NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

PAULA BECK

BSc in Chemical and Biochemical Engineering

UNRAVELING THE ANIONIC PROPERTIES ALONG THE MOLECULAR WEIGHT DISTRIBUTION OF EXTRACELLULAR POLYMERIC SUBSTANCES EXTRACTED FROM SEAWATER-ADAPTED AEROBIC GRANULAR SLUDGE

MASTER IN CHEMICAL AND BIOCHEMICAL ENGINEERING

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Unraveling the Anionic Properties along the Molecular Weight Distribution of Extracellular Polymeric Substances extracted from Seawater-adapted Aerobic Granular Sludge.

To my family, which always encouraged me to go beyond.

ACKNOWLEDGMENTS

To my supervisor Dr. Yuemei Lin, I would like to acknowledge her great insights, guidance, and expertise, as well as her kindness and warm welcoming. My sincere gratitude for providing me the opportunity to develop my thesis within the Environmental Biotechnology Group at the Faculty of Applied Sciences. It was a privilege to do research at the Delft University of Technology, where I had access to well-equipped facilities and cutting-edge technology.

To my co-supervisor Dr. Mario Pronk, I thank the openness, the insightful ideas, and the knowledge conveyed, which were crucial for approaching the thesis from a distinct perspective.

I would like to express my sincere thanks to Lemin Chen for all the support, availability, and willingness to help me, as well as for encouraging me to think critically and to look more in-depth into the research topics. This guidance was fundamental for the development of this thesis.

My sincere gratitude to Prof. Mark Van Loosdrecht for the remarkable insights and for providing me a thriving environment with a brilliant group of colleges and researchers.

Finally, to my family, I will be forever grateful for the constant support and motivation, just as for helping me to chart the path that led me to where I am. Without them, I would not be capable of so many achievements.

"Nature is the source of all true knowledge. She has her own logic, her own laws, she has no effect without cause nor invention without necessity." (Leonardo da Vinci).

ABSTRACT

Unraveling the complete biochemical profile of extracellular polymeric substances (EPS) is still a substantial challenge. A deeper understanding on the EPS composition could foster the development of new applications of these biopolymers in high-value market niches, such as pharmaceuticals. This thesis assessed the anionic properties of EPS extracted from seawater-adapted aerobic granular sludge (AGS) along its apparent molecular weight (aMW) distribution, focusing on sulfated glycosaminoglycans (sGAG) and at the binding capacity of EPS with histones. Moreover, the influence of seawater conditions on EPS anionic properties was investigated. The fractionation of EPS through size exclusion chromatography (SEC) enabled its enrichment for negatively charged groups in the higher aMW fractions, ranging from 54 to 5 000 kDa, which represented 58% of the fractionated EPS. The assay with 1,9- dimethyl methylene blue (DMMB) indicated the possible presence of sulfated groups in EPS, more concentrated in the higher aMW fractions, which increased up to 115% relative to unfractionated extract. Through agarose gel-electrophoresis the effective binding of histones with EPS and with the enriched fractions was confirmed. Results point to an equivalence of seawater EPS around 0.167 (w/w) of heparin regarding histone neutralization, which implies the potential of these polymers in future research for the treatment of sepsis. Moreover, the aMW chromatographic profile changed progressively with increasing contact time of granules to seawater. The DMMB and histone binding tests indicated a gradual enhancement of the anionic properties of EPS with a longer exposure time of AGS to seawater. These findings suggest that the EPS anionic properties change under seawater conditions and that it seems to be unnecessary the complete renewal of the biomass to observe different anionic properties in EPS. This thesis provides basis to future research on EPS anionic properties and its potential applications.

Keywords: Extracellular polymeric substances, aerobic granular sludge, seawater, anionic properties.

RESUMO

Desvendar o perfil bioquímico completo das substâncias poliméricas extracelulares, conhecido como EPS, ainda é um desafio substancial. Um entendimento mais profundo sobre a composição do EPS pode fomentar o desenvolvimento de novas aplicações desses biopolímeros em nichos de mercado de alto valor, como o farmacêutico. Essa avaliou as propriedades aniónicas do EPS extraído de lodo granular aeróbio (LGA) adaptado à água do mar, ao longo de sua distribuição de massa molecular aparente (aMM), com foco nos glicosaminoglicanos sulfatados (sGAG) e na capacidade de ligação do EPS com histonas. A influência da água do mar nas propriedades aniónicas do EPS também foi investigada. O fracionamento do EPS por cromatografia por exclusão de tamanho (SEC) possibilitou seu enriquecimento para grupos negativamente carregados nas frações de maior aMM, variando de 54 a 5 000 kDa, que representam 58% do EPS fracionado. O ensaio com azul de 1,9-dimetilmetileno (DMB), indicou a possível presença de grupos sulfatados no EPS, mais concentrados nas maiores frações, com um aumento de até 115% relativo ao extrato não-fracionado. Através de eletroforese em gel de agarose foi confirmada a interação efetiva de histonas com o EPS e suas frações enriquecidas. Os dados obtidos apontam para uma equivalência de EPS de água marinha em torno de 0.167 (m/m) de heparina quanto à neutralização de histonas, o que implica o potencial desses polímeros em pesquisas futuras para o tratamento da sepse. Além disso, o perfil cromatográfico da aMM mudou progressivamente com o aumento do tempo de contato dos grânulos com a água do mar. Além disso, os testes com DMB e interação com histonas indicaram um aumento gradual das propriedades aniónicas do EPS com uma exposição prolongada do LGA à água marinha. Esses resultados sugerem uma mudança das propriedades aniónicas do EPS sob as condições salinas e indicam ser desnecessária a renovação completa da biomassa para obter diferentes propriedades aniónicas no EPS. Esta tese fornece base para futuras pesquisas sobre as propriedades aniónicas do EPS e suas potenciais aplicações.

Palavras chave: Substâncias poliméricas extracelulares, lodo granular aeróbio, água do mar, propriedades aniónicas.

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ACRONYMS

AS	Activated Sludge.
AGS	Aerobic Granular Sludge.
aMW	Apparent Molecular Weight
cv	Column Volume
COD	Chemical Oxygen Demand
CS	Chondroitin Sulfate
DS	Dermatan Sulfate
DMMB	1,9- dimethyl methylene blue
GAO	Glycogen Accumulating Organisms
EPS	Extracellular Polymeric Substances.
FISH	Fluorescent in situ Hybridization
FTIR	Fourier Transform Infrared
GAG	Glycosaminoglycans
GS	Granular Sludge.
HP	Heparin
HS	Heparan Sulfate

KS	Keratan Sulfate
MW	Molecular Weight
PAO	Polyphosphate Accumulating Organisms
SBR	Sequencing Batch Reactor
SEC	Size Exclusion Chromatography
sGAG	Sulfated Glycosaminoglycans
SRT	Sludge Retention Time
TSS	Total Suspended Solids
UV	Ultraviolet
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plants

SYMBOLS

µ	Micro: a unit prefix in the metric systems that denote a factor of 10^{-6}.
m	Milli: a unit prefix in the metric systems that denote a factor of 10^{-3}.
M	Mega: a unit prefix in the metric systems that denote a factor of 10^{6}.
L	Liter: a metric unity of volume.
Da	Dalton: a unit of mass.
g	Gram: a unit of mass.
ºC	Degree Celsius: a unit of temperature on the Celsius scale.
h	Hour: a unity of time.
min	Minute: a unity of time.
M	Molar mass: mass of a compound divided by the amount of substance.
$\mathbf{t_0}$	Samples referent to freshwater-adapted granules.
$\mathbf{t_1}$	Samples referent to intermediate seawater-adapted granules.
$\mathbf{t_2}$	Samples referent to seawater-adapted granules.
X^R	Biomass concentration in the reactor.
M_X^S	Amount of Dry Weight in the Sample.

V_X^S	Volume of the Settled Bed in the Sample.
V_X^R	Volume of the Settled Bed in the Reactor.
V_L^R	Total Volume of the Reactor.
K_{av}	Partition Coefficient.
V_e	Elution Volume.
V_0	Void Volume.
V_t	Column Total Volume.

1

INTRODUCTION

The recovery of biomaterials from waste sludge and further characterization and application can enhance the sustainability and economics of wastewater treatment (Y. M. Lin et al., 2015). The most commonly used biological wastewater treatment is Activated Sludge (AS), which poses many limitations in comparison with the upcoming Aerobic Granular Sludge (AGS) technology. An important property of the Granular Sludge (GS) is the production of Extracellular Polymeric Substances (EPS) by microorganisms, which offers various benefits to the stability of granules, including higher tolerance to adverse conditions, such as salinity (Pronk, 2016). It has been reported adaptation of AGS to saline wastewater, as well as a consequent change in its composition (Campo et al., 2018; Corsino et al., 2017; Wan et al., 2014). Previous research with seawater-adapted AGS in our research group identified the presence of strongly negatively charged compounds in EPS and observed their interaction with cationic histones (Danny R. de Graaff et al., 2019; Sebastian, 2021). In addition, sulfated sugars, including sulfated glycosaminoglycans (sGAG), have been found in marine organisms (Pomin & Mourão, 2014) and in granular sludge (Boleij et al., 2020; Felz et al., 2020). These sulfated sugars are well studied for their polyanionic feature, which enables the interaction with proteins responsible for the balance of health and disease (Pomin & Mourão, 2014). Thus, investigating the presence of these anionic compounds in EPS extracted from seawater-adapted AGS, as well as their capability of interacting with histones, is of great interest mainly because of its potential for future application in high-value market niches, including pharmaceuticals. In the upcoming section, these concepts are going to be further exploited.

1.1 Salinity in Wastewater

The increase of salinity in water bodies poses a remarkable challenge in wastewater treatment. Wastewater systems can contain considerable amounts of inorganic salts resulting from agro-industrial effluents, such as aquaculture, food processing, textile, petroleum, and mining industries ((Li et al., 2017; Lefebvre & Moletta, 2006; Ahmad et al., 2021). Furthermore, in coastal regions, the intrusion of seawater or saline groundwater into sewer networks and the use of

seawater for toilet flushing are means of increasing salinity in wastewater treatment plants (WWTP) (Lu et al., 2011; Pronk, 2016).

1.2 Conventional Wastewater Treatment

Currently, Activated Sludge is the most implemented biological process for wastewater treatment at full scale (D. R. de Graaff, 2020). Nevertheless, these systems present drawbacks, namely large area requirements and limitations when it comes to complex effluents. High salinity conditions, as well as toxicity and other stress factors, generally have an inhibition effect on suspended cell cultures. These adverse conditions can be better tolerated by granular sludge (Pronk et al., 2014).

1.3 Aerobic Granular Sludge (AGS)

Aerobic Granular Sludge is a promising technology for the treatment of wastewater, presenting multiple advantages relative to the conventional systems. The large and dense shape of the granules promotes higher biomass retention, better settling capacity and more effective sludge-effluent separation (Feng et al., 2021). The greater resistance to adverse conditions is also an advantage of this technology, compared to the conventional AS (Pronk et al., 2014). Moreover, the removal of phosphorous, nitrogen and chemical oxygen demand (COD) can occur in one single reactor. This simultaneous conversion is possible due to the different redox condition within the granules, promoted by the combination of aerobic/anaerobic process phases and the mass transfer limitation that occurs inside the granules (Pronk, 2016). During aeration, an oxygen gradient occurs inside the granules, creating an aerobic outer layer, where ammonium is converted by nitrifying bacteria into nitrite and nitrate. Meanwhile, the inner core remains anoxic, in which denitrifying bacteria convert these compounds into nitrogen gas. The enhanced biological phosphorous removal is then performed by polyphosphate accumulating organisms (PAO) through the anaerobic uptake of volatile fatty acids, followed by aerobic/anoxic uptake of phosphate (D. R. de Graaff, 2020).

Full scale AGS technology for industrial and domestic wastewater treatment is currently operational in various countries worldwide, by the trade name of Nereda®. The technology requires 25% less investment and operational costs, 75% lower footprint and half the electricity of conventional systems (Inocencio P. et al, 2013).

1.4 Extracellular Polymeric Substances (EPS)

The sludge granules are aggregates of different microbial cells fixed in a self-made matrix of Extracellular Polymeric Substances (EPS), defined as biofilm (H. C. Flemming et al., 2016). The formation of biofilms is an advantage over free-living cells due to the range of EPS adaptational functions. These biopolymers are known for providing mechanical stability by creating scaffold, cohesion within the biofilm, and surface adhesion. These conditions enable the proximity of cells, creating synergetic micro consortia, where cell-to-cell interaction and communication occur. This complex matrix enhances nutrient uptake and water retention; besides it provides protection against predators, antibiotics, metallic cations, and radiation, leading to a constant adaptation. Moreover, this protective and adaptative environment is able to retain extracellular enzymes, creating an external digestion system that promotes the sorption of dissolved nutrients and recycling of components of the lysed cells (H. C. Flemming & Wingender, 2010).

The generation and maintenance of the biofilm are strongly dependent on the amount and composition of the EPS, where factors such as concentration, charge, specificity, sorption capacity, and nature of the extracellular polymers' components determine the biofilm development. Furthermore, nutrient concentration, hydrodynamic conditions, bacterial mobility, exopolysaccharides, and proteins influence the biofilm architecture. As an example, the interaction of anionic EPS with multivalent cations affects biofilm structure (H. C. Flemming & Wingender, 2010).

The complex composition of EPS hinders its analysis, the reason why it has been called "the dark matter of biofilms" (H.-C. Flemming et al., 2016). Despite decades of research, there is still much to be unraveled about EPS molecular composition and the compounds' functions and interactions, which hampers the efficient control and manipulation of biofilm (Seviour et al., 2019). A deeper understanding of the EPS could enhance the development of innovative biotechnological solutions.

1.4.1 EPS Characterization

The identification and characterization of EPS components depend on the isolation methods implemented. The whole isolation procedure consists of extraction, optional purification step, enrichment, and recovery (Feng et al., 2021). Nevertheless, there is no universal polymer

recovery approach – the process has to be adapted depending on the origin and type of biofilm under investigation and the desired goal (H. C. Flemming & Wingender, 2010).

The extraction can affect the EPS yield, composition, biochemical properties, and functional groups (Caudan et al., 2012; Felz et al., 2016; Feng et al., 2021). The methods can consist of physical, chemical, or biological treatments or a combination of physical and chemical techniques. Physical approaches include heating, centrifugation, sonification, or blending to disrupt the polymers from the extracellular matrix and dissolve them into solution. Chemical techniques involve cation exchange resins, chelating agents, alkaline, acid or aldehydic reagents to solubilize the polymers and are usually more efficient, resulting in a higher EPS yield. Biological methods involve enzymatic treatments (Feng et al., 2021). The combination of methods is recommended for an efficient extraction (Felz et al., 2016).

Something to be considered is the cellular damage caused by the extraction, which might release intracellular content. An optimal isolation procedure should be effective, causing no damage to EPS structure and minimal cell lysis (Sheng et al., 2010). Nevertheless, depending on the research purpose, a strategy might be using harsh physical methods such as high temperature, breaking the cells instead of maintaining them intact, with the objective to unravel the role of complex and unknown biopolymers (Felz et al., 2016; Feng et al., 2021).

In extraction studies of AGS (Felz et al., 2016) and anammox GS (Feng et al., 2019) the most efficient method in terms of EPS yield was the 80 ºC heating under alkaline conditions, in which the increase of pH enhanced the solubility of acid groups in EPS. The implementation of alkaline reagents such as Na_2CO_3 or NaOH targets mostly ionic polymer interactions (Feng et al., 2021) and can break disulfide bonds in proteins, making them easily extracted (Boleij et al., 2018). Thus, aiming to extract acidic polymers, the use of an alkaline treatment may be a good strategy.

To obtain a clear analysis of the EPS content and its functions, efficient recovery of the exopolymers with sufficient purity is essential. The recovery can be attained through dialysis purification, solvent, ethanol or acid precipitation, electrophoretic or chromatographic techniques, or membrane centrifugal filter (Feng et al., 2021).

Moreover, the EPS complexity and the lack of suitable methods hampers the assessment of the properties and functions of these polymers. Various real-time and in situ biochemical characterization methods have been exploited. Commonly used colorimetric techniques result in quantitative information, being necessary the use of additional qualitative methods to better characterize EPS (Bhatia et al., 2013). Fractionation of EPS according to their structural

interactions, and chemical properties could be valuable to unravel the polymers' complexity (Caudan et al., 2012). One method for fractionation is size exclusion chromatography (SEC). SEC can provide EPS fingerprints, revealing important information about its apparent molecular weight (aMW) distribution (Bourven et al., 2013), and fractionate the EPS by differences in sizes (Barth & Boyes, 1990).

1.4.2 EPS Composition

Many compounds have been identified in EPS, such as nucleic acids, lipids, humic-like substances, uronic acids, amyloids-like substances, glycoconjugates and some inorganic compounds (Feng et al., 2021; Liu et al., 2004; Seviour et al., 2019). Nevertheless, proteins and carbohydrates are the most studied components in EPS (Caudan et al., 2012; Sheng et al., 2010). These molecules are mainly quantified separately by colorimetric assays, causing an overlook on the presence of glycoproteins, which might be commonly present in EPS, given the abundance of glycosylated proteins in extracted EPS (Y. Lin et al., 2018). In the last decades, distinct methodologies have been implemented, identifying complex, including sulfated, glycoconjugates in EPS from different granular sludge. As an example, it was found in EPS recovered from AGS enriched with ammonium-oxidizing bacteria, glycosylated amyloid-like proteins, having glycoconjugates of neutral sugar, carboxyl groups, and sulfate groups (Y. Lin et al., 2018). In EPS from anaerobic GS, it was detected the presence of high molecular weight proteoglycan-like and sulfated proteoglycan-like substances, with signs of uronic acid residues, carboxylic groups, and sulfate groups (Bourven et al., 2015). In EPS extracted from anaerobic ammonium oxidation (anammox) granules, abundant glycoprotein with heterogeneous O-glycan structure and acidic (including sulfated) glycoconjugates have been identified (Boleij et al., 2018). In EPS from full-scale AGS, glycosaminoglycans (GAG) were found, such as hyaluronic acid-like, chondroitin sulfate-like and heparan sulfate/heparin-like polymers (Felz et al., 2020). The referred study pointed to the possibility of GAG-like polymers covalently bound to proteins being a common phenomenon in granular sludge, which might play a role in their stability. Moreover, in anammox GS EPS, sulfated glycosaminoglycans (sGAG) and sialic acids were found (Boleij et al., 2020). Sialic acids are negatively charged monosaccharides usually bound to glycoconjugates (Schauer & Kamerling, 2018). In previous studies developed in our laboratory, polyanionic properties, more specifically sulfated groups (Sebastian, 2021), and sialic acids (Danny R. de Graaff et al., 2019) were identified in EPS recovered from seawater-adapted AGS. Thus, it seems reasonable the presence of anionic compounds in EPS.

The quantity and composition of the EPS depend not only on the extraction method implemented but also on factors such as microbial community, growth phase, oxygen and substrate

limitation, shear force, temperature, ionic strength, toxic compounds, salinity, among others (Feng et al., 2021; Liu et al., 2004). It is known that environmental stress can promote enhanced EPS production (Feng et al., 2021). It has been reported that adaptation of AGS into high saline conditions leads to a change in EPS composition. An increased hydrophobicity, higher protein fraction and extra EPS production have been observed in saline conditions (Campo et al., 2018; Corsino et al., 2017; Wan et al., 2014). In anaerobic sludge, a high salinity exposure has led to an increased production and change in the chemical composition of extracellular polysaccharides (Vyrides & Stuckey, 2009). A significant shift in the glycoconjugate pattern was observed in AGS when adapted to different saline conditions (Sudmalis et al., 2020).

