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Abdo Bou Sarkis

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# THÈSE

## En vue de l'obtention du DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par l'Université Toulouse 3 - Paul Sabatier

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Présentée et soutenue par  
**ABDO BOU SARKIS**

Le 1 juin 2023

**Caractérisation des exopolymères de granules aérobies  
épuratifs : vers leur valorisation en tant que matériaux  
biosourcés issus du traitement des eaux usées**

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## List of communications

### Publications:

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Detection of Gel-Forming Polymers via Calcium Crosslinking, Applied to the Screening of Extracellular Polymeric Substances Extracted from Biological Aggregates. Gels (9) 157. 2023.

Benedetta Pagliaccia, Sidonie Durieux, Yolaine Bessiere, Mansour Bounouba, **Abdo Bou-Sarkis**, Elisabeth Girbal-Neuhauser, Emiliano Carretti, Claudio Lubello, Tommaso Lotti, Etienne Paul. Insights on the hydrogel-forming ability and post-gelling mechanical properties of structural extracellular polymeric substances (sEPS) from aerobic granular sludge (AGS): A comparison with model biopolymers. Journal of Water Process Engineering (49) 103076. 2022.

**Abdo Bou-Sarkis**, Benedetta Pagliaccia, Audrey Ric, Nicolas Derlon, Etienne Paul, Yolaine Bessiere, Elisabeth Girbal-Neuhauser. Effects of alkaline solvents and heating temperatures on the solubilization and degradation of gel-forming Extracellular Polymeric Substances (EPS) extracted from aerobic granular sludge. Biochemical engineering (185), 108500. 2022.

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Durieux, S., Simon, A., Chiramberro, N., **Bou-Sarkis, A.**, Pagliaccia, B., Dubos, S., Bounouba, M., Moya, E., Neuhauser, E., Bessièrè, Y. and Paul, E., 2021. Granular biofilm for the production of exopolymer substances". IWA Biofilm Reactors Conference (Notre Dame, USA (Online)).

Durieux, S., Simon, A., Dubos, S., Bounouba, M., Moya-Leclair, E., Pagliaccia, B., **Bou-Sarkis, A.**, Neuhauser, E., Bessièrè, Y., Paul, E. Evaluation of the EPS production in non-nitrifying reactor: study of the whole production chain. 13th European Congress of Chemical Engineering and 6th European Congress of Applied Biotechnology, virtual conference, September 2021, oral communication (Online).

Pagliaccia B., Durieux S., Bessiere Y., Bounouba M., **Bou-Sarkis A.**, Girbal-Neuhauser E., Lubello C., Lotti T., Paul E. Post-gelling mechanical behaviour of structural extracellular polymeric substances (EPS) from aerobic granules (AGS). 4th IWA resource recovery conference, Turkey, September 2021, oral communication (Online).

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## **List of abbreviations**

aa: Amino acids

ACaS: Cumulated Area of the Spectrum with calcium

ADA: Alginate dialdehyde

AGS: Aerobic Granular Sludge

AIS: Cumulated Area of the Initial Spectrum without calcium

ALE: Alginate Like Exopolysaccharides

ALV: Alginate Low Viscosity

AOB: Ammonium Oxidizing Bacteria

AS: Activated Sludge

AsFIFFF: Asymmetrical Flow Field Flow Fractionation

AU: Absorbance Units

BG: Biochemical Group

BSA: Bovine Serum Albumin

Cat/Neut: Cationic/Neutral

CER: Cationic Exchange Resin

CLSM: Confocal Laser Scanning Microscopy

COD: Chemical Oxygen Demand

Deni: Denitrifiers

DNA: Deoxyribonucleic Acid

DRIFT: Diffuse Reflectance Infrared Fourier Transform

E: Young's modulus

EPS: Extracellular Polymeric Substances

FTIR: Fourier Transform Infrared

G: Guluronic acid

G': Loss modulus

GAGs: Glycosaminoglycans

GAO: Glycogen Accumulating Organisms

GEL: Gelatin

GF: Gelling Factor

GG: Polyguluronic

GI: Gelling Index  
HAC: High Anionic Charge  
HMW: High Molecular Weights  
Ip: Isoelectric point  
LAC: Low Anionic Charge  
LMW: Low Molecular weights  
LS: Light Scattering signals  
M: Mannuronic acid  
m: Mass  
MAC: Medium Anionic Charge  
MALS: Multi Angle Light Scattering  
MG: Heteropolymeric  
MLSS: Mixed Liquor Suspended Solids  
MM: Polymannuronic  
MMW: Medium Molecular weights  
 $M_n$ : Number average molar mass  
MS: Mass spectrometry  
MW: Apparent molecular weight  
 $M_w$ : Weight average molar Mass  
NOB: Nitrite Oxidizing Bacteria  
OHO: Ordinary Heterotrophic Organisms  
P1: Pellet after alkaline extraction  
P2: Freeze-dried EPS after acidic precipitation and dialysis  
PAO: Phosphate Accumulating Organisms  
PN: Proteins  
PolyP: Polyphosphates  
PS: Polysaccharides  
r: Radius  
R: Reactivity  
 $R_g$ : Gyration radius  
S'1: Supernatant after acidic precipitation

S1: Supernatant after alkaline extraction

S2: Freeze-dried EPS after dialysis resuspended in sodium hydroxide

SEC: Size Exclusion Chromatography

SEM: Scanning Electron Microscopy

sEPS: Structural Extracellular Polymeric Substances

TS: Total Solids

UV-vis: Ultraviolet-visible

V<sub>g</sub>: Gel volume

VHAC: Very High Anionic Charge

V<sub>s</sub>: Liquid volume after calcium addition (supernatant after centrifugation)

VS: Volatile solids

V<sub>t</sub>: Initial total volume

WRRF: Wastewater Resource Recovery Facilities

WWTP: Wastewater Treatment Plants

$\epsilon$ : Strain

$\rho$ : Density of the solvent

$\rho_p$ : Density of the particle

$\sigma$ : Stress

$\omega$ : Angular force

$\phi$ : Centrifugal force

## Résumé

Les granules aérobies épuratifs sont des biofilms sphériques et denses, composés de bactéries entourées par une matrice de substances extracellulaires polymériques (EPS). L'utilisation de ces biofilms pour le traitement des eaux usées est en plein essor en raison de leur efficacité à traiter les polluants tout en étant plus compacts et moins consommateurs en énergie que les procédés à boues activées. Leur valorisation à travers l'extraction de différents sous-produits à haute valeur ajoutée représente un enjeu économique et sociétal pour les années à venir. La production de biopolymères gélifiants récupérés à partir des EPS de la matrice granulaire est une voie prometteuse qui a été étudiée dans ce travail de thèse.

Les EPS sont biochimiquement très diversifiés car composés d'un mélange de polysaccharides, de protéines, de lipides, d'acides humiques et d'ADN. Ils jouent un rôle important dans la structuration du biofilm granulaire et contribuent de façon essentielle au caractère dense et très cohésif de la matrice extracellulaire. De ce fait, la méthode d'extraction des EPS gélifiants qui a été adoptée repose sur une solubilisation alcaline à température élevée suivie d'une étape de précipitation acide. Les effets des solvants et des températures de chauffage sur la récupération et les propriétés des EPS gélifiants ont été étudiés. L'hydroxyde de sodium a permis une meilleure solubilisation des EPS par rapport au carbonate de sodium (respectivement  $711,3 \pm 38,0$  et  $408,4 \pm 4,0$  mg/g) mais la capacité à précipiter les EPS gélifiant à pH acide était plus élevée dans le carbonate de sodium (22 % contre 6 % dans de la soude à 80 °C). L'augmentation de la température de 20 °C à 80 °C a permis un meilleur rendement de solubilisation des EPS mais la dégradation des polymères dans l'hydroxyde de sodium a été mise en évidence, ce qui a orienté le choix vers le carbonate de sodium (0,2 M, 1h à 80 °C).

Diverses stratégies expérimentales ont ensuite été développées (i) une méthode miniaturisée capable de détecter la gélification induite par le calcium et (ii) le fractionnement des molécules extraites par voie alcaline et précipitation acide. La méthode miniaturisée utilisant seulement 1 à 3 mg d'échantillon pour évaluer la capacité de gélification a été mise au point sur des molécules modèles pures ou en mélange, telles que l'alginate, l'albumine de sérum bovin et l'ADN. La méthode a consisté à calculer un pourcentage de réactivité (%R) à travers les spectres UV-vis et à déterminer le pourcentage de volume de gel (%Vg) formé après l'ajout de calcium. Les deux facteurs ont été combinés pour donner un facteur de gélification (GF) permettant de classer les solutions selon leur capacité de gélification.

Les EPS extraits par voie alcaline et précipitation acide ont été fractionnés en fonction de leur charge à l'aide d'une colonne de chromatographie d'échange d'anions préparative. Le GF a été déterminé pour chaque fraction éluée et il a été montré que le GF des EPS anioniques adsorbés était corrélé avec leur charge négative. La distribution en taille des molécules présentes au sein des fractions et participant à la gélification a été analysée par chromatographie d'exclusion stérique : la majorité des hauts poids moléculaires détectés participent à la gélification alors que la participation des bas poids moléculaires est limitée. Enfin, il a été montré que même après fractionnement, les molécules anioniques participant à la gélification restent biochimiquement diversifiées et que les gels formés par les EPS isolés des granules sont des hydrogels fortement composites.

Dans le but de valoriser les EPS gélifiants, les résultats obtenus soulignent la nécessité de moduler les méthodes d'extraction et de fractionnement vers la sélection de molécules ayant la charge négative appropriée et également vers la préservation de leur poids moléculaire lors des étapes d'extraction.

**Mots-clés:**

Granules aérobies épuratifs, Substances Extracellulaires Polymériques, Extraction, Biopolymères, Gélification, Valorisation.

## **Abstract**

Aerobic granular sludges are dense spherical structured biofilms, composed of bacteria embedded in a matrix of extracellular polymeric substances (EPS) used for treating wastewater. Their use for treating wastewater is booming due to their efficiency in terms of pollutants removal while being more compact and less energy consuming than activated sludge processes. Their valorization through the extraction of various added value by-products represents an economic and societal challenge for the years to come. The production of gel-forming biopolymers recovered from the EPS of the granular biofilm is a promising valorization route that has been studied in this thesis.

EPS are biochemically very diverse as they are composed of a mixture of polysaccharides, proteins, lipids, humic acids and DNA. They play an important role in structuring the granular biofilm and contribute in an essential way to the dense and very cohesive nature of the extracellular matrix. Therefore, the extraction method of gel-forming EPS which has been developed relies on alkaline solubilization at high temperature followed by an acidic precipitation step. The effects of solvents and heating temperatures on the recovery and degradation of gel-forming EPS were determined. Sodium hydroxide allowed a better EPS solubilization compared to sodium carbonate ( $711.3 \pm 38.0$  and  $408.4 \pm 4.0$  mg/g respectively) but the capacity to precipitate gel-forming EPS at acidic pH was higher in sodium carbonate (22% compared to 6% in sodium hydroxide at 80 °C). Increasing the temperature from 20 °C to 80 °C allowed a better solubilization of EPS but the degradation of polymers in sodium hydroxide was evidenced and therefore the choice of extraction conditions was oriented towards sodium carbonate 0.2 M, during 1h at 80 °C.

Various experimental strategies were then developed (i) a miniaturized quick method capable of detecting gelation and (ii) the fractionation of the extracted molecules by alkaline and acidic precipitation. The miniaturized method (using only 1 to 3 mg of sample) for gelation capacity screening has been developed on pure or mixed model molecules, such as alginate, bovine serum albumin and DNA. The method consisted of calculating a percentage of reactivity (%R) through UV-vis spectra and determining the percentage of gel volume (%Vg) formed after the addition of calcium. Both factors were combined to give a gelling factor (GF) allowing the classification of solutions according to their gelation capacity.

EPS extracted by alkaline and acid precipitation were fractionated according to their charge using a preparative anionic exchange chromatography column. The GF was determined for each eluted fraction and it was shown that the GF of the adsorbed anionic EPS correlated with their global negative charge. The size distribution of the molecules within the fractions and those participating in the gelation was analyzed by size exclusion chromatography: the majority of detected high molecular weights participate in gelation whereas low molecular weights' participation in gelation is limited. Therefore, the obtained results show that both charge and molecular size are important factors for gelation. Finally, it was shown that even after fractionation, the anionic molecules involved in gelation remain biochemically diversified and that the hydrogels formed with EPS isolated from granules are strongly composite hydrogels.

With the goal of valorizing gel-forming EPS, the obtained results underline the necessity to modulate the extraction and purification methods towards the selection of molecules having the appropriate negative charge and also towards the preservation of their molecular weight during the extraction steps.

**Keywords:**

Aerobic granular sludge, Extracellular Polymeric Substances, Extraction, Biopolymers, Gelation screening method, Valorization.

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## **General introduction**

In the light of the recent global challenges facing humanity such as climate change, pollution, and biodiversity loss, the scientific community is encouraging the development of greener solutions and the application of circular economy concepts. In this context, the treatment of wastes should be ensured to reduce the impact of anthropogenic activities on the environment. Wastewater treatment aims to purify the water after its use, allowing to discharge it into the environment while avoiding eutrophication and the contamination of the receiving water body. The most common wastewater treatment plants nowadays are those using activated sludges, which are bacterial aggregates capable of treating the pollutants present in water. Recently, the use of aerobic granular sludge which is a more dense structured biofilm, composed of bacteria embedded in a matrix of extracellular polymeric substances (EPS) started gaining momentum (Seviour et al., 2009). Those EPS, are made of a variety of biochemical groups, such as proteins, polysaccharides, DNA, and humic-like substances amongst others (Oliveira et al., 2020) and play many roles within aerobic granules, for example they allow maintaining the integrity and stability of granules (Liu et al., 2004), as well as ensuring protection against viruses (Campo et al., 2022). In addition, EPS play a role in mass transfer within the granule, in their settleability (Sheng et al., 2010), water holding capacity, and nutrient sorption (Campo et al., 2022).

In 2022, there are over 90 wastewater treatment plants, using aerobic granular sludge technology around the world, and this number is increasing quickly throughout the years (source: royalthaskoningdhv.com). This technology is gaining interest because it allows the treatment of wastewater in a more economical, ecological and efficient way (Winkler et al. 2018). Indeed, in addition to their capacity to treat water efficiently, the bacterial aggregates involved during the process can be valorized through the extraction of valuable by-products, such as the Kaumera gum that is currently commercialized for various applications (Tan et al., 2021). This will promote a circular economy concept based on the transition from wastewater treatment plants (WWTP) to wastewater resource recovery facilities (WRRF). Recovering EPS from WWTP presents several benefits, beginning with the fact that it reduces the quantity of excess sludge to be treated which is very costly (Campo et al., 2022). In addition, selling the extracted polymers could be a source of revenue for WWTP. Many uses for the by-products extracted from granular sludges have been identified in literature such as their use as

waterproof material, biosorbents, flame retardants, and hydrogels among other uses (Tan et al., 2021). In 2022, two full-scale EPS recovery plants were established in the Netherlands, with an estimated recovery yield of 22.5% (18 kilos of EPS are extracted from 80 kg of granular sludge) (Campo et al., 2022). One ton of extracted gel-forming EPS generates a final revenue of 1000 to 2000 € (after the extraction and refining costs). The total Dutch production for the year 2030 is expected to be at 85 000 tons of EPS thus generating between 85 and 170 million € (Tavares Ferreira et al., 2021). In this thesis a focus will be done on the hydrogel forming capacity of EPS extracted from aerobic granular sludge.

Hydrogels have many applications in different sectors including the biomedical, agricultural, pharmaceutical, tissue engineering, and food additives sector amongst others. These applications depend on the characteristics of the produced hydrogels, such as their mechanical strength and biodegradability (Ahmed, 2015). An example of a widely used polymer for its gel forming capacity is alginate, which is a polysaccharide extracted from brown seaweeds but also secreted by some bacteria (Fang et al., 2007). This polymer is made of guluronic and mannuronic acid residues that contain carboxylate ions allowing the formation of hydrogels through ionic-crosslinking with calcium ions. Alginate has a wide variety of applications; it has been found very useful in the food industry as a stabilizer and emulsifier and was also used in the textile/paper-printing industry as a shear thinning agent. Many biomedical applications have also been proposed for this polymer because of its biocompatibility and non-immunogenicity. The properties of the formed hydrogel influence greatly its applications and therefore there is a necessity to control these properties. The modulation of the mechanical properties (swelling, stiffness...) of alginate hydrogels can be driven by intrinsic properties such as the molecular weight or charge distributions or achieved by varying operating conditions during the gel formation such as the cross-linker type, the cross-linking density and by chemically modifying the polymer (Augst et al., 2006).

One of the many by-products that can be extracted from aerobic granular sludges is structural EPS (sEPS) consisting the subset of the EPS matrix that provides the physical structure of the granule. They are classically recovered after drastic granule solubilization and show the capacity of forming gels *in vitro* through ionic cross-linking with divalent cations. Many questions remain unanswered concerning these gel-forming EPS and a better understanding of the physico-chemical characteristics that are linked with their hydrogel properties is

needed. A prerequisite is to design a suitable extraction protocol maximizing the yield of sEPS recovery while conserving their gel forming capacity. The protocols used for the extraction of sEPS are based on alkaline (NaOH or Na<sub>2</sub>CO<sub>3</sub>) solubilization of dried or wet granules and most often at high temperatures (80 °C). These methods maximize the EPS extraction yield without considering the effect of the treatment on the targeted molecules; understanding the effects of the extraction protocol such as degradation/denaturation on the gel-forming EPS remains a key point to be investigated. In addition, fractionation of the sEPS polymers is needed for a better characterization of the key gel forming molecules and identification of their physico-chemical and molecular properties. Understanding which molecular properties are involved in the gelation mechanisms is required because these molecular properties can influence the characteristics of the obtained hydrogels and hence their possible applications. Therefore this thesis will try to answer some of these points and is composed of five chapters in addition to this introduction.

At first, a literature review focusing on aerobic granular sludge and gel-forming EPS is presented. The questions arising from this review are summarized and the resulting scope of the thesis is given.

A second chapter treating the question of the extraction of gel-forming EPS is proposed. This chapter will focus on the effects of alkaline solvents and heating temperatures on the polymers extracted from aerobic granular sludge both in terms of quantities and biochemical properties.

The third chapter consisted of developing a miniaturized quick method to detect the gelling capacity of solutions in comparison with alginate (a well-known gelling commercial agent). The goal was to develop a test capable of detecting a solution's capacity to form gels in the presence of divalent cations, and compare solutions with each other while lowering the quantity of EPS needed to perform the tests. This test was intended to be used during the fractionation of EPS, as a screening method, to detect the fraction responsible for the gelling capacity of EPS.

The fourth chapter gives insights on the molecular properties of the gel-forming EPS, particularly determining which molecular size and apparent charge are the most implied in the gel formation. In addition, the biochemical groups that participated in the gel formation

were analyzed. The obtained results will help in understanding which molecular properties are important for the hydrogel formation, and therefore affect many steps in the recovery of the gel forming EPS, such as their extraction, further purification, and sought applications.

The fifth chapter summarizes the findings of this thesis and recalls the different conclusions from the previous chapters. Finally, various perspectives to this thesis are proposed.

# Chapter I: Literature review

## 1 Aerobic granular sludge technology

### 1.1 Towards sustainable sanitation

Most wastewater treatment plants (WWTP) currently use activated sludge to ensure water depollution. These plants rely on spatial or temporal separation of the different biological reactions required for the treatment of water. In addition, large clarifiers are needed for the liquid-solid separation of biomass leading to high investment costs and to sludge recirculation between the basins and/or between the clarifier and the biological reactor. Recently, many WWTP are starting to use the Nereda technology utilizing aerobic granular sludge that are dense spherical and self-aggregating biofilms (source: [royalhaskoningdhv.com](http://royalhaskoningdhv.com)) because they present many advantages over the conventional activated sludge. By definition, a biofilm is made of bacteria embedded in an extracellular polymeric substances (EPS) matrix that acts like a cement holding the biofilm together, and is usually secreted by the bacteria (Ni et al., 2015). Granules differ from flocs by their density and degree of microbial aggregation, conferring to this densified aggregate many advantages. Indeed, aerobic granular sludge has a particle size greater than 0.2 mm implicating denser and more compact aggregates than activated sludge. This specificity compared to flocs allows (i) diffusional gradients and transport limitation allowing aerobic and anoxic zones to coexist and thus to perform different reactions in only one reactor; (ii) a higher settling velocity of 30-90 m/h for granules in comparison to 8-10 m/h for activated sludges (Winkler et al. 2018) that permits performing the separation in the reaction tank itself (Bengtsson et al., 2019). These characteristics result in smaller size reactors (and thus investment costs) and remove the need for sludge recirculation (and thus smaller operational costs). To that is added their ability to treat industrial effluents with high organic charge in carbon, nitrogen and phosphorus and also to withstand toxic loadings. All these characteristics allow aerobic granular sludge to achieve a more efficient, economical and sustainable wastewater treatment (Winkler et al., 2018).

During the wastewater treatment in sequenced batch reactors (SBR) the granules are formed due to different selection pressures applied on the initial bacterial inoculum leading to its aggregation and the formation of stable dense aggregates. The factors influencing granulation

are the reactor's configuration (aeration, mixing, settling time) and its operational conditions such as the feeding characteristics, pH or temperature. For example, selecting slow growing microorganisms has been shown to be favorable for the aggregation and that can be done through alternating aerobic/anaerobic conditions (Weissbrodt et al., 2013). Lowering the settling time, which means selecting microorganisms that settle fast is a key factor for selecting microorganisms that are capable of granulation (Winkler et al., 2018). The alternation of feast and famine phases together with shear forces applied in the reactor have been found to promote the secretion of EPS which sustain the structural integrity of the aggregate and therefore promote granulation (Dulekgurgen et al., 2008). Many types of SBR cycles exist and they depend on the type of wastewater being treated. An example of such SBR cycles can be seen in Figure I-1. It consists of the succession of (i) a feeding period during which the organic pollutants are brought into the reactor (quantified as chemical oxygen demand or COD), (ii) an anaerobic reaction followed by (iii) an aerobic reaction period, (iv) a settling and (v) a purge period allowing the selection of dense aggregates with a high settling velocity.

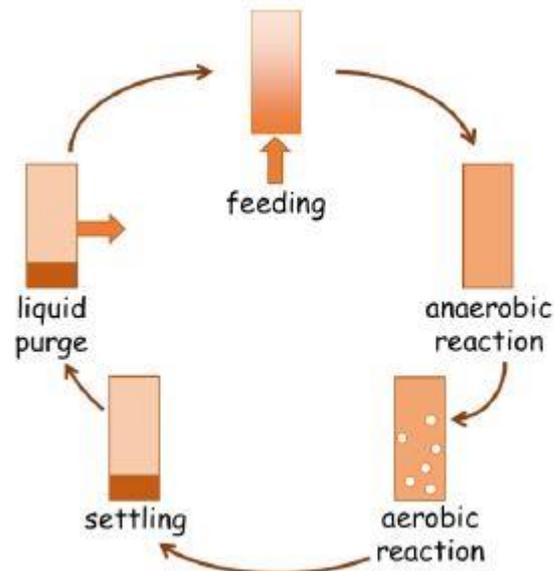
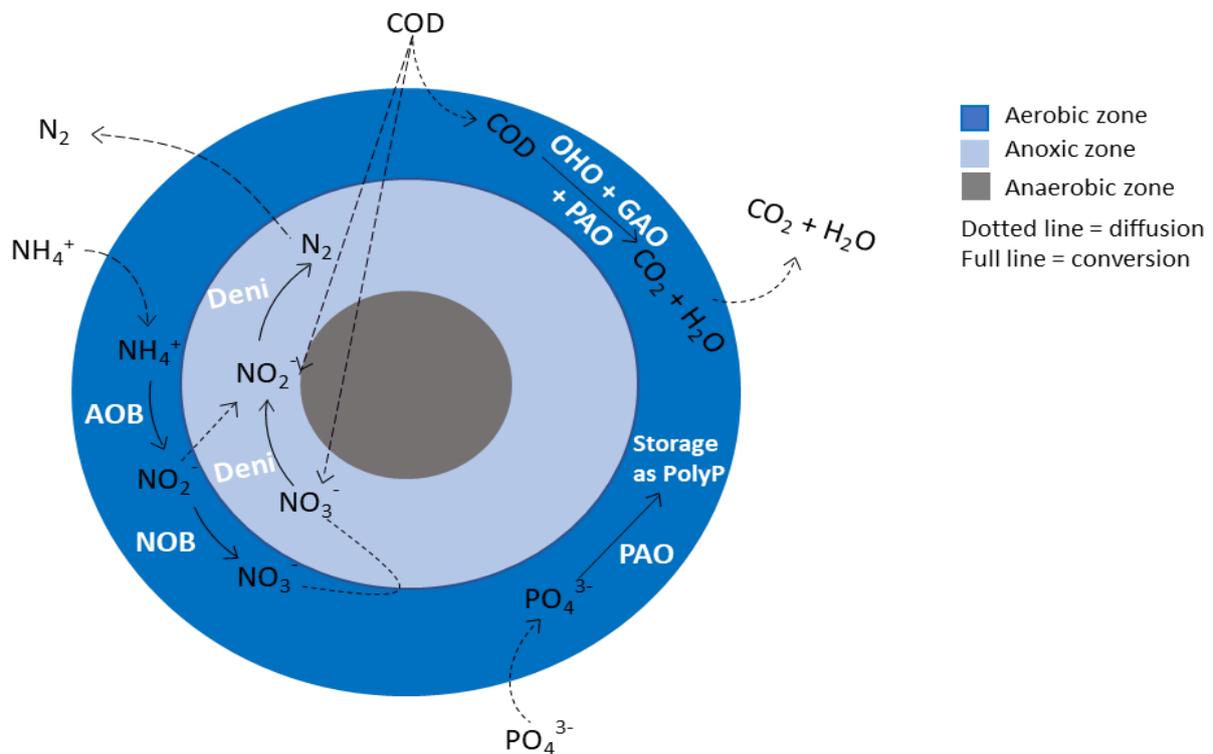


Figure I-1: The aerobic granules SBR cycle (Durieux thesis, 2022).

Aerobic granular sludge is assimilated to microreactors due to the presence of many bacterial populations working together to achieve the goal of water depollution. Indeed, granules are made from many layers of microorganisms and their organization inside the granule depends on the diffusion gradients of oxygen and nutrients within the EPS matrix. The simultaneous

removal of COD, nitrogen and phosphorus depends on the structure of the granule and the localization of bacteria within this biofilm. As schematically presented in Figure I-2, aerobic autotrophic nitrifiers are expected to be mainly located on the outer side (aerobic zone of Figure I-2) while anaerobic denitrifiers are more located in the deep core of the granule (anoxic zone of Figure I-2). The treatment of nitrogen is done in two steps: aerobic nitrification and anoxic denitrification. Ammonium is oxidized to nitrite by ammonium oxidizing bacteria (AOB) and later on to nitrate by nitrite oxidizing bacteria (NOB) which consists the aerobic nitrification step. Both populations are autotrophic and therefore require inorganic carbon to grow. The denitrification is the step leading to the formation of molecular nitrogen (Nancharaiah and Kiran Kumar Reddy, 2018). Denitrifiers (Deni) are able to use nitrites and/or nitrates as electron acceptors under anoxic conditions and are heterotrophic. COD treatment is performed by ordinary heterotrophic organisms (OHO) or storing heterotrophic organisms such as glycogen and phosphorus accumulating organisms (GAO and PAO respectively) leading to the production of  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . The treatment of phosphorus relies on the presence of PAO capable of storing polyphosphates (PolyP) as an energy source, during aeration (Bunce et al., 2018).



**Figure I-2: Biological reactions inside a granule. The roles of microorganisms during the wastewater treatment were shown particularly during nitrogen treatment through AOB, NOB and denitrifiers (Deni); COD treatment by OHO, GAO, and PAO; in addition to phosphorus removal by PAO.**

The particular spatial distribution of microorganisms within the aerobic granule is highly dependent on the physico-chemical interactions linking microbial cells and EPS. These polymers are made of a variety of macromolecules such as proteins, polysaccharides, humic substances, and nucleic acids among others (Ding et al., 2015; Schambeck et al., 2020). A growing interest was given to these polymers, since it was found that they play many interesting and important roles within the granule. Polysaccharides have been noted to be important in the granulation due to the formation of cross-networks with cells (Zhu et al., 2012). On the other hand, an increase in proteins content in the granule was correlated with an increase in cell surface hydrophobicity which encourages granulation (Zhang et al., 2007). It was shown by Caudan et al. in 2014 through enzymatic digestion of granules with specific hydrolases that proteins as well as glucans have a very important role in the cohesion of the granules. In addition, cationic bridging between polymers was found to be a major aggregative mechanism that can lead to the formation of capsular hydrogels involved in the cohesion and protection of micro-colonies against detrimental or toxic environments. Another role of EPS is to build a dense and strong matrix that will influence the settling properties and the surface charge of granules, along with playing an important role in the diffusion limitation inside the granule. Therefore, the EPS matrix is a key component for the structural integrity of granules and their protection against the environment (Ding et al., 2015; Seviour et al., 2019; Sheng et al., 2010). Considering this architectural function, the EPS extracted after complete solubilization of the granule are considered as structural EPS (sEPS) including both polymers loosely bound and tightly bound to the granular matrix (Ding et al., 2015; Tan et al., 2021). Extraction and recovery of the sEPS from granules is of real interest since they have an important valorization potential as biosourced materials. Furthermore, if their valorization scheme is mastered, they would help the transition from WWTP to WRRF, transforming these stations from economical burdens to a source of income through resource recovery (Duque et al., 2021).

## **1.2 Towards resource recovery**

WWTP have a high valorization potential and the recovery of a variety of resources including water, energy, nutrients, polymers and other products provides multiple issues to contribute to a more circular economy (Foglia et al., 2021). With the increasing prices of fuel and the limitation of this resource, searching for a more sustainable energy production is a must. Many

types of energy can be produced from WWTP including biogas, hydrogen, and thermal energy... (Zarei, 2020). The recovery of fertilizer-used nutrients is an example of resources recovered from WWTP i.e. ammonium sulfates, phosphates, and struvite amongst others (Duque et al., 2021; Ye et al., 2020).

The main waste product in WWTP is the excess sludge produced (excess biomass) during the water treatment, and its processing cost accounts for approximately half of the total operational cost (De Valk et al., 2019). Extracting biopolymers from this excess sludge could generate revenue from WWTP and fits within the scope of a more sustainable economy since the biopolymers produced will be from renewable sources. Microbial polymers can be divided into two groups: intracellular and extracellular. Examples of intracellular polymers valorized from sludge are glycogen and polyhydroxyalkanoate which can be used to produce ultra-strong fibers (Amorim de Carvalho et al., 2021; Feng et al., 2021). During the last ten years, the valorization of aerobic granules was directed towards the recovery of EPS due to the versatility of potential applications and their ease of production.

Actually, the commercial interest of EPS is related to properties such as biodegradability, biocompatibility and acceptable levels of toxicity. In comparison with oil-based polymers their extraction is easier and they have a higher production rate (Feng et al., 2021; Lima-Tenório et al., 2015). EPS have the potential to be used in a wide variety of applications, for example it was shown that they have amphiphilic properties due to a small fraction of lipids allowing their use as a surface coating material for paper packages giving them water resistance characteristics (Lin et al., 2015). In another study, EPS extracted from granules have shown flame retarding properties when tested on flax fabric coated with EPS (Kim et al., 2020). Heavy metal adsorption by using EPS has also been demonstrated particularly for Pb, Cd and Zn (Liu et al., 2015; Pagliaccia et al., 2022a). In addition, EPS have been commercialized as a product for the construction market because they enhance cement curing (Karakas et al., 2020).

As previously explained, sEPS play an important role in the cohesion and formation of the granule. Seviour et al. in 2009 showed that EPS extracted from granules have pH dependent hydrogel forming capacity; in fact, below pH 9 the extracted EPS showed the properties of a strong gel. Later on, some of these hydrogel forming EPS have been identified: one was named granulan and is a complex heteropolysaccharide able to form strong gels with or without divalent cations like  $\text{Ca}^{2+}$  (Seviour et al., 2009, 2012a). The other ones were called Alginate Like

Exopolysaccharides (ALE) due to their similarity with alginate because it contains some uronic acids i.e. aldoses of which the primary alcohol is oxidized into a carboxylic acid function (Codée et al., 2010). These ALE can form gels by interacting with calcium (Lin et al., 2010) but even if many bacterial species were found capable of secreting alginate (Clementi, 1997; Codée et al., 2010), their chemical composition is much more diversified and not yet established.

Many operational factors can influence the production of ALE, but until now a consensus on the optimal way for ALE production has not been found. This is due to the complexity of granules from a microbiological point of view and the multitude of operational factors to control. In literature, some operational factors were identified to have an impact on the ALE yield: for example, rapid fluctuations of the organic loading rate may cause an increase in ALE production, and a moderate salinity around 1% NaCl may also trigger ALE synthesis. It was also shown that the COD/N ratio (which is the amount of carbon over the amount of nitrogen present in the feeding) influences ALE quantities in granules (Amorim de Carvalho et al., 2021; Durieux et al., 2021). In addition, the feeding type also influences the quantities of ALE: it was shown that a simple synthetic feeding composed of equal COD fractions of propionate and acetate, grants the highest quantity of ALE in comparison with complex synthetic effluents or real wastewater, this observed difference was attributed to specific microbial and physical attributes of the granules (Layer et al., 2019; Schambeck et al., 2020). In particular, it was found that granules fed with simple synthetic wastewater were dominated by two bacterial species *Candidatus accumulibacter* and *Ca. competibacter*. These types of bacteria, capable of using volatile fatty acids (VFA), might have a better capacity to produce uronic acids and hence gel forming exopolysaccharides (Layer et al., 2019; Seviour et al., 2011).

The results can sometimes be contradictory, in particular due to biases introduced by the extraction protocols. Indeed, it was shown that the extraction protocol influences the composition of the extracts in terms of biochemical nature of the extracted molecules and of their concentration (Feng et al., 2019; Schambeck et al., 2020). Looking for the optimal extraction protocol implies an understanding of the structure of gel-forming molecules, as well as of the mechanisms used for the gel formation. Therefore, the next part will discuss more in depth the molecular characteristics of hydrogels, to understand later on the necessities and the pitfalls to avoid during the extraction of gel-forming EPS.



## 1. Highlights on “Aerobic granular sludge technology”

Section 1 showed the utility and efficiency of aerobic granular sludges in treating wastewater in comparison with the frequently used activated sludges. These granules are self-assembled spherical dense biofilms made of bacteria embedded in an EPS matrix. The main waste product of WWTP is excess sludge, therefore valorizing this excess sludge through the extraction of valuable by-products can help in resolving this problem and reducing its economical impact on WWTP.

Many by-products for granular sludges are described in literature among which the gel-forming polymers that are elucidating the most interest due to the diversity of their potential applications. The optimized recovery of gel-forming EPS depends on the efficiency of their extraction from granular sludges and also on understanding the gel-formation mechanisms and molecular properties implied in the gelation.

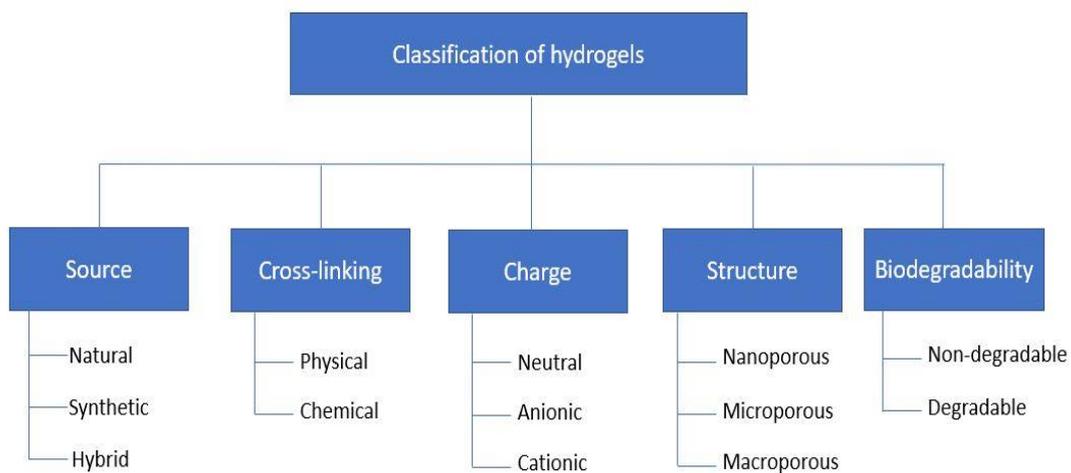
## 2 Biopolymers as hydrogels: structure, mechanism of gelation, and applications

In the upcoming paragraphs, the interest will be on polysaccharides and proteins that are known to form gels. The representatives of polysaccharides will be alginate (§2.2.1) and carrageenan (§2.2.2). The first was chosen to be studied due to its composition of uronic acids like some of the EPS extracted from aerobic granules, whereas carrageenan was studied to illustrate other mechanisms that might be implicated in the gelation of EPS. For the protein group, BSA (§2.3.1) which is a globular protein was studied to illustrate the possible participation of proteins in the gelation of EPS, and a focus was done on its capacity to form gels in the presence of divalent cations. Gelatin (§2.3.2) which is a fibrous protein shows the capacity of proteins to form hydrogels upon heating through a mechanism that does not rely on the presence of divalent cations, therefore showing the diversity of gelation mechanisms and interactions between molecules that can be possibly found within the EPS extracts. Factors affecting the gelation of each polymer were elucidated to understand factors that might affect the gelation of EPS.

## 2.1 Definition and classification

Hydrogels can be defined as a cross-linked polymeric network retaining water within its structure. The polymeric network is produced by one or many polymers and once formed will not dissolve in water. Their ability to absorb water comes from the hydrophilic groups present on the backbone of polymers while their resistance to dissolution arises from the cross-links between network chains (Ahmed, 2015). The classification of hydrogels depends on many properties and a non-exhaustive summary of some of these criteria can be seen in Figure I-3. Classification can be performed according to the source that could be natural, synthetic or a combination of both (hybrid); the type of cross-linking that can be based on physical (transient junctions based on weak bonds like hydrogen or van der Waals bonds) or chemical (based on covalent bonds) junctions. It can be also possible to discriminate hydrogels according to the charge of the involved polymers (neutral, anionic or cationic), their pores' structures (nanoporous, microporous, or macroporous), and their biodegradability amongst others (Ahmed, 2015; Labowska et al., 2021; Ullah et al., 2015).

Many uses for hydrogels have been cited in literature in different areas such as drug delivery, cosmetics, agriculture, food additives, coal dewatering, water industry, artificial snow, wound dressing, tissue engineering, and regenerative medicine among other uses (Ahmed, 2015; Labowska et al., 2021).



**Figure I-3: A non-exhaustive summary of the classification of hydrogels.**

Since EPS are made from a variety of different biochemical groups including polysaccharides, proteins, hyaluronic acid-like molecules, and sulfated glycosaminoglycans (Felz et al., 2020b, 2019; Schambeck et al., 2020), a focus will be done on some well characterized natural

polymers because they show different mechanisms of gelation that may be implied in the hydrogel formation of EPS.

## 2.2 Polysaccharidic hydrogels

### 2.2.1 Alginate

Alginate consists of two hexuronic acids: mannuronic acid (M) and guluronic acid (G); these two uronic acids can be arranged in homopolymeric blocks which means polymannuronic (MM) or polyguluronic (GG) or heteropolymeric (MG) blocks with a random distribution within the macromolecule (Figure I- 4).

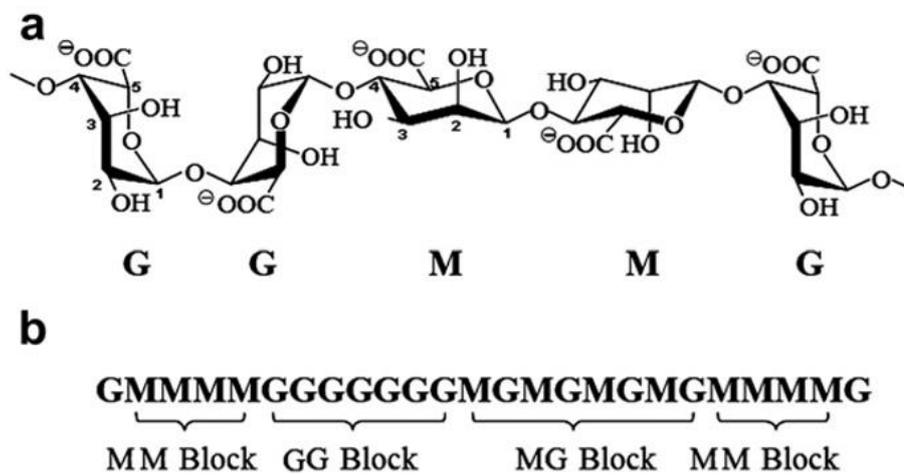


Figure I- 4: Structure of alginate (a) and its block fractions (b) consisting of mannuronic acid (M) and guluronic acid (G) (Lin et al., 2010).

The most common method used for the gelation of alginate is the ionic cross-linking using divalent cations i.e.  $\text{Ca}^{2+}$ . The calcium ions are thought to bind solely to the guluronate of the alginate main chain because it allows a high degree of coordination of the divalent cations. The G blocks of one chain form therefore junctions with the G blocks of the polymer adjacent to it, in what is called the egg-box model of cross linking resulting in the formation of a gel (Abdul Khalil et al., 2017; Lee and Mooney, 2012; Sobeck and Higgins, 2002) as seen in Figure I-5. In addition to the gelling by cross-linking with calcium ions, alginate has the capacity to form gels if the pH gets below the pka (3.6) of the carboxylic group present in guluronic acids (Gurikov and Smirnova, 2018; Tavassoli-Kafrani et al., 2016). However, care must be taken to lower the pH slowly to obtain homogeneous alginate acid gels, and this can be done by the addition of glucono- $\delta$ -lactone. If the pH lowering happened abruptly, through the addition of mineral acids for example, it would rather be a precipitation. This type of acidic alginate gel is

less studied and has fewer applications than the gels formed through ionic cross-linking (Draget, 2009). The swelling behavior of alginate gels obtained through cross-linking is nevertheless affected by the pH since alkaline environments are necessary to maintain negatively charged carboxylic groups. Then, the carboxylate ions repel each other while absorbing water to fill the voids within the alginate calcium network. On the contrary, in an acidic medium the carboxylic groups are protonated which lowers the repulsive forces and therefore causes water loss or syneresis. However, the gel strength is not affected significantly during the pH variation (Ramdhan et al., 2019).

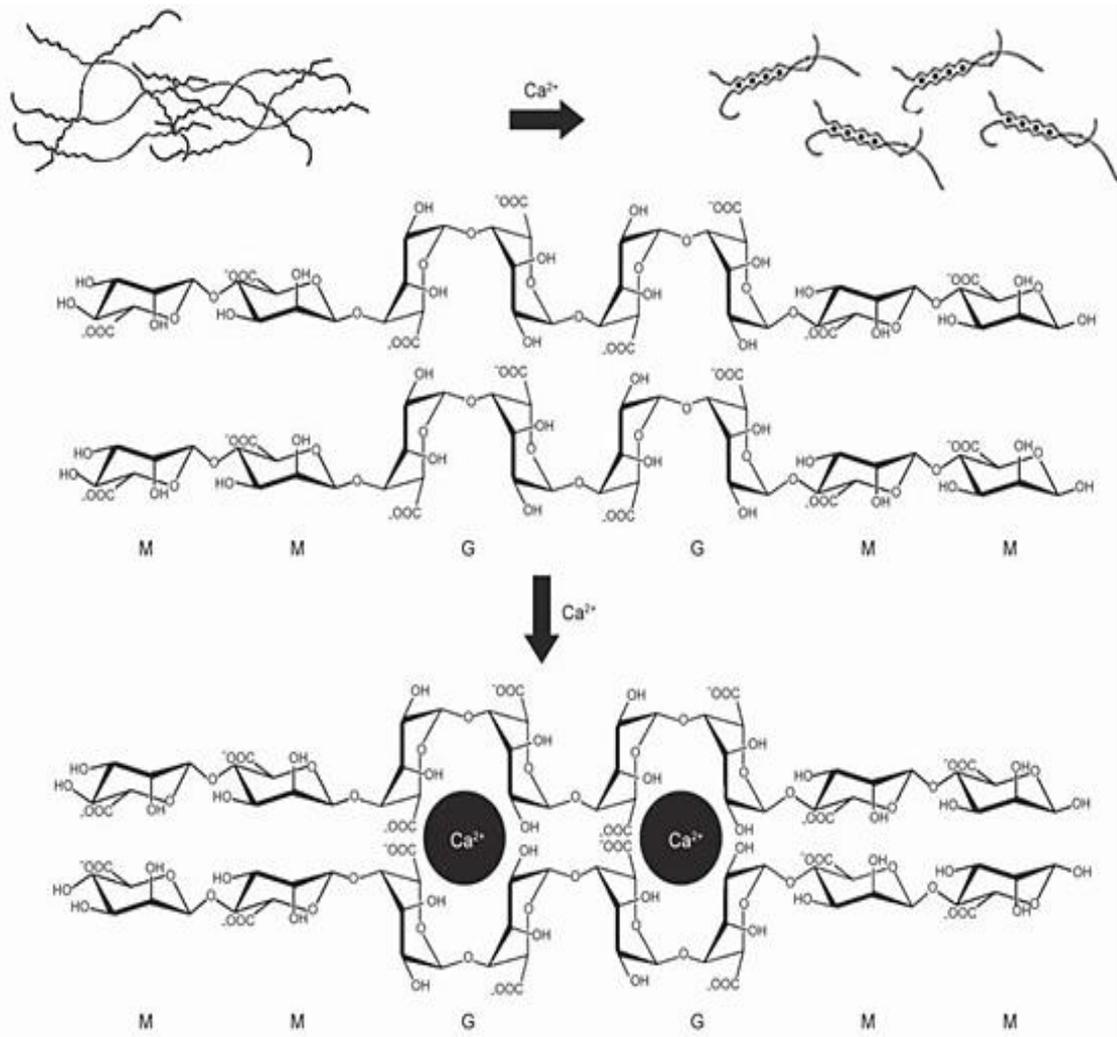


Figure I-5: Mechanism of gelation of alginate through the egg-box model (Abdul Khalil et al., 2017).

Alginate gels can be evaluated using the M/G ratio; an M/G ratio less than 1 indicates a large amount of guluronic acids capable of forming strong junctions with calcium giving brittle and stiffer gels whereas M/G ratios over one, indicate a lower amount of guluronic acids and therefore gives softer and more elastic gels (Fenoradosoa et al., 2010; Labowska et al., 2021;

Tavassoli-Kafrani et al., 2016). Moreover, high contents of G blocks reduced the rate of swelling and the amount of leaching from formed hydrogels (Draget et al., 1996).

The molecular weight plays an important role in the gel formation of alginate. Indeed, the pre-gel solution viscosity and the gel stiffness after its formation can be manipulated through the molecular weight of alginate and its distribution (Labowska et al., 2021). In addition, Draget et al. (1994) evaluated the Young's modulus of gels obtained from algal alginate of different origins (stipe and leaf of *Laminaria hyperborea*) and different molecular weights ranging from 60 to 710 kDa as seen in Figure I-6. For the alginate of stipe, they showed a positive correlation between the molecular weight and the gel's strength that increased from approximately 3 to 22 kPa with the increase of molecular weight from 60 to 400 kDa, however this was not the case for the leaf alginate that showed a constant Young's modulus at around 5 kPa for samples above 400 kDa. The differences between leaf and stipe are probably due to differences in the polymer composition. Indeed, alginate extracted from the stipe had a higher ratio of guluronic acids and had larger average guluronic acid blocks length than that of alginate extracted from the leaves (Draget et al., 1994).

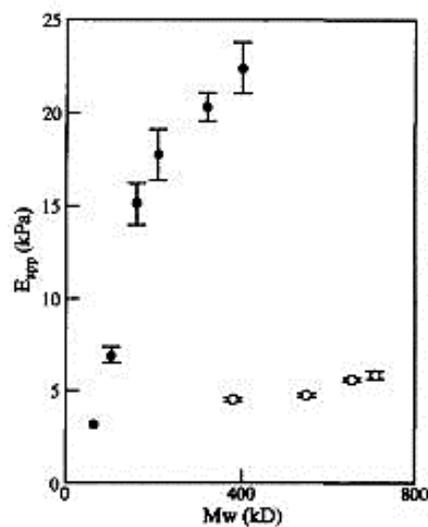


Figure I-6: The effect of molecular weight on alginate gel's strength extracted from the stipe (black dot) or leaf (white dot) of *Laminaria hyperborea* (Draget et al., 1994).

The mechanical properties of the formed gels depend also on the gelling rate, the slower it is the more homogeneous the gel is and therefore the better its mechanical properties are. The gelling rate can be controlled either by the temperature used during gelation or the used cross-linker, for example using  $\text{CaCO}_3$  or  $\text{CaSO}_4$  which are less soluble than  $\text{CaCl}_2$  can cause a slower gelling rate and therefore a more homogeneous gel (Lee and Mooney, 2012).

Many applications exist for this hydrogel in different fields such as the food industry, agriculture or medicine. An application for alginate hydrogels in agriculture relies on the preparation of microcapsules of microbial fertilizers containing *Klebsiella oxytoca* Rs-5 (Wu et al., 2011). These hydrogels were used to control salinity in soils used for the cultivation of cotton because the presence of this bacterial strain could help through the absorption of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Fe}^{2+}$ . However, this bacterium cannot colonize easily the soils due to its hostile environment, and therefore the encapsulation of the bacteria in alginate hydrogels can provide a better environment for their survival. It was shown in this study that the concentration of alginate has many influences on the properties of the formed microcapsules (swelling rate, particle size...) and therefore on controlling the release of bacteria in the soil and the colonization near the roots. For example, the released viable cells count was the highest for hydrogels made with 1.5% and 2% alginate compared to 1% alginate hydrogels that presented lower viable cells count (Wu et al., 2011). In the same way, He et al. (2015) administered plant growth promoting bacteria that help maintain soil fertility and plant growth. These bacteria were encapsulated in alginate hydrogels to insure slow bio-fertilizer release upon diffusion from pores or erosion and degradation of the capsules, reducing the risk of bacteria being washed out by water. Since sodium alginate is known for its degradability, non-toxicity, and simplicity of handling it is chosen for such agricultural applications. However, alginate matrices do not possess a high mechanical strength in presence of monovalent cations in the field due to ion exchanges precisely between the divalent and monovalent cations (He et al., 2015; Lee and Mooney, 2012) promoting the formation of large macropores which causes a quick diffusion of encapsulated materials. Different pore sizes (going from nm to  $\mu\text{m}$ ) have been reported in literature depending on the method of preparation, the cross-linker concentration, and other factors (Eiselt et al., 2000; Jang et al., 2014; Lee and Mooney, 2012). A relation with the calcium concentration has been established where the increase of calcium concentration caused smaller pore sizes (going from 247.5  $\mu\text{m}$  with 36 mM calcium to 30  $\mu\text{m}$  with 144 mM calcium) and a higher compressive modulus increasing from 3 to 40 kPa approximately with the increase of calcium concentration from 36 mM to 144 mM (Jang et al., 2014).

Biomedical applications for alginate have also been well studied and documented. Alginate hydrogels are often used for tissue regeneration, while simultaneously releasing growth

factors or cytokines. Protein delivery by alginate hydrogels has also been reported in literature. However, since the mechanical properties and pore size of the produced hydrogels are hard to control, the possibilities to tailor protein release are limited. Therefore, some researchers investigated structural modifications, such as the sulfonation of alginate, that can help in controlling these properties and therefore adapt to the desired application. Another option for a better release is the combination with other polymers that can improve the stability of the formed hydrogel and the release of entrapped proteins (Vermonden et al., 2012). As previously explained, the use of alginate with higher molecular weight increases the gel's strength. However, high molecular weight alginate solutions are highly viscous which is often undesirable in processing, or for injection of solutions containing cells or proteins for biomedical purposes especially because of the increase of shear forces that would cause damage to the proteins and cells in question. Manipulating the molecular weight and its distribution can control the pre-gelling viscosity of solutions while increasing the post-gelling stiffness. In fact, by using a combination of high and low molecular weight alginates the elastic modulus can increase significantly while the viscosity of the solution minimally raises (Kong et al., 2002; Lee and Mooney, 2012).

### 2.2.2 Carrageenan

Carrageenan polymers are linear hydrophilic polysaccharides, extracted from different types of red seaweeds, that mainly consist of D-galactose and 3,6-Anhydro-D-galactose units bound together with alternating  $\alpha$ -1,3 and  $\beta$ -1,4 linkages. Three types of carrageenan exist and are differentiated by the number of sulfates present on the disaccharide units and their positions (Chauhan and Saxena, 2016): one for Kappa ( $\kappa$ ), two for iota ( $\iota$ ) and three for lambda ( $\lambda$ ) (Figure I-7).

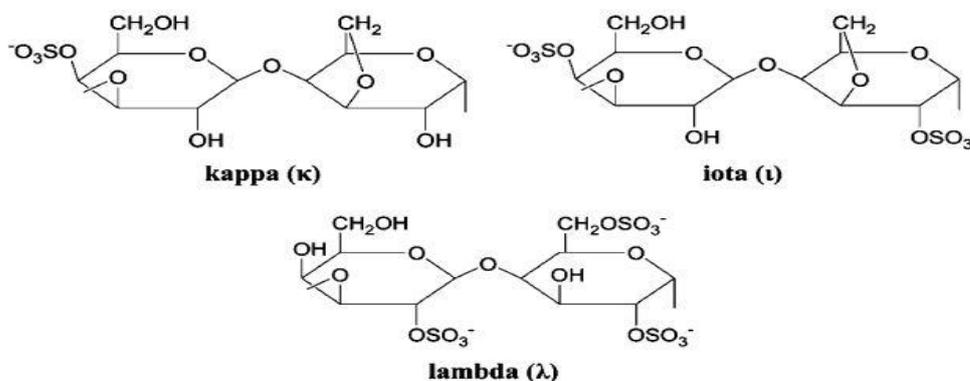
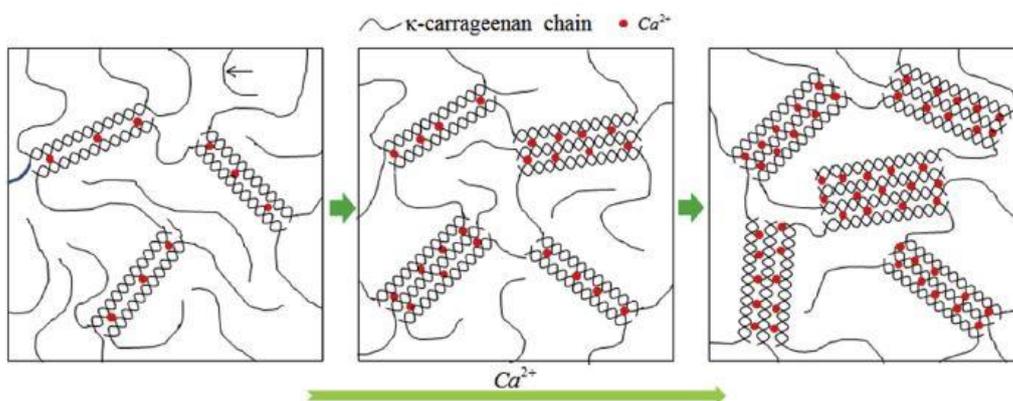


Figure I-7: Structures of the repeating units forming Kappa ( $\kappa$ ), iota ( $\iota$ ) and lambda ( $\lambda$ ) carrageenan (Chauhan and Saxena, 2016).

