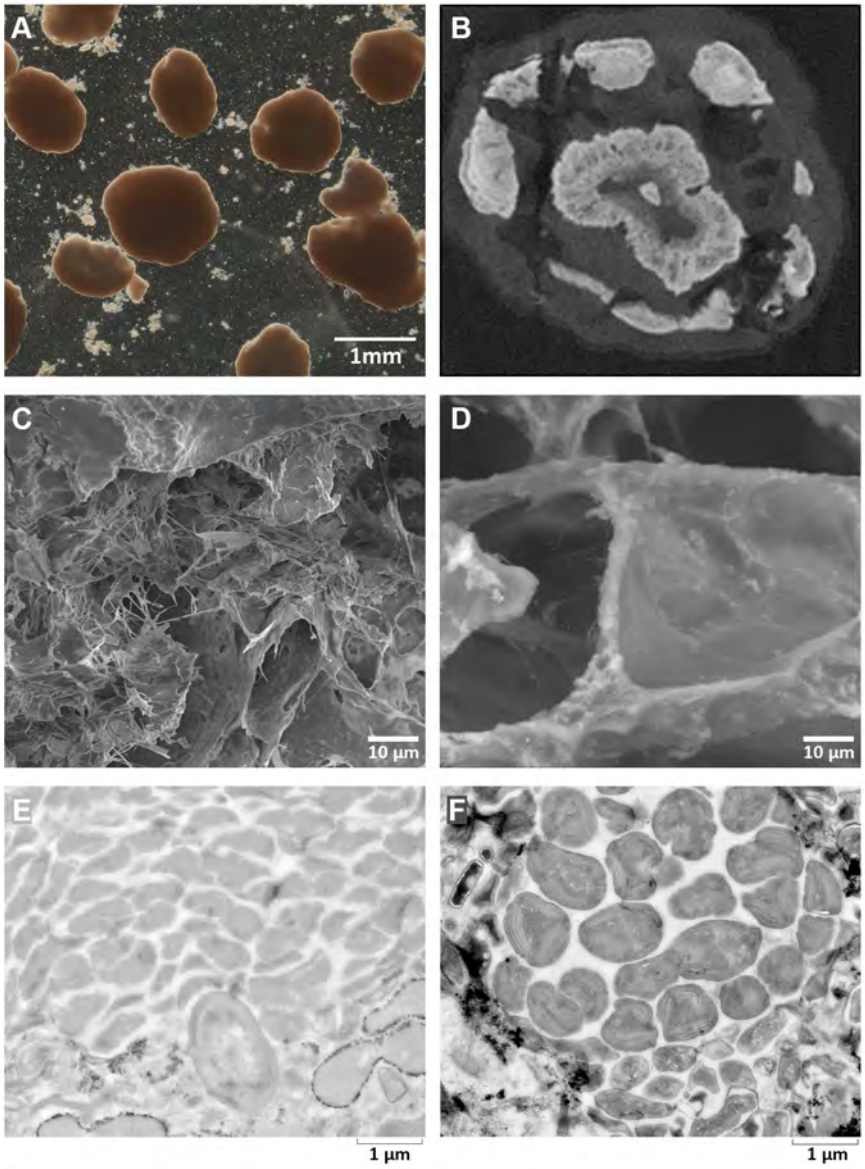



Figure 2.3: Visualization of anammox granular sludge. A) Optical microscope image. B) micro-CT scan of one intact anammox granule. The bright part is the mineral hydroxyapatite, and the grey part is the organic matrix. C) and D) are scanning electron microscopy pictures of the inside of the granules where the structure of the matrix of granules enriched with "*Ca.* Brociadia sapporoensis" and "Ca Brocadia sinica" respectively. E) and F) are transmission electron microscopy pictures of cells that are glued to each other by their EPS, in granules enriched with "*Ca.* Brocadia sapporoensis" and "Ca Brocadia sinica" respectively.

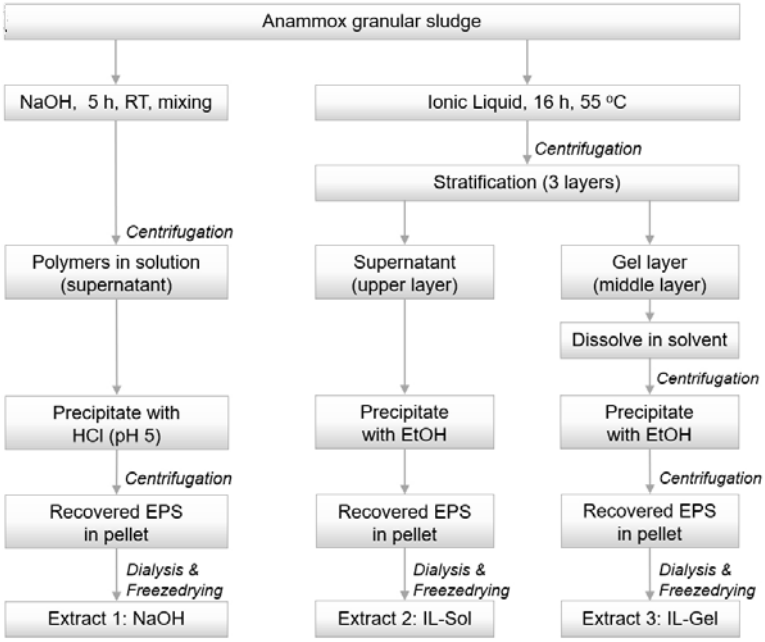



Figure 2.4: Workflow of the 2 different extraction methods, leading to the 3 extracts; NaOH, IL-Sol, IL-Gel.

## SOLUBILIZATION OF ANAMMOX GRANULES BY ALKALINE (NAOH) AND IONIC LIQUID (IL) TREATMENTS

The granular sludge from all four anammox reactors was subjected to alkaline (NaOH) and ionic liquid (IL) extractions. The extractions were performed according to the workflow scheme in Figure 2.4. Following NaOH treatment, the granular shape was lost (Figure 2.5A), indicating the dissolution of structural EPS. To precipitate the solubilized polymers, HCl was added dropwise until the pH was decreased to pH 5. This resulted in the formation of gel-like films, which could be recovered by centrifugation. After IL treatment there is a stratification of the granular matrix into a mineral part (lower layer), a gel layer (middle layer) and a soluble layer upper layer) (Figure 2.5A). The upper and the middle layers were recovered. The upper layer was soluble in water (IL-Sol) while the middle layer was insoluble in water (IL-Gel).

Using the NaOH extraction method, approximately 20% of the VSS is extracted, whereas with the IL extraction method up to 30% of the VSS is extracted (Figure 2.5B). Both treatments caused disintegration of the granular shape. The VSS components that could not be solubilized stayed in the pellet together with the inorganic part of the granules. Compared to the NaOH and IL-Gel fractions, the IL-Sol fraction is only a minor fraction of the EPS. Interestingly the dried IL-Sol fraction was a white powder while the NaOH and the IL-Gel fractions were both red.

Harsh extraction methods were used to solubilize the granular sludge, possibly lead-

ing to cell death. Live/dead staining of the treated anammox cells (Figure 2.5C) shows that IL and NaOH treatments resulted in more staining of the biomass by propidium iodide (red) than the control, which indicates either greater cell permeability or DNA release due to lysis. However, even before extraction there is a significant amount of dead cells present in the granular sludge.

In summary, the results of the extractions showed that both extraction methods fulfil the first requirement of EPS extraction, namely the solubilization of the granular matrix. Because ionic liquid and NaOH treatments damage the cells, it can not be excluded that intracellular proteins are released and co-extracted. For convenience, we will refer to the total extracted material by 'extracted EPS'. The extracted EPS will be characterized with a focus on potential extracellular properties (e.g. high amount of beta-sheets as was found in the granular matrix), and assigning intra-and extracellular proteins using mass spectrometry. Hereby aiming at identification of extracellular proteins and additionally validating the recovery of extracellular polymers with these extraction methods.



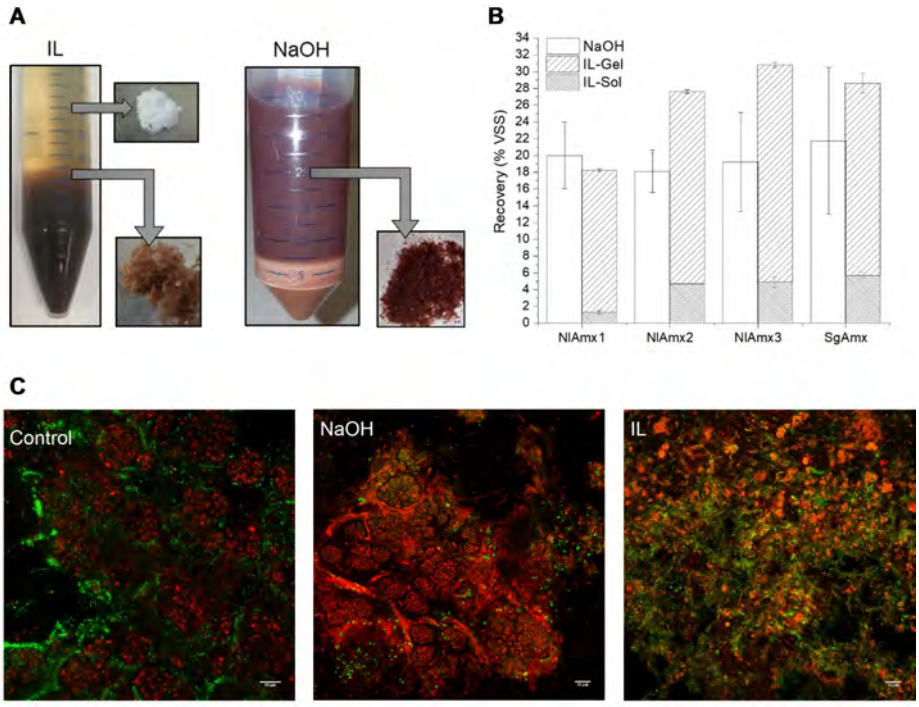



Figure 2.5: EPS extractions by IL and NaOH treatment. A) Images of the solubilisation of the granules in IL and NaOH, and the three recovered fractions B) Yield of the NaOH and IL extraction on the different biomass samples. C) Live/dead staining before treatment (left), and after NaOH (middle) or IL treatment (right). Staining by propidium iodide (red) indicates dead cells and staining with SYTO (green) indicates living cells. (Scale bar is 10 $\mu$m.)

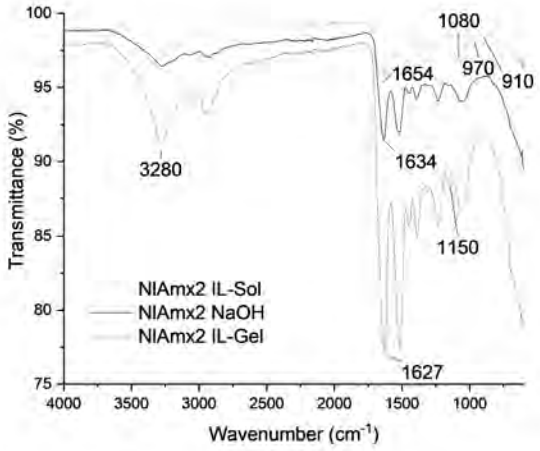



Figure 2.6: FTIR spectra of 3 different fractions, extracted from NlAmx2 ("*Ca.* Brocadia sinica").

### $\beta$-SHEETS DOMINATE THE SECONDARY STRUCTURE OF EXTRACTED PROTEINS

FTIR was applied in order to analyse the protein secondary structure and to explore functional groups in the extracted EPS. The FTIR profiles of the EPS from the different reactors were comparable (see supplementary Figure S3). Thus, based on the FTIR the functional groups look similar. In Figure 2.6 the FTIR spectra of extracted EPS from "*Ca.* Brocadia sinica" (NlAmx2) are shown as an example for the comparison of the EPS extracts NaOH, IL-Gel and IL-Sol. The FTIR spectra of NaOH extracted EPS and IL-Gel EPS are similar: A narrow band with a relatively sharp peak at 3280 $cm^{-1}$, which is a typical peak of –NH group in proteins. A peak at 3050 $cm^{-1}$, indicating the presence of aromatic amino acids (tryptophan, tyrosine, and phenylalanine) [54]. The dominant protein secondary structure are $\beta$-sheets with the peak at 1635 $cm^{-1}$ of NaOH extracted EPS and the peak at 1627 $cm^{-1}$ of IL-Gel EPS (Barth, 2007). The peak at 1150 $cm^{-1}$ implies there are C-O-C bonds due to the crosslinking of acidic sugars (e.g. sugars with –COOH group). A band at 1200-940 $cm^{-1}$ with two peaks, one peak at 1080 $cm^{-1}$ due to the presence of $-PO_4^{3-}$, and the other one at 1040 $cm^{-1}$, indicating carbohydrates.

In comparison, the spectrum of IL-Sol is different in the following peaks: a broad band at 3700-3100 $cm^{-1}$ with peak value at 3280 $cm^{-1}$, which is assigned to hydroxyl group (–OH); a peak at 1654 $cm^{-1}$ indicating the dominant protein secondary structure is $\alpha$-helix (Barth, 2007); a band at 1200-940 $cm^{-1}$ with the peak value at 1080 $cm^{-1}$, implying that there are carbohydrates and $-PO_4^{3-}$ groups, the strong signal of $-PO_4^{3-}$ covers the peak of carbohydrates. There are two extra peaks at 970 $cm^{-1}$and 910 $cm^{-1}$ from $-PO_4^{3-}$ group, matching with the strong signal of phosphate. In addition, no peak at 3050 $cm^{-1}$, indicating there is little aromatic amino acids (tryptophan, tyrosine, and phenylalanine) [54]; and no peak at 1150 $cm^{-1}$ implying there is little C-O-C bond from the crosslinking of acidic sugars.

Thus, NaOH extracted EPS and IL-Gel EPS are dominated by proteins with $\beta$-sheet secondary structure, while proteins in the IL-Sol fraction is dominated with $\alpha$-helix secondary structure, and phosphate groups (especially with hydroxyl groups, such as hydroxyapatite). The high amount of $\beta$-sheets are consistent with what was observed with the ThT staining.

In addition to the FTIR, the EEM spectra of the three extracts were measured (Figure 2.7 and supplementary Figure S4). While both NaOH and IL-Gel have a major peak at 275/340 (ex/em), the IL-Sol has a very small signal at those wavelengths, and a major peak at 240/360 instead. The 275/340 nm peak is reported to represent aromatic amino acid tryptophan. The 240/360 peak is the same as the peak for the autofluorescence of hydroxyapatite [55]. The high amount of tryptophan is in accordance with the FTIR spectra, where the 3050 $cm^{-1}$ peak showed phenolic amino acids. Tryptophan plays an important role in the formation of $\beta$-sheets. As $\beta$-sheet structures is dominant in the NaOH and IL-Gel samples, more tryptophan is present in these fractions than in the IL-Sol sample.



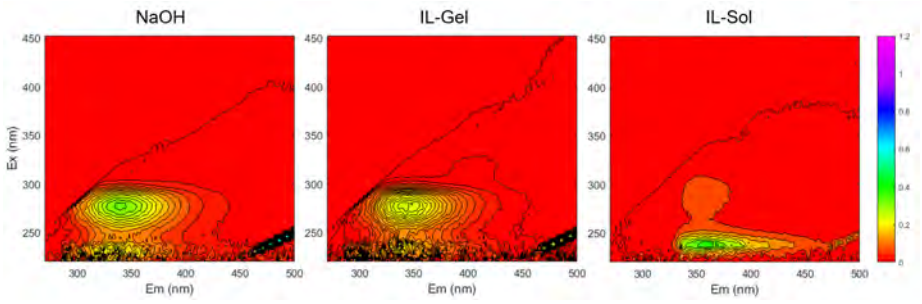



Figure 2.7: Excitation Emission spectra of the different fractions (NlAmx 2, "*Ca.* Brocadia sinica"). The NaOH (left) and the IL-Gel (middle) fraction have a peak at 275/340 nm while the IL-Sol (right) has the major peak at 240/360 nm.

## PROTEIN PROFILE OF EXTRACTED EPS DEPENDS MORE ON DOMINANT POPULATION THAN EXTRACTION METHOD

The protein profiles of the different extracts were compared by using SDS-PAGE analysis, in combination with different stains. Proteins, neutral glycans and acidic glycans, that were indicated with FTIR, were stained with Coomassie Blue, Periodic acid Schiff (PAS) and Alcian Blue, respectively. In Figure 2.8, pictures of SDS-PAGE gels with extracted EPS of NlAmx1 (representative for "*Ca.* Brocadia sapporoensis") and NlAmx2 (representative for "*Ca.* Brocadia sinica") are shown (see supplementary Figure S5 for all sludges). IL and NaOH extractions both extract glycoproteins, as indicated by the bands that stained positive for both Coomassie Blue and PAS stains. For NlAmx1 glycoproteins were observed at 80, 12 and 10 kDa, regardless of the extraction methods used. For NlAmx2, IL extracted EPS showed glycoprotein bands at 200, 150, 50 and 8 kDa. NaOH-extracted EPS also showed the 8 kDa band while the higher molecular weight glycoproteins appeared less resolved. Thus, the protein profile of the extracted EPS depended more on

dominant population than on the extraction method.

In addition, the Alcian Blue staining, that is specific for glycoconjugates with an acidic character (carboxylated or sulfated), showed that the NaOH extraction recovered more acidic polymers than the IL-Gel, which is indicated by the 'smear' in the high molecular weight range (>235 kDa). This was the case for both NlAmx1 and NlAmx2. For the IL-Sol, a larger sample amount needed to be applied to visualize the bands, indicating a lower presence of proteins in this fraction.