1.4.2.1 Sulfated Glycosaminoglycans (sGAG)

Sulfated glycosaminoglycans are highly sulfated polysaccharides, found within the extracellular matrix of cell surfaces, that exhibit a variety of biological functions, including mediating interactions and providing structural support, as well as anticoagulation, cartilage's ability to sustain stress during compression and the creation of structures capable of performing functional selectivity (Jones et al., 2010; Muthana et al., 2012). These molecules are associated with cell signaling and adhesion functions in most eukaryotes and pathogenic bacteria (DeAngelis, 2002). Within their properties, the intrinsic negative charge is substantial (Costa et al., 2017). Some of these anionic molecules have been reported to bind positively charged proteins, such as histones (Wang et al., 2020). These polysaccharides can be linked to proteins by glycosylation, forming negatively charged proteoglycans (Muthana et al., 2012). Sulfated glycosaminoglycans can be classified into 3 groups (figure 1.1): heparin (HP)/heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS), and keratan sulfate (KS) (Zhang et al., 2010). Hyaluronic acids are part of the non-sulfated glycosaminoglycans group. Of these molecules, heparin has the highest negative charge density, containing 3 sulfate ester groups per disaccharide sequence (Weiss et al., 2017). Heparin and heparan sulfate contain N-sulfated hexosamines, while some CS, DS and KS contain O-sulfated hexosamines (Felz et al., 2020). Thus, it is possible to distinguish between heparin/ heparan sulfate by determining the ratio between O- and N-sulfated glycosaminoglycans. Although sGAG have been mostly reported in mammalians and some pathogenic bacteria, these polysaccharides have also been found in marine organisms (P. Mourao, 2005; Pomin & Mourão, 2014).

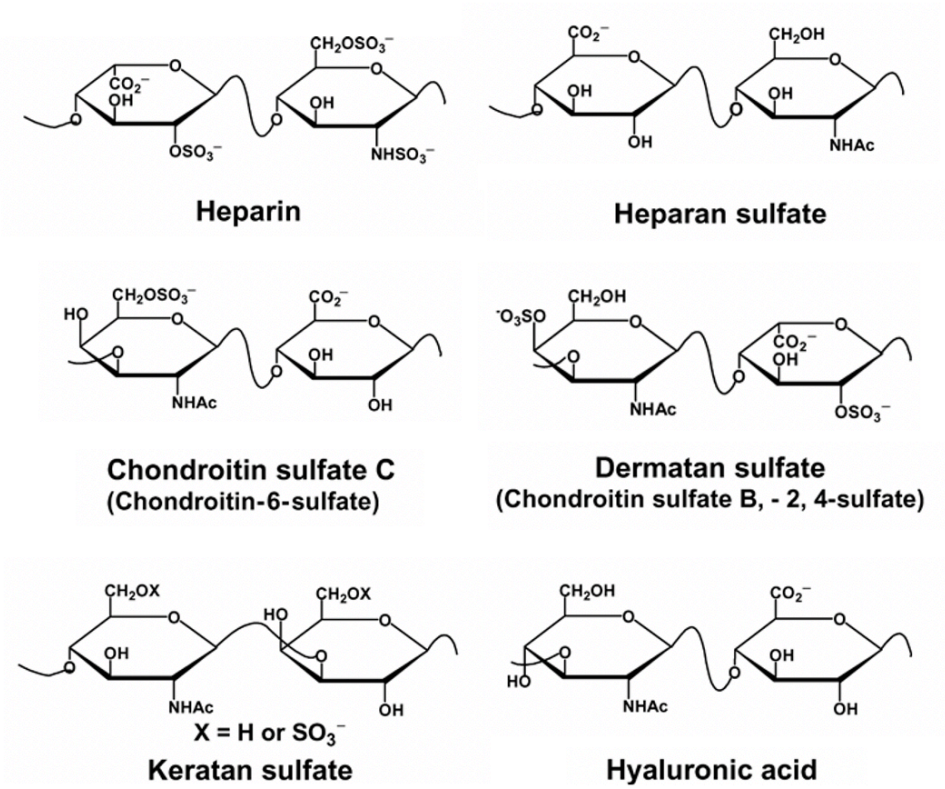

Figure 1.1. Structures of the disaccharide repeating units of heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid. Adapted from (Zhang et al., 2010).

1.5 Marine Organisms

The marine ecosystem has gained attention from the scientific community due to the diversity of organisms with unique molecular structures (Dufourcq et al., 2013). These organisms, such as bacteria, macro and micro-algae, sponges, sea cucumber, and fish have evolved to be adapted to a complex habitat with variable salinity, temperature, light, and pressure conditions (Ruocco et al., 2016). Many adaptation mechanisms of these species are based on metabolic pathways and the use of diverse molecules (Ruocco et al., 2016; Dufourcq et al., 2013), such as the synthesis of exopolysaccharides with distinctive and varied composition (Roca et al., 2016). In this environment, microbial EPS is widely distributed, and its production is influenced by the variable conditions (Bhaskar & Bhosle, 2005).

Therefore, marine organisms constitute a promising source of biomolecules for innovative biotechnological applications. Proteins, glycoproteins, and carbohydrates are present in these

organisms, being polysaccharides recognized for their potential therapeutic properties (Ruocco et al., 2016). Sulfated polysaccharides have been identified in marine organisms. Among them, glycosaminoglycans, sulfated galactans and sulfated fucans have been characterized (P. Mourao, 2005; Pomin, 2009; Pomin & Mourão, 2014). These anionic molecules were mostly isolated from brown, green, and red macroalgae, sea cucumber, sea urchins, and tunicates (Pomin, 2009). As mentioned previously in section 1.4.2.1, microbial sGAG were also reported. Sulfated polysaccharides have a potential role in the pharmaceutical industry due to their polyanionic characteristic, which enables their interaction with functional proteins. These molecules' interactions are mostly promoted by electrostatic interactions, which can be influenced by both the sulfation content and the structural characteristics of the sulfated sugars (Pomin & Mourão, 2014). Among the properties of sulfated sugars, the anticoagulant and antithrombotic activities are the most studied (P. A. S. Mourao et al., 1996).

1.6 EPS Applications

The extracellular polymeric substances can constitute a large fraction of sludge dry weight (Feng et al., 2019), which in turn is the main waste product in WWTP. The costs of handling and disposal of the sludge can account up to half of the total operational costs (Kroiss, 2004). Therefore, the application of biomaterials recovered from sludge waste can contribute to ecological and economical improvements in wastewater treatment (Y. M. Lin et al., 2015).

There is a broad range of potential applications for sludge-extracted EPS, such as agriculture, horticulture, paper and construction industries (van Leeuwen et al., 2018). The hydrogel-forming capacity of EPS can be used as coating material in paper industry (Y. M. Lin et al., 2015). In the construction field, the hydrophilic property of these biomaterials can be applied as a cement curing material (Karakas et al., 2020). Furthermore, EPS generally have a negative charge, which enables its application as a biosorbent material for the removal of heavy metals or organic pollutants in wastewater (Dobrowolski et al., 2017). Moreover, the ion exchange nature of EPS could be applied as a selective membrane towards the transport of monovalent ions (Sudmalis et al., 2020). Nevertheless, the potential of physicochemical properties of EPS could be better harnessed through its application in high-value market niches, such as pharmaceutical, biomedical and cosmetic (Freitas et al., 2011).

1.6.1 Sepsis Treatment

Sepsis is a syndrome which consists of a systemic inflammatory response to infection, potentially leading to death (O'Brien et al., 2007). Among the factor of developing sepsis, a major mediator of death is the release of extracellular histones, being these molecules potential targets for treating the syndrome (Wang et al., 2020; J. Xu et al., 2009). Histones are highly positively charged proteins, which in normal circumstances are bound to the negatively charged DNA (Park & Kim, 2020). Anionic polymers such as heparin and polysialic acids have been reported to neutralize histones (Xu Li & Ma, 2017; Mishra et al., 2010; Wang et al., 2020; Weiss et al., 2017; Zlatina et al., 2017).

Heparin is one of the most frequent pharmacotherapeutics used due to its anticoagulant property (Beurskens et al., 2020). Despite its highly anionic property and binding capacity, the potential bleeding risk in septic patients due to its anticoagulant property (Wildhagen et al., 2014), as well as the complex biosynthesis process, involving extraction from animal tissue (Beurskens et al., 2020), represent a drawback of heparin application for sepsis treatment.

The neutralization of histones was reported to have a protective effect on septic cases (Wang et al., 2020), thus the investigation into the efficiency of negatively charged polymers in neutralizing the positive histones is substancial. For the reasons mentioned above, the implementation of microbial anionic polymers, such as some reported EPS, as a possible treatment for sepsis could be an advantage. A previous study in our laboratory investigated the potential of histone binding of EPS from seawater-adapted AGS for the cure of sepsis. Results indicated an interaction between EPS and histones, which could be further explored (Sebastian, 2021).

1.7 Thesis Scope

The challenge of characterizing EPS is evident, due to its complexity. A clearer picture of the EPS composition could enable better control of the biofilm formation and the development of new applications. Therefore, our research group in the Environmental Biotechnology department at the Delft University of Technology is investigating this matter by diverse approaches. The presence of strongly negatively charged components in EPS and its interaction with histones observed in our previous studies with seawater-adapted AGS, raised the interest in further investigating the effect of seawater on the anionic composition of EPS. In addition, the presence of sulfated glycosaminoglycans in marine organisms and in granular sludge EPS, raised the question if there are sulfated glycans in EPS recovered from seawater-adapted AGS.

Therefore, the research aims at unraveling the distribution of anionic properties along the aMW of EPS, focusing on the presence of sulfated groups and the capacity of binding with histones. Consequently, elucidating the potential application of these biopolymers in high value market niches such as the pharmaceutical industry.

For this purpose, in the present study (illustrated in fig. 1.2), AGS was cultivated in a bubble column reactor in synthetic seawater. EPS was then extracted under heat and alkaline conditions and fractionated by aMW through SEC. The ionic properties of unfractionated EPS and their fractions were further analyzed through both quantitative and qualitative methods, 1,9-dimethyl methylene blue (DMMB) binding and fourier transform infrared (FTIR), respectively. Binding with cationic histones was performed to assess the potential of EPS for the use in pharmaceutical applications. In addition, all the above methods were also performed on freshwater AGS and the results obtained were compared with the EPS extracted from granules grown in seawater.

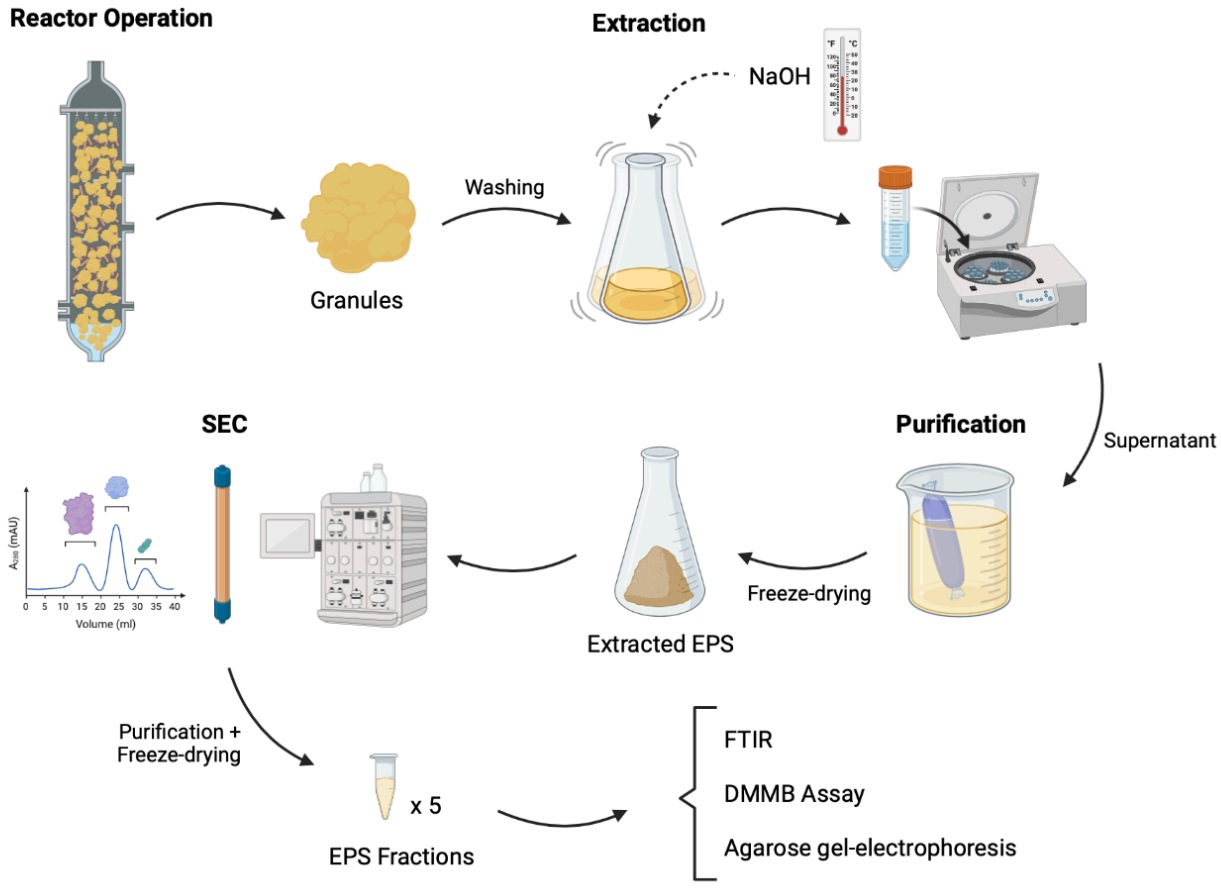

Figure 1.2. The roadmap of EPS obtention from AGS, isolation and characterization.

2

MATERIAL AND METHODS

2.1 Reactor Operation

A bubble column (6.5 cm diameter) was operated with a working volume of 2.8 L as a sequencing batch reactor (SBR). The reactor was inoculated with approximately 5 g VSS/L of aerobic granular sludge obtained from a stable lab-scale reactor treating glycerol under freshwater. Room temperature was about 20 ºC and pH was maintained at 7.3 ± 0,1 by dosing 1 M HCl or 1 M NaOH. The cycles were 3 h long, comprising 5 min settling phase, 5 min effluent discharge, 5 min N_2 sparging, 5 min anaerobic bottom feeding in a plug-flow regime, 50 min anaerobic phase (N_2 mixing) and 110 min aerobic phase (pressurized air). During aeration phase, dissolved oxygen (DO) was kept at 80% air saturation, by a mass flow controller. The average sludge retention time (SRT) was 12 days, controlled by non-selective sludge removal.

The feed of 1.5 L consisted of 1.2 L of artificial seawater (Instant Ocean® with a final concentration of 35 g/L), 150 mL of medium A and 150 mL of medium B. Medium A was composed of 62.5 mM of sodium acetate trihydrate. Medium B contained 41.13 mM of NH_4Cl, 0.34 mM of K_2HPO_4, 0.27 mM of KH_2PO_4, 0.07 mM of Allythiurea and 10 mL/L of trace elements solution similar to (Vishniac & Santer, 1957), but using 2.2 g/L of $ZnSO_4.7H_2O$ instead of 22 g/L and 2.18 g/L of $Na_2MoO_4.2H_2O$ instead of $(NH_4)_6Mo_7O_{24}.4H_2O$ (Pronk et al., 2015). The combination of these feed streams led to influent concentrations of 400 mg/L COD, 50 mg/L NH_4 -N and 12.2 mg/L PO_4 -P.

For the acclimation of freshwater granules into seawater, the reactor started operating under 10 g/L seawater, for 4 days. Then the concentration was increased to 12 g/L, for 3 days. For 6 days, 20 g/L was implemented. Finally, 35 g/L was achieved and maintained until the end.

Total organic and inorganic content were measured weekly with some adaptations of standard methods (Federation, 1954). The determination of Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) can be challenging in a bubble column reactor due to the stratification

of aerobic granules, which prevents homogeneous sampling. Therefore, to determine the biomass concentration in the reactor, the following method, described by (Pronk, 2016) is used. After the biomass has settled, the volume of the settled bed (mL) inside the reactor is recorded. A sample is taken while reactor is mixed and put into a volumetric cylinder (mL). The volume of the settled bed in the cylinder is recorded. The biomass in the volumetric cylinder is then washed three times, by adding demineralized water in a volume four times the volume of sludge collected and then dried at 105 °C overnight. Samples are subsequently placed in a 550 °C oven for 2 hours to determine the ash content. The result on the TSS and VSS of the cylinder can be coupled to the reactor biomass concentration by relating it to the ratio between de volume of settled aerobic granules in the volumetric cylinder and that in the reactor. The formula used to calculate the biomass concentration (X^R, g L^{-1}) in the reactor is

$$X^R = \frac{M_X^S}{V_X^S} \frac{V_X^R}{V_L^R}, \tag{2.1}$$

where M_X^S is the amount of dry weight in the sample (g), V_X^S is the volume of the settled bed in the sample (L), V_X^R is the volume of the settled bed in the reactor (L), V_L^R is the total volume of the reactor (L).

Cycle measurements were performed by collecting samples in five different time slots from the beginning of the anaerobic phase: 0 min, 25 min, 50 min, 105 min and 160 min. Samples were filtered through 0.22 µg PVDF filters. Acetate concentration was measured through high-performance liquid chromatography (Thermo Scientific Vanquish HPLC) at 50 ºC, flowrate of 0.75 mL/min with 1.5 mM phosphoric acid as eluent. Meanwhile, phosphate and ammonia were detected by a discrete analyzer (Thermos Scientific Gallery Discreate Analyzer). The granules morphology was observed through Leica Microsystems Ltd stereo zoom microscope (M205 FA).

2.2 Microbial Community Analysis by Fluorescent in situ Hybridization (FISH)

The handling, fixation and staining of Fluorescent in situ Hybridization (FISH) samples were performed as described in (Bassin et al., 2011). To stain all bacteria, a mixture of EUB338,

EUB338 -II and EUB338 -III probes were used (Amann et al., 1990; Daims et al., 1999). A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) were used for visualizing polyphosphate accumulating organisms (PAO) (Crocetti et al., 2000). A mixture of GAOQ431 and GAOQ989 probes (GAOmix) were used to target glycogen accumulating organisms (GAO) (Crocetti R. et al., 2002). The samples were examined with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 5515e565) for Cy5, Cy3 and fluos respectively.

2.3 EPS Extraction by Alkaline and Heat Conditions

The isolation procedure implemented in this study encompassed a combination of chemical and physical extraction methods, in which alkaline and heat conditions were applied, followed by a centrifugation step and dialysis purification.

Granules were taken from the reactor at the end of aerobic phase and washed three times, by adding demineralized water in a volume four times the volume of sludge collected. The granules were then frozen for 2 h in –80 ºC and then freeze dried overnight. The ash content of the granules was measured after burning 0.2 g of dried biomass (total suspended solids (TSS)) for 2 h under 550 ºC, to obtain the volatile suspended solids (VSS).

For the extraction, 1 % w/v VSS of 0.1 M NaOH was added to a baffled flask, which was placed in a water bath until 80 ºC was achieved. The dried granules were then added into the flask and the mixture was stirred for 30 min at 80 ºC in the water bath and then centrifuged at 4 000 × *g* and 4 ºC for 20 min. The supernatant was collected, and the pellet was discarded. The extracted EPS was dialyzed overnight against demineralized water in dialysis tubing (Sigma Aldrich) with molecular weight cut-off of 3 500 Da, frozen for 2 h at –80 ºC and freeze-dried overnight. Extracted EPS yield was defined as the VSS fraction of dialyzed extracts relative to the initial granules VSS.

2.4 EPS Fractionation and Apparent Molecular Weight Distribution by Size Exclusion Chromatography (SEC)

The Buffer utilized for dissolving the samples, elution and equilibration of the column consisted in 0.15 M NaCL, 50 mM Glycine, pH 10. Extracted, purified and dried EPS was dissolved

overnight with gentle stirring in Buffer at a concentration of 5 mg/mL. This solution was filtered in 0.45 µg PVDF filters and 5 mL were applied at the column. Size exclusion chromatography was performed with HiLoad 16/600 Superose 6 prep grade (Cytiva) column, 120 mL column volume (cv), at room temperature. The column separates molecules in a range from 5 to 5 000 Kilo Daltons (kDa), according to the manufacturer. Detection was monitored by following the absorbance of the eluted molecules at 280 nm, by an ultraviolet (UV) detector. The flowrate, volume and compounds utilized for each step are listed in table 2.1.