Gelation of  $\kappa$ - and  $\iota$ -carrageenan occurs with similar mechanisms. The gelation happens in two steps (Figure I-8): (i) a coil to a helix structure transition leading to the formation of double helices upon cooling, (ii) the helix formation is followed by further aggregation dependent on the presence of mono or divalent cations to neutralize the negative charges on the polymers causing repulsion and reducing interactions between polymers. In the case of  $\iota$ -carrageenan, due to the presence and position of the 2 sulfates, the aggregation is limited due to a stronger electrostatic repulsion between the carrageenan chains. The difference in sulfate content influences the gelation and therefore leads to hydrogels with different properties; in fact,  $\kappa$ -carrageenan hydrogels are strong and brittle while  $\iota$ -carrageenan hydrogels are softer (Beaumont et al., 2021; Campo et al., 2009; Chauhan and Saxena, 2016; Liu and Li, 2016).  $\lambda$ -carrageenan is usually described in literature as a thickening agent instead of a gel forming agent (Beaumont et al., 2021).

Concerning  $\kappa$ -carrageenan, it was found to have a better gelation in presence of monovalent cations in comparison with divalent cations (Beaumont et al., 2021). In addition, amongst monovalent cations, a better elastic modulus was shown for potassium, rubidium, or cesium ions in comparison with lithium or sodium ions. For  $\iota$ -carrageenan some studies suggested that divalent cations such as calcium are more effective than monovalent cations for the formation of hydrogels (Beaumont et al., 2021).



**Figure I-8: The sol-gel transition of  $\kappa$ -carrageenan with increasing calcium concentration (Liu and Li, 2016).**

Carrageenan uses in the food industry are well established (Hotchkiss et al., 2016). As an example,  $\kappa$ -carrageenan has a high synergy with milk protein in particular casein (positively charged), resulting in the formation of a milk-gel. Only low doses are needed (100 to 400 ppm or  $\mu\text{g/g}$ ) in the presence of  $\text{Ca}^{2+}$  or  $\text{K}^+$  to form a weak gel network that can act as a stabilizer and a suspending agent. Gelling properties with calcium were also described for other marine

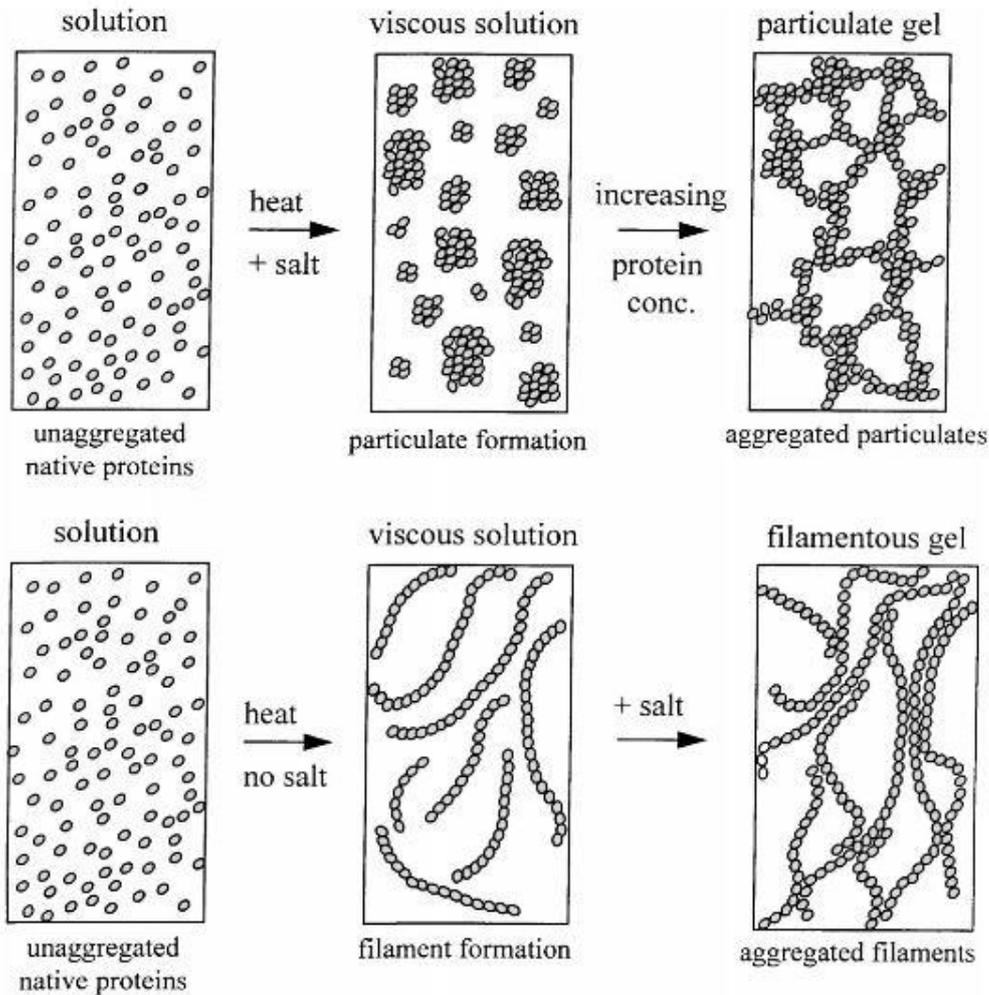
anionic polysaccharides such as a bacterial polysaccharide called infernan to perform delivery systems (Makshakova et al., 2022a).

$\kappa$ -carrageenan hydrogels can also be used as a matrix for therapeutic enzyme immobilization (Makshakova et al., 2022b). Indeed, three different lipases, from different sources with different sizes, net charge, and surface topology were used with spheres of  $\kappa$ -carrageenan hydrogels as their carriers. As expected, the lipase with the highest negative charge was the quickest to be released from the hydrogel due to repulsion between the charges of the enzyme and  $\kappa$ -carrageenan. Overall, this study showed that the interaction between the enzyme and the carrier is very important and depends on many factors such as their charge and size, and showed promising results for such applications (Makshakova et al., 2022b).

### 2.3 Protein hydrogels

Concerning protein hydrogels, two types of molecular networks were explained by Clark, in 1996, which are the associative and particulate networks. Associative networks, such as gelatin hydrogels, are the ones where macromolecular random coils undergo coil to helix transition which gives rise to the hydrogel formation directly or allows the network to develop through lateral helix association (Figure I-9). Particulate gel networks, such as milk casein hydrogels, arise from the association of globular biopolymers that form either well-ordered filamentous structures or randomly branched aggregates as seen in Figure I-9 (Clark, 1996). Most protein gelation methods rely on the unfolding of the protein chain and the aggregation of the denatured polypeptidic chain into a structure capable of forming gels. The network can be stabilized through the formation of non-covalent bonds such as hydrophobic interactions, electrostatic interactions, hydrogen bonds and/or covalent bonds such as disulfide bonds (Le et al., 2017). The gelation of proteins can be achieved through physical (heating, high pressure), enzymatic or chemical treatments (addition of salts, acidification) (Le et al., 2017) which cause the unfolding of the polypeptide chain and the exposure of hydrophobic areas as well as the breaking of disulfide bridges that can associate to form a well ordered structure. However, some proteins can precipitate instead and it all depends on the protein and the applied treatment. In fact, proteins' aggregation is a complex phenomenon that happens in phases (Navarra et al., 2016); first the protein partially unfolds and goes towards molten globule forms or amorphous pre-aggregates (intermediate state). If the pH, protein concentration, temperature, salts concentration were favorable for the formation of protein

hydrogels, this step is followed by forming larger amorphous aggregates, fibrils or fibers (Bryant and Julian McClements, 1998). These aggregates can form the bricks for protein hydrogels as seen in Figure I-9.



**Figure I-9: Particulate and filamentous protein hydrogels formation (Bryant and Julian McClements, 1998).**

As previously said, after aggregation some proteins might precipitate depending on their concentration in the solution. At low concentrations, gravity might cause the collapse of the weak network of aggregates formed and cause their sedimentation, but at high concentrations of proteins, a self-supporting gel can be formed (Nicolai and Durand, 2013). The minimal concentration needed to form hydrogels by proteins is specific to each protein and is dependent on its structure and on the gelation conditions. For gelatin the minimal concentration is 0.6%, whereas for albumin it is 3% and varies between 4 and 12% for whey proteins depending on pH and ionic strength (Le et al., 2017). In fact, ionic strength can influence hydrogel properties and can be a way to induce hydrogel formation through ionic

strength variation (Feng et al., 2012). For example, troponin C isolated in its  $\beta$ -sheet conformation self assembles to form  $\beta$ -sheet fibrils, these fibrils undergo physical cross-linking to form hydrogels as a function of ionic strength (Katyal et al., 2020).

The functional properties (gel strength, water holding capacity, elasticity...) of the formed hydrogels depend on many parameters such as the protein's intrinsic characteristics, its concentration, ion type and concentration, the pH of the solution, as well as the gelation temperature and times (Le et al., 2017). A model protein that has been extensively studied will be developed furthermore in the coming paragraph: gelatin.

### 2.3.1 Gelatin

Gelatin shows interesting gelling properties and it is produced through the hydrolysis of collagen from various animal origins (porcine, fish, or bovine collagen). It is the most abundant protein in mammals, birds, and fish (Eysturskard et al., 2009a; Labowska et al., 2021). In the human body there are at least 16 types of different collagen, the most prominent types are type I, II, and III. Type I collagen consists of three spiral polypeptides chains (two  $\alpha$ - and one  $\beta$ -chain(s)) about 30 nm in length. The transformation from collagen to gelatin is done in three stages: pretreatment of raw material, followed by gelatin extraction, and finally its purification/drying. The pretreatment can be done under acidic or alkaline conditions (sodium carbonate, or sodium hydroxide) which gives different types of gelatin called respectively type A (isoelectric point 6 to 9) and type B (isoelectric point 5) (Alipal et al., 2021; Wang et al., 2017). Therefore different structures of gelatin exist depending on their source, and hydrolytic treatment meaning that the amino acid composition and sequence in the single chains vary between gelatins (Wang et al., 2017). Generally, gelatin is made of a variety of 18 amino acids with a majority of glycine, proline and 4-hydroxyproline residues (up to 57% of amino acids) (Alipal et al., 2021; Deshmukh et al., 2017). The abundance of certain amino acids, such as proline and hydroxyproline, is associated with a higher melting point, higher gelling temperature, higher thermal stability and lower viscosity (Wang et al., 2017). A schematic illustration of collagen hydrolysis into gelatin and their respective structures can be seen in Figure I-10.

Globally, gelatin is soluble in water and it can absorb 5 to 10 times its weight of water upon gelation. Gelatin has the capacity to form hydrogels at temperatures of 20°C to 30°C upon cooling. During the gelation process, locally ordered regions with a structure in triple helix



that a homogeneous weight distribution (i.e. removing low molecular weights from a gelatin sample) can improve the mechanical properties of the formed gels (Eysturskard et al., 2009b).

### **2.3.2 Bovine serum albumin (BSA)**

The BSA molecule is a globular non-glycosylated multifunctional protein made of 583 amino acids containing 17 cystine residues and therefore forming 8 disulfide bridges in addition to one free thiol group. It has a molecular weight of 66.4 kDa. The amino acid chain consists of three domains arranged in a heart-shaped structure (Figure I-11). Each domain is divided into two sub-domains. The secondary structure of the protein is mainly  $\alpha$ -helical (74%) while the rest of the protein occurs in turns of extended or flexible regions connecting the sub-domains. BSA is known for being poor in tryptophan (only two), methionine, glycine, and isoleucine while being rich in ionic amino acids (aa) such as glutamic acid and lysine (185 aa) conferring to the native globular protein a high solubility (Oliva et al., 2020; Topalá et al., 2014).

As mentioned earlier, the first step for the initiation of gelation for most proteins relies on a first step of denaturation. BSA denaturation begins at temperatures above 56°C at pH 8. However, a heating of 15 min at 65°C was not sufficient for the formation of a self-supporting hydrogel, and this was only possible above a heating of 15 min at 70°C and with the increase of temperature the hardness of the obtained gels increased (Matsudomi et al., 1991). In addition, a minimal concentration of 4% is needed for BSA to be able to form gels. Matsudomi et al., in 1991 showed that the presence of low amounts of calcium ions (5 mM), during heating, can yield gels with a higher hardness due to calcium bridging between proteins (Matsudomi et al., 1991).

Later on, Navarra et al. (2009) have reported a study on the gelation of BSA using a method called metal induced cold gelation. The method consisted of two steps, the first one was a heating step at 58°C for 2 hours, to induce denaturation. The second step consisted of cooling the mixture to room temperature and then adding copper or zinc ions to initiate gel formation.

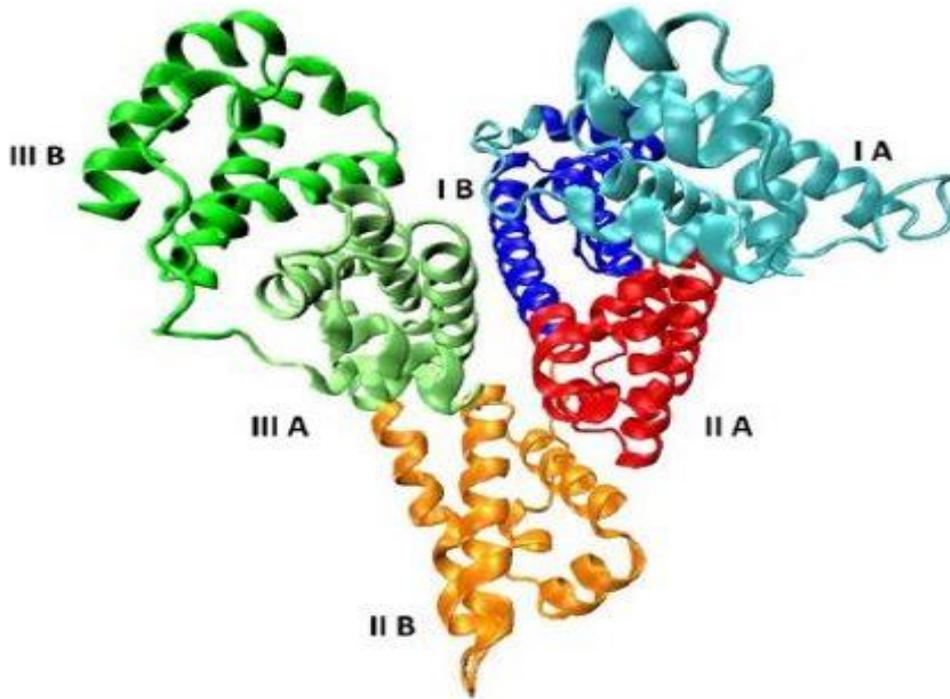


Figure I-11: BSA structure. The subdomains are indicated in different colors. Adapted from Oliva et al., (2020).

Concerning the ionic cross-linker effects, it was noted that for BSA at 1 mM the elastic moduli increased with the increase of the concentration of cations ( $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ ) until reaching 10 mM where it started decreasing (Navarra et al., 2009). The explanation given for this observation was that at pH values far from the isoelectric point ( $\text{I}_p$ ) of BSA (around 5) and at low ionic strength, the ions present in the solution are not sufficient to shield the charges present at the surface of the protein. These charges lower the formation of large aggregates and therefore favor the expansion of the network in a linear direction leading to a network with fine and filamentous structures. This structuration in a filamentous network gives gels with high elastic moduli that are transparent because of the small dimension of aggregates present in the solution. On the opposite, adding ions at higher concentrations ( $> 10$  mM for 1 mM BSA) decreases the energy barrier between proteins, and hence facilitates their contact. Under these conditions, the gels are formed of random spherical aggregates connected together in a structure called particulate gel. Therefore, the obtained gels are coarse, opaque, and brittle with a low elasticity. At intermediate protein/ions ratio, both filamentous and random spherical networks can exist together. Concerning this metal induced cold gelation

reported by Navarra et al. (2009), the effect of cations could be resumed in two steps: the first being a neutralization of the charges allowing proteins to interact with each other, and the second is a bridging between two adjacent molecules.

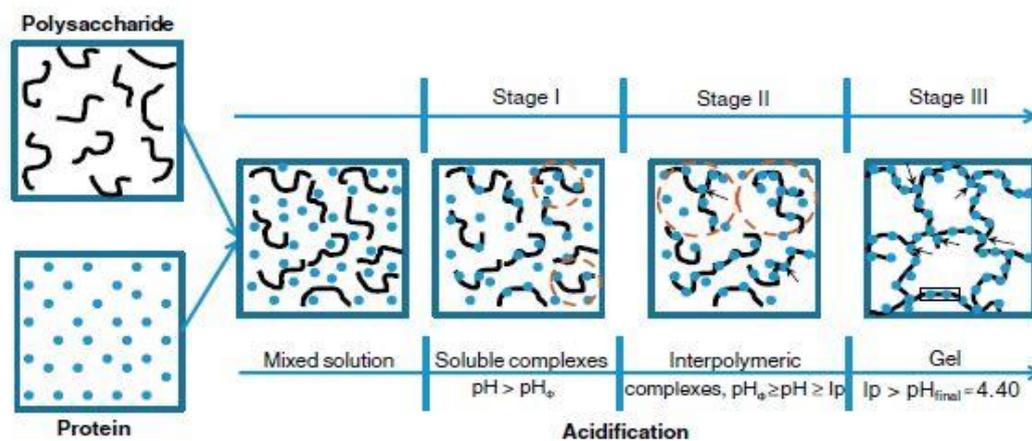
BSA hydrogels can be used in various applications such as in biotechnology, pharmaceutical, and food applications. They might be used in medical applications particularly in therapy/understanding of some illnesses like the Menkes and Wilson disease due to their capacity to interact with  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  (Navarra et al., 2009). Potential applications have also been discussed in literature concerning their capacity for encapsulation, protection, and delivery of some bioactive compounds including drugs and probiotics (Chen et al., 2019).

#### **2.4 Mixed hydrogels**

Interest has risen in combining polysaccharides and proteins with gelling capacities together to improve the characteristics of the formed hydrogels. The combination of alginate with gelatin was done through the oxidation of alginate to form alginate dialdehyde (ADA) which is later on covalently cross linked with gelatin (GEL). The combination called ADA-GEL shows a higher biodegradability, better cell adhesion, proliferation and migration properties in comparison with alginate alone, allowing more utilities in the medical field for these two polymers (Labowska et al., 2021).

Forming hydrogels consisting of BSA and alginate have also been tested by Neiser et al. (1999). Heat denaturated gels of BSA with alginate were prepared and compared to pure BSA hydrogels. The BSA/Ca-Alginate hydrogel had a higher Young's modulus than the pure BSA hydrogel (without calcium) at the tested pH range between 5.7 and 7.1. Further results elucidated the possibility of interaction between BSA and alginate networks through electrostatic cross-links or entanglements. The study concluded that the strengthening effect of alginate on BSA hydrogels could be called a synergism that is enhanced by the presence of calcium (Neiser et al., 1999). Another study led by Zhao et al. (2009) showed that BSA in both its native and denatured forms was able to interact with alginate through electrostatic linkages between its positively charged amino acids and the anionic polysaccharidic molecules.

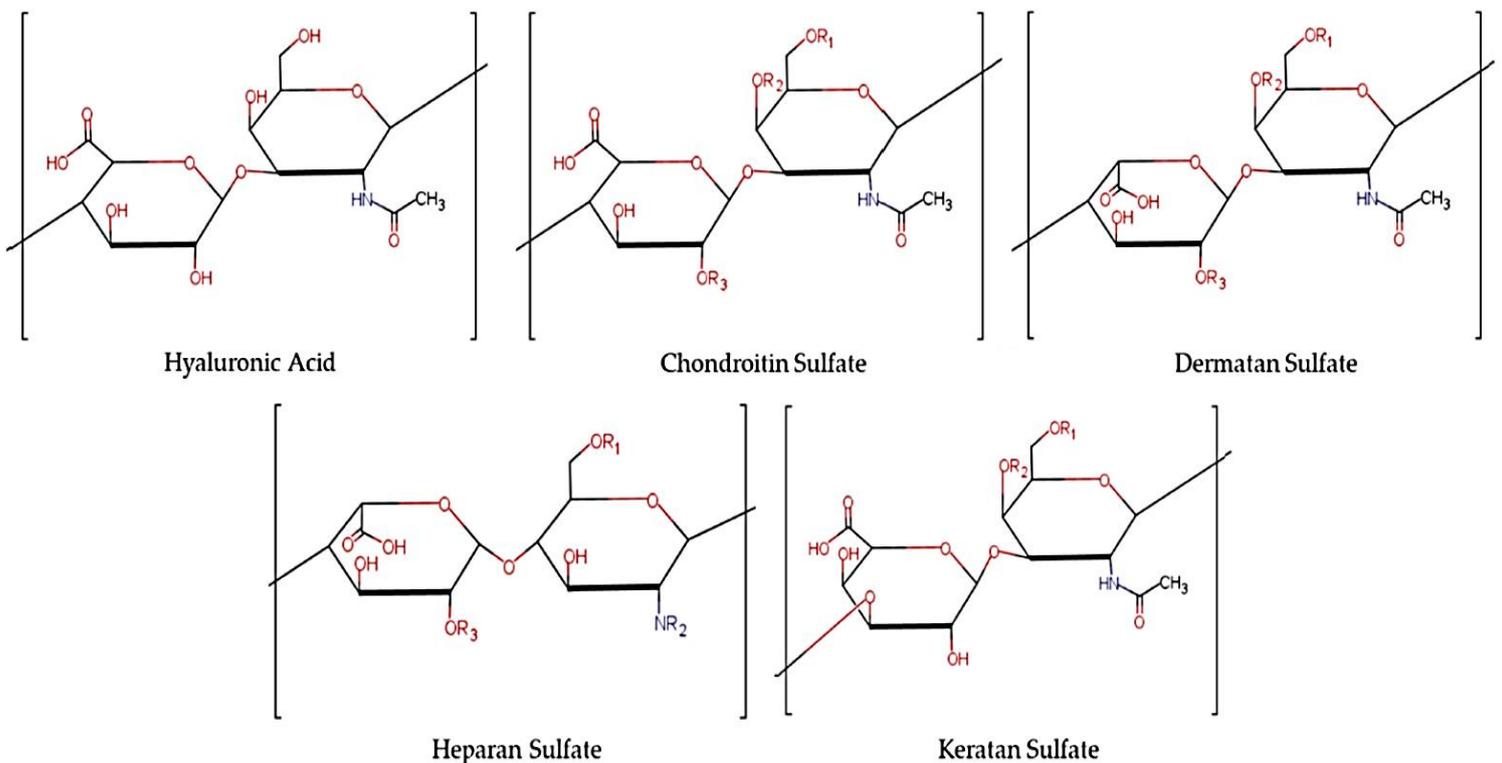
Another example of mixed hydrogels consists of forming gels using xanthan gum (a glucopyranose glucan with a side chain of glucuronic acid and mannose) with  $\beta$ -lactoglobulin (Patel et al., 2020; Le et al., 2017). The gelation of the  $\beta$ -lactoglobulin-xanthan gum mixture happened in three stages (Figure I-12). During initial acidification and as the pH approaches the isoelectric point of the protein, the interaction between the positively charged groups of  $\beta$ -lactoglobulin with the negatively charged groups ( $\text{COO}^-$ ) of the xanthan gum results in the formation of soluble complexes (stage 1). With further acidification, stage two begins where more proteins can aggregate on the xanthan gum chains reducing therefore the net charge of the soluble complexes which tend to form interpolymer aggregates due to the formation of junction zones between two xanthan gum polymers. In this case, the  $\beta$ -lactoglobulin molecule may be considered as the crosslinker. During stage 3, the electrostatic associative interactions increase, resulting in the transition from sol to gel (Le et al., 2017).



**Figure I-12: Stages of the formation of the mixed  $\beta$ -lactoglobulin-xanthan gum during acidification with pH: pH of the mixture,  $\text{I}_p$ : isoelectric point of the protein,  $\text{pH}_\phi$ : pH of formation of interpolymeric networks complexes (Le et al., 2017).**

Mixed hydrogels can also be composed by mixing two types of polysaccharides, each one providing some specific mechanical properties. The water retention ability and elastic properties of alginate make it a relevant candidate to use in mixed hydrogels. Chitin is a polymer of N-acetyl-D-glucosamine and its deacetylated form is called chitosan. Mixing alginate with chitosan to form hydrogels through calcium cross-linking has been extensively used for its controlled drug release capacities in the pharmaceutical industries (Abreu et al., 2008; Rinaudo, 2006). Another example is mixing alginate with glycosaminoglycans (GAGs) which are a family of glycans constituted of long, linear anionic polysaccharidic chains sharing common repeating disaccharide units composed of sulfated or non-sulfated uronic acids

(glucuronic or iduronic acid) or galactose, coupled to amino sugars (glucosamine or galactosamine). GAGs can occur with different sulfation degrees and patterns that affect their biological roles. Some GAGs can be non-sulfated like hyaluronic acid and some can be sulfated such as heparin, heparan sulfate, and chondroitin sulfate (Figure I-13) which can be found in both vertebrates and invertebrates either on the cell surface or in the extracellular matrix and play a key role in cell-cell interactions as well as cell-matrix interactions (Collic-Jouault et al., 2023; Neves et al., 2020; Sodhi and Panitch, 2021). GAGs can interact with various proteins present in their environment and these interactions are mainly responsible for their biological roles. GAGs-like polymers were speculated to have the same functions as those in vertebrates such as cellular signaling, binding cations, forming hydrogels... (Felz et al., 2020b).



**Figure I-13: Structure of the repeating disaccharide unit of major GAGs. R indicates a potential sulfation point (Sodhi et al., 2021).**

The sulfation patterns of GAGs not only affect their biological roles but also their binding strength with proteins and also with calcium (Collic-Jouault et al., 2023; Gandhi and Mancera, 2008). Combining alginate with chondroitin sulfate allowed the formation of hydrogels in presence of calcium ions through the egg box mechanism. Depending on the sulfate position in the chondroitin sulfate molecule, the interactions with calcium ions were different. The sulfation at the 6-position of the N-acetyl-glucosamine enhanced the Ca<sup>2+</sup> binding compared

with sulfonation at the 4-position. Alginate with chondroitin sulfate hydrogels exhibited a better mechanical stability than alginate hydrogels (Liu et al., 2016).



## 2. Highlights on “Hydrogels from biopolymers”

As a conclusion to section 2, it can be noted that hydrogels have interesting potential applications and can be obtained through the use of polysaccharides and proteins alone or in mixtures.

The characteristics of the obtained gels depend on the polymer used and the external conditions as well. Intrinsic factors influencing the gelation include the molecular weight, the polymers' monomer composition, the charge density, and the conformation among other molecular characteristics.

Concerning polysaccharides, both the occurrence of negatively charged groups and their position inside the chain are important factors for the formation of a gel through calcium bridging. Sulfate and/or carboxylic groups are key functional groups in the gelation.

Protein-based hydrogels require a first step of denaturation leading to ordered molecular structures tending to form a network if the necessary conditions are present. A high number of variables control the protein-based hydrogels such as protein concentration, appropriate divalent cation, and stabilization of the unfolded forms.

The importance of extrinsic factors, such as pH, temperature, and the presence of specific counter ions on gelation cannot be neglected. Variations of these extrinsic factors might provoke changes in the conformation. The variations in these intrinsic and extrinsic factors lead to hydrogels with different properties and hence different application potentials.

Combining polymers with interesting gelling properties can lead to the formation of mixed hydrogels with improved hydrogels' characteristics and the possibility to diversify applications.

EPS extracted from aerobic granular sludge show promising hydrogel forming capacities, but understanding their chemical structure and diversity is a must before diving deeper into their hydrogel forming abilities.

### **3 Structural Extracellular polymeric substances: chemical diversity and structural function**

Densified aggregates such as biofilms or granules are three-dimensional structured communities made of two major components which are the bacterial cells and the EPS matrix in which cells are embedded (Flemming and Wingender, 2010). In the case of granules, these EPS can have different origins: they can originate from the effluents being treated, from the bacterial excretion or from intracellular material due to cellular lysis (Liu et al., 2004; Tan et al., 2021). Exploring the chemical diversity of EPS within the aerobic granule and the polymers that were identified in this matrix is crucial because it allows to define the roles of EPS within the granules, and helps in guiding the choice of extraction methods able to target EPS with specific functions such as EPS having cohesive and aggregative properties. Therefore, the next paragraph will be dedicated for the EPS biochemical diversity within granular sludges.

#### **3.1 EPS chemical diversity within granular sludges**

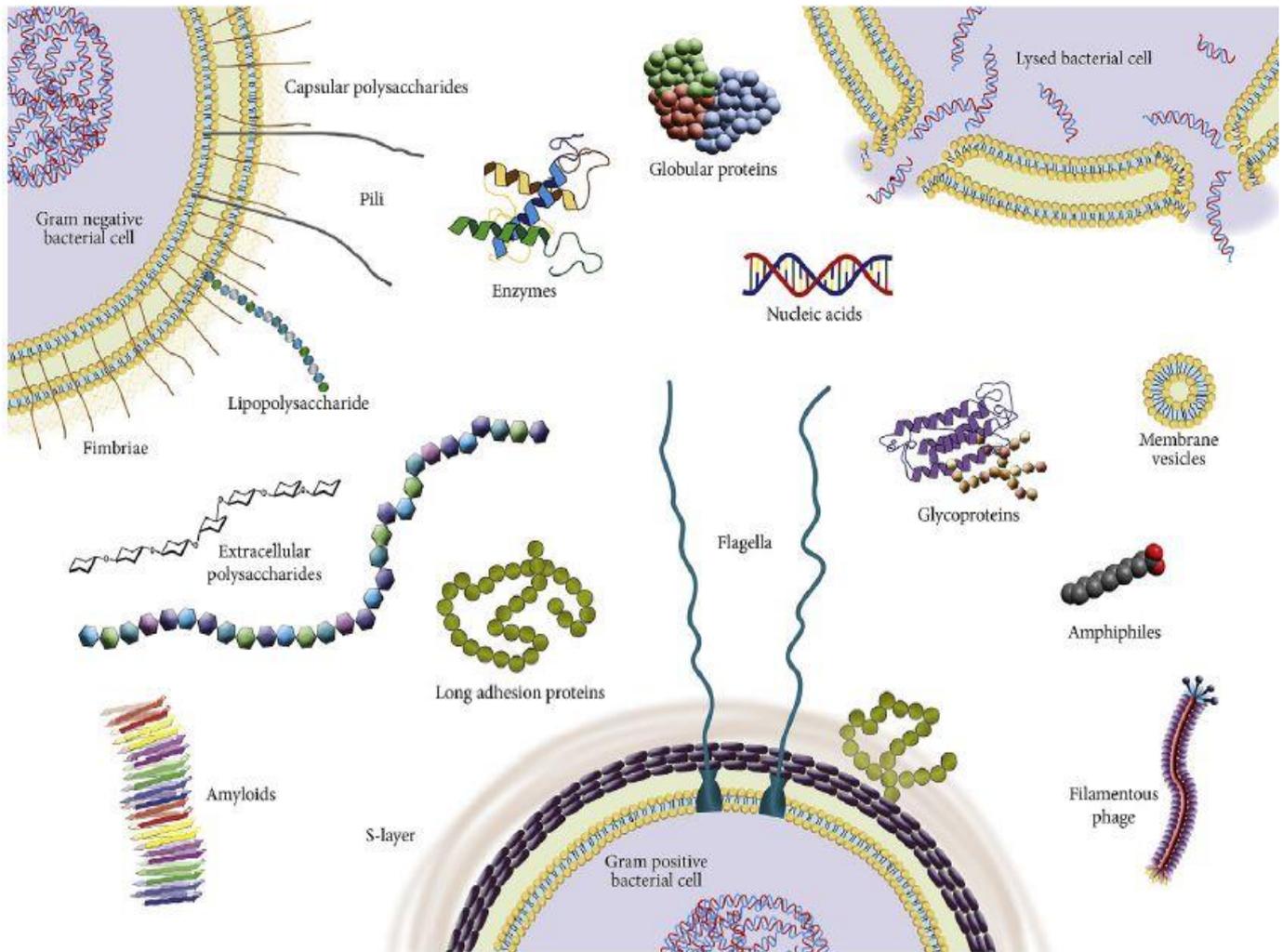
The diversity of EPS is due to many factors among which the microbial populations that are present within the biofilm, their physiological state or the external conditions (Liu et al., 2004; Seviour et al., 2019). In fact, it was shown that within granular sludges a large number of different bacterial communities can coexist, and this bacterial diversity can reflect the EPS diversity. This was proven through 16S rRNA gene sequencing of the bacterial communities present within aerobic granules (Layer et al., 2019). Moreover operational conditions can influence the produced EPS (Schambeck et al., 2020) and a wide variety of molecules have been reported to be present within the EPS matrix, including polysaccharides, proteins, lipids, nucleic acids, and humic acids among others (Felz et al., 2020b; Lin et al., 2010; Schambeck et al., 2020; Seviour et al., 2019) (Figure I-14). Since many factors control the production of EPS, this would explain their high diversity in terms of the quality/quantity reported in literature.

An evidence of the molecular diversity within the EPS matrix is the high diversity in the molecular weight of EPS extracted from aerobic granules. Indeed, Caudan et al. (2012) have shown that the EPS extract is composed of molecules that have a molecular weight ranging from more than 1000 kDa to less than 1 kDa. Even though many different compounds have been found to compose the EPS matrix, two essential components are reported in the studies regarding the EPS matrix, which are polysaccharides and proteins (Caudan et al., 2014; Lin et al., 2010; Oliveira et al., 2020; Schambeck et al., 2020; Seviour et al., 2012b). Caudan et al.

(2012) showed that the protein to polysaccharide ratio (PN/PS) is equivalent to 3.6, meaning that there are 3.6 more proteins than polysaccharides in the extracted EPS. The same observation was done by Oliveira et al., in 2020 through the use of the widely used colorimetric methods and noted a higher PN/PS ratio varying between 6 and 8 approximately. Moreover depending on the extraction method the PN/PS values can differ for the same granular sludge, using NaOH for the extraction yielded a PN/PS of 10.9 while extraction performed with a DOWEX cationic exchange resin gave a PN/PS of 7.6 (Mcswain et al., 2005). All these results show that proteins are an important part of the EPS matrix and these molecules are supposed to participate to the hydrophobicity properties of aggregates (Sheng et al., 2010). Their structural role in the granule by linking with each other was evidenced by enzymatic treatment with a protease (savinase), upon which granules showed cohesion disturbances (Caudan et al., 2014). It was reported that proteins forming the EPS matrix are composed of about 25% negatively charged amino acids, hence they can participate to the globally negative surface charge of aggregates (Sheng et al., 2010). Interactions could exist between positively charged amino acids with negative charges of carboxyl or phosphate groups from other polymers leading to neutral structures. In fact, proteins are made of a sequence of amino acids called the primary structure. Each amino acid has a side chain "R" that confers different properties and the amino acids can be charged positively or negatively, hydrophobic or hydrophilic, polar or apolar. These amino acids assemble in  $\alpha$ -helices or  $\beta$ -sheets constituting the secondary structure. The tertiary structure of proteins is the three-dimensional shape of the proteins that depends on the positioning of the amino acids inside the final protein structure leading to the formation of globular proteins such as BSA or fibrous proteins like gelatin. The association of different polypeptidic chains together forms the quaternary structure of proteins, an example of such association is amyloid proteins.

Amyloid proteins, are usually thin fibrils, insoluble and extremely resistant to denaturants and they are formed by  $\beta$ -sheets stacked perpendicularly along the fibril axis (Figure I-14). Amyloid-like adhesins were identified in the EPS matrix of activated sludge and are thought to play a role in the adhesion of bacteria together (Larsen et al., 2008). Glycosylated amyloid-like proteins were also found in aerobic granules enriched with ammonium oxidizing bacteria (Lin et al., 2018). Moreover, the presence of amyloid-like aggregates was established in anammox granular sludges and their role in determining the mechanical properties of the parent biofilm

was elucidated using Thioflavin T assays (Lotti et al., 2019). The presence of glycoproteins was also found in activated and anaerobic granular sludge through their detection by periodic acid Schiff after SDS-PAGE electrophoresis (Bourven et al., 2012).



**Figure I-14: The biochemical diversity within the EPS matrix of biofilms (Seviour et al., 2019).**

In addition, complex polymers extracted from aerobic granular sludges have been described in literature such as hyaluronic acid like polymers and sulfated glycosaminoglycans like polymers. Evidence for the presence of chondroitin sulfate-like and Heparan sulfate-like polymers was based on the sensitivity of polymers to enzymatic digestion with their respective specific enzymes (Felz et al., 2020b). The presence of sialic acids and sulfated-GAGs was also reported in EPS extracts from anammox granular sludge. An amount of 1.6 % of the volatile solids (VS) was attributed to sialic acids by using the N-acetylneuraminic acid as standard and 2.4 % of VS as sulfated-GAGs using a chondroitin standard (Boleij et al., 2020). As indicated in Figure I-15, glycosaminoglycans can be implied in many functions within the granule including adhesion, protection of the microorganisms and scavenging of other components present

within the matrix. Concerning sulfated polysaccharides they can also be implied in the adhesion and have a role in the regulation of receptors (Tan et al., 2021).

The presence of lipids have also been reported in aerobic granular sludges (Meng et al., 2020). Lipids are a diverse and ubiquitous group of compounds, a precise definition of this group is hard to give, and they have been defined loosely as organic compounds that are not soluble in water but in organic solvents. They are usually hydrophobic or amphiphile (Fahy et al., 2011).

DNA are the polymers that carry the genetic information within the cells, and are found in the EPS matrix of granular sludges, through secretion by bacterial cells or after their lysis (Oliveira et al., 2020; Sheng et al., 2010). They are hydrophilic molecules that are highly negatively charged due to the presence of many phosphate groups. It was found that for aerobic granular sludge the highest concentration of DNA was in tightly bound EPS extracted using cation exchange resin (Rusanowska et al., 2019). A structural role has been attributed to these molecules and DNA is particularly known to be implied in the fixation of cells to surfaces facilitating the development of biofilms (Monticolo et al., 2020).

Hence, the EPS matrix is far more complex than it was once thought to be, and different biochemical groups exist in this matrix (Figure I-14). These EPS contribute to different functionalities within the EPS matrix such as the adhesion to surfaces, cohesion between bacteria forming the biofilm among other roles (Tan et al., 2021) as seen in Figure I-15. Indeed, through the interactions between the polymers described above, the EPS matrix gets to contribute to the stability and cohesiveness of the biofilm as well as its characteristics. However, the lack of knowledge about EPS composition and functions remain significant and, as raised by several authors in 2019, more research is needed to have a more holistic view of the EPS matrix composition (Seviour et al., 2019).

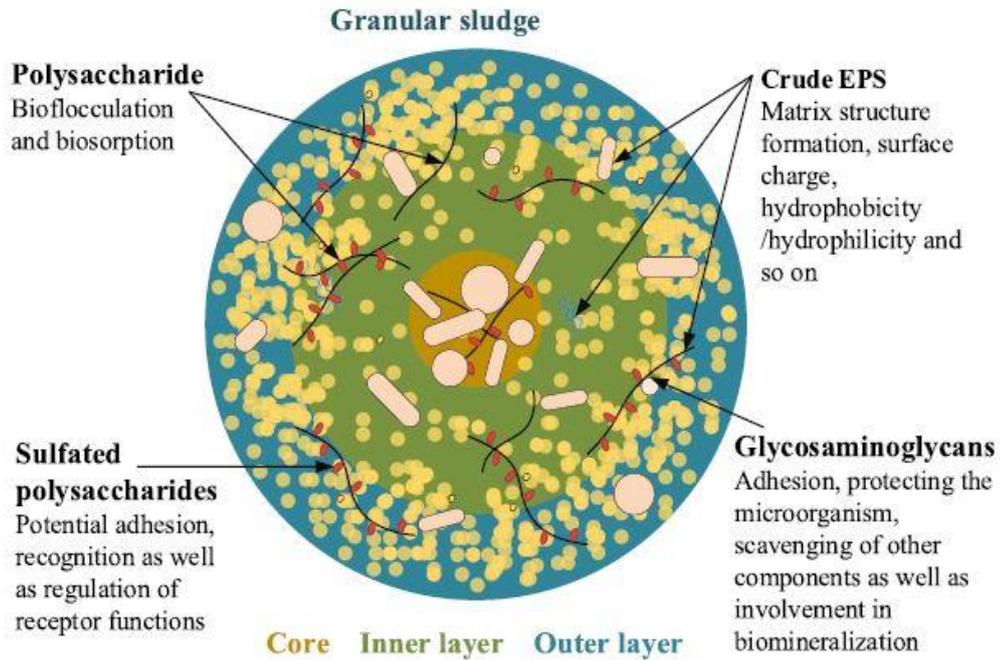


Figure I-15: Roles and distribution of EPS within granular sludges (Tan et al., 2021).

### 3.2 Chemical interactions and spatial distribution within granular sludges.

A model of the EPS matrix was proposed by Nielsen and Jahn in 1999 where two layers of EPS can be noticed additionally to soluble EPS (Figure I-16). The loosely bound EPS region is less dense/compact than the tightly bound EPS layer. This model is hence related to the strength of the bonds linking EPS within the granular matrix and the capacity of extraction methods to detach them.

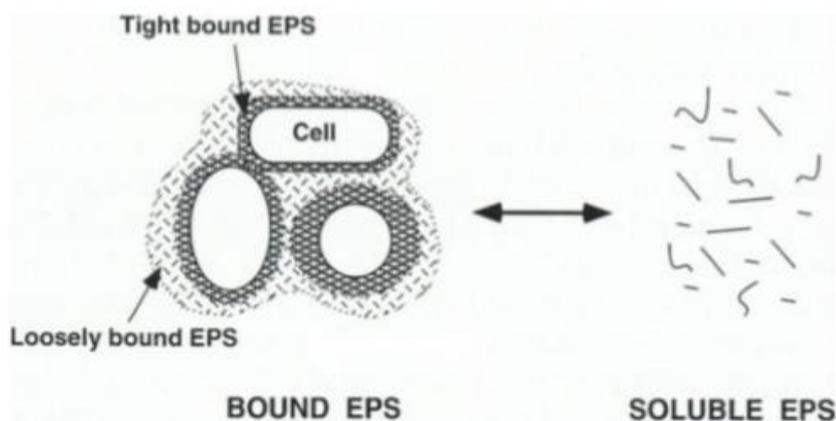


Figure I-16: EPS distribution around cells, and their degree of attachment to them (Nielsen and Jahn, 1999).

Polymers tend to form weak bonds within the EPS matrix. Their binding is mainly due to low energy linkages such as hydrogen bonds, van der Waals, electrostatic and hydrophobic

interactions (Wingender et al., 1999). Divalent cations bindings have also been shown to play a major role in the structure of the EPS matrix (Ras et al., 2008a). Indeed, they are thought to be responsible for the aggregation of anionic proteins (Caudan et al., 2014, 2012) and in another study the implication of covalent bonds such as disulfide bonds have been noted (Emerson and Ghiorse, 1993). A summary of the most common bonds within the EPS matrix can be seen in Figure I-17.

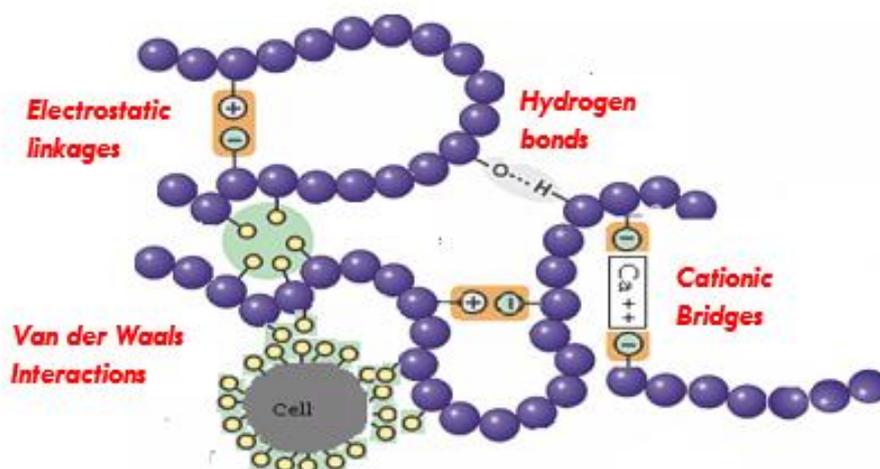
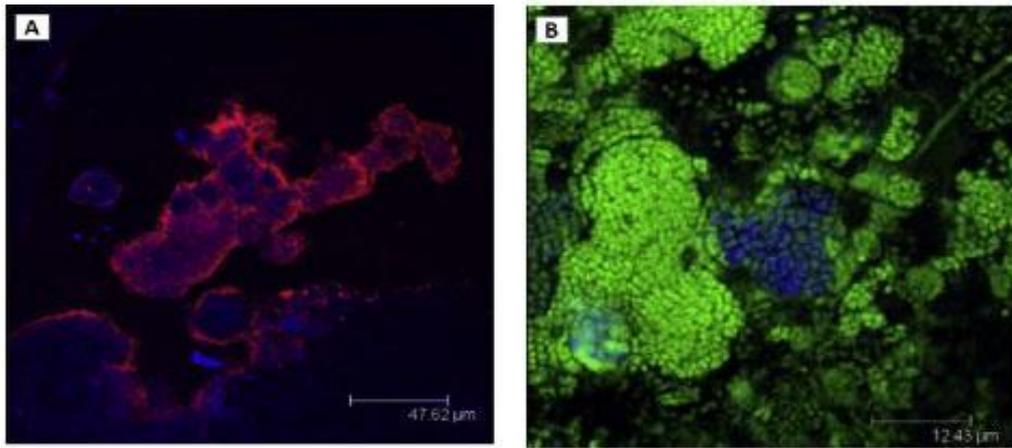


Figure I-17: Chemical interactions within the EPS matrix (Monique Ras: unpublished).

A study done by Caudan et al. (2014) using confocal laser scanning microscopy (CLSM) tried to determine the spatial distribution of EPS within the extracellular matrix of the aerobic granule. The granule was first fixated and then cut at  $-21\text{ }^{\circ}\text{C}$  into  $40\text{ }\mu\text{m}$  slices using a cryostat. They used fluorescent markers including FITC that is an amine reactive dye staining all proteins and amino-sugars, ConA conjugated with tetramethyl rhodamine was used to stain  $\alpha$ -mannopyranosyl and  $\alpha$ -glucopyranosyl sugar residues along with other fluorescent probes for bacteria detection. The microscopy results (Figure I-18) showed that carbohydrates containing glucose or mannose residues formed a capsular layer surrounding some clusters of bacteria and that proteins seemed to form an intercellular cement uniformly distributed inside the clusters and in direct contact with bacteria (Caudan et al., 2014).



**Figure I-18:** CLSM images of granules cut by a cryomicrotome and stained by Con A in red for carbohydrates and Eub mix FTIC targeting all bacteria in blue (A) or FITC staining all amine groups in green and NOS mix Cy3 marking ammonium oxidizing bacteria in blue (B) (Caudan et al., 2014).

The study continued by doing enzymatic attacks on the granule targeting  $\alpha$ -(1-4)-glucans and proteins among others. Upon the attack by these enzymes the authors showed that  $\alpha$ -(1-4)-glucans and proteins are key polymers for granule cohesion. The use of the chelator EDTA on granules showed that cationic bridging plays a major role in the aggregation of granules and that calcium is especially involved in bridging proteins. As suggested by the above-mentioned microscopic observations and enzymatic digestions, it was concluded that proteins might constitute a sort of intercellular cement that reinforces cohesion inside bacterial clusters, whereas carbohydrates were arranged in a capsular layer surrounding bacterial clusters in a hydrogel-like barrier. A model was proposed for the structure of the aerobic granule as seen in Figure I-19 (Caudan et al., 2014). Another study, attacked aerobic granular sludges with different types of enzymes including a Lipase, RNase, DNase, protease,  $\alpha$  and  $\beta$ -amylases. They found through the measurements of the loss modulus ( $G'$ ) that proteins and  $\alpha$ -polysaccharides were the major granule structural materials and granules were described therefore as protein–polysaccharide composite hydrogels. Another study conducted on aerobic granules dominated by *Deffluviococcus* II showed the presence of an acid soluble EPS that was present as tightly bound capsular EPS around bacterial cells (Pronk et al., 2017).

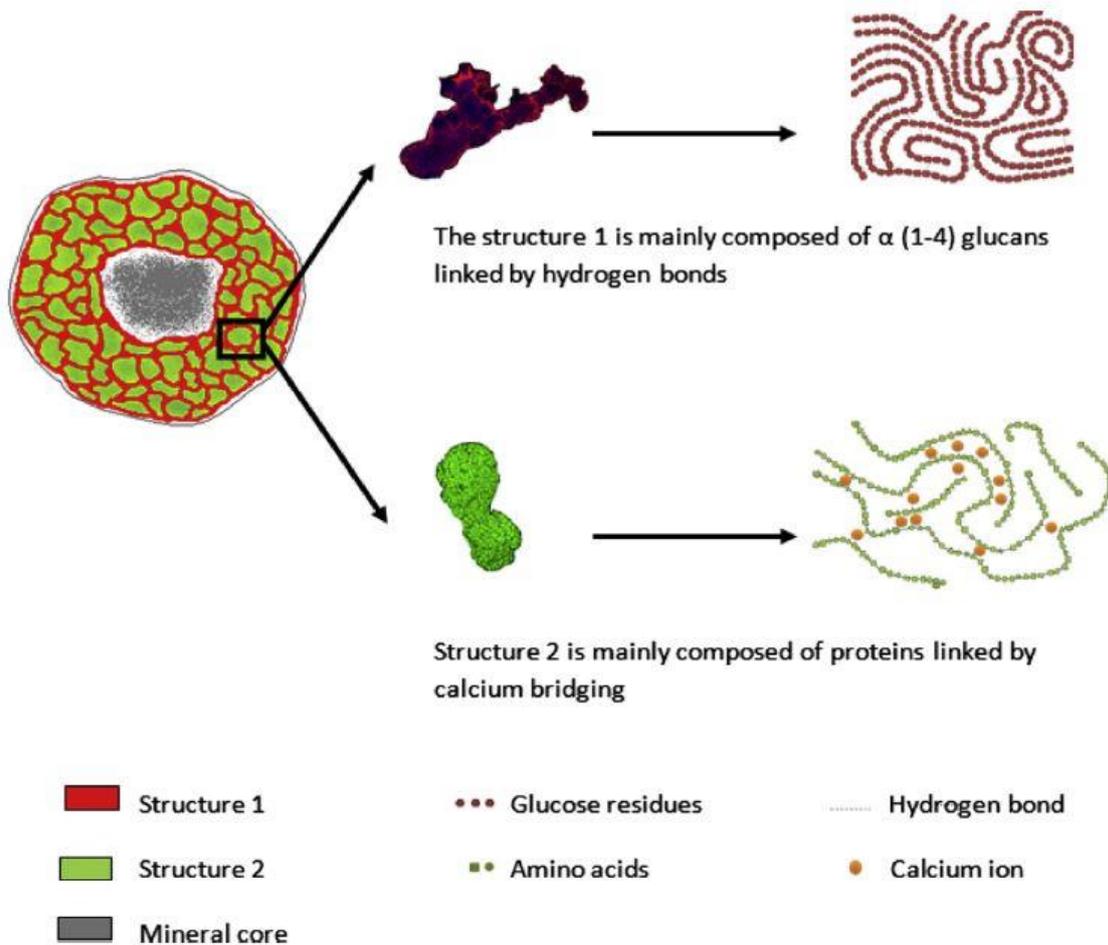
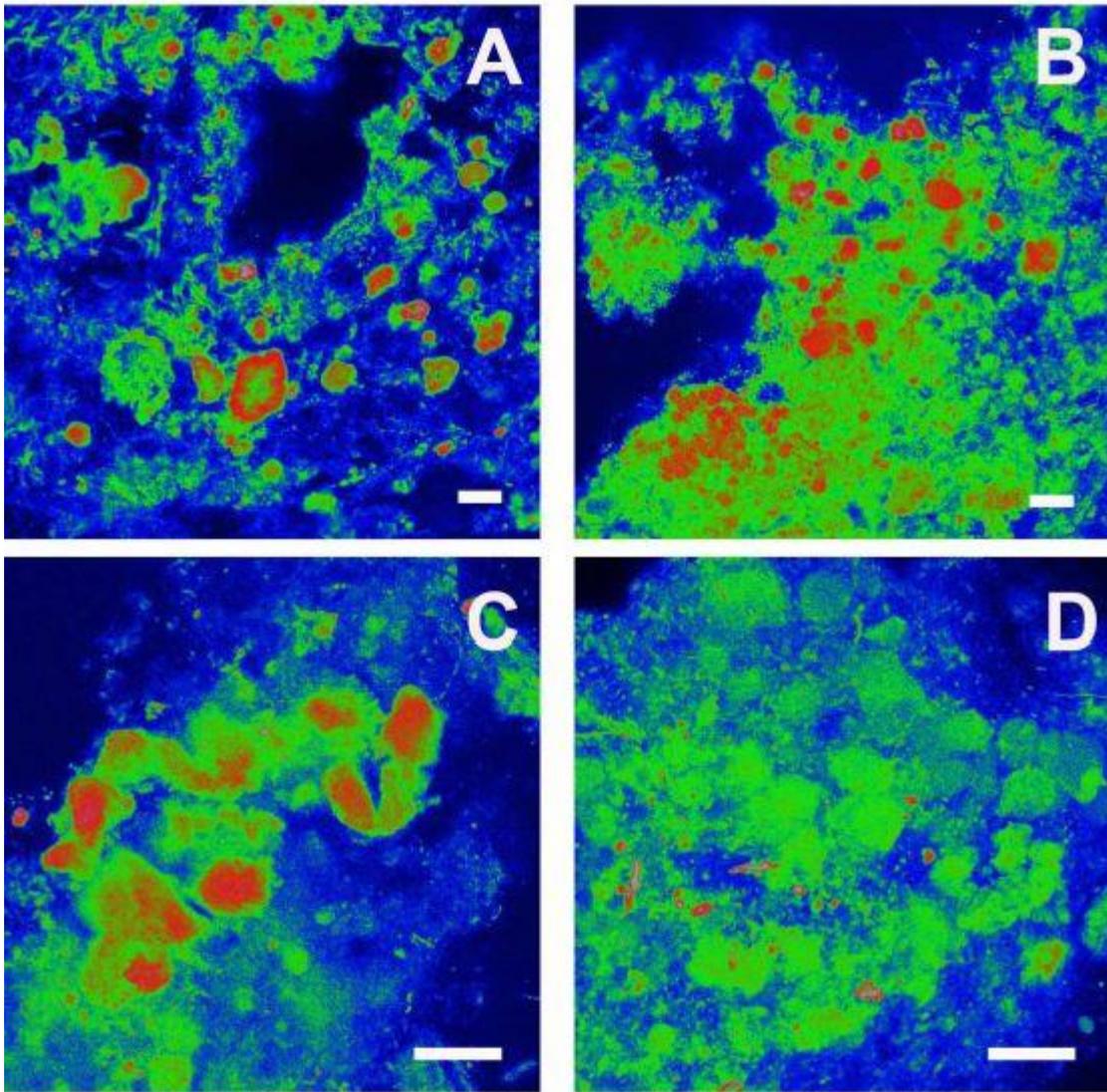


Figure I-19: A model showing the interaction of polymers and their spatial distribution within the general structure of an aerobic granule (Caudan et al., 2014).