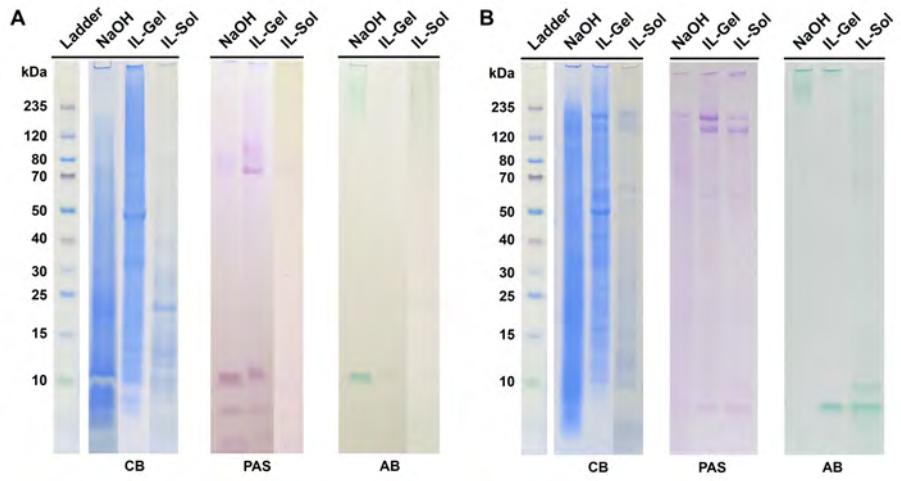



Figure 2.8: The different extracted EPS of A) "*Ca.* Brocadia sapporoensis" (NlAmx1) and B) "*Ca.* Brocadia sinica" (NlAmx2) were analysed on a SDS-PAGE gel using different staining's. Coomassie Blue (CB) was applied to stain proteins, Periodic acid Schiff's (PAS) stain for glycans and Alcian Blue (AB) for acidic sugars.

### IN SEARCH OF STRUCTURAL EXTRACELLULAR (GLYCO)PROTEINS

To identify proteins with a potential function in the structural matrix, the extracted EPS from NlAmx1 ("*Ca.* Brocadia sapporoensis") and SgAmx ("*Ca.* Brocadia sinica") were analysed using mass spectrometry (MS). All fractions from the extractions were analysed to compare the results of the NaOH and IL methods. The obtained spectra were matched against a "Candidatus Brocadia" wide database. Of the detected proteins in the NaOH extracted EPS, 59% and 62% was also detected when EPS was extracted with the IL method, for NlAmx1 and SgAmx respectively. Of the total detected proteins 41.5% and 37.1% are overlapping for NlAmx1 and SgAmx respectively, as illustrated in Figure 2.9. The amount of detected proteins in NaOH and IL extracted samples are comparable in the case of NlAmx1, while for SgAmx the amount of detected proteins was higher for the IL extracted EPS. Of the IL soluble proteins, 63% and 6% for NlAmx1 and SgAmx respectively are also detected in the IL gel proteins (supplementary Figure S6).

The emPAI method was applied to find the relative abundance of the proteins in the extracted EPS. A list with abundant proteins in the different fractions is shown in Table 2.2. Considering abundant proteins in the different extracts, similar predicted func-

tions could be found. Various enzymes, proteases, superoxide dismutases, chaperones, a heme transporter and elongation factor Tu, and uncharacterized proteins were found. The emPAI analysis shows similar types of proteins that are abundant in both "*Ca.* Brocadia sapporoensis" and "*Ca.* Brocadia sinica" (supplementary Tables S1-6).

The sequences of the abundant proteins were analysed with online tools (ExPaSy, ProtParam and PredictProtein) that predict various physical and chemical parameters like subcellular localization, secondary structure, grand average of hydropathicity (GRAVY), aliphatic index and instability index of the proteins. The proteins that were predicted to be extracellular proteins (including secreted and fimbrium proteins) all belong to the uncharacterized proteins, which means that the function is still unknown. Among the predicted secreted proteins, especially A0A1V6LWI7 was noticed for its secondary structure was predicted to contain no $\alpha$-helix, but only $\beta$-sheet (42%) and loop structures (58%). This is similar as in the previously identified glycoprotein in "*Ca.* Brocadia sapporoensis", which was proposed to be a surface layer protein by Boleij *et al.* [36], and which contains 47% $\beta$-sheet and 53% loop structures. Looking at the conserved domains present in the sequence of A0A1V6LWI7, it contains a WD40/YVTN repeat domain. Structurally, both the WD40 and the YVTN repeated motifs form a circularised $\beta$-propeller structure, which consist of seven 4-stranded $\beta$-sheets. Looking to the aligned PDB (protein database bank) homologs, the structure of the protein A0A1V6LWI7 is related to the structure of a hydrazine synthase (E-value: 9e-84) and to a surface layer of archaea bacteria (E-value: 7e-54) (supplementary Figure S7).

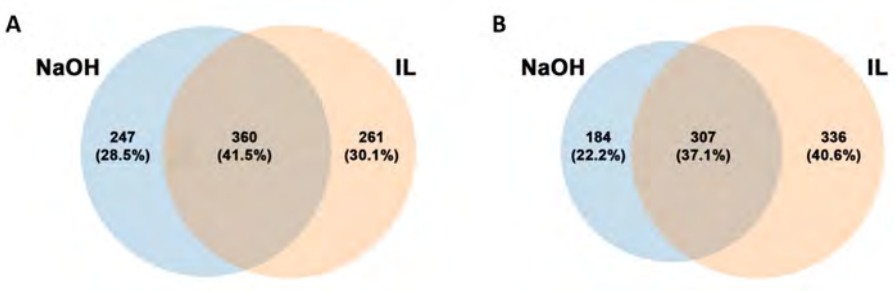



Figure 2.9: Venn diagram of the total detected proteins in the NaOH and IL extracts (IL-Gel and IL-Sol taken together), using a *Ca.* Brocadia wide database, detected in A) NlAmx1 ("*Ca.* Brocadia sapporoensis") and in B) SgAmx ("*Ca.* Brocadia sinica").

Table 2.2: Abundant proteins in extracted EPS of NlAmx1 based on the emPAI scores analysis, and their predicted subcellular location and secondary structure. All are Ca. sapporoensis except the one indicated with the asterix (*), which is sinica JPN1 (n.d = not detected)

| **Accession** | **Annotated function** | **(%) emPAI** | | | **Predicted** | | |
|---|---|---|---|---|---|---|---|
| | | **NaOH** | **IL-Gel** | **IL-Sol** | **Location** | **$\beta$-sheet (%)** | **$\alpha$-helix (%)** |
| AA0A1V6M2T4 | Nitrate oxidoreductase subunit | 4.8 | 5.5 | 0.1 | Periplasm | 8 | 16 |
| A0A1V6LWQ0 | Uncharacterized protein | 3.5 | 4.7 | 3.8 | Periplasm | 20 | 5 |
| A0A1V6LWN1 | Heme transporter CcmC | 3.4 | 1.6 | 6.8 | Periplasm | 4 | 29 |
| A0A1V6LY92 | ATP synthase subunit beta | 3.3 | 3.2 | 0.4 | Cytoplasm | 16 | 34 |
| A0A1V6M2W3 | Uncharacterized protein | 2.7 | 1.4 | 9.4 | **Secreted** | 3 | 71 |
| A0A1V6M077 | Superoxide dismutase | 2.4 | 1.1 | 0.4 | Cytoplasm | 7 | 58 |
| A0A0C9NKJ8 | Nitrate reductase subunit beta | 2.2 | 1.4 | n.d. | Periplasm | 8 | 18 |
| A0A1V6LYV6 | Serine protease | 1.9 | 1.1 | n.d. | Outer Membrane | 26 | 13 |
| A0A1V6LZP8 | 60 kDa chaperonin | 1.8 | 5.9 | 5.8 | Cytoplasm | 12 | 47 |
| A0A1V6LZQ8 | Uncharacterized protein | 1.5 | 1.9 | 0.6 | Periplasm | 31 | 8 |
| A0A1V6LWI7 | Uncharacterized protein (Fragment) | 1.4 | 1.6 | 2.1 | **Secreted** | 42 | 0 |
| A0A1V6LXE2* | Uncharacterized protein | 1.3 | 0.7 | n.d. | Periplasm | 0 | 29 |
| A0A1V6LYC0 | Cysteine synthase A | 1.3 | 1.3 | n.d. | Cytoplasm | 12 | 35 |
| A0A1V6M345 | Hemerythrin | 1.3 | n.d | 0.3 | Cytoplasm | 0 | 20 |
| A0A1V6LZD4 | Thioredoxin peroxidase | 1.1 | n.d. | n.d. | Cytoplasm | 25 | 25 |
| A0A1V6LZX1 | ATPase | 1.1 | n.d. | n.d. | Cytoplasm | 9 | 47 |
| A0A1V6LZI3 | 60 kDa chaperonin | 1.0 | 2.3 | 2.3 | Cytoplasm | 13 | 47 |
| A0A1V6M3P3 | Hydroxylamine oxidoreductase | 1.0 | 0.6 | 0.5 | Periplasm | 1 | 27 |
| A0A1V6M1T3 | Glutamate synthase (NADPH), homotetrameric | 1.0 | 0.6 | n.d. | Cytoplasm | 18 | 27 |
| A0A1V6LZY1 | Probable transaldolase | 0.9 | 0.2 | n.d. | Cytoplasm | 13 | 45 |

## 2.4. DISCUSSION

### EXTRACTING EPS FROM ANAMMOX GRANULES WITH TWO DIFFERENT EXTRACTION METHODS

The EPS are responsible for the stability of biofilms and granular sludge. Knowledge on the composition of the EPS is therefore valuable, because it can aid in development of methods to monitor (e.g. FTIR online measurement), and ultimately control EPS production. Still, it is a poorly characterized material. This is partly due to limitations in extraction methods as well as the analytical methods [35, 37]. Here we analysed EPS of granular sludge of various anammox reactors. Although these four reactors are operated under different conditions, two sets of dominant species were found. The observation of EPS characteristics in this study could be related to the dominant species present, and not to specific reactor conditions. Both the NaOH and IL extractions method could solubilize the anammox granules used in this study, which indicated that at least a part of the structural polymers of the granules was solubilized. In general, both NaOH and IL extractions recovered a range of proteins, glycosylated proteins (with neutral and acidic glycoconjugates). High molecular weight acidic glycoconjugates were more abundant in NaOH than in IL-Gel extracts. There were relatively little differences between the NaOH and IL-Gel extracts, that were both recovered after the granular gel matrix was disaggregated was not solubilized yet, was only a minor fraction and had more differences compared to the two fractions above. This indicates that extraction biases may be reduced when the extraction of EPS is associated with solubilizing the granular matrix.

Looking more in detail to the different fractions, the proteins in both the NaOH and IL-Gel fractions had $\beta$-sheets as dominant secondary structure while the proteins in the IL-Sol fraction had $\alpha$-helixes as the dominant secondary structure. The $\beta$-sheets structure was found to be abundant in the granule, indicated by ThT staining. This was also earlier observed for anammox granules [42]. In addition the NaOH and IL-Gel fraction contained proteins with a high amount of tryptophan as opposed to IL-Sol (Figure 2.7 and supplementary Figure S8). Tryptophan-rich EPS was observed in previous studies on EPS of aerobic ammonium oxidizing (AOB) granules [44], where it was proposed to play a structural role. Furthermore, phosphate was present in all the fractions, which might relate to the hydroxyapatite accumulation in anammox granules.

Looking at the extraction mechanisms, alkaline treatment can hydrolyse sugars by beta-elimination, and can break disulphide bonds in proteins, which can aid in the disintegration of the EPS matrix [20]. Solubilization by ionic liquids is based on disruption of the hydrogen bonding and its kosmotropic and chaotropic effects. They are also used to stabilize proteins. Wong *et al.* [45] showed that the ionic liquid EMIA solubilizes neutral (cellulose) and cationic polysaccharide (chitosan), basic proteins, and to a lesser extend acidic proteins. It does not extract acidic polysaccharides (alginate) as opposed to NaOH, in which acidic polysaccharides are usually more soluble [56]. Consistent with these observations, in this study NaOH extracted more acidic glycans from the anammox granules than IL. The fact that the gel matrix was still present after IL treatment, and could only be completely solubilized after addition of the solvent (dimethylacetamide), could indicate a structural role of acidic glycans in the EPS matrix.

Concerning EPS extraction, the choice of the extraction method is dependent on the

type of biofilm and on the components that are targeted. To study the structural EPS, it is very important to solubilize the biofilm first. When the composition of the biofilm is unknown, it is suggested to perform a screening of extraction methods to see which one can dissolve the matrix of the biofilm. In the current case, both extraction methods satisfy the requirement of disaggregating the granular structure. The choice is then dependent on the targeted components and follow-up analyses. When anionic components are the target of interest, NaOH is likely more suitable than the ionic liquid EMIA. (Other ionic liquids than EMIA could be more effective for solubilization of acidic glycans.) Since ionic liquids are also used to stabilize proteins, if tertiary structures need to be preserved for analysis, the IL treatment can have advantages over NaOH treatment, which is more prone to cause some hydrolysis of proteins and sugars, and deacetylation of sugars. Here both methods have shown useful to solubilize and characterize the EPS of anammox granular sludge.

Considering the observed damage to the cells during EPS extraction, both methods are likely to cause significant damage. It is worth pointing out that in order to solubilize the structural part of the biofilm, cell damage might be unavoidable during EPS extraction, especially when the granules have compact and strong structures (cell lysis will be further discussed in the next section).

### IN SEARCH OF STRUCTURAL EXTRACELLULAR PROTEINS

Besides the characterization of the structural features and functional groups, MS was applied in order to identify the extracted proteins. Looking at the list of the most abundant identified proteins, it contains intracellular proteins and uncharacterized proteins. Remarkably, various metabolic enzymes, chaperones and elongation factor Tu have been reported in literature as moonlighting proteins [57]. A 'moonlighting' protein is a single protein that can have two or more functions [58]. They can have different intra- and extracellular roles. Many of the reported moonlighting proteins work on the cell surface as an adhesin and can bind to structural components like fibronectin, laminin, collagen, or to mucin [57]. These kind of moonlighting proteins were also observed in *Clostridium acetobutylicum* [50] and *Staphylococcus aureus* [59] biofilms, and were proposed to play a role in biofilm formation.

In this study we cannot differentiate between possible 'moonlighting proteins' or intracellular proteins that were released during the extractions. However, the possibility of intracellular proteins having an extracellular role underlines that it is very difficult to quantify cell lysis during EPS extraction. During the natural development of granular sludge, intracellular proteins, whether through active secretion or as a result of cell death, can end up in the extracellular matrix [3]. Hence, they could already be present before the extraction is applied, and could not be distinguished as intracellular or extracellular proteins. Nevertheless, it is highly likely that there are lysis products released with both extraction methods. Therefore, as proposed by Seviour *et al.* [35], the extracellular location of the isolated polymers should always be verified. Location of potential targets can be verified with specific stains, antibodies or lectins (as was for done glycoproteins for example in Boleij *et al.* [36].

### CHARACTERIZING THE UNCHARACTERIZED PROTEINS

With the MS analysis no match was obtained that directed to obvious structural extracellular proteins. Likely the proteins that belong to the matrix polymers are among the 'uncharacterized proteins'. In order to clarify the potential function of uncharacterized proteins, online tools can be used to predict structure, subcellular localization and conserved domains [60]. The protein A0A1V6LWI7 was noticed because it was predicted to be secreted, and to have a secondary structure with a high percentage of $\beta$-sheets. The protein has a WD40/YVTN repeat domain, which in general forms structures called $\beta$-propellers [61]. These $\beta$-propellers can have various amounts of blades. For this protein a 7-bladed $\beta$-propeller structure is predicted, which is reported to have significantly high thermostability and/or thermodynamic stability [62]. Looking to the aligned PDB structures, the structure of the protein A0A1V6LWI7 is related to hydrazine synthase [63]. On the other hand, the structure was also related to a surface layer protein of archaea bacteria, which was proposed to have a role in cell-cell interactions [64]. The latter would be interesting considering a role in extracellular matrix. The protein A0A1V6LWI7 is a potential target to be analysed in more detail, regarding the function in granular sludge. Moreover, localization using e.g. antibodies should be performed to verify it is indeed a secreted protein, or an intracellular protein (or perhaps both). In general, confidence scores for the localization of the proteins, using the prediction tool were low (supplementary Table S7). This could be due to anammox bacteria having a unique cell compartmentalization with intercellular membranes, unique ladderane membrane lipids, and no standard gram-negative or gram-positive cell wall.

The MS analysis might have not detected, or underestimated part of the extracted proteins. This could be due to recalcitrant proteins not entering the SDS-PAGE gel or due to glycosylation protecting the protein against tryptic digestion, resulting in fewer detected peptides. Another issue is that the database is limited, since it consists of proteins that were annotated from a draft genome. For example, the sequence entry matching the previously identified abundant glycoprotein of "*Ca.* Brocadia sapporoensis" [36] is retracted from the database. When matching the sequence of this protein against the MS spectra of the EPS in this study, it does not come up as an abundant protein, while the band on SDS-PAGE was very abundant. To increase the chance to detect structural matrix proteins, the samples preparation needs to be optimised specifically for the more recalcitrant EPS proteins, e.g. by including a deglycosylation step.

### CONCEPT OF THE ANAMMOX EXTRACELLULAR MATRIX AND OUTLOOK

Based on what is currently known, we can make a concept of the organization of the extracellular matrix of anammox granules. In this study and also in previous studies [47, 65], hydroxyapatite is observed as a part of anammox granules. The EPS matrix may contain the proteins that function as a template for hydroxyapatite formation and thereby provide the 'mineral skeleton' within granules. For example, phosphorylated proteins and acidic groups are both reported to be involved in biomineralization [66, 67]. Furthermore, $\beta$-sheet rich proteins present on the cell surface forming the glue in between the cells and the matrix, may be S-layer proteins and/or adhesins. To elucidate the extracellular matrix of anammox granules, the individual components need to be analysed in more detail. For example the glycoprotein for "*Ca.* Brocadia sapporoensis"

[36] and for "*Ca.* Brocadia sinica" [45] were already characterized in more detail.