Table 2.1. Buffer, washing and storing solutions and their flowrate and volume implemented in SEC.

	Compound	Flowrate (mL/min)	Volume (cv)
Equilibration	Buffer	0.2 – 0.5	5
Elution	Buffer	1	1.5
Washing	0,1 M NaOH	1	1
Storing	25% Ethanol	0.2 – 0.5	1.5

After fractionation, fractions were collected, dialyzed, and freeze dried following the procedures mentioned previously. For the freeze-drying, the liquid fractions were disposed in petri dishes to obtain a greater surface area and speed up the drying process. After drying, the samples were transferred to previously weighed tubes and then weighed.

The aMW for the column was calibrated using six proteins standards (Sigma-Aldrich) having molecular range from 43 to 669 kDa: Ovalbumin - 43 kDa; Conalbumin - 75 kDa; Aldolase - 158 kDa; Ferritin - 440 kDa; Thyroglobulin - 669 kDa. The elution volume (V_e) is the volume of eluent from the point of injection to the center of the elution peak. To determine the void volume (V_0), Blue Dextran - 2 000 kDa (Sigma-Aldrich) was used, in which the elution volume of Dextran is the same as the void volume. The partition coefficient (K_{av}) is calculated through

equation 2.2, where V_t is the column's total volume (120 mL). The logarithm of MW (log (MW)) was plotted as a function of K_{av}, leading to a calibration equation (eq. 2.3).

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}, \tag{2.2}$$

$$Log(MW) = 1{,}71 - 0{,}24Kav\,. \tag{2.3}$$

Chromatogram profiles were recorded with UNICORN 5.1 software (GE Healthcare). Peak retention times and areas were directly calculated by the program.

2.5 Molecular Fingerprint by Fourier Transform Infrared (FTIR)

The fourier transform infrared (FTIR) spectra of the granules, unfractionated EPS and EPS fractions were recorded using a FTIR spectrometer (Perkin Elmer, Shelton, USA) with a wave-number range from 500 to 4 000 cm^{-1}, at room temperature. A resolution of 2 cm^{-1} and accumulation of 8 scans were applied to each sample.

2.6 Sulfated Glycosaminoglycans Quantification by DMMB Assay

The Sulfated Glycosaminoglycans (sGAG) content measurement was performed through a quantitative dye-binding method, Blyscan™ Sulfated Glycosaminoglycans (Biololor, UK). The provided assay protocol was followed, differing in the amount of sample added, which was 1.2 mg of EPS instead of 20 – 50 mg and the volume of papain extraction reagent added to each sample of 0.5 mL instead of 1 mL. For all EPS and fractions samples, the papain extraction was performed for 3 h. Moreover, another assay was performed, win which the extraction was done for 18 h.

The assay is based on the binding of a dye reagent (1,9- dimethyl methylene blue (DMMB)) to polyanionic substances, such as sGAG, in the sample. The DMMB – polyanion complex

precipitates, which is then dissolved in a dissociation reagent, resulting in different shades of blue according to polyanion content. A calibration curve was obtained by performing the assay with reference standard (bovine tracheal Chondroitin-4-sulfate) and blanks (demineralized water). The absorbance of each sample was measured at 656 nm and concentrations of total sGAG obtained from the calibration curve. O-sGAG content was measured through the same assay, with an additional nitrous acid cleavage pretreatment, which reacts with N-sulfated d-glucosamine and cleaves the N-sulfated site. N-sGAG was obtained by subtracting O- from total-sGAG content, enabling the determination of O- and N- sulfated glycosaminoglycan ratio within the test samples.

2.7 Histone Binding by Agarose Gel-Electrophoresis

The interactions between the histones H_1, H_2A and H_2B (Sigma Aldrich) with EPS was tested through native agarose gel electrophoresis. Solutions of EPS in 50 mM Tris were prepared with a concentration of 2.5 mg/mL. For the binding, a ratio between histone and EPS of 1:2 was used, where 4 µL of the EPS solution (2.5 mg/mL) was added to 5 µL of histone (1 mg/mL). As positive control, heparin was added to histone in the same ratio as EPS. As negative controls, heparin and EPS samples were diluted in tris. 5 µL of histone was added to 4 µL of tris and 4 µL of each EPS sample were added to 5 µL of tris. Furthermore, the interaction of H_2B with EPS t_0, t_1 and t_2 was tested in rations 1:3 and 1:4. As positive controls, histone was added to heparin (from porcine intestinal mucosa, Sigma Aldrich) in ratios 1:0.5; 1:1 and 1:2.

All the samples were incubated at 30 ºC and 300 rpm for 1 h and, then 1 µL of glycerol was added. Then, 10 µL of each sample were added to 0,8% agarose gel (500 mM Tris/HCl, 160 mM boric acid, 1 M urea, ph 8.5). The electrophoresis was performed with a running buffer (90 mM Tris/HCl, 90 mM boric acid, ph 8.5) at 80 V for 90 min. Staining was performed using Coomassie blue staining (SimplyBlue, Invitrogen), slowly mixing during 1 h. Distaining was done by adding demineralized water, changing the water after 1 h and leaving the gel immersed overnight. Through ChemiDoc MP Imaging System, images were obtained, and peaks were measured. The peak reduction caused by the histone-EPS binding was calculated by comparing the original histone peak size with each histone-EPS complex peak sizes, obtaining a peak reduction percentage caused by each unfractionated EPS and their fraction, relative to the unbound histone.

3

RESULTS AND DISCUSSION

3.1 SEC is a suitable method for the separation of EPS by apparent molecular weight

3.1.1 Reactor Operation and Microbial Community

Freshwater and seawater-adapted aerobic granular sludge were performing complete removal of COD and phosphate. A typical reactor cycle performance is shown in figure 3.1. During anaerobic phase, acetate was completely consumed within the first 25 min, while phosphate was released up to 88.13 mg PO_4^{3-} -P/L, corresponding to 0.49 P mol/C mol of phosphate release per carbon uptake, comparable to ratios obtained in polyphosphate accumulating organisms (PAO) enriched cultures (M.K. de Kreuk, J.J. Heijnen, 2015; Welles et al., 2015), indicating that most of acetate was consumed by PAO (Pronk, 2016). This amount of phosphate was then consumed in the aerobic period. The reactor's biomass concentration was roughly constant at around 7 g VSS/L and the VSS/TSS around 76%.

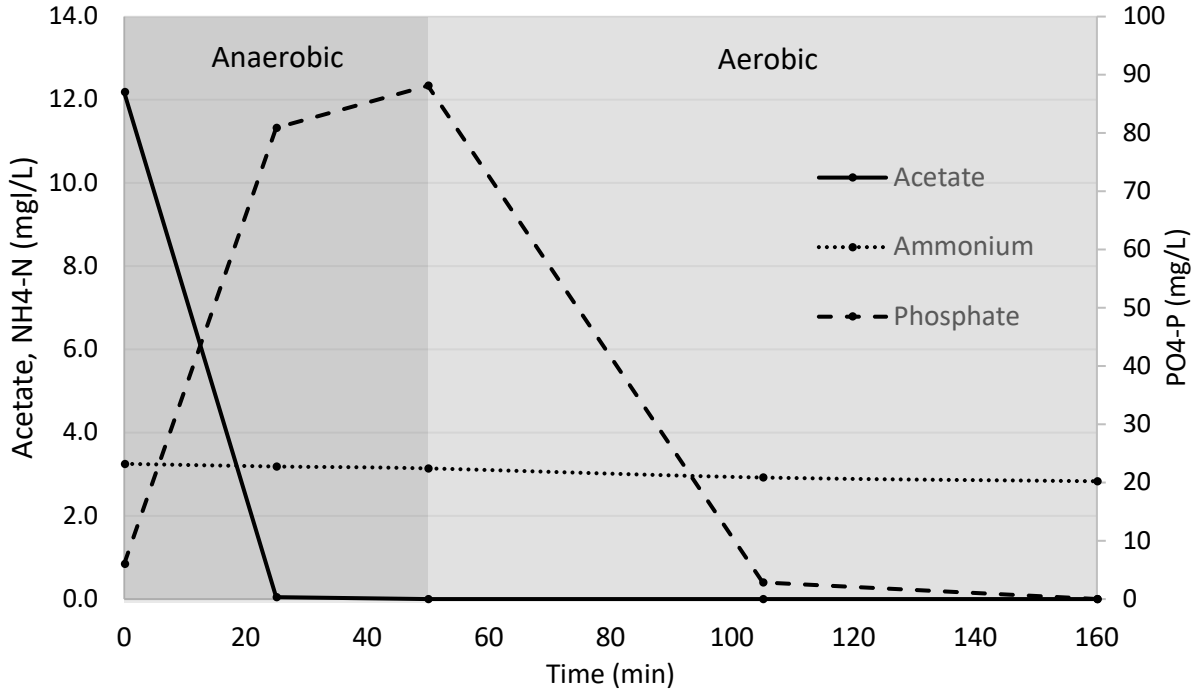

Figure 3.1. Typical reactor cycle of a seawater-adapted aerobic granular sludge, showing concentrations of phosphate, ammonium, and acetate, with 50 minutes of anaerobic phase and 110 minutes of aerobic phase.

Aiming the understanding of seawater influence on the anionic properties of AGS based extracellular polymeric substances (EPS), granules were collected in three different time slots: t_0, t_1 and t_2 (fig. 3.4). The sample t_0 refers to the inoculum, grown in freshwater. The second sample, t_1, was collected 5 days after the seawater concentration achieved 35 g/L (before the start of SRT control), representing the granules in an intermediate stage. The sample t_2 was taken 17 days from the start of 35 g/L seawater (12 days from the start of the 12 days SRT control), representing seawater-adapted granules.

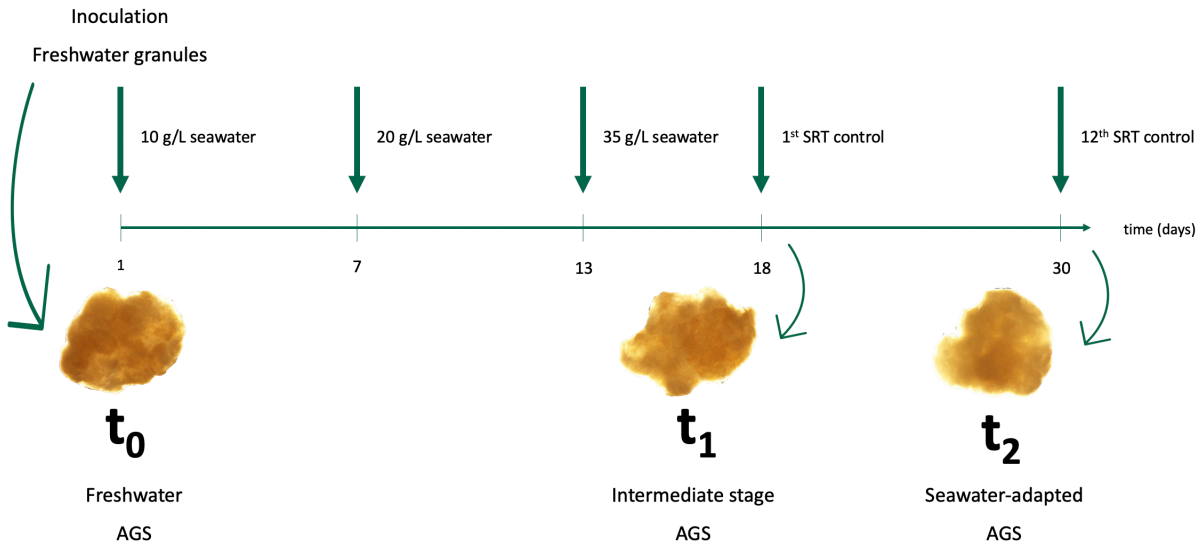

Figure 3.2. Roadmap of granules collection time over different seawater exposure periods.

Good granulation was achieved (fig. 3.3), with no filamentous growth observed and adequate settling capacity of granules. Moreover, no significant difference in granule morphology was observed between the three samples (figure A.1 in appendix). Fluorescent in situ Hybridization (FISH) was performed to analyze the relative amounts of polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO). High quantity of PAO were observed in seawater-adapted and freshwater aerobic granular sludge (t_0, t_1 and t_2), while GAO were detected in a minority (fig. 3.4). Distinct and uniform PAO cells were distinguished and seen in cell clusters. These results signal a dominance of PAO over GAO in the systems.

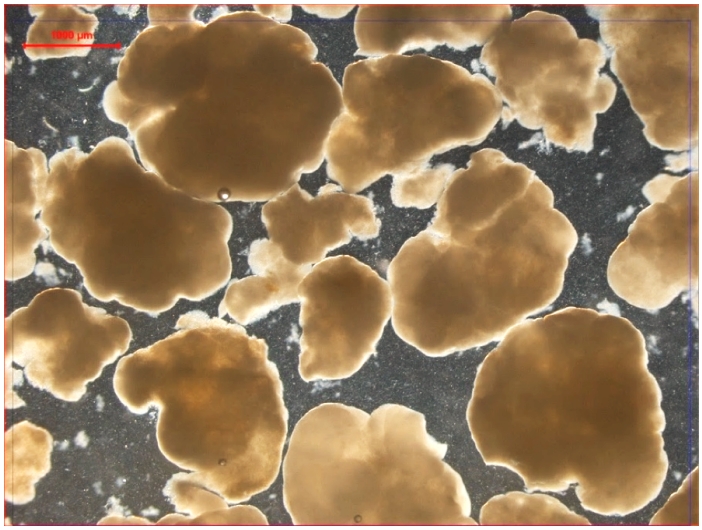

Figure 3.3. Image analyzer picture of seawater-adapted aerobic granular sludge. Scale bar equals to 1 000 µm.

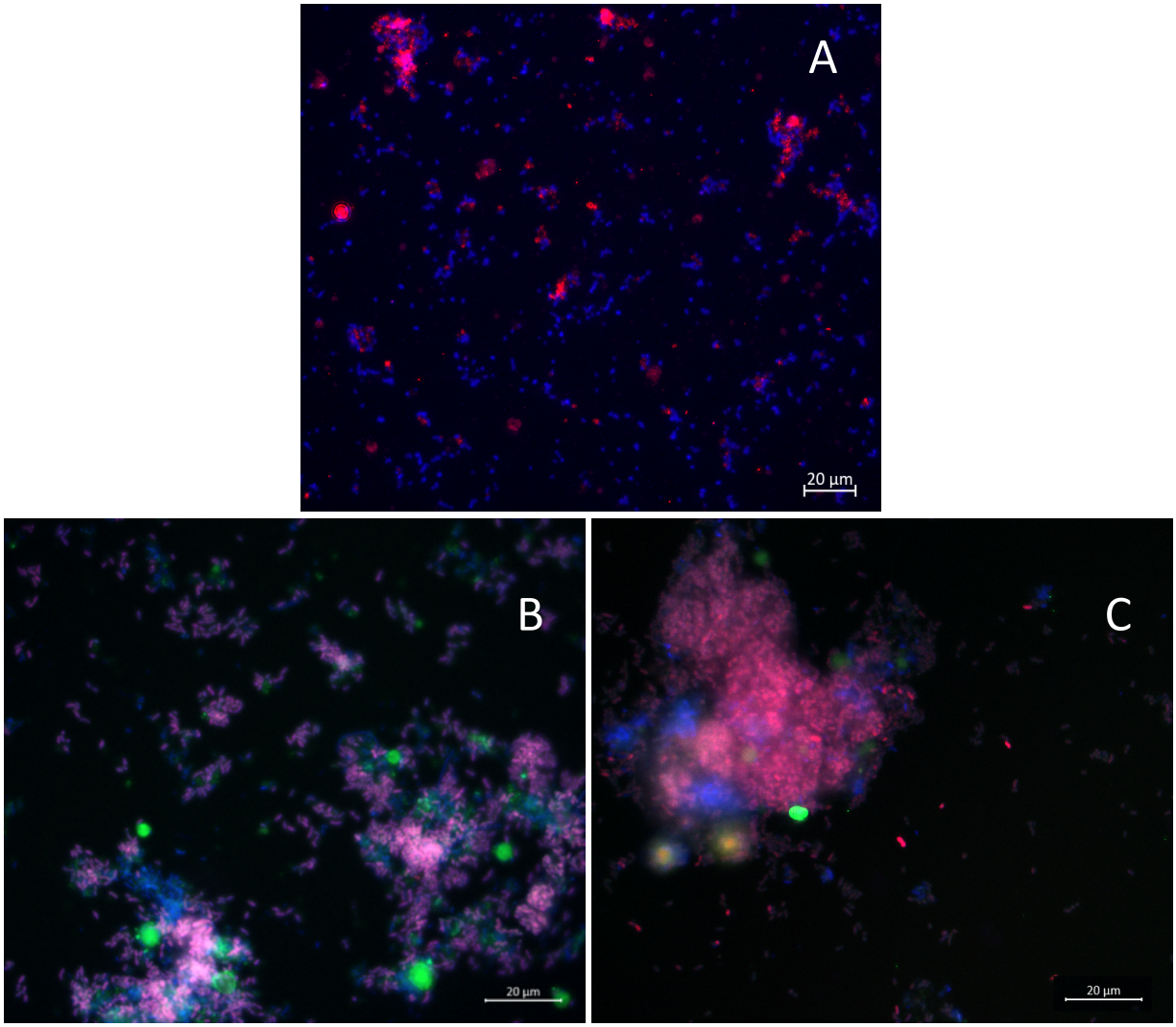

Figure 3.4. Fluorescence in situ hybridization (FISH) images of aerobic granular sludge (AGS) stained by EUB338, Cy5/blue, PAOmix, Cy3/red and GAO, fluos/green. Magenta color is an overlao between eubacteria (blue) and PAOmix.Scale bar equals to 20 µm. A: Freshwater AGS based EPS (t_0); B: Intermediate seawater-adapted AGS based EPS (t_1); C: Seawater-adapted AGS based EPS (t_2).

However, before the granules were exposed to seawater conditions were exhibiting good granulation and performing complete COD and phosphate removal, two other startups were necessary. The first inoculation was performed directly with 35 g/L of seawater. After some period, filamentous growth was observed, which increased over time. Moreover, the acetate was not being taken up in the anaerobic phase, but was partially consumed in the aerobic phase, which indicated low abundance of PAO. Hypothesis about the problem were outlined: there could be oxygen or nutrient limitation. All oxygen sources, measurement, and control were verified, new trace elements and medium A and B were prepared and a complete restart of the reactor with fresh inoculum was necessary. The second startup started with 35 g/L seawater as well and no COD removal was observed in the following period. Another explanation

for this behavior then arose: either the inoculum grown in glycerol was not able to grow under acetate, which would then require further investigation into the microbial community, or the abruptly transition of the granules from freshwater to 35 g/L seawater was giving them a shock. An experiment was then performed, in which the inoculum was placed into acetate in the absence of seawater and samples were taken to monitor the COD uptake. Results showed fast COD consumption, which excluded the first hypothesis. Therefore, the third inoculation started under 10 g/L seawater, which was increased over time according to the granules' adaptation, that was monitored through the carbon anaerobically uptake, until 35 g/L was achieved. This, when inoculating the reactor with freshwater-adapted AGS in seawater conditions, a few adjustments may be needed for the biomass acclimation.

3.1.2 EPS Extraction

The goal of the extraction was to recover as much acidic polymers as possible, by separating the EPS from the cells at 80 °C and solubilizing them into basic solution (NaOH) and then purifying through dialysis. The alkaline and heat extraction method resulted in an EPS with the same yellow color as the aerobic granules. Extracted EPS t_0, t_1 and t_2 yielded 191 mg/g, 220 mg/g and 682 mg/g (VSS ratio), with a VS/TS ratio of 69%, 70% and 86% respectively. Using sodium carbonate and heat, Felz et al., 2020 reported a structural EPS from AGS yield of 253 ± 14 mg/g VSS and Feng et al., 2019 yielded 210 ± 5 mg/g VSS EPS from anammox GS. Meanwhile, the extraction yield of EPS from AGS through the same method implemented in the present study was reported to vary between 640 and 740 mg/g VSS (Chen, 2019) and from 370 to 580 mg/g VSS (Hof, 2021).