The distribution of anionic polymers can be mapped through the use of Heparin Red staining and results are shown in Figure I-20. This staining allows the visualization of polyanionic molecules and the obtained color is based on the negative charge density (Felz et al., 2020b). It was observed that the negative charges were not only present in the microcolonies but also on the outer boundaries (with a high negative charge density) and in the extracellular matrix in between the colonies. The study continued by staining hexosamine containing glycoconjugates such as GAGs with different lectins that were visualized in the microcolonies (capsular region or in between capsules) and in the extracellular matrix.



**Figure I-20: Heparin Red staining of aerobic granules. Blue= no binding of heparin, red= binding, green= strong binding (high density). A and B show the globular substructure of a granule, while C and D are closeups on colonies (Felz et al., 2020b).**

A study led on anammox granular sludge proved a high abundance of glycoproteins within the granule. Particularly an 80 kDa glycoprotein was studied furthermore and it was attributed to the S-layer. Indeed, S-layer proteins are often glycosylated and are present on the cell surface of prokaryotes allowing interactions between the cell and its environment (Boleij et al., 2018).

### 3.3 Structural EPS as gel-forming polymers

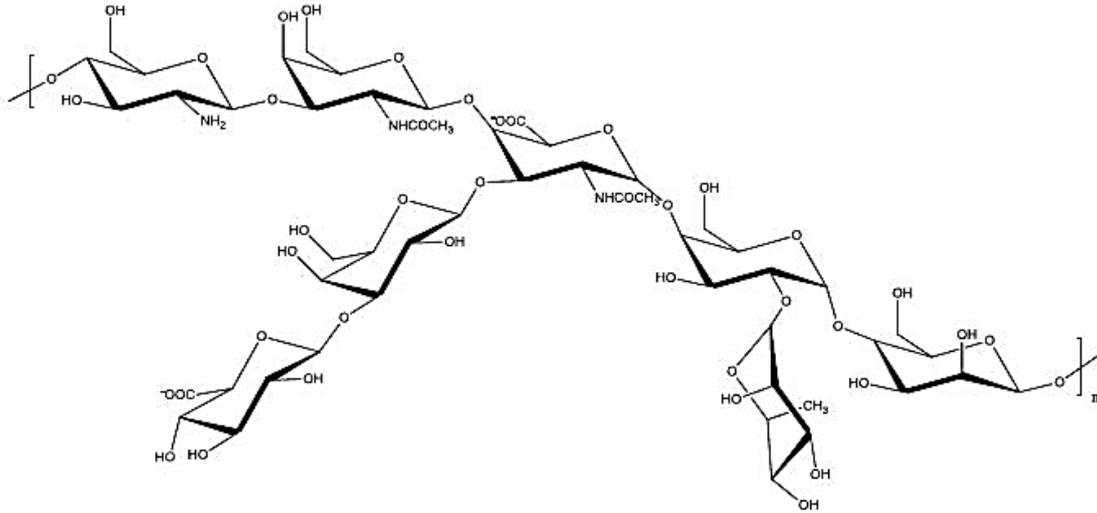
Recently, emerging criteria have been proposed for the denomination of EPS based on their specific chemical, physical and structural properties; for example, they can be divided according to their charge into anionic/cationic; they can also be classified as polar or non-polar; acidic/alkaline. Because of the high cohesiveness of granules, another recent denomination was “structural EPS” for polymers able to form hydrogels in vitro and supposed

to be largely involved in the aggregation and structuration of aerobic granules (Feng et al., 2021; Tan et al., 2021). Indeed, sEPS are the subset of the EPS matrix that provide the physical structure of the granule and are responsible for the mechanical properties of the biofilm (Boleij et al., 2018; Lotti et al., 2019).

The cohesiveness of the EPS matrix was associated with the presence of gel-forming polymers like ALE and granulan which are considered as a major part of sEPS. Those sEPS are very complex and contain polysaccharides, proteins, DNA, humic substances among others (Oliveira et al., 2020; Schambeck et al., 2020). The extraction protocol using an alkaline extractant (NaOH or Na<sub>2</sub>CO<sub>3</sub>) is not strictly specific for gel-forming polymers explaining the big diversity of polymers extracted. Further enrichment in gel-forming polymers, particularly ALE can be done through an acidic precipitation step that allows the enrichment in gel-forming polymers followed by a precipitation using cold absolute ethanol (Lin et al., in 2010). These operations performed during the purification of seaweed alginate, were supposed to select the polysaccharidic fraction but no evidence was clearly provided to confirm such purification. Nevertheless, the ALE fraction showed maximum absorbance at 485 nm with the phenol-sulfuric acid assay, indicating that it's made of a majority of uronic acids; in addition, they used the ruthenium red staining to detect the presence of C-O-O<sup>-</sup> groups and the results indicated the presence of many of these groups. Fourier transform infrared spectroscopy (FTIR) was used for the detection of functional groups present and the presence of O-H, C-O-C, C-O, C-H groups were noted, going in favor of a structure similar to alginate. MALDI-TOF MS also confirmed the presence of uronic acids (Lin et al., 2010).

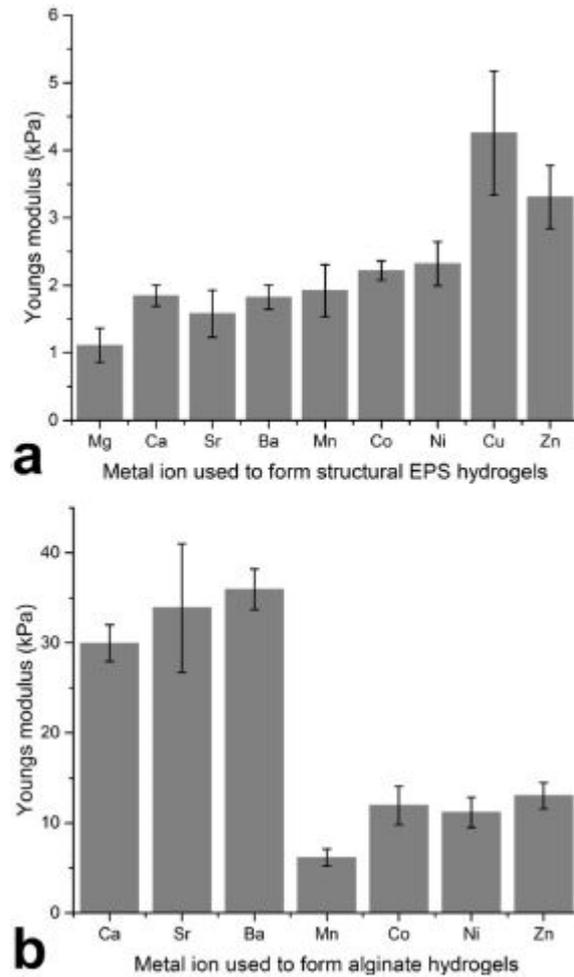
Another sEPS described in literature with gel-forming capacities is granulan, a complex heteropolysaccharide with a repeating sequence of galactose, mannose, glucosamine, N-acetylgalactosamine and 2-acetoamido-2-deoxygalactopyranuronic acid (Figure I-21) and a disaccharide branch of  $\beta$ -galactose and  $\beta$ -glucuronic acid attached to the 2-acetoamido-2-deoxy- $\alpha$ -galactopyranuronic acid (Seviour et al., 2012b). The presence of C-O-O<sup>-</sup>, and O-H, among other groups give to the polymers a hydrophilic character allowing their solubilization in an aqueous medium, especially at high pH because it will favor the negative charges on the carboxylic groups increasing repulsion between polymers and the interaction with water

molecules. The gel formation mechanism of granulan is based on the formation of symmetrical antiparallel helices stabilized by hydrogen bonds between the identical chains. The presence of calcium ions facilitates bridging between helices through their  $\beta$ -galactose- $\beta$ -glucuronic branches and leads to the formation of stronger gels (Seviour et al., 2012a).



**Figure I-21: The chemical structure of granulan (Seviour et al., 2012b) consists of a main chain from left to right of  $\beta$ -glucosamine, N-acetyl- $\beta$ -galactosamine, 2-acetoamido-2-deoxy- $\alpha$ -galactopyranuronic acid,  $\alpha$ -galactose,  $\beta$ -mannose. It had a disaccharide branch of  $\beta$ -galactose and  $\beta$ -glucuronic acid attached to the 2-acetoamido-2-deoxy- $\alpha$ -galactopyranuronic acid.**

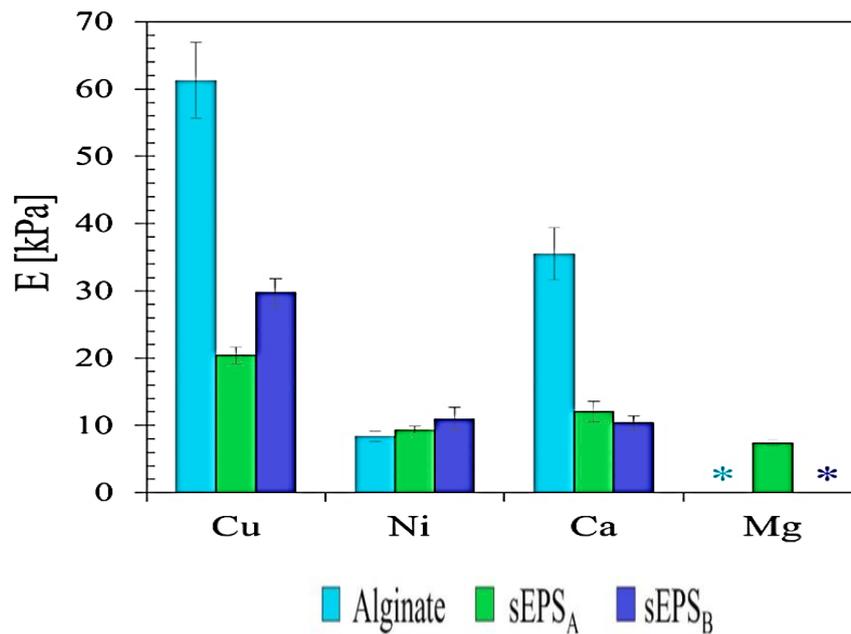
The characteristics of the gels obtained with sEPS depend on many factors and in particular, the type of ion used for the gel formation can greatly influence the gel's mechanical strength (Felz et al., 2020a; Pagliaccia et al., 2022b). In the study led by Felz et al. (2020a), a comparison was done between 2.5 % (w/v) alginate and 10 % (w/v) sEPS hydrogels using different cations: sEPS hydrogels formed using copper and zinc ions had the highest Young's modulus equivalent to approximately 3.7 kPa (Figure I-22a) whereas alginate had a greater affinity to calcium, strontium and barium ions with a Young's modulus around 35 kPa (Figure I-22b).



**Figure I-22: Young's modulus of sEPS (a) and alginate (b) hydrogels using different divalent cations (Felz et al., 2020a).**

These differences can be explained by molecular differences between the two tested samples since the ion selectivity is influenced by the available functional groups and the conformation of the polymers (Felz et al., 2020a). Indeed, alginate interacts with calcium only through the carboxyl groups present on its backbone (preference for G residues) (Lee and Mooney, 2012) whereas sEPS are more biochemically diverse and hence different functional groups could participate in the gelation such as phosphates, sulfates, amino and hydroxyl groups (Felz et al., 2020a; Pagliaccia et al., 2022b). Proteins have been shown to bind with copper and zinc, and many amino acids have a higher stability constant with transition metal ions ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ...) than with alkaline earth metal ions ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ...). Considering chemical groups, phosphates were shown to bind with alkaline earth metals and polyphosphates were shown to favor transition metals over alkaline earth metals (Felz et al., 2020a). It was also demonstrated by another study that sEPS from different origins could react differently to the same divalent

cations (Figure I-23). The same mechanical strength was observed with calcium and nickel, but with copper significant differences were noticed between the two sEPS tested. With copper and calcium, alginate had a higher stiffness than the tested sEPS, but with Nickel this observation was inverted. Only sEPS<sub>A</sub> were capable of forming hydrogels with magnesium ions. These results indicate a heterogeneity within the sEPS composition of aerobic granules from different reactors, and the different reactivities of the polymers with divalent cations (Pagliaccia et al., 2022b).



**Figure I-23: Young's modulus of alginate and sEPS from different origins with various divalent cations (Pagliaccia et al., 2022b).**

In addition, it was shown that sEPS were not able to form stable hydrogels at concentrations below 0.1 M of calcium while alginate was capable of forming stable hydrogels at very low calcium concentrations of 1 mM (Figure I-24). Concerning sEPS<sub>A</sub> the increase of calcium concentration caused an increase in the mechanical strength; however, it was noted for sEPS<sub>B</sub> that upon reaching 1 M of calcium the mechanical strength decreased and this observation was attributed to the formation of precipitates on the surface of the hydrogel hindering the diffusion of calcium ions. This experiment showed that the choice of the calcium concentration is of great influence on the mechanical strength (Pagliaccia et al., 2022b). Finally, it was also noted that increasing the polymer's concentration led to an increase in the hydrogels mechanical strength (Pagliaccia et al., 2022b).

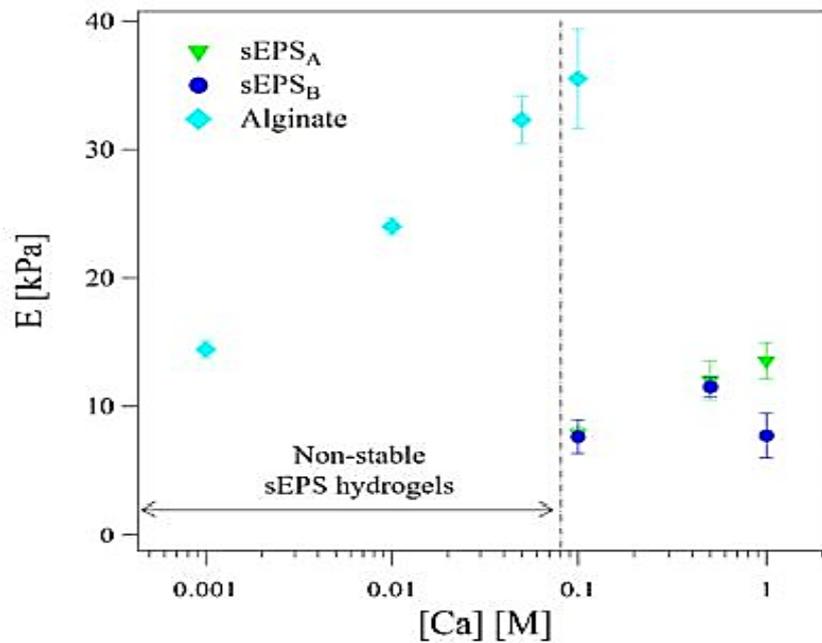


Figure I-24: The effects of the calcium concentration on the mechanical strength of sEPS from different origins and alginate (Pagliaccia et al., 2022b).

### 3.4 Potential applications of gel-forming EPS

Many potential applications have been reported in literature for gel-forming EPS. An example consists of using sEPS as additives to cement to improve their curing (Karakas et al., 2020), and they can also be used as heavy metal adsorbing polymers due to their proved capacity to bind to different heavy metals, metallic ions, dyes, antibiotics among others due to the presence of negatively charged groups within their structure (Cyzdik-Kwiatkowska et al., 2022; Liu et al., 2015; Tan et al., 2021).

It was shown that the water holding capacity of a 1% w/w sEPS based hydrogel is equivalent to  $99 \text{ g}_{\text{H}_2\text{O}}/\text{g}_{\text{sEPS}}$ . This water-holding capacity is typical of super absorbent polymers that are compounds able to absorb water equivalent to many times their original weight. This feature allows their use as raw materials alone or in combination with other materials for a myriad of applications; for example in paper and textile industries as a coating agent, as well as a soil enhancer/conditioner to increase the water holding capacity of soils affected with water scarcity or to reduce the leakage of fertilizers and to allow the slow release of agrochemicals in soils such as nutrients or phytostimulants (Campo et al., 2022; Pagliaccia et al., 2022b). In fact, sEPS based hydrogels can replace poly(acrylic acid)/ polyacrylamide-based material that are nonrenewable, containing potential toxic materials (acrylamide), and only biodegradable

to a certain extent (Pagliaccia et al., 2022b). Progressing towards the use of sEPS based hydrogels in agro-practices requires many peculiar characteristics (i) compositional properties meaning the presence or not within sEPS materials of phytotoxic elements like heavy metals and micro/macro nutrients; (ii) ability to reversibly sorb and desorb water; (iii) biodegradability and stability under natural soil conditions (Pagliaccia et al., 2022b). Hence it can be seen, that an approach based only on the mechanical properties of the formed hydrogels is not sufficient and a synergic approach combining many parameters must be applied assessing biochemical properties, physico-chemical properties of polymers, biodegradability etc.

Another application for ALE hydrogels, was their use as novel and innovative biosorbents for methylene blue dyes from aqueous solutions. Dye removal up to 65% was achieved at a temperature of 35°C and a contact time of 60 min. The dye removal is possible since it is a cationic molecule that will be captured by the negatively charged functional groups present on ALE molecules (Ladnorg et al., 2019). Alginate like exopolymers hydrogels were also used for the removal of phosphorus from aqueous solutions. Interestingly, alginate like exopolymers beads had a higher phosphorus removal in comparison with alginate (49.5% and 36.7% respectively). The study suggested the use of these phosphorus rich alginate like exopolymers beads in agriculture as a slow release fertilizer or in animal feed after detailed toxicity and nutritional composition studies (Dall' Agnol et al., 2020). Another study confirmed this aspect and proved that alginate like exopolymers hydrogels could be used to remove phosphate from municipal wastewater with efficiencies going up to 90.8% and elucidated the possible use of these phosphate rich hydrogels as fertilizers directly or after burning them (Schambeck et al., 2021).

Since the applications of sEPS are very affected by the properties of the polymers, the extraction procedure should be optimized according to application related criteria; which means that each step of the extraction procedure should be carefully studied and optimized in terms of used chemicals, choice of method, purification procedure...(Pagliaccia et al., 2022b). For example, the uses of sEPS are considered to be limited when it comes to applications that require consumption/direct contact with humans such as food additives and drug delivery systems since they require a high degree of purity (Karakas et al., 2020) contrary to applications in the domains of agriculture and building materials. Therefore, it only seems

logical and important to continue this literature review with a part on the extraction and characterization of EPS allowing to understand the effects of the extraction methods on the extracted polymers and their physico-chemical properties.



### 3. Highlights on “Structural EPS from granular sludges”

Section 3 showed the biochemical diversity of polymers composing the EPS matrix that are called Structural EPS. Indeed, polysaccharides, proteins, humic acid-like substances, DNA, glycoproteins, sialic acids, glycosaminoglycans were all proven to be a part of the EPS matrix of granular sludges. This diversity in biochemical groups and molecular properties is essential to ensure the different functional properties of the EPS matrix and opens up many potential valorization routes.

A focus was done on sEPS having the capacity to form hydrogels with different cations and in particular on alginate like exopolymers and granular that are the most studied components of sEPS. Although granular structure is characterized, the composition of alginate like exopolymers and the molecular mechanisms implied in the hydrogel’s formation remain to be elucidated. It is nevertheless established that complex anionic hetero-polysaccharides with carboxylic and/or sulfate groups were involved in the gelation while the participation of proteins in the hydrogel formation has been hypothesized, knowing that it was evidenced that proteins are a major part of the EPS extract and are often more abundant than polysaccharides. The cation type and concentration were determinants for the strength of EPS gels. Due to the complexity of these hydrogel-forming EPS, more studies are required to tailor their extraction and understand the molecular properties involved in the gelation. Such investigations are a prerequisite to offer industrial applications and a viable source of revenue reducing the economic burden of the actual WWTP.

## 4 EPS extraction and characterization

As previously discussed, the polymers forming the EPS matrix are held together through weak bonds such as hydrogen bonds, van der Waals bonds, etc. The extraction techniques of EPS usually depend on disrupting these interactions between polymers. One should keep in mind that differences exist from one EPS matrix to the other, so different extraction techniques

must be tried to determine which one is the most suitable for the specific EPS matrix being handled (Wingender et al., 1999).

Generally, the EPS matrix extraction is considered a liquid/solid extraction: the desired compounds are usually adsorbed on the surface of the solid sample or present in various forms as a mixture in the sample. Many factors can influence the extraction efficiency; (i) a smaller particle size is better because there is a better contact between the solvent and the molecules to extract, (ii) a larger solvent volume increases the extractability of the solute from the solid matrix due to maintaining a high diffusion coefficient, (iii) an adequate extraction time is required, and (iv) a low solvent's viscosity is preferred (Moldoveanu and David, 2015).

Many methods of extraction have been applied to EPS matrices in literature and in general they can be grouped into three categories: physical, chemical, and biological. Depending on the goal of the extraction and the targeted fraction of EPS the chosen method of extraction could differ. If the goal was to extract loosely bound EPS, gentle physical techniques are used such as centrifugation or mixing, whereas for tightly bound EPS more drastic chemical, or a combination of chemical and physical extraction techniques are used such as alkaline solubilization under heating, cationic exchange resin (CER)... Multistep extraction protocols have been also used to first separate loosely bound EPS using gentle extraction techniques followed by more drastic extraction conditions to extract tightly bound EPS (Ding et al., 2015; Feng et al., 2021). Multi-method extraction techniques can be also used to separate EPS according to their molecular properties such as hydrophobicity and anionic charge (Caudan et al., 2012). Another goal of the extraction could be the valorization of EPS and in this case drastic extraction techniques that give an optimal yield are used such as the use of sodium carbonate coupled with heating at 80 °C (Felz et al., 2016; Schambeck et al., 2020). The three extraction categories will be developed furthermore in the upcoming paragraphs.

## **4.1 Methods for EPS extraction: mechanisms and molecular effects**

### **4.1.1 Physical methods**

Physical extraction methods are defined as methods applying a shear to the matrix to extract the molecules of interest (Wingender et al., 1999). In addition, physical methods (except CER) are not based on disruption and solubilization of specific types of chemical groups. Some of the methods used for the extraction of EPS from granules which are centrifugation, sonication,

and heating will be further developed. The mechanism of extraction will be explained and examples of their uses for EPS extraction will be given.

#### 4.1.1.1 Centrifugation

Centrifugation is often used to separate soluble EPS from the cell mass. Speeds used in literature vary usually from 2 000 g to 20 000 g for about 10 to 30 min (Ding et al., 2015; Feng et al., 2021; Tan et al., 2021).

During centrifugation two forces are acting on the particles, the gravitational and centrifugal forces. The gravitational force is homogeneous over a sample and in general much smaller than the centrifugal force, that's why it is often neglected in centrifugation processes (Moldoveanu and David, 2015).

The centrifugal force formula proposed by Moldoveanu and David (2015) links the centrifugal force  $\phi$  (in N) to the radius  $r$  (in m) of the point in the rotating sample (distance to the rotation axis), the angular force  $\omega$  (expressed in radians per time unit), the mass  $m$  of the particle (in kg) and the density  $\rho$  of the solvent over the density  $\rho_p$  of the particle.

$$\Phi = \left(1 - \frac{\rho}{\rho_p}\right) m\omega^2 r$$

By analyzing this formula, it can be noticed that the higher the density and the mass of the particle are, the greater the centrifugal force  $\phi$  is, while if the density of the solvent is high the centrifugal force gets lower. The increase in angular force and radius causes also an increase in the centrifugal force. A higher centrifugal force might lead to a better separation of soluble EPS, but it must be noted that centrifugation as a method of extraction yields low amounts of EPS and extracts only to a limited extent bound EPS (Tan et al., 2021). Indeed, separating loosely bound EPS from tightly bound EPS can be achieved by using centrifugation, however the yield of EPS is low between 5.3 and 17.3 mg/gVS (Felz et al., 2016; Tan et al., 2021).

#### 4.1.1.2 Sonication

Ultrasound technology is based on mechanical waves having a frequency above the human hearing threshold (>16KHz). Ultrasonication is used for the extraction of molecules because it can (i) increase mass transfer, and (ii) allow better penetration of the solvent in the solid matrix due to the partial desorption of polymers. The sonication used to extract EPS falls

usually in the low frequency ultrasound category at around 20 kHz, possibly causing alterations to the extracted molecules (Caudan et al., 2012; Feng et al., 2021; Ras et al., 2008b). It can destroy cell walls releasing the contents of cells (Soria and Villamiel, 2010). This last effect is not really desired during the analysis of EPS because it contaminates the polymers with intracellular components.

The effect of ultrasounds on liquid systems is due mainly to the cavitation phenomenon causing the formation of bubbles from gas nuclei present within the fluid. These bubbles reach a critical size and implode violently (Patist and Bates, 2008; Soria and Villamiel, 2010). The conditions within these imploding bubbles can be very extreme with a pressure reaching 1000 atm and a temperature of 5000 K.

On a molecular level, during sonication, sonolysis of water can lead to the generation of H<sup>•</sup> and OH<sup>•</sup> radicals. These radicals either combine forming H<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, and water or attack molecules that are either oxidized or reduced (Riesz and Kondo, 1992). For example, pectin, composed of uronic acids, was submitted to ultrasonication at 1 MHz and showed signs of degradation upon the treatment whereas DNA seemed to be more stable to this treatment (Henglein and Gutiérrez, 1988).

The effects of sonication on proteins should be considered because it can drastically alter their structure. The sonication of structurally diverse proteins results in their unfolding causing the possible apparition of beta sheets structures leading to the association of monomers of proteins forming aggregates similar to amyloid fibers (Stathopoulos et al., 2008). Moreover, the generated free radicals during sonication can undergo a variety of reactions with amino acids, peptides, and proteins. Reactive oxygen species can react with different chemical moieties on proteins, producing therefore protein radicals that undergo further reactions such as cleavage reactions, crosslinking, and oxidation (Hawkins and Davies, 2001).

#### *4.1.1.3 Heating*

Heating the EPS matrix for extraction purposes is commonly used and the technique relies on different mechanisms to enhance the extraction of molecules from the matrix. The first mechanism proposed by Wingender et al. (1999) is based on shear forces provided by heating. Another extraction mechanism consistent with this technique is that the temperature increase causes the molecules to vibrate more quickly which enhances their solubility in the solvent

(Sheng et al., 2010). Furthermore, the energy given by an increase of temperature corresponding at least to 250 KJ/mol theoretically allows for breaking all kinds of low energy bonds/interactions. As an example, an increase of 60°C would bring 4.5 KJ/mol of water. The energy of bonds can be checked in Figure I-25 (Mezenov et al., 2019).

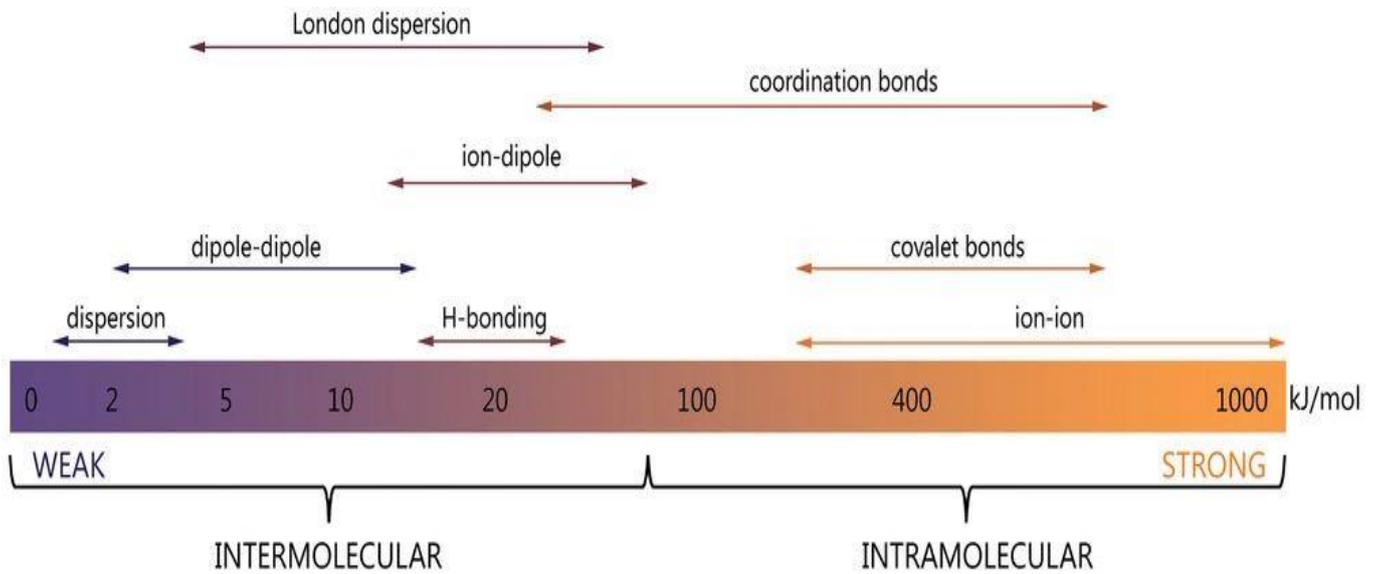


Figure I-25: Energy scale of weak and strong chemical bonds from (Mezenov et al., 2019).

Polysaccharides seem to be more stable than proteins to heat treatments in general. However, an experiment done on alginate at 3% w/v showed that the viscosity of solutions measured after 15 minutes of heating started decreasing at temperatures above 100°C (Figure I-26). This decrease was attributed by authors to a marked level of depolymerization (Leo et al., 1990).

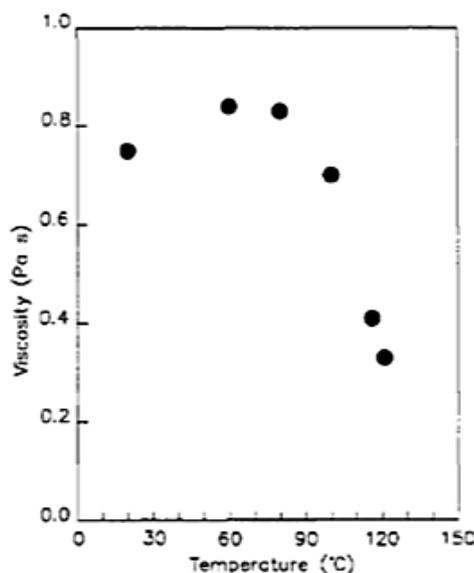


Figure I-26: Effect of temperature on the viscosity of 3% sodium alginates solutions (15 min exposure time) (Leo et al., 1990).

Although heating may be an efficient and easy method to extract EPS, it can greatly impact the stability of some polymers. According to Matsuura et al. in 2015, hydrophobic interactions make the largest contribution to general protein thermal stability. During heating intact proteins are gradually unfolded and sufficient energy is given to prevent the proteins from getting back to their initial conformation, therefore increasing the chances of interaction between hydrophobic regions of different proteins leading to their aggregation (Park & Russell, 2000). Consequently, proteins that express a higher surface hydrophobicity after denaturation are the most susceptible to aggregation (Avanza and Anon, 2007). The protein aggregation will be the most pronounced if there is a high protein concentration, and near isoelectric point conditions (Park and Russell, 2000).

In order to better understand this process of thermal denaturation and aggregation, Honda et al. (2000) have studied the behavior of the model protein BSA towards heating conditions. They proposed two thermal aggregation stages: the first one consists of monomers of BSA merging together into an aggregate, while during the second step, the reaction between inter-aggregates takes place rapidly increasing the size of aggregates. The increase of the particle radius is shown in Figure I-27.

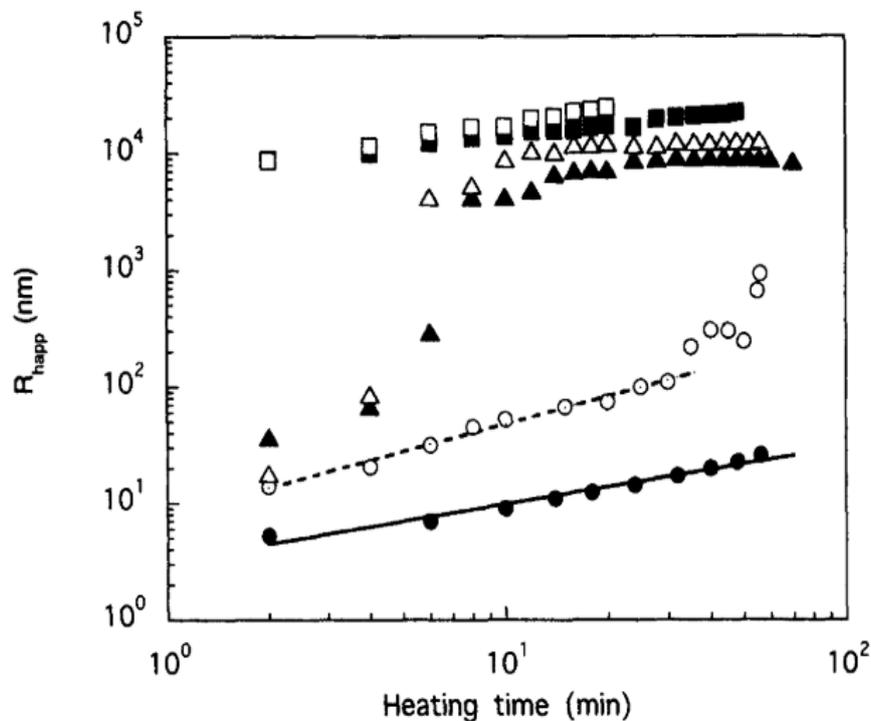


Figure I-27: The increase of particles radii for aggregates of BSA (1%) as a function of heating time at a pH of 5.5, temperatures tested going from down upwards are: 56,58,59,61,62,67°C (Honda et al., 2000).

It can be concluded that the mechanism of extraction using heat relies on several factors: the applied shear forces, the vibration of molecules increasing their solubility, and the energy given by heating leading to the cleavage of some weak bonds. However, aggregation of proteins might happen leading to a lower solubility. This phenomenon is more pronounced in the presence of more hydrophobic amino acids in the protein, high protein concentration, and near isoelectric point conditions.

#### **4.1.2 Chemical methods**

Chemical methods for the extraction of EPS are defined as treatments that include the addition of various chemical compounds that can break linkages in the EPS matrix facilitating therefore their extraction (Wingender et al., 1999). These methods are interesting because they allow the extraction of molecules with particular properties such as their polarity, charge, etc. However, these extractants can interfere with analytical methods and often a step of dialysis is needed to get rid of them. The main interest in the coming parts will be on the following methods: alkaline and acidic conditions as well as chelators. These methods are widely used for EPS extraction from microbial biofilms and aggregates.

##### *4.1.2.1 Alkaline and acidic conditions*

Before developing this technique, an important thing to remember about the EPS matrix of granules is that it contains a lot of negatively charged polymers including polysaccharides as well as proteins. Indeed, it was shown that 25% of the amino acids present in EPS matrices tend to be negatively charged i.e. aspartic and glutamic acids (Sheng et al., 2010).

Usually, in order to increase the solubility of molecules bearing chemical groups with isoelectric points above 7 (amine and guanidine residues) buffers with  $\text{pH} < 7$  are used in order to increase their positive charges. On the contrary, buffers with a  $\text{pH} > 7$  are used to increase the solubility of acidic polymers (carboxyl, sulfate residues) due to repulsion between charges and consequently their extraction (Moldoveanu and David, 2015). As previously described, alkaline solvents are the most used in literature for EPS extraction (Adav and Lee, 2008; Felz et al., 2016; Lin et al., 2010; Pagliaccia et al., 2022b; Schambeck et al., 2020). Alkaline conditions cause many charged groups, such as carboxylic groups in proteins and polysaccharides, to be ionized. Indeed, their isoelectric points are generally below  $\text{pH}$  4-6 which results in a strong repulsion between EPS providing a higher water solubility at high  $\text{pH}$  (Sheng et al., 2010; Wingender et al., 1999).

During the extraction, the choice of the alkaline solvent used (potassium hydroxide, sodium hydroxide, or sodium carbonate) is based on many criteria. Sodium hydroxide is usually easier to handle because it does not involve the production of carbon dioxide as a byproduct. Sodium salts tend also to have a better solubility than their respective protonated forms. However, if compounds are sensitive towards strong bases or nucleophiles (such as polymers with aldehydes, ester, and ketones...) a weaker base should be used like sodium carbonate.

An article studied the degradation of alginic acid with different concentrations of sodium hydroxide (0.1, 0.5, and 2M) at 2 different temperatures of 95°C and 135°C for a treatment of 2.5 hours. Depending on the conditions of treatment the amounts of carboxylic acids (degradation products) formed correspond to 30-70% of the initial alginic acid. They obtained a variety of dicarboxylic acids and monocarboxylic acids indicating the presence of several competing routes of degradation (Klaus and Eero, 1985).

#### 4.1.2.2 *Chelators and cationic exchangers*

As previously explained, divalent cations have an important role in maintaining the EPS matrix because they can make a link between the negatively charged polymers of the extracellular matrix (Caudan et al., 2014; Kończak et al., 2014; Sajjad and Kim, 2016). Removing these cations will increase the repulsion between EPS polymers and therefore the EPS matrix will tend to fall apart.

The removal of these cations can be done by using chelators such as EDTA, and EGTA. However, some studies have shown that the use of EDTA can have some drawbacks if used for EPS extraction, mainly because it can remove divalent cations from cell walls leading to its destabilization and release of lipopolysaccharides and cellular macromolecules. Other methods have also been used to carry out cation exchange such as the use of high NaCl concentrations (Salama et al., 2015).

Sodium oxalate was also used as a cation exchanger because oxalate anions have a strong binding affinity with divalent cations. In addition, the sodium cation will enhance the ratio of monovalent/divalent cations which will lead to the displacement of divalent cations and disintegration of the structure, thereby increasing extractability (Sajjad and Kim, 2016).

CER is a method that combines physical shear due to agitation of anionic particles (due to the presence of sulfonic or carboxylic acids on them) at speeds between 600 and 1000 rpm with

the removal of cations from the EPS matrix (Ras et al., 2008a; Wingender et al., 1999). The negatively charged resin will fix various cations, hence allowing the extraction of EPS polymers. One important advantage is that the EPS extract recovered by centrifugation is not contaminated by any chemical. One inconvenience is the possible adsorption of biopolymers on the resin.

### **4.1.3 Biological methods**

Biological methods consist of using enzymes for the extraction of EPS. This type of technique is not widely used for global EPS extraction in literature but it can give interesting information about the function of specific polymers. Sesay et al. in 2006 used three different enzymes (proteinase, amylase, and cellulase) to extract EPS from activated sludge. An interesting finding of this study was that polysaccharides and proteins are linked together within the EPS matrix, since using proteinase allowed the extraction of some polysaccharides and the opposite is also true. The advantages of such methods are that they are specific for the desired polymers because each enzyme only attacks specific bonds, and they also allow an understanding of the types of predominant interactions. In addition, they are environmentally friendly because of the low energy requirement and the low temperature used during the treatment. The main disadvantages are (i) that due to the big diversity of EPS and the specificity of enzymes, only a small minority of the polymers composing the EPS matrix are extracted, (ii) the high cost of the enzymes, (iii) the sensitivity of enzymes to several factors (Sesay et al., 2006; Tan et al., 2021).

## **4.2 Extraction of EPS from dense granules**

Two main goals are predominant concerning EPS extraction from granular sludges and may result in different extraction strategies: the first is understanding the role of EPS in the granulation and increasing the scientific knowledge concerning this complex EPS matrix (§4.2.1); the second has for target the valorization of EPS for various industrial applications and particularly for their gel-forming abilities (§4.2.2).

### **4.2.1 Gaining insights on the granules' EPS matrix through extraction methods**

Despite decades of research, the scientific community's knowledge about the EPS matrix remains limited (Seviour et al., 2019). An in depth-study of EPS is imperative and can be

achieved through in-situ chemical analyses which are still limited or through the extraction of EPS and their analysis (Pan et al., 2016).

Understanding the EPS matrix present within granular sludges can be achieved through multi-steps EPS extraction methods that are used to separate fractions of EPS according to their molecular properties. Such method was applied on aerobic granular sludge and consisted of three successive steps: a mechanical disintegration step through the use of ultrasonication, followed by the use of non-ionic detergent Tween targeting hydrophobic interactions and finally the use of the chelator EDTA targeting cationic bridges. The three previously cited steps constituted a sequence, and the sequence was repeated four times in succession on the granular sludge (Caudan et al., 2012). The polysaccharidic fraction was extracted mostly through ultrasonication (94.4%) indicating that most of the polysaccharidic fraction is hydrophilic since polysaccharides were extracted only to a limited extent by Tween. On the contrary proteins, that were major components of the EPS matrix (232 mg per g of volatile soluble solids), required all three different methods for their extraction. The mechanical extraction using ultrasonication allowed the extraction of 47% of proteins, followed by EDTA (43%) and Tween (only 10%). This agrees with the complexity of proteins formed by ionic, hydrophobic, and neutral amino acids (Caudan et al., 2012).

A study led on aerobic granules dominated by *Deffluviicoccus*, showed that the extraction of sEPS wasn't possible using alkaline extraction ( $\text{Na}_2\text{CO}_3$ , 80°C, 30 min) since the yield of sEPS was less than 1% of the organic fraction, and the granules weren't solubilized. The granules were then subjected to an acidic extraction (acetic acid, 95°C, 20h) and they were solubilized efficiently meaning that the sEPS of this type of granule are not similar to ALE. This study showed that depending on the type of granule, the EPS content and composition could be different and hence there is no fit for all concerning extraction methods (Pronk et al., 2017).

Another study compared many extraction methods used on anammox granules and found that EDTA destabilized the most granules' cohesiveness (microscopic observation). Anammox granules are richer in proteins than polysaccharides with PN/PS ratios of 3.4 to 7.2; only acid treatment yielded more polysaccharides than proteins with a PN/PS ratio of 0.6. The content of proteins and carbohydrates in the EPS obtained from the Formamide-NaOH extraction method was significantly higher than in the Formaldehyde-NaOH treatment. This is due to cell shrinkage by formamide, but both solvents are criticized for their environmental footprint.

Based on the EPS yield, as well as the PN and PS contents the extraction methods efficiencies were as follows: Heating- $\text{Na}_2\text{CO}_3$  > Formamide- $\text{NaOH}$  > Formaldehyde- $\text{NaOH}$  > CER > EDTA > Acid > Sonication (Feng et al., 2019).

#### 4.2.2 Extraction methods for the recovery of gel-forming EPS.

Alkaline extraction methods are the most widely used in literature for the extraction of gel-forming EPS from granules since these polymers are supposed to have negative charges to be able to link with divalent cations and form hydrogels (Lin et al., 2010; Schambeck et al., 2020; Seviour et al., 2010a). As explained, alkaline extraction techniques favor repulsion between negatively charged polymers allowing their solubilization (Feng et al., 2021; Sheng et al., 2010). The reported pKa of granulan was approximately 9 and that of ALE was 4.5 which explains the extractability of these molecules under alkaline conditions (Seviour et al., 2012b).

Sodium carbonate is usually coupled with heating whereas sodium hydroxide is not (Schambeck et al., 2020; Seviour et al., 2010a) as seen in Table I-1. In literature yields of gel-forming EPS varied between 15 and 290 mg/gVS (Meng et al., 2019; Rollemberg et al., 2021; Schambeck et al., 2020). This variability in yields can be attributed to the differences in operational conditions amongst other factors such as the feeding type, as well as the yield determination method. The yield can be determined using colorimetric methods subject to interferences or by gravimetry which is more precise. It can be clearly noticed in Table I-1 that there is no consensus on the extraction method in the scientific community. The solvent can be different ( $\text{Na}_2\text{CO}_3$  or  $\text{NaOH}$ ), the treatment temperature can vary between 4°C and 80°C, the heating time varies between 35 min and 3.5 hr and finally the concentration of the extractant can vary between 0.05 and 0.2 M. In addition, some studies stop the enrichment in gel-forming EPS at the acidic precipitation step while others continue by doing an ethanol precipitation. These differences make comparisons between results from different studies difficult and there is rarely an explanation for the chosen extraction parameters in the reported studies. Moreover, the differences in yields can be attributed to the presence of insoluble molecules within different types of granules, for example some high molecular weight polysaccharides can form helical structures through hydrogen bonds that render them highly insoluble. Therefore, granules containing such molecules would be expected to have low extractable yields of EPS (Seviour et al., 2012b). Globally, there is a need to understand the effects of extraction methods on gel-forming EPS to be able to choose the most suitable

method for the extraction of these valuable polymers while conserving their native structure and gel-forming properties. Once the extraction of these gel-forming EPS is done, their properties could be analyzed via various techniques that will be developed furthermore in the upcoming paragraphs.

**Table I-1: Comparison of the extraction and enrichment methods for the recovery of gel-forming EPS between various studies**

Aerobic granule feeding type	Extraction method	Solubilized sEPS Yield (mg/gVS)	Acid precipitated sEPS yield (mg/gVS)	Acid and ethanol precipitated sEPS yield	Authors
abattoir wastewater	NaOH 0.1 M – 4°C - 2 hr	-	-	-	(Seviour et al., 2009)
real wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.2 M - 80°C- 1 hr	-	-	160±4 (mg/gVS) <sup>1</sup>	(Lin et al., 2010)
Synthetic wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.2 M - 80°C- 3.5 hr	-	-	100 (mg/gVS) <sup>2</sup>	(Yang et al., 2014)
real wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.05 M - 80°C- 35 min	≈325 <sup>1</sup>	≈205 <sup>1</sup>	-	(Felz et al., 2016)
synthetic saline wastewater	Formaldehyde-NaOH 1 N - 4°C - 3 hr	-	≈27-53 <sup>2</sup>	-	(Li et al., 2017)
synthetic saline wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.2 M - 80°C- 1 hr	-	≈15-50 <sup>2</sup>	-	(Meng et al., 2019)
simple synthetic wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.05 M - 80°C- 35 min	598±37 <sup>1</sup>	261±33 <sup>1</sup>	-	(Schambeck et al., 2020)
real wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.05 M - 80°C- 35 min	495±45 <sup>1</sup>	184±18 <sup>1</sup>	-	(Schambeck et al., 2020)
real wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.05 M - 80°C- 35 min	-	212.87±15.65 <sup>1</sup>	-	(Dall' Agnol et al., 2020)
real wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.05 M - 80°C- 35 min repeated 4 times	672-896 <sup>2</sup>	122-149 <sup>2</sup>	-	(Oliveira et al., 2020)
synthetic wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.2 M - 80°C- 1 hr	-	≈190-290 <sup>1</sup>	-	(Rolleberg et al., 2021)
real wastewater	Na <sub>2</sub> CO <sub>3</sub> 0.2 M - 70°C- 45 min	-	-	131 (mgTS/gMLSS) <sup>1</sup>	(Cydzik-Kwiatkowska et al., 2022)

With yield determination done by gravimetry (<sup>1</sup>) or colorimetric methods (<sup>2</sup>).

### 4.3 Methods for studying EPS

Many methods are used in literature to analyze EPS, some of them require a first step of extraction and others allow the analysis of extracellular polymers *in situ*. Due to the complexity of the EPS matrix, its analysis has proven difficult. However, the advances in analytical chemistry allowed some progress in this field (Sheng et al., 2010; Seviour et al., 2019). Some of the most frequently used techniques for studying the EPS matrix are developed in the upcoming paragraphs whether in terms of quantification (§4.3.1) physico-chemical properties (§4.3.2) or biochemical characterization using enzymatic digestion (§4.3.3).

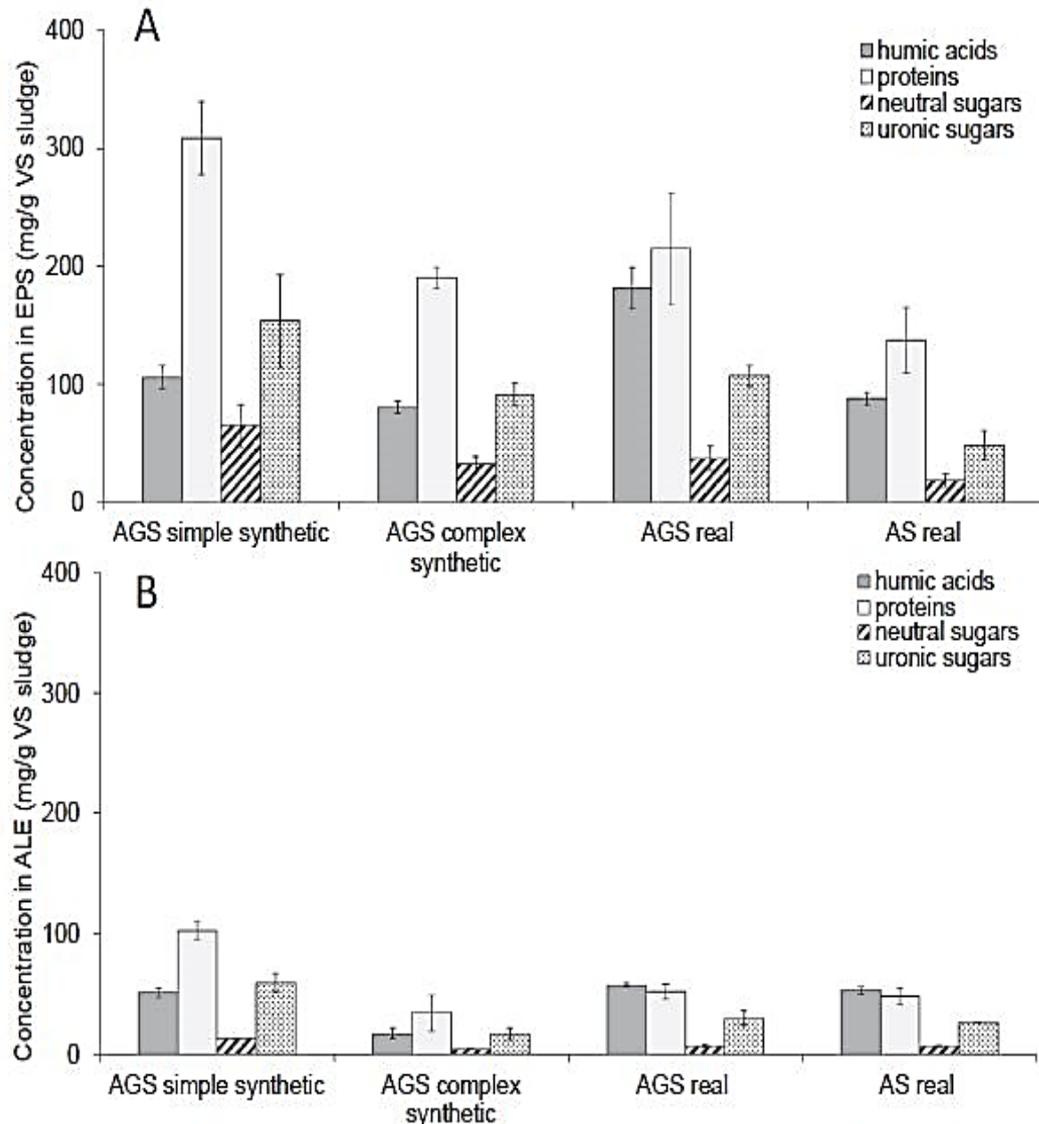
#### 4.3.1 EPS Quantification

##### 4.3.1.1 Gravimetry

The most used method for the quantification of EPS is gravimetry consisting of drying the EPS sample at 105°C overnight and then measuring the dry weight. This step is followed by burning at 550°C for 2 hours and measuring the remaining weight which consists the mineral fraction. The difference between the two measured weights is the organic fraction or the VS content (Felz et al., 2016).

##### 4.3.1.2 Colorimetric methods

Colorimetric methods are widely used for the quantification and characterization of EPS. Indeed, they are easy, fast, and cheap methods that allow the detection of proteins, saccharides, uronic acids, humic substances... The study led by Schambeck et al. (2020) used these techniques to compare the effect of feeding in reactors on the extracted gel-forming EPS biochemical groups. They found that aerobic granules fed with simple synthetic wastewater (propionate and acetate) presented the highest content of uronic acids and proteins in comparison with granules fed with complex synthetic or real wastewater. The difference subsisted after acidic precipitation to select alginate like exopolymers (Figure I-28). Another study used these colorimetric methods to follow the biochemical composition within the reactors over time. The extracted EPS, during all the sampling campaigns had more or less a homogeneous composition (Oliveira et al., 2020).



**Figure I-28: Comparison of the feeding effects on the biochemical composition of EPS and alginate like exopolymers extracted from aerobic granular sludge (AGS) and flocs from activated sludge (AS) (Schambeck et al., 2020).**

These results show that colorimetric methods can be used to have some insights on the composition of extracted EPS but they have their limitations. Indeed, they can be dependent on many factors such as the choice of standard and interferences with other compounds (Table I-2) present in the solution may give an over-estimation or an under-estimation of the compound being quantified, meaning that attention must be paid during the analysis of the results (Felz et al., 2019).