## 2.5. CONCLUSION

Global proteomics studies have shed huge insight into many biological processes, however their application to assigning major structural extracellular proteins is limited by extraction techniques, protein solubility and digestibility, and poor representation of extracellular proteins in the reference databases. We addressed the issue of extraction and solubility and observed that while using different extraction methods increases the number of proteins detected, nonetheless the main ones were largely common. We observed by microscopy that the matrix is dominated by $\beta$-sheet structures, and confirmed that the extracted proteins had predominately $\beta$-sheet structures. Hence, we believe that we extracted structural proteins. However, we could not assign any of the proteins identified from the different extracts by MS as being dominant structural proteins, due to the high number of uncharacterized proteins, potential for moonlighting functions among proteins, and the lack of biophysical data regarding the structures of many of these proteins. Nonetheless, the extraction methods described here enable the biochemical (e.g. isolation) and biophysical approaches needed to fill in the blanks in the protein databases regarding extracellular protein identity and structure.

## SUPPLEMENTARY INFORMATION

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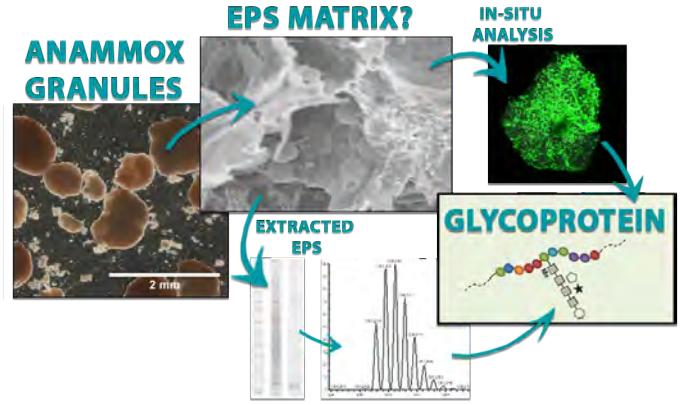
# 3

# Identification of glycoproteins in EPS of anammox granular sludge

This chapter has been published in *Environmental Science & Technology* as: 'Identification of glycoproteins isolated from extracellular polymeric substances of full-scale anammox granular sludge', by Marissa Boleij, Martin Pabst, Thomas R. Neu, Mark C. M. Van Loosdrecht, and Yuemei Lin (2018).

## Abstract

Anaerobic ammonium oxidation (anammox) is an established process for efficient nitrogen removal from wastewater, relying on anammox bacteria to form stable biofilms or granules. To understand the formation, structure and stability of anammox granules, it is important to determine the composition of the extracellular polymeric substances (EPS). The aim of this research was to elucidate the nature of the proteins, which are the major fraction of the EPS and were suspected to be glycosylated. EPS was extracted from full-scale anammox granular sludge, dominated by "*Candidatus* Brocadia", and subjected to denaturing polyacrylamide gel electrophoresis. By further analysis with mass spectrometry, a high abundant glycoprotein, carrying a heterogeneous O-glycan structure, was identified. The potential glycosylation sequence motif was identical to that proposed for the surface layer protein of "*Candidatus* Kuenenia stuttgartiensis". The heavily glycosylated protein forms a large fraction of the EPS and was also located by lectin staining. Therefore, we hypothesize an important role of glycoproteins in the structuring of anammox granules, comparable to the importance of glycans in the extracellular matrix of multicellular organisms. Furthermore, different glycoconjugates may have distinct roles in the matrix of granular sludge, which requires more in-depth characterization of different glycoconjugates in future EPS studies.

## 3.1. INTRODUCTION

Anammox (anaerobic ammonium oxidation) bacteria are a distinct phylogenetic group within the phylum of *Planctomycetes*, that can convert nitrite and ammonium into nitrogen gas [68]. They are widely occurring in nature and applied in wastewater treatment plants (WWTPs) to remove nitrogen from wastewater. This process has the advantage over the conventional nitrogen removal process that it requires no oxygen and no organic carbon source. The anammox process is applied in biofilm or granular sludge systems. Because of the high settling velocity of granules, these systems allow for sufficient biomass retention and require less space compared to reactors with flocculent sludge [6]. For the stability and efficiency of WWTP processes, the stability of the granular sludge is of high importance. Although the mechanism of granule formation is not well understood, it is generally accepted that extracellular polymeric substances (EPS) are a key factor [69, 70].

Similar as in biofilms, in granular sludge, EPS are the components that form the matrix wherein the microorganisms are embedded. EPS are reported as proteins (structural proteins and enzymes), polysaccharides, nucleic acids and lipids [1]. Many functions are assigned to EPS, including functions related to structure and stability, like promoting aggregation, maintaining the physical structure of granules, retaining water and serving as protective barrier for the cells [3, 71]. However, in most systems, knowledge on the exact composition of EPS and a link to the function of the individual components has not been established to date. To gain a better understanding of the structure and the stability of the granular sludge EPS matrix, establishment of methods and protocols for both extraction and proper characterization is required [21, 72, 73]. Especially the subset of EPS components that provide the physical structure (structural EPS) needs to be determined and characterized. Importantly, many of the traditional EPS extractions that are focussed on avoiding cell lysis, do not solubilize the structural polymers. Therefore they do not allow to analyse the structural polymers from biofilms and granular sludge [21]. In order to find new targets in the EPS matrix to study, it is required to solubilize the matrix. Once new targets are analysed, their extracellular origin should be verified. With this approach the release of intracellular components during extraction is not a problem.

Characterization of proteins and polysaccharides in EPS has been mostly limited to the use of colorimetric assays. Colorimetric assays have a relatively low specificity and may cause a significant over- or underestimation of individual components [72, 73]. Moreover, the use of those methods only allows for characterization of the separate classes of molecules, but provides no insight into the macromolecular structure of the components. Some studies speculate that the proteins and polysaccharides in EPS are not solely present as separate components, but also in various forms of glycoconjugates [74, 75]. While historically glycosylation of proteins used to be considered to occur exclusively in eukaryotes, today it is accepted that also prokaryotes can perform (complex) protein glycosylation [76]. In eukaryotes, glycoproteins fulfil important roles in the extracellular matrix, e.g. in cell-cell interactions, protecting the cells and providing a hydrated gel matrix [77]. Prokaryotic protein glycosylation however, is far from well-studied due to its complexity and enormous diversity. Furthermore it has been mainly studied in relation with pathogenic traits [78, 79]. Therefore, studying protein glycosylation in a purely environmental sample such as the extracellular polymers of granular

sludge is of special interest. Bourven *et al.* [80] found glycoproteins in anaerobic granular sludge and very recently, glycosylated amyloid-like proteins in the structural EPS of aerobic granular sludge was reported by Lin *et al.* [44]. However, presence of glycoproteins in EPS was only proposed by colocalization and orthogonal staining experiments, but never followed up by in-depth molecular characterization. To find direct proof for glycosylated proteins in EPS, more dedicated methodologies are required.

Here, we are presenting the identification and in-depth characterization of glycoproteins from anammox granular sludge which will aid in providing a better understanding of structural EPS. For this, granular sludge from a full-scale anammox WWTP was used to extract EPS by using an alkaline extraction. Proteins were analysed using SDS-PAGE (sodium dodecyl sulfate acrylamide gel electrophoresis) in combination with carbohydrate specific stains, followed by high-resolution mass spectrometry for protein identification and determination of the glycan attachment sites as well as the glycan composition. In addition, lectin staining was applied to localize glycoconjugates in the granules.

## 3.2. MATERIALS AND METHODS

### ANAMMOX GRANULAR SLUDGE

Anammox granular sludge was collected from the full-scale anammox reactor in Dokhaven (Sluisjesdijk), Rotterdam [17]. The VSS (volatile suspended solids) content of the granules was 0.71 g/g granules (determined in accordance with APHA, 2005 [81]). To identify the dominant anammox species in the granules, a clone library analysis was performed. This was complemented by Fluorescent In Situ Hybridization (FISH), which was performed as described by Johnson *et al.* [82]. More detailed information on dominant species analysis is provided in the supplemental materials.

### EXTRACTION OF EPS

Freeze-dried anammox granules were incubated in 0.1 M NaOH (50 mg/ml) for 5 hours, while being stirred with a magnetic stirrer at 400 rpm. After centrifugation at 4000 rpm for 20 min at 4°C, the pellet was discarded. Polymers in the supernatant were precipitated out by decreasing the pH to 5 using 1 M HCl. The precipitated polymers were collected by centrifugation at 4000 rpm for 20 min at 4°C and lyophilized.

### COMPOSITION ANALYSIS OF EXTRACTED EPS

The elemental composition of lyophilized EPS was analysed in terms of C, H, N, O and S. Carbon (C), hydrogen (H) and nitrogen (N) were determined by purge-and-trap chromatography (VARIO Elementar EL, Elementar), and sulfur (S) by ion chromatography (IC 883 Plus, Methrom). Oxygen was taken as the residual organic fraction. The proteins in the EPS were measured as BSA (Bovine Serum Albumin) equivalents using the bicinchoninic acid (BCA) method (Interchim Uptima BC assay quantitation kit). The carbohydrates were measured as glucose equivalents using the phenol-sulfuric acid assay [83].

### SDS-PAGE ANALYSIS AND STAINING EXPERIMENTS

SDS-PAGE was performed by using NuPage® Novex 4-12% Bis-Tris gels (Invitrogen). EPS samples were prepared in NuPAGE LDS-buffer and DTT (dithiothreitol) was added to a final concentration of 10 mM. The proteins were denatured by incubation at 70 °C for 10 min. Subsequently, 10 $\mu$l (16 $\mu$g EPS) sample was loaded per well. The Thermo Scientific Spectra Multicolor Broad Range Protein Ladder was used as molecular weight marker. The gel electrophoresis was performed at 200 V for 35 min. The gels were stained by three different stains afterwards.

For visualization of proteins, the Colloidal Blue staining kit (Invitrogen) was used according to manufacturer's instructions. For visualization of glycoproteins, the Thermo Scientific Pierce Glycoprotein Staining Kit was used, which is based on the periodic acid-Schiff (PAS) method. This method is specific for glycans bearing vicinal hydroxyl groups. Horseradish Peroxidase was employed as positive control and Soybean Trypsin Inhibitor was used as negative control. For staining of acidic glycoproteins, Alcian Blue 8GX (Fluka, Sigma Aldrich) was used. Alcian Blue is a cationic dye. It was employed to stain dissociated (ionic) acidic groups on the carbohydrates. To differentiate between the relatively weaker acidic groups like carboxylate (R-$COO^-$) and the stronger acidic group like sulfate (R-$OSO_3^-$ ), staining with Alcian Blue was performed at different pH values, namely pH 2.5 and pH 1.0. Due to the different dissociation constants (Ka) of the acidic groups, at pH 2.5 carboxylate and sulfate groups are stained by Alcian Blue, while at pH 1.0 only sulfate groups are stained [84]. For staining at pH 2.5, an adapted protocol of Møller and Poulsen [49] was used. After electrophoresis, the gels were extensively washed in solution I (25% (v/v) ethanol and 10% (v/v) acetic acid) for 2.5 hours while refreshing the solution 4 times. Subsequently, the gel was stained in 0.125% (w/v) Alcian Blue in solution I (the solution was stirred overnight to dissolve the Alcian Blue and centrifuged before use) for 30 min and washed in solution I overnight. For staining of sulfated groups at pH 1.0, the same protocol was performed except that solution I was replaced by solution II (0.1 M HCl and 25% (v/v) ethanol), according to [85].

### ENRICHMENT OF THE 80 KDA GLYCOPROTEIN

The enrichment of the glycoprotein stained at 80 kDa was achieved following a protocol for the extraction of S-layer proteins with LiCl [86]. 0.5 gram of the extracted EPS was added to 50 ml of 5 M LiCl and stirred on ice for 30 min at 400 rmp. The mixture was centrifuged for 15 min at 13000 rpm. The supernatant was dialyzed at 4°C against milli-Q water and subsequently lyophilized.

### IN-GEL PROTEOLYTIC DIGESTION AND PROTEIN IDENTIFICATION

Following SDS-PAGE analysis and Coomassie staining, the 80 kDa gel band originating from approximately 1-2 $\mu$g of protein, was cut from the gel. After destaining, an in-gel trypsin digestion was performed over-night at 37°C. Peptides were extracted and subjected to nanoLC-MS/MS analysis on an Ultimate 3000 HPLC (Dionex, San Donato Milanese, Milano, Italy) coupled to a LTQ-Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany). For each solution a volume of 5 $\mu$l was directly injected on a self-made nanocolumn packed with an Aeris Peptide XB-C18 phase (75 $\mu$m i.d. × 15 cm, 3.6 $\mu$m, 100 Å, Phenomenex, Torrance, CA, USA) and eluted at 300 nL/min flow rate. Solvent A

consisted of 3% acetonitrile in H2O containing 0.1% formic acid and solvent B of 80% acetonitrile in H2O containing 0.1% formic acid. The elution gradient program was: 0 min, 2% B; 40 min, 2% B; 68 min, 15% B; 168 min, 25% B; 228 min, 35% B; 273 min, 50% B; 274 min, 90% B; 288 min, 90% B; 289 min, 2% B; and 309 min, 2% B. Mass spectra were acquired in positive ion mode, setting the spray voltage at 1.8 kV. Data were acquired in data dependent mode with dynamic exclusion enabled; survey MS scans were recorded in the Orbitrap analyser in the mass range of 300–2000 m/z; then up to five of the most intense ions in each full MS scan were fragmented. Data was analysed against a bacteria-wide proteome database (SwissProt, 24/11/17), or a "*Candidatus* (*Ca.*) Brocadia" focused proteome database (TrEMBL, 24/11/17), using X!tandem (release ALANINE, The GPM) and PEAKS Studio 8.5 (for parameters see supplemental materials). The best match protein sequence was further analysed with the InterPro protein analysis tool [87].

For further analysis of the glycopeptides, the extracted peptides were injected to a LC-MS/MS system using reverse phase chromatography (ACQUITY M class ultraperformance liquid chromatography (UPLC) connected to an ESI-Q-TOF Premier mass spectrometer, Waters). Solvent A consisted of 3% acetonitrile in H2O containing 0.1% formic acid and solvent B of 90% acetonitrile in H2O containing 0.1% formic acid. A linear gradient was performed from 5 to 75% solvent B over 30 min at constant flow rate of 5 $\mu$L/min. Data depended analysis was performed selecting the 2-3 most intense peaks from each scan for collision-induced dissociation (CID) [88]. Data were analysed manually using MassLynx 4.1. Peak lists were exported using the msconvertGUI (ProteoWizard) and analysed with X!tandem (The GPM, release ALANINE) using a "*Ca.* Brocadia" protein specific database.

### IN-GEL GLYCAN RELEASE AND COMPOSITION ANALYSIS

As described in the previous section, following SDS-PAGE analysis and Coomassie staining, the 80 kDa gel band originating from approximately 1-2 $\mu$g of protein, was cut from the gel. The O-glycans were further released from the protein backbone by in-gel reductive $\beta$-elimination using 1 M NaBH in 0.5 M NaOH at 50°C and overnight incubation [89]. Prior to MS analysis, released O-glycans were purified using a Hypercarb™ SPE cartridge (Thermo Fisher Scientific) [90]. The purified fraction was further analysed using LC-ESI-MS/MS with a Hypercarb™ Porous Graphitic Carbon (PGC) stationary phase (0.32 x 150 mm, 5 $\mu$m) [89, 91]. Solvent A consisted of 3% acetonitrile in H2O containing 0.1% formic acid and solvent B was 90% acetonitrile in H2O containing 0.1% formic acid. A linear gradient was performed from 5 to 70% solvent B within 15 min maintaining a constant flow rate of 9 $\mu$L/min using a UPLC pump system (ACQUITY M class, Waters). The UPLC system was coupled to an ESI-Q-TOF mass spectrometer (Waters Premier), which was operated in positive ion mode (ES+). Fragmentation experiments were performed on the identified glycan peaks using CID in separate analysis runs. Data were analysed using MassLynx 4.1 and annotation of fragment ion peaks was done by using Glycoworkbench 2.1 [92].

### IMAGING OF GLYCOCONJUGATES

The granules were stained and mounted in coverwell chambers with a 1 mm spacer in order to avoid squeezing of the samples. Glycoconjugates of the anammox granules were examined by means of fluorescence lectin bar-coding [93]. Thus all commercially available lectins (FITC or Alexa488) were applied as an individual probe to one granule. For 3d imaging a TCS SP5X confocal laser scanning microscope (Leica, Germany) was employed. The upright microscope was equipped with a super continuum light source and controlled by the software LAS AF 2.4.1. The confocal datasets were recorded by using 25x NA 0.95 and 63x NA 1.2 water immersion lenses. Excitation was at 490 nm (laser power 70 % at laser, 50% in software), emission signals were detected simultaneously with two photomultipliers from 485-495 nm (reflection) and 505-600 nm (fluorescence). Image data sets were deconvolved with Huygens version 16.05 using the CMLE algorithm (SVI, The Netherlands) and projected with Imaris version 9.1.2 (Bitplane, Switzerland).

## 3.3. RESULTS

### EXTRACTION AND COMPOSITION ANALYSIS OF EPS FROM ANAMMOX GRANULAR SLUDGE

The granular sludge collected from the wastewater treatment plant Dokhaven was enriched with a species that was very close to "*Ca.* Brocadia sp. 40" (See supplemental Figure S1), which is renamed to "*Ca.* Brocadia sapporoensis" [94]. For solubilisation of the granular sludge, the granules were incubated in a stirred 0.1 M NaOH solution for 5 hours at room temperature. This procedure fully disintegrated the granular structure (Figure 3.1). A mixture of sol-like liquid and mineral particles was formed, indicating the organic matrix of the granules was solubilized. After precipitating the polymers with HCl, 0.20 ± 0.04 g/g organic dry weight was recovered from the granular sludge. In Table 3.1 the basic characterization of the composition of the recovered EPS is given. The main part of the recovered EPS consists of proteins, which is in agreement with the high content of nitrogen from the elemental composition analysis. Interestingly also the sulfur content was relatively high (in comparison with the average sulfur content in protein, which is 0.3%).