Later on, a second extraction in the same conditions was performed in our laboratory of EPS from granules t_1, yielding 388 mg/g VSS. This value is higher than what obtained previously, nevertheless it is still much lower than the EPS t_2 yield. It should be mentioned as well that EPS samples t_0 and t_1 partially melted in the freeze-drying process, causing the sample to boil, which could have resulted in loss of some material.

Furthermore, during the extraction procedure, the color of solubilized EPS t_2 turned red (figure A.2 in appendix), turning back to yellow during dialysis (figure A.3 in appendix), while EPS t_0, t_1 were yellow in the extraction solution. According to Feng et al., 2019, the darker reddish color obtained during alkaline extraction could potentially indicate a higher solubility of biopolymers.

The higher yield of EPS from seawater-adapted AGS could mean either that more EPS was produced with the longer exposure of AGS to seawater, which is in accordance with the increase in EPS observed in AGS exposed to salinity (Corsino et al., 2017); or that the alkaline extraction was more efficient with the seawater EPS, maybe due to a more acidic EPS. Polyacids in basic conditions gain anionic nature due to deprotonation, resulting in higher solubilization (Kocak et al., 2017), thus yielding in more EPS extracted. It could also be a higher solubilized content of cell debris, single cells or inner cell plasma, resulted from the heat damage on cells (Hee-deung et al., 2015).

3.1.3 EPS Fractionation and Apparent Molecular Weight Distribution

Size exclusion chromatography (SEC) has been used to determine sludge extracted EPS apparent molecular weight (aMW) distribution as well as to separate it into fractions, to better characterize their biochemical composition (Simon et al., 2009). At a wavelength of 280 nm, mainly proteins and humic-like substances are detected (Bhatia et al., 2013; Garnier et al., 2006).

The chromatographic peaks obtained through SEC give us the EPS fingerprint. Differences in the fingerprint can be related to variation in the molecular chemical composition and the amount of each type of molecule in the EPS (Simon et al., 2009). The EPS fingerprint significantly changes when they originate from different types of sludge (Simon et al., 2009). EPS extracted from AGS collected in three different contact period with seawater were characterized by SEC. EPS t_0 was extracted from the inoculum grown in freshwater, t_1 represents an intermediate point and EPS t_2 was extracted from seawater-adapted AGS.

The SEC enabled the separation of EPS in five fractions with different morphologies. Fractions 1 and 2 were whitish, light and sticky, similar to a cotton candy appearance and behavior. Fractions 3 and 4 where similar to the other two, but with a shiny aspect and less sticky. Fraction 5 was the most different, with a yellow color, very sticky and slimy. An illustration of the fractions appearance and the aMW distribution of the EPS from t_0, t_1 and t_2 obtained through SEC is shown in fig. 3.5. Although differences can be observed between the three chromatographic profiles, their fingerprint have a similar pattern, with two larger peaks and two very smooth peaks in between them (retention time of 72 and 82 min). The last and higher peak, illustrating the smaller molecular weight molecules that elute at the end, have a retention time between 108 and 110 min. Since the wavelength of 280 nm enables mainly protein detection, the higher peak having the longest retention time (fraction 5), indicates that this fraction contains molecules with smaller molecular weight and higher protein content.

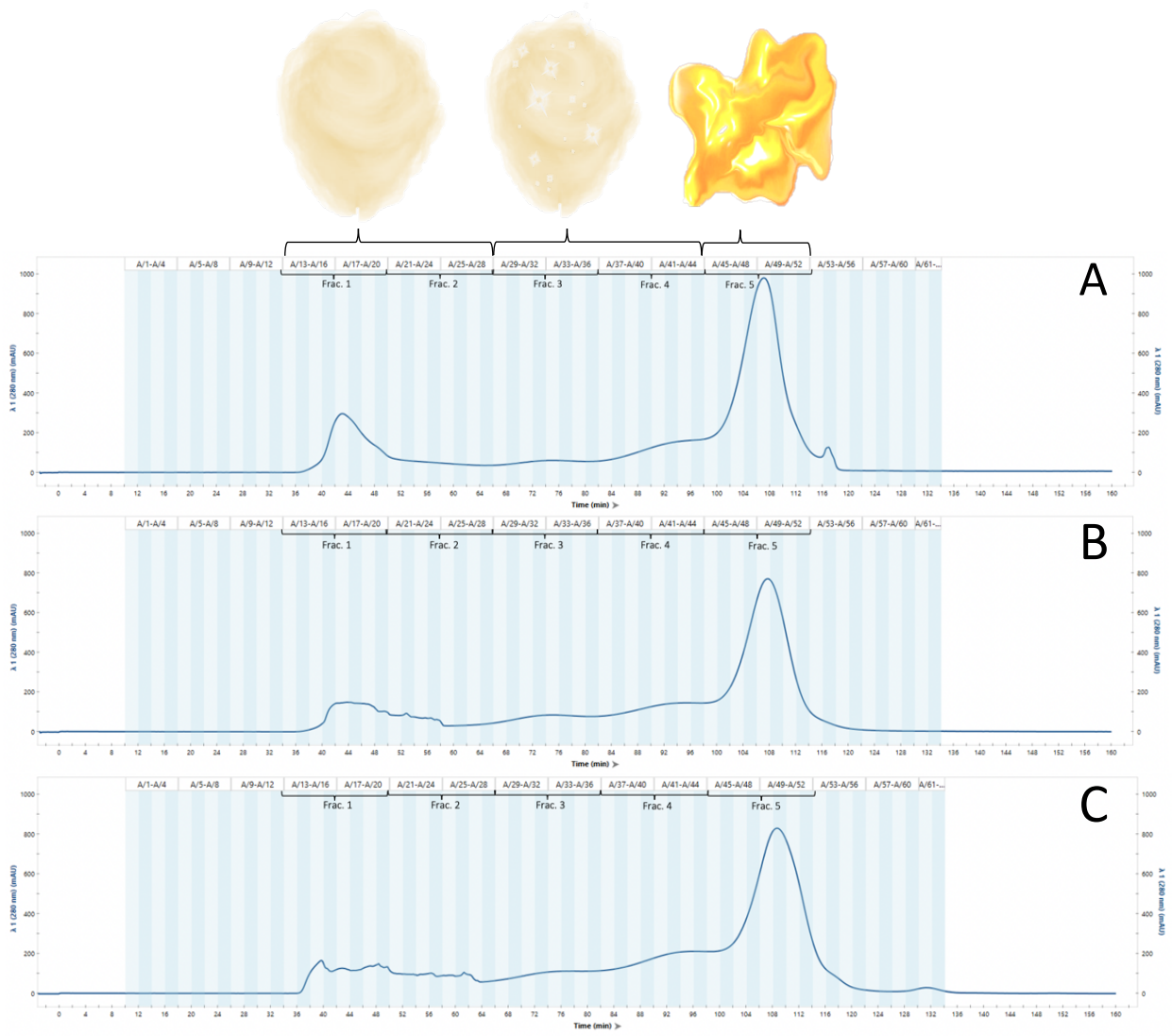

Figure 3.5. Size exclusion chromatography chromatograms of extracellular polymeric substances (EPS) from aerobic granular sludge (AGS) with 280 nm UV detection. A: Freshwater AGS based EPS (t_0); B: Intermediate seawater-adapted AGS based EPS (t_1); C: Seawater-adapted AGS based EPS (t_2). Illustrative representation of fractions morphology observed after freeze-drying is presented.

Nevertheless, the main difference between the profiles is seen in the first peak, representing the first molecules to elute and thus a higher apparent molecular weight. In EPS t_0 this peak is well defined (fraction 1), while in EPS t_1 and t_2 it is a broader plateau-like peak, divided into fractions 1 and 2. This difference already observed in the intermediate seawater-adapted AGS based EPS could indicate that it is unnecessary the complete renewal of the biomass for EPS molecular characteristics change under a seawater environment.

The first peak starts around 36 min for all samples, but it gets increasingly broadened with the longer exposure time of AGS to seawater conditions. The broadening of the peaks means that

those molecules took a longer time to elute (16 min and 28 min for t_0 and t_2 respectively), which represents a higher interaction with the column. The area calculated under each fraction's peak is listed in table A.1 in appendix. The area under the first peak of t_0 and t_2 are 1.9×10^3 and 2.09×10^3 mL mAU respectively, which are numbers in the same order of magnitude. Since the area under the peak represents the amount of component in the sample, a similar area with different elution period implies that a similar amount of molecules had a different interaction with the column.

The longer interaction with the column could be due to smaller molecules, that more readily get entrapped into the column pores, or because of a greater viscosity of the molecules, causing resistance to elute the column. A possibility for this event could be that the increased exposure time to seawater, resulted in a higher sugar content in fractions 1 and 2, maybe due to glycosylation. The enhanced carbohydrate content could lead to higher viscosity, as reported in many biofilms (Sutherland, 2001), but also increase the chances of negatively charged molecules being present in these factions. A strong negative charge of sulfate ester groups can have a function in preventing cellulose nano crystals to aggregate (Beck-Candanedo et al., 2005; Y. Lin et al., 2018). Thus, it could be possible that the higher anionic content contributed to higher repulsion, which reduced aggregation – resulting in smaller molecules. This hypothesis of higher sugar and anionic groups in fractions 1 and 2 will be assessed in section 3.2.2. This profile change can already be observed in the intermediate EPS extracted from AGS collected before SRT was being controlled (t_1). Thus, it seems likely that it is not necessary one complete SRT for the EPS anionic characteristics change under seawater conditions and be potentially more negatively charged.

For the calibration curve (figure A.4 in appendix), a high MW protein standard kit was used (range 43 to 669 kDa) with an additional polysaccharide Blue Dextran (2 000 kDa); chromatograms illustrated in figures A.5 and A.6 in appendix respectively. Blue Dextran had a retention time of 41 min, while EPS started eluting around 36 min, thus it seems probable that the apparent molecular weight of the first molecule to elute is at least 2 000 kDa. Nevertheless, based on extrapolation of the calibration standards, an aMW of 11 350 kDa was calculated in the highest MW fraction of EPS. The calculated aMW range of each fraction collected as well as the yield of EPS recovered from the column and respective fractions are listed in table 3.1. The fractions yield refers to the recovered fraction dry weight over the total EPS fractionated and recovered from the column. The total yield is the total recovered EPS from the column over the (unfiltered) amount of EPS applied to the column. Observing the yields of EPS t_2, the higher molecular weight fractions (1, 2 and 3) represent 58% of the fractionated EPS. Nevertheless, the weighed dry fractions were previously transferred from petri dishes to tubes, which

caused content loss. In addition, mass of EPS was retained in the filter before being applied into the column. These losses are represented in the unrecovered EPS percentage, which explains such low yields obtained in SEC. Thus, these yields are underestimated values.

Table 3.1. Apparent molecular weight (aMW) range of fractions from extracellular polymeric substances (EPS) obtained through size exclusion chromatography (SEC) and respective yields regarding total fractionated EPS.

Fractions	aMW range (kDa)	EPS t_0 (% of fractionated EPS)	EPS t_1 (% of fractionated EPS)	EPS t_2 (% of fractionated EPS)
1	2 800 – 5 000	18	20	20
2	436 – 2 800	2	14	19
3	54 – 436	18	18	19
4	8 – 54	29	16	26
5	1 – 8	33	31	16
Total yield (%)		55	66	63
Unrecovered EPS (%)		45	34	37

EPS t_0: Freshwater AGS based EPS; EPS t_1: Intermediate seawater-adapted AGS based EPS; EPS t_2: Seawater-adapted AGS based EPS.

A molecular weight above 1 000 kDa was observed in exopolysaccharide recovered from saline condition (Roca et al., 2016) and an aMW over 5 000 kDa was estimated for EPS extracted from full scale AGS (Chen et al., n.d.). In the present study, the EPS has an aMW of at least 2 000 KDa and with the estimations obtained, presumably it could be 11 350 kDa. However, the column separation range is up to 5 000 KDa, according to the manufacturer's description. Although a higher aMW value was extrapolated, it is unclear yet how big these polymers could be, thus, it is more realistic to suppose an EPS aMW of above 5 000 kDa. Nevertheless, in nature, high molecular weight biopolymers can occur, such as gel-forming mucins, complex glycoproteins that can present a molecular weight as high as 200 000 kDa (Kesimer et al., 2009).

3.2 EPS extracted from seawater-adapted AGS contain anionic properties in the higher molecular weight fractions

3.2.1 Molecular Fingerprint

The fourier transform infrared (FTIR) was used to obtain a better comprehension of the molecular fingerprint of the different EPS samples (t_0, t_1 and t_2) and their fractions. The FTIR spectra of the fractionated EPS t_2 are illustrated in figure 3.6. It has been reported that peaks between 1 250 – 900 cm^{-1} indicate polysaccharides, that can be attributed by C-O-H and C-O-C bonds, while absorption bands near 1 600 – 1 700 cm^{-1} and 1 520 – 1560 cm^{-1} represent amide I and II respectively, belonging to proteins (Farooq & Ismail, 2014). Peaks around 3 400 – 3 070 cm^{-1} can represent N-H and O-H stretching (Farhat et al., 1998), in which a broad absorption band in this region suggests hydroxyl group (R.-B. Xu et al., 2012). Judging from the broad peak in the X-H stretching region and the strong peak at 1 031, fractions 1 and 2 are sugar dominant. Meanwhile, fractions 3 and 4 have a stronger vibration in amide peaks relative to the carbohydrate region, suggesting protein dominance.

In fraction 5, the peak at 1 068 cm^{-1} may indicate the presence of phosphate groups (Masaki et al., 2017), in addition, observing the high peak in the SEC chromatogram at 280 nm (fig 3.5), it can be presumed a protein dominance in this fraction. These findings suggest that there could be phosphorylated proteins in this fraction. Moreover, considering that the samples analyzed referred to granules collected at the end of aerobic phase, it must be considered the polyphosphate accumulated inside the bacteria. The cells could have been disrupted in the extraction process, releasing the polyphosphate, which could be related to the phosphate groups identified in fraction 5. The same pattern described above can be seen in EPS t_0 and t_1 fractions (figures A.7 and A.8 in appendix). Other complementary FTIR spectra are illustrated in figures A.9 to A.11 in appendix.

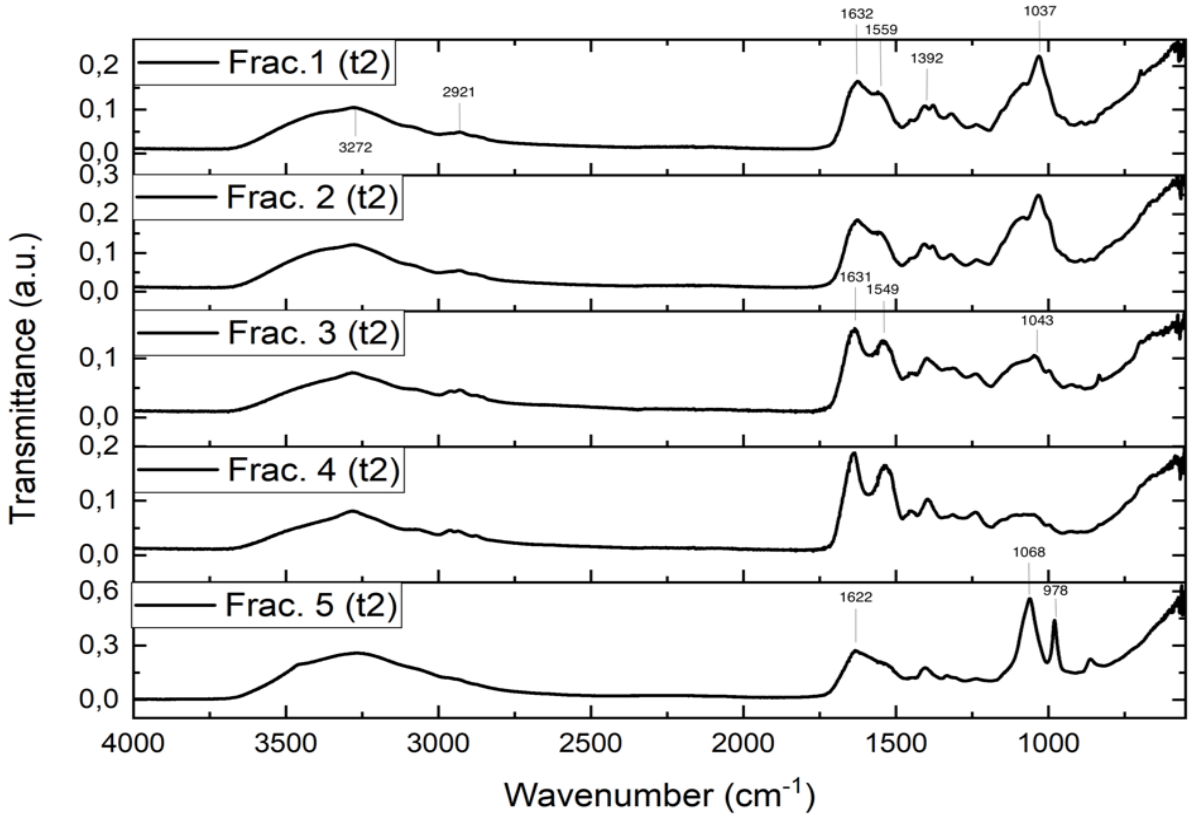

Figure 3.6. Fourier transform infrared (FTIR) spectra of seawater-adapted AGS based EPS (t_2) fractions (1 – 5).

Observing the infrared spectra of the whole EPS t_0, t_1 and t_2 (fig. 3.7), EPS t_0 and t_1 have a similar profile, while the main difference in EPS t_2 seems to be in the sugar portion. The peak around 1 062 in EPS t_2 seems to be broadened and the band around 895 cm^{-1} almost disappeared. This suggests there might be a difference in the carbohydrate composition between extracts from seawater-adapted AGS and EPS from freshwater. Gagliano et al. (2018) studied differences between EPS recovered from high and low salinity conditions, identifying a significant change in glycoconjugates between them, such as mannose and galactose enrichment in the high salinity EPS.

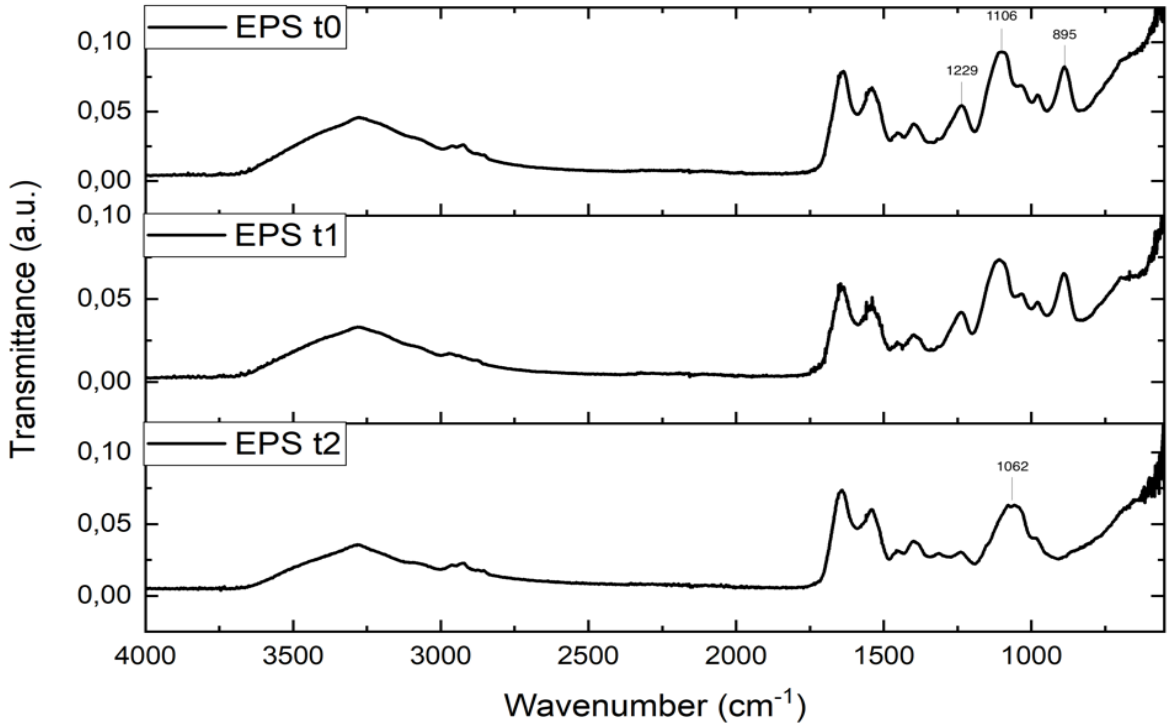

Figure 3.7. FTIR spectra of freshwater AGS based EPS (t_0), intermediate seawater-adapted AGS based EPS (t_1) and seawater-adapted AGS based EPS (t2).