**Table I-2: Cross-interferences between the compound being measured and compounds that might be present in the solution. The darker the grey color is the higher the cross-interference is (Felz et al., 2019).**

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

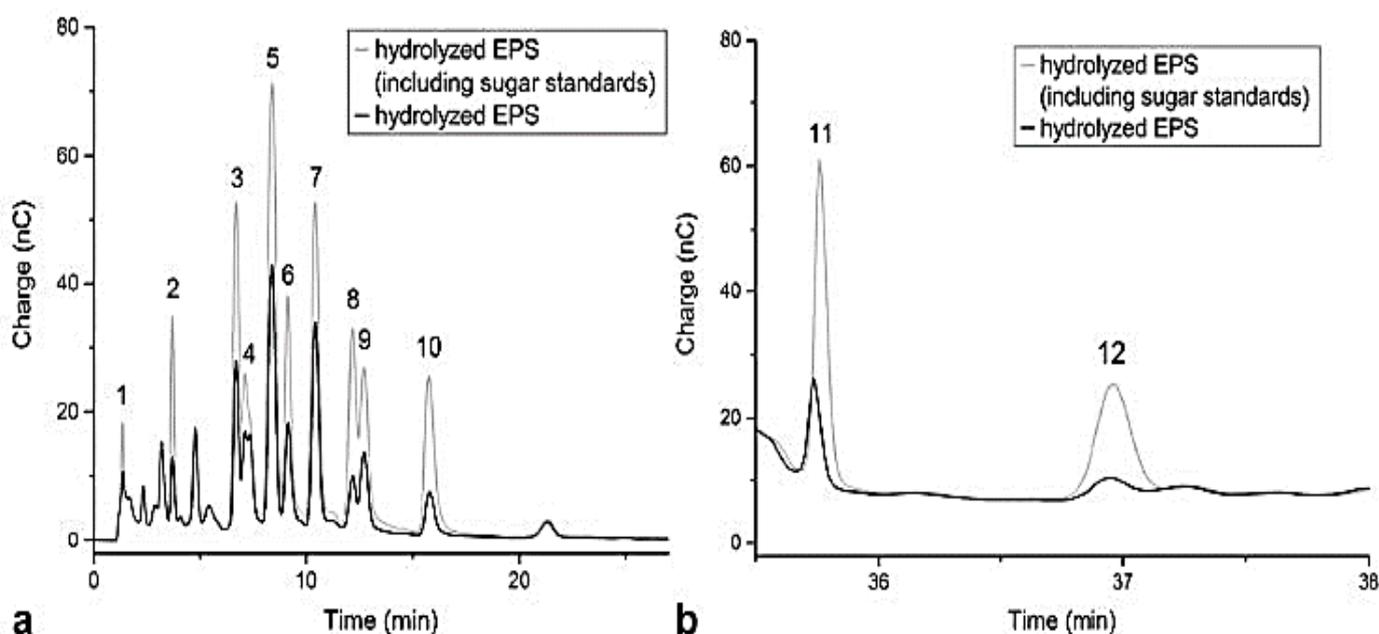
### 4.3.2 Characterization of the physico-chemical properties of EPS

#### 4.3.2.1 Chromatography methods

Liquid Chromatography is a technique used to separate molecules solubilized in a complex mixture. Depending on the type of chromatography used, the separation of molecules can be done according to size, charge or hydrophobicity. Caudan et al. (2012) have used size exclusion chromatography as well as anionic exchange chromatography on fractions of EPS extracted from granular sludge using a multimethod extraction technique (sonication, tween, EDTA). Depending on the method of extraction used, different chromatograms are obtained, indicating that the method of extraction influences the molecular characteristics of the extracted molecules. They also showed the presence of high molecular weight polymers attaining more than 1000 kDa. Concerning the charge, they compared the anionic profiles from flocculent and granular sludges and noticed different profiles between the two types of aggregates with much more highly anionic molecules in the EPS extracted from granular sludge (Caudan et al., 2012). Another study done on aerobic granules showed the size distribution through the use of gel permeation chromatography of polymers or association of polymers with apparent molecular weights attaining several millions were observed and they were thought to be polysaccharidic since no UV signal but only a RI signal was detected (Seviour et al., 2010a). Size exclusion chromatography was also used by Lin et al., (2015) to

determine the size of polysaccharidic EPS (precipitated by ethanol) used as a coating material and showed that one-third of that fraction had molecular weights higher than 70 kDa. It can be noticed that there is a lot of heterogeneity between the reported molecular weights in literature and this is possibly due to different extraction methods and different origins of granules.

Through the use of high-performance anionic exchange chromatography coupled with pulsed amperometric detection (Figure I-29), monosaccharides have been identified including sugar alcohols like glycerol, amino sugars like galactosamine and glucosamine, neutral sugars like fucose, rhamnose, galactose, glucose, xylose, mannose, and ribose. In addition, anionic polysaccharides containing uronic acids like galacturonic acid and glucuronic acid were confirmed in the EPS matrix (Felz et al., 2019).



**Figure I-29:** Analysis of monosaccharides composition in acid hydrolyzed EPS using high performance anionic exchange chromatography coupled with pulsed amperometric detection. The elution with 2mM sodium hydroxide revealed the presence of sugar alcohols, neutral sugars and amino sugars (a). Detected compounds were (1) glycerol, (2) fucose, (3) galactosamine, (4) rhamnose, (5) glucosamine, (6) galactose, (7) glucose, (8) xylose, (9) mannose, (10) ribose. Further elution with 50mM sodium acetate and 200mM sodium hydroxide (b) showed the presence of galacturonic acid (11) and glucuronic acid (12).

#### 4.3.2.2 Fourier transform infrared spectroscopy

The FTIR technique relies on the absorption of infrared radiation by the molecules. The wavelengths absorbed by atoms depend on their electronic surroundings, allowing therefore, the detection of functional groups on the spectrum. The chemical functions are therefore

associated with frequencies as shown in Table I-3. However, the analysis of a spectrum can be difficult, especially if the sample is complex and if similar chemical functions have different vibration modes and show several absorption bands.

**Table I-3: Functional groups assigned to their respective frequency (Oliveira et al., 2020).**

Frequency (cm <sup>-1</sup> )	Assignment
3285	O–H stretching of hydroxyl groups
2930–2910	C–H stretching asymmetric (fatty acids)
1633	Amide I (C=O and C=C stretching in proteins)
1538	Amide II (N–H deformation, C–N stretching in proteins)
1450	C–H deformation of –CH <sub>2</sub>
1400	C=O stretching symmetric of COO <sup>-</sup>
1300	Amide III (N–H deformation, C–N stretching in proteins)
1233	N–H deformation, C–N stretching
1075–1048	C–O–C and C–H stretching (polysaccharides and/or nuclei acids)
833	Tri-substituted alkene sp <sup>2</sup> C–H deformation (in lipids) or para di-substituted aromatic sp <sup>2</sup> C–H deformation (in proteins)
900–600	“fingerprint region”

Oliveira et al. (2020) used this technique to compare EPS before and after acidic precipitation. Globally they found no major differences before and after precipitation in terms of functional groups except for one band at 833 cm<sup>-1</sup>, thus meaning that the acidic precipitation step was responsible for eliminating some proteins or lipids or both from the initial extract. They also noted the presence of carboxylic acid groups, amide, and carbohydrate functional groups.

In addition, this technique can be used to determine the presence of  $\alpha$ -helices,  $\beta$ -sheets or random coils in proteins. This can be done through further analysis of the amide I region (derivative spectra and curve fitting). Shi and Liu (2021) found that EPS extracted using the formamide-NaOH method presented aggregated strands and antiparallel  $\beta$ -sheets that were less dominant than other secondary structures in granular sludge. This study was also able to correlate granule size and the granulation process with the abundance of  $\beta$ -sheets, suggesting that these secondary structures play an important role in granulation (Shi and Liu, 2021). A similar analysis was done by Lin et al., (2018) to demonstrate the presence of amyloids in EPS extracted using 0.1% SDS at pH 9 with a heating of 100°C for 30 min from aerobic granular sludge enriched with ammonium-oxidizing bacteria.

FTIR can also be used to detect the adsorption of heavy metals on gel-forming EPS extracted from aerobic granules. Indeed, the study led by Cydzik-Kwiatkowska et al., (2022) showed that cadmium was able to adsorb on alginate like polymers through the use of FTIR amongst other techniques.

Therefore, FTIR allows obtaining information on the contents of the EPS matrix extracted from the granules. The limitations of this technique are the possibility of the presence of many functional groups under each peak especially when the sample is complex. In addition, the observation of the spectra does not allow to characterize the polymers with precision and to differentiate polymers from one another.

#### 4.3.2.3 *Zeta potential*

The Zeta potential is an important parameter to characterize surface charge. A study showed that the lower the absolute value of the zeta potential of EPS is, the better the granulation is. This is due to a lower repulsion between microorganisms due to negative charges allowing their aggregation and the formation of granules (Liu et al., 2021; Su et al., 2014). Moreover, the lower absolute value of the zeta potential seemed to correlate with a high PN/PS ratio, because proteins can neutralize the negative charges on the polysaccharides (Liu et al., 2021).

A study done on EPS extracted from anammox granules showed that at pH 12, EPS dispersions had the highest absolute value of zeta potential forming a colloidally stable dispersion with no precipitation. At pH 4, the overall charge is neutral decreasing the affinity for water and facilitating EPS aggregation (determined by the hydrodynamic radius) and precipitation (Lotti et al., 2019).

Hence, this method can be used to determine the effects of the surface charge on the granulation process as well as on the extraction protocol by determining which pH is the most suitable for the acidic precipitation step to obtain the highest yield of gel-forming EPS.

#### 4.3.2.4 *Nuclear magnetic resonance (NMR) spectroscopy*

NMR has been used by many studies to characterize the extracted molecules. The structure of granulan has been elucidated thanks to NMR (Seviour et al., 2010b). Analysis of the acid soluble EPS extracted from aerobic granules enriched with *Deffluviicoccus* II was found to predominantly contain  $\alpha$ -(1-4) polysaccharide linkages. The NMR analysis also showed the presence of pyranose rings containing an O-CH<sub>3</sub> substitution within the molecule (Pronk et al., 2017).

This technique has also helped in showing the differences between sEPS recovered from two different aerobic granular sludges extracted using sodium hydroxide. The first had methanol as a carbon source and nitrate as electron acceptor, and the second was enriched with

*Competitibacter*. The NMR spectra of the EPS extracted from the first granular sludge indicated the presence of lipids and proteins but not of granulan since there was a lack of the anomeric  $\alpha$ -galactose peak found in granulan. The granular sludge enriched with *Competitibacter* showed the presence of the anomeric  $\alpha$ -galactose peak indicating the presence of granulan (Seviour et al., 2011).

Another interesting use for NMR spectroscopy is for the determination of the degree of branching of polysaccharides. As an example, the degree of branching can be determined from the ratio between the integrals of the distinct signals given by the  $\alpha$ -1,4- and  $\alpha$ -1,6-glycosidic bonds (Wang et al., 2020).

Globally this method can be very helpful in determining the structure of molecules and their building blocks. However, one major weakness is its low sensitivity and the fact that studying some samples requires long-lasting experiments going up to several weeks and not all samples can be studied using NMR (Bouleau et al., 2015). In fact, the sample purity is a major setback for applications in the EPS related field. The preferred purity for NMR analysis is 90% or greater but advances in the technique are rendering the feasibility of NMR on more complex samples possible (Kwan et al., 2011).

#### 4.3.2.5 Mass spectrometry (MS)

This technique can be very useful for the identification of molecules present within the EPS matrix of granular sludges. After the enrichment in glycoproteins of EPS from anammox granular sludge using a LiCl extraction protocol for S-layer proteins, SDS-PAGE electrophoresis was performed and the glycoprotein band was selected and subjected to digestion by trypsin. The peptides were then analyzed by tandem mass spectrometry and they uncovered that its structure is close to the protein sequence of a hypothetical protein of "*Ca. Brocadia sapporoensis*". Furthermore, they confirmed that the tested molecule is a glycoprotein because they found glycans attached to at least four fragments of peptides. To determine the composition of the discovered glycan,  $\beta$ -elimination was done to release the O-linked glycan from the protein. The glycan had a mass of 1456.6 Da and its structure is as follows: a methylated *N*-acetyl hexosamine backbone, substituted with a pentose and a dideoxyhexose residue. The glycan carried a 350 Da large terminal residue that is unique and has not been reported before and therefore could not be further specified by mass (Boleij et al., 2018). Moreover, sialic acids were identified in anammox granular sludge using MS in the form of *N*-

acetyl neuraminic acid, deaminated neuraminic acid, and pseudaminic acid/legionaminic acid (Boleij et al., 2020).

### 4.3.3 Enzymatic digestion of EPS

The use of enzymes for the digestion of the EPS matrix can give important insights on its composition. As previously discussed, it was shown in aerobic granules through enzymatic treatment of aerobic granules with amylase that  $\alpha$ - (1,4) glucans constituted of glucose monomers are present in the granules (Caudan et al., 2014).

Another study treated aerobic granules first with neuraminidase which cleaves N-acetylneuraminic acid (Neu5Ac), the most widespread form of sialic acids, and then with  $\beta$ -galactosidase to measure the content of galactose. Results underlined that sialic acids located in the outer layer of granular sludge are bound to galactose (De Graaff et al., 2019).

Structural EPS extracted from aerobic granules were digested using chondroitinase ABC from *Proteus vulgaris* and a blend of Heparinase I&III from *Flavobacterium heparinum*. The results indicated the presence of sulfated GAGs-like polymers, such as chondroitin sulfate-like and heparan sulfate/heparin-like polymers in aerobic granular sludge that are different than those found in mammals (Felz et al., 2020b).

Finally, enzymatic degradation tests were also applied to EPS hydrogels formed with calcium to detect the chemical groups/bonds involved in the hydrogel formation. Different enzymes were tested including papain and savinase which are proteases with different specificities. Papain hydrolyzes amide type peptide bonds of basic amino acids, leucine and glycine, and also hydrolyzes esters and amides. Savinase was applied for the digestion of amide type linkages in proteins and peptides. Lysozyme was used for the digestion of  $\beta$  (1  $\rightarrow$  4) linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in N-acetyl amino glycan. Finally, alginate lyase was employed for the digestion of osidic linkages found in uronic sugars like alginate. The hydrogel beads after the enzymatic treatment with active savinase, lysozyme, and papain visually maintained their structure. On the other hand, the disintegration of EPS hydrogels exposed to alginate lyase was very clear, confirming the key role of uronic acids in the formation of EPS hydrogels (Schambeck et al., 2020).

This technique allows the detection of the presence of specific molecules in the intact granules or in the extracted EPS. However, many factors could limit its uses such as the sensitivity of

enzymes to environmental factors, especially the pH since EPS are usually solubilized in alkaline solvents, particularly NaOH.



#### **4. Highlights on “EPS extraction and characterization methods”**

The literature review of this last section showed that each extraction method either physical or chemical, has a mechanism of action that is unique and its effects on the extracted molecules depend on the sensitivity of the molecules including aggregation, alteration, degradation, precipitation... However, extraction of EPS is essential to study the EPS matrix and gain insights on its properties and composition. For the valorization of gel-forming EPS, the extraction method of choice is based on increasing the negative repulsive forces between polymers due to their negatively charged functional groups i.e. alkaline extraction. However, there is no unified protocol for their extraction. Parameters such as the choice and concentration of the base, the heating temperature and time vary from one study to another without a clear explanation for these choices. Therefore, it is important to understand the effects of these parameters on the yield and quality of extracted molecules with the goal of optimizing the yield and preserving the gel-forming properties to have an efficient valorization of EPS. Once extracted, the characterization of EPS is done through different techniques. Although each technique has its limitation, the use of different techniques together allows to reach important insights on the EPS matrix.

#### **5 Conclusions of the literature review and aims of the thesis**

This literature review falls within the context of the recovery and valorization of waste resources. More and more countries will adopt wastewater treatment stations working with granular sludge over activated sludge. This is due to the higher efficiency and economic advantages of the granular sludge process over conventional wastewater treatment processes. However, this means that excess aerobic granular sludge produced in these wastewater treatment stations will constitute an important waste in the years to come. Valorization of the EPS composing the extracellular matrix of the granule appears thus as an important issue for the transition from WWTP to WRRF. Indeed, the EPS composing the matrix of granular sludges have been proven to have a versatility of potential industrial applications due to their huge molecular diversity. Indeed, EPS extracted from aerobic granular sludge

were composed of polysaccharides, proteins, DNA, and humic-like substances among other molecules, attesting to a big biochemical diversity. To that is added the diversity in the extracted molecules' properties such as molecular weight, charge, hydrophobicity... This diversity is essential to ensure the multiple functions occupied by the EPS matrix, among which the structuration of the granule. The fraction of EPS participating in the structuration of the granule was named sEPS among which some polymers were proven to have a hydrogel forming capacity in the presence of divalent cations.

Through the literature review concerning model polymers (polysaccharides and proteins), it was noticed that many mechanisms for gelation exist. For example, the hydrogel formation for alginate relies on cross-linking with calcium ions, whereas that of gelatin relies on hydrogen bonds. The formed gels' properties depend greatly on the polymer being used such as its molecular weight, spatial conformation, charge, functional groups present on the backbone as well as the conditions of the medium where the gelation is taking place. For example, it was shown in the literature review that the pH of the medium impacts the charge of polymers and therefore their capacity for gelation, the presence of divalent cations as well as their concentration has an impact on the properties of the formed hydrogels, amongst other important factors. Afterward, a focus was done on sEPS having the capacity to form hydrogels, particularly ALE and granulan. It is established that complex anionic heteropolysaccharides with carboxylic and/or sulfate groups were involved in the gelation while the participation of proteins in the hydrogel formation has been hypothesized. The molecular characteristics that have a great impact on the capacity of the molecules to form hydrogels depend greatly on the extraction protocol being used. Therefore, the literature review continued with a detailed study of the effects of the extraction techniques on the extracted polymers.

Extraction techniques for EPS can be divided into three large groups: physical, chemical, and biological methods. Valorization of granules through their EPS potential needs both a good extraction efficiency (solubilization and precipitation of gel-forming polymers) while preserving the gel-formation capacity of the targeted polymers that can be both polysaccharides or proteins. The literature review helped in understanding some of the effects of extraction methods on some model polymers. Depending on the used method of extraction the effects on the polymers were different: pectin was degraded upon sonication and alginate showed a decrease of viscosity attributed to a degradation upon heating above 95°C in alkaline

conditions, some proteins like BSA went through conformational changes leading to the formation of aggregates upon heating above 56°C. Globally it was noticed that polysaccharides are somehow resistant to heating and alkaline conditions and if the targeted fraction is polysaccharidic a combination of chemical and physical methods can be used to enhance the negative repulsive forces on the polymeric backbones and favor solubilization. One of the objectives set for this thesis was attaining an efficient extraction of gel-forming EPS while understanding the effects of the extraction techniques on the resulting properties. This aspect was addressed in chapter II by studying the effects of different solvents and heating temperatures on the extraction efficiency and molecular properties of gel-forming EPS (Table I-4).

The complexity of the extracted EPS makes studying it a hard task and using different analytical techniques is a must to have inputs from different sources allowing the construction of conclusions that are the closest to the reality of the EPS matrix. Both in situ and in vitro approaches were adopted for the analysis of EPS. In situ approaches such as microscopy allow an understanding of the organization of bacteria as well as polymers inside the granule. For a valorization approach, in vitro analyses are more adapted to characterize the extracted polymers. However, to obtain detailed information and decipher which polymers participate in the hydrogel formation, it is mandatory to go through a fractionation step since the obtained raw extracts are very complex. Therefore, chapter III focused on the development of a miniaturized method for the detection of gelation through calcium cross-linking and gaining insights about interactions that might happen between polymers from different biochemical groups during the formation of hydrogels. Chapter IV, focused on the fractionation of sEPS using an anionic exchange chromatography column to enhance the knowledge on the characteristics of gel-forming EPS (charge, molecular weight, and biochemical groups). Table I-4 summarizes the scientific questions addressed in this thesis.

Table I-4: Summary of the thesis scope

<b>Questions addressed</b>	
<b>Chapter II</b>	<ul style="list-style-type: none"> <li>• What are the effects of the extraction solvent on the amounts of solubilized and acid precipitated EPS?</li> <li>• What are the effects of the extraction solvent on the biochemical composition of solubilized and acid precipitated EPS?</li> <li>• What are the effects of the heating temperature on the amounts and molecular size of extracted EPS?</li> </ul>
<b>Chapter III</b>	<ul style="list-style-type: none"> <li>• How to develop a miniaturized technique capable of detecting gelation?</li> <li>• Are there synergies or antagonist effects between the biochemical entities forming the EPS extracts during gelation?</li> </ul>
<b>Chapter IV</b>	<ul style="list-style-type: none"> <li>• Does the charge of the extracted polymers affect their capacity to form hydrogels?</li> <li>• Is the molecular weight of EPS an important factor for gel formation?</li> <li>• Which biochemical and functional groups are the most implied in the gel formation?</li> </ul>

The document will end with an exhaustive summary of the main findings and conclusions followed by the perspectives arising from this work.

# Chapter II:



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## **Effects of alkaline solvents and heating temperatures on the solubilization and degradation of gel-forming Extracellular Polymeric Substances (EPS) extracted from aerobic granular sludge.**

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**Abstract:** The effects of solvents and heating temperatures on the recovery and degradation of gel-forming EPS (Extracellular Polymeric Substances) extracted from aerobic granular sludge were investigated. Sodium hydroxide has allowed a better EPS solubilization compared to sodium carbonate ( $711.3 \pm 38$  and  $408.4 \pm 4$  mg VS /g VS respectively) but the capacity to precipitate gel-forming EPS at acidic pH was higher in sodium carbonate (22% compared to 6% in sodium hydroxide at 80 °C). Increasing the temperature from 20 °C to 80 °C during EPS solubilization has multiplied the amount of solubilized matter by 4 in sodium carbonate and 2.2 in sodium hydroxide. Similar biochemical composition but different molecular weight profiles were observed for the two solubilized EPS extracts, evidencing partial degradation of the polymers in sodium hydroxide. Furthermore, coupling molecular weight analysis and precipitation yield measurement revealed that the lower precipitation of sodium hydroxide solubilized EPS is related to the size reduction due to degradation. Altogether these results help to guide towards a unified protocol for the extraction of gel-forming EPS.

**Keywords:** Extracellular polymeric substances (EPS), Aerobic granular sludge, Alkaline solubilization, Heating temperature, Precipitation, Gel-forming polymers.

## 1 Introduction

The use of aerobic granular sludge for the treatment of wastewater has elicited more interest over the past ten years. Granular sludges present many advantages over activated sludges among which a higher settling velocity *in fine* leading to a reduction in reactor size, energy consumption and environmental footprint. From an economical point of view, granular sludges are also interesting because they lower investment and installation costs [1,2]. Aerobic granular sludges are currently investigated as potential resources for the recovery of high value-added biomaterials to reduce the amount of wastes produced during water treatment processes. Extracellular polymeric substances (EPS) insuring the mechanical properties and stability of the granules [3] can provide interesting renewable materials for industrial applications [4–6]. The extracted material was shown to have amphiphilic properties due to the presence of a small fraction of lipids, allowing the use of EPS as a surface coating material for paper packages thus enhancing their water resistance [5]. Flame retarding properties [6] and heavy metal adsorption, particularly for Pb, Cd and Zn, have also been demonstrated for EPS extracted from aerobic granules [7] or anammox granules [8]. Among the existing applications, hydrogel-forming capacity has received the most attention. Indeed, EPS secreted by aerobic granular sludge can form hydrogels with ionic cross linkers such as calcium ions and precipitate at low pH [4]. Due to similar extraction methods and the presence of carboxyl groups, these structural EPS were later on named Alginate Like Exopolysaccharides (ALE) [4]. Extracted molecules value and their significance as alternatives to industrial products remain however dependent to the downstream granules biorefinery [9]. Indeed, due to the molecular diversity of the EPS extracted from aerobic granules, the precise composition and chemical nature of the structural EPS remain unknown. Recent works have confirmed this complex chemical composition including proteins, neutral sugars, uronic acids, phenolic compounds, hyaluronic acid-like and sulfated glycosaminoglycan-like polymers [10–12]. Extraction technologies have thus to be adapted to the diversity of the secreted molecules and a better understanding of their impacts on the recovered polymers (both in terms of quantities and properties) is needed to ensure the economic feasibility of this resource recycling. The lack of information on the chemical and physical identities of EPS greatly limits the potential to rationally propose relevant EPS extraction protocols. In addition, the extraction procedures have to be defined depending if the entire spectrum of the EPS is

required for further comprehension of the diversity and biological significance of EPS or if specific functional EPS are targeted [13].

The aggregation of the EPS matrix is due to interactions between the polymers forming it; the main forces responsible of maintaining this structure are van der Waals forces, hydrogen bonds, electrostatic linkages, hydrophobic interactions and covalent bonds [14–16]. Solubilizing these polymers requires to break these interactions using extraction methods that can focus on one or multiple types of EPS bonds in the matrix. Considering stratified and dense aggregates such as fixed biofilms or granular sludges, harsh extraction methods are required to solubilize the extracellular matrix and recover EPS. Physical methods (sonication, heating, centrifugation...), chemical methods (alkaline extraction, chelators, detergents...) or a combination of both can be used to attain this objective of maximal extraction yield whether on biofilms [17] or granular sludges [18,19]. The extraction techniques are thus very heterogeneous and there is no unified protocol. Understanding the factors influencing the extraction is therefore essential to propose relevant extraction protocols.

Alkaline conditions are widely used for the extraction of structural EPS showing gel-forming properties because such conditions allowed high extraction yields [20]. Due to the fact that EPS isoelectric points are generally below 6 [14], high pH conditions cause carboxylic groups in proteins and polysaccharides to be ionized which results in a strong repulsion between EPS and provides a higher water solubility of the compounds [21,22]. Different alkaline extraction procedures were tested previously which mainly relied on the use of sodium hydroxide or sodium carbonate at various concentrations [4,12,23,24]. High temperatures of 80 °C were used in combination with sodium carbonate while sodium hydroxide solubilization was generally performed at a lower temperature of 4 °C but no clear justification of the choice of such extraction conditions were provided by the authors [4,12,21,23]. Seviour et al. [24] suggested that granule solubilization could influence the properties and characteristics of the putative structural gel-forming agents extracted either with sodium hydroxide (as a complex hetero-polysaccharide named granulan) or with sodium carbonate (as uronic acids including ALE). Comparison between studies remains also difficult since the type of feeding may have a direct effect on the EPS extraction yield depending on the carbon sources and nutrient concentrations and an indirect effect via changes in microbial diversity: for example, some granular sludges are fed by municipal wastewater others are fed by synthetic wastewater,

which influences the amount of EPS produced by aerobic granules [12]. In addition, using the same type of biomaterial but changing the method of extraction gives different yields [20,23]. Alkaline conditions are not efficient for structural EPS extraction from aerobic granular sludge enriched with ammonium-oxidizing bacteria [25] or with *Deftuviicoccus* sp. [26]. Therefore, there is a need to understand what really influences the yield of extraction and the molecules selected by the extraction protocol.

The global solubilization efficiency cannot be considered as the only relevant parameter, particularly for the purpose of recovering polymers with specific functional properties. In the case of alginates from seaweed, a higher molecular weight was shown to give the compound a better gel formation kinetics and enhanced viscosities [27]. A molecular weight dependency of alginic acid has been proved to influence the gel strength since Young's modulus values were increased 5 times when the molecular weight of algal alginates increased from 60 to 400 kDa [28]. With the objective of recovering gelling biomaterials from granular sludges, preserving the entire native size of the polymers may be a key issue. Thus, attention must be drawn to the extraction conditions that could lead to depolymerization/alteration of the molecules forming the EPS matrix.

Two main objectives were set for this study. The first one was to compare the effect of chemicals and heating on the yields of solubilization and precipitation of gel-forming EPS extracted from granular sludge. Particular attention was paid for the adverse effect of the degradation/alteration of biopolymers on their recovery efficiency. The second objective was to evaluate the selectivity of the extraction methods on EPS in terms of molecular weight and biochemical composition of extracted molecules that are relevant parameters to drive aerobic granular sludge valorization towards gel-forming biomaterials.

## **2 Materials and methods**

### **2.1 Biomaterial**

EPS were extracted from aerobic granular sludges cultivated in 13 L column Sequencing Batch Reactors (SBR) fed with 100%-VFA synthetic wastewater composed of equal COD fractions of acetate and propionate [29]. All systems were operated at a constant volume and SBR cycles were as follows: anaerobic plug-flow feeding (1.5 h), aerobic phase (4 h), settling (variable time), and excess sludge removal after settling (critical settling velocity of 1.7 to 5.1 m/h). A

detailed characterisation of the influent composition as well as the physical properties of the granules used for organic pollution, nitrogen and phosphorus removal is provided in Layer et al. [29,30].

## 2.2 EPS extraction

The extraction protocol was divided into two steps: (i) a solubilization step of polymers from aerobic granules using different solvents and (ii) a precipitation step of the solubilized polymers in the form of a gel pellet. As usually defined by many authors [12,20,31], the extracted material was named EPS for the sake of clarity, even if the presence of some intracellular contamination cannot be excluded. For EPS solubilization, a ratio of 0.2 g of freeze-dried granules to 32 mL of the chosen solvent at 0.2 M (urea, sodium carbonate, sodium hydroxide, and sodium chloride considered as a control) was kept for the rehydration of granules under stirring at 300 rpm for 30 minutes. Afterwards, the granule suspension was homogenized using the ultra-turrax T25 (Janke & Kunkel from IKA-Labortechnik) at a speed of 13500 rpm for 3 cycles of 1 minute separated by 45 seconds of resting phases. Then the disrupted granules were heated for 60 min in a water bath at 80 °C. After centrifugation (22 000 g, 4 °C, 15 min) the supernatant was recovered (named S1) and the pellet (named P1) was analyzed for its content in volatile solids (VS). The extraction protocol was also performed at different temperatures (20 °C, 50 °C, 70 °C and 80 °C) with sodium hydroxide or sodium carbonate to determine the effect of the heating temperature on the yield and properties of extracted EPS.

As previously described for the acidic precipitation of ALE [4], the pH of the S1 extract was adjusted to  $2 \pm 0.02$  with 1 M HCl, and after an incubation of 30 minutes in a water-ice bath, the precipitate was collected by centrifugation at 22 000 g, 4 °C for 15 minutes. The supernatant after acidic precipitation was recovered and named S'1. The precipitate was solubilized in 0.1 M sodium hydroxide then dialyzed for 36 hours against distilled water by using a dialysis membrane with a cutoff of 3500 Da (Spectrum labs). All the salts formed by the previous acidic precipitation and alkaline solubilization were eliminated thanks to this dialysis step, allowing direct evaluation of the recovered EPS by gravimetry after their lyophilization. The freeze-dried material (named P2) was finally resuspended in 0.1 M sodium hydroxide (named S2) for further analysis.

Efficiency of EPS solubilization was calculated by subtracting the VS amount remaining in the pellet P1 from the initial amount of VS present in the granules and dividing it by the initial amount of VS in the granules. EPS precipitation efficiency was evaluated by the dry mass of VS contained in the P2 lyophilizate. All the extraction protocols and yields evaluation were performed in triplicate and standardized by the initial amount of VS in the granules.

### **2.3 Size exclusion chromatography (SEC)**

Chromatography analysis, to determine the apparent molecular weight of molecules, was performed using the AKTA purifier system (Cytiva) equipped with an on-line spectrophotometer.

The SUPERDEX 200 column was used to separate molecules between 10 and 600 kDa (Cytiva). The exclusion volume corresponding to the volume of liquid located outside the pores of the column is equal to 7.7 mL and the total inclusion volume corresponding to the volume of liquid included outside and inside the pores of the column is equal to 24 mL. The injection volume was set to 250  $\mu$ L. The used mobile phase was Tris HCl 50 mM with 0.1 M NaCl, pH  $9 \pm 0.02$  with a flow of 0.5 mL/min. According to their elution volume and apparent size, the peaks composing the chromatograms were divided into three regions: HMW=high molecular weights that were eluted first between 7.7 and 12 mL, MMW= medium molecular weights eluted from 12 to 17 ml and LMW= low molecular weights that were eluted lastly after 17 ml. The P2 samples were solubilized in 2 mL of 0.1 M sodium hydroxide and filtered through a regenerated cellulose membrane of 0.2  $\mu$ m. Molecules were detected by monitoring the absorbance at 280 nm (AU). The peak area (AU $\times$ mL) was processed using the UNICORN 5.31 software.

For each molecular weight category, the percentage of precipitation was calculated by dividing the peak area in the S2 chromatogram with the peak area in the S1 chromatogram and multiplying by 100.

### **2.4 Asymmetrical Flow Field Flow Fractionation (AsFIFFF)**

AsFIFFF was used to determine some molecular characteristics of the S2 polymers such as size and gyration radius. The setup was an Eclipse Dualtec separation system (Wyatt technology Europe) with a UV detector (Ultimate 3000, Thermo Scientific). Multi Angle Light Scattering (MALS) measurements were performed with a DAWN-HELEOS II (Wyatt technology

Corporation) coupled to a differential Refractive Index detector (Optilab rEX, Wyatt technology Corporation). Size distribution was determined by studying the dependency of the intensity of the scattered light as a function of the scattering angle with a laser source at a wavelength of 658 nm. Astra software package, version 7.1.2 was used to provide molecular weight ( $M_w$ ) and gyration radius ( $R_g$ ).

For the experiments, 50  $\mu$ L solutions of acid precipitated EPS (S2), at 2 mg/mL in 0.1 M sodium hydroxide, were injected. The eluent for the AsFIFFF experiments was prepared from ultrapure water (Milli-Q), and consisted of 50 mM of Tris-HCl, 0.1 M NaCl,  $\text{NaN}_3$  0,02% at a pH of 9. Elution mode was started with a crossflow rate of 3 mL/min for 10 min, and then reduced linearly for 10 min to a rate of 0.09 mL/min.

## **2.5 EPS biochemical composition**

To evaluate the selectivity of the most efficient solvents, S1 and S2 extracts obtained with sodium hydroxide and sodium carbonate at 80 °C, were analyzed in triplicate for proteins, humic acid-like substances, neutral sugars, uronic acid-like and DNA components. All measurements were done in 96 well microplates, using the FLUOstar OPTIMA (BMG, Labtech) microplate reader.

### **2.5.1 Protein and humic acid-like substances quantification**

The modified Lowry assay was used for the quantification of proteins and humic acid-like substances according to Frølund et al. [32] and the used standards (0-500 mg/L) were respectively BSA (Sigma-Aldrich) and humic acid (Fluka-Aldrich). The measurement of absorbance was done at 750 nm, with or without  $\text{CuSO}_4$ . When  $\text{CuSO}_4$  is omitted, the color development is due mainly to humic substances and chromogenic amino acids, and BSA color development decreased to 20%. Therefore, concentration of proteins and humic acid-like substances can be calculated by using the equations described in Frølund et al. [32].

### **2.5.2 Neutral and uronic acid-like sugars quantification**

The Anthrone method was used for the quantification of both neutral and uronic acid-like sugars as adapted from Rondel et al. [33] with respectively glucose (0 to 75 mg/L) and glucuronic acid (0 to 750 mg/L) as standards. The Anthrone reagent was prepared with a 2 mg/mL concentration in sulfuric acid 96% (v/v) and 150  $\mu$ L of this reagent were added to 75  $\mu$ L of samples/standards in 96 well microplates. Incubation at 60 °C for 30 minutes followed

by cooling at room temperature for 10 minutes were done. The absorbance was read at 544 nm and 620 nm. The slopes of the four equations linking the standard solutions with glucose or glucuronic acid concentrations were calculated. Afterwards, the concentrations of the unknown samples can be calculated by solving a system of two equations as described in Rondel et al. [33].

### **2.5.3 DNA quantification**

The Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen) was used for the DNA quantification. Lambda DNA (100 µg/mL) was used for the DNA standard curve going from 0 to 2000 ng/mL. In a 96 well microplate, 100 µL of the reagent prepared according to the manufacturer, were added to 100 µL of standards/samples. Fluorescence intensity was measured at an excitation emission wavelength pair of 485/520 nm.

### **2.6 Young's modulus determination**

In order to determine the gel stiffness a protocol was adapted from Felz et al. [34]. The dialyzed and freeze-dried material (named P2) was resuspended in 0.1 M sodium hydroxide at a final concentration of 8% w/v (mg VS / 100 mL). Hydrogels were then prepared via ionic cross-linking in the presence of calcium ions. The calcium ions (0.1 M CaCl<sub>2</sub>) diffused into the EPS solution through a dialysis membrane (3.5 kDa MWCO, 36h dialysis). The Young's modulus (E, Pa) was selected as a sensitive parameter to analyze the polymers mechanical response. The mechanical tests were performed using a Mars III rheometer (Thermo Scientific) in a stress-controlled mode. The samples were subjected to consecutive compression-decompression cycles (n. 17) increasing the maximum deformation in the loading step up to 20%. The Young's modulus (E) was calculated from the linear regression of the stress ( $\sigma$ , Pa) – strain ( $\epsilon$ , -) data in the linear elasticity domain of the material (i.e.  $\sigma=E\cdot\epsilon$ ). Each test was performed in triplicate and the Young's moduli were reported as average values  $\pm$  standard deviations.

## **3 Results and discussion**

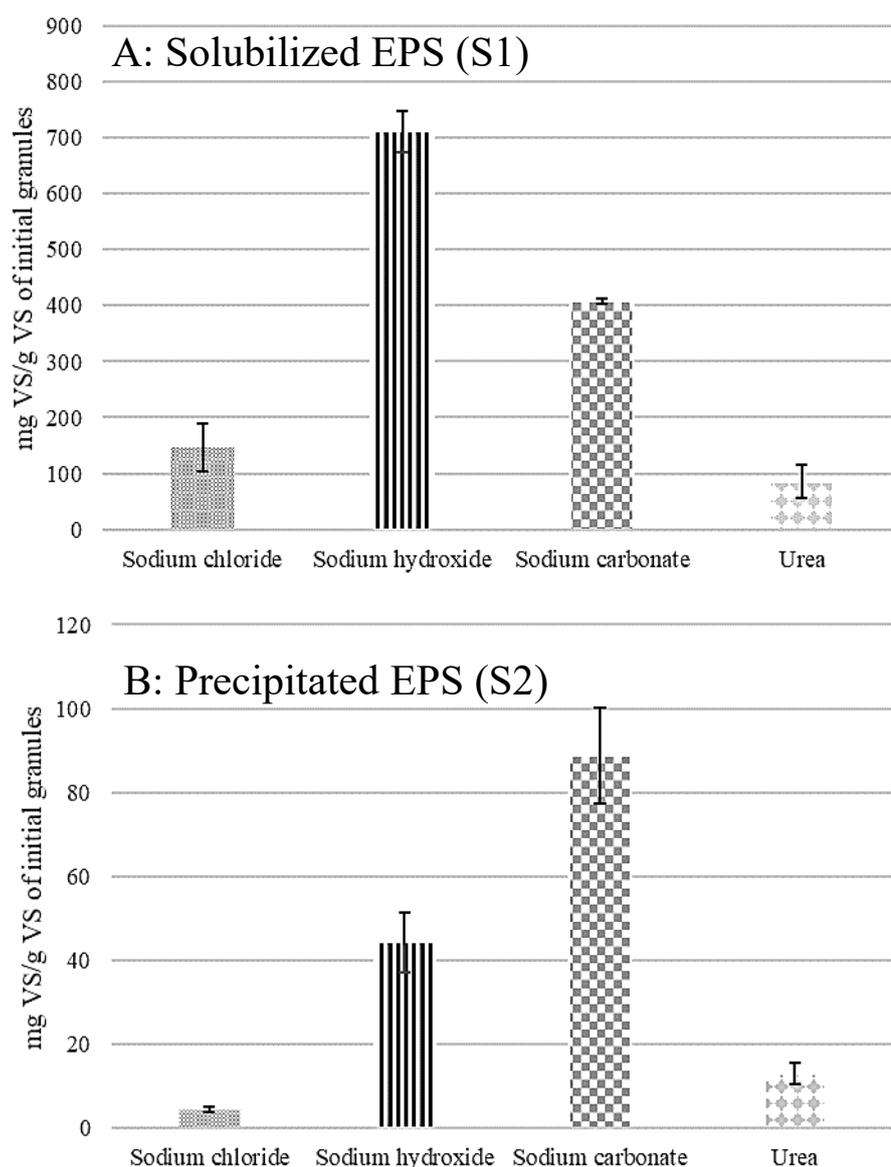
### **3.1 Effects of chemical solvents on EPS solubilization and precipitation**

#### **3.1.1 Comparison of extraction efficiency between solvents**

Results of the solubilization with the used solvents (urea, sodium carbonate, sodium hydroxide, and sodium chloride) followed by the precipitation step are shown in Figure II-1.

At 80 °C sodium hydroxide was the most efficient for solubilizing granules with  $711.3 \pm 38.0$  mg VS solubilized/g VS followed by sodium carbonate with a solubilizing power diminished to  $408.4 \pm 4.0$  mg VS solubilized/g VS as seen in Figure II-1A. Urea and sodium chloride were clearly less efficient with a solubilization efficiency of  $85.2 \pm 30.0$  mg VS solubilized/g VS and  $146.7 \pm 43.0$  mg VS solubilized/g VS respectively. Those different solvents rely on contrasted mechanisms: alkaline solubilization, i.e. sodium carbonate or sodium hydroxide, is based on the increase of negative repulsive forces of the charged polymers while urea is known to facilitate the exposure of hydrophobic residues and to break hydrogen bonds [35,36]. The pH value is  $11.30 \pm 0.02$  for sodium carbonate 0.2 M and  $13.40 \pm 0.02$  for sodium hydroxide 0.2 M. In the case of proteins, a higher pH is likely to induce a higher negative charge of the chains containing acidic amino acids which will favor electrostatic repulsions between molecules. This repulsion contributes to the improvement of proteins solubility thanks to increased protein–water interactions [37]. Due to the ionization of carboxyl groups, some polysaccharides such as pectin or functional acidic polysaccharide extracted from okra pods, also show a better solubilization with the increase of pH [38,39].

Our results therefore suggest that the EPS matrix is composed of a majority of negatively charged polymers and that switching solvents used for extraction significantly affects the solubilization of the organic material extracted from aerobic granules. This is in agreement with results obtained in previous studies showing that alkaline treatments provide a higher solubilization yield in comparison with other physical or chemical methods [20].



**Figure II-1. Effect of the initial solvent on the efficiency of EPS solubilization (A) and EPS precipitation (B). Efficiency is expressed in mg of recovered VS in S1 (A) or mg of precipitated VS in P2 (B) and referred to the initial amount of VS in granules. Extractions and acidic precipitations were performed in triplicate.**

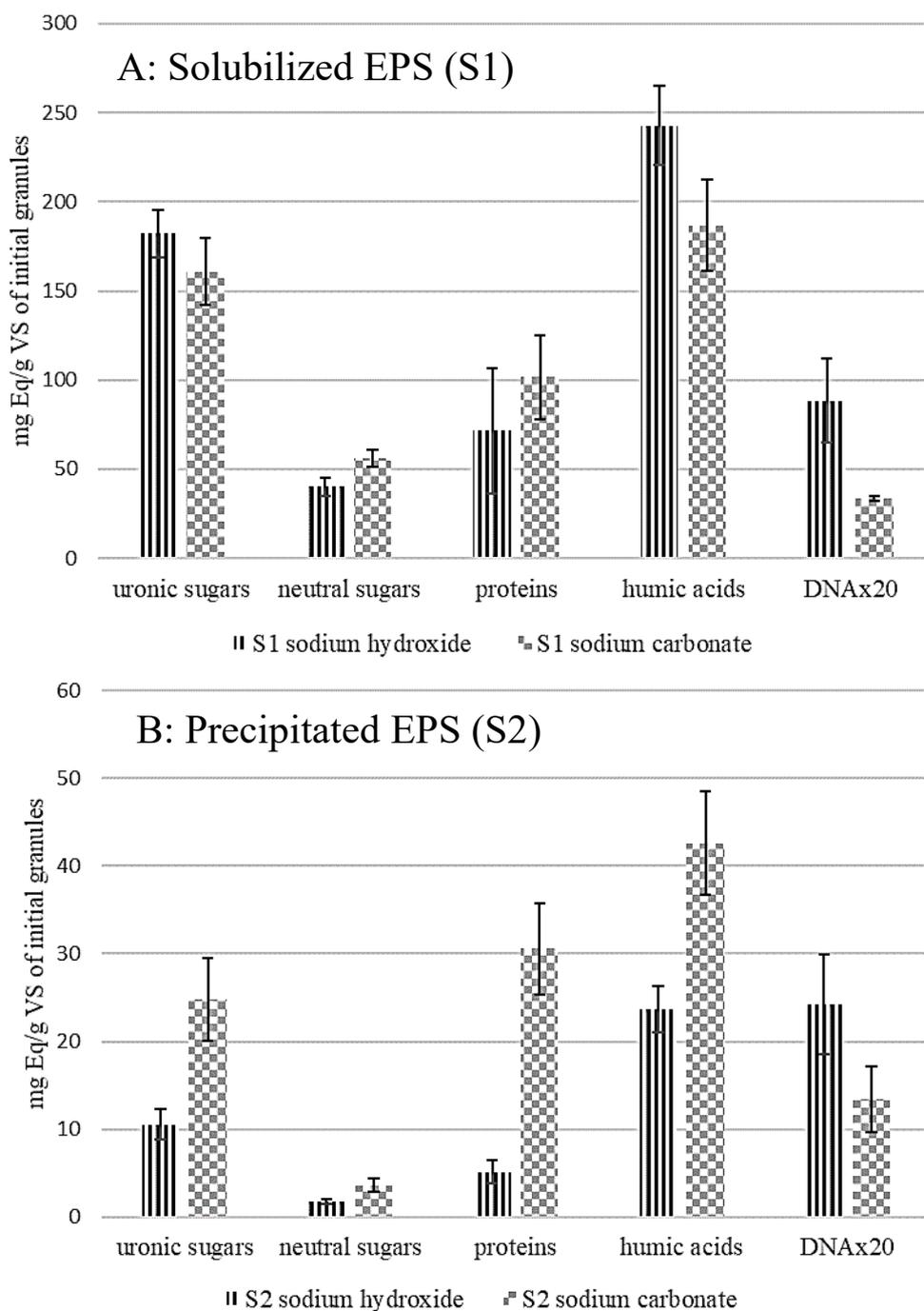
After acidic precipitation an inversion in the final yield happened, leading to the recovery in P2 final samples of  $44.3 \pm 7.1$  and  $88.8 \pm 11.5$  mg VS precipitated/g VS of initial granules for sodium hydroxide and sodium carbonate respectively (Figure II-1B). Materials recovered in S1 by sodium carbonate and sodium hydroxide can be precipitated by 21.7% and 6.2% respectively. Previous analysis of the zeta potential of EPS solubilized by sodium hydroxide from anammox granules, revealed that EPS precipitation should be done at a pH around 4 corresponding to a global neutral charge facilitating aggregation of polymers [31]. Indeed, biopolymers have minimal interactions with water at their isoelectric point, while molecule-

molecule interactions are at their maximum promoting precipitation [37]. As described previously by many authors, a big amount of the solubilized EPS is lost after acidic precipitation similarly to what is observed in this study [12,31,40]. Therefore, initial solubilization should not be the only parameter considered to choose the extraction solvent and further investigation on the biochemical and molecular characteristics of the extracted molecules must be done in order to better understand this difference in precipitation yields between solvents. Based on the high yields of solubilization and precipitation, only sodium hydroxide and sodium carbonate will be investigated furthermore in this study.

### **3.1.2 Biochemical composition of extracted EPS**

To evaluate possible selectivity of the two alkaline solvents on the recovery of a type of biopolymer (neutral or uronic acid-like polysaccharides, proteins or humic acid-like substances), S1 and S2 samples obtained using sodium hydroxide or sodium carbonate extraction at 80 °C were analyzed for their biochemical composition (Figure II-2).

The solubilization of neutral sugar (Figure II-2A) was slightly higher in S1 sodium carbonate compared to S1 sodium hydroxide ( $56.0 \pm 4.9$  mg/g VS compared to  $40.1 \pm 5.3$  mg/g VS), solubilization of uronic acid-like molecules and proteins were approximately the same in both solvents. However, after precipitation (Figure II-2B), 15.4% of the S1 uronic acid-like molecules solubilized in sodium carbonate precipitate (from  $160.9 \pm 18.5$  to  $24.8 \pm 1.7$  mg/g VS in S1 and S2 respectively) while only 5.8% precipitate for sodium hydroxide (from  $182.2 \pm 13.4$  to  $10.6 \pm 1.7$  mg/g VS in S1 and S2 respectively).



**Figure II-2. Biochemical composition of S1 samples containing EPS solubilized in alkaline solvents (A) and of S2 samples containing EPS recovered by acidic precipitation (B).** Neutral sugars and uronic acid-like molecules quantification were done using glucose and glucuronic acid as equivalents, respectively. Protein and humic acid-like substances quantification were done using BSA and humic acid as equivalents respectively. DNA quantification was done using Lambda DNA as equivalent and values were multiplied by 20 for representation in the graphs. Analytical (3 assays) and biological (3 extractions) replicates were performed.

The same trend was observed for proteins: for sodium carbonate 33.7% of the proteins solubilized in S1 have precipitated ( $90.4 \pm 14.2$  mg/g VS in S1 to  $30.5 \pm 5.1$  mg/g VS in S2) in

comparison to only 4.6% of proteins solubilized in S1 sodium hydroxide ( $113.7 \pm 40.5$  mg/g VS in S1 to  $5.2 \pm 1.3$  mg/g VS in S2) (Figure II-2B).

Sodium carbonate solubilizes humic acid-like substances less than sodium hydroxide (Figure II-2A) but the molecules present in sodium carbonate are more precipitable and therefore the quantity of humic acid-like substances in S2 is approximately the double (Figure II-2B).

Altogether these results indicate that S1 sodium hydroxide and sodium carbonate samples have rather similar biochemical diversity and the inversion of yield observed from S1 to S2 after acidic precipitation cannot be attributed to the prevalence of a specific biochemical fraction. Precipitation requires molecules/molecules interactions that are favored by various polymer properties such as high molecular weight, low charge and the presence of apolar and hydrophobic zones [41]. Extreme alkaline pH used during solubilization may thus influence the precipitation ability of polymers because of denaturation or alteration of the polymer structure leading to modification of their molecular weight and/or spatial conformation.

As previously mentioned the molecular weight of molecules impacts precipitation. On one hand, polysaccharides are generally relatively stable to alkali; an exception is present for uronic sugars (for example alginate) that have a glycosidic linkage in a beta position in relation to a carbonyl group which makes them more sensitive to  $\beta$ -elimination and oxidative-reductive depolymerization [42]. As recently shown by Bai et al. [39] studying the influence of pH on the extraction and physico-chemical properties of galacturonic acid containing polysaccharides from okra pods, both the degree of esterification and the length of the polymers regularly decreased while increasing the pH from 2 to 12. On the other hand, proteins are known to be sensitive to degradation in alkaline conditions, as shown by Hou et al. [36]. The authors reported that when sodium hydroxide concentration was higher than 0.07 M at 35 °C, peptide bonds might be hydrolyzed and the size of the polypeptides reduced. To check if proteins are degraded in the conditions used in this study, BSA was submitted to both conditions and the resulting solutions were analyzed by SEC (Figure II-S1 of the supplementary material). While only a slight shift was observed for the peaks of BSA incubated in sodium carbonate, more than 80% of the peaks were found at lower elution volumes for BSA incubated in sodium hydroxide. Higher degradation in sodium hydroxide may thus explain the observed differences in precipitation yield between the two alkaline solvents. To support

this, S1 SEC profiles were compared to SEC profiles of the remaining acidic supernatant S'1 after acid precipitation and it was observed that acidic precipitation is selective for HMW and MMW molecules whereas only a small fraction of LMW molecules is precipitated (Figure II-S2 of the supplementary material) reinforcing the hypothesis that molecular size influences the precipitation behavior of molecules.

Another factor influencing the precipitation is denaturation and the presence of apolar/hydrophobic zones. Among the effects induced by extreme pH, unfolding of proteins leads to substantial changes by the exposition of some hydrophobic areas that are buried at neutral pH. It can be supposed that denaturation occurred in both solvents but as described by Kristinsson and Hultin [43], different properties of the proteins were obtained after refolding. The differences in precipitation ability of proteins between sodium hydroxide and sodium carbonate solvents (4.6% and 33.7% respectively) suggest that not the same type of proteins is extracted with both solvents and/or that major changes happened to the structure of proteins in sodium hydroxide hindering their recovery in acidic conditions. A higher release of intracellular proteins during sodium hydroxide solubilization may be also hypothesized since DNA concentration (Figure II-2) was approximately 2.5 times higher in S1 and 2 times higher in S2 ( $4.4 \pm 1.2$  and  $1.2 \pm 0.3$  mg/g VS respectively in S1 and S2) compared to DNA in sodium carbonate ( $1.7 \pm 0.1$  and  $0.7 \pm 0.2$  mg/g VS in S1 and S2 respectively). However, for the sodium hydroxide fractions, the obtained values are not correlated with a significant increase in the protein contents, eliminating thus a big contamination with intracellular proteins in sodium hydroxide in comparison with sodium carbonate. The presence of DNA may be attributed to both extracellular and intracellular origin.

The differences in gel forming polymers (S2) recovery and in particular in the precipitation efficiency observed for the two alkaline solvents cannot be thus related to their biochemical composition but rather to enhanced denaturation or degradation of the extracted polymers with sodium hydroxide. To confirm this, AsFIFFF fractionation was then performed in order to compare the molecular characteristics of the recovered gel forming polymers in each extraction condition.

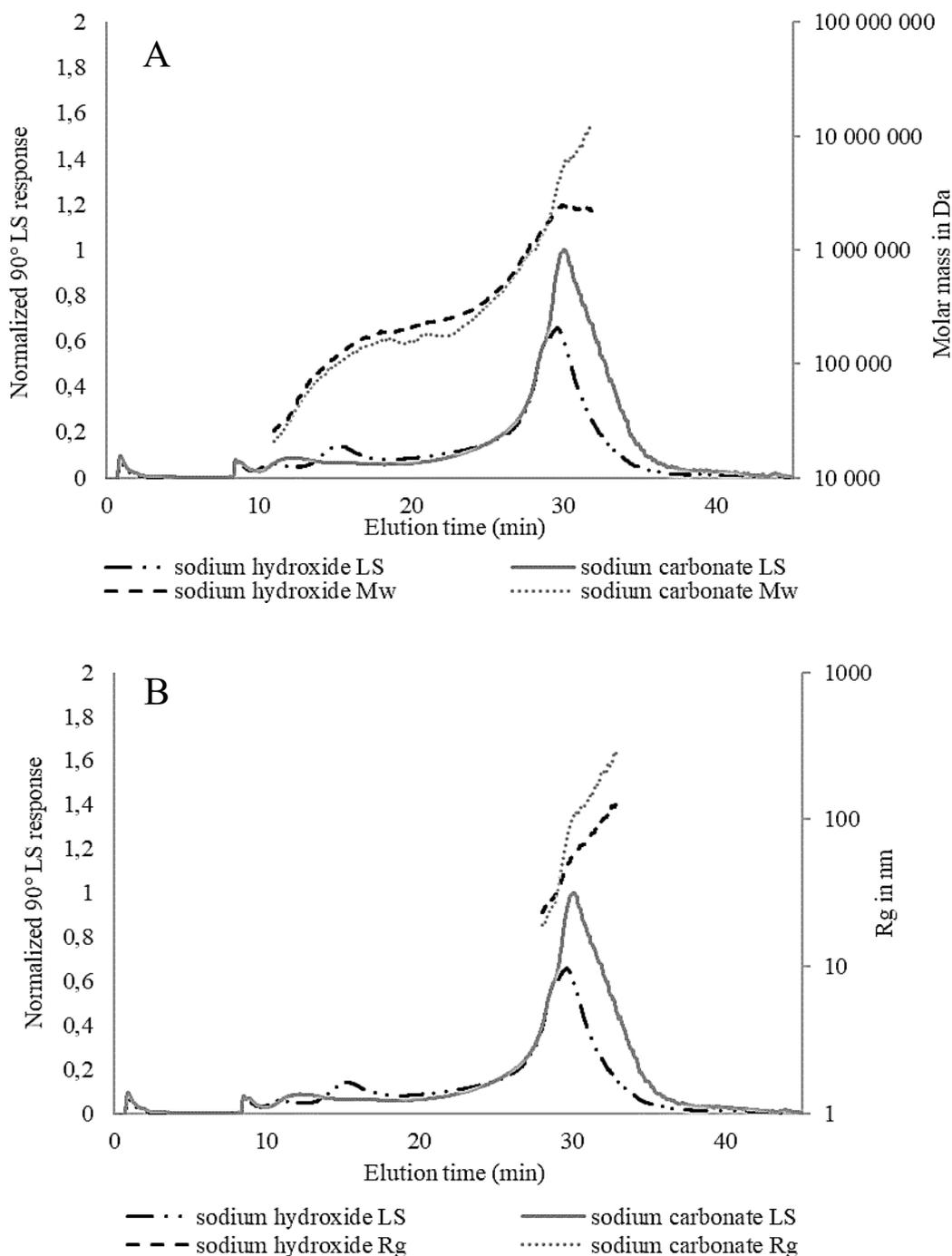
### 3.1.3 Molecular characteristics of alkaline extracted EPS

The S2 samples obtained after acidic precipitation were analyzed by AsFIFFF coupled with MALS detection. This method provides a continuous separation of particles as a function of their diffusion coefficient, and allows determining parameters such as real molecular weight and gyration radius of the molecules.