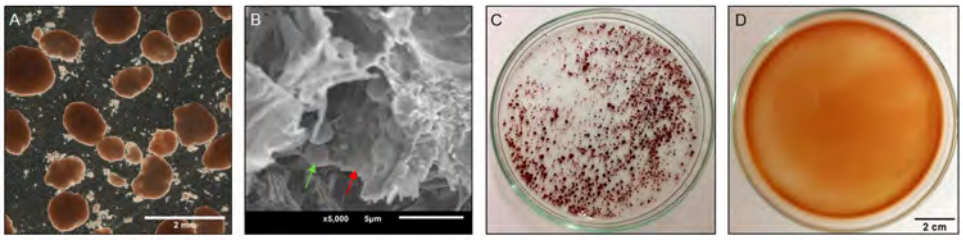



Figure 3.1: A) Optical microscope image of anammox granular sludge from the WWTP. B) Scanning electron microscope image of the inside of a broken granule where bacteria (green arrow) can be seen, embedded in the EPS matrix (red arrow). C) Anammox granules before and D) after incubation for 5 hours in 0.1 M NaOH.

Table 3.1: Protein and carbohydrate contents, and the elemental composition of the extracted EPS from anammox granules.

| proteins mg/g EPS | carbohydrates mg/g EPS | elemental composition (weight-%) | | | | |
|---|---|---|---|---|---|---|
| | | C | H | N | O | S |
| 599 ± 4 | 49.0 ± 2 | 40.5 | 6.6 | 9.0 | 35.7 | 1.4 |

## SDS-PAGE ANALYSIS

The proteins in the extracted EPS were further characterized using SDS-PAGE in combination with different stains. Following Coomassie Blue staining, bands were observed at molecular weights of ca. 80, 55, 35, 20 and 12 kDa (Figure 3.2, lane 1). Although proteins were the major component of the extracted EPS material, only a few predominant bands were observed. This is different from intracellular protein profiles, which typically show bands over the whole molecular weight range. PAS stained 2 bands, at approximately 80 kDa and 12 kDa, confirming the presence of carbohydrates in the protein extract (Figure 3.2, lane 2). In addition, Alcian Blue staining was applied with pH 2.5 (Figure 3.2, lane 3) and pH 1.0 (Figure 3.2, lane 4). With pH 2.5, carboxylate and sulfate groups are stained, while with pH 1.0 only the sulfate groups are stained. It was observed that at pH 1.0, only the band at 12 kDa was stained by Alcian Blue, indicating the presence of sulfate groups. This is in correspondence with the high sulfur content that was measured by the elemental composition analysis. It was also observed that a smear appeared at the high molecular weight range (above 235 kDa), when the gel was stained with Alcian Blue at pH 2.5. However, no band was observed at this position when using Coomassie Blue or PAS, indicating either a heavily negatively charged carbohydrate polymer or aggregate-like structure. Carbohydrate branches may shield the protein backbone from staining by Coomassie Blue. Furthermore, the large amount of acidic carbohydrate residues may lead to an unevenly distributed charge on the molecules, inhibiting the formation of a dense band on the gel [49]. This smeared band in the high molecular weight range is similar to what is observed for mucin-like proteins, when stained with Alcian Blue at pH 2.5 [95, 96].

In summary, besides a few major bands stained with Coomassie Blue, carbohydrate and acid specific staining revealed three bands in the solubilized EPS extract: i) a high molecular weight smeared band containing acidic groups, ii) a band at 80 kDa with both protein and carbohydrate positive staining and iii) at approximately 12 kDa a band stained positive for sulfate groups. Based on these results, our further analysis was focused on the protein band with an apparent molecular weight of 80 kDa because it showed an abundant band with a clear staining with both Coomassie and PAS. The protein appeared at a molecular weight which fits in the range of surface layer (S-layer) proteins, making it an interesting target. The high molecular weight smear may be associated with a large aggregate or a mucin-like structure. The relatively low molecular weight 12 kDa band on the other hand, may originate from a carbohydrate/peptide like structure, or another highly sulfated polymer, rather than from a glycoprotein.

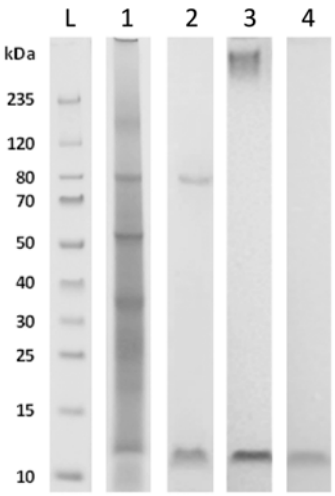



Figure 3.2: Proteins of EPS from anammox granules were analysed by SDS-PAGE and stained with Coomassie Blue for proteins (lane 1), with PAS for (neutral) carbohydrates (lane 2), with Alcian Blue at pH 2.5 for acidic carbohydrates (lane 3) and with Alcian Blue at pH 1.0 for the presence of sulfate groups (lane 4). Besides bands only stained by Coomassie Blue, there was one major band at 80 kDa which also showed a strong PAS staining. A smeared band above 235 kDa marker was only visible with Alcian Blue staining at pH 2.5. A low molecular weight band at 12 kDa was stained with Alcian Blue also at pH 1.0 indicating the presence of sulfate groups. Lane L is the molecular weight ladder.



## ENRICHMENT AND MS ANALYSIS OF THE 80 KDA GLYCOPROTEIN

The strategy to characterize the target glycoprotein that appeared at 80 kDa is illustrated in Figure 3.3. First, the glycoprotein was enriched using a LiCl based surface-layer protein extraction protocol (Figure 3.4A). The Coomassie stained band was excised from the gel and subjected to proteolytic digestion using trypsin. The extracted peptides were analysed by tandem mass spectrometry using a LTQ-Orbitrap mass spectrometer. There was no match found with any known S-layer protein from the database. Instead, the database search using a bacteria-wide and further a "*Ca.* Brocadia" focused proteome database, uncovered the protein sequence of a hypothetical protein of "*Ca.* Brocadia sapporoensis" (WP_070066018.1) as strongest match, as well as a sequence identical with a small C-terminal fragment of WP_070066018.1 (OQD46794.1). (For the summary of the database search results see supplemental Tables S1-S4.) Unfortunately, it was noticed that the sequence for WP_070066018.1 was retracted from the protein database recently. On the other hand, the same protein shows high sequence homology to another protein from "*Ca.* Brocadia sapporoensis" (WP_070066019.1). (For Results for sequence alignment see supplemental Figure S2.)

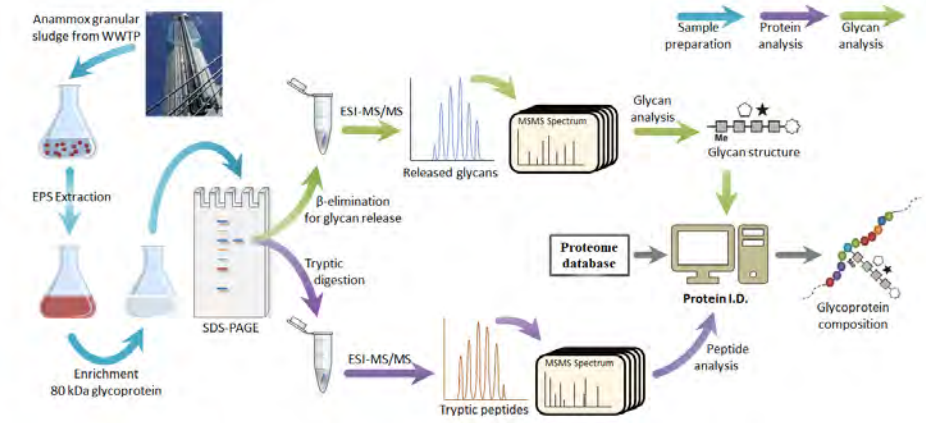



Figure 3.3: Workflow for the identification of glycoproteins from granular sludge sample, following recently established protocols [89, 97].

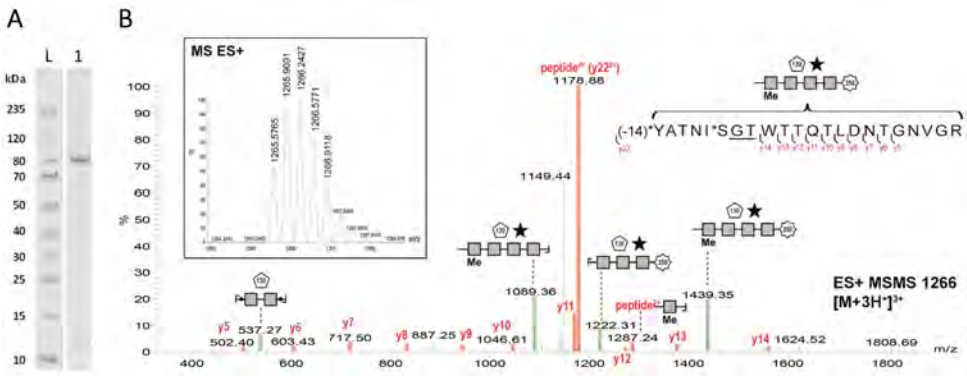



Figure 3.4: A) Coomassie Blue staining shows the enrichment of the glycoprotein at approx. 80 kDa from the bulk EPS following a protocol of Lortal *et al.* [86], established for LiCl based S-layer protein extraction (lane 1). Lane L is the molecular weight ladder. B) Fragmentation spectrum of the glycopeptide matching the sequence WP_070066018.1 of *"Ca.* Brocadia sapporoensis". The peak at 1287.24 represents a fragment that contains both a part of the protein and the glycan.

## ANALYSIS OF GLYCOPEPTIDES AND GLYCAN STRUCTURE FOLLOWING IN-GEL GLYCAN RELEASE

Following a closer investigation of the tandem-MS fragmentation spectra of the 80 kDa gel band, an approximately 1.5 kDa glycan was found attached to at least four different peptides, all matching the sequence of WP_070066018.1 very closely. The fragmentation spectrum of one of the glycopeptides is shown in Figure 3.4B. The peak at 1287.24 represents a fragment that contains both the protein and the glycan and therefore confirms the identification of a glycoprotein. In addition, following further manual investigation of the spectra, indications for additional glycosylation sites were found, which could not

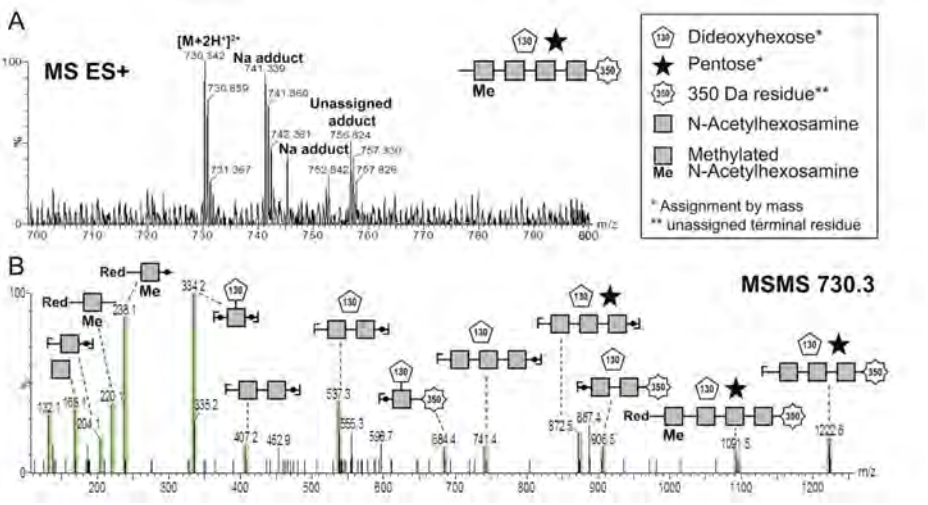



Figure 3.5: Analysis of the glycan structure composition following in-gel glycan release via $\beta$-elimination. A) shows the precursor ion and B) shows the spectrum after collision-induced dissociation (CID).

be clearly assigned to any annotated protein sequence due to a limited number of fragments. In all four glycopeptides identified, the sequence motif GTX (Glycine-Threonine-any amino acid) was present. Overall, the GTX motif was found fifteen times within the sequence of WP_070066018.1 which would indicate the potential for a very high degree of glycosylation (see supplemental Figure S5). The molecular weight of the best matched protein is 53 kDa, while the apparent molecular weight of the glycoprotein on the SDS-PAGE was approximately 80 kDa. A substantially higher apparent molecular weight on SDS-PAGE compared to the theoretical molecular weight has been frequently observed for glycosylated proteins.

To determine the composition of the discovered glycan, in-gel $\beta$-elimination was applied to release the presumed O-linked glycan from the protein backbone. Subsequent PGC-MS/MS analysis of the released structure confirmed the presence of a glycan with a monoisotopic mass of 1456.6 Da. Fragmentation experiments further showed that the glycan is a heptameric structure with 4 different types of residues, as indicated in Figure 3.5 (and supplemental Figure S7). A methylated N-acetyl hexosamine (HexNAc) backbone, substituted with a pentose and a dideoxyhexose residue were annotated by mass. In addition, the glycan carried a 350 Da large terminal residue. The 350 Da terminal residue is unique and to the best of the knowledge of the authors, has not been reported before and therefore could not be further specified by mass.

### LECTIN STAINING

To demonstrate that glycoproteins are located outside the cell or within the EPS matrix the granules were subjected to fluorescence lectin staining. After screening all commercially available lectins, *Vicia graminea* (VGA) labeled with fluoresceine isothiocyanate (FITC) was selected as it showed a strong signal of the granule sub-structure. VGA is mostly known from medical applications for detecting O-linked Galactose/GalNAc epitopes. Interestingly, VGA is reported to only recognize peptide linked carbohydrate conjugates, while it does not react with free carbohydrates [98, 99]. In Figure 3.6 a maximum intensity projection of 53 images recorded by confocal laser microscopy is presented indicating the high abundance of glycoproteins throughout the granule. It is reasonable to assume VGA binding to the HexNAc constituents of the 80 kDa protein identified here, as it is a highly abundant O-glycosylated protein.

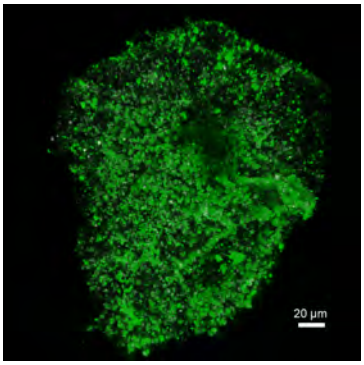



Figure 3.6: Confocal laser scanning microscopy showing a maximum intensity projection of 53 optical sections 1 $\mu$m apart. The anammox granule was stained with the lectin from *Vicia graminea* (VGA-FITC). VGA is reported to be specific for peptide linked glycan conjugates while it does not bind to the free carbohydrates [98, 99].

## 3.4. DISCUSSION

EPS are the key factor when it comes to understanding the structure and the stability of anammox granular sludge. In the present study, EPS was extracted from granular sludge from a full-scale anammox WWTP. An alkaline extraction was used, because it solubilised the granular shape, indicating that a significant part of the structural polymers was solubilized. Because the EPS is a complex network, there is no single method to extract all the EPS components. To study other possible EPS components, different extraction methods are required. Alkaline treatment can extract negatively charged components and can break disulphide bonds in proteins, making it easier to extract them [20]. Due to the acidic nature of the EPS, solubilizing them can cause a decrease in pH. A high concentration of NaOH was used to maintain a high pH during the whole extraction. The alkaline extraction resulted in new targets to study. Glycoproteins and acidic (including sulfated) glycoconjugates were found with the appropriate staining experiments. A relatively high abundant glycoprotein with an apparent molecular weight of 80 kDa was

further characterized. It was found to be heavily glycosylated with a heterogeneous O-glycan. To our knowledge, little or no literature provides direct evidence of glycoproteins in granular sludge or natural biofilms. In previous EPS studies, glycoproteins would have been overlooked due to the fact that proteins and polysaccharides are often studied separately, and unspecific colorimetric assays are commonly used.

The apparent molecular weight of the 80 kDa glycoprotein fitted in the molecular weight range for S-layer proteins and the glycoprotein was successfully enriched with a protocol for S-layer extraction. This suggested the targeted glycoprotein to be an S-layer protein. S-layer proteins are often glycosylated and are one of the most commonly observed cell surface structures of prokaryotes [100]. Since they form the outermost layer of the cells, they are directly involved in the interactions between the cell and its environment, which makes it an interesting target in biofilm research. Mass spectrometric analysis of the 80 kDa glycoprotein showed that the sequence of the target protein was not identical to any known anammox S-layer protein annotated in the database. This could be due to the fact that there is in general low homology amongst S-layer proteins [101]. The identified glycoprotein might be a not yet annotated protein from a "*Ca.* Brocadia" species, since there were strong indications for single amino acid polymorphism within the matched sequence regions and some other segments of the sequence were not matched at all. Mass spectrometric analysis revealed a previously retracted protein sequence from "*Ca.* Brocadia sapporoensis" (WP_070066018.1) and a C-terminal fragment of the same protein (OQD46794.1) as closest match.