3.2.2 Sulfated Glycosaminoglycans Quantification

Sulfated glycosaminoglycans (sGAG) have been found in marine organisms, among other sulfated polysaccharides such as sulfated fucans and galactans. These sulfated sugars have been studied for their capability to interact with proteins responsible for health/ disease balance, fostered by their polyanionic feature (Pomin & Mourão, 2014). To understand whether the AGS grown under seawater conditions could contain a similar polyanionic feature, the sulfated sugars were quantified by the 1,9- dimethyl methylene blue (DMMB) Assay.

The Assay is based on the binding of the dye to polyanionic substances. Thus, it is worth mentioning that besides sGAG, other polyanions such as hyaluronic acid (HA), DNA and RNA could be detected by this dye (Zheng & Levenston, 2015).

The total content of polyanionic substances measured in EPS t_0, t_1 and t_2, with a 3 h long papain extraction of the EPS, was 13 ± 0,3 mg/g, 10 ± 5 mg/g and 20±0,7 mg/g respectively. The total sGAG in the unfractionated EPS from t_0, t_1 and t_2 and the respective fractions are represented in fig 3.8. Total sGAG measured in EPS t_2 is higher than in EPS t_0. Moreover, in fractions 1, 2 and 3, the detected polyanionic molecules gradually increased from t_0 to t_2. Fractions 1 and 4

are the most abundant in sGAG for t_0, while in the case of t_1 and t_2, fractions 1 and 2 have the highest amount of sGAG per weight of fraction. These fractions (1 and 2) from EPS extracted from seawater-adapted AGS had up to 115% sGAG increase relative to the unfractionated EPS. This represents a possible polyanionic substances enrichment of over 2-fold in the higher molecular weight fractions.

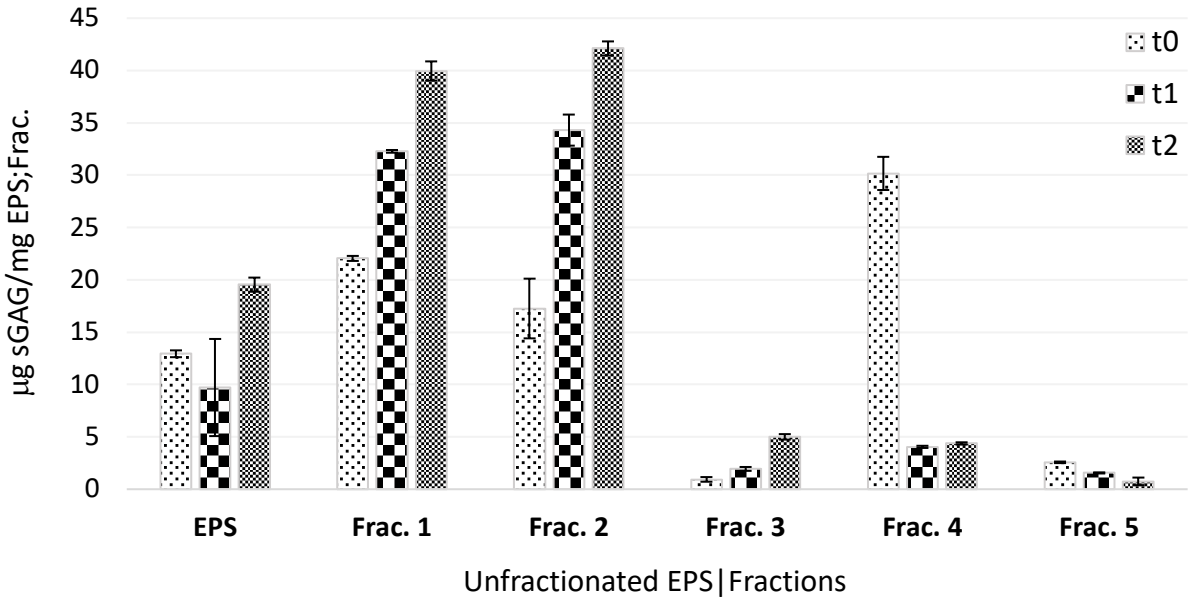

Figure 3.8. Total sulfated glycosaminoglycans in unfractionated extracellular polymeric substances and respective fractions (1 – 5). EPS: unfractionated extracellular polymeric substances; t_0: freshwater AGS based EPS/fractions; t_1: intermediate seawater-adapted AGS based EPS/fractions; t_2: seawater-adapted AGS based EPS/fractions.

The ratio of O- and N-sulfated GAG in seawater-adapted EPS is illustrated in fig. 3.9. It can be observed that in EPS t_2, fractions 1, 2 and 3, as well as the unfractionated EPS, have a much higher ratio of N-sulfated GAGs than O-sulfated. The ratios of O- and N-sulfated GAG of EPS t_0 and t_1 are illustrated in figure A.12 and A.13 in appendix. Furthermore, as seen in figure 3.10, the N-sulfated portion seems to increase from EPS t_0 to t_2. The fact that chondroitin sulfate, keratan sulfate and dermatan sulfate contain O-sulfated hexosamines while heparin and heparan sulfate hold N-sulfated hexosamines and heparin being the most negatively charged biomolecule known, imply that a higher N-sulfated glycosaminoglycan fraction points to a higher anionic charge density. Fractions 1 and 2 from EPS t_2 revealed a FTIR spectrum indicating sugar predominance. Thus, having in mind that up-to-date sulphated groups have only been described in carbohydrates, it is likely that the sulfated groups are present in these higher molecular weight fractions of seawater EPS, which are sugar dominant.

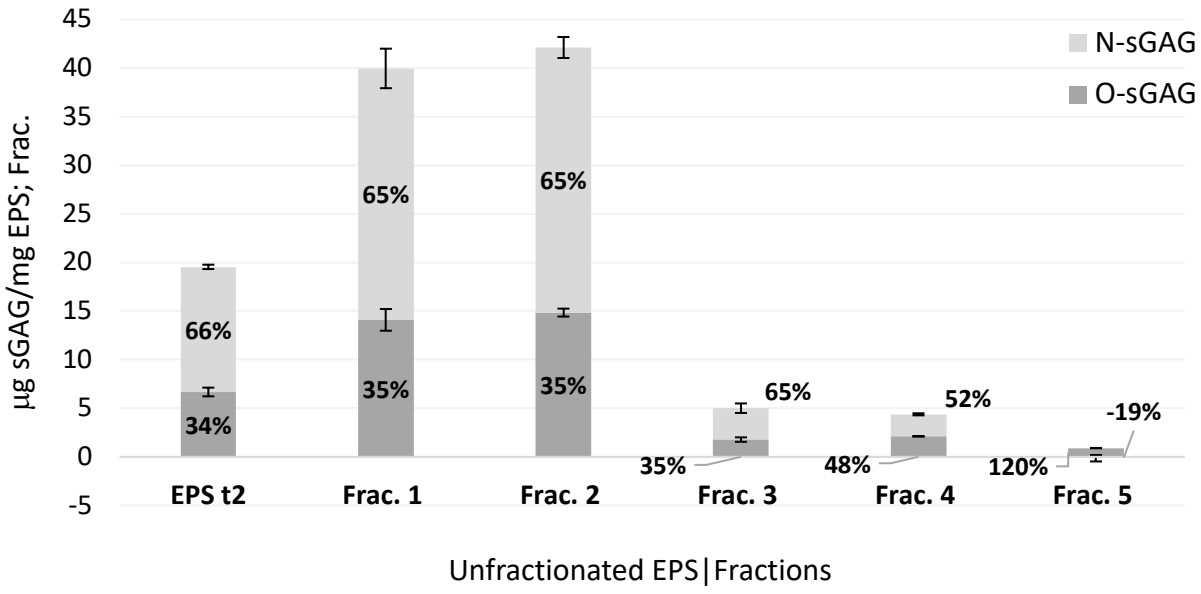

Figure 3.9. O- and N- sulfated glycosaminoglycans in seawater-adapted AGS based EPS (t_2) and its fractions (1 – 5).

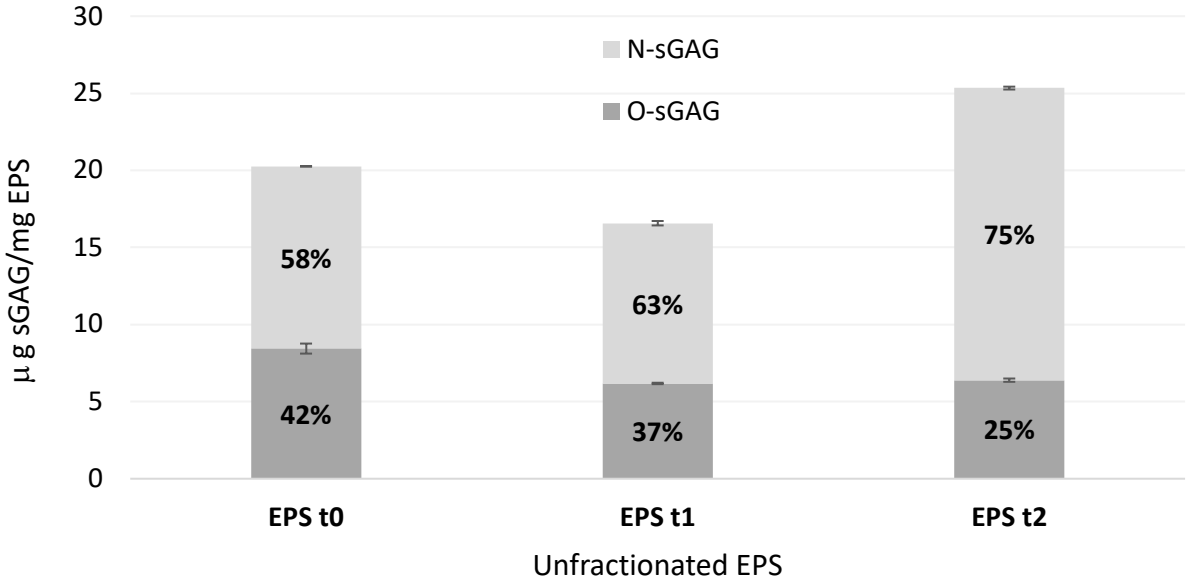

Figure 3.10. O- and N- sulfated glycosaminoglycans in AGS based EPS. EPS t_0: Freshwater AGS based EPS; EPS t_1: Intermediate seawater-adapted AGS based EPS; EPS t_2: Seawater-adapted AGS based EPS.

This assay gives a sign of the presence of sulfated sugars in EPS from seawater-adapted AGS. Although lower amounts of s-GAG were measured in comparison with the traced in Kaumera EPS in the order of 8 to 93 mg/g (Chen et al., n.d.), a higher content of polyanionic substances was obtained in EPS t_2 than in the inoculum (t_0), as well as a higher N-sulfated GAG fraction. This could be an indication of the enhanced presence of sulfated carbohydrates in EPS extracted from seawater-adapted AGS. Glycosaminoglycans have been identified in EPS from

anammox granular sludge (Boleij et al., 2020) and full-scale granular sludge (Felz et al., 2020). In saline anaerobic granular sludge, glycoconjugates have been found (higher than in low salinity) (Gagliano et al. 2018). In addition, Sudmalis et al., 2020 reported a significant shift in the glycoconjugate pattern of EPS with increasing salinity. Furthermore, the progressive increase in the sGAG content from t_0, t_1 and t_2 in fractions 1 to 3 suggests that the EPS from the granules before the SRT control, still in adaptation to seawater conditions, already revealed a change in the ionic composition in the higher molecular weight fractions. Studies with anaerobic granules under saline conditions showed that in two weeks the ionic equilibria of granules changed under exposure to different salt concentrations (Sudmalis et al., 2020).

With the attempt to verify if the longer papain extraction would enable a higher detection of polyanions, a 18 h long extraction was performed with the three unfractionated EPS. Total sGAG values slightly increased, although not significantly (figure A.14 in appendix).

In section 3.1.3, it was hypothesized the reason for the longer interaction with the SEC column observed in fractions 1 and 2 from EPS t_2, relative to t_1 and t_0 (fig 3.5). It was speculated that the increased exposure time to seawater may be related to an increased carbohydrate content in these fractions, maybe due to glycosylation. As previously mentioned, fractions 1 and 2 exhibited a sugar dominance in the FTIR spectra and possible sulfated glycosaminoglycans revealed by DMMB assay, which support the hypothesis.

The reason why bacteria would produce more negatively charged EPS is still uncertain, although it could be hypothesized a protective mechanism against the unfavorable seawater environment. The toxicity of high chloride concentrations to aerobic microorganisms was reported, due to the flux of Cl^- into the cells (Suzuki et al., 1999). Nevertheless, the marine environment has been recognized for its rich microbial diversity, thus these organisms should have mechanisms to cope with high ion concentrations (Sudmalis et al., 2020). It has been reported the protection function of anionic groups in bacteria against environmental stress (Roberts, 1996). The negative charge on EPS can enable the absorption of multivalent heavy metals (d'Abzac et al., 2013; Dobrowolski et al., 2017). The cation sorption through binding and coordination reactions with the negatively charged groups of EPS can alleviate metals toxicity towards microorganisms (H. C. Flemming & Wingender, 2010; Gail M. Teitzel and Matthew R. Parsek, 2003; Sudmalis et al., 2020). Studies with pure culture marine bacteria revealed that cultivation under seawater ionic strength conditions lead to exopolymers with higher capacity to sequester monovalent cations than those grown under nonionic conditions (Gutierrez et al., 2008). EPS showed, even in high salinity, protective role against monovalent cations, such as sodium, by preventing their diffusion into the cells (Sudmalis et al., 2018). In

Sudmalis et al., 2020 studies with EPS from salt adapted anaerobic GS, results suggested that the negative charge of EPS functions as a protection against anions, lowering the osmotic pressure experienced by the microorganisms in high salinity environments, by hindering the transport of cations into the cells. Gagliano et al., 2018 speculated the need for different cation/polysaccharides interactions to protect cells from Na^+ accumulation, stemmed from the observed enrichment of mannose and galactose in EPS grown in high salt conditions. Moreover, it is known that the biofilm architecture can be strongly influenced by the anionic interactions of EPS with multivalent cations, as an example, the development of thick and compact biofilms with increased mechanical stability can be provided by bridges between Ca^+ and polyanionic alginate molecules (H. C. Flemming & Wingender, 2010). Y. Lin et al., 2010 speculated that alginate-like polysaccharides could be a major constituent in EPS from AGS. Thus, it seems reasonable the speculation that the observed increase of anionic substances in seawater EPS compared to freshwater extracts, could be a protective mechanism to cope with the osmotic pressure caused by the seawater conditions.

3.3 Application of EPS from seawater-adapted AGS as Histone Neutralizer

3.3.1 Histone Binding

To understand whether the anionic properties of the EPS could have potential pharmaceutical application, such as the treatment for sepsis by neutralizing histones, the interaction between EPS and different histones were studied by the effect in their electrophoretic mobility. Histones are positively charged proteins and should migrate to the negative pole of the agarose gel. If an interaction occurs between the negative EPS and the positive histone, there should be a band reduction or even a migration block or shift to the positive pole (Zlatina et al., 2017). Previous study carried in our laboratory confirmed some interaction of histones and EPS extracted from seawater-adapted AGS (Sebastian, 2021).

Initially H_1, H_2A and H_2B were tested with the whole extracted EPS t_0, t_1 and t_2. Histone H1 seemed to have the less efficient interaction with EPS (figure A.15 in appendix), therefore only H_2A and H_2B were further tested with the five fractions obtained from each EPS. The applied ratio between histone and EPS/fractions was 1:2 (w/w). The bands lengths were measured and the reduction rate of each band regarding the migration of the histone was calculated.

Through the gel images obtained (figure 3.11) and the calculated reduction, it can be observed that most of the fractions and unfractionated EPS had some effect in the histone band migration. Overall, there seems to be a slightly stronger interaction of EPS with H_2B, although the binding with H_2A also occurs efficiently. In the case of H_2A, it is evident the increased binding capacity from EPS t_0 to t_1 and t_2. These results along with the increase of sGAG and a higher N-sGAG content from t_0 to t_2, point to an enhanced anionic property of EPS when adapted to seawater conditions. Thus, two hypothesis arise: there might be an overall change, meaning there is a higher quantity of negatively charged polymers, or there might be a change in the charge density of EPS, meaning the polymers are more negatively charged.

Moreover, the higher molecular weight fractions present a better binding capacity with both histones. In general, fractions 1, 2 and 3 had the most efficient binding, even better than the unfractionated EPS, being fraction 3 the most outstanding, in most cases. Meanwhile, mostly fraction 5 had the poorest interaction. These results indicate that the higher molecular weight fractions of EPS are more negatively charged than the smaller fractions.

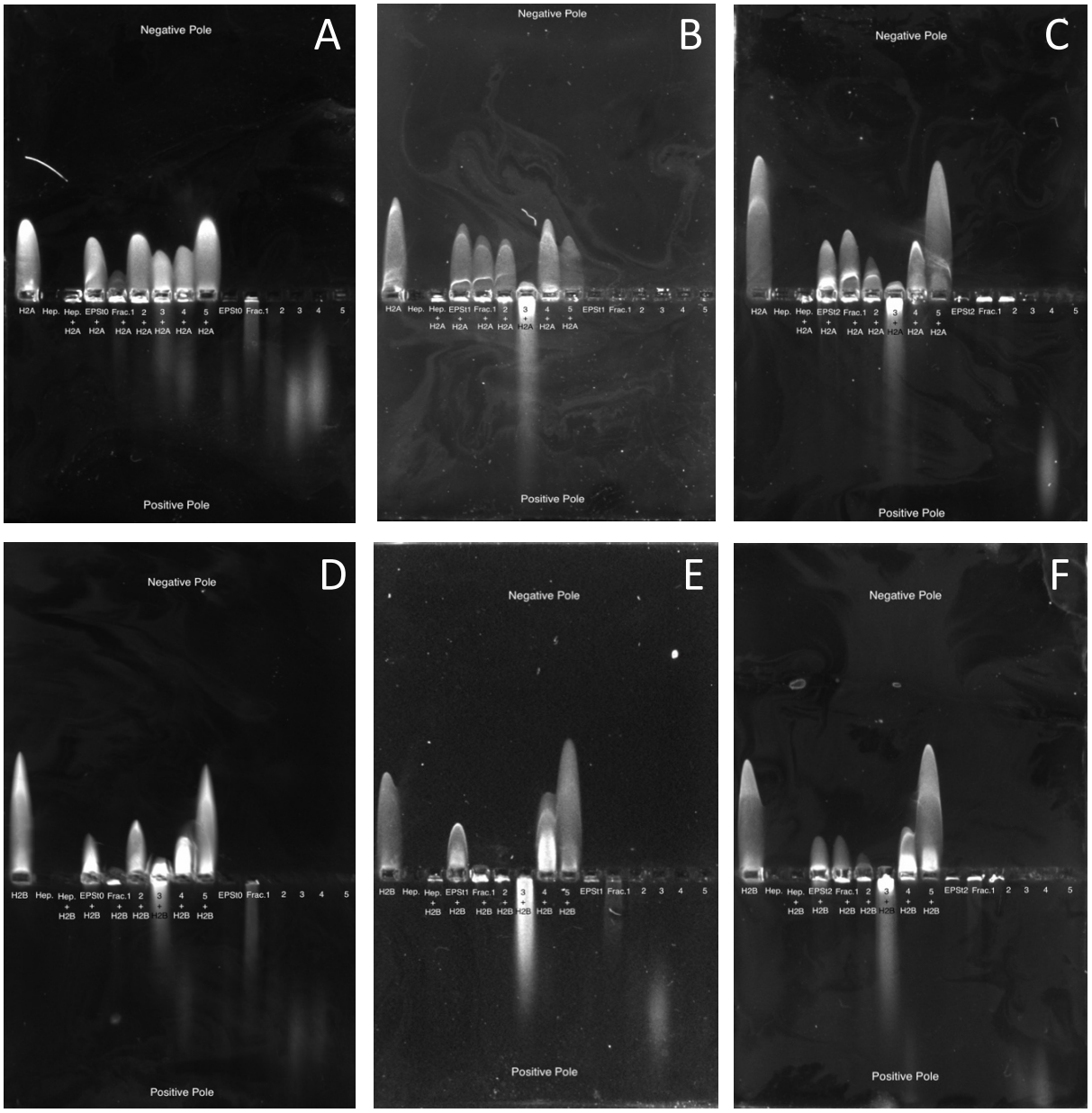

Figure 3.11. Agarose gel-electrophoresis to assess the interaction of histones H_2A and H_2B with extracellular polymeric substances (EPS) and their fractions. Heparin was used as a positive control. Histones were incubated with EPS, fractions, and heparin in 1:2 (w/w). EPS and respective fractions without histone interaction were used as negative control. Cationic histones should migrate to the negative pole and if binding is successful, the migration should be reduced or go to the positive pole. A: H_2A with EPS/fracs. t_0; B: H_2A with EPS/fracs. t_1; C: H_2A with EPS/fracs. t_2; D: H_2B with EPS/fracs. t_0; E: H_2B with EPS/fracs. t_1; F: H_2B with EPS/fracs. t_2. Hep: Heparin; EPS t_0: Freshwater AGS based EPS; EPS t_1: Intermediate seawater-adapted AGS based EPS; EPS t_2: Seawater-adapted AGS based EPS. Frac. (1 – 5): fractions relative to the EPS in question.