The fractograms reveal molecular size heterogeneity within the samples with  $M_w$  increasing steadily with the elution time as expected for the normal mode of AsFIFFF operation (Figure II-3A). Similar molecular size profiles were observed for sodium hydroxide and sodium carbonate extracted molecules eluted between 10 and 27 min. On the other hand, quite different profiles were noticed for the lastly eluted polymers (after 27 min): increasing molecular size reaching approximately 10 million Da was obtained for sodium carbonate extracted polymers while a pool of polymers of much smaller sizes (around 2 million Da) are eluted after sodium hydroxide extraction (Figure II-3A). The radius of gyration was also determined for the lastly eluted polymers and it can be noticed that the gyration radius of polymers extracted in sodium carbonate can reach  $277.2 \pm 3.2$  nm and is higher than that of polymers extracted in sodium hydroxide limited to  $126.1 \pm 2.2$  nm (Figure II-3B).

AsFIFFF analysis was used to focus on the HMW defined for elution time ranging from 27.2 min to 32 min (Table II-S1 of the supplementary material). For the HMW molecules eluted from sodium carbonate S2, the average value of Rg is  $118.6 \pm 1.6$  nm,  $M_w$  was  $5.57 \times 10^6 \pm 3.34 \times 10^5$  Da with a number average molar mass  $M_n$  of  $2.2 \times 10^6 \pm 4.6 \times 10^4$  Da leading to a polydispersity index ( $M_w/M_n$ ) of 2.5. Lower values were found for sodium hydroxide S2 fraction with an average Rg of  $61.7 \pm 1.3$  nm,  $M_w$  of  $1.88 \times 10^6 \pm 3.70 \times 10^4$  Da and an average  $M_n$  of  $1.67 \times 10^6 \pm 2.60 \times 10^4$  Da leading to a lower polydispersity index of 1.1. In other studies, the molecular weight of commercial alginates was found to vary on average between 115 000 and 321 700 Da and having slight fractions reaching approximately 1 million Da [44]. Another study carried out on ultra-high viscosity alginates found that the molar mass ranged between 330 000 Da and 2.9 million Da with their gyration radius varying between 98 and 300 nm [45]. In the case of sodium carbonate solubilization, some of the particles eluted after 30 min had molecular weights approximately 3 times higher than those observed in the previously cited study and might be formed *in vitro* by the association of several polymers. Indeed, the precipitated EPS have a very complex biochemical composition, interactions between

different types of molecules could be elicited during acidic precipitation as for example proteins with polysaccharides which are preponderant components of the precipitated EPS. Although the molecules eluted after 30 minutes for sodium carbonate have a higher  $M_w$ , they have similar  $R_g$  to that observed in the study of Storz et al. [45], implying a more compact structure.



**Figure II-3.: Molar mass (A) and gyration radius (B) distribution of the S2 precipitated EPS after initial solubilization in sodium hydroxide or sodium carbonate. The data was obtained using AsFFFF coupled with a MALS detector. Light scattering signals (LS) were represented for each tested sample.**

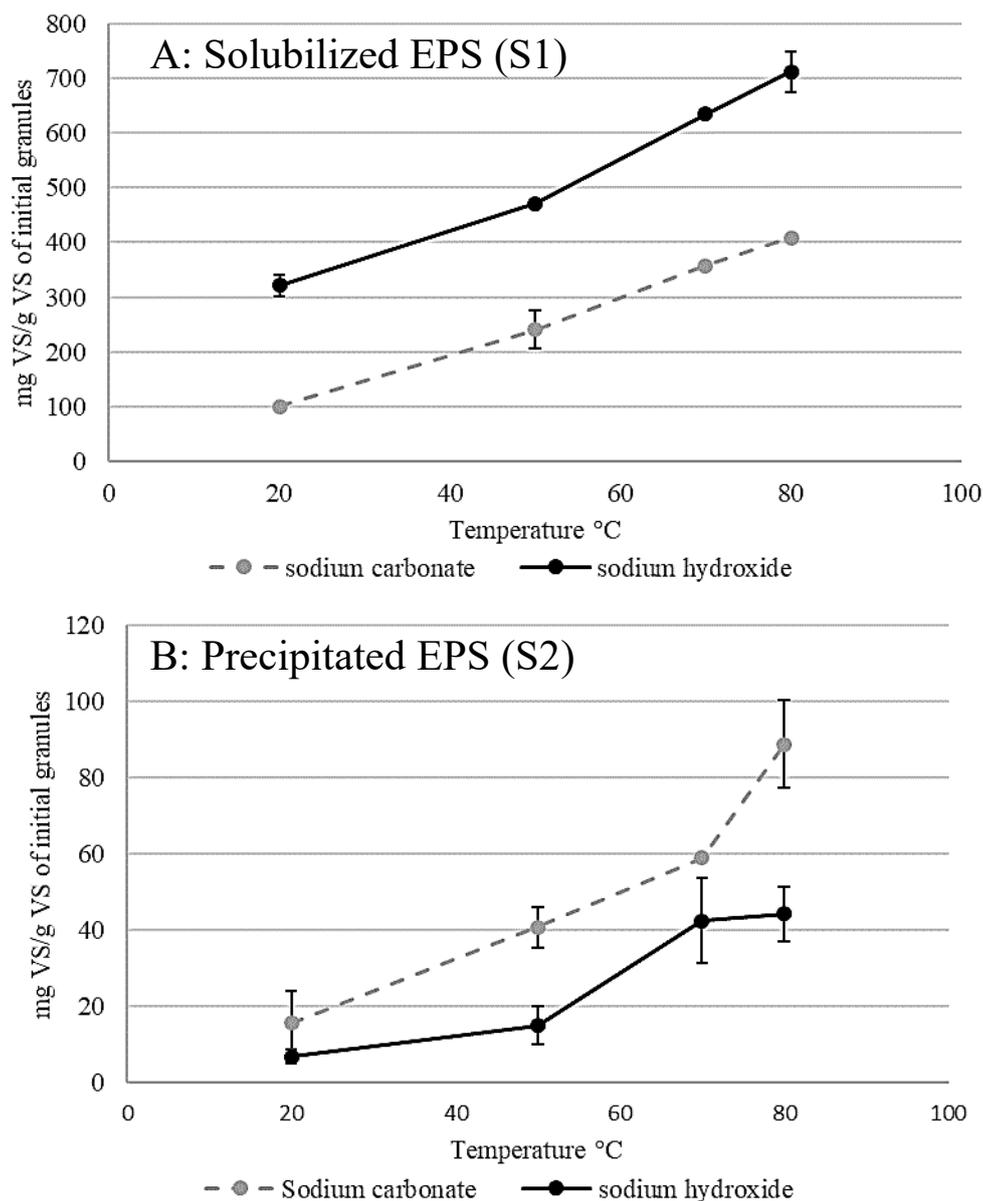
Altogether, these data confirm that initial solubilization conditions influence the physico-chemical properties of the recovered precipitated EPS and that molecules recovered after sodium carbonate solubilization have a higher molecular weight and gyration radius in comparison with those extracted with sodium hydroxide. At this stage of the study, an adverse effect of the degradation/alteration of biopolymers on their recovery efficiency can be reasonably suspected since the final yield of EPS is higher for the extraction with sodium carbonate. The last aspect of this study has consisted of generating more or less denatured EPS during the solubilization step and studying the consequences on the molecular size and capacity of EPS for acidic precipitation.

## **3.2 Effect of heating temperature on EPS solubilization and precipitation**

### **3.2.1 Comparison of extraction efficiency at different temperatures**

Temperature was selected as a relevant parameter to modulate the degree of EPS denaturation. Moreover, this parameter is important for the economic feasibility of future fractionation/biorefinery processes of granular sludges leading to the valorization of biopolymers.

Considering the solubilization step performed at temperatures ranging from 20 °C to 80 °C, the amount of organic matter solubilized with sodium hydroxide remains systematically higher (Figure II-4A). The heating has allowed to increase the amount of solubilized matter by a factor of 4 in sodium carbonate and 2.2 in sodium hydroxide. This better solubilization is explained by the fact that heating causes molecules to vibrate more quickly, which improves their solubility in the solvent [22]. In addition, the energy given during heating causes the cleavage of some low energy bonds which are an important part of the EPS matrix [46].



**Figure II-4. Effect of temperature on the efficiency of EPS solubilization (A) and EPS precipitation (B).** The yields are expressed in mg of recovered VS in S1 (A) or mg of precipitated VS in P2 (B) and referred to the initial amount of VS in granules. Extractions and acidic precipitations were performed in triplicate.

The inversion of solubilization and precipitation efficiency between the two solvents is also confirmed even at low temperatures where the yield for final EPS recovery is always higher in sodium carbonate compared to sodium hydroxide (Figure II-4B). Moreover, it can be noticed that for both solvents, the temperature increase during the initial solubilization step has a similar influence on the precipitation efficiency (Figure II-S3 of the supplementary material).

Altogether, these data indicate that the temperature affects the solubilization of EPS from granules while it does not affect significantly their precipitation ability in acidic conditions. They also confirm that the choice of the solvent and associated pH values are relevant

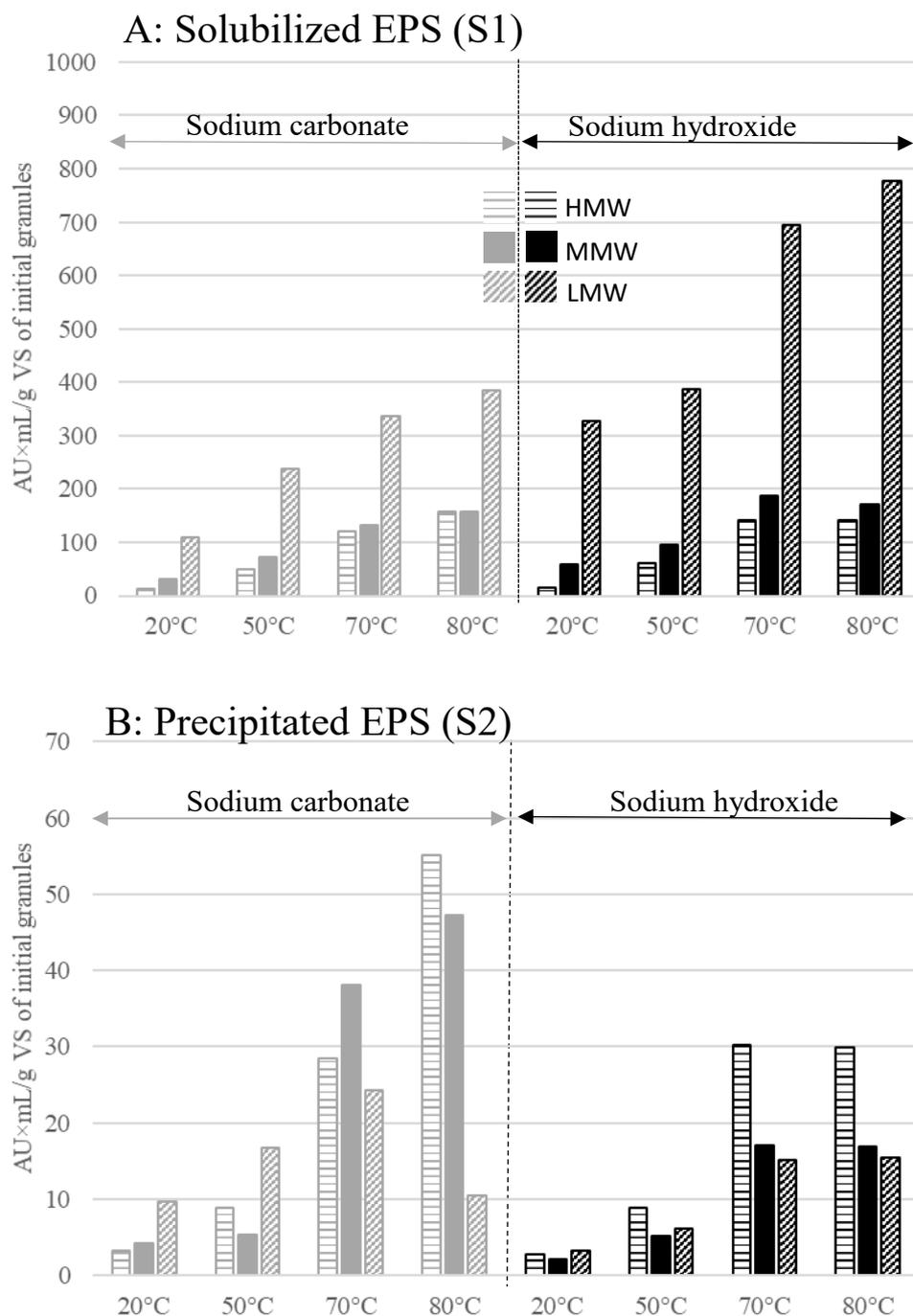
parameters since for all the tested temperatures, the yield for final EPS recovery is always higher in sodium carbonate compared to sodium hydroxide (Figure II-4B).

### 3.2.2 Size distribution of alkaline extracted EPS

In order to better understand such low precipitation ability for sodium hydroxide extracted molecules, the molecular weight distribution of the S1 (solubilized) and S2 (precipitated) molecules were explored by size exclusion chromatography.

The EPS solubilized in S1 samples were divided into three classes of High, Medium and Low Molecular Weight molecules based on the integration of SEC peaks after various solubilization steps performed at temperatures ranging from 20 °C to 80 °C (SEC chromatograms provided in Figure II- S4 of the supplementary material). It can be noticed that the temperature increase has impacted the solubilization of all categories of polymers in both solvents (Figure II-5A). However, the proportion of LMW polymers per g VS of initial granules remains much higher in sodium hydroxide compared to sodium carbonate, suggesting gradual degradation of the solubilized molecules.

After precipitation, the recovered polymers in S2 samples showed various molecular weight distributions depending on the temperature and the solvent used for solubilization (Figure II-5B and Figure II-S4 of the supplementary material). At low temperatures (20 °C, 50 °C), LMW polymers are the major components of S2 sodium carbonate but at high temperatures (70 °C, 80 °C), the molecular weight distribution is in favor of high and medium sized polymers. The amounts of HMW reach their maximum at 80 °C and are in higher proportions than those observed with sodium hydroxide at similar temperatures.

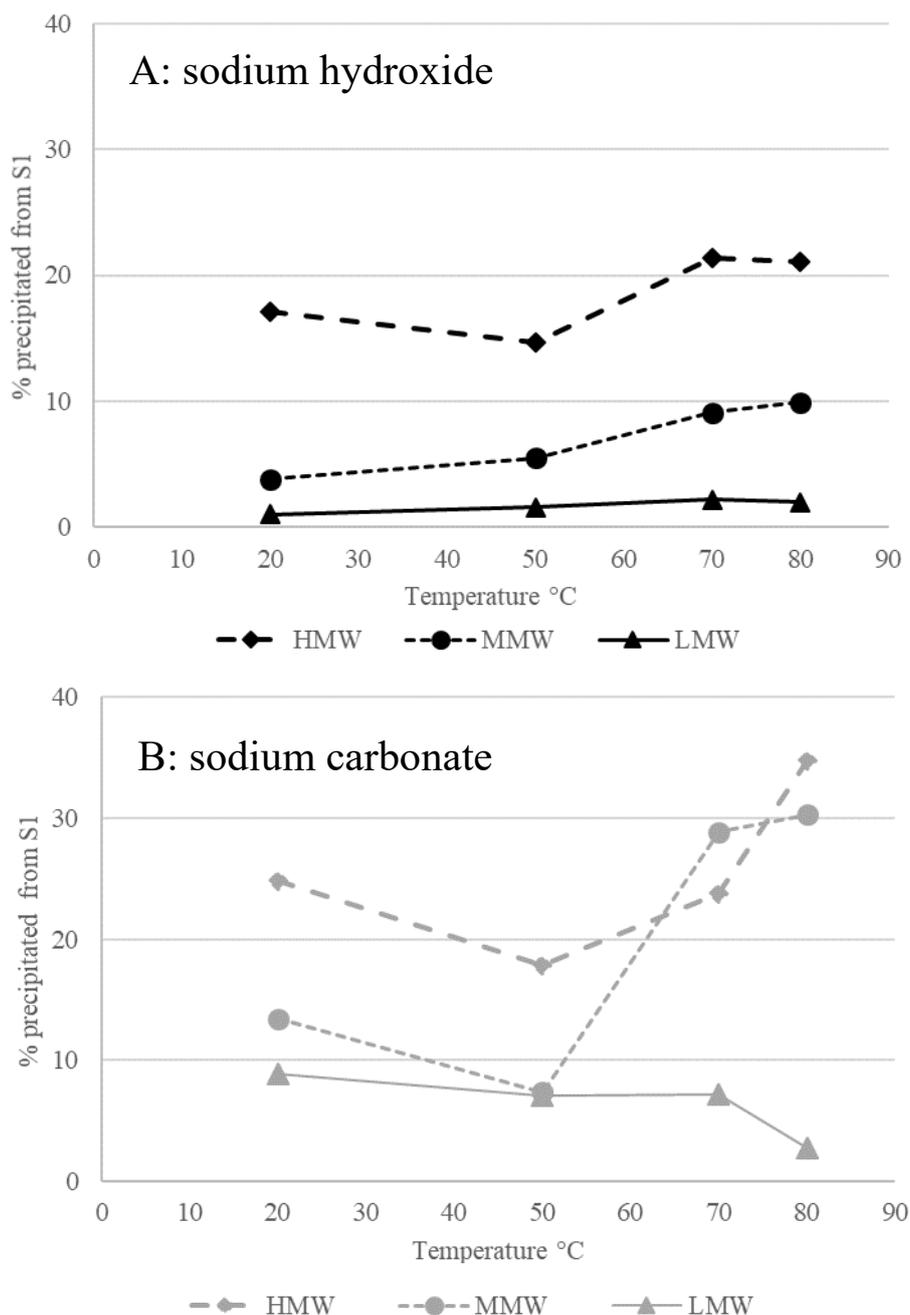


**Figure II-5.** Size distribution of the S1 solubilized EPS (A) and of the S2 precipitated EPS (B) in regard to the temperature used for the initial alkaline solubilization. The analysis was done using a Superdex 200 SEC column. The molecules were detected at 280 nm (AU: absorbance unit) and peak areas were integrated ( $AU \times mL$ ) and referred to the initial amount of VS in granules.

The adverse effect of the degradation of biopolymers on their precipitation ability was further investigated studying the efficiency of precipitation for each category of polymers (Figure II-6). After sodium hydroxide solubilization, acidic precipitation efficiency is correlated to the size of molecules since the percentages of molecules precipitated from S1 are always higher for HMW compared to MMW and LMW and this is true for all the tested temperatures (Figure

II-6A). This selectivity of precipitation towards high molecular weight polymers is less pronounced after sodium carbonate solubilization and varies with temperature: at 50 °C, LMW and MMW are precipitated with similar efficiency, at 70 °C, MMW are more efficiently precipitated than HMW and at 80 °C, the best precipitation is observed for HMW with a precipitation percentage of 34.7% (Figure II-6B). In such sodium carbonate conditions, the precipitation ability of HMW and MMW molecules appears positively correlated with an increase in temperature between 50 °C and 80 °C while this is not the case for the lowest temperatures between 20 °C and 50 °C. This suggests that partial denaturation or alteration of the high and medium sized polymers by thermal treatments may be favorable for their precipitation. Interestingly, the positive influence of thermal action on the precipitation ability of HMW and MMW solubilized with sodium hydroxide is less obvious and this may be related to the fact that the polymers were previously drastically altered or denatured during the initial step.

This SEC analysis of solubilized and precipitated polymers after increasing extraction temperatures reinforces the hypothesis that there are greater signs of degradation in sodium hydroxide than in sodium carbonate. In addition, the loss of LMW molecules that represents a majority of the compounds extracted with sodium hydroxide, and the low precipitation ability of MMW (below 10%) explain the inversion of yields between the solubilization and acidic precipitation observed in Figure II-1.



**Figure II-6.** Percentage of precipitated polymers by molecular weight category using SEC for sodium hydroxide (A) and sodium carbonate (B). Molecules were detected at 280 nm. For each molecular weight category, the percentage of precipitated polymers was evaluated by comparison of the peak areas in S1 and S2.

Since it was proven previously that acidic precipitated EPS have a gel forming capacity through ionic cross-linking with calcium ions [34], the S2 sodium hydroxide sample obtained at 70 °C and the S2 sodium carbonate at 80 °C were analyzed for their gelling capacity in the presence of divalent calcium.

Using similar amounts of acidic precipitated EPS, the gel formed with S2 sodium carbonate has a Young's modulus of  $10.40 \pm 0.75$  kPa slightly higher than that of sodium hydroxide which is equivalent to  $8.82 \pm 0.85$  kPa. These values are very close and indicate that both alkaline solvents used at high temperature, allow the recovery of gel-forming EPS. The HMW/MMW and LMW distributions were respectively: 48.7% / 41.9% / 9.4% for sodium carbonate and 48.4% / 27.4% / 24.2% for sodium hydroxide. The equivalent percentage in HMW could explain their similar gelation behavior. This also indicates that despite some EPS degradation occurring during the solubilization step, the HMW molecules recovered by acidic precipitation show a gel-forming capacity, whether they have been extracted with sodium hydroxide or sodium carbonate. However, the study performed in this paper shows that temperature and pH conditions have to be carefully managed in order to find optimal conditions avoiding degradation and reduction of the polymer molecular weight since this property appears to be very impacting on the final EPS recovery.

#### **4 Conclusions**

- Alkaline chemicals coupled with heating provide efficient solubilization of aerobic granular sludges.
- Acidic precipitation of the solubilized EPS is influenced by their molecular weight distribution (HMW molecules are the most precipitable) but not by their biochemical composition.
- For the recovery of gel-forming EPS by acidic precipitation, care must be taken when using a strong base as sodium hydroxide coupled with a high temperature (above 50 °C) in regards to the degradation of the biopolymers.
- For valorization purposes, 0.2 M sodium carbonate used at 80 °C offers a good yield of acid precipitated EPS and good gel-forming capacities.

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## References:

- [1] M. Pronk, B. Abbas, S.H.K. Al-zuhairy, R. Kraan, R. Kleerebezem, M.C.M. van Loosdrecht, Effect and behaviour of different substrates in relation to the formation of aerobic granular sludge, *Appl. Microbiol. Biotechnol.* 99 (2015) 5257–5268. <https://doi.org/10.1007/s00253-014-6358-3>.
- [2] M.K.H. Winkler, C. Meunier, O. Henriot, J. Mahillon, M.E. Suárez-Ojeda, G. Del Moro, M. De Sanctis, C. Di Iaconi, D.G. Weissbrodt, An integrative review of granular sludge for the biological removal of nutrients and recalcitrant organic matter from wastewater, *Chem. Eng. J.* 336 (2018) 489–502. <https://doi.org/10.1016/j.cej.2017.12.026>.
- [3] S. Wang, X. Huang, L. Liu, P. Yan, Y. Chen, F. Fang, J. Guo, Insight into the role of exopolysaccharide in determining the structural stability of aerobic granular sludge, *J. Environ. Manage.* 298 (2021) 113521. <https://doi.org/10.1016/j.jenvman.2021.113521>.
- [4] Y. Lin, M. de Kreuk, M.C.M. van Loosdrecht, A. Adin, Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant, *Water Res.* 44 (2010) 3355–3364. <https://doi.org/10.1016/j.watres.2010.03.019>.
- [5] Y.M. Lin, K.G.J. Nierop, E. Girbal-Neuhauser, M. Adriaanse, M.C.M. van Loosdrecht, Sustainable polysaccharide-based biomaterial recovered from waste aerobic granular sludge as a surface coating material, *Sustain. Mater. Technol.* 4 (2015) 24–29. <https://doi.org/10.1016/j.susmat.2015.06.002>.
- [6] N.K. Kim, N. Mao, R. Lin, D. Bhattacharyya, M.C.M. van Loosdrecht, Y. Lin, Flame retardant property of flax fabrics coated by extracellular polymeric substances recovered from both activated sludge and aerobic granular sludge, *Water Res.* 170 (2020) 115344. <https://doi.org/10.1016/j.watres.2019.115344>.
- [7] W. Liu, J. Zhang, Y. Jin, X. Zhao, Z. Cai, Adsorption of Pb(II), Cd(II) and Zn(II) by extracellular polymeric substances extracted from aerobic granular sludge: Efficiency of protein, *J. Environ. Chem. Eng.* 3 (2015) 1223–1232. <https://doi.org/10.1016/j.jece.2015.04.009>.
- [8] B. Pagliaccia, E. Carretti, M. Severi, D. Berti, C. Lubello, T. Lotti, Heavy metal biosorption by Extracellular Polymeric Substances (EPS) recovered from anammox granular sludge, *J. Hazard. Mater.* 424 (2022) 126661. <https://doi.org/10.1016/j.jhazmat.2021.126661>.
- [9] X. Tan, G.J. Xie, W.B. Nie, D.F. Xing, B.F. Liu, J. Ding, N.Q. Ren, High value-added biomaterials recovery from granular sludge based wastewater treatment process, *Resour. Conserv. Recycl.* 169 (2021) 105481. <https://doi.org/10.1016/j.resconrec.2021.105481>.
- [10] S. Felz, P. Vermeulen, M.C.M. van Loosdrecht, Y.M. Lin, Chemical characterization

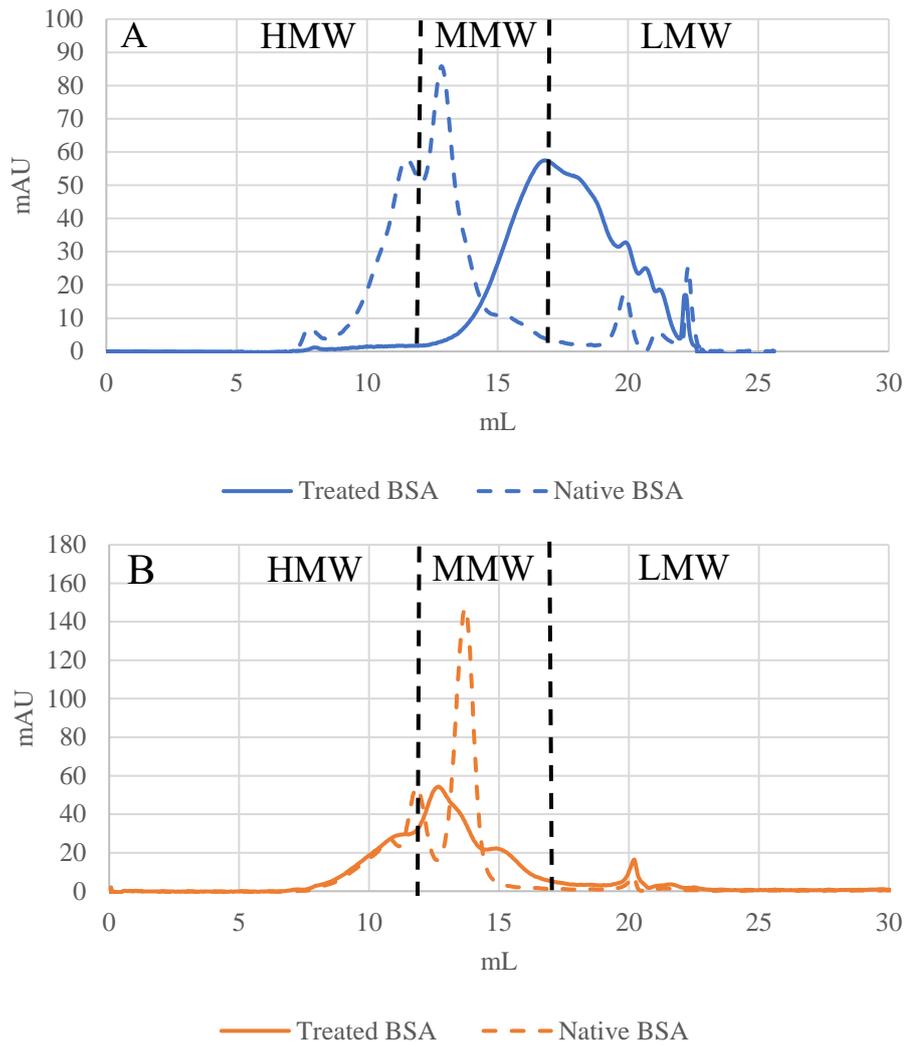
- methods for the analysis of structural extracellular polymeric substances (EPS), *Water Res.* 157 (2019) 201–208. <https://doi.org/10.1016/j.watres.2019.03.068>.
- [11] S. Felz, T.R. Neu, M.C.M. van Loosdrecht, Y. Lin, Aerobic granular sludge contains Hyaluronic acid-like and sulfated glycosaminoglycans-like polymers, *Water Res.* 169 (2020) 115291. <https://doi.org/10.1016/j.watres.2019.115291>.
- [12] C.M. Schambeck, E. Girbal-Neuhauser, L. Böni, P. Fischer, Y. Bessièrè, E. Paul, R.H.R. da Costa, N. Derlon, Chemical and physical properties of alginate-like exopolymers of aerobic granules and flocs produced from different wastewaters, *Bioresour. Technol.* 312 (2020) 123632. <https://doi.org/10.1016/j.biortech.2020.123632>.
- [13] T. Seviour, N. Derlon, M.S. Duelhom, H. Flemming, E. Girbal-neuhauser, H. Horn, S. Kjelleberg, M.C.M. Van Loosdrecht, T. Lotti, M.F. Malpei, R. Nerenberg, T.R. Neu, E. Paul, H. Yu, Y. Lin, Extracellular polymeric substances of biofilms : Suffering from an identity crisis, *Water Res.* 151 (2019) 1–7. <https://doi.org/10.1016/j.watres.2018.11.020>.
- [14] P.H. Nielsen, A. Jahn, Extraction of EPS, in: Wingender J., Neu T.R., Flemming HC. *Microb. Extracell. Polym. Subst.*, Springer, Berlin, Heidelberg, 1999: pp. 49–72. [https://doi.org/10.1007/978-3-642-60147-7\\_3](https://doi.org/10.1007/978-3-642-60147-7_3).
- [15] M. Ras, E. Girbal Neuhauser, E. Paul, D. Lefebvre, A high yield multi-method extraction protocol for protein quantification in activated sludge, *Bioresour. Technol.* 99 (2008) 7464–7471. <https://doi.org/10.1016/j.biortech.2008.02.025>.
- [16] N. Pfaff, J.M. Kleijn, M.C.M. Van Loosdrecht, A.J.B. Kemperman, Formation and ripening of alginate-like exopolymer gel layers during and after membrane filtration, *Water Res.* 195 (2021) 116959. <https://doi.org/10.1016/j.watres.2021.116959>.
- [17] M. Ras, D. Lefebvre, N. Derlon, J. Hamelin, N. Bernet, E. Paul, E. Girbal-Neuhauser, Distribution and hydrophobic properties of Extracellular Polymeric Substances in biofilms in relation towards cohesion, *J. Biotechnol.* 165 (2013) 85–92. <https://doi.org/10.1016/j.jbiotec.2013.03.001>.
- [18] P. D’Abzac, F. Bordas, E. Van Hullebusch, P.N.L. Lens, G. Guibaud, Extraction of extracellular polymeric substances ( EPS ) from anaerobic granular sludges : comparison of chemical and physical extraction protocols, *Appl. Microbiol. Biotechnol.* 85 (2010) 1589–1599. <https://doi.org/10.1007/s00253-009-2288-x>.
- [19] C. Feng, T. Lotti, Y. Lin, F. Malpei, Extracellular polymeric substances extraction and recovery from anammox granules: Evaluation of methods and protocol development, *Chem. Eng. J.* 374 (2019) 112–122. <https://doi.org/10.1016/j.cej.2019.05.127>.
- [20] S. Felz, S. Al-Zuhairy, O.A. Aarstad, M.C.M. van Loosdrecht, Y.M. Lin, Extraction of structural extracellular polymeric substances from aerobic granular sludge, *J. Vis. Exp.* 2016 (2016). <https://doi.org/10.3791/54534>.
- [21] T. Seviour, M. Pijuan, T. Nicholson, J. Keller, Z. Yuan, Gel-forming exopolysaccharides explain basic differences between structures of aerobic sludge granules and floccular sludges, *Water Res.* 43 (2009) 4469–4478. <https://doi.org/10.1016/j.watres.2009.07.018>.

- [22] G.P. Sheng, H.Q. Yu, X.Y. Li, Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review, *Biotechnol. Adv.* 28 (2010) 882–894. <https://doi.org/10.1016/j.biotechadv.2010.08.001>.
- [23] S.S. Adav, D.J. Lee, Extraction of extracellular polymeric substances from aerobic granule with compact interior structure, *J. Hazard. Mater.* 154 (2008) 1120–1126. <https://doi.org/10.1016/j.jhazmat.2007.11.058>.
- [24] T. Seviour, Z. Yuan, M.C.M. van Loosdrecht, Y. Lin, Aerobic sludge granulation: A tale of two polysaccharides?, *Water Res.* 46 (2012) 4803–4813. <https://doi.org/10.1016/j.watres.2012.06.018>.
- [25] Y. Lin, C. Reino, J. Carrera, J. Pérez, M.C.M. van Loosdrecht, Glycosylated amyloid-like proteins in the structural extracellular polymers of aerobic granular sludge enriched with ammonium-oxidizing bacteria, *Microbiol. Open.* 7 (2018) 1–13. <https://doi.org/10.1002/mbo3.616>.
- [26] M. Pronk, T.R. Neu, M.C.M. van Loosdrecht, Y.M. Lin, The acid soluble extracellular polymeric substance of aerobic granular sludge dominated by *Deffluviococcus* sp., *Water Res.* 122 (2017) 148–158. <https://doi.org/10.1016/j.watres.2017.05.068>.
- [27] I. Fernández Farrés, I.T. Norton, Formation kinetics and rheology of alginate fluid gels produced by in-situ calcium release, *Food Hydrocoll.* 40 (2014) 76–84. <https://doi.org/10.1016/j.foodhyd.2014.02.005>.
- [28] K.I. Draget, G. Skjåk Bræk, O. Smidsrød, Alginic acid gels: the effect of alginate chemical composition and molecular weight, *Carbohydr. Polym.* 25 (1994) 31–38. [https://doi.org/10.1016/0144-8617\(94\)90159-7](https://doi.org/10.1016/0144-8617(94)90159-7).
- [29] M. Layer, A. Adler, E. Reynaert, A. Hernandez, M. Pagni, E. Morgenroth, C. Holliger, N. Derlon, Organic substrate diffusibility governs microbial community composition, nutrient removal performance and kinetics of granulation of aerobic granular sludge, *Water Res.* X. 4 (2019) 100033. <https://doi.org/10.1016/j.wroa.2019.100033>.
- [30] M. Layer, M.G. Villodres, A. Hernandez, E. Reynaert, E. Morgenroth, N. Derlon, Limited simultaneous nitrification-denitrification (SND) in aerobic granular sludge systems treating municipal wastewater: Mechanisms and practical implications, *Water Res.* X. 7 (2020) 100048. <https://doi.org/10.1016/j.wroa.2020.100048>.
- [31] T. Lotti, E. Carretti, D. Berti, M.R. Martina, C. Lubello, F. Malpei, Extraction, recovery and characterization of structural extracellular polymeric substances from anammox granular sludge, *J. Environ. Manage.* 236 (2019) 649–656. <https://doi.org/10.1016/j.jenvman.2019.01.054>.
- [32] B. Frølund, T. Griebe, P.H. Nielsen, Enzymatic activity in the activated-sludge floc matrix, *Appl. Microbiol. Biotechnol.* 43 (1995) 755–761. <https://doi.org/10.1007/BF00164784>.
- [33] C. Rondel, C.E. Marcato-Romain, E. Girbal-Neuhausser, Development and validation of a colorimetric assay for simultaneous quantification of neutral and uronic sugars, *Water Res.* 47 (2013) 2901–2908. <https://doi.org/10.1016/j.watres.2013.03.010>.
- [34] S. Felz, H. Kleikamp, J. Zlopasa, M.C.M. van Loosdrecht, Y. Lin, Impact of metal ions on structural EPS hydrogels from aerobic granular sludge, *Biofilm.* 2 (2020) 100011.

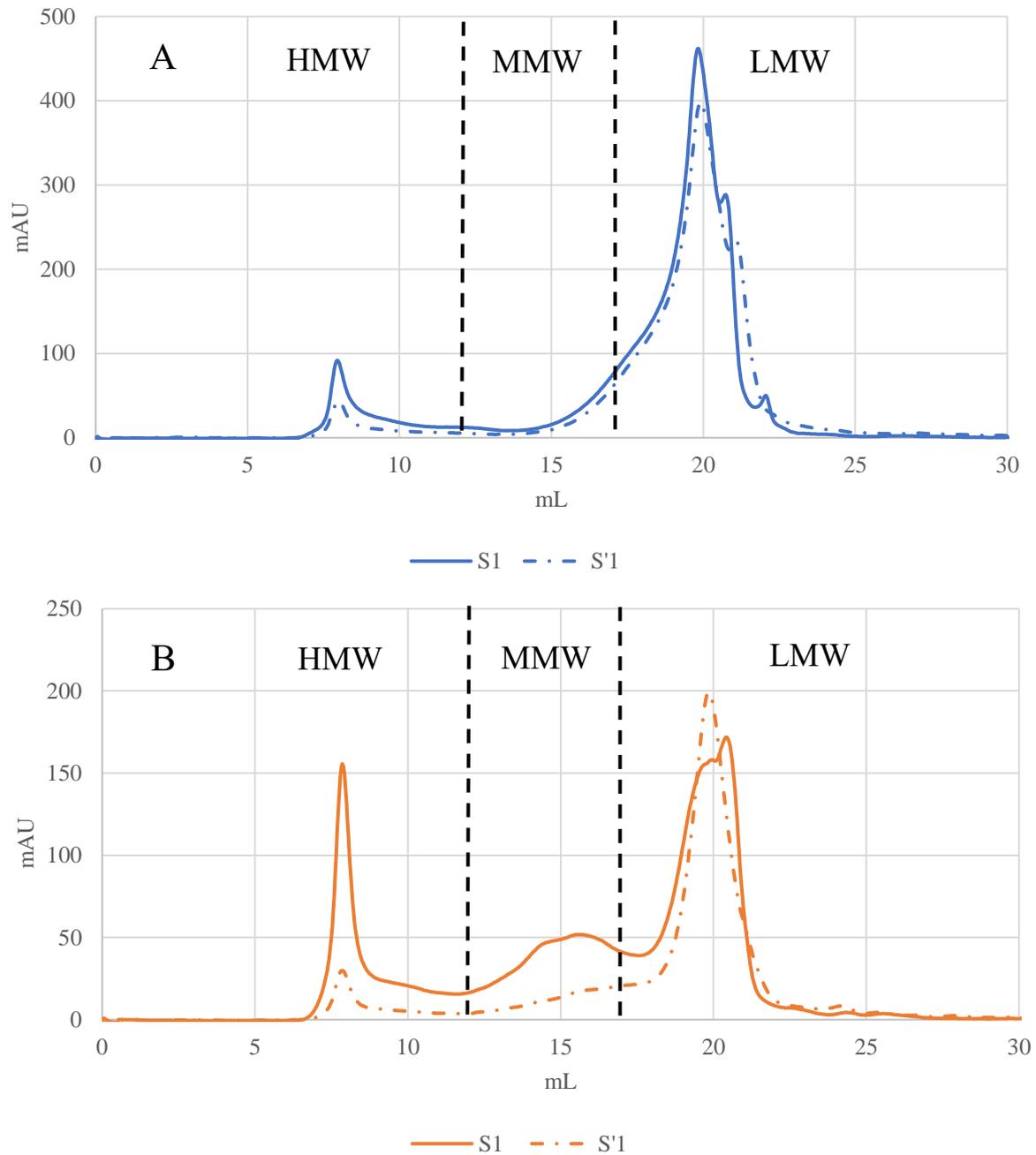
- <https://doi.org/10.1016/j.bioflm.2019.100011>.
- [35] B.J. Bennion, V. Daggett, The molecular basis for the chemical denaturation of proteins by urea, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 5142–5147. <https://doi.org/10.1073/pnas.0930122100>.
- [36] F. Hou, W. Ding, W. Qu, A.O. Oladejo, F. Xiong, W. Zhang, R. He, H. Ma, Alkali solution extraction of rice residue protein isolates: Influence of alkali concentration on protein functional, structural properties and lysinoalanine formation, *Food Chem.* 218 (2017) 207–215. <https://doi.org/10.1016/j.foodchem.2016.09.064>.
- [37] V.K.R. Surasani, Acid and alkaline solubilization (pH shift) process: a better approach for the utilization of fish processing waste and by-products, *Environ. Sci. Pollut. Res.* 25 (2018) 18345–18363. <https://doi.org/10.1007/s11356-018-2319-1>.
- [38] J.D. Pérez-Martínez, M. Sánchez-Becerril, J.J. Ornelas-Paz, M.M. González-Chávez, V. Ibarra-Junquera, P. Escalante-Minakata, The effect of extraction conditions on the chemical characteristics of pectin from *Opuntia ficus indica* cladode flour, *J. Polym. Environ.* 21 (2013) 1040–1051. <https://doi.org/10.1007/s10924-013-0602-2>.
- [39] L. Bai, P. Zhu, W. Wang, M. Wang, The influence of extraction pH on the chemical compositions, macromolecular characteristics, and rheological properties of polysaccharide: The case of okra polysaccharide, *Food Hydrocoll.* 102 (2020). <https://doi.org/10.1016/j.foodhyd.2019.105586>.
- [40] A.S. Oliveira, C.L. Amorim, M.A. Ramos, D.P. Mesquita, P. Inocêncio, E.C. Ferreira, M. Van Loosdrecht, P.M.L. Castro, Variability in the composition of extracellular polymeric substances from a full-scale aerobic granular sludge reactor treating urban wastewater, *J. Environ. Chem. Eng.* 8 (2020) 104156. <https://doi.org/10.1016/j.jece.2020.104156>.
- [41] R. Yasothai, R. Giriprasad, Acid/alkaline solubilization method of processing protein, *Int. J. Sci. Environ. Technol.* 4 (2015) 96–100. [www.ijset.net](http://www.ijset.net).
- [42] B.E. Christensen, Physical and chemical properties of extracellular polysaccharides associated with biofilms and related systems, in: Wingender J., Neu T.R., Flemming H.C. *Microb. Extracell. Polym. Subst.*, Springer, Berlin, Heidelberg, 1999: pp. 143–154. [https://doi.org/10.1007/978-3-642-60147-7\\_8](https://doi.org/10.1007/978-3-642-60147-7_8).
- [43] H.G. Kristinsson, H.O. Hultin, Changes in conformation and subunit assembly of cod myosin at low and high pH and after subsequent refolding, *J. Agric. Food Chem.* 51 (2003) 7187–7196. <https://doi.org/10.1021/jf026193m>.
- [44] T. Turquois, H. Gloria, Determination of the absolute molecular weight averages and molecular weight distributions of alginates used as ice cream stabilizers by using multiangle laser light scattering measurements, *J. Agric. Food Chem.* 48 (2000) 5455–5458. <https://doi.org/10.1021/jf000003v>.
- [45] H. Storz, K.J. Müller, F. Ehrhart, I. Gómez, S.G. Shirley, P. Gessner, G. Zimmermann, E. Weyand, V.L. Sukhorukov, T. Forst, M.M. Weber, H. Zimmermann, W.M. Kulicke, U. Zimmermann, Physicochemical features of ultra-high viscosity alginates, *Carbohydr. Res.* 344 (2009) 985–995. <https://doi.org/10.1016/j.carres.2009.02.016>.
- [46] C. Caudan, A. Filali, M. Spérandio, E. Girbal-Neuhauser, Multiple EPS interactions

involved in the cohesion and structure of aerobic granules, *Chemosphere*. 117 (2014) 262–270. <https://doi.org/10.1016/j.chemosphere.2014.07.020>.

## 5 Supplementary material



**Figure II-S1: size exclusion chromatography profiles of native BSA (2 mg/mL) solubilized in sodium hydroxide (A) and sodium carbonate (B) and of the treated BSA at 80°C for an hour in 0.2 M of sodium hydroxide (A) and sodium carbonate (B).**

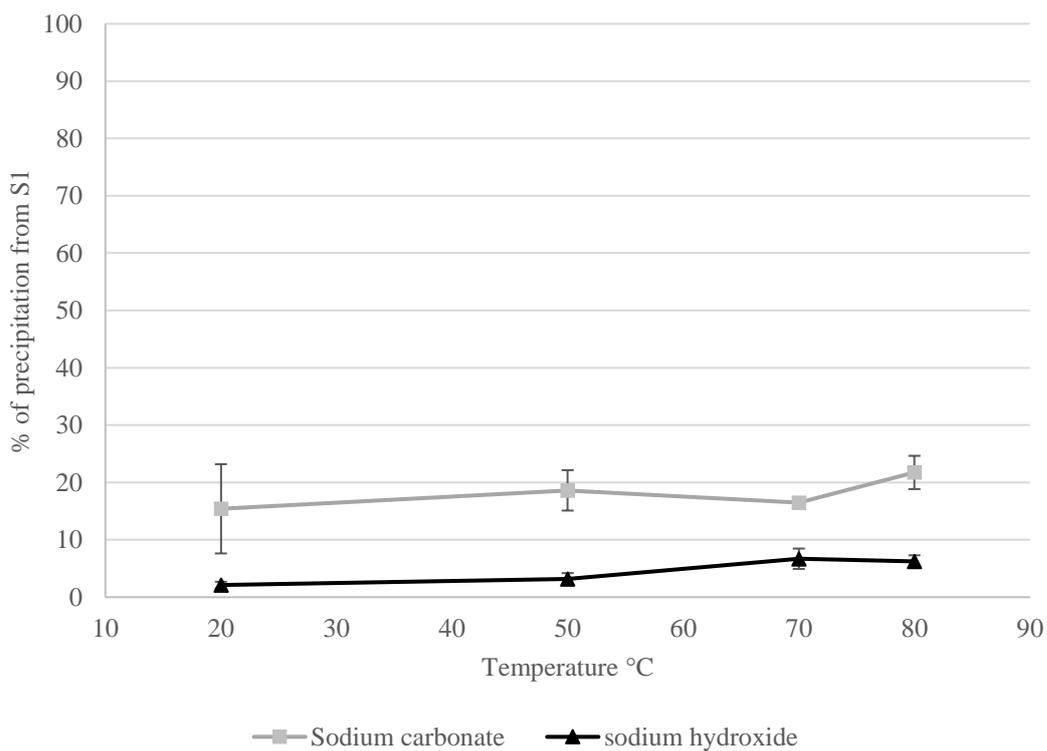


**Figure II-S2: SEC showing the selectivity of precipitation between S1 (solubilized EPS) and S'1 (the supernatant after acidic precipitation) for sodium hydroxide (A) and sodium carbonate (B). The acidic precipitation is selective towards HMW and MMW more than LMW. Detection wavelength was set to 280 nm.**

**Table II-S1: Physico-chemical parameters of the HMW molecules eluted between 27 min and 32 min on S2 fractograms obtained through AsFIFFF (Figure II-3).**

HMW	Weight average molar Mass (Da) ( $M_w$ )	Number average Molecular mass ( $M_n$ )	Weight average Gyration radius ( $R_g$ )	Polydispersity (PI) <sup>1</sup>
S2 sodium carbonate	$5.57 \times 10^6$	$2.20 \times 10^6$	118.60 nm	2.50
S2 sodium hydroxide	$1.88 \times 10^6$	$1.67 \times 10^6$	61.70 nm	1.10

<sup>1</sup>  $M_w/M_n$



**Figure II-S3: Effect of temperature on the percentage of precipitation from S1. The percentage of precipitation was calculated by dividing the VS precipitated by the VS in the initial granules and multiplying by 100. The experiments were done in triplicate.**

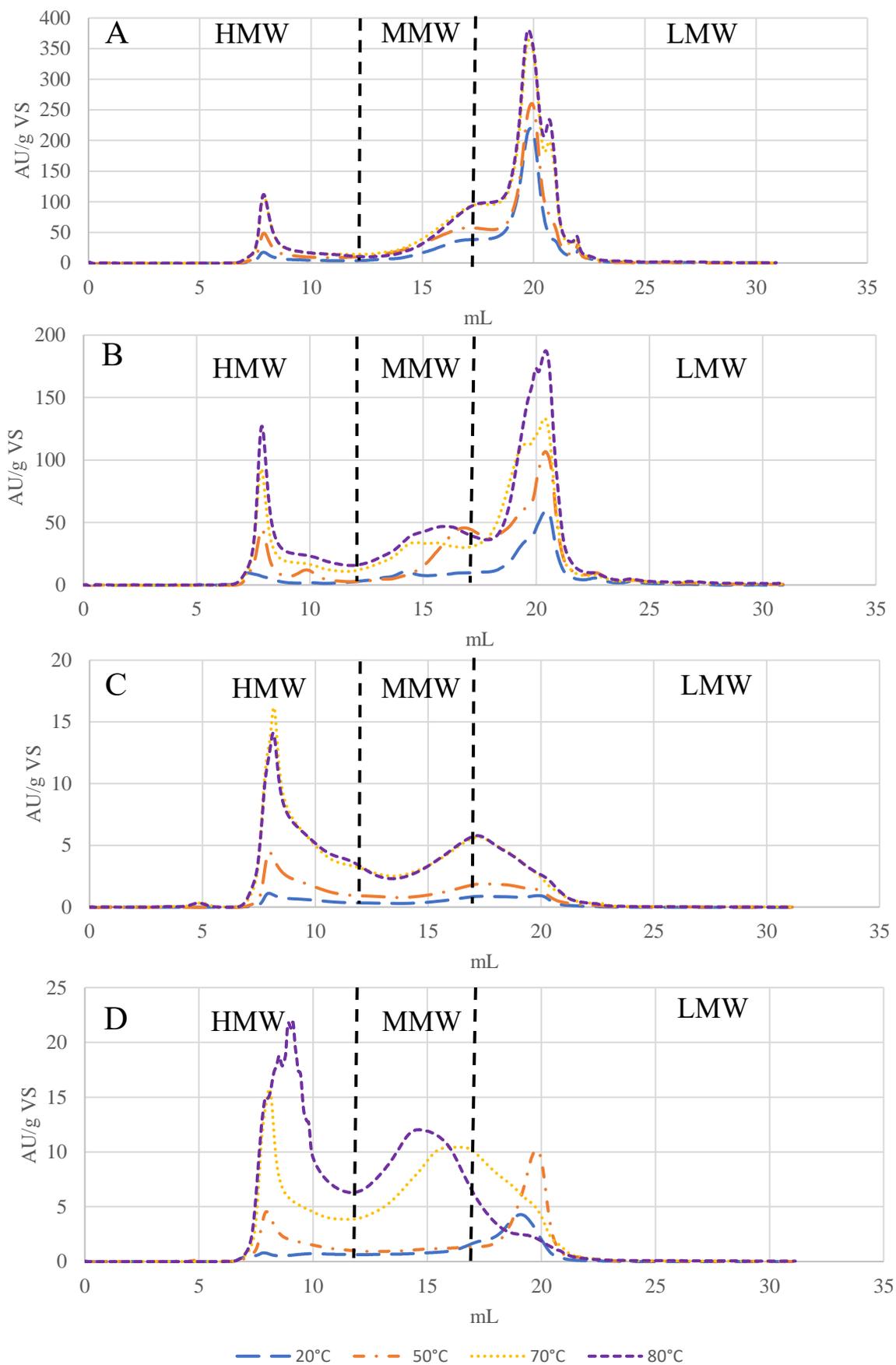


Figure II-S4: Size exclusion chromatography profiles for S1 sodium hydroxide (A), S1 sodium carbonate (B), S2 sodium hydroxide (C) and S2 sodium carbonate (D).

## 6 Conclusions of Chapter II

With the objective of recovering gelling biomolecules while preserving the native size of the polymers, attention was paid in this chapter to the extraction conditions of sEPS from granular sludges.

The experimentations aimed to compare different extraction protocols using four different solvents at a fixed concentration of 0.2 M and heating temperatures from 20°C to 80°C to determine their quantitative and qualitative effects on the recovered sEPS. Indeed, although drastic protocols based on both high pH and high temperature are classically used for granules solubilization, no analysis has yet been made on the effects of the initial solubilization conditions on the yield and quality of the structural EPS recovered after acidic precipitation.

It was shown that the best solvents for obtaining the highest yields of acid precipitated EPS were sodium carbonate and sodium hydroxide. However, the precipitation efficiency was different between the two solvents and more precipitable polymers, at pH 2, were present after initial solubilization of granules in sodium carbonate. The biochemical composition and molecular characteristics (precisely molecular weight and gyration radius) of EPS extracted with these two solvents were determined. Concerning the biochemical composition, both extracts proved to be complex and the acidic precipitation step caused the precipitation of all the present biochemical groups. This aspect can be caused either by associations between molecules hence causing their coprecipitation or by the non-specificity of the proposed precipitation technique. A conclusion that may be drawn from this observation is that it may be necessary to consider further efficient steps to isolate the gelling EPS from the set of structural EPS in order to increase the gelling performances.

First signs of degradation of polymers were noticeable using field flow fractionation when the size of polymers detected with sodium hydroxide was smaller than those in sodium carbonate. This led to studying EPS degradation by varying the heating temperature. The study on the effect of heating showed that solubilization of EPS kept increasing with the increase of temperature for both alkaline solvents. However, for sodium hydroxide the acidic-precipitated-EPS yield stabilized after 70°C whereas that of sodium carbonate kept on increasing till 80°C. More precisely, a correlation between the molecular weight and the acidic precipitation yield was observed since high molecular weights and medium molecular weights precipitate better than low molecular weight molecules. In addition, a remarkable increase in

low molecular weight molecules was noticed during solubilization at high temperatures with sodium hydroxide, evidencing a possible degradation of polymers. Therefore, this work proved a degradation of polymers during the extraction with sodium hydroxide at high temperatures and thus, preserving the molecules from degradation could be a key to improve acid precipitation yields and possibly the gelling performances of the recovered EPS. Additional information on the effects of the extraction protocol on the extracted EPS could be proved by doing supplementary experiments. The degradation aspect can be further understood by knowing which type of polymers is the most affected by this phenomenon. Following the amount of free amino acids with the increase of temperature in the samples could be a way to determine the extent of degradation of proteins for example.