The draft genome of "*Ca.* Brocadia sp. 40" was published in 2016 by Ali *et al.* [102]. In 2017 the bacteria were analysed in more detail and was renamed "*Ca.* Brocadia sapporoensis" [94]. Although the closest match sequence (WP_070066018.1) is retracted, it is very similar to WP_070066019.1 "*Ca.* Brocadia sapporoensis" and also other "*Ca.* Brocadia species" have a similar protein (see supplemental Figure S8). Therefore, the sequence WP_070066018.1 was considered representative and was used to predict structural aspects of the identified glycoprotein. These aspects were found to be comparable to the 250 kDa S-layer glycoprotein Kustd1514. This glycoprotein was recently identified in another anammox species, "*Ca.* Kuenenia stuttgartiensis", van Teeseling *et al.* [103, 104]. (The similarities are also valid for WP_070066019.1.) Interestingly, using the InterPro protein analysis tool, the 80 kDa glycoprotein was predicted to contain an Immunoglobulin-like (Ig-like) domain. This is also found for Kustd1514 (See supplemental Figure S3 and S4 for sequence analysis.) Ig-like domains are observed in cell surface proteins and have different functions, including cell-cell recognition and cell surface receptor functions [105]. The highly abundant amino acids, 16% threonine, 9% serine and 9% glycine, suggest a structural role, similar as for Kustd1514 (14% threonine, 12% serine and 10% glycine). Also in terms of glycan attachment site (GTX) and glycan structure the 80 kDa glycoprotein was comparable with Kustd1514.45 In this study at least 4 O-glycan attachment sites could be identified. Considering the number of GTX motifs within the proposed protein sequence, an even higher degree of glycosylation could be assumed. The HexNAc backbone, substituted with a pentose and a dideoxyhexose residue was also comparable to Kustd1514 [104].

Based on the findings of this study, we hypothesize that the found glycoprotein is an S-layer protein. Although S-layer proteins are one of the most commonly observed cell

surface structures of prokaryotes [101], there is no general function assigned. One of the functions that are proposed in literature is a role in biofilms. For example, S-layer proteins of *Tannerella forsythia*, have been found to be up-regulated when grown as biofilms [89]. It would be of interest to study the role of S-layers in the structure of the matrix of granules. The best way to identify S-layers is by visualizing their specific pattern on the cell surface with microscopy. However, because of the compact EPS around the cells it is more difficult to visualize the S-layer proteins in mature granules than in suspended biomass from lab-scale reactors. In addition, S-layers can be shed of the cells [106], and therefore can be potentially integrated in the matrix. To elucidate the potential role of the identified glycoprotein in granules, more specific studies on the localization of the protein are required to study whether it is attached to the outside of the cell wall or integrated within the matrix. Apart from a potential structural role in supporting the formation of a gel matrix, the heterologous glycan structure which is linked to the protein via a methylated HexNAc may provide a very efficient protective layer against degradation [107]. This could be of high importance for slow growing micro-organisms.

In addition to the O-glycosylated 80 kDa protein, the staining experiments showed the presence of other glycoconjugates: a large conjugate (>235 kDa) or polymer-like structure with carboxylate residues, and a sulfate containing structure of approximately 12 kDa. Currently the extracellular matrix of biofilms is recognized to be a highly complex and organized structure, and sometimes even considered comparable to the extracellular matrix of multicellular organisms [108, 109]. Glycoproteins and (sulfated) proteoglycans are major components of the extracellular matrix of mammalian cells [77]. In the current research, glycoconjugates with both carboxylate and sulfate groups are present in the recovered EPS, and glycoproteins were detected in situ throughout the whole granule. In addition, the high molecular weight smear that appeared on the SDS-PAGE, resembled properties of mucin-like compounds. Mucins are glycoproteins which form mucus gels as a protective barrier around the epithelial cells and aid against infection and dehydration [110]. Likewise, the EPS matrix of granules is considered as a hydrogel-like matrix which protects the cells and allows nutrients to diffuse to the cells [111]. The finding of the various glycoconjugates in the anammox granules reflects the idea of similarity between biofilms and multicellular organisms, regarding the extracellular matrix.

Remarkably, most of the prokaryotic protein glycosylation with higher complexity have been associated with pathogenic traits of bacteria but were rarely described in natural biofilm communities. The results presented in this study demonstrate that it is necessary to include glycoproteins as a major target in the EPS research field. The proteins and glycans should be studied in the context of an integrated structure, since the physical properties and the biological function of glycoproteins are determined by the combination of both parts [112]. To achieve this, in-depth molecular analyses that will allow a deeper understanding of the matrix structure in biofilms and granular sludge are significantly required.

## SUPPLEMENTARY INFORMATION

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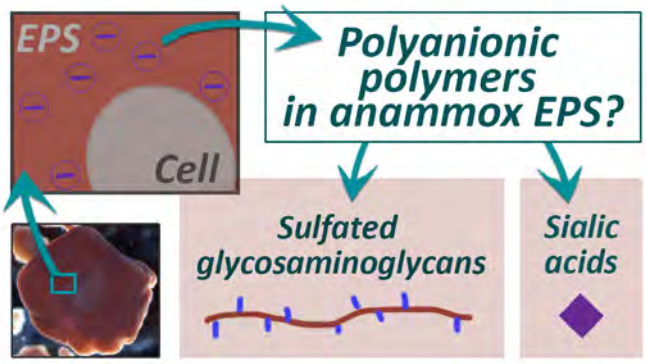
# 4

# Decorating the Anammox House: Sialic acids and Sulfated Glycosaminoglycans in the Extracellular Polymeric Substances of Anammox Granular Sludge

This chapter is under review for publication in *Environmental Science & Technology* as: 'Decorating the Anammox House: Sialic Acids and Sulfated Glycosaminoglycans in the Extracellular Polymeric Substances of Anammox Granular Sludge', by the authors Marissa Boleij, Hugo Kleikamp, Martin Pabst, Thomas R. Neu, Mark C. M. Van Loosdrecht, and Yuemei Lin

## ABSTRACT

ANAMMOX (anaerobic ammonium oxidation) bacteria are important for the nitrogen cycle in both natural environments and wastewater treatment plants. These bacteria have a strong tendency to grow in aggregates like biofilms and granular sludge. To understand the formation of anammox aggregates, it is required to unravel the composition of the extracellular polymeric substances (EPS), which are produced by the bacteria to develop into aggregates and granules. Here we investigated anionic polymers in anammox granular sludge, focussing on sialic acids and sulfated glycosaminoglycans. Quantification assays and fluorescent stains indicated that sialic acids and sulfated glycosaminoglycans were present in the anammox EPS (1.6% equivalents of sialic acids and 2.4% equivalents of sulfated glycosaminoglycans). Staining specific for strongly polyanionic components indicated that the sulfated glycosaminoglycans are located around the cell walls of the bacteria in anammox granules. Finally, the potential genes for the biosynthesis of sialic acids and sulfated glycosaminoglycans were analyzed in the anammox draft genomes. The finding of these components in anammox granular sludge and previously other non-pathogenic bacteria, point out that sialic acids and sulfated glycosaminoglycans are worth investigating in the context of a broader function in microbial communities and biofilm systems in general.



## 4.1. INTRODUCTION

In the early nineties the anaerobic ammonium oxidation (anammox) process, in which ammonium and nitrite are converted into nitrogen gas, was discovered [113]. The bacteria that were found to perform this process are referred to as anammox bacteria, and they belong to a separate order named '*Candidatus* Brocadiales', within the phylum *Planctomycetes*. They can perform the anammox process without the need of an organic carbon source or oxygen. In natural environments, anammox bacteria contribute significantly to the global nitrogen cycle [114]. Moreover, the anammox process is applied in wastewater treatment to remove nitrogen from wastewater. Anammox bacteria have a strong tendency to grow in aggregated form [8]. This trait is exploited in anammox wastewater treatment plants, where the bacteria are grown in granular sludge form. Granular sludge systems provide high sludge settling velocities, ensuring a high biomass retention and utilization of relatively small reactors, costs and footprint.

Similar as in other kinds of biofilm, in granular sludge, bacteria are immobilized in a self-produced matrix. This matrix consists of a complex mixture of components which is referred to as extracellular polymeric substances (EPS). In biofilm research, unravelling the EPS composition and function is important to move towards a comprehensive understanding and better control of biofilm formation. Due to limitations in methodology for extraction and characterization, the EPS composition is still far from fully characterized [35]. Great efforts have been taken along this research line in recent years, and progress has been made with respect to solubilization of the biofilm and discovery of new components in EPS [36, 43, 44, 115].

In our previous study, presence of strongly anionic groups in EPS of anammox granules was indicated by means of staining with the cationic dye Alcian Blue [36]. Alcian Blue stained two targets after gel electrophoresis of alkaline extracted EPS, which could be distinguished by molecular weight and strength of negative charge: A high molecular weight component (> 235 kDa) stained at pH 2.5, indicating acidic groups (i.e. carboxyl group and/or sulfate group), and a component with an apparent molecular weight of around 12 kDa stained at pH 1.0, indicating an even stronger negative charge. Because of the strong acidic character, combined with the high S-content (1.4%) measured in the extracted EPS, this strongly negatively charged component was assumed to be a polymer with sulfate groups. However, the nature and functions of these acidic components remain unknown.

Negatively charged groups are reported to play an important role in the adhesion capacity of EPS [116], and protection of bacteria against environmental stresses [117]. Moreover, negatively charged polysaccharides have been a target of interest in a few biofilm studies, e.g. alginates and uronic acids [1]. However, existence of polysaccharides with other negatively charged groups is also possible.

Sialic acids are a group of negatively charged nine-carbon monosaccharides which are mostly found as the terminal residue of glycoconjugates in eukaryotes or pathogenic bacteria, where they play important roles in mediating cellular recognition, adhesion processes, and protecting underlying tissue [118, 119]. There are different types of sialic acids. The most known is N-acetyl neuraminic acid, among many other (more than 50) derivatives. Sialic acid types were found in prokaryotes, as well. These are present in different isomeric forms, named after pseudaminic acid and legionaminic acid. These

sialic acids are synthesized by bacteria via (partly) different pathways in comparison to their eukaryotic counterpart [120]. They were also referred to as bacterial sialic acids in literature.

Sulfated polysaccharides are mostly known from the extracellular matrix of animal as sulfated glycosaminoglycans. Sulfated glycosaminoglycans are complex linear polysaccharides that can be classified into three major groups: (1) chondroitin sulfate and dermatan sulfate, (2) heparin and heparan sulfate, and (3) keratan sulfate. These molecules are associated with functions in mediating adhesion and cell signalling in mostly eukaryotes and pathogenic bacteria [121]. The presence of the strongly negatively charged components in combination with the high sulfur content in our previous study, raised the question: Is the strong anionic component detected in the EPS of anammox granular sludge due to presence components like sulfated glycosaminoglycans?

Here, we present a study in which the previously revealed anionic components in anammox EPS are further analysed, focussing on identification, quantification and localization of sialic acids and sulfated glycosaminoglycans. To this end, alkaline extracted EPS was subjected to quantification assays and to mass spectrometry. In addition, specific fluorescent stains were applied to the intact anammox granules. Finally, genome database searches were performed in order to find potential pathways that could be involved in the formation of these anionic components by anammox bacteria.

## 4.2. MATERIALS AND METHODS

### ANAMMOX GRANULAR SLUDGE AND EPS EXTRACTION

Anammox granular sludge was collected from the full-scale anammox reactor in Sluisjesdijk, Rotterdam [17]. The VSS (volatile suspended solids) content of the granules was 0.71 g/g granules (determined in accordance with APHA, 2005 [81]). The dominant anammox species in the granules was '*Ca.* Brocadia sapporoensis' (according to FISH, clone library analysis and protein analysis in Boleij *et al.* [115]). EPS extraction was performed as described in Boleij *et al.* [36]. The granules were solubilized in 0.1 M NaOH for 5 hours while being stirred with a magnetic stirrer (IKA, C-MAG HS7), using a magnet with a diameter that covers the surface of the bottle, at 400 rpm. After centrifugation at 4000 rpm for 20 minutes at 4°C, the pellet was discarded. Polymers in the supernatant were precipitated out by decreasing the pH to 5 using 1M HCl. The precipitated polymers were collected by centrifugation at 4000 rpm for 20 minutes at 4°C and directly lyophilized.

### SIALIC ACID QUANTIFICATION ASSAY

To measure sialic acids, the Sialic Acid Quantitation Kit (Sigma-Aldrich) was used according to manufacturer's instruction. The protocol was performed as described by de Graaff *et al.* [122]. The assay was applied on crushed lyophilized granules, and N-acetylneuraminic acid (NeuAc) was used as a standard. This assay measures NeuAc after its release by enzymatic cleavage using neuraminidase. Therefore it is suitable to measure the amount of NeuAc in either free form, or in glycoproteins, cell surface glycoproteins, polysialic acids, and capsular polysaccharides.

### SIALIC ACID MEASUREMENT WITH MASS SPECTROMETRY

Mass spectrometric sialic acid analysis was performed following physical disruption and homogenization of 2.5 mg of freeze-dried granule material, using a lab mortar and pestle, and hydrolysis using diluted (2M) acetic acid solution Released sialic acids were further labelled directly from the speed vac dried lysate using DMB (1,2-diamino-4,5-methylene dioxybenzene dihydrochloride). Labelled sialic acid derivatives were further analysed by reverse phase chromatography Orbitrap mass spectrometry (QE plus quadrupole Orbitrap, Thermo, Germany). Sialic acids were identified by comparison to commercial standards and mass.

### EPS EXTRACTION FOR SULFATED GLYCOSAMINOGLYCANS MEASUREMENT

In the previous study where anionic polymers were discovered [36], an alkaline extraction using precipitation at pH 5 was applied. For the measurement of sulfated glycosaminoglycans in this study, the same extraction method (as described in section 2.1) was used as reference. In addition, the alkaline extraction was applied with precipitation at pH 2.5, in order to investigate if there are more sulfated glycosaminoglycans extracted when the precipitation was performed at a lower pH. As a control, the alkaline protocol was applied, using dialysis to remove the NaOH, instead of precipitation. In theory this would include all polymers that are solubilized, with a molecular cut-off of 3 kDa. Samples were lyophilized directly after extraction until further analysis. Hence, three extracted samples: extract 1 (pH 5), extract 2 (pH 2,5), extract 3 (dialyzed) were subjected to the sulfated glycosaminoglycan assay and the preceding treatment.

### SULFATED GLYCOSAMINOGLYCAN ASSAY

As a pre-treatment for the glycosaminoglycan assay, samples were denatured and treated with proteinase K (Sigma Aldrich, 30 units/mg of protein). For denaturation, 4 mg/ml lyophilized sample was solubilized in a solution of 6 M Urea, 25 mM $NH_4HCO_3$ and 10 mM DTT, and incubated at 65 °C for 30 min. After cooling to room temperature, iodoacetamide was added to a final concentration of 40 mM, and samples were incubated for 30 min at room temperature. For the enzyme treatment, the samples were 4x diluted in TRIS buffer (50 mM TRIS, 5 mM $CaCl_2$ and 10 mM EDTA) to decrease the urea concentration, avoiding inhibition of the enzyme treatment. Proteinase K was added to a final concentration of 125 $\mu$g/ml and the samples were incubated overnight at 37 °C.

### PRE-TREATMENT FOR SULFATED GLYCOSAMINOGLYCAN ASSAY

The Blyscan sulfated glycosaminoglycan assay was used to quantify sulfated glycosaminoglycans in the three extracts (described in an earlier section), as well as in the whole granules and the pellet remaining after alkaline solubilization. The assay is based on binding of 1,9-dimethyl-methylene blue (DMMB) to sulfated glycosaminoglycans at a low pH (measured pH in the DMMB solution was 1.7). The DMMB-sulfated glycosaminoglycan complex will precipitate and subsequently the non-bound soluble dye can be removed. Then, the bound dye is released using a dissociation reagent and the absorbance is measured to indicate the amount of dye that formed a complex with the sulfated glycosaminoglycans. The standard that is included in the kit is bovine tracheal chondroitin 4-sulfate. The protocol was performed according to manufacturer's instruc-

tions (Blyscan, Biocolor (UK)), after the pre-treatment was applied as described in the previous section.

### SDS-PAGE (SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS) AND STAINING WITH COOMASSIE BLUE AND ALCIAN BLUE

The extracted EPS were analysed by SDS-PAGE, as described in Boleij *et al.* [36]. SDS-PAGE was performed using NuPage® Novex 4-12% Bis-Tris gels (Invitrogen). EPS samples were prepared in NuPAGE LDS-buffer and DTT (dithiothreitol) was added to a final concentration of 10 mM. The proteins were denatured by incubation at 70 °C for 10 minutes. Subsequently, 10 $\mu$l sample was loaded per well. The Thermo Scientific Spectra Multicolor Broad Range Protein Ladder was used as molecular weight marker. The gel electrophoresis was performed at 200 V for 35 minutes. The gels were stained by three different staining procedures afterwards.

For visualization of proteins, the Colloidal Blue staining kit (Invitrogen) was used according to manufacturer's instructions. For staining of acidic glycoconjugates, the cationic dye Alcian Blue 8GX (Fluka, Sigma Aldrich) was used. To differentiate between the relatively weaker acidic groups like carboxylate (R-$COO^-$) and the stronger acidic group like sulfate (R-$OSO_3^-$), staining with Alcian Blue was performed at different pH values, namely pH 2.5 and pH 1.0. An adapted protocol of Møller and Poulsen [49] was used. After electrophoresis, the gels were extensively washed in solution I (25% (v/v) ethanol and 10% (v/v) acetic acid, pH 2.5) for 2.5 hours while refreshing the solution 4 times. Subsequently, the gel was stained in 0.125% (w/v) Alcian Blue in solution I (the solution was stirred overnight to dissolve the Alcian Blue and centrifuged before use) for 30 minutes and washed in solution I overnight. For staining of sulfated groups at pH 1.0, the same protocol was performed except that solution I was replaced by solution II (0.1 M HCl and 25% (v/v) ethanol, pH 1.0) according to [85].

### LECTIN STAINING

The glycoconjugates of the granules were studied after fluorescence lectin bar-coding and subsequent fluorescence lectin-binding analysis [93]. Consequently, all commercially available lectins (FITC, Fluorescein, Alexa488) were screened for binding to granule structures. Lectin staining was already described in several publications [19, 123]. In short, the fully hydrated, intact granules were incubated with a few droplets of lectins (0.1 mg/ml) for 20 min at room temperature in the dark and washed 3 times to remove the unbound lectins. The lectin-stained, hydrated, intact granules were mounted in coverwell chambers (Thermofisher) with 1 mm spacers in order to avoid compression of the granule. Samples were examined using a Leica SP5X instrument (Leica, Germany) equipped with an upright microscope and a super continuum light source (white laser). The setup was controlled by the LAS AF software version 2.4.1. Confocal images were recorded as serial scan and a step size of 1 $\mu$m using a 25x NA 0.95 water immersion lens. Laser excitation was at 490 nm, emission was from 485-495 nm (reflection) and 505-600 nm (lectins). For improvement of resolution and contrast, the image datasets were subjected to blind deconvolution with Huygens version 18.10.0 (SVI, The Netherlands). Data were projected using Imaris version 9.2.1 (Bitplane). All image data sets were finalized in Photoshop (Adobe).