Observing the calculated reduction of H_2A migration (fig. 3.12), for EPS t_0, only fraction 1 caused a strong reduction in the migration to the negative pole. Meanwhile, the band migration reduction was slightly stronger in EPS t_1 and, fraction 3 caused a strong shift migration to the positive pole. In the case of EPS t_2, all fractions and the unfractionated polymer caused a strong migration reduction, except for 5 with no reduction. For EPS t_2, interaction with fraction 3 also caused a migration to the positive pole. Considering the interaction with H_2B (fig. 3.13), all EPS and fractions caused a very strong reduction of migration to the negative pole, except for fraction 5, with almost no effect. In most cases, the higher molecular weight fractions had the bigger effect on migration. In EPS t_0, fraction 1 caused the best reduction, while in EPS t_1, fractions 1, 2 and 3 had a very efficient neutralization, with 3 migrating to the positive pole. In EPS t_2, fractions 2 and 3 had the best reduction, with the same shift pattern for fraction 3. Overall, a similar trend was observed in both histones, despite the slightly higher efficiency of H_2B. It is yet unclear the reason behind this difference, although their isoelectric points (pI) differ, in which H_2A and H_2B present pI equal to 10.95 and 9.76 respectively (Galuska et al., 2017). The pI is related to the proteins positive/negative net charge, according to the pH they are inserted in (Novák & Havlíček, 2016). Thus, it is possible that this difference could influence their binding capacity with different EPS fractions.

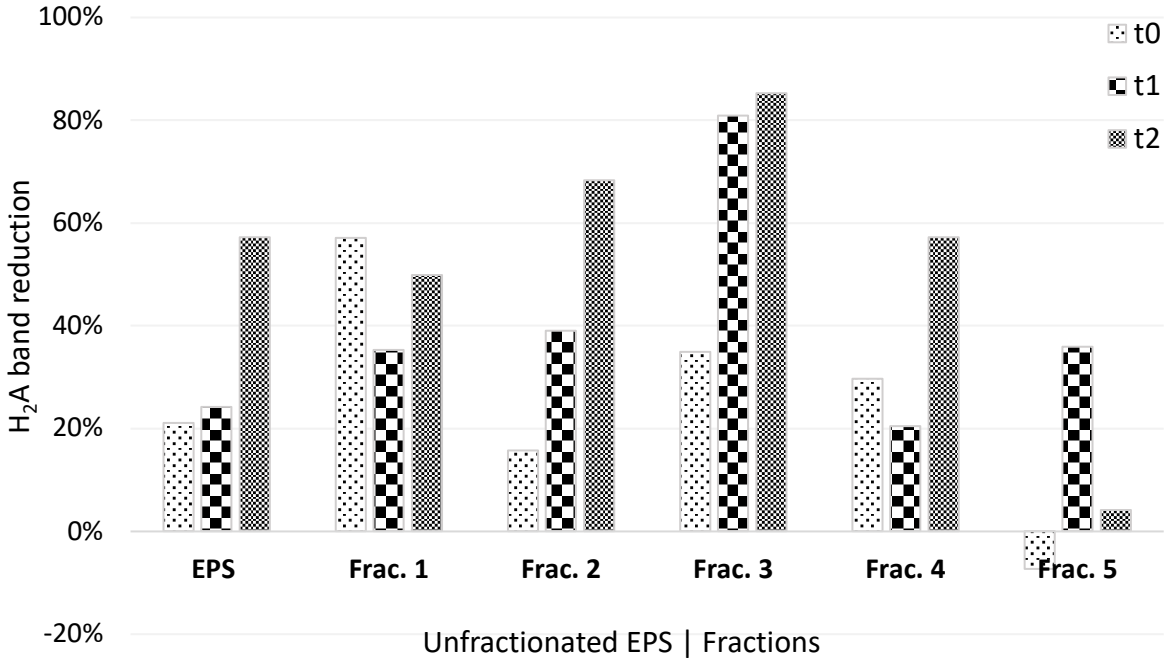

Figure 3.12. Band reduction relative to histone H_2A migration, caused by the interaction with unfractionated EPS and respective fractions. EPS t_0: Freshwater AGS based EPS; EPS t_1: Intermediate seawater-adapted AGS based EPS; EPS t_2: Seawater-adapted AGS based EPS. Frac. (1 – 5): fractions relative to the EPS in question.

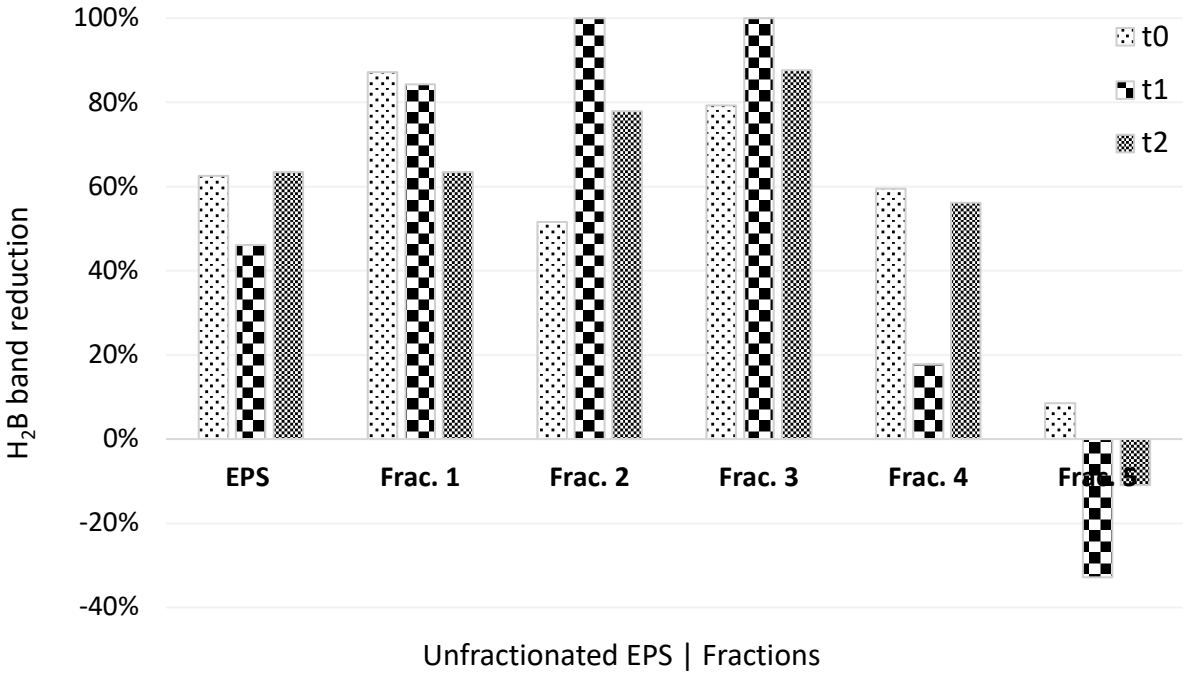

Figure 3.13. Band reduction relative to H_2B migration, caused by the interaction with unfractionated EPS and respective fractions (1 – 5).

Fraction 3 was the most efficient in neutralizing histones, although low sGAGs were measured. From this results, two hypotheses arise: fraction 3 does not contain sulfated sugars, but other type of highly negatively charged glycans; or there are sulfated groups that were underestimated in the assay performed to measure sGAGs. The fact that sialic acid has been found in EPS (Boleij et al., 2020; Danny R. de Graaff et al., 2019) and polysialic acid was proven to have good interaction with the histones H2A and H2B (Zlatina et al., 2017) sustain the assumption that polysialic acid might be present in fraction 3, although this should be further investigated. Moreover, genome analysis on *Candidus accumulibacter* indicated high probability of sialic acid synthesis by this PAO (Danny R. de Graaff et al., 2019; Tomás-Martínez et al., 2021). The second hypothesis arise from the fact that chondroitin sulfate (CS) was used as a standard in the assay; thus, a higher charge density of the sulphated glycan could fall out of the drawn calibration curve, resulting in a poor representation of the sulfated sugars content in fraction 3.

Considering the promising results obtained with the histone binding, in a 1:2 (w/w) ratio of histone to EPS, further investigation was done into the necessary amount of EPS to obtain a neutralization as efficient as with heparin. To this end, ratios of 1:3 and 1:4 were tested for the whole EPS t_2 and its fractions 1, 2 and 3. Ratios of 1:0.5; 1:1 and 1:2 for heparin were also performed to study the efficiency with lower ratios (figure 3.14).

From this study, it can be observed that all ratios applied blocked the migration to the negative pole, both for EPS and heparin. A ratio of 1:3 is already efficient in neutralizing histone H_2B with the unfractionated EPS t_2 and fractions 1, 2 and 3. In the case of heparin, a ratio of 1:0.5 already neutralized histones. Thus, based on the present results, charge density of seawater-adapted EPS would be equivalent to approximately 0.167 of heparin (w/w) regarding the neutralization of histone H_2B. Thus, the EPS t_2 can be compared to the heparin efficiency of binding histone H_2B. This indicates the potential application of high molecular weight EPS from seawater-adapted AGS in the treatment for sepsis.

Figure 3.14. Agarose gel-electrophoresis to assess the interaction of histone H_2B with seawater-adapted AGS based EPS and fractions 1, 2 and 3. Ratios of 1:3 and 1:4 (w/w) were tested for the whole EPS t2 and its fractions 1, 2 and 3. Ratios of histone to heparin were 1:0.5; 1:1 and 1:2 (w/w). Ratio of histones to EPS/fractions were 1:4 and 1:5 (w/w). The cationic histone should migrate to the negative pole and if binding is successful, the migration should be reduced or go to the positive pole. EPS t_2: Seawater-adapted AGS based EPS.

Ratios of histone to EPS lower than 1:3 could be further investigated. Moreover, according to Kitzis et al., 1976, a ratio of 1:0.25 of heparin is already enough for binding histone H_2B. Therefore, further studies with smaller ratios of EPS and heparin could be tested to obtain a more exact EPS/heparin equivalent.

The fact that fractions 1 and 2 present a higher N-GAG content than O- and their histone binding capacity comparable to heparin, might indicate the presence of heparin-like molecules in the higher molecular weight fractions of EPS. GAGs, including heparan sulfate/ heparin-like, were found in AGS (Felz et al., 2020). Research on EPS of anammox granular sludge pointed to a high chance of annamox bacteria to produce heparan sulfate (Boleij et al., 2020). Nevertheless, this hypothesis should be further investigated.

3.4 SEC is a suitable method to enrich EPS for anionic properties

Besides revealing the fingerprint, the molecular weight distribution and enabling a better characterization of EPS (Bhatia et al., 2013), SEC with a column MW range up to the order of Mega Daltons appears to be a prominent technique to enrich the EPS for anionic properties.

In this study, five fractions with different properties and characteristics were obtained through SEC. A change in the physical characteristics was observed from the small to high molecular weight. In general, the unfractionated EPS had a lower sGAG content and a less effective histone binding capacity than the higher molecular weight fractions, indicating enrichment in anionic property in these fractions.

Commonly, the molecular weight range used for SEC of EPS is up to kilo Daltons (Bhatia et al., 2013; Garnier et al., 2006; Simon et al., 2009), although Roca et al., 2016 determined the average MW of exopolysaccharides with a 10 MDa cut off. The fact that enriched anionic properties were found in higher molecular weight fractions, which represent 58% of the fractionated EPS, suggests that most of the previous methods implemented to fractionate EPS were excluding this very interesting portion of the EPS. Through a SEC with a molecular weight range of 5×10^{-3} to 5 MDa, the enriched fractions obtained from EPS extracted from seawater AGS can be further studied and analyzed and their anionic characteristic can be more effectively applied, for histone neutralization, for example. Therefore, SEC with a high molecular weight separation range appears to be a good method to purify and enrich EPS for its anionic properties.

4

CONCLUSION

The objective of the present thesis was to obtain a better comprehension on the anionic properties along the apparent molecular weight (aMW) distribution of extracellular polymeric substances (EPS) extracted from seawater-adapted aerobic granular sludge (AGS), focusing on the sulfated groups and the binding capacity with cationic histones. Moreover, the understanding of the seawater impact on the EPS charge was within this study scope. These elucidations aimed at exploring the future possibility of these negatively charged biopolymers being applied in the pharmaceutical industry, more specifically for the possible treatment of sepsis.

The AGS was successfully cultivated in seawater conditions, obtaining a polyphosphate accumulating organisms (PAO) dominant culture and EPS production. Alkaline and heat extraction was performed. The implementation of size exclusion chromatography (SEC) with a column separation range up to 5 MDa, enabled the enrichment of seawater-adapted EPS for negatively charged groups in the higher molecular weight fractions, representing 58% of the fractionated EPS. The aMW of EPS was supposed to be higher than 2 000 kDa and based on extrapolation of the calibration standards, 11 350 kDa in the highest MW fraction. The extracts were fractionated in five fractions, being fraction 1 the higher MW and 5 the smallest. The fourier transform infrared (FTIR) spectra showed a sugar predominance in fractions 1 and 2, while 3 and 4 were protein dominant and possible phosphate groups present in fraction 5.

The assay performed with 1,9- dimethyl methylene blue (DMMB) to identify sulfated glycosaminoglycans (sGAG) indicated the possible presence of sulfated groups in EPS. The sGAG detected was higher in extracts from seawater-adapted AGS than in EPS recovered from freshwater AGS. Moreover, not only these anionic glycans were more concentrated in the two higher molecular weight fractions (up to 115% greater than unfractionated EPS), but also the N-sulfated glycosaminoglycan content (related to more negatively charged molecules) was higher than O-sGAG, suggesting the enrichment of negatively charged groups in these fractions. The FTIR spectra showed a sugar predominance in the same two fractions, which supports the supposed presence of glycosaminoglycans. Nevertheless, the used assay is based on the binding of DMMB with polyanionic substances, not being fully specific to sulfated polymers. Thus, different methodologies to sustain this result should be implemented.

To explore the possibility of applying these anionic properties as a future treatment for sepsis, a lethal clinical condition that causes the release of histones, the neutralization of these cationic proteins by EPS was tested**.** The unfractionated EPS recovered from seawater-adapted AGS and the enriched fractions could effectively bind histones. Its binding capacity seemed higher than EPS from freshwater AGS. Based on the results obtained, the charge density of seawater-adapted EPS would be equivalent to about 0.167 of heparin (w/w) regarding the neutralization of histone H_2B. In addition, in agreement with DMMB and FTIR results, the higher molecular weight fractions had the most effective binding capacity. Thus, pointing to the potential of these anionic polymers in future research for the treatment of sepsis.

To study the effect of seawater in the EPS negative charge, granules were collected in three different time slots: before the introduction of seawater (t_0), at an intermediate point of seawater adaptation, before one sludge retention time (SRT) of 12 days (t_1), and 35 g/L seawater adapted granules (t_2). Extraction and analysis were performed, and results indicated that the EPS anionic properties change under seawater conditions. Moreover, complete renewal of the biomass seems not to be necessary to obtain different anionic properties in EPS, since changes in the negative charge properties of EPS t_1 were already observed from EPS t_0. The aMW chromatographic profile changed progressively from t_0 to t_1 and t_2. In addition, the DMMB assay and histone binding tests showed a gradual enhance of the anionic properties of EPS with the increasing time exposure of AGS to seawater conditions.

5

OUTLOOK

Considering that a six-month research period is not enough to explore all possible approaches, as well as the limitations of the methods implemented, future work could explore deeper and distinct aspects of the present thesis.

- Other isolation methods could be explored to understand their influence in the anionic composition of EPS. For example, acid precipitation is successfully used in Kaumera isolation process, achieving high yields. It was observed in our laboratory that EPS extracted from seawater-adapted AGS tends to not precipitate in acidic conditions. Thus, different pH values of acidic precipitation could be investigated and compared to the dialysis purification implemented, to have a better understanding of the isolation effect on the EPS anionic composition and yield.
- The cause of the higher extraction yield obtained for EPS t_2 could be further investigated by performing again the extraction procedure with the same granules. Doing it in duplicate could give a more accurate yield.
- The extrapolation of the EPS aMW could be improved by using more and larger proteins size for the calibration curve and by running them separately into the size exclusion chromatography column. For example, Roca et al., 2016 used twelve different pullulan standards between 342 Da and 2.56 MDa to perform the calibration.
- To support the indication of sulfated glycosaminoglycan (sGAG) in EPS, additional methodologies could be applied. The staining with Coomassie blue and Alcian blue could enable the differentiation between the relatively weaker acidic groups such as carboxylate and the stronger acidic groups like sulfate. In addition, the presence of heparin-like substances could be further investigated. The staining of granules with heparin red could identify negatively charged macromolecules such as heparin. Moreover, analysis of pathways for syntheses of sulfated glycosaminoglycans could be implemented (Boleij et al., 2020).
- The fact that low amount of sGAG was identified in fraction 3 while it was the most effective at binding histones, raised the hypothesis of another type of negatively charged molecule present, such as polysialic acid, that was reported to neutralize histones (Zlatina et al., 2017). Thus, the presence of different highly anionic substances could be further explored. The sialic acid quantification kit based on the release of N-acetylneuraminic acid by enzymatic treatment could be implemented (Boleij et al., 2020; Danny R. de Graaff et al., 2019).
- The accuracy of the value obtained for the EPS equivalence to heparin (0,167 (w/w)) could be improved through the testing of different EPS/ heparin over histone ratios.

6

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A

COMPLEMENTARY RESULTS

A.1 Reactor Operation

A bubble column reactor was operated under aerobic and anaerobic cycles. The reactor was inoculated with freshwater-adapted aerobic granular sludge (AGS). The seawater concentration started at 10 g/L and was gradually increased until 35 g/L seawater. To understand the seawater influence on the anionic properties of extracellular polymeric substances (EPS) extracted from AGS, granules were collected in three different time slots: t_0, t_1 and t_2. The sample t_0 refers to the inoculum, grown in freshwater. The second sample, t_1, was collected 5 days after the seawater concentration achieved 35 g/L (before the start of SRT control), representing the granules in an intermediate stage. The sample t_2 was taken 17 days from the start of 35 g/L seawater (12 days from the start of the 12 days SRT control), representing seawater-adapted granules. Good granulation was achieved and no significant difference in granule morphology was observed between the three samples (figure A.1).