Concerning the gel formation capacity, two extracts were chosen (based on molecular weight profiles and the obtained yields) for Young's modulus test, i.e. extracts using sodium carbonate at 80 °C and sodium hydroxide at 70 °C. The two extracts had a similar Young's modulus; however, it would have been interesting to test the gelation capacity with all the tested heating temperatures but this was not possible due to the fact that the Young's modulus test consumed a lot of EPS. From here, the necessity of developing a miniaturized screening gelation capacity arose.

Chapter II deepens the knowledge about the extraction of EPS and the implications of these extraction methods at the molecular level. The findings of this chapter can give valuable information in terms of compromise between the conditions for i) efficient initial granule solubilization, ii) efficient EPS acid precipitation, and iii) preservation of functional EPS properties. These findings can help the orientation towards a unified extraction protocol of gel-forming EPS and can have an important impact on the industrial extraction and the future applications of EPS. For the rest of the chapters, the sodium carbonate extraction method at 80°C was chosen since it showed a good yield with less signs of degradation.

The next chapter aimed to develop a miniaturized screening method allowing the detection of the gelling ability of any solution or fraction containing gel-forming polymers. The need for developing such test rises from many aspects. First of all, the variable yields of extracted gel-forming EPS (Felz et al., 2016; Lin et al., 2010; Meng et al., 2019; Schambeck et al., 2020) require a gelling screening test that allows the detection of gelation even at very low concentrations and with a quantification based on the gelling function and not on the overall

quantity of extracted sEPS. Secondly, the biochemical diversity of the fraction recovered after alkaline extraction and acidic precipitation that is considered as gel-forming EPS requires fractionation and separation of the different polymers in order to understand which chemical motif or molecular group is responsible for the gelation. These steps of purification and fractionation often reduce the yield furthermore, and bring us back to the necessity of having a method capable of detecting gelation with small quantities of molecules. The developed method needs to be capable of following the enrichment of gelling molecules to help in guiding the decision-making in the extraction/purification process and quantify the enrichment of gelling-polymers.

As a summary, the next chapter proposes a methodology for the detection of solution's gel formation capacities with a limited quantity of sEPS. This test allows to enrich the understanding of possible interactions between pure molecules and their effects on the gelation. Moreover, this method could be of utmost help especially in the area of fractionation/purification of gel-forming EPS.



# Chapter III:

## Detection of Gel-Forming Polymers via Calcium Crosslinking, Applied to the Screening of Extracellular Polymeric Substances Extracted from Biological Aggregates

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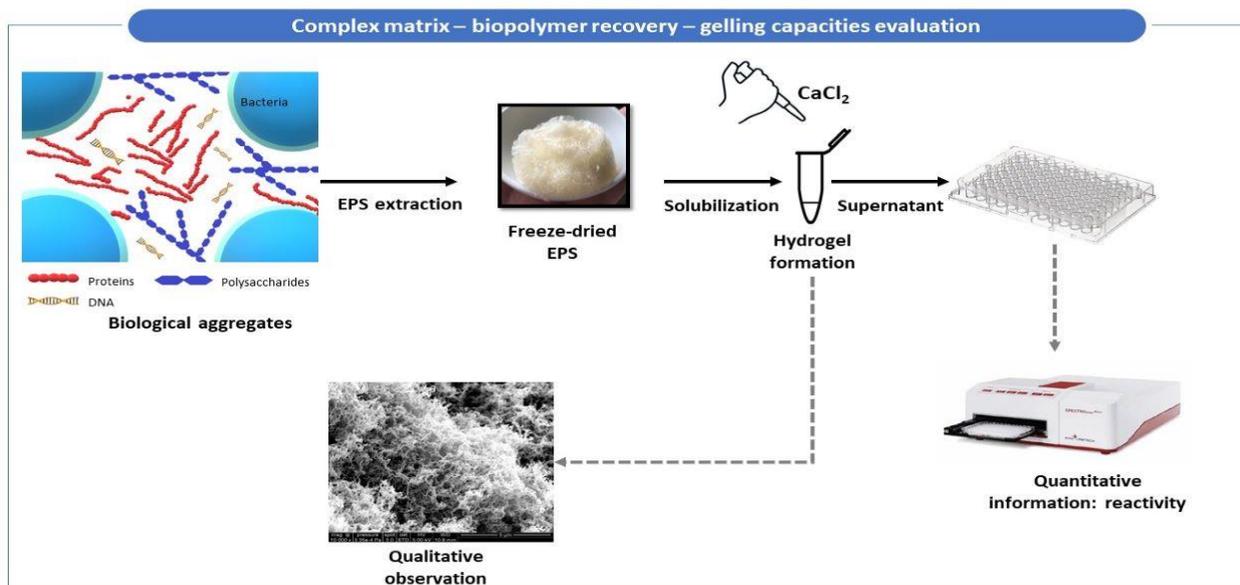
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**Abstract:** The valorization of biological aggregates through the extraction of hydrogel-forming polymers can enhance the economics and sustainability of various processes in which bacteria are involved in organic waste transformation, such as wastewater treatment. Achieving these goals requires the development of a method capable of detecting the presence of gel-forming polymers in complex mixtures containing biopolymers that are most often unknown and uncharacterized. A miniaturized screening method capable of detecting gelation via ionic crosslinking using only 1 to 3 mg of the tested samples (commercial molecules or extracellular polymeric substances, EPS) is proposed. The method consists of calculating a percentage of reactivity (%R) through UV-vis spectra and determining the percentage of gel volume (%Vg) formed after the addition of calcium. Both factors were combined to give a gelling factor (GF), and the test was applied to pure commercial molecules (BSA, DNA, alginate (ALV), and a mixture of them), allowing the classification of the following solutions according to their gel-forming capacity:  $GF_{(ALV)} > GF_{(ALV+DNA)} > GF_{(BSA+ALV+DNA)} > GF_{(BSA+ALV)} > GF_{(DNA)} > GF_{(BSA+DNA)} > GF_{(BSA)}$ . As a relevant tool for screening hydrogel-forming solutions, the method was applied to the EPS extracted from aerobic granular sludge. The EPS (0.5% w/v) had a GF of  $0.16 \pm 0.03$ , equivalent to approximately half of the GF of ALV ( $0.38 \pm 0.02$  at 0.5% w/v). The developed test pushes the limits of the existing gel-detection techniques because it allows for quicker, less consuming, and more informative gelation detection through the use of simple methods that do not require sophisticated equipment.



**Keywords:** hydrogels; gelling capacity; calcium crosslinking; aerobic granules; alginate-like exopolymers; extracellular polymeric substances.

## 1 Introduction

Aerobic granular sludges are self-assembled spherical biofilms composed of micro-organisms embedded in an extracellular polymeric substances (EPS) matrix. These biofilms are used in wastewater treatment and present many benefits over activated sludges [1]. Extracted EPS have the potential to be used in a wide variety of applications [2–5] mainly due to their capacity to form stable hydrogels with divalent cations, like calcium [6–8]. A hydrogel is defined as a crosslinked polymeric network that has the capacity to absorb water while maintaining resistance to dissolution. In this sense, hydrogels are swollen by the solvent forming a one-phase system, whereas precipitates are dense, solid structures that make two-phase systems [9,10]. The extraction of gel-forming EPS from aerobic granular sludge is conventionally carried out by alkaline treatment, followed by acidic precipitation, giving a yield varying from 1.5% to 29% of organic matter [11–14]. This underlines the necessity for a gel formation screening method that is able to detect the presence of gel-forming polymers at the beginning of the extraction protocol and to follow their loss during extraction (if any).

Some of the gel-forming polymers extracted from aerobic granules were named alginate-like exopolysaccharides (ALE) due to their functional and chemical similarities with alginate [7]. In the presence of  $\text{CaCl}_2$ , the gelation mechanism relies on calcium ions playing the role of a crosslinker through bridging two carboxylic acid functions (negatively charged) from adjacent alginate polymers, specifically through their guluronic acids [15,16]. Different functional groups are implied in the (ionic) crosslinking reactions of biomolecules with gel-forming capacities, such as primary amines, alcohols, thiols, carbonyls, and carboxylic acids [16]. In fact, it was proven that the ALE extracted from aerobic granules, whatever their origin, are made of a wide variety of molecules, including polysaccharides, proteins, lipids, and humic acids, among other molecules [13,14]. Further fractionation steps are required to obtain the fractions enriched with gel-forming EPS to disclose the chemical groups that are responsible for the gelation mechanisms. Therefore, the development of a miniaturized method that is able to detect gel-forming capacity with low quantities of polymers either in liquid raw extracts and/or in the enriched soluble fractions obtained through diverse fractionation techniques (liquid chromatography, selective precipitation, etc.) is required.

In literature, the detection of the gelling capacity of EPS is carried out through different methods. The first technique consists of extruding concentrated EPS in a calcium solution and checking if a gel bead forms, therefore giving only a qualitative (gel formation or not)

appreciation of the gel-forming capacity [17]. A study carried out by Seviour et al. (2009) used a rheometer equipped with a Couette cell to follow the storage and loss modulus of the sample (224 mg per test) [18], necessitating the sampling of large volumes of aerobic sludge, which could be problematic, especially for lab-scale reactors, as well as the availability of a rheometer being necessary. The most used method is based on the formation of cylindrical gels through dialysis for 24 h with divalent cations and then measuring the deformation of the formed hydrogels by dynamic mechanical analysis to obtain the corresponding Young's modulus [6]. This generally requires 10% (w/v) EPS solutions and, therefore, 40 mg for each test. Efforts have been made by Pagliaccia et al. (2022) to limit the consumption of EPS through the optimization of the gels' geometry, the concentration of the EPS solutions, and the calcium ions being used. It was found that at 0.1 M  $\text{Ca}^{2+}$ , the minimal concentration of EPS required is 5% (w/v) to obtain stable hydrogels and, therefore, 13.5 mg per test. The dependency of the hydrogel stiffness on the crosslinker concentration has also been underlined [8].

This article aims to study the feasibility and performance of a miniaturized screening method using a simple technique (UV-visible spectroscopy) to measure the reactivity of the samples to calcium ions, with the goal of ensuring the availability of gelation screening methods to a wider audience of scientists and industrials through the use of simple techniques. The test aims to detect the presence of the molecules capable of interacting with calcium and forming gels and compare the gel formation capacity of different solutions. This objective will be attained through the development of the method using commercial standard molecules alone or in mixtures and then through its application through the different steps of the extraction protocol of EPS performed on granular sludge to check its efficiency in selecting gel-forming polymers. The test will help monitor the efficiency of gel-forming EPS extraction and will therefore allow the development of more targeted extraction protocols for a better recovery of gel-forming biopolymers [14]. On an industrial level, the test will allow for the examination of the richness of wastewater treatment plants with gelling polymers for valorization purposes.

## 2 Materials and Methods

### 2.1 Samples Preparation

Since the EPS extracted from microbial biofilms or aggregates are made of a majority of polysaccharides and proteins [13,19], a representative polymer was selected for each group. Alginate with low viscosity (ALV) from brown algae (9005-38-3, Sigma-Aldrich, St. Louis, USA) was selected for the polysaccharide group because gel-forming molecules extracted from EPS were found to exhibit similar properties to alginate, as previously explained [7]. BSA (9048-46-8, Sigma-Aldrich, St. Louis, USA) was chosen as a representative for the protein group since it is a well-studied protein that is known to be rich in ionic amino acids (like glutamic acid, which is negatively charged) [20] and does not have a gel-forming capacity unless it is heated beforehand [21,22], and was therefore chosen as a negative control for the gelation test. Since DNA is also commonly found in the alkaline extracts of granular sludges [14,19] it was also used. Indeed, DNA is a highly negatively charged polymer, which goes in favor of an interaction with calcium ions. Therefore, commercial DNA from salmon testes (438545-06-3, Sigma-Aldrich, St. Louis, USA) was elicited as a representative molecule possibly involved in the gelation process of complex biological samples. The volatile solids (VS) contents were determined for the powder of each commercial molecule, and they were dissolved in 0.1 M sodium hydroxide at final VS concentrations of 3%, 2%, and 1% (w/v). Mixtures by pairs of commercial molecules (BSA+ ALV, BSA+DNA, ALV+DNA) were prepared to obtain solutions at 2% (w/v) VS concentration, with a ratio of 1:1 for each molecule, and a mixture of the three molecules (BSA+ ALV+ DNA) was also prepared so to obtain a solution of 3% (w/v) with a ratio of 1:1:1.

Finally, the test was applied to the three solutions recovered when performing the extraction of gel-forming EPS from aerobic granules, as explained in Bou-Sarkis et al. (2022) and summarized in supplementary data (Figure III-S1). Briefly, S1 contains all the polymers solubilized in 0.2 M sodium carbonate after the incubation of freeze-dried aerobic granules for 1 h at 80 °C; S'1 was the supernatant after the acidic precipitation of S1 raw extract, and S2 was the solution containing the gel-forming EPS corresponding to the acidic precipitated polymers. All the fractions were dialyzed against distilled water for 36 h and were freeze-dried. Samples were solubilized in sodium hydroxide 0.1 M at final VS concentrations ranging from 1% to 3% (w/v) and were submitted to the test.

## 2.2 Test Methodology

A volume of 100  $\mu\text{L}$  of the sample obtained (as described in Section 2.1) was diluted by a factor of two through the addition of either 100  $\mu\text{L}$  of distilled water (sample without calcium) or 100  $\mu\text{L}$  of 0.2 M calcium chloride (sample with calcium), leading to obtaining a final solution with 0.1 M  $\text{CaCl}_2$  at the desired concentration of the tested molecule (1.5%, 1%, and 0.5% w/v). The sample was homogenized by a vortex to ensure homogeneous calcium and polymer distribution. The incubation time was set to 1 h since it was shown in the literature that this time is sufficient for the formation of hydrogels [23]. All the samples were then centrifuged at  $14000\times g$  for 15 min. The supernatants were recovered, and their volumes ( $V_s$ ) were measured, allowing us to obtain the volume of the remaining pellet ( $V_g$ ), i.e., the formed gel corresponding to the difference between the initial total volume of liquid ( $V_t$ ) and the volume of the supernatant, which is the liquid remaining above the gel ( $V_s$ ). A dilution of the supernatant should be carried out directly after centrifugation to avoid the formation of  $\text{Ca}(\text{OH})_2$  precipitate. The lowest dilution, allowing for the obtainment of a value of absorbance between 0 and 1 (for the sample without calcium), was adopted for both samples (with and without calcium). A volume of 200  $\mu\text{L}$  of each diluted supernatant was put in a 96-well UV transparent microplates (Microtiter), and a UV-visible spectrum was acquired between 220 and 500 nm using the nanoSpectrostar spectrophotometer (BMG-LABTECH). All the absorbance values (Absorbance Units AU) were multiplied by  $V_s$  over  $V_t$  to take into consideration the liquid volume change due to gelation after the addition of calcium. A sodium hydroxide spectrum was also measured in triplicate at the used dilution and was subtracted from the spectra of samples performed with and without calcium. All samples were analyzed in triplicates, and their averages were reported with the standard deviations.

## 2.3 Evaluation of Gel-Forming Capacity

To determine the percentage of reactivity to calcium, the trapezoidal method (with a step of 1 nm) was used to calculate the area below each spectrum, and the % of reactivity (%R) was obtained through Equation (1):

$$\% R = \frac{(V_t * AIS - V_s * A_{CaS}) * 100}{V_t * AIS} \quad (1)$$

Where AIS is the cumulated area of the initial spectrum without calcium [AU.nm], AcaS is the cumulated area of the spectrum with calcium [AU.nm],  $V_t$  is the initial total volume [ $\mu\text{L}$ ], and  $V_s$  the liquid volume after calcium addition (supernatant after centrifugation) [ $\mu\text{L}$ ]

The % of apparent gel volume (%Vg) was obtained by Equation (2):

$$\% Vg = \frac{(V_t - V_s) * 100}{V_t} \quad (2)$$

This parameter will allow for the distinction between a precipitate and a gel since precipitates do not usually tend to adsorb water and swell (contrary to hydrogels).

Using these two parameters, a gelling factor (GF) was proposed to quantify the capacity of a solution to form gels and compare them with each other (see Equation (3)):

$$GF = \%R * \%Vg \quad (3)$$

Finally, the gelling index (GI), defined in Equation (4), was used to compare the relative capacity of a solution to form hydrogels with a model gel-forming molecule (here ALV) in similar tested conditions:

$$GI = \frac{GF \text{ of the sample}}{GF \text{ of ALV}} \quad (4)$$

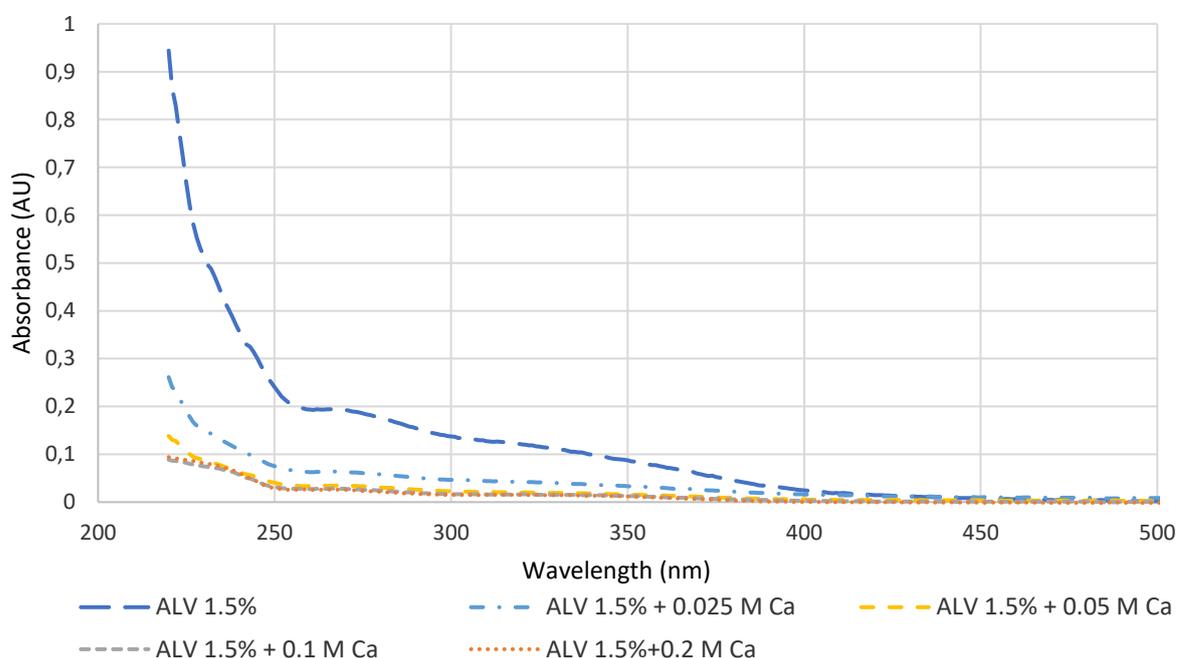
## 2.4 Scanning Electron Microscopy (SEM)

After the gelation protocol (see Section 2.2), the obtained gel pellets were washed with distilled water and then dehydrated using ethanol. After critical-point drying, the samples were mounted on microscope stubs and sputtered with platinum (8 nm, 1 min, 60 mA). Samples were examined on a FEI Quanta 250 FEG scanning electron microscope at an accelerating voltage of 5 KV.

### 3 Results and Discussions

#### 3.1 Calcium Requirements for Gel Formation

The detection of the calcium requirements for gel formation was performed to ensure that the molecules capable of reacting with calcium have the necessary amount of calcium to form gels. This was carried out by comparing the UV-vis spectra of supernatants obtained for 1.5% w/v alginate with low viscosity (ALV) in various  $\text{Ca}^{2+}$  concentrations (0; 0.025; 0.05; 0.1; 0.2 M). ALV was chosen because its density of negative charges is very high in comparison with other natural biopolymers due to the alternation of mannuronic and guluronic monomers bearing a negative carboxylic charge.



**Figure III-1: Effect of calcium concentration using UV-vis spectroscopy for ALV at 1.5% (w/v). For each calcium concentration, the spectrum shown is the average of three supernatant spectra obtained after gelation and includes the correction by the volume of supernatant/initial total volume ( $V_s/V_t$ ).**

Figure III-1 presents the spectra with and without calcium. It is clearly noticed that ALV can react with small amounts of calcium since, even at 0.025 M, the spectrum decreased drastically because of gel formation. However, upon reaching 0.1 M in calcium, it can be seen that the spectrum stabilized and no further decrease in the signal was noticed in comparison with the addition of 0.2 M calcium. In agreement with this, other studies underlined that alginate solutions with concentrations varying between 1 and 5% were able to react even at very low concentrations of calcium (i.e., 0.001 M). However, the stiffness (Young's modulus) of the formed alginate hydrogels at 2.5% w/v with 0.001 M calcium was approximately 2.3

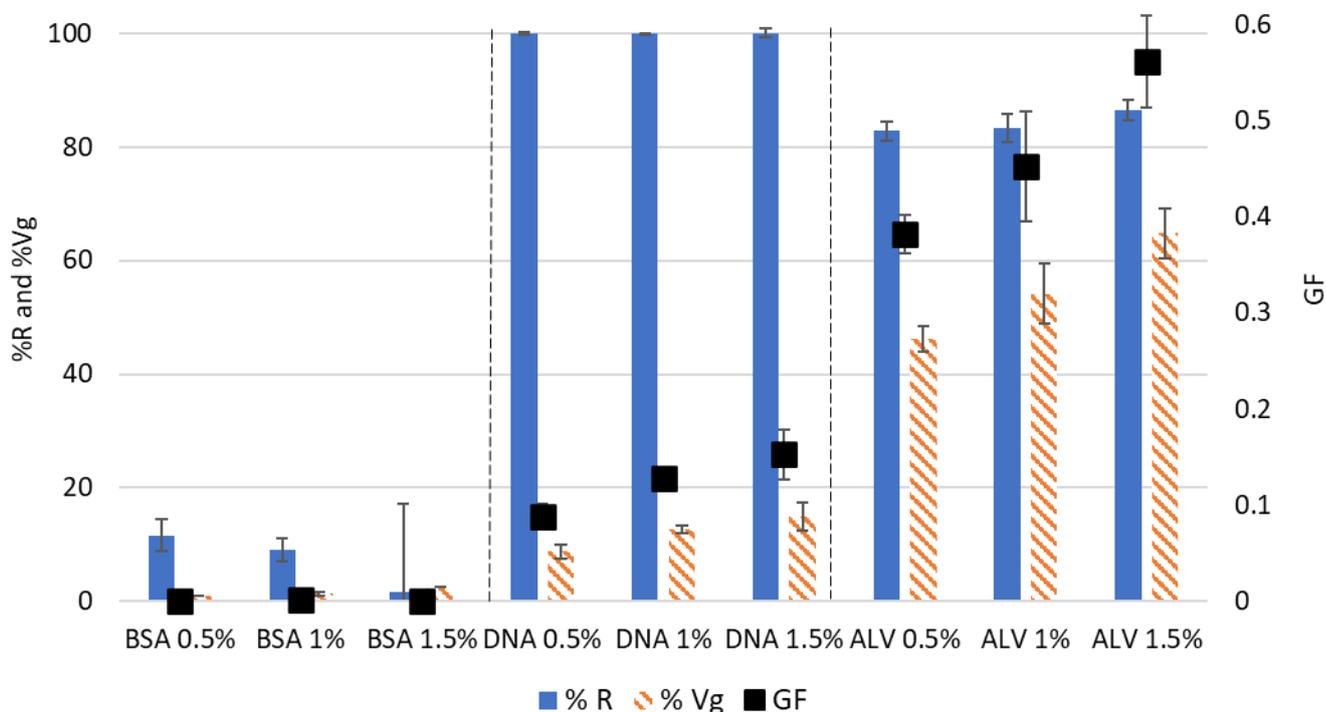
times lower compared to the highest tested concentration of calcium (0.1 M) [8]. Therefore, the concentration of 0.1 M was chosen for the overall test.

### 3.2 Gelling Capacity of Standard Molecules Solutions

ALV, Bovine Serum albumin (BSA), and deoxyribonucleic acid (DNA) were used for the development of the test to represent the biochemical diversity within the EPS matrix. Whatever the tested polymer concentrations (0.5%, 1%, and 1.5% w/v), the BSA solutions exhibited a very low reactivity to calcium, with a %R of around 10% (Figure III-2), and no significant differences were noted between the tested concentrations. After calcium addition, there was no or minimal decrease at the 280 nm wavelength specific to proteins, while a decrease in absorbance was specifically localized around the 220 nm wavelength (Supplementary Data Figure III-S2). This can be explained by changes in the secondary structure of BSA [20]. Another explanation could be the formation of  $\text{Ca}(\text{OH})_2$  precipitates due to the interaction of calcium ions with the sodium hydroxide used to dissolve the samples. For the three tested BSA concentrations, the gel volume (Vg) was lower than 2.5% and did not increase significantly with an increase in BSA concentration (Figure III-2), reinforcing the hypothesis that no gel formation occurred and that only a small pellet of  $\text{Ca}(\text{OH})_2$  precipitate was formed. As expected, the GF of BSA (Figure III-2) is very low, indicating that the solutions of BSA did not exhibit a measurable gelling capacity.

Concerning DNA, whatever the tested concentrations, all the molecules present in the solutions reacted with calcium, leading to %R values of around 100 % (Figures III-2 and III-S2). The %Vg parameter showed an increase from 8.3% to 15%, with an increase in the DNA concentration from 0.5% to 1.5% w/v. Hence, it can be noted that the GF of DNA increases with an increase in concentration to reach  $0.15 \pm 0.03$  at 1.5% w/v. This capacity of DNA molecules to form gels can be explained by their backbone composed of negatively charged phosphates that allow for interactions with positively charged biomolecules and ions. Hence, nucleic acids can function as strong hydrophilic polyelectrolytes with the ability to absorb water. In the literature, DNA was described as an attractive material for hydrogel matrices due to its functional properties, such as biocompatibility, biodegradability, and permeability, among others [24]. In addition, the high affinity of DNA to calcium has been proven even in

the presence of competing cations, such as monovalent cations ( $\text{Na}^+$  or  $\text{K}^+$ ) or divalent cations like  $\text{Mg}^{2+}$  [25].



**Figure III-2: Effect of the polymer concentration (% w/v) of pure standard molecules on the %R, %Vg, and GF. The results are obtained through three gelation replicates and the standard deviations were represented as bars.**

The ALV solutions exhibited a high reactivity to calcium since the percentage of the initial area that disappeared was higher than 80% for all the tested concentrations (Figures III-2 and III-S2). In addition, a high percentage of gel volume was obtained, evolving from 46.1% to 68.1% with an increase in concentration from 0.5% to 1.5% w/v (Figure III-2). The highest GF was noted for alginate at 1.5% (w/v) and was equal to  $0.56 \pm 0.04$ . Indeed, alginate is a well-known gelling molecule capable of forming gels with different ions and particularly with calcium [26]. Therefore, the capacity to form gels of the various solutions in terms of both molecular interaction with calcium and water swelling was as follows: ALV > DNA > BSA, whatever the tested concentrations.

### 3.3 Scanning Electron Microscopy (SEM) Characterization

The gels formed with Alginate and DNA were observed using SEM, and, in both cases, the 3D network responsible for gel formation was detected (Figure III-3). However, different structures were observed; indeed, alginate hydrogels are more compact in comparison with

DNA hydrogels that appear more filamentous with larger pores. The difference in porosity influences the properties of the hydrogels formed, such as their elastic properties. When compression is applied to a hydrogel, fluid is pushed through the pores, and the polymeric chains realign and then reach a point where the fluid's ability to pass through the pores is reduced. Hydrogels with larger pores have a greater ability to recover once the mechanical load is reduced due to the greater ability of fluids to be redistributed within the polymeric network and, therefore, are more elastic [8].

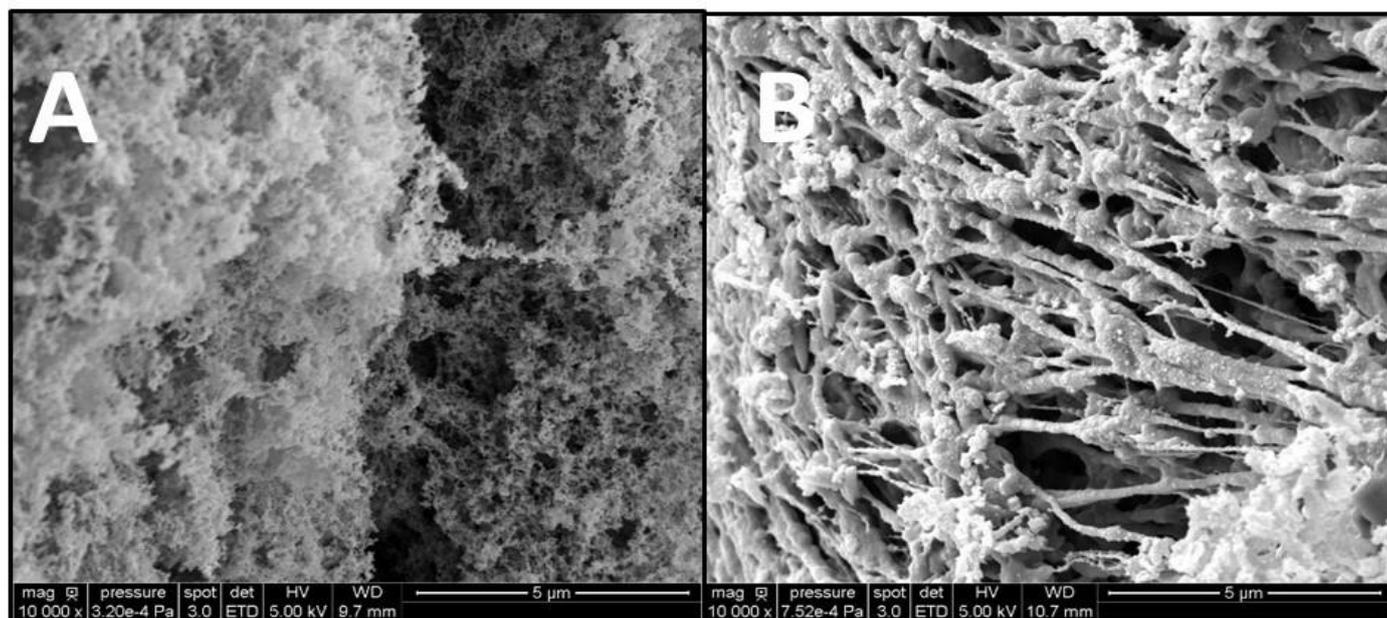


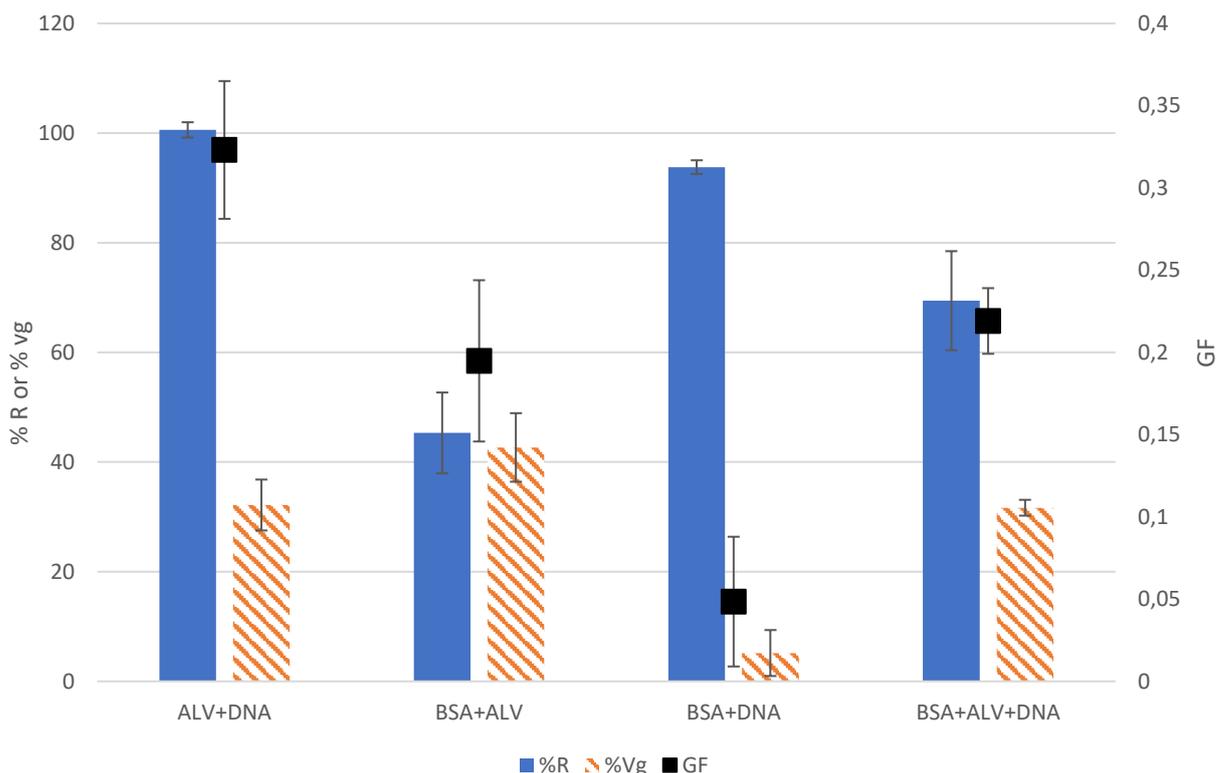
Figure III-3: SEM observation of gels made of ALV (A), and DNA (B) at 1.5% (w/v) and 0.1 M Calcium.

### 3.4 Gelling Capacity of Mixtures of Standard Molecules

The developed method was tested on mixes of standard molecules, with the concentration of each molecule present in the final mixture set to 0.5% (w/v). This low concentration was chosen in order to evaluate the screening capacity for gelling molecules by respecting low material consumption and to check the capacity of the test to detect gelation, even in complex mixtures.

As shown in Figures III-4 and III-S3, the three solutions composed of two types of molecules exhibited GF values ranging from  $0.05 \pm 0.04$  to  $0.32 \pm 0.04$ , the highest being obtained with the mixture of ALV+DNA and the lowest corresponding to the binary solution of BSA+DNA. The mixture containing the three standard molecules (BSA+ALV+DNA) had an intermediate GF of  $0.22 \pm 0.02$  that decreased by one-third compared to the GF of the binary ALV+ DNA solution ( $0.32 \pm 0.04$ ). This is consistent with the fact that BSA did not harm the capacity of ALV and

DNA to form gels (%Vg remained the same between ALV+DNA and BSA+ALV+DNA). However, the addition of BSA to the mixture has reduced the ratio of the gelling molecules and, therefore, has caused a %R decrease in the tested conditions. In the same way, combining BSA with ALV reduced the GF by half, going from  $0.38 \pm 0.02$  for ALV at 0.5% (Figure III-2) to  $0.19 \pm 0.05$  for the mixture of BSA+ALV. A similar reduction in the GF was also observed after the addition of BSA to a solution of pure DNA that decreased from  $0.09 \pm 0.01$  to  $0.05 \pm 0.04$ .



**Figure III-4: The %R, %Vg, and GF of the mixtures of the standard molecules, calibrated at 0.5% (w/v) for each molecule with 0.1 M calcium. The results are obtained through three gelation replicates and the standard deviations were represented as bars.**

Concerning the effects of DNA in the mixtures, it can be noticed that a decrease in %Vg is observed after the addition of DNA to a solution (from a %Vg of  $46.1 \pm 2.3\%$  for ALV down to  $32.1 \pm 4.6\%$  in the case of ALV+DNA). The same observation can be made upon comparing BSA+ALV with BSA+ALV+DNA. A hypothesis explaining this observation could be that DNA is a highly negatively charged molecule, which increases the negative repulsive forces between molecules in the solution, thus possibly hindering gelation. In addition, interactions between DNA and ALV could lead to the formation of a more compact hydrogel with a lower water retention capacity.

In order to summarize the different cases when performing this test, a classification based on the GF, %R, and %Vg is proposed in Figures III-5 and III-S4. The previously tested solutions could be classified as samples with high gelling potential ( $GF > 0.4$ ) resulting from a high %R and high %Vg or with low-gelling potential ( $GF < 0.1$ ) or with an intermediate gelling potential ( $0.1 < GF < 0.4$ ), divided into two categories depending on the %R (higher or lower than 50%) and the %Vg (higher or lower than 35%).

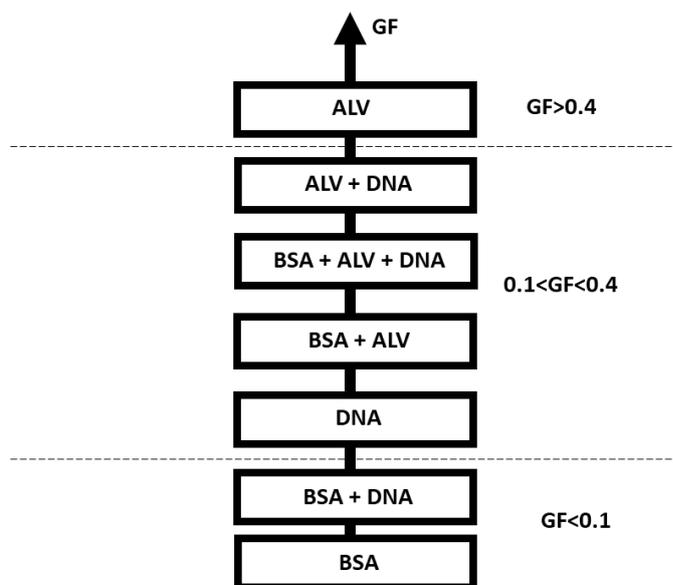


Figure III-5: Classification of solutions according to their GF, %R, and %Vg.

According to this classification, BSA and the binary of BSA+DNA are the representatives of the low GF group. As previously mentioned, BSA does not have a gel-forming capacity in the tested conditions. Although the %Vg of the binary BSA+DNA was similar to that of DNA, the binary BSA+DNA was classified as a low gel-forming solution because the ratio of gelling molecules present in the solution was reduced due to the presence of BSA (a non-gelling molecule). The high GF group consisted of the pure ALV solution, a gelling molecule with a high capacity for gel formation.

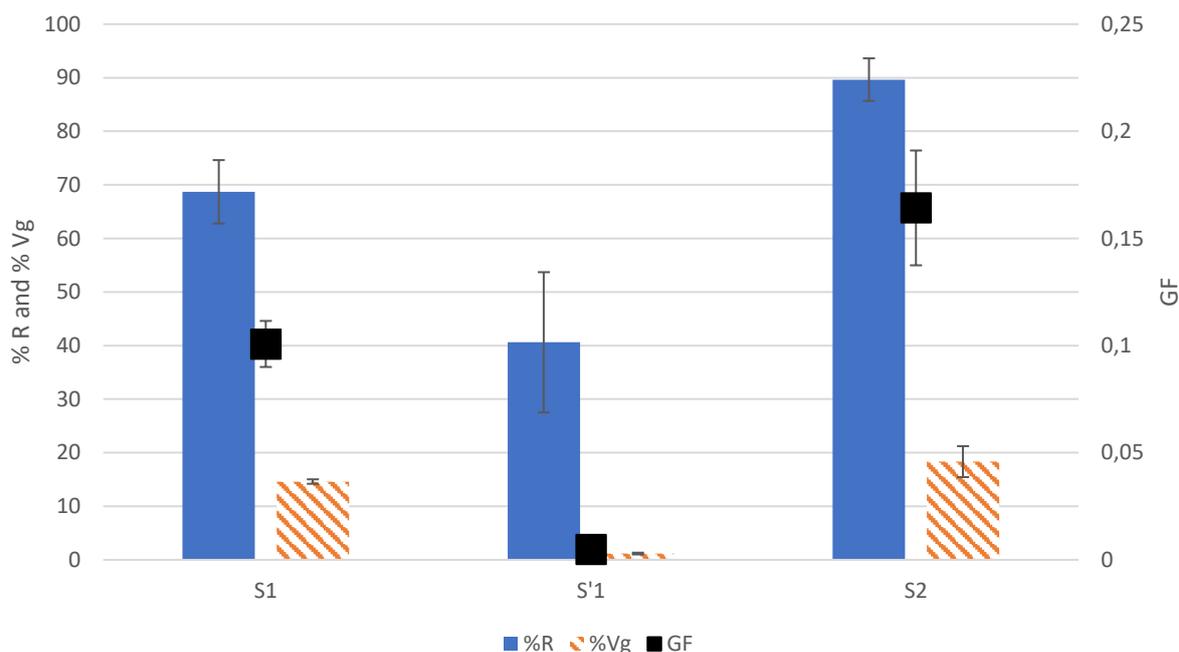
Particular attention should be paid to the solutions with a medium or intermediate GF, which could correspond to two categories. The first category is based on high %R (>50%) and low %Vg (<35%). In this case, the studied solution might be exhibiting precipitation behavior since it is reactive but does not have the capacity to retain water, which is a characteristic of hydrogels. Another possibility is that a gelling molecule that has a low water retention capacity is present in the solution. It can be noticed that the majority of solutions containing DNA are

present in this category (as noticed previously, the presence of DNA reduced the %Vg hence decreasing the gel formation capacity).

The second profile that resulted from a low %R (<50%) but with a high %Vg (>35%) is due to a swelling behavior, therefore meaning the presence of a gelling molecule. In this case, the low %R might be due to a low extinction coefficient, therefore masking the reactivity of the gelling molecule. In this category, the BSA+ALV solutions are the only representatives. Indeed, as previously discussed, BSA did not hinder gelation, and this was previously shown in the literature where there was no influence of BSA on the post-gelling stiffness of alginate [8]. However, the presence of BSA reduced the ratio of gelling molecules in the solution, causing a decrease in %R. In addition, the extinction factors of BSA and ALV are very different, and this shows that even with quite different extinction factors, the test was still capable of detecting gelation. Attention must be paid when comparing solutions with the same GF since it can be possible to obtain a similar GF but with different %R and/or %Vg. This is clearly shown when comparing the BSA+ALV and BSA+ALV+DNA solutions that exhibited similar GF values ( $0.22 \pm 0.02$  and  $0.19 \pm 0.05$ , respectively) but different reactivities and gel volumes leading to different interpretations.

### **3.5 Application on Complex EPS Mixtures**

The developed test was applied to the three EPS samples that were recovered during gel-forming EPS extraction from aerobic granular sludges [7,14]. The three tested fractions corresponding to the S1 (raw extract), S2 (solubilized acidic precipitate), and S'1 (supernatant of the acidic precipitation) were submitted to the test at a final concentration of 0.5% w/v, and the results can be seen in Figures III-6 and III-S5.



**Figure III-6: Comparison of the efficiency of the extraction protocol for the selection of gelling molecules using the developed method. The %R, %Vg, and GF are used to detect the capacity of solutions at 0.5% (w/v) and 0.1 M calcium to form gels. The results are obtained through three gelation replicates and the standard deviations were represented as bars.**

The highest GF was for S2 at  $0.16 \pm 0.03$ , followed by S1 at  $0.10 \pm 0.01$ , and finally, S'1 at  $0.004 \pm 0.002$ . The acidic precipitation is meant to select the gel-forming molecules [7,13,14], therefore explaining that the S2 solution had the highest GF. Since S1 is made by the totality of the extracted molecules (i.e., the molecules that have the capacity to form gels and those that do not), a lower GF was expected due to a lower ratio of gelling molecules in the solution. The gelling molecules are not supposed to be present in the supernatant of the acidic precipitation, S'1; hence, the lowest value of GF was obtained in this solution. Altogether, these results show that the protocol of extraction and acidic precipitation is indeed capable of extracting and concentrating the gelling molecules in one fraction (S2), where it can be noticed that the GF increased by a factor of 1.6 only through this step of acidic precipitation. As previously mentioned, biochemical groups often recurring in gel-forming EPS are proteins, polysaccharides, and DNA [13,14,19]. However, they are not equally abundant in the mixture; for example, Bou-Sarkis et al. (2022) showed that there are equal amounts of proteins and polysaccharides in the EPS extracted using sodium carbonate, whereas it had approximately 40 times less DNA. Another study using anammox granules showed that EPS are composed, to a lesser extent, of DNA in comparison with polysaccharides and proteins [27]. This means

that the effects of DNA on the GF of S2 are most probably limited, but other contaminants were present, such as non-gelling proteins that could reduce the GF of S2, as was the case when mixing BSA with ALV.

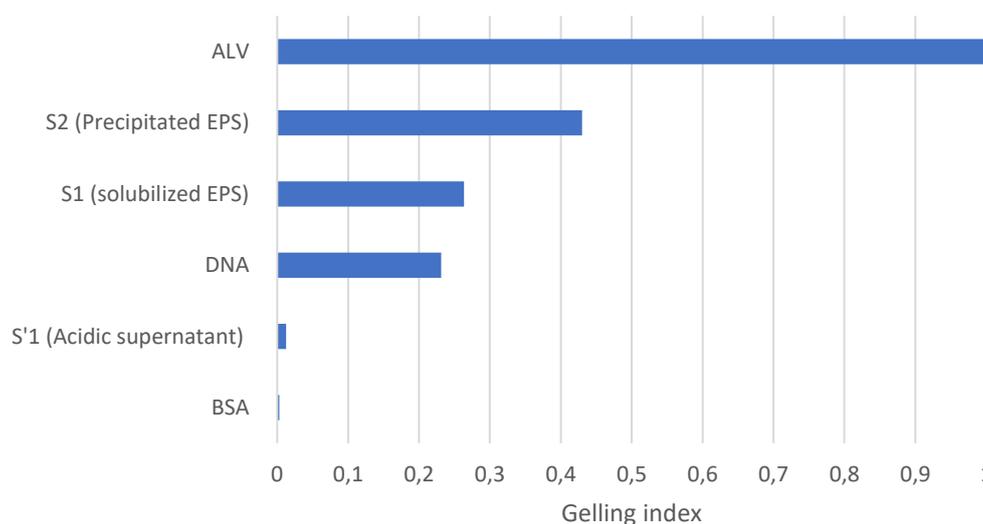
The effect of the concentration on the GF values for S2 was quantified. As can be seen in Supplementary Data Figure III-S6A, in the tested range (0.5 to 1.5% w/v), the GF increased linearly (from  $0.16 \pm 0.03$  to  $0.23 \pm 0.03$ ).

A more filamentous and porous structure for S2 (Supplementary Data Figure III-S6B) was obtained in comparison with alginate gels (Figure III-3). Pfaff et al. (2021) noticed that the gels formed by gel-forming EPS are rather fibrous and filled with voids, and they attributed this observation to many factors, including the calcium concentration and the concentration of the gelling polymers; hence, these factors can be used to control the properties of the gels [28]. In 2022, Pagliaccia et al. attributed more significative linear elastic behavior to EPS hydrogels in comparison to alginate, and this could be due to a more porous structure.

### **3.6 Gelling Index: Comparing Solutions' Gelling Capacity to a Reference**

Finally, a new parameter was proposed and named "gelling index", with the goal of comparing the gelling capacity of a solution to an alginate solution at the same concentration (a well-known gelling molecule). This parameter will allow for the determination of the gelling capacity in reference to a well-studied gelling polymer and hence obtain a more tangible notion of the gelling capacity of the solution being studied in comparison with that of alginate. The GI of both the standard molecules and the EPS samples described in this study was calculated for the concentration of 0.5% (w/v). As shown in Figure III-7, the evaluation of the GI allows for the classification of solutions according to their capacity to form gels. It can be noticed rapidly that none of the solutions' gelling index is close to 1 (alginate). Among the compared solutions, precipitated EPS in S2 has the highest gelling index: 0.43, followed by the S1 sample containing alkaline extracted EPS, with a GI of 0.26. This technique allows for a quick comparison between solutions' capacities to form gels by using a reference (ALV). Indeed, when compared to the obtained results within the literature, it can be noticed that alginate has a better gel formation capacity than EPS; in particular, the value of alginate's Young's modulus is approximately 10 times higher than that of EPS at 5% w/v [8]. In addition, it was shown that alginate (at a concentration of 2.5% w/v compared to EPS at 10% w/v) had a Young's modulus approximately 15 times higher than calcium [6]. Thus, Young's modulus has a better sensitivity to detect the differences between hydrogels, but the proposed test

remains interesting since it can provide information about the presence of nongelling molecules within a mixture and can be coupled with other methods to have a global view on the gelation capacity of a solution. The lower sensitivity compared to Young's modulus is acceptable since the primary purpose of the developed test is to screen for the gelling capacity. In comparison to the literature [6,8,17,18], this screening method allows for reduced sampling from bioreactors due to the reduced amount of EPS needed per test, and offers a quick screening using simple equipment. These results show that the test is capable of detecting gel formation as a screening method to select interesting solutions for further characterization. The test showed that more purification of the extracted EPS is needed in order to obtain a solution of polymers with more interesting properties closer to that of alginate.



**Figure III-7: The GI of different solutions at 0.5% (w/v) with 0.1 M calcium, allowing for a comparison of their gelling capacities (between each other and to a reference molecule ALV).**

Therefore, the proposed test (Supplementary Data Figure III-S4) allows for a quick comparison of the gelling potential of the biological samples assayed at the same final concentration. The test can be used to monitor the increase or decrease in gelling capacity during the purification of biopolymers in order to quickly identify the relevant fractions. In addition, activating or inhibiting effects could be detected, thus offering the possibility of formulating new preparations or mixtures of biopolymers. In fact, the mixtures of molecules that give composite hydrogels were shown previously in the literature to have interesting properties. Hydrogels made by using xanthan gum and  $\beta$ -lactoglobulin have been described in which  $\beta$ -

lactoglobulin plays the role of a crosslinking agent between the xanthan polymers when the pH conditions are convenient [29]. Hence, this test can be used to study composite hydrogels since it proved its capacity to detect gelation even in complex mixtures.

#### 4 Conclusions

A method was developed to quickly screen the capacity of solutions to form gels in the presence of calcium. This method can be used to understand the role of contaminants in the hydrogel formation and by being coupled with microscopic observations (SEM), can provide knowledge on the structure of the hydrogel network formed. The method has the advantage of being less material and time-consuming in comparison to the conventional methods used in the literature and allows for the detection of gelation through the use of simple methods (UV-vis spectroscopy). Screening tests can be carried out using only 1 mg of the sample to be tested, leading to less sampling volumes and, therefore, less perturbation of lab-scale reactors.

The direct application of the method will be as a screening process for gelling capacity and to compare extracts' capacities of gelation. The method was applied to the EPS extraction protocol as a proof of concept, and it was found that the protocol allowed for the selection of gel-forming molecules, but the obtained gelling solution still needs further purification to attain a GF closer to alginate and, therefore, a GI closer to 1. This test can be further used to follow the enrichment in gelling polymers during purification/fractionation processes. In conclusion, due to its ease of application, low consumption of materials, and speed, this test can be very helpful in the progression toward efficiently selecting gel-forming molecules from aerobic granules. In the future, trying to detect the participation of specific biochemical groups in gelation through the coupling of this test with fluorescent detection using specific probes can be tested. Moreover, this test can help in modulating and understanding the parameters affecting the gelation of different solutions, such as pH, divalent ions, the concentration of polymers, etc., and hence allow for the more efficient valorization of EPS.

**Author Contributions:** Conceptualization, A.B.-S., E.G.-N., and Y.B.; methodology: A.B.-S.; Validation, A.B.-S.; Formal analysis, A.B.-S.; Investigation, A.B.-S.; Visualization, A.B.-S.; Resources, N.D.; Writing—Original Draft, A.B.-S.; Writing—Review & Editing, A.B.-S., E.P., E.G.-N., N.D., and Y.B.; Funding acquisition, E.P., E.G.-N., and Y.B., Supervision E.G.-N. and Y.B.,

Project administration E.G.-N. and Y.B. All authors have read and agreed to the published version of the manuscript.

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## References

1. Winkler, M.K.H.; Meunier, C.; Henriot, O.; Mahillon, J.; Suárez-Ojeda, M.E.; Del Moro, G.; De Sanctis, M.; Di Iaconi, C.; Weissbrodt, D. An integrative review of granular sludge for the biological removal of nutrients and recalcitrant organic matter from wastewater. *Chem. Eng. J.* **2018**, *336*, 489–502. <https://doi.org/10.1016/j.cej.2017.12.026>.
2. Lin, Y.M.; Nierop, K.; Girbal-Neuhauser, E.; Adriaanse, M.; van Loosdrecht, M.C.M. Sustainable polysaccharide-based biomaterial recovered from waste aerobic granular sludge as a surface coating material. *Sustain. Mater. Technol.* **2015**, *4*, 24–29. <https://doi.org/10.1016/j.susmat.2015.06.002>.
3. Kim, N.K.; Mao, N.; Lin, R.; Bhattacharyya, D.; van Loosdrecht, M.C.M.; Lin, Y. Flame retardant property of flax fabrics coated by extracellular polymeric substances recovered from both activated sludge and aerobic granular sludge. *Water Res.* **2020**, *170*, 115344. <https://doi.org/10.1016/j.watres.2019.115344>.
4. Liu, W.; Zhang, J.; Jin, Y.; Zhao, X.; Cai, Z.; Pb, A. Cd(II) and Zn(II) by extracellular polymeric substances extracted from aerobic granular sludge: Efficiency of protein. *J. Environ. Chem. Eng.* **2015**, *3*, 1223–1232. <https://doi.org/10.1016/j.jece.2015.04.009>.
5. Cydzik-Kwiatkowska, A.; Gusiatin, M.Z.; Zielińska, M.; Wojnowska-Baryła, I.; Kulikowska, D.; Bernat, K. Alginate-like polymers from full-scale aerobic granular sludge: Content, recovery, characterization, and application for cadmium adsorption. *Sci. Rep.* **2022**, *12*, 22260. <https://doi.org/10.1038/s41598-022-26743-5>.
6. Felz, S.; Kleikamp, H.; Zlopasa, J.; van Loosdrecht, M.C.M.; Lin, Y. Impact of metal ions on structural EPS hydrogels from aerobic granular sludge. *Biofilm* **2020**, *2*, 100011. <https://doi.org/10.1016/j.biofilm.2019.100011>.
7. Lin, Y.; de Kreuk, M.; van Loosdrecht, M.; Adin, A. Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant. *Water Res.* **2010**, *44*, 3355–3364. <https://doi.org/10.1016/j.watres.2010.03.019>.
8. Pagliaccia, B.; Durieux, S.; Bessiere, Y.; Bounouba, M.; Sarkis, A.B.; Girbal-Neuhauser, E.; Carretti, E.; Lubello, C.; Lotti, T.; Paul, E. Insights on the hydrogel-forming ability and post-gelling mechanical properties of structural extracellular polymeric substances (sEPS) from aerobic granular sludge (AGS): A comparison with model biopolymers. *J. Water Process Eng.* **2022**, *49*, 103076. <https://doi.org/10.1016/j.jwpe.2022.103076>.
9. Ahmed, E.M. Hydrogel: Preparation, characterization, and applications: A review. *J. Adv. Res.* **2015**, *6*, 105–121. <https://doi.org/10.1016/j.jare.2013.07.006>.
10. Kallala, M.; Julien, R.; Cabane, B. Crossover from gelation to precipitation. *J. Phys. II* **1992**, *2*, 7–25. <https://doi.org/10.1051/jp2:1992110>.
11. Meng, F.; Liu, D.; Pan, Y.; Xi, L.; Yang, D.; Huang, W. Enhanced amount and quality of alginate-like exopolysaccharides in aerobic granular sludge for the treatment of salty wastewater. *BioResources* **2019**, *14*, 139–165. <https://doi.org/10.15376/biores.14.1.139-165>.
12. Rollemberg, S.L.D.S.; Dos Santos, A.F.; Ferreira, T.J.T.; Firmino, P.I.M.; Dos Santos, A.B. Evaluation of the production of alginate-like exopolysaccharides (ALE) and tryptophan in aerobic granular sludge systems. *Bioprocess Biosyst. Eng.* **2021**, *44*, 259–270. <https://doi.org/10.1007/s00449-020-02439-w>.
13. Schambeck, C.M.; Girbal-Neuhauser, E.; Böni, L.; Fischer, P.; Bessière, Y.; Paul, E.; da Costa, R.H.R.; Derlon, N. Chemical and physical properties of alginate-like exopolymers of aerobic granules and flocs produced from different wastewaters. *Bioresour. Technol.* **2020**, *312*, 123632. <https://doi.org/10.1016/j.biortech.2020.123632>.
14. Bou-sarkis, A.; Pagliaccia, B.; Ric, A.; Derlon, N.; Paul, E.; Bessiere, Y.; Girbal-neuhauser, E. Effects of alkaline solvents and heating temperatures on the solubilization and degradation of gel-forming Extracellular Polymeric Substances (EPS) extracted from aerobic granular sludge. *Biochem. Eng. J.* **2022**, *185*, 108500. <https://doi.org/10.1016/j.bej.2022.108500>.
15. Guo, X.; Wang, Y.; Qin, Y.; Shen, P.; Peng, Q.; Structures, properties and application of alginic acid: A review. *Int. J. Biol. Macromol.* **2020**, *162*, 618–628. <https://doi.org/10.1016/j.ijbiomac.2020.06.180>.
16. Klein, M.; Poverenov, E. Natural biopolymer-based hydrogels for use in food and agriculture. *J. Sci. Food Agric.* **2020**, *100*, 2337–2347. <https://doi.org/10.1002/jsfa.10274>.
17. Felz, S.; Al-Zuhairy, S.; Aarstad, O.; van Loosdrecht, M.C.M.; Lin, Y. Extraction of structural extracellular polymeric substances from aerobic granular sludge. *J. Vis. Exp.* **2016**, *115*, e54534. <https://doi.org/10.3791/54534>.
18. Seviour, T.; Pijuan, M.; Nicholson, T.; Keller, J.; Yuan, Z. Gel-forming exopolysaccharides explain basic differences between structures of aerobic sludge granules and floccular sludges. *Water Res.* **2009**, *43*, 4469–4478. <https://doi.org/10.1016/j.watres.2009.07.018>.