### HEPARIN RED STAINING

The fluorescent probe Heparin Red (RedProbes, Münster, Germany) was employed for staining negatively charged macromolecules (such as Heparin) in the granular matrix. For staining the protocol of the supplier's data sheet was followed. In short, 8.8 $\mu$l Heparin Red and 1 ml enhancer solution were mixed and added to the fully hydrated, intact granules for 1 hour. The Heparin Red-stained, fully hydrated, intact granules were immediately examined by CLSM using the instrument described above. Image data sets were recorded as single or serial scan (step size 1, 0.5, or 0.17 $\mu$m) using a 63x NA 1.2 water or a 100x NA 1.4 oil immersion lens at various zoom settings. Laser excitation was at 480 nm and 567 nm, emission was from 470-490 nm (reflection) and 590-650 nm (Heparin Red). Image data handling was already explained above. In addition, Heparin Red data sets were loaded in Fiji (https://fiji.sc/) and color coded with the lookup table called "rainbow RGB". For better color separation of pixel intensities, the contrast was set to auto. By this treatment three pixel intensities are color coded as: red = strong signal, green = intermediate signal, blue = weak signal.

### BLAST (BASIC LOCAL ALIGNMENT SEARCH TOOL) ANALYSIS OF PATHWAYS FOR SYNTHESIS OF SIALIC ACID TYPES AND SULFATED GLYCOSAMINOGLYCANS

BLAST analysis was applied in order to find homologous biosynthesis pathways of sialic acids and sulfated glycosaminoglycans. The BLAST tool at the NCBI website was used to BLAST reference proteins against the draft genomes included in the database of '*Candidatus* Brocadiales' (taxid:1127829). Matches were viewed as significantly similar when E-values were below 1E-20.

## 4.3. RESULTS

### SIALIC ACIDS IN ANAMMOX GRANULAR SLUDGE

Previously, a high amount of proteins (around 60%) was measured in the extracted EPS of anammox granules, and various glycoconjugates were found [36]. Glycoconjugate modifications with acidic groups such as sulfate (sulfation) and/or sialic acid (sialylation) on the polysaccharides part are common phenomena in the extracellular matrix of eukaryotes [124]. In this previous study, the presence of polyanionic groups in glycoconjugates was indicated by staining with a cationic dye (Alcian blue). Here, analysis is performed on the strongly negatively charged polyanionic components such as sulfates and sialic acids.

Sialic acids were measured in the anammox granules, using a sialic acid quantitation kit, which is based on the release of N-acetylneuramic acid (NeuAc) by an enzymatic treatment. According to this assay, the anammox granules contain 1.6% sialic acids (weight-% of gram of N-acetylneuraminic acid equivalents per gram VSS of granular sludge). Hence, the quantification assay data indicates the presence of sialic acids. To identify which kinds of sialic acids are present in anammox granules, mass spectrometry (MS) was applied. With MS, sialic acids were detected in the form of N-acetyl neuraminic acid (NeuAc), deaminated neuraminic acid (KDN) and pseudaminic acid/legionaminic acid (Pse/Leg, which have the same molecular weight) (see supplementary Table S1).

Hence, there are three different kinds of sialic acids widely distributed in anammox granules.

The draft genome of "*Ca.* Brocadiales" was analysed for genes present that could code for the pathways for the biosynthesis of sialic acids, reference pathways from known sialic acid producers were taken (see supplemental Figure S1). The genes of the required enzymes were aligned with the draft genomes of species in the order "*Ca.* Brocadiales" that are available in the database of NCBI. Because *Campylobacter jejuni* has characterized pathways for biosynthesis of NeuAc, Leg and Pse, these pathways were used as reference genes [125]. Those pathways were partly matching with proteins in the "*Ca.* Brocadiales" database (supplementary Table S2). Many of the genes of Brocadiales that matched the legionaminic acid synthase were in common with the genes that matched with pseudamiminc acid synthase and the N-acetylneuraminic acid synthase. Even though these pathways are distinct, the enzymes are homologous.

In the halophile archaea *Halorubrum sp.* PV6, a legionaminic acid was reported to be attached to its surface layer (S-layer) glycoprotein [126]. Therefore, the reported genes of *Halorubrum sp.* PV6 were also used as a reference to BLAST the draft genome of "*Ca.* Brocadiales". All enzymes for biosynthesis of legionaminic acid were found to have a significant match with a database protein in the order of "*Ca.* Brocadiales" (Table 4.1). Hence, the biosynthesis pathway of *Halorubrum* matched better with anammox than the synthesis pathways in *Campylobacter jejuni*.

Table 4.1: Blast search for the pathway for synthesis of legionaminic acid. The reference pathway is from halophile *Halorubrum sp.* PV6 [126]. The best match shown is the best match with '*Ca.* Brocadia sapporoensis'. *When there was no match with '*Ca.* Brocadia sapporoensis', the best match of any '*Ca.* Brocadiales' is shown.

| **Reference gene and protein** | **Accession nr. best match** | **Name protein and species** | **Identity** | **E-value** |
|---|---|---|---|---|
| HrrPV6_1047 AYD49518.1 (LegB) | WP_070067840.1 | NAD-dependent epimerase/ dehydratase family protein [*Ca.* Brocadia sapporoensis] | 38.0 % | 1.8E-55 |
| HrrPV6_1046 AYD49517.1 (LegC) | WP_070067943.1 | DegT/DnrJ/EryC1/StrS family aminotransferase [*Ca.* Brocadia sapporoensis] | 35.4% | 1.2E-66 |
| HrrPV6_1014 AYD49510.1 (LegH) | WP_070066607.1 | hypothetical protein [*Ca.* Brocadia sapporoensis] | 32.7% | 3.1E-51 |
| HrrPV6_1049 AYD49520.1 (LegG) | TFG47122.1 | UDP-N-acetylglucosamine 2-epimerase (hydrolyzing) [*Ca.* Brocadiae bacterium] * | 37.5% | 2.3E-72 |
| HrrPV6_1048 AYD49519.1 (LegF) | TLD41229.1 | N-Acetylneuraminate cytidylyltransferase [*Ca.* Jettenia ecosi] * | 34.6% | 8.0-42 |
| HrrPV6_1053 AYD49523.1 (LegI) | TFG47123.1 | N-acetylneuraminate synthase [*Ca.* Brocadiae bacterium] * | 50.1% | 1.5E-109 |

Table 4.2: Measured amount of sulfated glycosaminoglycans (GAG) (chondroitin sulfate equivalents), compared to the amount that was recovered from the granular sludge. The percentages of sulfated GAG equivalents are based on the measured amount of VSS in samples before pretreatment.

| | Extraction yield (VSS EPS/granules) | Sulfated GAG equivalents measured | Sulfated GAG in granules (calculated) |
|---|---|---|---|
| **Extract 1** | 24% | 5.4% | 1.3% |
| **Extract 2** | 22% | 6.9% | 1.5% |
| **Extract 3** | 31% | 5.1% | 1.6% |
| **Granules** | - | 2.4% | 2.4% |
| **Pellet** | - | - | 0.9% |

### QUANTIFICATION OF SULFATED GLYCOSAMINOGLYCANS

In order to investigate the strongly anionic polymers, the presence of sulfated glycosaminoglycans was investigated by using the Blyscan sulfated glycosaminoglycan assay. This assay is based on a dye-binding method, in which 1,9-dimethylmetylene blue (DMMB) is used to estimate the amount of sulfated glycosaminoglycans. DMMB can bind free sulfated glycosaminoglycans or sulfated polysaccharides that are bound to a protein backbone (proteoglycans).

In the EPS extraction process, the anammox granules were first solubilized in NaOH. Afterwards, the EPS was recovered from this alkaline solution by three methods in parallel: precipitation with acid at pH 5 (extract 1), precipitation with acid at pH 2.5 (extract 2) and with dialysis (extract 3). The different recovery methods yielded 24, 22 and 31% of the initial VSS amount, respectively (Table 4.2). The recovered materials were subjected to the sulfated glycosaminoglycan assay. In addition, the whole granules and the pellet that remained undissolved after alkaline treatment were analysed.

For application of the glycosaminoglycan assay the recovered EPS were solubilized in TRIS buffer. When the solution of the extracted EPS was directly applied to the assay, 1.2% of the VSS fraction was measured as sulfated glycosaminoglycans. It was noticed that a huge amount of precipitation formed once the EPS solution was mixed with the dye solution, presumably due to the high protein content in combination with the low pH of the dye solution. Formation of protein precipitation was suspected to hinder the binding of DMMB to the sulfated glycosaminoglycans. To avoid the interference of proteins, a pre-treatment was applied in which the EPS was denatured and treated with proteinase K. After pre-treatment, most of the proteins were successfully removed (no more binding of Coomassie blue in SDS-PAGE). The 12 kDa band in the SDS-PAGE gel was still stained by Alcian Blue after pre-treatment. The pre-treatment significantly increased the outcome of the quantification assay. The sulfated glycosaminoglycans measured in extract 1, 2 and 3 was 5.4%, 6.9% and 5.1% of the VSS fraction of the extracted EPS, respectively (see Table 4.2. When whole anammox granules were subjected to pretreatment and quantification assay, instead of the extracted EPS, 2.4% of the VSS fraction was measurement as sulfated glycosaminoglycans.

Since the quantification assay is based on precipitation of the DMMB-sulfated glycosaminoglycan complex, it also served as a method for sulfated glycosaminoglycans isolation. The precipitated DMMB-sulfated glycosaminoglycan complex was collected,

re-solubilized in the NuPAGE LDS-buffer and DTT (dithiothreitol), and applied on SDS-PAGE to check what components were precipitated in the DMMB complex. The profiles are shown in Figure 4.1. The target component was the band at 12 kDa. In Figure 4.1 it can be seen that the 12 kDa band is indeed present in the precipitated DMMB complex. Hence, the DMMB assay targets the 12 kDa band.

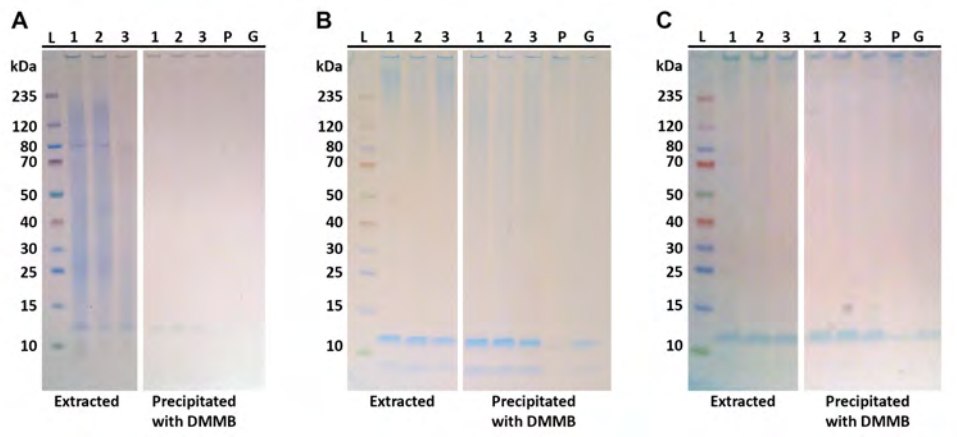



Figure 4.1: Images of SDS-PAGE gels with extracts 1, 2, 3, the pellet (P) and the whole granules (G). Lane L contains the molecular weight ladder. Gels were stained with A) Coomassie Blue, B) Alcian blue at pH 2.5 and C) Alcian blue at pH 1.0. In all panels the right part contains the profile of the material after EPS extraction and the left part is the fraction that formed a complex with the DMMB dye in the glycosaminoglycan assay. (Note that before pre-treatment, the pellet and the whole granules cannot be applied on a gel since they are not soluble.)

### ANALYSIS OF ANAMMOX GENOME FOR SYNTHESIS OF SULFATED GLYCOSAMINOGLYCANS

Sulfotransferases are the enzymes that transfer sulfo groups to polysaccharides [127]. For sulfated glycosaminoglycan sulfotransferases there is little literature available about the encoding genes in bacteria. Searches in the NCBI database to chondroitin, heparan and keratan sulfotransferase resulted in bacterial genes only for the first two types. Examples for known genes are the chondroitin 4-O-sulfotransferase gene in *Pseudomonas fluorescens F113* and heparan sulfate glucosamine 3-O-sulfotransferase in bacteria *Sinorhizobium fredii NRG234* [128]. The corresponding protein sequences were blasted against the '*Ca.* Brocadiales' database. For chondroitin 4-O-sulfotransferase (accession number WP_014336261.1) no significant similarity was found (i.e. E-values were higher than 2E-05). Interestingly, blasting against the heparan sulfate glucosamine 3-O-sulfotransferase (accession number YP_002823420.1) resulted in significant similarity with several proteins, shown in Table 4.3, which means there is a higher chance for anammox bacteria to produce heparan sulfate.

Table 4.3: List with significant hits (E-value<1E-20) of blast search against heparan sulfate glucosamine 3-o-sulfotransferase (accession number YP_002823420.1).

| Accession number | Name protein and species | Identity | E-value |
|---|---|---|---|
| RZV93996.1 | hypothetical protein EX341_03835 [*Ca.* Scalindua sp. SCAELEC01] | 31.8% | 6.04E-33 |
| RZV93978.1 | hypothetical protein EX341_03745 [*Ca.* Scalindua sp. SCAELEC01] | 29.8% | 4.76E-31 |
| GAN32649.1 | hypothetical protein BROSI_A1164 [*Ca.* Brocadia sinica JPN1] | 30.2% | 9.45E-31 |
| WP_082059063.1 | hypothetical protein [*Ca.* Brocadia sinica] | 30.2% | 2.72E-30 |
| KXK32771.1 | sulfotransferase [*Ca.* Brocadia sinica] | 28.9% | 7.13E-29 |
| KXK32773.1 | sulfotransferase [*Ca.* Brocadia sinica] | 28.8% | 3.62E-28 |
| WP_052562786.1 | hypothetical protein [*Ca.* Brocadia sinica] | 29.6% | 1.95E-27 |
| TLD42416.1 | Sulfotransferase [*Ca.* Jettenia ecosi] | 27.3% | 3.66E-24 |
| WP_099326166.1 | sulfotransferase [*Ca.* Kuenenia stuttgartiensis] | 29.0% | 6.33E-24 |
| WP_007223162.1 | sulfotransferase [*Ca.* Jettenia caeni] | 26.4% | 2.60E-23 |
| KHE93325.1 | sulfotransferase [*Ca.* Scalindua brodae] | 27.1% | 1.80E-22 |
| TLD42415.1 | Sulfotransferase [*Ca.* Jettenia ecosi] | 28.4% | 1.26E-21 |
| KXK28276.1 | sulfotransferase [*Ca.* Brocadia sinica] | 25.8% | 1.30E-21 |
| ODS30602.1 | sulfotransferase [*Ca.* Scalindua rubra] | 27.3% | 4.78E-21 |
| OQZ03151.1 | hypothetical protein B6D35_00200 [*Ca.* Brocadia sp. UTAMX2] | 24.8% | 6.16E-20 |
| KXK28277.1 | sulfotransferase [*Ca.* Brocadia sinica] | 29.1% | 7.05E-20 |

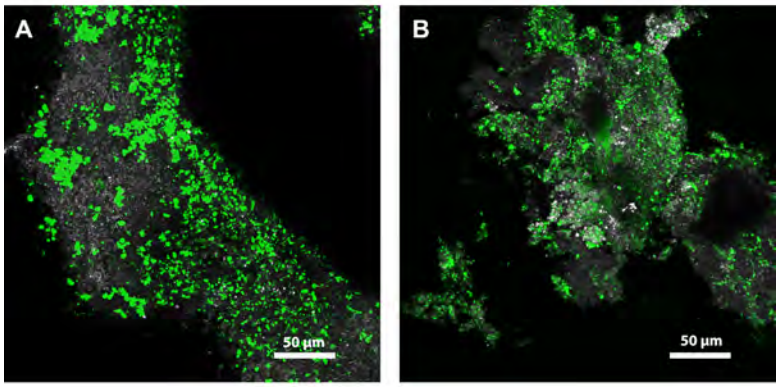



Figure 4.2: Confocal laser scanning microscopy of fully hydrated, intact anammox granules stained with fluorescently labelled lectins shown as maximum intensity projections. A) WGA (Wheat germ agglutinin, 40 optical sections) and B) HAA (Helix aspersa agglutinin, 66 optical sections). Colour allocation: grey – reflection signal, green – lectin staining.