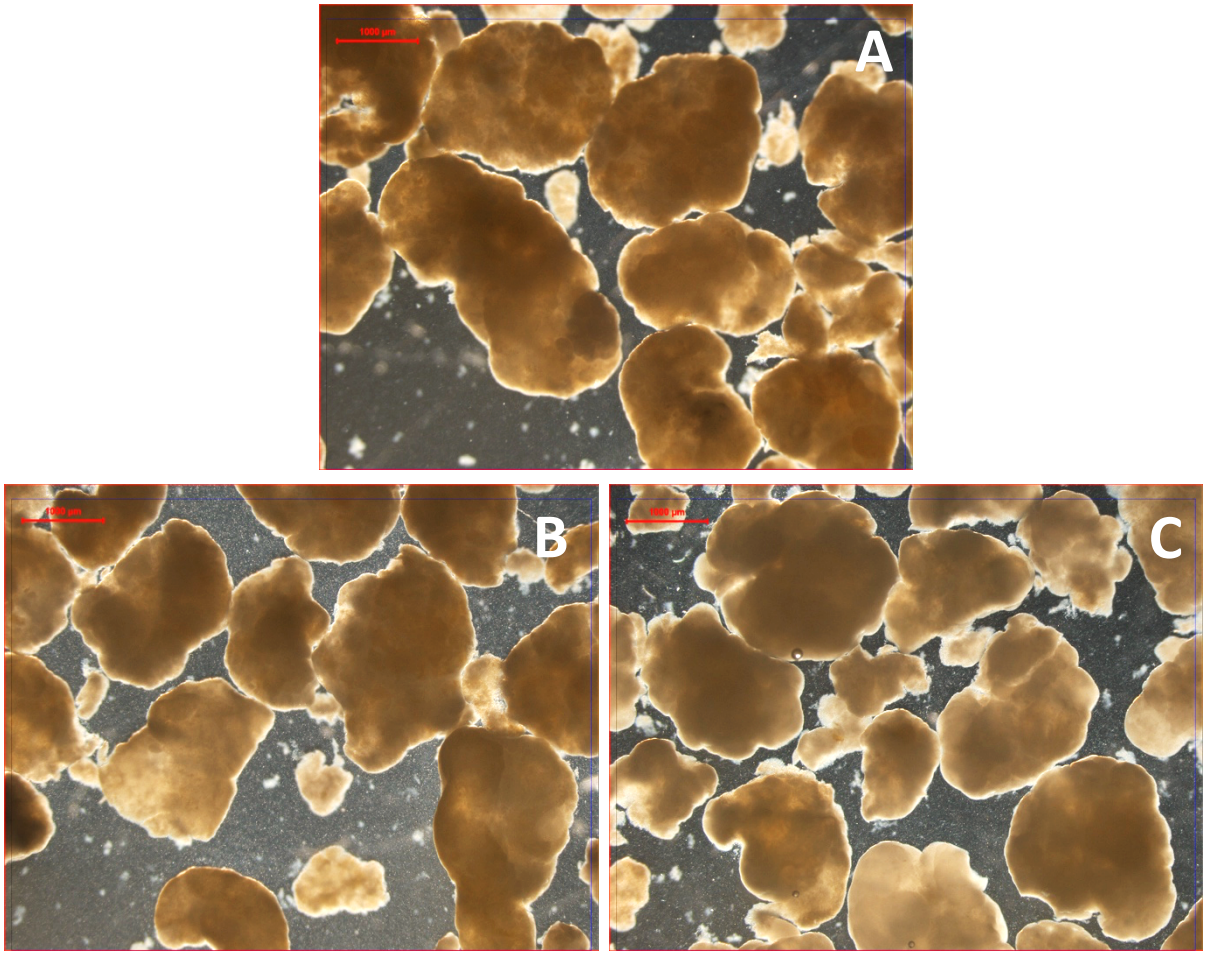

Figure A. 1. Image analyzer picture of aerobic granular sludge (AGS). Scale bar equals to 1 000 µm. A: Freshwater AGS (t_0); B: Intermediate seawater-adapted AGS (t_1); C: Seawater-adapted AGS (t_2).

A.2 Extracellular Polymeric Substances Extraction

Alkaline (NaOH) and heat (80 °C) extraction and further dialysis were performed with EPS t_0, t_1 and t_2, aiming to recover as much acidic polymers as possible. During the extraction, the color of solubilized EPS t_2 turned red (figure A.2), turning back to yellow during dialysis (figure A.3), while EPS t_0, t_1 were yellow in the extraction solution.

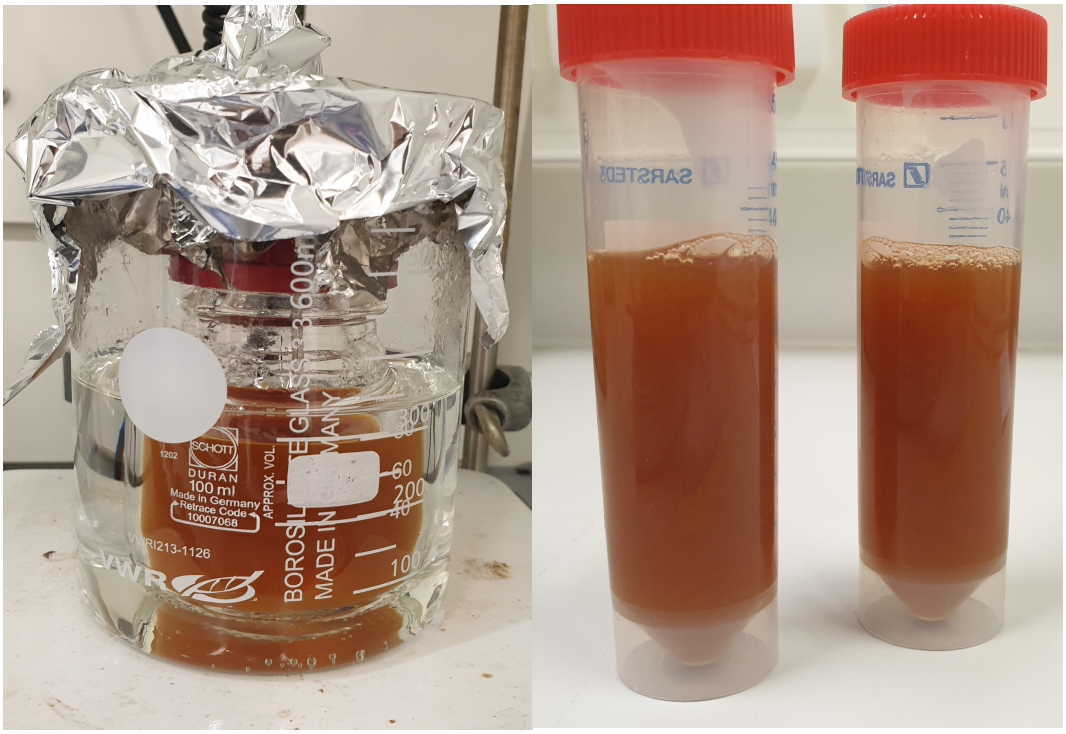

Figure A. 2. Alkaline and heat extraction of extracellular polymeric substances from seawater-adapted aerobic granular sludge.

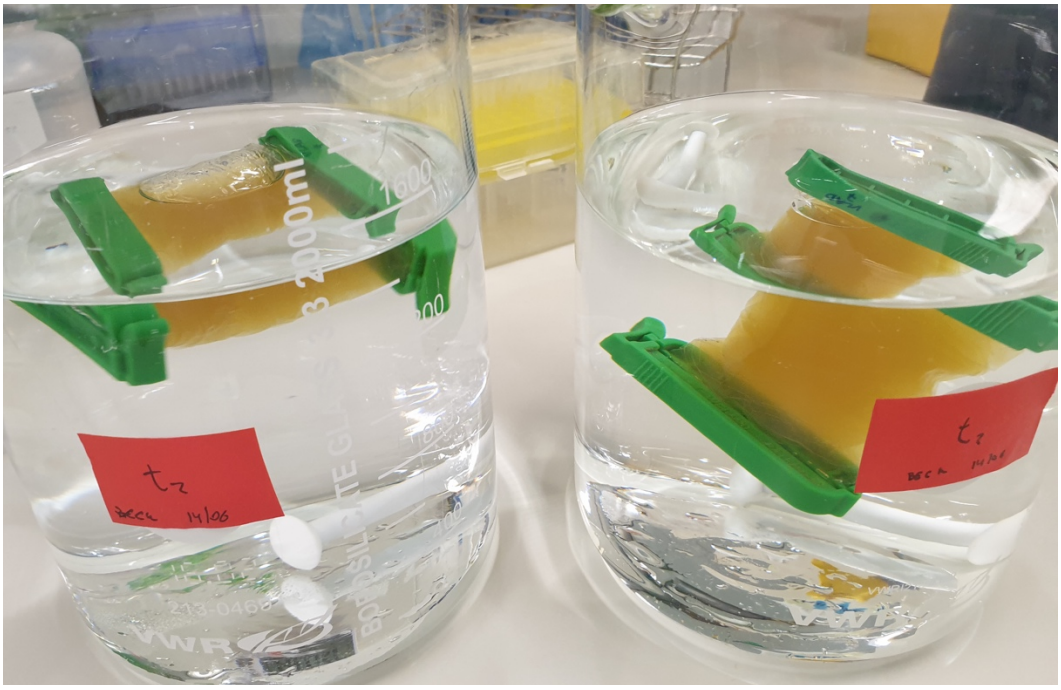

Figure A. 3. Dialysis of extracted extracellular polymeric substances from seawater-adapted aerobic granular sludge.

A.3 Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) was implemented to determine sludge extracted EPS apparent molecular weight (aMW) distribution as well as to separate it into fractions. The SEC enabled the separation of EPS in five fractions, in which fraction 1 has the highest aMW and 5 the smallest. The chromatographic peaks obtained through SEC give us the EPS fingerprint. The area calculated under each fraction's peak is listed in table A.1.

Table A. 1. Area calculated under each peak of fractions obtained through size exclusion chromatography (SEC) of extracellylar polymeric substances (EPS) extracted from aerobic granular sludhe (AGS). EPS t_0: Freshwater AGS based EPS; EPS t_1: Intermediate seawater-adapted AGS based EPS; EPS t_2: Seawater-adapted AGS based EPS. Fractions (1 – 5): fractions relative to the EPS in question.

	Area (10^3 mL mAU)		
Fraction	EPS t_0	EPS t_1	EPS t_2
1	1.90	1.19	1.41
2	0.36	0.47	0.68
3	0.12	0.92	0.95
4	1.47	1.79	2.37
5	7.27	6.18	7.50
Total	11.11	10.54	12.90

For the calibration curve (figure A.4), a high MW protein standard kit was used (range 43 to 669 kDa) with an additional polysaccharide Blue Dextran (2 000 kDa). The chromatogram of proteins standards solution and of blue dextran are represented in figures A.5 and A.6.

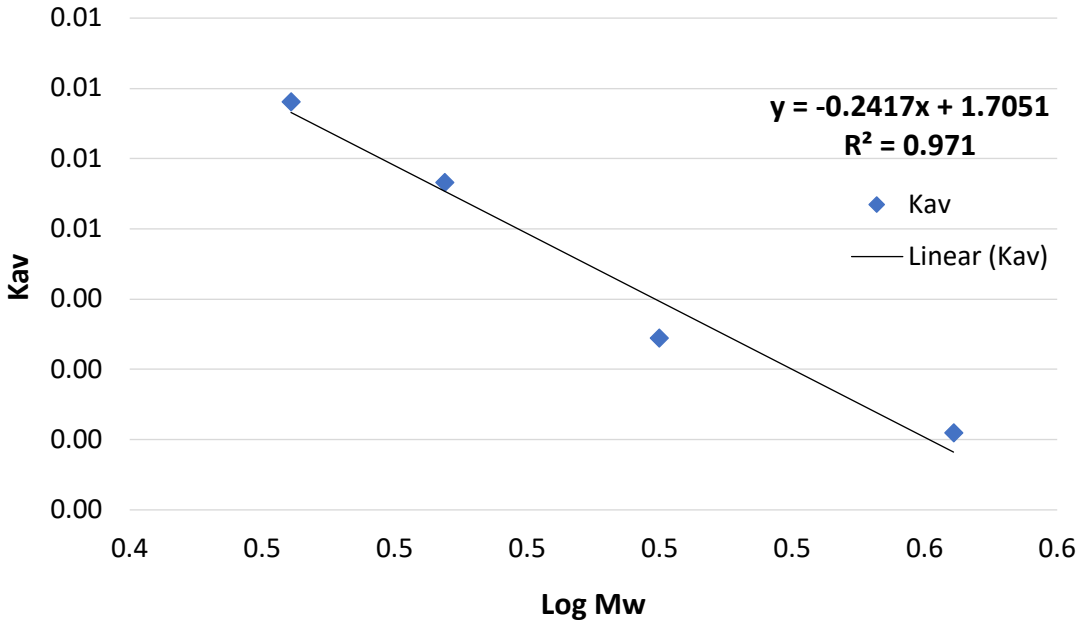

Figure A. 4. Apparent molecular weight calibration curve for size exclusion chromatography, with six protein standards.

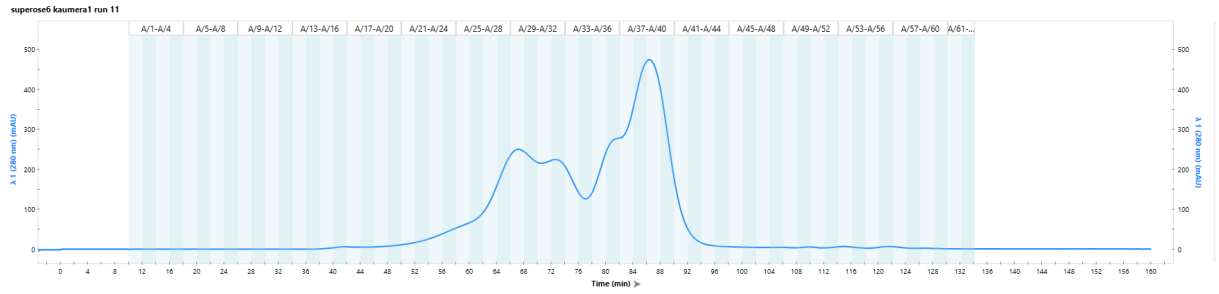

Figure A. 5. Chromatogram of proteins standards solution with molecular range from 43 to 669 kDa: Ovalbumin - 43 kDa; Conalbumin - 75 kDa; Aldolase - 158 kDa; Ferritin - 440 kDa; Thyroglobulin - 669 kDa.

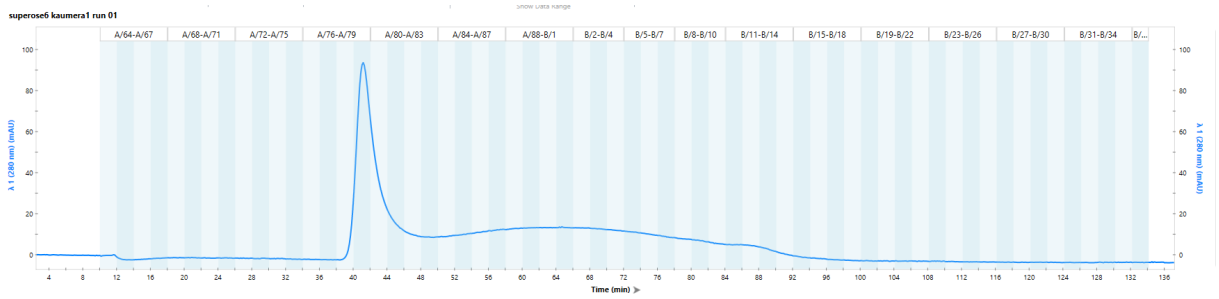

Figure A. 6. Chromatogram of Blue dextran – 2 000 kDa.

A.4 Fourier Transform Infrared (FTIR)

The fourier transform infrared (FTIR) was used to obtain a better comprehension of the molecular fingerprint of the different EPS samples (t_0, t_1 and t_2) and their fractions. The FTIR spectra of EPS t_0 and t_1 fractions are represented in figures A.7 and A.8. Other complementary FTIR spectra are illustrated in figures A.9 to A.18.

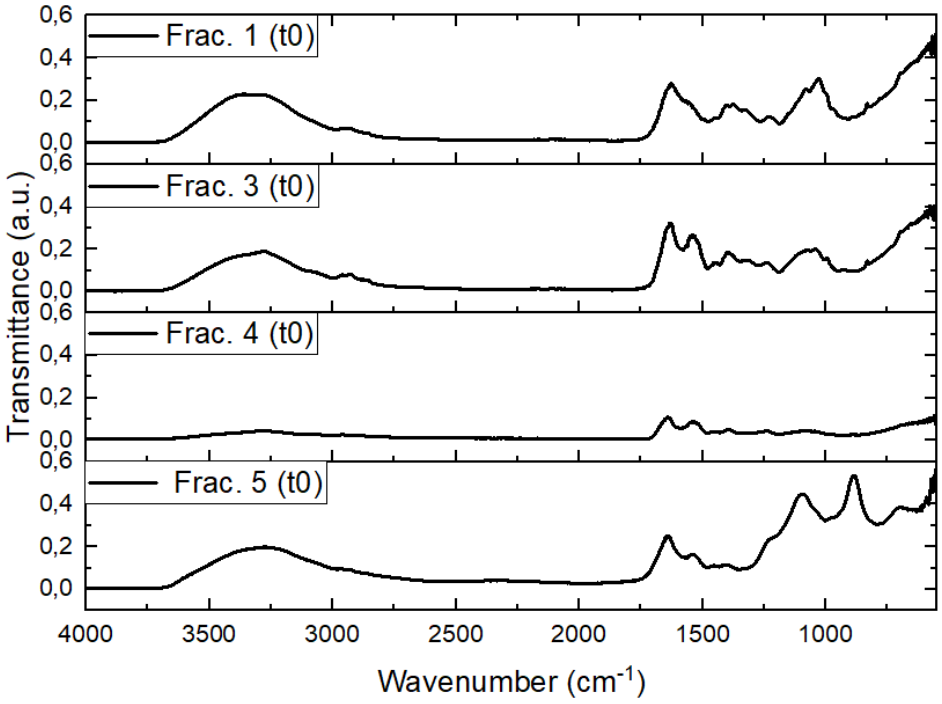

Figure A. 7. Fourier transform infrared (FTIR) spectra of freshwater-adapted AGS based EPS (t_0) fracns (1 – 5); the amount obtained from fraction 2 had not enough for the FTIR measurement.

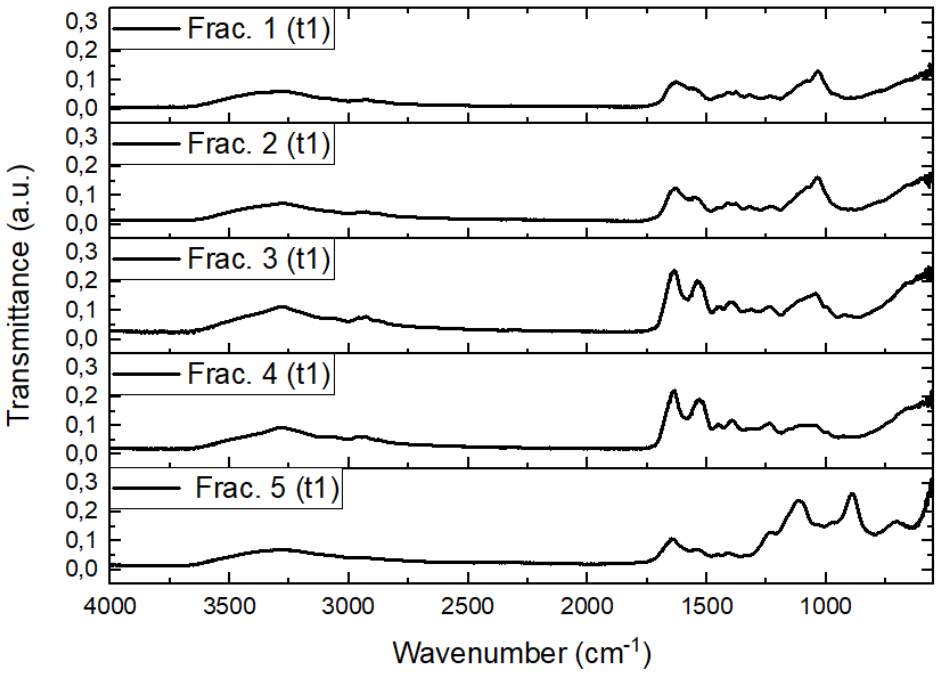

Figure A. 8. Fourier transform infrared (FTIR) spectra of intermediate seawater-adapted AGS based EPS (t_1) fractions (1 – 5).