19. Oliveira, A.S.; Amorim, C.L.; Ramos, M.A.; Mesquita, D.P.; Inocência, P.; Ferreira, E.C.; Van Loosdrecht, M.; Castro, P.M.L. Variability in the composition of extracellular polymeric substances from a full-scale aerobic granular sludge reactor treating urban wastewater. *J. Environ. Chem. Eng.* **2020**, *8*, 104156. <https://doi.org/10.1016/j.jece.2020.104156>.
20. Topalã, T.; Bodoki, A.; Oprean, L.; Oprean, R. Bovine serum albumin interactions with metal complexes. *Clujul Med.* **2014**, *87*, 5. <https://doi.org/10.15386/cjmed-357>.
21. Matsudomi, N.; Rector, D.; Kinsella, J. Gelation of bovine serum albumin and  $\beta$ -lactoglobulin; effects of pH, salts and thiol reagents. *Food Chem.* **1991**, *40*, 55–69. [https://doi.org/10.1016/0308-8146\(91\)90019-K](https://doi.org/10.1016/0308-8146(91)90019-K).
22. Navarra, G.; Giacomazza, D.; Leone, M.; Librizzi, F.; Militello, V.; Biagio, P.S. Thermal aggregation and ion-induced cold-gelation of bovine serum albumin. *Eur. Biophys. J.* **2009**, *38*, 437–446. <https://doi.org/10.1007/s00249-008-0389-6>.
23. Ramdhan, T.; Ching, S.; Prakash, S.; Bhandari, B. Time dependent gelling properties of cuboid alginate gels made by external gelation method: Effects of alginate-CaCl<sub>2</sub> solution ratios and Ph. *Food Hydrocoll.* **2019**, *90*, 232–240. <https://doi.org/10.1016/j.foodhyd.2018.12.022>.
24. Kim, E.; Jang, Y.; Kim, H.; Lee, D.Y.; Choi, J.G.; Kim, S.J. Calcium-driven DNA artificial muscle inspired by natural muscle contraction. *Sensors Actuators B Chem.* **2022**, *371*, 132556. <https://doi.org/10.1016/j.snb.2022.132556>.
25. Korolev, N.; Lyubartsev, A.; Rupprecht, A.; Nordenskiöld, L. Competitive binding of Mg<sup>2+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup>, and K<sup>+</sup> ions to DNA in oriented DNA fibers: Experimental and Monte Carlo simulation results. *Biophys. J.* **1999**, *77*, 2736–2749. [https://doi.org/10.1016/S0006-3495\(99\)77107-9](https://doi.org/10.1016/S0006-3495(99)77107-9).
26. Hu, C.; Lu, W.; Mata, A.; Nishinari, K.; Fang, Y. Ions-induced gelation of alginate: Mechanisms and applications. *Int. J. Biol. Macromol.* **2021**, *177*, 578–588. <https://doi.org/10.1016/j.ijbiomac.2021.02.086>.
27. Feng, C.; Lotti, T.; Lin, Y.; Malpei, F. Extracellular polymeric substances extraction and recovery from anammox granules: Evaluation of methods and protocol development. *Chem. Eng. J.* **2019**, *374*, 112–122. <https://doi.org/10.1016/j.cej.2019.05.127>.
28. Pfaff, N.; Kleijn, J.M.; Van Loosdrecht, M.C.M.; Kemperman, A.J.B. Formation and ripening of alginate-like exopolymer gel layers during and after membrane filtration. *Water Res.* **2021**, *195*, 116959. <https://doi.org/10.1016/j.watres.2021.116959>.
29. Le, X.; Rioux, L.; Turgeon, S. Formation and functional properties of protein–polysaccharide electrostatic hydrogels in comparison to protein or polysaccharide hydrogels. *Adv. Colloid Interface Sci.* **2017**, *239*, 127–135. <https://doi.org/10.1016/j.cis.2016.04.006>.

## 5 Supplementary material

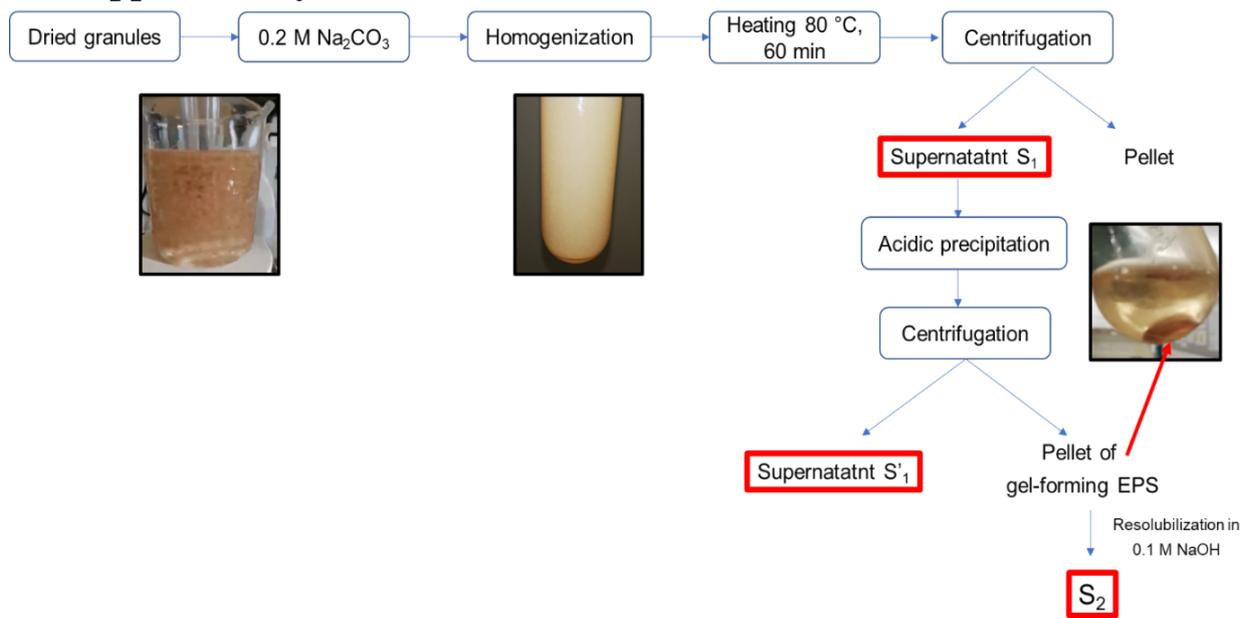


Figure III-S1: Summary of the extraction protocol of gel-forming EPS.

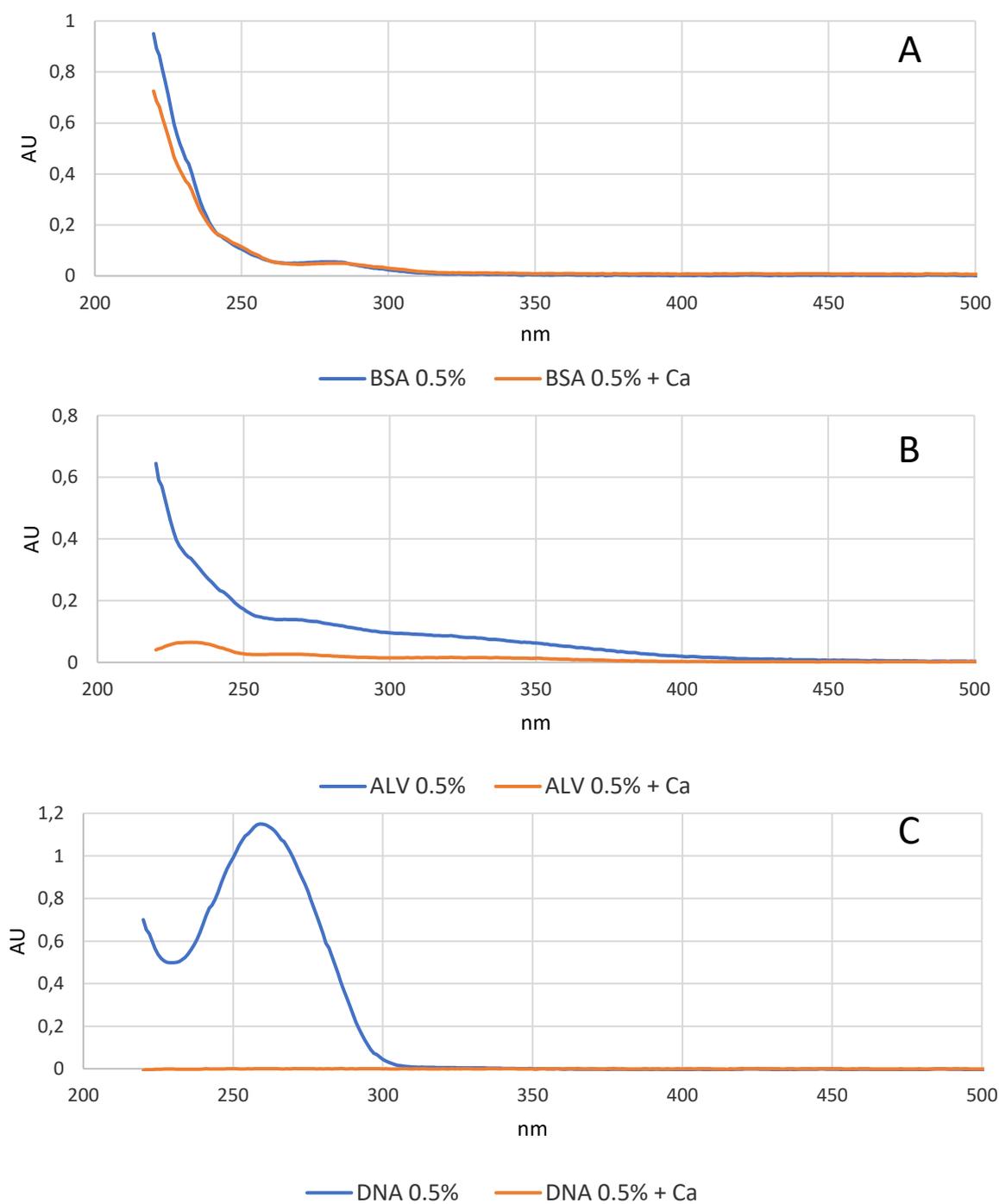
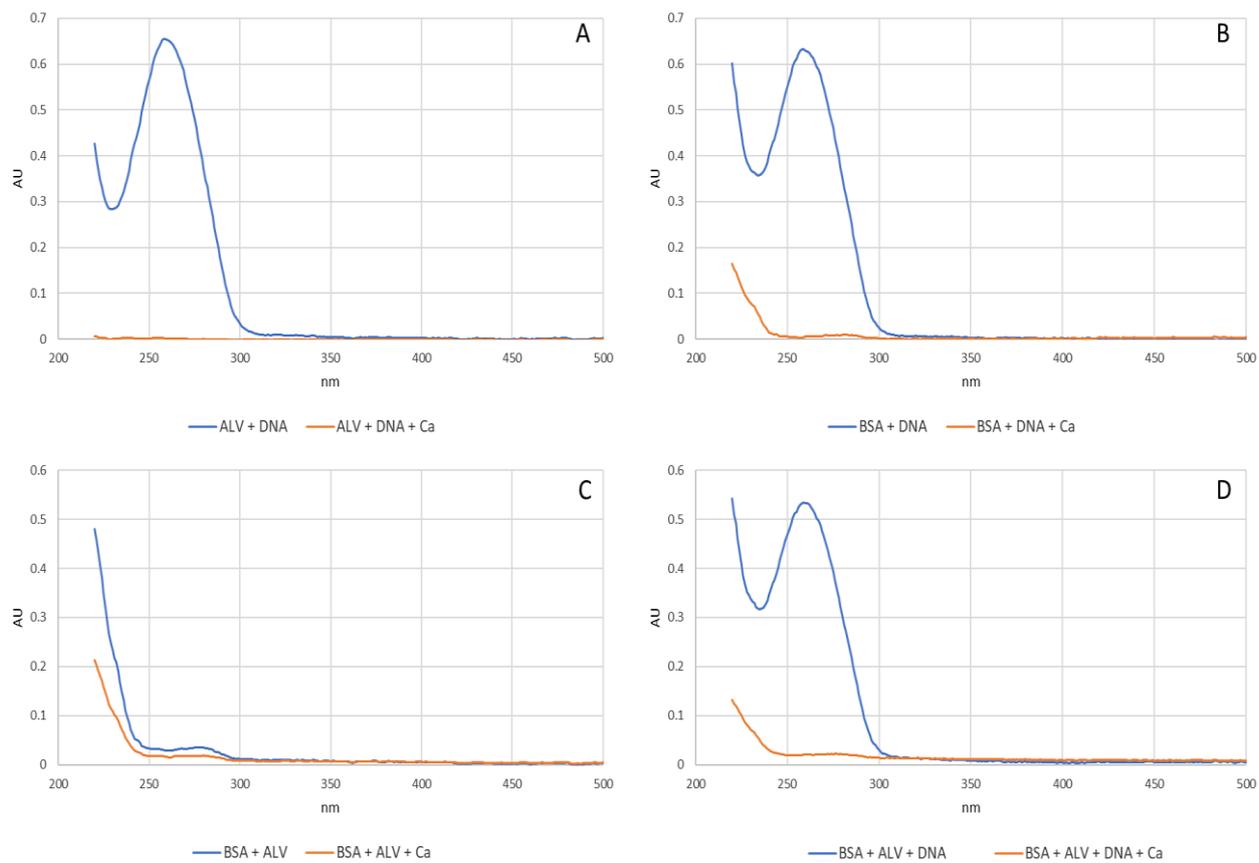
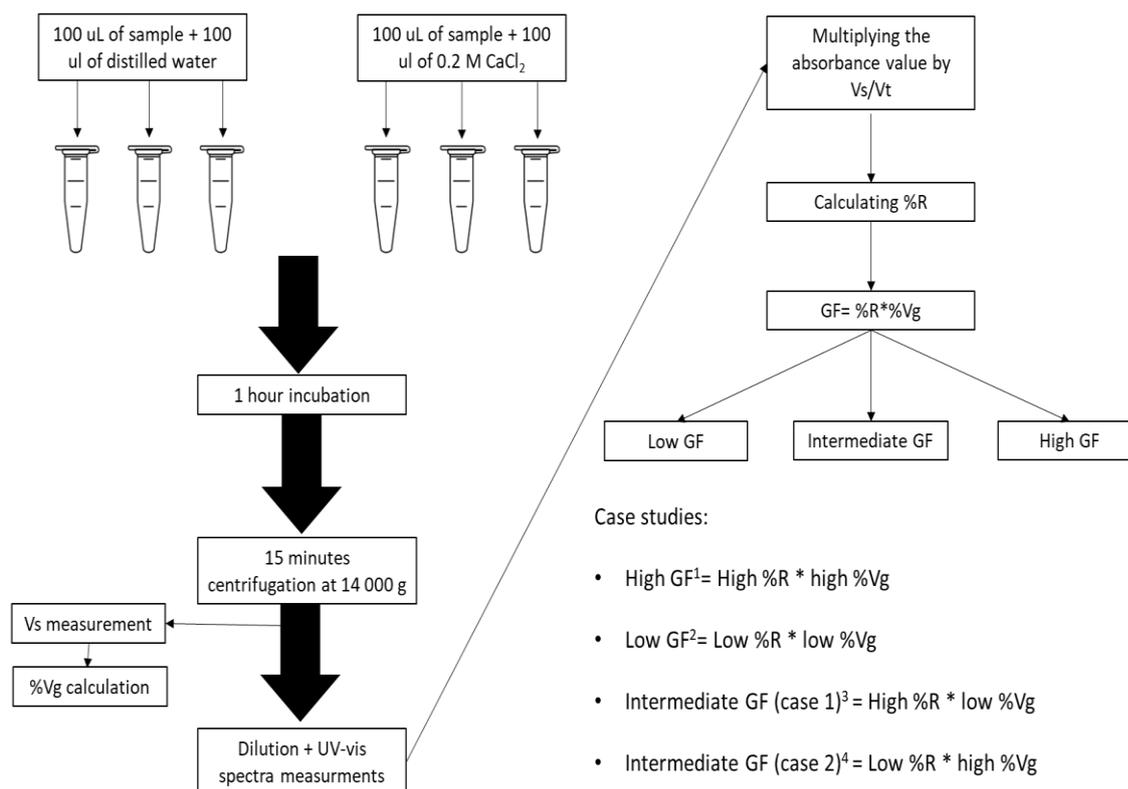


Figure III-S2: UV-vis spectra of BSA (A), ALV (B) and DNA (C) at 0.5% w/v with and without calcium at 0.1M with blue line for polymer alone and orange line for polymer with calcium.



**Figure III-S3: Uv-vis spectra of mixtures at 1% w/v final concentration of ALV+DNA (A), BSA+DNA (B), BSA+ALV (C), BSA+ALV+DNA (D) with and without calcium at 0.1M with blue line for polymer alone and orange line for polymer with calcium.**



**Figure III-S4: Summary of the proposed test, with case studies of the GF.**

<sup>1</sup>: in the case of a high  $GF > 0.4$ , resulting from a high %R and high %Vg, the solution being studied has a good gelation capacity such as ALV

<sup>2</sup>: in the case of a low  $GF < 0.1$ , resulting from a low %R and low %Vg, the solution being studied has no gelation capacity such as BSA

<sup>3</sup>: in the case of an intermediate GF ( $0.1 < GF < 0.4$ ), resulting from a high %R ( $> 50\%$ ) and low %Vg ( $< 35\%$ ), the solution being studied might be exhibiting a precipitation behavior or a gelling molecule that has low water retention capacity such as DNA, or a molecule with a high extinction coefficient and a small quantity in the mixture

<sup>4</sup>: in the case of an intermediate GF ( $0.1 < GF < 0.4$ ), resulting from a low %R ( $< 50\%$ ) and high %Vg ( $> 35\%$ ), the high %Vg is due to a swelling behavior therefore meaning a gelling molecule present, the low %R in this case is due to a low extinction coefficient therefore masking the reactivity of the gelling molecule.

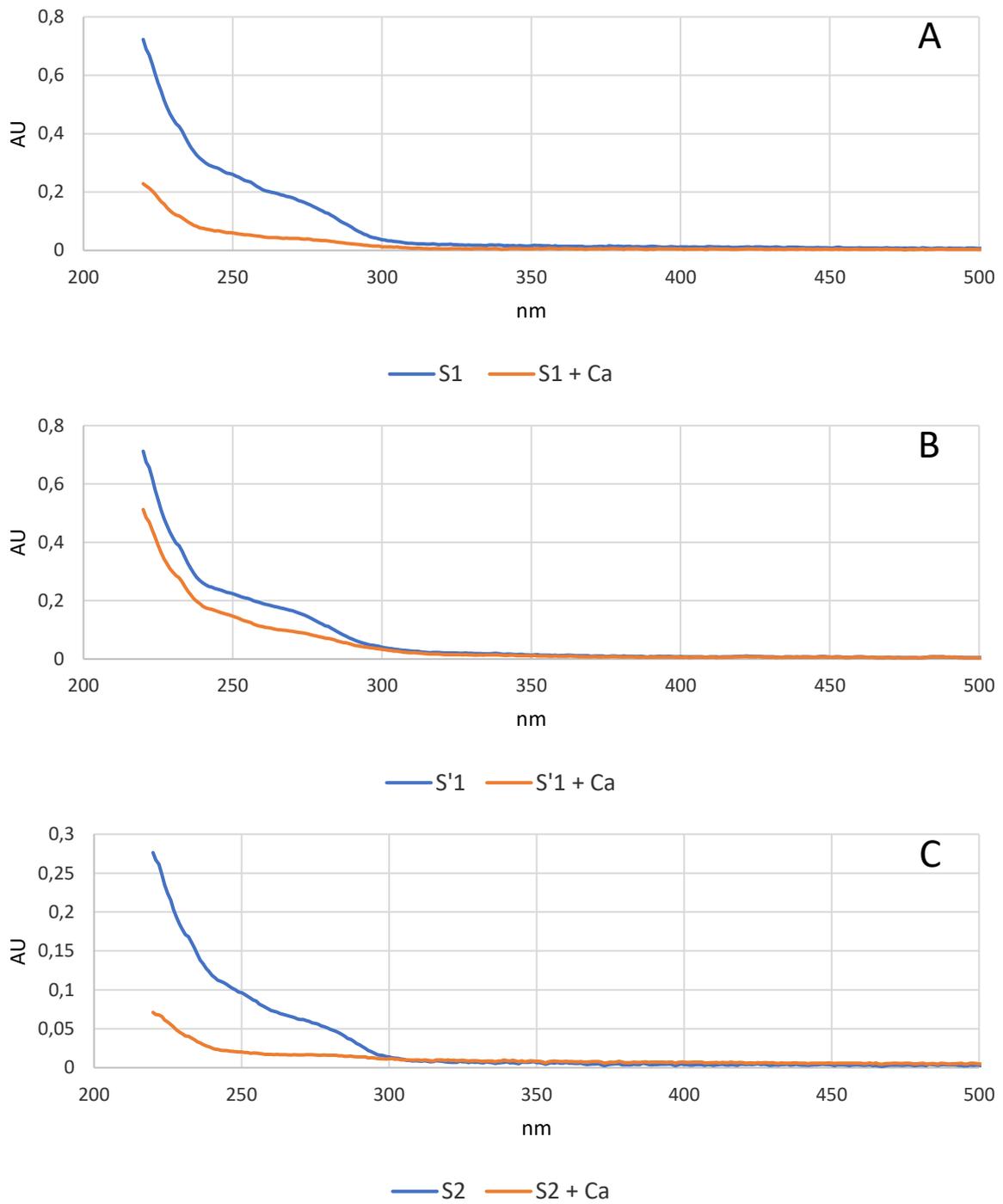
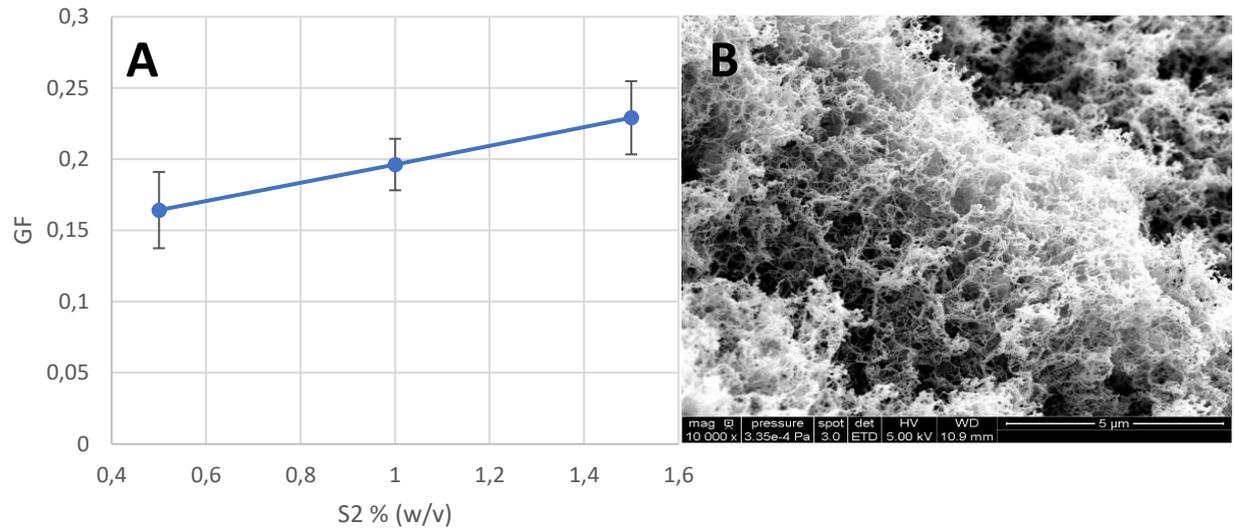


Figure III-S5: UV-vis spectra at 0.5% w/v of S1(A) S'1(B) S2(C) with and without calcium at 0.1M.



**Figure III-S6: The effect of the concentration % (w/v) of the solutions of S2 (precipitated EPS) on the GF (A) and SEM observation of a 1.5% (w/v) with 0.1 M calcium S2 hydrogel (B).**

## 6 Conclusion of Chapter III

Chapter III proposed a methodology for the screening of the gelation capacity of solutions using a limited quantity of molecules (1 to 3 mg) with calcium as the ionic cross linker. The test was developed using various commercial molecules that are commonly found in gel-forming EPS i.e. polysaccharides, proteins and DNA (Lin et al., 2010; Schambeck et al., 2020; Seviour et al., 2012b). The test had to be capable of detecting gelation in complex mixtures due to the biochemical complexity of gel-forming EPS (Oliveira et al., 2020; Schambeck et al., 2020). Moreover, a test capable of detecting synergies or antagonist effects between the biochemical entities forming the EPS extracts during gelation is needed.

The method proved efficient at detecting the presence of gelling molecules in complex mixtures. Moreover, the method can evidence the presence of contaminants (non-gelling molecules) in a solution shown by a reduction in the GF values due to a decrease in the reactivity with calcium (%R) as it was the case for the mixture of BSA+ALV. Analyzing furthermore the gel volume %Vg and %R can give indications on the effects of combining molecules together. For example, it was shown that combining BSA with ALV did not cause a decrease in %Vg and therefore BSA did not negatively impact ALV's capacity to form hydrogels but only reduced the %R because BSA did not have gelling capacity in the tested conditions. This observation is interesting in the case of EPS extracted from aerobic granules because they contain a big fraction of proteins (The content in S2 sodium carbonate of uronic acids was of  $24.8 \pm 1.7$  mg/g VS and proteins content was of  $30.5 \pm 5.1$  mg/g VS as shown in chapter II). On the contrary DNA reduced the %Vg of solutions upon combining it with other molecules. In addition, the gel volume %Vg can differentiate to a certain extent between a gelling and a precipitation process after the addition of calcium. Interestingly, comparison to an alginate solution can be done through a Gelling Index that allows the direct comparison of the gelling performances of the samples between each other's in regard to the standard alginate.

The developed test was applied on EPS extracted using sodium carbonate (0.2M, 80 °C, 1 h) precisely on the fractions corresponding to the S1 (raw extract), S2 (solubilized acidic precipitate), and S'1 (supernatant of the acidic precipitation) at a final concentration of 0.5% w/v. The test was capable of detecting the presence of gel-forming polymers in S1 and S2 and showed an enrichment of gelling polymers between S1 and S2 due to the acidic precipitation.

The gelling index of S1 was approximately 1.6 times lower than that of S2. This showed the capacity of the developed test to detect gelation even in complex biological samples.

This technique can have many applications, especially in lab scale experiments where the quantities of EPS can be very low; considering the research field on wastewater treatment with microbial aggregates, the sampling volume from the pilot-scale reactors is often limited to avoid disturbing the functioning of the reactor. In industries it could be a method used to follow the presence of gelling molecules in reactors for valorization purpose.

Focused to gain new insights on the key factors governing the gel forming properties of sEPS extracted from granular sludges, this test will be used in the next chapter to follow the enrichment in gel-forming EPS during fractionation. In fact, gel-forming EPS are a very complex mixture due to the presence of different biochemical groups (Oliveira et al., 2020; Schambeck et al., 2020) and their heterogeneity in molecular weight and charge has been proven (Caudan et al., 2012; Felz et al., 2020b; Lin et al., 2015). A specific focus on fractionation of the EPS recovered in the S2 sample according to their charge has been proposed and the developed test was applied to determine which collected fractions are being enriched in gelling molecules.

# **Chapter IV:**

## **Physico-chemical characteristics affecting the gelation of EPS extracted from aerobic granules**

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**Abstract:** Aerobic granular sludges containing highly cohesive Extracellular Polymeric Substances (EPS) constitute a promising resource for the production of gel-forming biopolymers. Preparative anionic chromatography was performed on the structural EPS extracted from granules in order to gain more insights on the charge distribution of these gel-forming EPS. A gelling factor (GF) based on the capacity of gelation after calcium addition was attributed to each eluted fraction and it was shown that the GF of the adsorbed anionic EPS correlated with their global negative charge. A GF of  $0.04 \pm 0.03$  was measured for molecules eluted at NaCl 0.25M and a GF of  $0.32 \pm 0.02$  for the molecules eluted at NaCl 0.75M. However, upon reaching a very high negative charge (with NaCl 1 M) the gelation capacity decreases probably due to increased repulsions between polymers hence hindering gelation. The size distribution of the molecules participating in the gelation was analyzed by Size Exclusion Chromatography. The majority of detected high molecular weights participate in gelation (between 65% and 100% of initial present molecules) whereas low molecular weights participation in gelation is limited (<40% of initial present molecules). Therefore, the obtained results show that both charge and molecular size are important factors for the gelation. Moreover, it was shown that molecules participating in the gelation are biochemically diversified with a major contribution of proteins and polysaccharides in the hydrogel formation. With the goal of selecting gel-forming EPS, these results underline the necessity to modulate the extraction and purification methods towards the selection of molecules having the appropriate negative charge and also towards the preservation of their molecular weight during the extraction steps.

**Keywords:** Aerobic granular sludge; calcium-induced hydrogels; gel-forming EPS; molecular weight; resource recovery; surface net charge.

## 1 Introduction

Biopolymers produced from renewable sources are becoming more and more attractive since they fall within the circular economy concept. Currently, biopolymers are the focus of intensive research efforts with the goal of reaching a production less dependent on petrochemical industries. Microbial polymers are an important source of renewable biopolymers and include intracellular polymers (polyphosphate, glycogen...) and Extracellular Polymeric Substances, EPS (proteins, carbohydrates, and others) (Feng et al., 2021). EPS have gained a lot of interest lately because of their biodegradability, their high productive rate and their easier recovery in comparison with petroleum-based polymers (Feng et al., 2021).

In the last ten years, aerobic granular sludge was recognized as a type of biofilm that proved efficient for the treatment of wastewater (Schambeck et al., 2020; Winkler et al., 2018). EPS play many roles within aerobic granules such as settleability, nutrient sorption or diffusion. Moreover, the subset of the EPS matrix that provide the physical structure of the granule is called structural EPS (sEPS). They are classically recovered after drastic granule solubilization and show the capacity of forming gels *in vitro* through ionic cross-linking with divalent cations (Felz et al., 2016; Feng et al., 2021; Tan et al., 2021; Winkler et al., 2018). Polysaccharides, proteins, humic substances, and DNA were all proven to be a part of the biochemical composition of sEPS (Adav and Lee, 2008; Mcswain et al., 2005; Oliveira et al., 2020). The EPS matrix of aerobic granular sludge contains highly anionic polymers and some hyaluronic acid-like polymers and sulfated glycosaminoglycans (GAGs)-like polymers were suspected as additional EPS components (Felz et al., 2020b). A study led on anammox granular sludge proved the presence of glycoproteins within the granule (Boleij et al., 2018) and glycosylated amyloid-like proteins were also detected in aerobic granules enriched with ammonium oxidizing bacteria (Lin et al., 2018).

This wide diversity in macromolecules paves the way for the valorization of granular sludges through the extraction of many high added-value byproducts (Tan et al., 2021). In particular, hydrogel forming polymers have elicited the most interest. EPS tend to form hydrogels in the presence of divalent cations like calcium. However, the molecular properties governing the gel-formation of EPS are still unclear, and understanding them would help in orienting the valorization of EPS.

Hydrogels are three-dimensionally cross-linked polymeric networks that have the capacity to retain water within their structure. The capacity to retain water depends on the presence of hydrophilic functional groups within the polymeric backbone, mainly OH, COOH, NH<sub>2</sub>, CONH, and HSO<sub>3</sub> groups (Labowska et al., 2021). The uses of hydrogels are very diverse and in literature, applications were described in the medical industry, food industry, agriculture, and water industry amongst others (Ahmed, 2015). These applications are dependent on the characteristics of the polymer being used as well as on the extrinsic factors such as temperature, pH, crosslinker type and concentration. It was reported that the molecular weight, chemical composition, charge density (sulfate/carboxylic groups), conformation (3D structure) related to either native or denaturated state and isoelectric point, all play important roles in the gelation process (Eysturskard et al., 2009b; Le et al., 2017; Navarra et al., 2016).

The effect of charge on gel formation can be seen clearly with the example of the three well-known types of carrageenan differing by their negative charge density due to the number of sulfates present on the disaccharide unit ( $\beta$ -D-galactose with 3,6-Anhydro-D-galactose or  $\alpha$ -D-galactopyranose) (Chauhan and Saxena, 2016). Contrary to  $\lambda$ -carrageenan bearing 3 sulfates per disaccharide unit,  $\kappa$ - and  $\iota$ -carrageenan bearing respectively 1 and 2 sulfates, are capable of forming hydrogels. The number and position of ester sulfate groups as well as the content of 3,6-Anhydro-D-galactose was shown to influence the gel properties since  $\kappa$ -carrageenan gives strong and stiff gels whereas  $\iota$ -carrageenan gives softer ones (Beaumont et al., 2021). Shielding the electronegative repulsions is necessary for gelation to happen and hence the presence of a lot of negative charges in  $\iota$ -carrageenan may cause repulsion and limits gelation (Chauhan and Saxena, 2016; Hotchkiss et al., 2016; Liu and Li, 2016; Morris, 1979).

The molecular weight of gelling polymers can also drive their capacity to form hydrogels and the final properties of the obtained hydrogels. Many studies have reported the gel formation of alginate in the presence of an ionic cross-linker such as calcium. Alginate is a linear polyuronic polysaccharide consisting of (1-4)-linked blocks of poly- $\beta$ -D-mannuronic acid (M) and poly- $\alpha$ -L-guluronic acid (G) residues at different proportions and with different sequential occurrences. Several results underlined that an increase in molecular weight was correlated with an increase in gel's strength (Draget et al., 1994; Labowska et al., 2021, Fernández Farrés and Norton, in 2014).

The gel-forming capacity of EPS has been mainly attributed to two polymers: (i) alginate-like exopolysaccharides (ALE) which were named like this due to their extraction method (sodium carbonate) and composition (rich in carboxyl groups attributed to uronic acids) that are similar to alginate (Lin et al., 2010) and (ii) granulan which is a complex heteropolysaccharide extracted using sodium hydroxide (Seviour et al., 2012b). However, recent studies have proved that the gel-forming EPS extracted using sodium carbonate have a very complex biochemical composition and are made of diversified polymers including polysaccharides, proteins, humic-like substances, DNA amongst others (Bou-sarkis et al., 2022; Oliveira et al., 2020; Schambeck et al., 2020). Examples of composite hydrogels obtained using polysaccharides and proteins are numerous and this combination allows the obtention of hydrogels with competitive characteristics. For example, a hydrogel with an enhanced water retention capacity was obtained through the association of  $\beta$ -lactoglobulin and xanthan gum (Bealer et al., 2020; Labowska et al., 2021; Le et al., 2017).

Several studies have shown the capacity for EPS to form hydrogels with different types of divalent cations other than calcium and in particular with copper and zinc which are known to interact with proteins (Felz et al., 2020a). Gel-forming EPS have also the capacity to form hydrogels upon lowering the pH below 9, which are the conditions in wastewater plants (pH between 6 and 8.5) that can therefore trigger the formation of granular sludges (Seviour et al., 2009). Concerning the physico-chemical properties of EPS, it was proven that EPS extracted from aerobic granular sludge are a mix of molecules with different anionic properties and a wide distribution in molecular weight. Indeed, low, medium and high molecular weight polymers going up to several million Da were reported (Caudan et al. 2012; Bou-sarkis et al., 2022).

Efforts must be made to fractionate the gel-forming EPS with the aims of purifying and deciphering which chemical groups participate in the gel formation in order to understand the mechanisms implied in the gelation of these EPS. Since sEPS are a very complex mixture of molecules with different properties, the effects of molecular weight and charge on the gelation of EPS should be elucidated. Especially the combination of both molecular charge and size parameters was not examined yet although controlling these aspects could orient the valorization of EPS.

In this context, this article aims to explore which molecular factors among global charge, molecular weight and chemical composition, are the most implied in the gel formation of sEPS

extracted from aerobic granules. This task will be achieved through EPS fractionation according to their net surface charge using a preparative liquid chromatography. The recovered fractions, exhibiting diversified anionic charges will be analyzed for their gel-forming potential in relation to their elemental composition, molecular weight and charge distributions. These experiments will allow to deepen the knowledge about the key determinants that govern the gelation of sEPS extracted from granular sludge.

## **2 Materials and methods**

### **2.1 Biomaterial and sEPS extraction**

Aerobic granular sludge was recovered from sequencing batch reactors fed with 100%-VFA synthetic wastewater (equal COD of acetate and propionate) as described in Layer et al. (2019).

The extraction protocol detailed in Bou-sarkis et al. (2022) was used for the extraction of sEPS. Briefly, the extraction protocol consisted of rehydrating the freeze-dried granules for 30 minutes in 0.2 M sodium carbonate, followed by a homogenization step using an ultra-turrax and a heating phase at 80 °C for one hour. After centrifugation at 14 000 g for 15 minutes, the supernatant was recovered and an acidic precipitation step was done through the addition of HCl to reach pH 2 as described by Lin et al. (2010). The pellet obtained after the acidic precipitation step was solubilized in sodium hydroxide 0.1 M, dialyzed for 36 hours, and freeze-dried. The obtained powder was dissolved in 0.1 M sodium-hydroxide and named sEPS extract.

### **2.2 Preparative liquid chromatography**

#### **2.2.1 Fractionation according to the net surface charge**

The fractionation of structural EPS was performed using an AKTA purifier system (Cytiva) equipped with an on-line spectrophotometer. The used column was the 6 mL Resource™ Q (Cytiva) filled with a cationic stationary phase grafted with quaternary ammonium allowing the separation of polymers according to their charge density and surface charge distribution. Equilibration of the column was done using Tris HCl 50 mM at a pH of  $12 \pm 0.02$ , and elution was performed by sequential addition of NaCl 1 M, so to obtain five steps corresponding to various NaCl concentrations in the elution buffer. A solution of sEPS with a VS content of 0.2% (w/v) was injected (5 mL) and the flow was set to 1 mL/min during the initial step (0 M NaCl)

to allow optimal fixation of negatively charged polymers on the stationary phase. Then, the flow speed was increased to 2 mL/min for the following steps. Molecules were detected by monitoring the absorbance at 280 nm (mAU). The chromatograms were divided into five regions corresponding to the NaCl concentration of the elution buffer: Cat/Neut for Cationic/Neutral (0 M NaCl), LAC for low anionic charge (NaCl 0.25 M), MAC for medium anionic charge molecules (NaCl 0.5 M), HAC for high anionic charge molecules (NaCl 0.75 M) and VHAC for very high anionic charge (NaCl 1 M) molecules. The peak area (mAU\*mL) was processed using the UNICORN 5.31 software. All the fractions were collected, and the fractions belonging to the same step were pooled together and dialyzed for 36 hours against distilled water using a dialysis membrane with a cutoff of 3500 Da (Spectrum labs). Afterwards, the dialyzed fractions were freeze-dried and used for further analysis.

An injection of the Cat/Neut fraction (250  $\mu$ L) was done in the cationic exchange chromatography HiScreen™ Capto™ S Impact (Cytiva). The sample was solubilized in NaOH 0.1 M to obtain a VS concentration of 0.4 % (w/v). The elution protocol and the used detection were the same as the ones described for the anionic exchange chromatography.

### 2.2.2 Size exclusion chromatography

The fractions collected after anionic chromatography were solubilized in 0.1 M sodium hydroxide so to obtain solutions at a VS concentration of 1% (w/v) and 200  $\mu$ L were mixed either with 200  $\mu$ L of calcium at 0.2 M or with 200  $\mu$ L of distilled water. After an hour, both samples (with and without calcium) were centrifuged 15 min at 14 000 g to collect the supernatant for further analysis with the size exclusion chromatography column SUPERDEX 200. According to the manufacturer, this column separates molecules having apparent molecular weights (MW) between 10 and 600 kDa (Cytiva). The injection volume was set to 100  $\mu$ L and the mobile phase was Tris HCl 50 mM with 0.1 M NaCl, pH  $12 \pm 0.02$  with a flow of 0.5 mL/min. According to their elution volume and apparent size, the peaks composing the chromatograms were divided into three regions: HMW for high molecular weights that were eluted first between 7.7 and 12 mL, MMW for medium molecular weights eluted from 12 to 17 mL and LMW for low molecular weights that were eluted lastly after 17 mL. Molecules were detected by monitoring the absorbance at 280 nm (mAU). The peak area (mAU\*mL) was processed using the UNICORN 5.31 software.

Comparison of the chromatograms obtained with or without calcium allowed to evaluate the % of reactivity to calcium in each MW category using equation (1):

$$MW \text{ Reactivity } (\%) = \frac{\text{Reactive area} * 100}{\text{Total area without calcium}} = \frac{(\text{Total area without calcium} - \text{total area with calcium}) * 100}{\text{Total area without calcium}} \quad (1)$$

### 2.3 Test for gelation capacity

The test previously described by Bou-Sarkis et al. (2023) was used to compare the gelation capacity of the freeze-dried sEPS extract and collected fractions using solutions at final VS concentrations of 1% (w/v). As described in supplementary data (Figure IV-S1), 100  $\mu$ L of the tested sample at 2% was mixed either with 100  $\mu$ L of calcium at 0.2 M or distilled water. After 1 hour, both samples (with and without calcium) were centrifuged and the volume of the supernatant was measured; therefore the gel volume ( $V_g$ ) was deduced and the gel volume percentage (% $V_g$ ) was calculated using Equation (2):

$$\% V_g = \frac{(V_t - V_s) * 100}{V_t} \quad (2)$$

Where  $V_t$  is the initial total volume ( $\mu$ L), and  $V_s$  the liquid volume ( $\mu$ L) after calcium addition (supernatant after centrifugation). Then, UV-vis spectra between 220 and 500 nm were monitored for both samples (with and without calcium) to determine the percentage of reactivity (% $R$ ) of the sample through equation (3):

$$\% R = \frac{(V_t * AIS - V_s * ACaS) * 100}{V_t * AIS} \quad (3)$$

Where AIS is the cumulated area of the initial spectrum without calcium (AU.nm), ACaS is the cumulated area of the spectrum with calcium (AU.nm). Both factors, i.e. % $V_g$  and % $R$  were combined to obtain a gelling factor (GF) (equation (4)):

$$GF = \%R * \%V_g \quad (4)$$

### 2.4 Fourier Transform Infrared (FTIR) spectroscopy

Following the gelation procedure of the sEPS extract, the three types of samples corresponding to the gel pellet, the liquid supernatant, and the initial sEPS were freeze-dried, diluted at 3% (w/w) final in KBr and submitted to FTIR using a spectrometer (PerkinElmer) equipped with a Diffuse Reflectance Infrared Fourier Transform (DRIFT) module. The resolution was set to 1  $\text{cm}^{-1}$  and a total of 32 scans were done on each sample. A mathematical

model was applied on all the spectra using the Spectrum 65 software (Kubelka Munk) and the spectra were normalized according to the total surface of the peaks.

## 2.5 Biochemical composition

Triplicates of fractionated sEPS solutions with a final VS concentration of 0.1 % (w/v) without or with 0.1 M calcium, were prepared in NaOH 0.1 M. After 1 hour, the samples were centrifuged for 15 min at 14 000 g. The supernatants were dialyzed against distilled water for 6 hours and then against NaOH 0.05M for another 6 hours to remove residual calcium. The dialyzed samples were assayed for their biochemical composition using different colorimetric tests. All samples were assayed in triplicates in 96 well microplates, using the FLUOstar OPTIMA (BMG, Labtech) microplate reader. Comparison of the concentrations determined in the solutions with or without calcium allowed to evaluate the % of reactivity with calcium of each biochemical group (BG) using equation (5):

$$BG \text{ reactivity } (\%) = \frac{\text{Reactive BG} \times 100}{\text{Concentration without calcium}} = \frac{(\text{Concentration without calcium} (\frac{mg}{L}) - \text{Concentration with calcium} (\frac{mg}{L})) \times 100}{\text{Concentration without calcium} (\frac{mg}{L})} \quad (5)$$

### 2.5.1 Neutral and uronic-acid like sugars quantification

The Anthrone method used for the quantification of neutral and uronic acid-like sugars was adapted from Rondel et al. (2013). The standards used for neutral sugars and uronic acids are glucose (0 to 75 mg/L) and glucuronic acid (0 to 750 mg/L) respectively. The Anthrone reagent was prepared at 2 mg/mL in sulfuric acid 96% (v/v) and 150  $\mu$ L of this reagent were added to 75  $\mu$ L of samples/standards in 96 well microplates. Incubation at 60 °C for 30 minutes followed by cooling at room temperature for 10 minutes was done. The Anthrone reagent gives chromogenic compounds depending on the nature of osidic monomers (neutral or uronic) and the absorbances were read at 544 and 620 nm. The slopes of the four equations linking the standard solutions with glucose or glucuronic acid concentrations were calculated. Afterwards, the concentrations of the unknown samples can be calculated by solving a system of two equations as described in Rondel et al. (2013).

### 2.5.2 Proteins and humic acid quantification

The modified Lowry assay was used for the quantification of proteins and humic acid-like substances according to Frølund et al. (1995) and the used standards (0-500 mg/L) were respectively BSA (Sigma-Aldrich) and humic acid (Fluka-Aldrich). The measurement of absorbance was done at 750 nm, with or without CuSO<sub>4</sub>. The measurement of absorbance

was done at 750 nm, with or without CuSO<sub>4</sub>. With CuSO<sub>4</sub> both proteins and humic acids absorb, however when CuSO<sub>4</sub> is omitted, the color development is due mainly to humic substances and chromogenic amino acids, and BSA color development decreased to 20% but no decrease was observed for humic acids. The respective concentration of proteins and humic acid-like molecules can be calculated by using the equations described in Frølund et al. (1995).

### **2.5.3 DNA quantification**

The Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen) was used for DNA quantification. DNA from phage Lambda (100 µg/mL) was used for the DNA standard curve going from 0 to 2000 ng/mL. In a 96 well microplate 100 µL of the reagent prepared according to the manufacturer were added to 100 µL of standards or samples. Fluorescence intensity was measured at an excitation emission wavelength pair of 485/520 nm.

### **2.6 Elemental analysis**

The freeze-dried fractions collected during anionic chromatography were analyzed for their content of C, N, H and S using the flash2000 (ThermoFisher scientific) instrument and their oxygen content was analyzed using the NA2100 Protein (CE instruments) so to obtain the percentage of each element in the studied fractions. The samples were compared to Alginate with low viscosity from brown algae (Sigma-Aldrich), and BSA (Sigma-Aldrich).

## **3 Results and discussions**

### **3.1 What is the FTIR chemical fingerprint of the sEPS involved in gelation?**

A global biochemical fingerprint of complex samples can be obtained through the analysis of FTIR spectra. This technique was used to get a glimpse of the biochemical groups participating in the gelation of EPS. FTIR analysis was performed on the three samples obtained after calcium addition to the solubilized sEPS: the gelled pellet, the non-gelled supernatant along with the initial sEPS extract (Figure IV-1).

**Table IV-1: FTIR band assignments (Campo et al., 2022; Cheah and Chan, 2022; Karakas et al., 2020; Lin et al., 2010), with the observed peaks in the gel pellet, supernatant and structural EPS.**

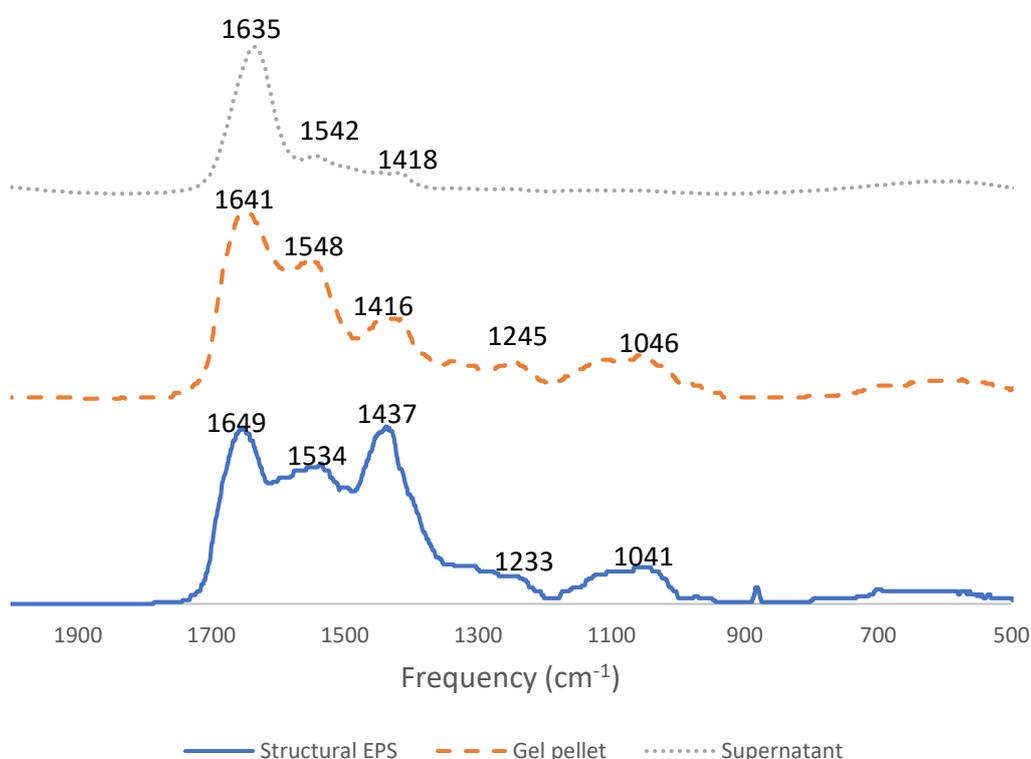
Wavenumber $\text{cm}^{-1}$	Assignment	Wavenumber ( $\text{cm}^{-1}$ ) detected in the:		
		Structural EPS	Gel pellet	Supernatant
1600-1700	Amide I region of proteins	1649	1641	1635
1500-1600	Amide II region of proteins	1534	1548	1542
1400-1500	Carboxyl groups, hydrocarbon-like molecules	1437	1416	1418
1200-1300	Amide III region of proteins	1233	1245	-
1000-1200	Polysaccharides C-O-C, C-O	1041	1046	-

As seen in Table IV-1, the amide I and amide II region of proteins were visible in the three samples. As reported by Campo et al. (2022), the observed shifts of amide I and amide II peaks between samples could be attributed to cross-links between proteins and calcium. Moreover, it was discussed that the peaks in these regions could be attributed to amino-sugars and N-acetylated derivatives. The presence of glycosylated amyloids and sulphated glycosaminoglycans were also attributed to these regions and the possibility of their involvement in the gelation was previously elucidated for sEPS extracted from aerobic granular sludge (Campo et al., 2022). Concerning the amide III region between 1200 to 1300  $\text{cm}^{-1}$ , it was detectable in the sEPS sample and within the gel pellet but not in the supernatant remaining after gelation. These results indicate that the extracted proteins participate in the hydrogel formation and are in agreement with the model of the granular structure proposed by Caudan et al. (2014). In this model, anionic proteins may constitute some intercellular cement playing a major role in the cohesion of the aerobic granule through cationic bridging with calcium.

Carboxylic groups and hydrocarbon-like molecules were also detected between 1400 and 1500  $\text{cm}^{-1}$  in the initial sEPS and in the gel and peak shifts were noted between the FTIR profiles. These peaks can be attributed to the very diversified monosaccharides composing sEPS including glucosamine, galactosamine and seven other neutral sugars (Felz et al., 2019). In addition, the detection of carboxyl groups in this region may be due to the presence of uronic acids (Lin et al., 2010). Therefore, the presence of these groups in the gel pellet was expected; however, a small fraction remained in the supernatant after calcium addition. Finally, polysaccharides, extending at 1041  $\text{cm}^{-1}$  in the sEPS extract (Lin et al., 2010), got

transferred into the gel pellet (at  $1046\text{ cm}^{-1}$ ) after calcium addition, confirming the polysaccharidic nature of some gel-forming EPS.

Although FTIR confirmed definitively that proteins and polysaccharides both participate in the formation of sEPS hydrogels, the FTIR signature of the sEPS gels is also compatible with the involvement of more complex composites such as glycoproteins or hetero-polysaccharides. However, FTIR approach remains not enough resolutive and quantitative and further purification of structural EPS is needed in order to better identify the physico-chemical characteristics of the molecules that are the most implied in the gel formation.