### LECTIN STAINING AND HEPARIN RED STAINING

To localize the distribution of sialic acids in granules, lectin staining was applied. A screening with 70 lectins was performed, and several that bind to sialic acid were found to bind to the matrix of the anammox granules. In Figure 4.2, images with anammox granules stained by fluorescent lectins Wheat germ agglutinin (WGA) and Helix aspersa agglutinin (HAA), respectively, are shown. WGA binds sialic acids (N-acetyl neuraminic acid) and N-acetyl glucosamine (GlcNAc) [129]. HAA binds to O-linked glycans composed of GalNAc [130]. The visualization of the fluorescent signal of these lectins provides the possible distribution of sialic acids in the granules. Figure 4.3 shows anammox granules that were stained with Heparin Red, which is a fluorescent molecular probe. It is used for direct detection of Heparins in blood plasma. Heparin Red is polycationic. It forms complex aggregations with polyanionic components and it was used to localize polyanionic components. When Heparin Red is bound to polyanionic components, which have a charge density more negative than 0.81 per monosaccharide, it emits a fluorescent signal. On top of that, the fluorescence intensity of Heparin Red is quenched when it forms stable aggregates on the polyanionic chains. The higher the charge density, the more quenched the fluorescent signal is [131]. Since both heparin (1.8-2.4 sulfate per disaccharide) and heparan sulfate (0.8-1.8 sulfate per disaccharide) are highly negatively charged macromolecules [132, 133], their binding to Heparin red results to a quenched signal. Figure 4.3 shows the cells in the granules, after Heparin Red staining. Figure 4.3A and C are in white and black in order to see the shape of the cell clearly. In comparison, Figure 4.3B and D are the same image data sets as Figure 4.3A and C respectively, but using a look up table in which the pixel intensities are colour coded as: very low pixel intensities in blue: no binding of Heparin Red, high pixel intensities in red: binding of Heparin Red, and intermediate pixel intensities in green: strong binding of Heparin Red due to quenching. The black and white images show that after staining

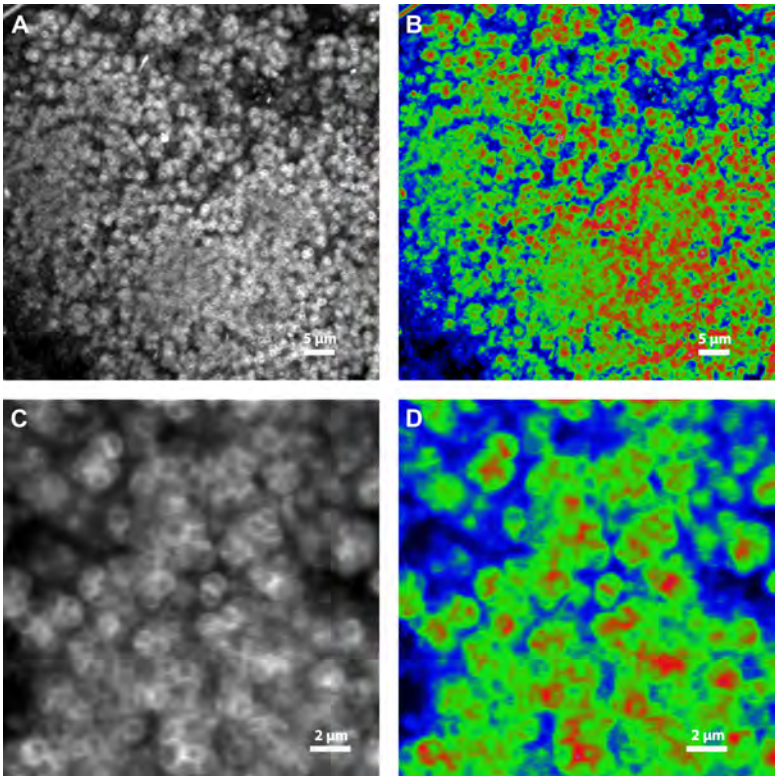



Figure 4.3: Confocal laser scanning microscopy of Heparin Red, bound to components in the fully hydrated, intact anammox granule. A) and C) are greyscale images of the fluorescent signal. A) and B) represents an image series of 45 optical sections at 0.17 $\mu$m step size shown as maximum intensity projection, C) and D) represents a single image. B) and D) are the images of A and C, respectively, converted to color images with three pixel intensities: red = binding of Heparin Red, green = quenched signal, implying high negative charge density, and blue = no binding of Heparin Red (see detailed explanation in Result section).



the measured signal looks like the shape of the cells. This suggests that the polyanionic components are located around the cell wall or in the capsule of the bacteria.

## 4.4. DISCUSSION

In EPS of anammox and other biofilm forming bacteria, presence of proteins/glycoproteins and polyanionic groups are reported [80, 122]. As both sulfate groups and sialic acids are anionic, and both sulfation and sialylation are common modifications of (glyco)-proteins and polysaccharides in the extracellular matrix of eukaryotes, the polyanionic components of anammox EPS were studied, with a focus on these two acidic modifications. Looking at the function of similar structures in known systems can direct us in finding potential functionalities of sulfation and sialylation in biofilm systems.

With the colorimetric quantification assays, around 1.6% of the VSS was measured as sialic acids (NeuAc equivalents) and 2.4% of the VSS was measured as sulfated gly-

cosaminoglycans (chondroitin equivalents). Since the samples that we analysed are not well-defined mixtures containing possibly interfering components, and we do not have the information to know the exact appropriate standard, these assays are not valid for an absolute quantification in EPS of anammox granules. Possible biases in these assays were not evaluated. In addition, the applied treatments might not release all target components. The lectin and heparin staining intensity appeared very intense in comparison with the amount that was measured with the assays. However, the assays did indicate the presence of sialic acids and sulfated glycosaminoglycans, respectively, and therefore they are worth to further look into.

In anammox granules, the presence of sialic acids in the form of NeuAc, KDN and Pse/Leg was confirmed with MS measurements. The genes encoding for sialic acid synthesis enzymes are partly present in '*Ca.* Brocadia sapporoensis' genomes. The enzymes that are necessary for sialic acids synthesis but could not be found particularly in '*Ca.* Brocadia sapporoensis' genome were found in other '*Ca.* Brocadiales' species. There are more than 50 different variants of sialic acids. In animals, sialic acids are often found as terminal residue of (mucin-)glycoprotein, which have a function in protection against proteases, cell signalling/recognition and adhesion processes [107]. In bacteria, various nine-carbon sugars like NeuAc are known, e.g. Pse and Leg. These are mostly reported in pathogenic bacteria and are thought to mimic the host with the purpose of invading the host [134]. However, this image may be biased due to the increased focus of studies on those species that are involved in host-microbe interactions. Recent examples of non-pathogenic organisms carrying sialic acid derivatives are e.g. seawater-adapted aerobic granular sludge, dominated by "*Candidatus* Accumulibacter" [122] or the surface layer (S-layer) glycoprotein of haloarchaea *Halorubrum sp.* PV6, which has a sialic acid as terminal residue [126].

Anammox bacteria are known to produce S-layer glycoproteins as well [103]. Specifically, the anammox granular sludge used in this study contains a putative S-layer glycoprotein, visualized with SDS-PAGE at 80 kDa [36]. The glycan structure of this glycoprotein was determined to be composed of a methylated N-acetyl hexosamine (HexNAc) backbone, substituted with a pentose and a dideoxyhexose residue, and carrying an unknown terminal residue of 350 Da. Hence, the S-layer glycoprotein is a possible source of the measured sialic acids in anammox EPS. However, the band at 80 kDa was not stained by Alcian Blue. If the terminal residue would be a derivative of sialic acid, the overall negative charge of the molecule is not strong enough for it to be stained by Alcian Blue.

The smear at > 235 kDa was only stained by Alcian Blue at pH 2.5, indicating carboxylic groups. The profile resembles high molecular weight polysaccharides or glycoproteins. It is also possible that both the S-layer proteins and other high molecular weight polysaccharides or glycoproteins are differently sialylated. For example in *Campylobacter jejuni*, different NeuB genes were found to be involved in biosynthesis of lipo-oligosaccharides and flagella [135]. Based on the results, sialic acids are confirmed to be present, but the exact molecular location remains to be determined. Determining the saccharide sequence that the sialic acids are attached to would reveal more about the nature of these components. In known eukaryotic systems, there are many biological roles for sialic acids, of which cellular recognition is a very important one [136]. In granular sludge and biofilms, sialic acids might also play a role in masking bacterial cells

in order to protected against invaders.

The other targeted component in this study were sulfated glycosaminoglycans. In order to determine the exact type of sulfated polysaccharide (or glycoprotein), the component should be isolated and the molecular structure should be determined. From the analyses presented here: the staining with Alcian Blue at pH 1.0, the complexing with DMMB and the staining with Heparin Red, the component resembles sulfated glycosaminoglycans. Regarding the homology with the sulfotransferases in anammox draft genomes, it fits best with heparan sulfate glucosamine 3-O-sulfotransferase. Heparin Red staining indicated that the strongly polyanionic components, suggested to be sulfated glycosaminoglycans are located at the outside of the cell walls. In comparison, the sulfated glycosaminoglycans content in aerobic granular sludge is around 3.1% of the VSS. However, the negatively charged macromolecules which were stained by Heparin Red seem to be differently distributed. While in the aerobic granular sludge the signal was observed in the space in between capsules within the microcolony and also in the extracellular matrix in between the colonies [137], in the anammox granules it appears as the shape of the cells, indicating the strongly polyanionic components are located around the cell walls or in the capsules of the anammox cells.

In the extracellular matrix of eukaryotes, heparan sulfates are located at the outside of cell surfaces, with the function of cellular recognition and adhesion to extracellular matrix components [138]. Interestingly, heparan sulfate is the most ancient of all known glycosaminoglycans [139]. With this in mind, it may be suggested that heparan sulfate might have a similar function in anammox granules as well. To confirm what kind of sulfated polysaccharides are present, the monosaccharide sequence should be determined. Knowing the exact structure would aid in predicting the function of these molecules by comparing with known systems. In addition, since bacteria don't have an endoplasmatic reticulum and Golgi system as used to synthesize sulfated glycosaminoglycans in eukaryotes, the machinery that is needed for the synthesis remains to be determined.

Currently, both sialic acids and sulfated glycosaminoglycans that are found in prokaryotes are mostly related to microbe-host interaction in the context of mimicking host extracellular matrix components, in order to bypass its immune system. However, recently they are also found in prokaryotic, non-pathogenic organisms [80, 122, 140], as well as in this study. This indicates they could be more broadly present, with a differential functionality in microbial communities and biofilms in general. Proposed functions of these negatively charged components include; protecting the cells, cell-cell or cell-matrix adhesion, scavenging of other components and involvement in biomineralization. Looking into the question if these polymers could also have similar roles in the biofilm extracellular matrix as in the multicellular eukaryotic cells, will improve the understanding of the composition and functioning of the biofilm matrix. In addition, analysing the more complex components of the biofilm matrix paves the way to the production of biopolymers, that currently need to be extracted from higher organisms. (e.g. heparin, which is used as anticoagulant, is extracted from porcine mucosa.)

# SUPPLEMENTARY INFORMATION

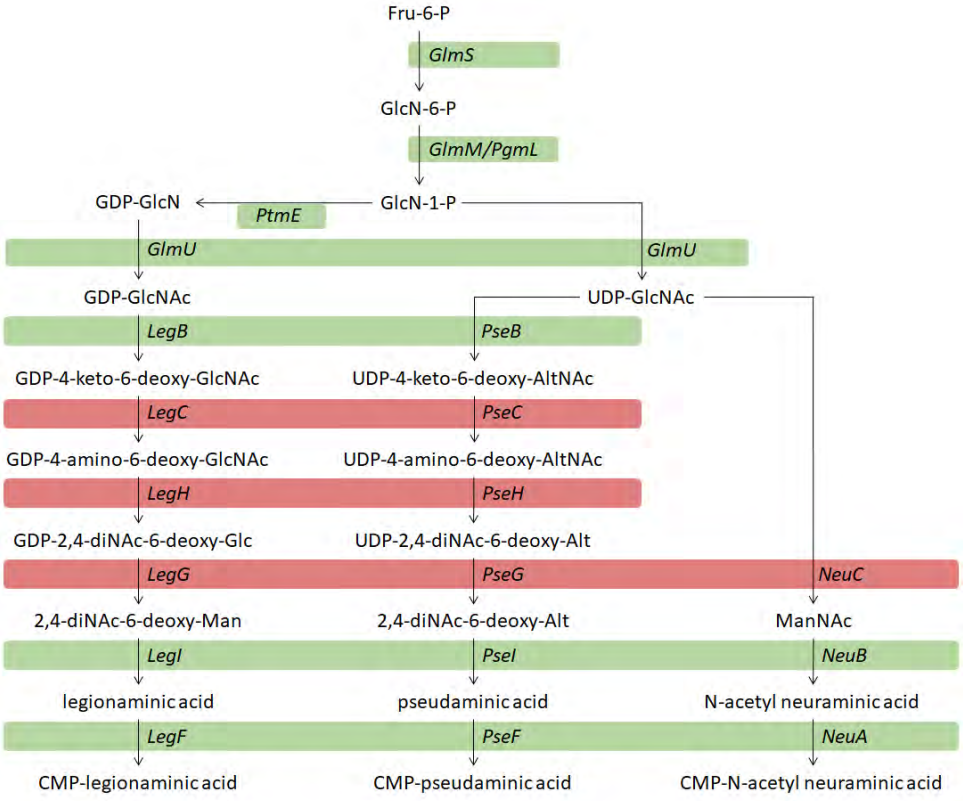



Figure S1: Pathways for the biosynthesis of legionaminic acid, pseudaminic acid and N-acetyl neuraminic acid in *Campylobacter jejuni*. Reference pathways were taken from Schoenhofen *et al.* [125]. The green have a match with *Ca.* Brocadiales (Table S1)

Table S1: Mass spectrometric sialic acid analysis. The obtained (DMB-labelled) masses matched with; Pse/-Leg (Pseudaminic acid/legionaminic acid), KDN (deaminated neuraminic acid), and NeuAc (N-acetyl neuraminic acid).

| Structure | Sum formula | monoisotopic mass | mass delta (av, ppm) | run 1 | run2 |
|---|---|---|---|---|---|
| Pse/Leg | C20H26N4O8 | 450.1750638 | -0.130837989 | x | x |
| KDN | C16H20N2O9 | 384.1168802 | 2.31439972 | x | x |
| NeuAc | C18H23N3O9 | 425.1434293 | -0.705173782 | x | x |

Table S2: Enzymes from the pathways (depicted in Figure S1) were BLASTed against the *Ca.* Brocadiales database (NCBI). The best match is shown in this table.

| Gene | Enzyme | Accession nr. *C. jejuni* | Best match *Ca.* Brocadiales | E-value | ID (%) |
|---|---|---|---|---|---|
| PseB | dehydratase/epimerase | CAL35407.1 | WP_034409924.1 | 4E-131 | 56.6 |
| PseC | aminotransferase | CAL35408.1 | WP_034409923.1 | 5E-73 | 37.6 |
| PseH | N-acetyl transferase | CAL35427.1 | - | - | - |
| PseG | UPD-sugar hydrolase | CAL35426.1 | - | - | - |
| PseI | pseudaminic acid synthase | CAL35431.1 | TVL99718.1 | 4E-92 | 44.4 |
| PseF | CMP-pseudaminic acid synthetase | CAL35425.1 | RIK01952.1 | 8E-44 | 37.2 |
| LegB | dehydratase | CAL35433.1 | TLD41235.1 | 2E-136 | 59.4 |
| LegC | aminotransferase | CAL35434.1 | OQY98965.1 | 1E-138 | 48.7 |
| LegH | N-acetyl transferase | CAL35412.1 | - | - | - |
| LegG | Hydrolyzing 2-epimerase | CAL35441.1 | - | - | - |
| LegI | legionaminic acid synthase | CAL35440.1 | TVL99724.1 | 4E-120 | 54.6 |
| LegF | CMP-legionaminic acid synthetase | CAL35444.1 | TVL99729.1 | 3E-35 | 35.6 |
| NeuC | Hydrolyzing 2-epimerase | CAL35259.1 | - | - | - |
| NeuB | sialic acid synthase | CAL35258.1 | TVL99718.1 | 3E-54 | 33.0 |
| NeuA | CMP- sialic acid synthatase | CAL35260.1 | TVL99729.1 | 4E-32 | 38.3 |

# 5

# Integration & Outlook

## 5.1. GENERAL CONCLUSIONS

The aim of this PhD-thesis was to improve the understanding of the EPS composition of anammox granular sludge. Analysing the EPS as a bulk material is complex due to the low solubility, the heterogeneity of the mixture and the lack of standardized methods. Therefore, the approach was to search for candidate EPS components and characterize components one by one. In order to obtain candidate components, it was required to first achieve the disintegration of the granular sludge and to have the structural components in solution. In chapter 2, two different extraction methods, alkaline and ionic liquid extractions, dissolved the granular matrix and recovered a range of components. Proteins were found to be the dominant fraction in the extracted material. The obstacles in searching obvious extracellular candidates among the extracted proteins, were the lack of annotated functionalities in the anammox protein database and the possibility of moonlighting protein. However, various dominant glycoconjugate components were discovered which provided a new direction in the search for the anammox EPS composition. Glycosylated proteins and glycoconjugates with a strong negative charge were observed in the extracellular matrix and selected as targets for further investigation in this thesis. These targets could be distinguished on the SDS-PAGE gel by different stains: a band at 80 kDa that stained positive for both protein and carbohydrates, a high molecular weight smeared band containing acidic groups, and at approximately 12 kDa a band stained positive for sulfate groups. In chapter 3, the first target was isolated and identified as glycoprotein. The glycoprotein showed high similarity with a surface layer (S-layer) protein. The other two targets being anionic indicated the presence of carboxylic and/or sulfate groups and therefore the possibility of glycan modifications sialyation and sulfation was investigated in chapter 4. A sialic acid assay based on enzymatic cleavage showed the presence of sialic acids. Furthermore, various sialic acids derivatives were identified with mass spectrometry. The sulfated glycosaminoglycan assay showed presence of sulfated glycosaminoglycan equivalents in the extracted EPS. Blasting of important enzymes in the pathways for synthesis of sialic acids and heparan sulfate demonstrated a positive match with anammox bacteria genomes. Excitingly, all the new targets: glycoproteins, sialic acids and highly charged polymers (like heparan sulfate) could be traced back and visualized in the extracellular space in anammox granular sludge with in-situ staining of the granules.

To summarize, the thesis presents a compositional analysis of various components in EPS. In the next section a proposed image of the anammox granular sludge matrix is presented, although much remains to be investigated regarding their exact location and function. The findings are presented as an integrated view with a discussion on the knowledge gaps.