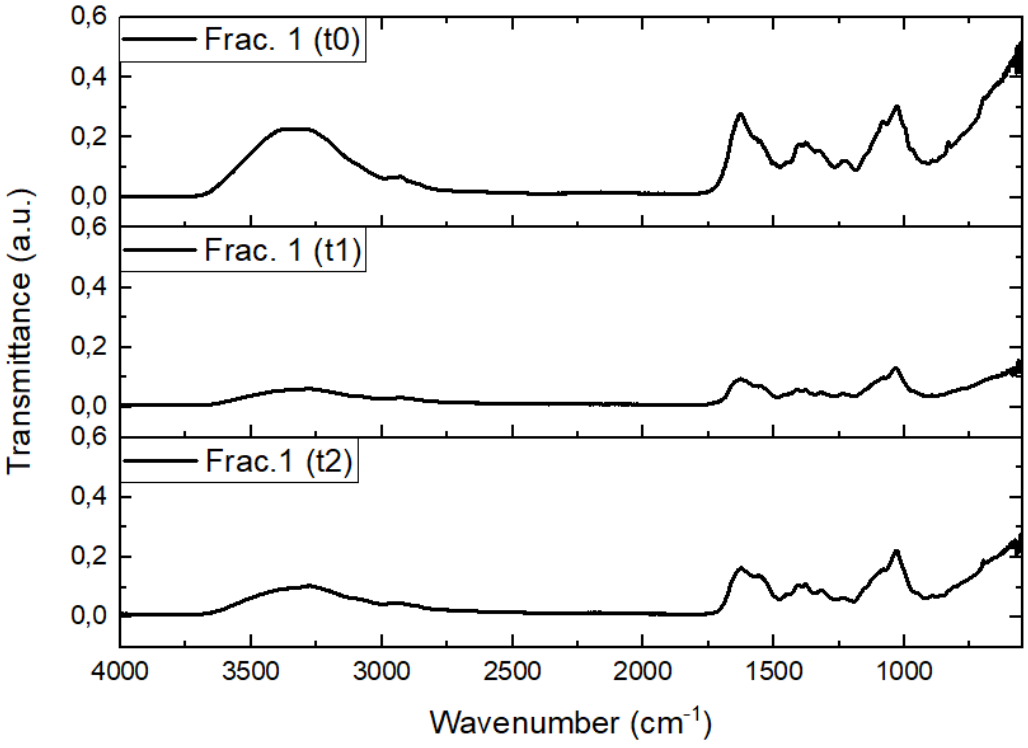

Figure A. 9. Fourier transform infrared (FTIR) spectra of fraction 1 from extracellular polymeric substances (EPS). t_0: Freshwater AGS based EPS; t_1: Intermediate seawater-adapted AGS based EPS; t_2: Seawater-adapted AGS based EPS.

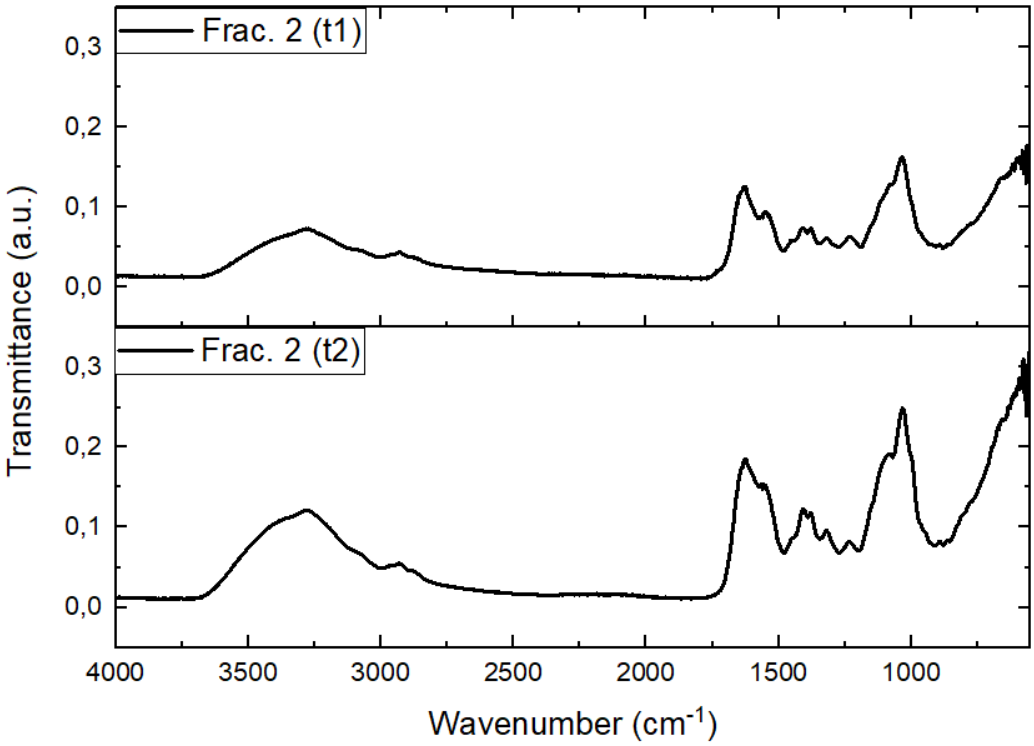

Figure A. 10. FTIR spectra of fraction from extracellular polymeric substances (EPS). T_1: Intermediate seawater-adapted AGS based EPS; t_2: Seawater-adapted AGS based EPS.

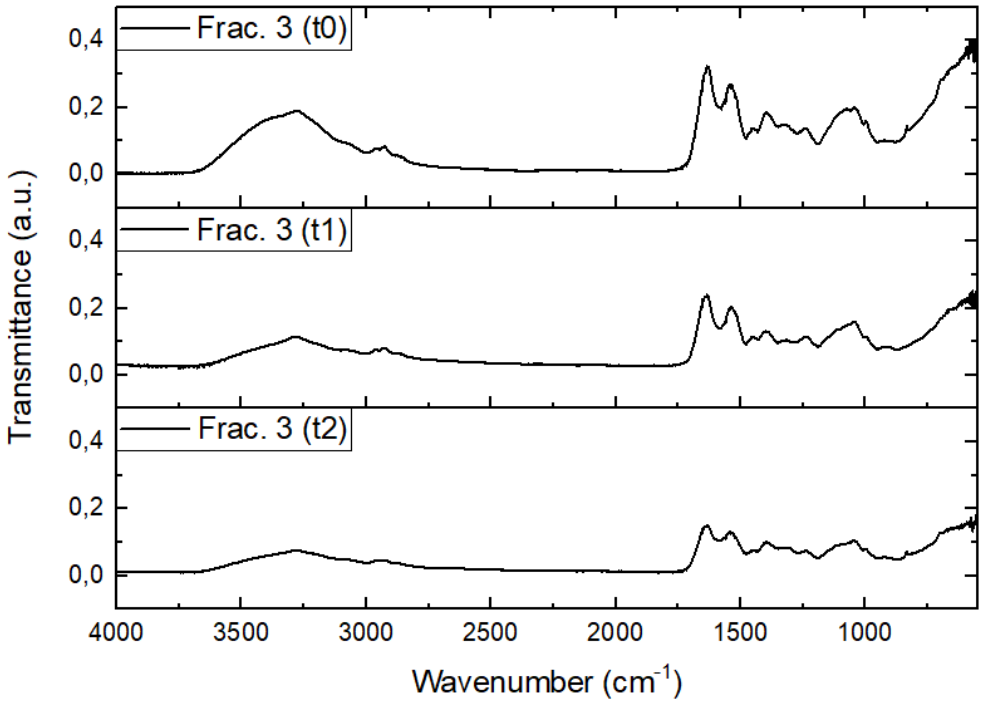

Figure A. 11. FTIR spectra of fraction 3 from extracellular polymeric substances (EPS). t_0: Freshwater AGS based EPS; t_1: Intermediate seawater-adapted AGS based EPS; t_2: Seawater-adapted AGS based EPS.

A.5 1,9- dimethyl methylene blue (DMMD) Assay

The 1,9- dimethyl methylene blue (DMMB) Assay was implemented to quantify sulfated glycosaminoglycans (sGAG) in EPS t_0, t_1 and t_2 and their fractions. The Assay is based on the binding of the dye to polyanionic substances. The O- and N-sulfated ratios of EPS t_0 and t_2 are represented in figures A.19 and A.20. With the attempt to verify if the longer papain extraction would enable a higher detection of polyanions, a 18 h long extraction was performed with the three unfractionated EPS (fig. A.21).

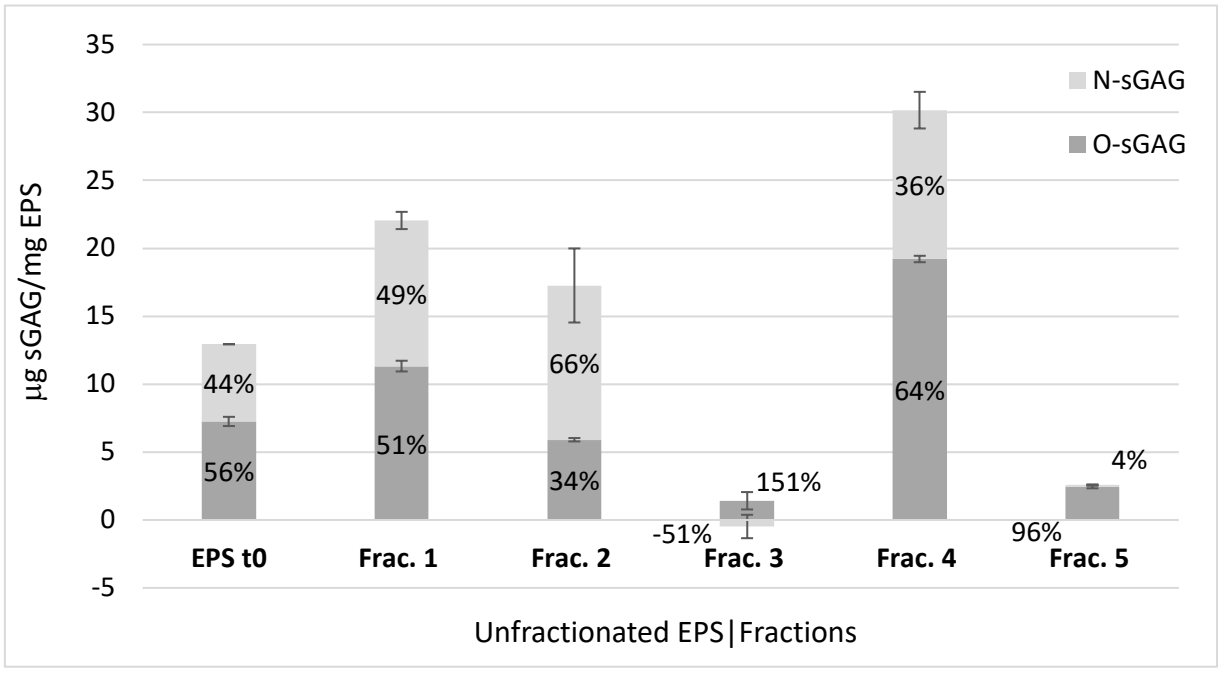

Figure A. 12. O- and N- sulfated glycosaminoglycans in freshwater-adapted AGS based EPS (t_0) and its fractions (1 – 5).

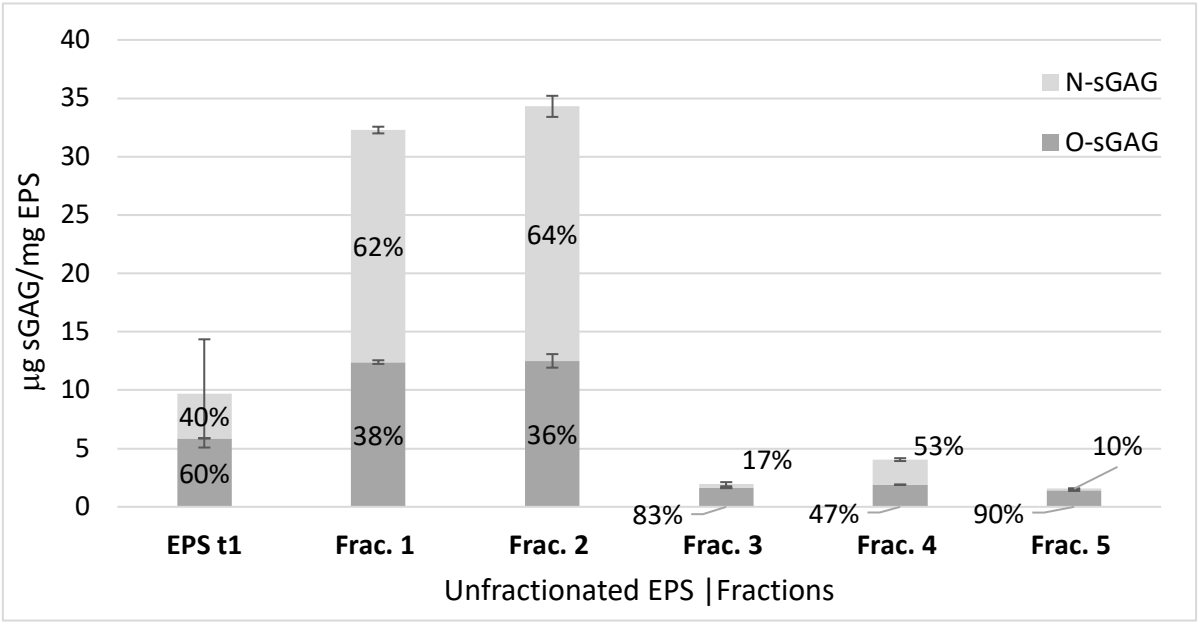

Figure A. 13. O- and N- sulfated glycosaminoglycans in intermediate seawater-adapted AGS based EPS (t_1) and its fractions (1 – 5).

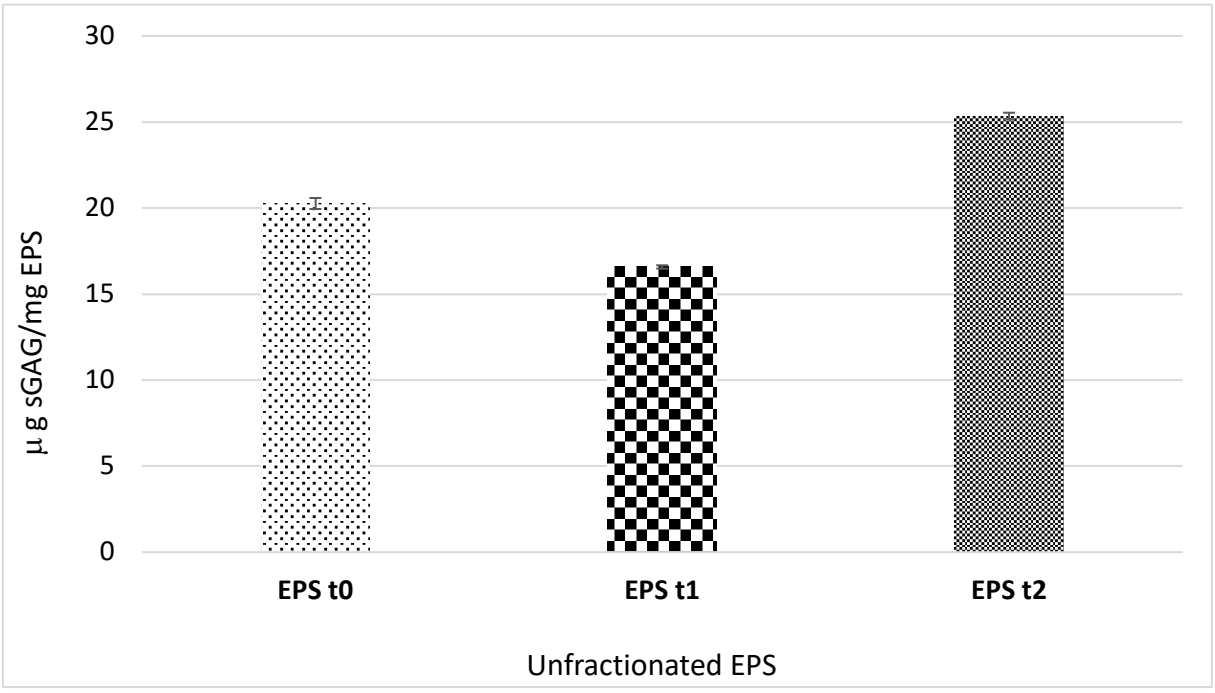

Figure A. 14. Total sulfated glycosaminoglycans measured after 18 h papain digestion in AGS based EPS. EPS t_0: Freshwater AGS based EPS; EPS t_1: Intermediate seawater-adapted AGS based EPS; EPS t_2: Seawater-adapted AGS based EPS.

A.6 Agarose Gel-Electrophoresis

Initially H_1, H_2A and H_2B were tested with the whole extracted EPS t_0, t_1 and t_2. Histone H1 seemed to have the less efficient interaction with EPS (figure A.22), therefore only H_2A and H_2B were further tested with the five fractions obtained from each EPS.

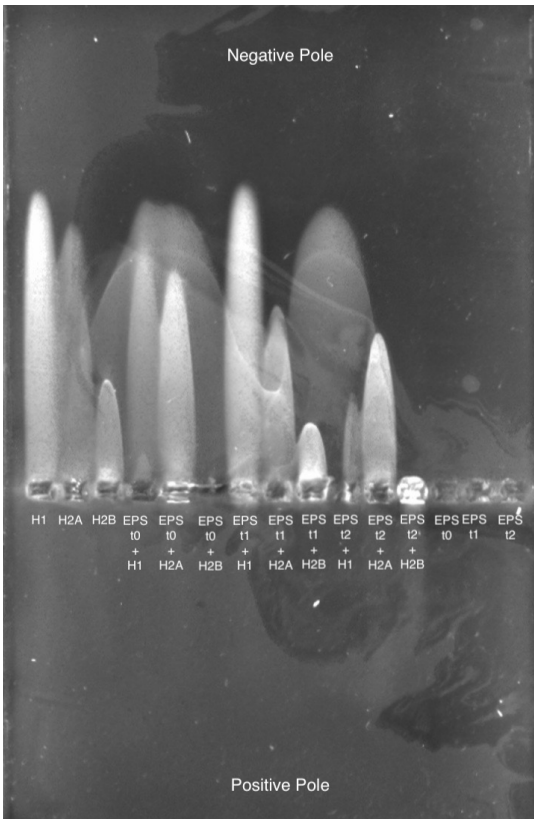

Figure A. 15. Agarose gel-electrophoresis to assess the interaction of histone H_1, H_2A and H_2B with AGS based EPS. Histones were incubated with EPS, fractions, and heparin in 1:2 (w/w). EPS without histone interaction were used as negative control. Cationic histones should migrate to the negative pole and if binding is successful, the migration should be reduced or go to the positive pole. EPS t_0: Freshwater AGS based EPS; EPS t_1: Intermediate seawater-adapted AGS based EPS; EPS t_2: Seawater-adapted AGS based EPS.