## 5.2. PROPOSED IMAGE OF THE EXTRACELLULAR MATRIX OF ANAMMOX GRANULAR SLUDGE

Based on the combined information that was presented in chapter 2-4, a proposed image of the extracellular matrix of anammox granular sludge is shown in Figure 5.1. The three main targets in the extracted EPS that were investigated are highlighted in the SDS-PAGE picture and in the proposed schematic image of the extracellular matrix.

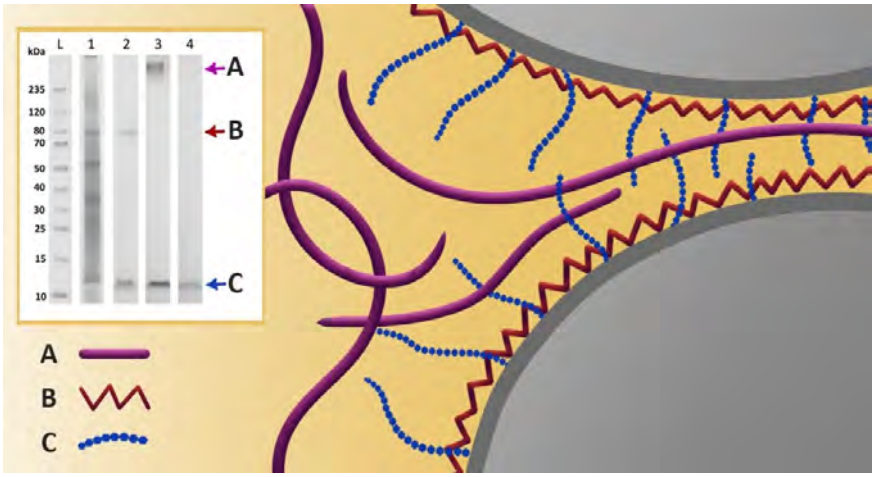



Figure 5.1: Proposed image for the extracellular matrix of anammox granular sludge. The three main targets that were explored are indicated in the SDS-PAGE gel (see Figure 3.2 for details of the gel and different staining's), and in the drawing. A) High molecular weight anionic components are proposed to be large polysaccharides or heavily glycosylated glycoproteins (pink). B) A glycoprotein that is probably a surface layer protein (red) and C) A strongly anionic polymer that is probably a linear sulfated polysaccharide which is located around the cell envelopes (blue).

The glycoprotein that was identified in chapter 3, is probably a surface layer (S-layer) protein (indicated in red in Figure 5.1). A 1.5 kDa glycan with methylated N-acetyl hexosamine backbone, substituted with a pentose and a dideoxy hexose residue was found. The 350 Da terminal residue of the glycan could not be assigned by mass. In the case of the S-layer protein of *Tanneralla forsythia*, a terminal residue was suggested to be a nonulosonic acid (derivative of legionaminic acid) [141]. Possibly the terminal sugar of the S-layer protein of anammox is another derivative of a nonulosonic acid, which may have a role in the biofilm formation and the interaction with the surroundings (e.g. protection and recognition of the anammox cells). Though attempts were made to image the surface layer protein with freeze-etching and TEM (transmission electron microscopy), these experiments didn't reveal the typical organized pattern of S-layers. The fractures were through the cells or over the membrane, and not over the outside of the S-layer. This indicates that the S-layer has a strong interaction with the surrounding EPS. For now, we can only assume that the S-layer forms the outer layer of the cells, as it does in known systems. Future research should focus on localization of the glycoprotein, for example with immunogold labelling experiments, to confirm the S-layer proteins are present around the cells. Previously S-layer were discovered in anammox bacteria around the cells from a suspended culture [103]. It is an interesting question why the bacteria also form this layer around their cell whilst they are surrounded by an extracellular matrix. As the S-layer is on the boarder of the cell and the extracellular matrix, its function related to the extracellular matrix is an important remaining question. S-layer are found in most archaea and many bacteria as outermost layer of the cells and there is no general function. Possibly that is the result of the lack of studies on biofilms instead

of suspended cells.

The band that was stained with Alcian Blue is proposed to be a linear sulfated polysaccharide (indicated in blue in Figure 5.1). The high sulfur amount, in combination with the strong anionic character raised the hypothesis of presence of sulfated components. In chapter 4, the component could be targeted with a glycosaminoglycan quantification assay. Therefore, we hypothesize it is a linear sulfated polysaccharide, like sulfated glycosaminoglycans. It has similarities with heparan sulfate, which has a function in higher organisms to bind to many different extracellular components. Remarkably, strongly polyanionic components were found around the cell envelope. This led to the hypothesis that the sulfated polysaccharides are surrounding the S-layer and bind to other extracellular matrix components. S-layers have no general function assigned. They could provide the anchor for the sulfated polysaccharides. This image would fit with earlier proposed arrangements of the glycocalyx by Costerton *et al.* [142]. They present the S-layer as a possible link between acidic polysaccharides and the cells. In general, S-layers are reported to be relatively loosely bound to the underlying cell, while more strongly bound to the neighbouring S-layer protein subunit Sleytr and Beveridge [106]. For many bacteria the S-layer can be shed of, while for anammox it is currently unclear if the S-layer is crucial for the cell integrity. However, for cells in biofilms it seems like a good strategy to be surrounded by a layer that is strongly fixed to the matrix while being able to shed it without compromising the cell integrity.

The last target on the SDS-PAGE are the high molecular weight negatively charged components (indicated in pink in Figure 5.1). These were least characterized of the three. Due to their high molecular weight and anionic character, it is proposed these are large polysaccharides or heavily glycosylated proteins. The SDS-PAGE profile of the Alcian Blue staining are reminiscent of mucins [95]. Sialic acids are widely present in mucins. In chapter 4 sialic acids were identified in anammox granules. It remains to be determined if the sialic acids are indeed part of this component. In Figure 5.1 the high molecular weight acidic component is depicted as long polymers, but possibly have branched groups. The high molecular weight suggest they have the potential to form a tangled network, which is a common mechanism for gel formation. This way they could form the matrix in between the cells. Probably it is not one component that forms the matrix. There may be a main gel-forming component, which requires other components to form a gel (e.g. other (glyco)proteins, polysaccharides and ions).

## 5.3. RECOMMENDATIONS FOR FUTURE RESEARCH

### ALTERNATIVE EXTRACTION METHODS AND ADDITIONAL TARGETS

The presented image in Figure 5.1 is based on the targets that were analysed in the extracted EPS, and their hypothesized function. The SDS-PAGE showed also various protein bands. As discussed in chapter 2, many proteins don't have an annotated function yet, and it is not easy to assign if the proteins are intra- or extracellular. Therefore, here we first investigated the glycosylated and anionic components in more detail, because these were more obvious targets to be related to the extracellular matrix. Future research should also focus on more detailed investigation of the proteins. For example Faria *et al.* [143] reported exceptionally large proteins (6000 to 10,000 amino-acids) in the extra-

cellular, periplasmic or membrane-associated locations for *Planctomycetes*. Such large proteins would have been overlooked in the SDS-PAGE gel used in this thesis.

Besides the further investigation of the above discussed targets, to complement and improve the image, additional targets are needed in order to fill in the image further. The undissolved pellet after the alkaline extraction may contain other polymers of interest. Therefore, additional solubilization methods based on different mechanisms are recommended. Along this way, piece by piece the image of the anammox extracellular matrix can be constructed.

### FIBRIL FORMATION OF EPS

One type of component with relevance for further investigation are fibril forming components. In the extracted EPS, fibrils were observed in the extracted EPS with atomic force microscopy (Figure 5.2, unpublished data). These fibrils were observed after extracted EPS was dialyzed against $Ca^{2+}$, while they could not be found in the control samples without $Ca^{2+}$. Hence, the role of $Ca^{2+}$ in assembling of EPS polymers could be a direction in this research. In another study to EPS of anammox granular sludge, by Lotti *et al.* [42], fibrils were also found in intact granules. Fibril forming polymers are often discussed in the context of biofilm formation. In the form of protein (e.g. amyloids, pili/flagella/collagen) or polysaccharide (cellulose) can both form the fibrils that can fulfil a structural role in the extracellular matrix.

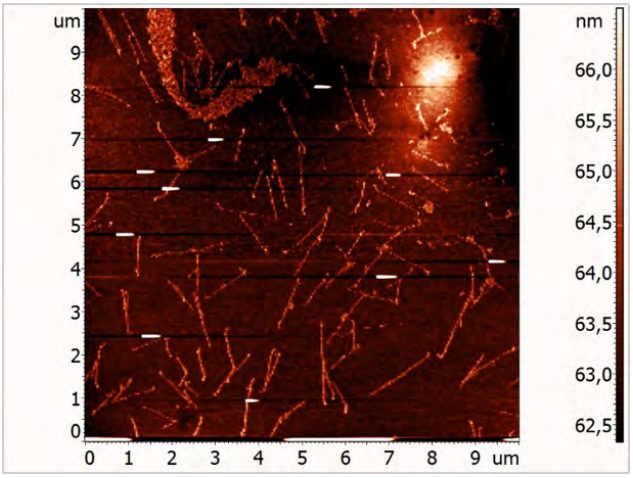



Figure 5.2: Atomic force microscopy (AFM) image. Extracted EPS was dialyzed against $Ca^{2+}$, to allow assembly of the polymers. After this treatment, fibril formation was observed as showed in this image. The fibrils could not be observed for the control samples without $Ca^{2+}$.

### ROLE OF EXTRACELLULAR POLYMERS IN BIOMINERALIZATION

Anammox granular sludge contains a high amount of the mineral hydroxyapatite. An interesting topic of research is the role of the different extracellular polymers in the formation of this hydroxyapatite. Biomineralization is widely occurring process in the environment. It is a known process from formation of mineralized tissues like bone, cartilage

and teeth. A variety of polymers are known to be involved in biomineralization processes, for example collagen, glycosaminoglycans, acidic polysaccharides and S-layer proteins can be involved. Looking into this aspect might aid in assigning functionality to one of the identified components from this thesis. Furthermore, this might reveal why the high amount of hydroxyapatite is present in the anammox granular sludge. In cyanobacteria and a Lysinibacillus species, it was observed that after mineralization of the S-layers, the encrusted S-layer can be shed of the cell as a survival mechanism [144].

### PHYSICAL/MECHANICAL PROPERTIES

The analysis in this thesis where mainly biochemical characterizations. These analyses should be complemented with physical mechanical characterization, in order to understand the composition to the structural function of the extracellular matrix. Linking composition and mechanical properties does not only aid in the understanding of the biofilm matrix, it also increases the possibility of producing biopolymers for application. Now the complexity of the extracellular matrix components is emerging, it also provides potential production of biopolymers. Especially granular sludge is a form of biofilm that provides potential production of biopolymer production, since biomass is not attached to any surface or carrier and therefore it is easy to collect the biomass.


Figure 5.3: Picture of gel formation of anammox EPS in a petri dish. After solubilization in NaOH, the polymers were recovered by precipitation by decreasing the pH. The precipitation formed films that were like a gel.

### BACK TO THE LIVING CELLS

After a start of the development of methods for the extraction and characterization, and some components of interest have been identified, it would be good to start relating it again to the living organism. Comparison of different morphologies e.g. biofilm, granules, flocs and planktonic cells would be relevant, with the targets defined in this thesis. Reactor experiments could be applied to test which factors influence the production of EPS and the morphology of the aggregates, and ultimately being able to direct the morphology. Parameters of interest could be for example reactor conditions that may influence the assembly of the polymers, like ions that are present in the feeding medium, different stress factors or different solid retention times. Linking the EPS composition and the biomass morphology, during different culturing conditions, will aid to understand the anammox biofilm formation strategy.

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# List of Publications

## Publications

### This thesis

- **Boleij, M.**, Seviour, T., Wong, L. L., van Loosdrecht, M. C. M. & Lin, Y., *Solubilization and characterization of extracellular proteins from anammox granular sludge.* Water Research 164, 114952 (2019).
- **Boleij, M.**, Pabst, M., Neu, T. R., Van Loosdrecht, M. C. M. & Lin, Y., *Identification of Glycoproteins Isolated from Extracellular Polymeric Substances of Full-Scale Anammox Granular Sludge.* Environmental Science and Technology 52, 13127–13135 (2018).

### Other

- Wong, L.L., Natarajan, G., **Boleij, M.**, Thi, S.S., Winnerdy, F.R., Mugunthan, S., Lu, Y., Lee, J.-M., Lin, Y., Loosdrecht, M. van, Law, Y., Kjelleberg, S., Seviour, T.,*Extracellular protein isolation from the matrix of anammox biofilm using ionic liquid extraction.* Applied Microbiology and Biotechnology. In press (2020).
- Wang, R., **Boleij, M.**, Yin, Q., Galjart, N., Lin, B., Yuan, N., Zhou, X., Tan, M., Ding, J., Liu, Z. & Abrahams, J.P., *Purification of Biotinylated Proteins Using Single Walled Carbon Nanotube-Streptavidin Complexes.* Journal of Nanoscience and Nanotechnology, 17(2), 926-931 (2017).
- van den Berg, E. M., **Boleij, M.**, Kuenen, J. G., Kleerebezem, R., & van Loosdrecht, M. C.M . *DNRA and Denitrification Coexist over a Broad Range of Acetate/N-$NO_3^-$ Ratios*, in a Chemostat Enrichment Culture. Frontiers in Microbiology, 7 (2016).

## Conference contributions

### Oral presentations

- **Boleij, M.**, van Loosdrecht, M. C. M. & Lin, Y., *Extraction and characterization of extracellular polymers of anammox granular sludge.* KNVM/NBV Microbial Biotechnology: MB 5.0 Fall meeting, 5 November 2018, Delft, The Netherlands
- **Boleij, M.**, Pabst, M., Neu, T. R., van Loosdrecht, M. C. M. & Lin, Y., *Identification of glycoproteins in EPS of anammox granular sludge.* SIAM Symposium, 12 Oktober 2018, Utrecht, The Netherlands
- **Boleij, M.**, van Loosdrecht, M. C. M. & Lin, Y., *Extracellular polymeric substances of anammox granular sludge contain glycoproteins and have a gel-forming property.* Biofilms7 conference, 26-28 June 2016, Porto, Portugal

- **Boleij, M.**, van Loosdrecht, M. C. M. & Lin, Y., *Extraction and characterization of extracellular polymers from anammox granular sludge.* Scientific Spring meeting KNVM & NVMM, 22-23 March 2016, Arnhem, The Netherlands

### Poster presentations

- **Boleij, M.**, Pabst, M., Neu, T. R., van Loosdrecht, M. C. M. & Lin, Y., *'Glycoproteins in anammox granular sludge'.* Granular Sludge Conference (IWA biofilms), 18-21 March 2018, Delft, The Netherlands
- **Boleij, M.**, van Loosdrecht, M. C. M. & Lin, Y., *Elucidating the composition and mechanical properties of extracellular polymeric substances of anammox granular sludge.* 9th European Symposium on Biopolymers, 5-7 July 2017, Toulouse, France
- **Boleij, M.**, van Loosdrecht, M. C. M. & Lin, Y., *Extraction and characterization of extracellular polymers from anammox granular sludge.* Young Water Professionals, 28-29 September 2015, Leeuwarden, The Netherlands
- **Boleij, M.**, van Loosdrecht, M. C. M. & Lin, Y., *The role of surface layer proteins in anammox granular sludge.* Scientific Spring meeting KNVM & NVMM 14-15 April 2015, Arnhem, The Netherlands

### Workshops

- *Extracellular polymers in anammox granular sludge*, EPS Workshop, Politecnico Milano, 24 November 2017, Milano, Italy
- *Methods and challenges for exopolymers extraction and analysis from granular sludge and biofilms*, Granular Sludge Conference (IWA biofilms), 18 March 2018, Delft, The Netherlands

## Awards

- Biofilms7 – SPM Young researcher award, 28 June 2016, Porto, Portugal

# CURRICULUM VITÆ

## Marissa BOLEIJ

Marissa Boleij was born on March 17th 1991 in Schiedam, the Netherlands. As a kid she was already looking through a microscope to the microbial life in the water from the fish tank. In high school at Erasmus College in Zoetermeer she liked the courses physics and math the most, but was also very much interested in the genetics part of the biology classes. That is how the choice to study Life Science and Technology at the TU Delft and the University of Leiden was made. After doing the minor and the bachelor thesis in Leiden, she was more drawn to the biotechnology part of the field and decided to do the Life Science and Technology master in TU Delft in the direction of Biochemical Engineering and Cell Factory. She did her master thesis in the Environmental Biotechnology group in which she studied the competition between two pathways in the nitrogen-cycle, namely denitrification and dissimilatory nitrate reduction to ammonium. After that she started a PhD in the same group, which resulted in the thesis you are reading now. After the PhD Marissa worked on the development of a TU Delft online course about aerobic granular sludge for wastewater treatment.

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Almost finished.. Those two words gained a new dimension for me during the past year. But now this booklet is *really* almost finished, and it's time to look back. The PhD can be frustrating, lonely, it is a long time on the same topic and all those clichés about a PhD, which are all partly true. But above all of that, it has been a privilege to do a PhD. To have the freedom to perform curiosity driven research for four years. For that I am grateful. For that and the unique environment with wonderful people that I have been working with.

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The last part of the PhD I changed office to, I guess, the most chaotic place in the building. But I actually felt quite at home there. Michel, Gerben, Jelmer, Jure (office mate through telephone) and Francesc, thanks for the good atmosphere and helping me to surviv my last year of PhD. I enjoyed the lively lunches with you and often with Udo, Robbert, Mario, Morez and Diana, which besides the actual food also gave me food for thought after the discussions about everything (and nothing).

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I think it is clear the PhD was a great experience for me. But of course, there also existed a life outside of the lab (luckily), where I'm lucky to be surrounded with amazing friends and family. Special thanks go to my parents, and brothers and sisters. The ones that are there since I remember, but also the bonus ones and in-laws. Thank you for your support. And of course, last but not least, the one who has experienced my ups and downs most intensely is Rogier. It is the best thing in the world to know there is someone you can always count on. Duizendmaal dank.