**Figure IV-1: FTIR spectra of the sEPS extract without calcium, the gel pellet and the supernatant after the addition of calcium.**

## 3.2 What is the favorable charge for EPS gelation?

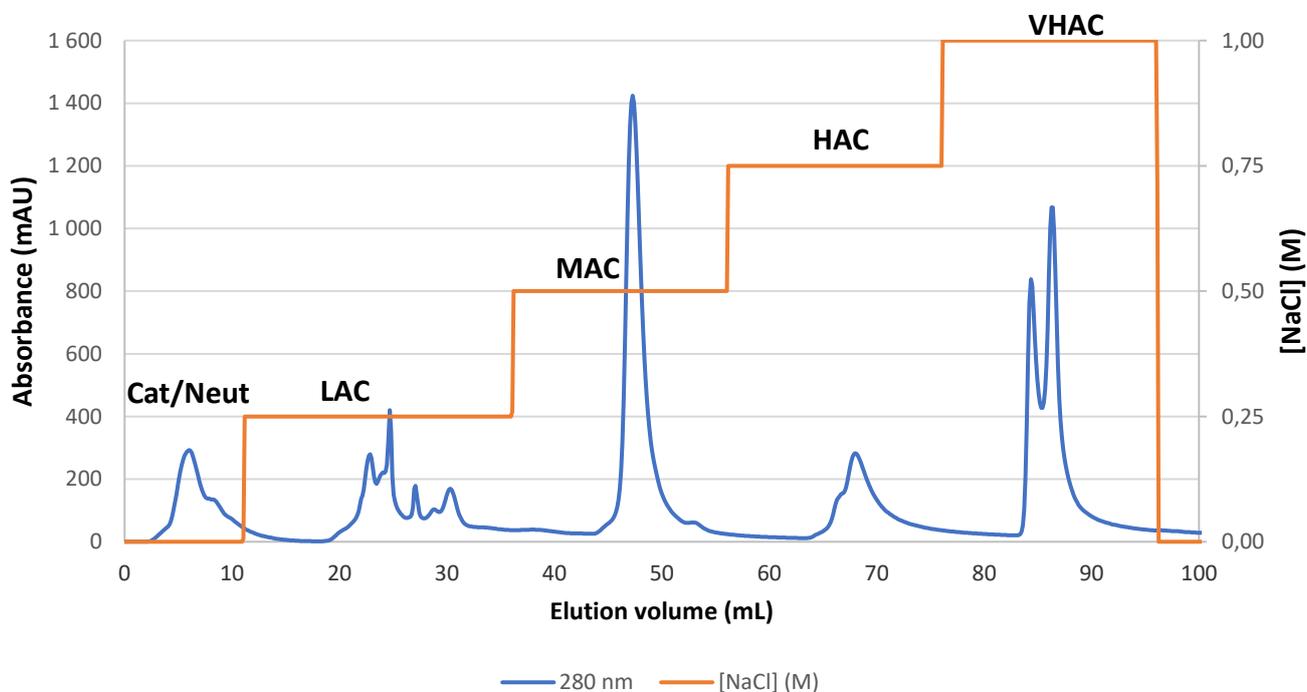
### 3.2.1 Ionic fractionation

sEPS extracted from granules were separated according to their net surface charge using anionic exchange chromatography. As shown in Figure IV-2, the sequential elution strategy allowed to clearly separate groups of molecules with a different negative surface charge thanks to the stepwise elution with increasing NaCl concentrations from 0 to 1 M. The eluted EPS were divided into five categories going from the cationic/neutral (Cat/Neut) that

corresponds to unbound molecules to the very high anionic charged (VHAC) molecules that were eluted with the highest NaCl concentration. To further explore the charge profile in the Cat/Neut group, this fraction was injected in a cationic exchange chromatography column to determine if it contains cationic, neutral molecules or a combination of both. The obtained chromatogram showed that this fraction could not bind at all to the negatively charged column (supplementary data Figure IV-S2) suggesting that it is composed of neutral polymers.

Upon quantification of the peak areas, it can be noticed that the majority of molecules present within the initial EPS extract ( $91.5 \pm 5\%$ ) are negatively charged in the tested conditions. In agreement, EPS extracted from aerobic granules with a different multi-method protocol (mechanical and chemical) but fractionated with similar anionic exchange chromatography were also found as mainly negatively charged since approximately 75% could be adsorbed on the cationic resin (Caudan et al., 2012).

Moreover, this technique was used for the enrichment of sulfated glycosaminoglycans which are highly negatively charged polymers allowing the presence of chondroitin- and heparan/heparin-like polymers to be elucidated in sEPS extract of aerobic granules (Felz et al., 2020b).



**Figure IV-2: Chromatogram showing the different fractions collected during structural EPS fractionation using anionic exchange chromatography.** The shown chromatogram is obtained through the mean of 10 separate injections. Cat/Neut=cationic/neutral molecules, LAC= low anionic charge molecules, MAC= medium anionic charge molecules, HAC= high anionic charge molecules and VHAC= very high anionic charge molecules.

### 3.2.2 Elemental analysis

The fractions collected after the anionic exchange chromatography were then analyzed for their content in C, H, N, S and O (Table IV-2). The Cat/Neut molecules and the HAC polymers eluted with NaCl 0.75 M have the lowest content of nitrogen being 8.5 and 7.0% respectively. The presence of nitrogen in these fractions could be attributed to the presence of aminosugars as it is the case for granulan which contains glucosamine and galactosamine (Seviour et al., 2012a). Interestingly, the Cat/Neut fraction has a S content equal to 2.6% that is higher than for the other groups. This suggests the presence of sulfate groups provided by other molecules than proteins since the globular model protein BSA, that contains a high ratio of 35 cysteine and 4 methionine residues upon 583 amino acids, exhibits a lower S content of 1.6 %. Indeed, analysis by a specific colorimetric assay has revealed the presence of sulfated GAGs in alkaline extracts recovered either from aerobic or anammox granules (Boleij et al., 2020; Felz et al., 2020b). In the same way, the low N content value of 8.5% in this Cat/Neut fraction may be attributed to hexosamine rather than proteins that are usually considered to have a N content ratio around 16% (Biancarosa et al., 2017). In consequence, the C/N ratio in the Cat/Neut group is equal to 4.7 and is much higher than the C/N ratio of the model protein BSA reaching 3.3. However, sulfated GAGs are considered as highly negative polymers and their presence in the fraction that was unable to bind with the positively charged quaternary ammonium is hard to explain at this stage.

Comparing the elemental composition of alginate with the ones of the fractionated groups shows that there is a lot of difference between them. The sample having the closest content of C equivalent with alginate is the HAC sample, however similarly to other tested samples its content of oxygen is quite different from that of alginate. Globally these results show that none of the fractions has a similar elemental composition as that of alginate and suggest that sulfate-containing molecules other than proteins are components of the sEPS.

**Table IV-2: Elemental analysis of the different fractions collected after anionic exchange chromatography, in comparison with alginate and BSA.**

Fractions (NaCl)	% N	% C	% H	% S	% O	C/N
Cat/Neut (0)	8.5	40	5.7	2.6	23.9	4.7
LAC (0.25M)	10.7	40.2	6.3	1.4	22.4	3.8
MAC (0.5 M)	12.1	47.2	7.1	0.7	24.3	3.9
HAC (0.75 M)	7	31.2	4.6	0.5	16.4	4.5
VHAC (1M)	7.4	23.7	4.8	1.5	22.17	3.2
Alginate	-	33.1	4.8	0.06	43.2	-
BSA	15	49.9	7.1	1.6	27	3.3

*Cat/Neut=cationic/neutral, LAC= low anionic charge, MAC= medium anionic charge, HAC= high anionic charge and VHAC= very high anionic charge molecules.*

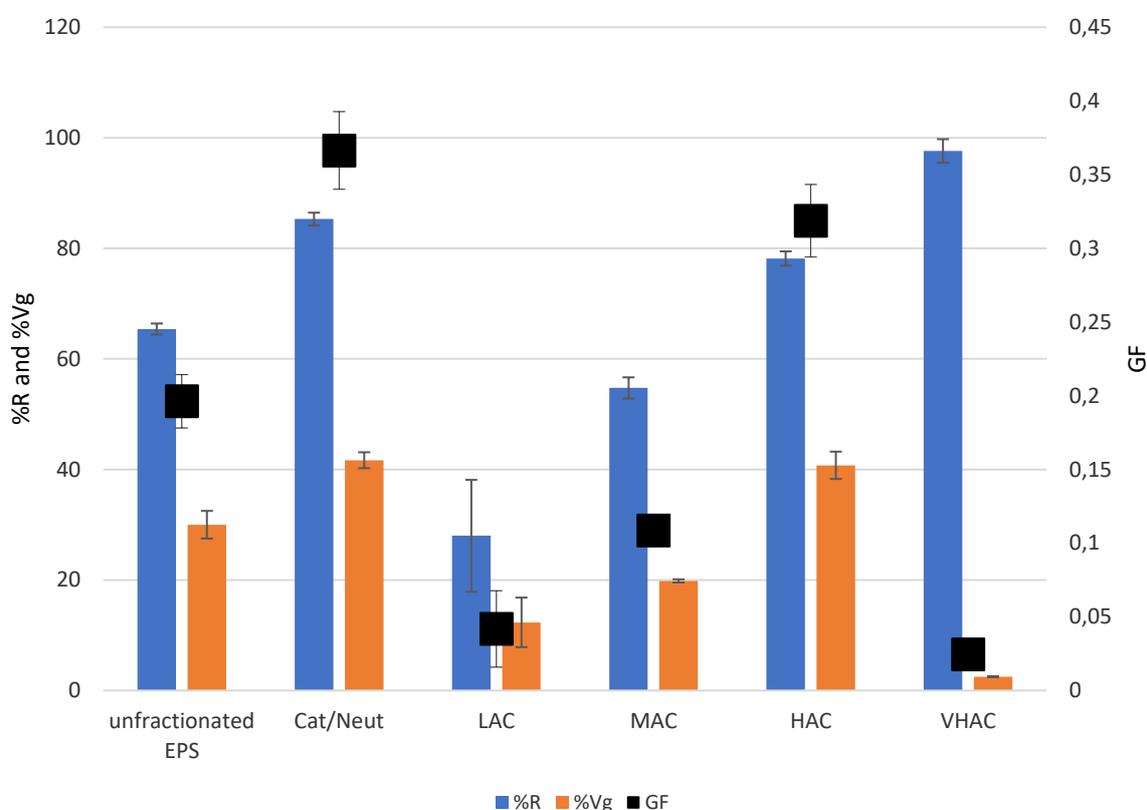
### 3.2.3 Gelling capacities

The collected fractions were analyzed for their gelling capacity and results can be seen in Figure IV-3. The unbound Cat/Neut group exhibited the highest GF at  $0.37 \pm 0.03$  due to a very good reactivity to calcium since more than 80% of the molecules were entangled in a gelled structure that represents 42 % of the total initial liquid volume. Reacting with calcium requires the presence of negatively charged functional groups. It was hence hypothesized that the Cat/Neut fraction is obtained through the aggregation of negatively charged polymers with positively charged ones and therefore lead to the formation of neutral structures. Both the presence of sulfated groups and the C/N ratio (Table IV-2) support the presence of GAGs-like polymers in this fraction, polymers that were previously reported as components involved in the cohesiveness of some granular structures (Boleij et al., 2020; Felz et al., 2020b). In another way, granulan can also be suspected as a component of this fraction since its complex structure allows the formation of an anti-parallel double helix conformation stabilized by hydrogen bonds between amine and carboxylic groups and/or by divalent cation bridging and thus leading to globally neutral aggregates (Seviour et al 2012). Alternatively, the association of polymers with remaining positively charged ions (i.e. sodium ions) that neutralized the charges might also explain their elution at this stage.

Concerning the negatively charged polymers, the GF increases with charge from  $0.04 \pm 0.03$  for LAC to  $0.32 \pm 0.02$  for HAC group, indicating that the gelation capacity increases with the net negative charge. However, upon reaching a very high negative charge (VHAC group), the gelation capacity decreases abruptly and reaches  $0.02 \pm 0.01$ . In the latter case, the high %R coupled to the low %Vg could be an indication of a precipitation instead of a gelation process

(Bou-Sarkis et al., 2023). The presence of very highly negatively charged polymers could hinder the hydrogel formation capacity due to an increased repulsion of polymers as described for carrageenan hydrogels (Liu and Li, 2016; Morris, 1979).

Upon comparison with the GF of the initial unfractionated sEPS ( $0.19 \pm 0.02$ ) some fractions exhibited a better gelling capacity up to  $0.37 \pm 0.03$  for the Cat/Neut sample and  $0.32 \pm 0.02$  for the HAC sample. This means that the fractionation according to charge has allowed to concentrate gel forming polymers in the Cat/Neut fraction as well as in the HAC fraction that constitute the most favorable charges of EPS for the formation of ionic hydrogels.

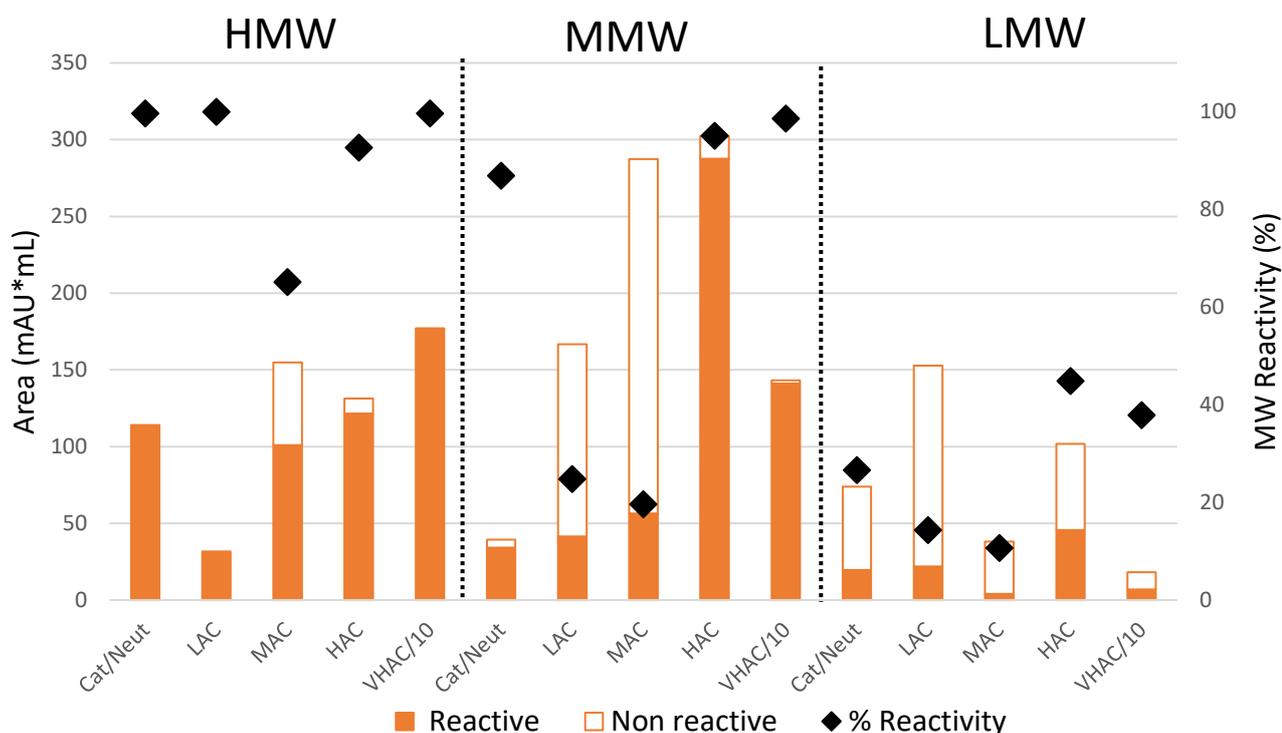


**Figure IV-3: The effect of net surface charge on the capacity of gelation for EPS fractions: Cat/Neut=cationic/neutral molecules, LAC= low anionic charge molecules, MAC= medium anionic charge molecules, HAC= high anionic charge molecules and VHAC= very high anionic charge molecules.**

### 3.3 Does the molecular weight of EPS affect their gelation capacity?

SEC was performed on the fractions collected after anionic chromatography and also on the supernatants remaining after their gelation. This allows to establish the molecular weight profiles of the gelling polymers that were divided into High, Medium or Low Molecular weight presented in Figure IV-4 along with the % MW Reactivity. This MW Reactivity corresponds to the proportion of molecules that have disappeared from the supernatant in each MW

category due to their reaction with calcium and the remaining molecules that didn't react with calcium constituted the non-reactive part. No matter what the net negative surface charge is, the HMW group always participates in the gelation with a percentage of MW Reactivity going from 65% for the MAC fraction to values as high as 100% for the LAC fraction (Figure IV-4). On the contrary, participation of MMW molecules to the hydrogel formation is dependent on the net negative surface charge. Indeed, for the LAC and MAC, the % of MW Reactivity is below 25% while for the fractions with high negative charge (HAC and VHAC), the participation of MMW is above 95%. The Cat/Neut fraction has a percentage of MW Reactivity for MMW equal to 87%, supporting the hypothesis that this fraction contains aggregates of charged polymers. When it comes to LMW molecules, their participation is always limited, reaching 27%, 14% and 11% respectively for Cat/Neut, LAC and MAC and around 40% for HAC and VHAC.



**Figure IV-4: Molecular weight groups participation in the gelation of EPS according to their charge during anionic exchange chromatography elution (Cat/Neut, LAC, MAC, HAC, VHAC).** Non-reactive and reactive areas were calculated by comparison of the SEC chromatograms obtained for the initial and the remaining solution after gelation. This allows to evaluate the % of MW Reactivity of each molecular weight group. The VHAC sample peak areas were divided by 10 for visual representation.

According to the reactive areas presented in Figure IV-4, the Cat/Neut, MAC and VHAC groups hydrogels are mostly formed by HMW molecules, whereas for the LAC and HAC hydrogels, it is the MMW molecules that mostly participate to the hydrogel.

It was previously shown that the gelation behavior could be impacted by the molecular weight of the gelling polymers. Fernández Farrés and Norton (2014) have compared two samples of alginate with contrasted molecular weights but having the same guluronic acids content, the latter, being, *a priori*, a driver for gelation. They observed that the formation of mono complexes with calcium is privileged for high molecular weight polymers because of higher flexibility allowing the polymer to contract/shrink more freely. In addition, the molecular weight impacts the mechanical properties of the formed hydrogels: the higher the molecular weight, the higher the gel's stiffness is (Draget et al., 1994; Kong et al., 2002; Labowska et al., 2021; Lee and Mooney, 2012). Since, it was reported that the drastic alkaline and heating treatments used for the extraction of sEPS can cause their degradation (Bou-sarkis et al., 2022); therefore attention must be paid to keep the highest proportion of molecules in their native size in order to improve their participation in the hydrogel network.

### **3.4 Which biochemical groups participate in EPS gelation?**

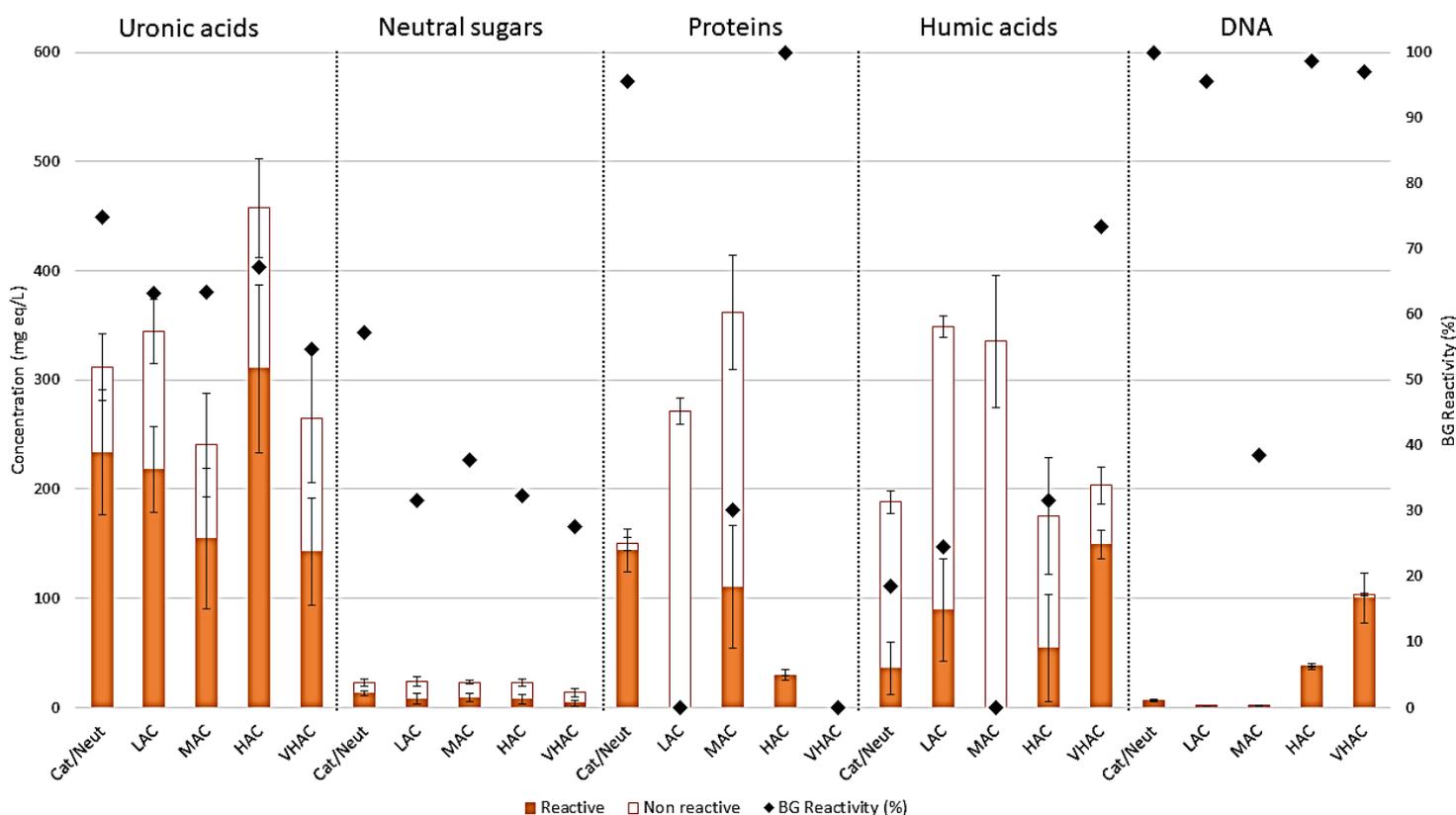
The fractions collected after anionic chromatography and also the supernatants remaining after their gelation were analyzed for their biochemical diversity. The molecules assayed in the supernatants after gelation were considered as non reactive, thus allowing to evaluate the reactive ones (Figure IV-5). Finally, this allows to establish the percentage of reactivity of each biochemical group (BG Reactivity) involved in the formation of the hydrogel after calcium addition on the various charged fractions.

As shown in Figure IV-5, all the gels exhibited the presence of all the tested biochemical groups, except the VHAC gel in which proteins were undetectable. On the other hand, this VHAC gel appeared to be particularly rich in DNA compared to the others fractions. Globally, it can be seen that uronic acids are those that participate the most in the formation of the hydrogels in terms of quantity and their BG Reactivity toward calcium was around 60% for the five fractions (Figure IV-5). This data confirms the structural involvement of uronic acids in calcium induced EPS gelation observed by Schambeck et al. (2020): indeed, once the hydrogels are formed, if subjected to an enzymatic digestion step by alginate lyase, they lose their structure which is a clear indication of the participation of uronic acids in the formation of EPS hydrogels. Neutral sugars are very little represented in the gel composition and their presence as reactive molecules with calcium is probably due to the complex sequence of some bacterial

polysaccharides built with alternating neutral and anionic sugars which is the case of granulan (Seviour et al., 2012a).

Proteins participate in the formation of the hydrogels for three out of the five fractions since they were undetectable in the VHAC fraction and were not quantitatively represented in the LAC hydrogel. In this study, granules were solubilized in sodium carbonate during 1h at 80 °C, and this may favor the denaturation of some proteins, enhancing their gel formation capabilities in the presence of divalent cations (Navarra et al., 2009; Sandra et al, 2012). Depending on the fractions, the participation of proteins in the hydrogel formation varied: Cat/Neut and HAC samples exhibit an almost total reactivity but the MAC fraction shows a reactivity of 30.1 %. The differences in reactivity observed for proteins are not really correlated with their global charge suggesting that other gelation mechanisms based on molecule/molecule interactions may have occurred such as hydrogen bonds and hydrophobic interactions (Deshmukh et al., 2017; Wang et al., 2017).

Humic acid like substances also participate in the formation of the hydrogel and their reactivity is more important in the VHAC group (73.4 %) than in others fractions (< 35 %). However, it was proven elsewhere that humic substances tend to form aggregates with calcium instead of gels (Kloster et al., 2013). Although they tend to form aggregates, they can possibly influence the formed gel properties. Indeed, Venezia et al. (2022), showed that humic acids can modulate the gel properties of gelatin depending on their concentration in the medium.



**Figure IV-5: Biochemical group participation in the gelation of sEPS according to their charge during anionic exchange chromatography elution (Cat/Neut, LAC, MAC, HAC, VHAC).** Non reactive and reactive subsets were calculated by comparison of the quantification of each biochemical group (Uronic and neutral sugars, proteins, Humic acids, DNA) in the initial and remaining solutions after gelation. This allows to evaluate the % of BG Reactivity for each biochemical group. Analytical (3 assays) and biological (3 gel formations) replicates were performed to get the means and the associated standard errors were represented as bars.

Lastly, DNA is more present in the HAC and VHAC gels than in other fractions and this agrees with the presence of phosphate groups that confer significant negative charges to these molecules. Interestingly, almost the totality of DNA molecules assayed in the initial solutions react with calcium, except for the MAC sample where only 40% of the DNA molecules have disappeared from the liquid supernatant. DNA is a polyanionic polymer and DNA hydrogels have been reported in literature and can be obtained using various method including interactions with calcium ions (Bou-Sarkis et al., 2023; Kim et al., 2022; Li et al., 2019; Wang et al., 2018).

## 4 Conclusions

This study provides new results on the process involved in ionic-induced gelation of the complex sEPS mixture extracted from aerobic granules. It was shown that having a negative

net surface charge increases the polymers capacity to form hydrogels whereas upon reaching a very high negative charge, this capacity could be decreased due to increased repulsion between polymers. In addition, it was proven that the high molecular weight fractions are those that have the highest reactivity and participate efficiently in the formation of hydrogels. The participation of different biochemical groups in the formation of hydrogels was shown. In terms of quantity, uronic sugars and proteins are both major constituents of the formed hydrogels and thus have both an important role in gelation. Interestingly, a subset of sEPS exhibiting 2.6% in S content and a 2 times higher gelation capacity than the initial unfractionated sEPS extract can be isolated by anionic exchange chromatography.

Therefore, a fractionation based on anionic exchange seems relevant to collect fractions enriched in gel-forming polymers such as the HAC and Cat/Neut fractions. Additional strategies to enhance the gelation of sEPS can include the elimination of LMW or the preservation of the native molecular weight during the extraction steps.

## 5 Supplementary material

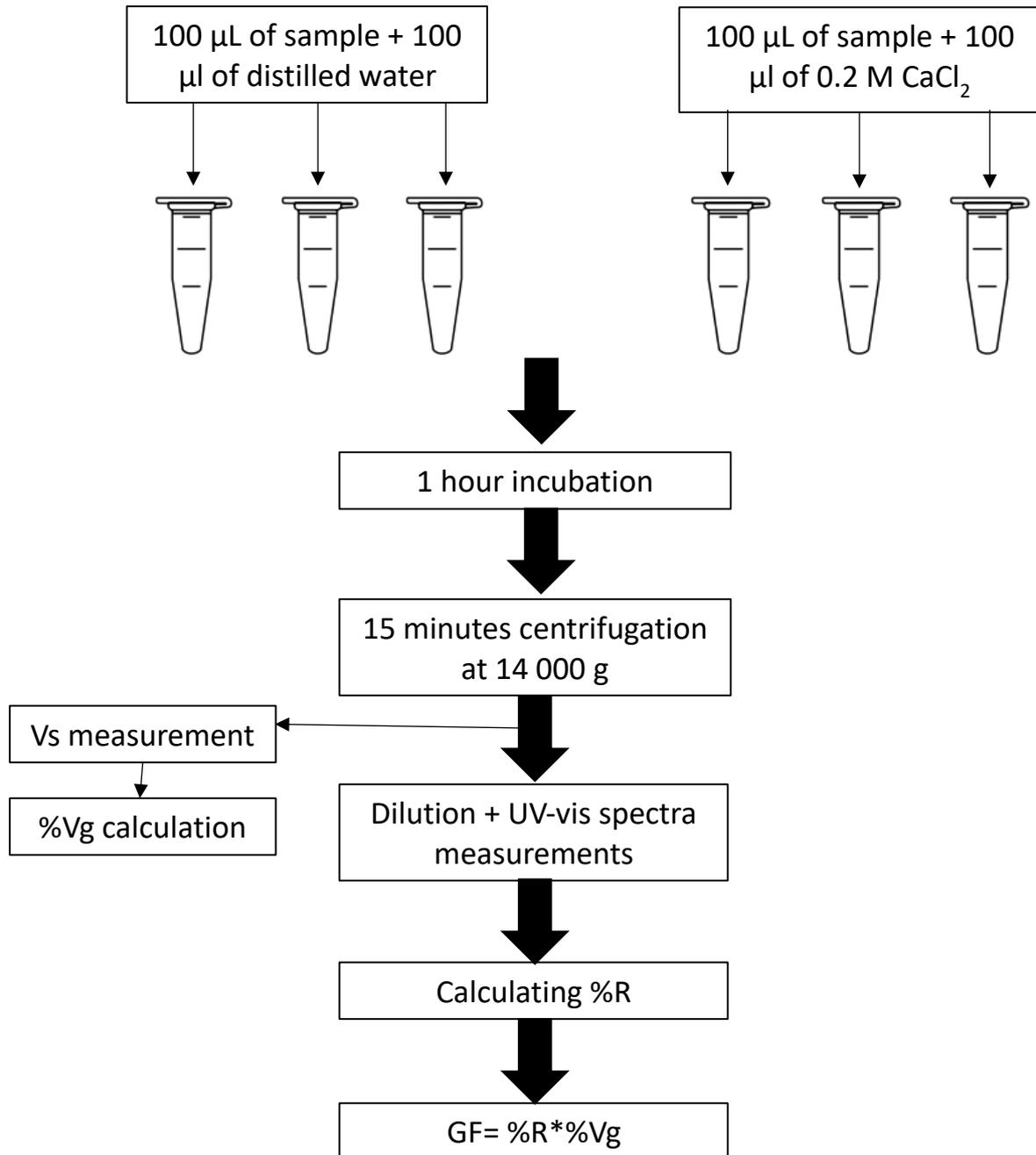


Figure IV-S1: gelation detection through a miniaturized screening method as adapted from (Bou-Sarkis et al., 2023).

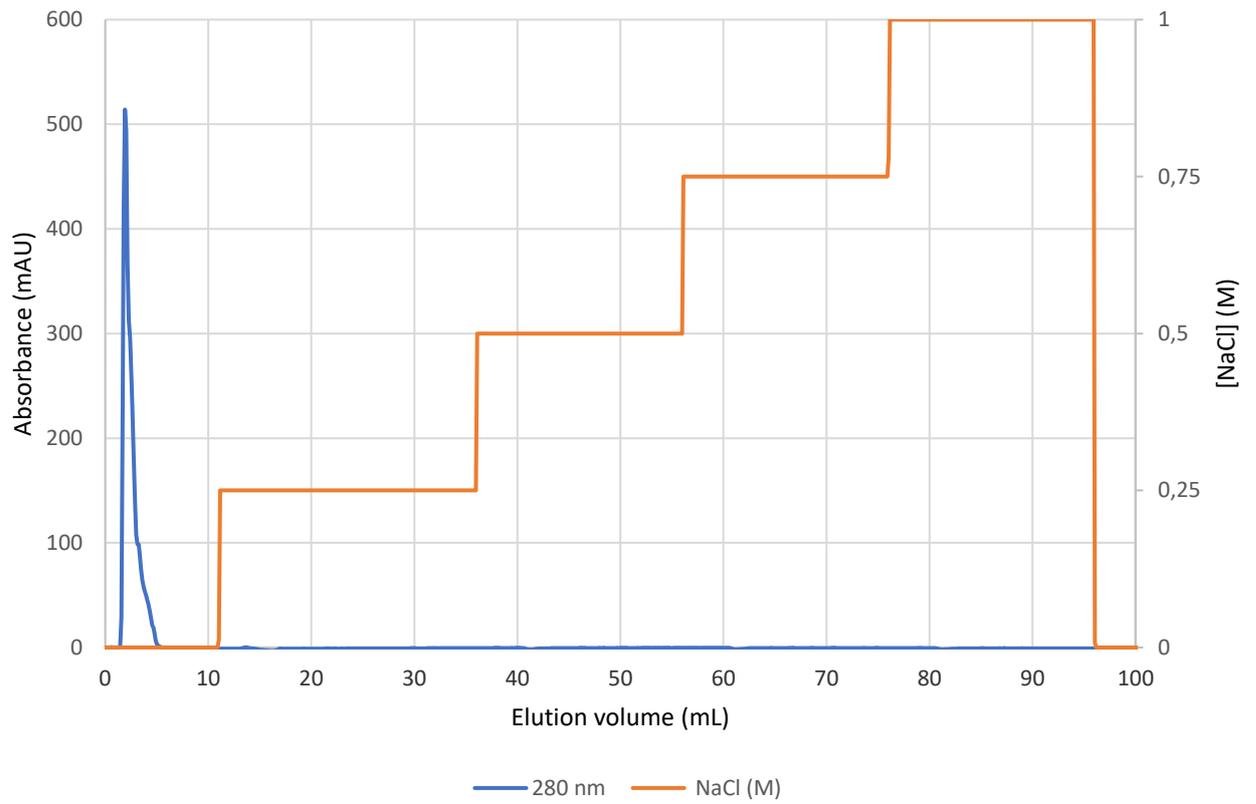


Figure IV-S2: Cationic exchange chromatography for the Cat/Neut fraction.

## 6 Conclusions of chapter IV

As previously seen in the literature review, the sEPS biochemical composition is very complex. In addition, the physico-chemical and molecular properties of the molecules forming the EPS matrix are quite diversified. Therefore, understanding interactions that are likely to occur within this matrix requires efforts to separate molecules and studying them independently. This was achieved through the fractionation of sEPS using anionic exchange chromatography with the goal of understanding the effects of molecular charge on the gelation capacity. The results showed a correlation between the global negative charge of polymers and their gelation capacity. However, upon reaching a very high anionic charge, the capacity to form hydrogels decreased drastically and this observation was attributed to excessive negative charges possibly hindering gelation but such hypothesis remains to be verified. The two fractions that showed the highest GF were the Cat/Neut and HAC fractions. Although the global net charge of the Cat/Neut fraction was proven to be neutral, unexpectedly, it showed the best capacity to form hydrogels with calcium. This observation is somewhat surprising, since forming hydrogels with calcium requires negatively charged polymers. Thanks to the data on the elemental composition showing the presence of sulfate groups in this fraction, it was hypothesized that this fraction might be composed of polymers that aggregated together through their positive and negative charges to form neutral aggregates. Upon addition of calcium, the negatively charged polymers present within this structure could dissociate from the positively charged polymers to form hydrogels with calcium. Further analysis on the chemical nature of the building blocks composing this fraction is needed to confirm the presence of sulfated monomers.

The collected fractions were also analyzed for their size distribution and the participation of each molecular size group in the gelation was determined. The results showed that low molecular weight molecules tend to participate to a limited extent in the formation of the hydrogels. Medium molecular weight and high molecular weight molecules were the most implied in the gelation. These observations tend to fortify the need of preserving the native molecular weight of gel-forming EPS during the extraction from aerobic granules as discussed in chapter II.

Finally, the analysis of the biochemical groups participating in the gelation was done to detect if only complex polysaccharides are responsible for the gelation or if different biochemical

groups participate in the gelation of EPS as well. Upon addition of calcium, it was found that polysaccharides and proteins both participate in the formation of hydrogels. As seen in the literature review, many proteins have the capacity to form hydrogels especially after undergoing a step of heating causing the denaturation of proteins (Le et al., 2017; Navarra et al., 2009). Heating at 80 °C was applied during the extraction of sEPS, so the participation of proteins to the gelation can be expected even if the mechanism inducing gelation is distinct.

These aspects gave important insights on the hydrogel formation of EPS because they impact many key parameters/decisions in the recovery of gel-forming EPS from aerobic granules. Indeed, understanding the targeted molecules' properties can allow a better recovery of these polymers through more precise extraction methods that consider the preservation of the molecular features responsible for the gelation of polymers.

Finally, a general conclusion of the thesis is proposed, grouping all the findings of the different chapters and the perspectives for future studies are given.

# Chapter V:

## General conclusion and perspectives

Wastewater treatment plants using aerobic granular sludge technology will be part of the future of sanitation due to their capacity to efficiently eliminate pollutants from water while reducing installation costs and energy consumption. Moreover, through the valorization of aerobic granules, wastewater treatment plants can fall within the circular economy concept and be transformed into wastewater resource recovery facilities. This thesis focused on the valorization of aerobic granular sludge through the recovery of EPS with gel-forming properties. In order to improve the impact of this valuation, two main issues have been raised: (i) the influence of the extraction and fractionation process on the composition and properties of the solubilized structural EPS, (ii) the EPS physico-chemical properties and molecular factors that are involved in their gel-forming ability.

The literature review summarized the recent findings in the domain and shed the light on the remaining unanswered questions concerning gel-forming EPS. Depending on the goal of extraction a multitude of protocols may be applied: soft extraction methods such as centrifugation can be applied or harsh methods such as alkaline solubilization coupled with heating can be used for valorization purposes. Concerning gel-forming EPS, alkaline solubilization coupled with heating followed by acidic precipitation is the method of choice. However, there is no consensus on the parameters (solvent concentration, heating time and temperature) of this extraction leading to heterogeneity in the obtained yields. The reasons behind the choices of the parameters of extraction remained unclear and their effects on the molecular level were not addressed previously. Although, these EPS recovered through alkaline extraction followed by acidic and ethanol precipitation were initially named “Alginate Like Exopolysaccharides”, the literature review emphasized the biochemical diversity of EPS extracted from aerobic granules as well as their molecular properties. Indeed, glycosaminoglycans, glycoproteins, amyloids, and many other complex molecules were detected within the EPS matrices of granular sludges. This diversity complicates the characterization of gel-forming EPS and the task of understanding which molecules are responsible for the gel-formation capacity. In fact, gel-forming EPS can be assimilated to mixed

hydrogels that were discussed in the literature review, forming hydrogels through the participation of different molecules. Therefore, with the goal of enlarging our understanding on the effects of extraction methods on the gel-forming methods as well as understanding which physico-chemical properties are responsible for the gel-formation capacity of EPS, four following questions are discussed in this conclusion section.

1. Does the choice of solvent affect the molecular characteristics of the extracted gel-forming EPS?

Chapter II compared four different solvents' capacity to extract gel-forming EPS. Urea and sodium chloride were eliminated on the basis of a low yield, whereas sodium hydroxide and sodium carbonate were analyzed furthermore. As can be noticed, sodium hydroxide used at 80 °C allowed a better solubilization of granules in comparison with sodium carbonate. Those different solvents rely on contrasted mechanisms of extraction: alkaline solubilization, i.e. sodium carbonate or sodium hydroxide, is based on the increase of negative repulsive forces of the charged polymers while urea is known to facilitate the exposure of hydrophobic residues and to break hydrogen bonds. This shows that from one hand the EPS matrix is composed of a majority of negatively charged polymers allowing an efficient extraction with alkaline solvents, and on the other hand it shows that hydrogen bonds do not constitute a major contributor to the granule's cohesion. However, during the enrichment step in anionic gel forming EPS that is done thanks to a pH decrease, the precipitation ability of polymers was influenced by the initial solvent being used for granule disintegration and the yields were inverted. Sodium carbonate showed a better capacity to precipitate gel-forming molecules than sodium hydroxide. Noticeably, our analysis of both the alkaline-solubilized EPS and the acid precipitated ones indicated that this better capacity to precipitate was not attributed to a particular biochemical group and that the precipitation method being used is not selective for a particular biochemical group. On the other hand, signs of degradation were noticeable on the sodium hydroxide extracts that were hypothesized to be the cause of this lower precipitation ability. These findings show that the choice of the solvent impacts the yield as well as the molecular characteristics of the extracted gel-forming polymers.

2. Does the heating temperature affect the integrity and yield of extracted polymers?

Chapter II continued by addressing this question by varying the temperature of extraction for sodium hydroxide and sodium carbonate between 20 °C and 80 °C. Whatever the temperature used, sodium hydroxide always allowed a better solubilization of granules, and sodium carbonate a better precipitation of gel-forming EPS. This effect of temperature was noticeable not only on a quantity aspect but also regarding the molecular weight of recovered molecules. In the case of sodium hydroxide, a global reduction of the EPS size was visible with the increase of heating temperature, whereas it was not the case for sodium carbonate. Moreover, a link between the size of molecules and their capacity to precipitate was established. These findings showed that temperature modulation is an important parameter when extracting gel-forming EPS and special attention must be paid to avoid the degradation of polymers that could influence negatively the yields of precipitated gel-forming EPS.

3. How to follow the enrichment of gel-forming polymers during fractionation?

The biochemical diversity of the acid precipitated EPS proved in chapter II clearly supports further fractionation in order to better understand the gel-formation capacity of these EPS. However, such fractionation requires a relevant process and also a quick test that is capable of detecting the presence of gel-forming molecules in each fraction. Hence, the aim of chapter III was to develop a miniaturized screening method to detect gel-formation. This method, using only between 1 to 3 mg of the tested sample, was developed based on two parameters which are the %R that allows to detect the reactivity of molecules to the calcium addition and %Vg which allows to obtain the volume of the hydrogel formed. Both factors combined together gave the gelling factor GF. The method proved to be efficient at detecting the gelation even in complex mixtures. In addition, the developed method showed the capacity to detect the interactions between molecules. This method can be hence used for lab scale monitoring of gelation or in industrial applications with the goal of valorizing gel-forming EPS. The developed test was applied on EPS extracted using sodium carbonate (0.2M, 80 °C, 1 h). The S1 (raw extract), S2 (solubilized acidic precipitate), and S'1 (supernatant of the acidic precipitation) fractions at a final concentration of 0.5% w/v were used. S1 and S2 fractions were found to contain gelling polymers and on the contrary S'1 did not show any gelling capacity. An enrichment in gelling polymers between S1 and S2 due to the acidic precipitation step was noted. It was shown in chapter III that this acidic precipitation eliminates some

polymers that apparently do not have a gelling potential since the gelling index of S1 was approximately 1.6 times lower than that of S2 attesting the enrichment in gelling polymers in S2. This showed the capacity of the developed test to detect gelation even in complex biological samples.

#### 4. What are the physico-chemical properties of gel-forming EPS?

The fourth chapter aimed at determining some of the key factors influencing the gelation of EPS. After the fractionation of EPS according to their charge, it was noted that the charge affects the gelation capacity of EPS. Indeed, the increase of negative charge caused an increase of the GF. However, two exceptions were present, the Cat/Neut which exhibited a high GF and this could be explained by either an aggregation between positively charged polymers with negatively charged polymers or the presence of counter ions neutralizing the structure. The VHAC fraction exhibited a low GF although it had a very high anionic charge and this is probably due to repulsion between polymers hindering the gelation that might be caused by the presence of DNA. Moreover, it was noted in this chapter that the MW has an impact on the gelation capacity since LMW (with an apparent molecular size under 20 kDa) did not participate efficiently in the gelation. Contrary to the initial postulate based on uronic-dependent gelation, proteins and polysaccharides were shown to participate in the gelation. However, due to this observation, one should consider that complex molecules containing all together amino acids, uronic acids and neutral monomers might also be considered as involved in the gelation process.

All these results, enlarge the scientific community understanding on the gel-forming EPS and allow the development of purification methods targeting the molecules of interest.

Globally this thesis provided a better understanding of the extraction protocol's effects on the gel-forming EPS, a miniaturized method for the detection of gelation, and a more in depth understanding of the physico-chemical characteristics of gel-forming EPS. However, many questions remain unanswered and more work should be done to have a better grasp on the valorization of EPS. Particularly, concerning the extraction parameters such as the couples of heating time/heating temperature, pH/heating, and concentration/type of the solvent should be studied furthermore to detect if they influence the yield and properties of the extracted molecules and their gelling capacity should be addressed with the developed test.

Complementary experiments could clarify the role of proteins in the gelation of EPS and to understand if they are a crucial component of the gelation or not. This can be achieved through an enzymatic digestion of proteins and then applying the gelation capacity test on the digested EPS to check if the GF actually decreases as it should if proteins are key for gelation. The same technique can be used to determine the role of DNA in the gelation especially for the HAC and VHAC samples.

Concerning the developed gelation detection test and its use to understand which molecules participate in the gelation a coupling with fluorescent probes, more specific than an absorbance wavelength, could be beneficial. Synergies between molecules are still to be elucidated, that might be achieved by mixing fractions (Cat/Neut, LAC, MAC, HAC, and VHAC) together in different proportions and checking how the GF evolves. A fractionation based on the molecular weight through the use of filtration membranes with different molecular weight cutoffs can be performed, to check which parameter is more determining concerning gelation (molecular weight or molecular charge?).

The fractions of interest (Cat/Neut and HAC) can be analyzed furthermore to determine their building blocks through high performance anionic exchange chromatography coupled with pulsed amperometric detection. The Cat/Neut fraction should be submitted either to chemical or enzymatic treatments allowing the dissociation of the potential formed aggregates to study them separately. After further purification, Mass spectrometry or NMR can be done to determine the molecular structure of the gel-forming polymers.

The work presented in this thesis contributes to advancing the knowledge about the valorization of aerobic granular sludges as gel-forming polymers and shows that particular attention should be paid to tailor the extraction protocol according to the targeted applications. An evaluation of the whole process of EPS recovery is necessary through a life cycle assessment to determine the environmental and economical impacts of this resource recovery and make sure that it falls within the scope of a sustainable development.

## References:

- Abdul Khalil, H.P.S., Tye, Y.Y., Saurabh, C.K., Leh, C.P., Lai, T.K., Chong, E.W.N., Nurul Fazita, M.R., Hafiidz, J.M., Banerjee, A., Syakir, M.I., 2017. Biodegradable polymer films from seaweed polysaccharides: A review on cellulose as a reinforcement material. *Express Polym. Lett.* 11, 244–265. <https://doi.org/10.3144/expresspolymlett.2017.26>
- Abreu, F.O.M.S., Bianchini, C., Forte, M.M.C., Kist, T.B.L., 2008. Influence of the composition and preparation method on the morphology and swelling behavior of alginate-chitosan hydrogels. *Carbohydr. Polym.* 74, 283–289. <https://doi.org/10.1016/j.carbpol.2008.02.017>
- Adav, S.S., Lee, D.J., 2008. Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. *J. Hazard. Mater.* 154, 1120–1126. <https://doi.org/10.1016/j.jhazmat.2007.11.058>
- Ahmed, E.M., 2015. Hydrogel: Preparation, characterization, and applications: A review. *J. Adv. Res.* 6, 105–121. <https://doi.org/10.1016/j.jare.2013.07.006>
- Alipal, J., Mohd Pu'ad, N.A.S., Lee, T.C., Nayan, N.H.M., Sahari, N., Basri, H., Idris, M.I., Abdullah, H.Z., 2021. A review of gelatin: Properties, sources, process, applications, and commercialisation. *Mater. Today Proc.* 42, 240–250. <https://doi.org/10.1016/j.matpr.2020.12.922>
- Amorim de Carvalho, C. de, Ferreira dos Santos, A., Tavares Ferreira, T.J., Sousa Aguiar Lira, V.N., Mendes Barros, A.R., Bezerra dos Santos, A., 2021. Resource recovery in aerobic granular sludge systems: is it feasible or still a long way to go? *Chemosphere* 274. <https://doi.org/10.1016/j.chemosphere.2021.129881>
- Augst, A.D., Kong, H.J., Mooney, D.J., 2006. Alginate Hydrogels as Biomaterials. *Macromol. Biosci.* 623–633. <https://doi.org/10.1002/mabi.200600069>
- Avanza, M.V., Anon, M.C., 2007. effect of thermal treatment on the proteins of amaranth isolates. *J. Sci. Food Agric.* 1243, 1237–1243. <https://doi.org/10.1002/jsfa>
- Bealer, E.J., Onissema-Karimu, S., Rivera-Galletti, A., Francis, M., Wilkowski, J., de la Cruz, D.S., Hu, X., 2020. Protein-polysaccharide composite materials: Fabrication and applications. *Polymers (Basel)*. 12, 1–28. <https://doi.org/10.3390/polym12020464>
- Beaumont, M., Tran, R., Vera, G., Niedrist, D., Rousset, A., Pierre, R., Shastri, V.P., Forget, A., 2021. Hydrogel-Forming Algae Polysaccharides: From Seaweed to Biomedical Applications. *Biomacromolecules* 22, 1027–1052. <https://doi.org/10.1021/acs.biomac.0c01406>

- Bengtsson, S., de Blois, M., Wilén, B.M., Gustavsson, D., 2019. A comparison of aerobic granular sludge with conventional and compact biological treatment technologies. *Environ. Technol. (United Kingdom)* 40, 2769–2778. <https://doi.org/10.1080/09593330.2018.1452985>
- Biancarosa, I., Espe, M., Bruckner, C.G., Heesch, S., Liland, N., Waagbø, R., Torstensen, B., Lock, E.J., 2017. Amino acid composition, protein content, and nitrogen-to-protein conversion factors of 21 seaweed species from Norwegian waters. *J. Appl. Phycol.* 29, 1001–1009. <https://doi.org/10.1007/s10811-016-0984-3>
- Boleij, M., Kleikamp, H., Pabst, M., Neu, T.R., van Loosdrecht, M.C.M., Lin, Y., 2020. Decorating the Anammox House: Sialic Acids and Sulfated Glycosaminoglycans in the Extracellular Polymeric Substances of Anammox Granular Sludge. *Environ. Sci. Technol.* 54, 5218–5226. <https://doi.org/10.1021/acs.est.9b07207>
- Boleij, M., Pabst, M., Neu, T.R., Van Loosdrecht, M.C.M., Lin, Y., 2018. Identification of Glycoproteins Isolated from Extracellular Polymeric Substances of Full-Scale Anammox Granular Sludge. *Environ. Sci. Technol.* 52, 13127–13135. <https://doi.org/10.1021/acs.est.8b03180>
- Bou-sarkis, A., Pagliaccia, B., Ric, A., Derlon, N., Paul, E., Bessiere, Y., Girbal-neuhauser, E., 2022. Effects of alkaline solvents and heating temperatures on the solubilization and degradation of gel-forming Extracellular Polymeric Substances ( EPS ) extracted from aerobic granular sludge. *Biochem. Eng. J.* 185, 108500. <https://doi.org/10.1016/j.bej.2022.108500>
- Bou-Sarkis, A., Paul, E., Girbal-Neuhauser, E., Derlon, N., Bessiere, Y., 2023. Detection of Gel-Forming Polymers via Calcium Crosslinking, Applied to the Screening of Extracellular Polymeric Substances Extracted from Biological Aggregates. *Gels* 9, 157. <https://doi.org/10.3390/gels9020157>
- Bouleau, E., Saint-Bonnet, P., Mentink-Vigier, F., Takahashi, H., Jacquot, J.F., Bardet, M., Aussenac, F., Pureau, A., Engelke, F., Hediger, S., Lee, D., De Paëpe, G., 2015. Pushing NMR sensitivity limits using dynamic nuclear polarization with closed-loop cryogenic helium sample spinning. *Chem. Sci.* 6, 6806–6812. <https://doi.org/10.1039/c5sc02819a>
- Bourven, I., Costa, G., Guibaud, G., 2012. Qualitative characterization of the protein fraction of exopolymeric substances (EPS) extracted with EDTA from sludge. *Bioresour. Technol.* 104, 486–496. <https://doi.org/10.1016/j.biortech.2011.11.033>
- Bryant, C.M., Julian McClements, D., 1998. Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends Food Sci. Technol.* 9,

143–151. [https://doi.org/10.1016/S0924-2244\(98\)00031-4](https://doi.org/10.1016/S0924-2244(98)00031-4)

Bunce, J.T., Ndam, E., Ofiteru, I.D., Moore, A., Graham, D.W., 2018. A review of phosphorus removal technologies and their applicability to small-scale domestic wastewater treatment systems. *Front. Environ. Sci.* 6, 1–15. <https://doi.org/10.3389/fenvs.2018.00008>

Campo, R., Carretti, E., Lubello, C., Lotti, T., 2022. Recovery of structural extracellular polymeric substances (sEPS) from aerobic granular sludge: Insights on biopolymers characterization and hydrogel properties for potential applications. *J. Environ. Manage.* 324, 116247. <https://doi.org/10.1016/j.jenvman.2022.116247>

Campo, V.L., Kawano, D.F., Silva, D.B. da, Carvalho, I., 2009. Carrageenans: Biological properties, chemical modifications and structural analysis - A review. *Carbohydr. Polym.* 77, 167–180. <https://doi.org/10.1016/j.carbpol.2009.01.020>

Caudan, C., Filali, A., Lefebvre, D., Spérandio, M., Girbal-Neuhauser, E., 2012. Extracellular polymeric substances (EPS) from aerobic granular sludges: Extraction, fractionation, and anionic properties. *Appl. Biochem. Biotechnol.* 166, 1685–1702. <https://doi.org/10.1007/s12010-012-9569-z>

Caudan, C., Filali, A., Spérandio, M., Girbal-Neuhauser, E., 2014. Multiple EPS interactions involved in the cohesion and structure of aerobic granules. *Chemosphere* 117, 262–270. <https://doi.org/10.1016/j.chemosphere.2014.07.020>

Chauhan, P.S., Saxena, A., 2016. Bacterial carrageenases: an overview of production and biotechnological applications. *3 Biotech* 6, 146. <https://doi.org/10.1007/s13205-016-0461-3>

Cheah, Y.T., Chan, D.J.C., 2022. A methodological review on the characterization of microalgal biofilm and its extracellular polymeric substances. *J. Appl. Microbiol.* 132, 3490–3514. <https://doi.org/10.1111/jam.15455>

Chen, X. min, Yuan, J. lan, Li, R. xia, Kang, X., 2019. Characterization and embedding potential of bovine serum albumin cold-set gel induced by glucono- $\delta$ -lactone and sodium chloride. *Food Hydrocoll.* 95, 273–282. <https://doi.org/10.1016/j.foodhyd.2019.04.050>

Clark, A.H., 1996. Biopolymer gels. *Curr. Opin. Colloid Interface Sci.* 1, 712–717. [https://doi.org/10.1016/s1359-0294\(96\)80072-0](https://doi.org/10.1016/s1359-0294(96)80072-0)

Clementi, F., 1997. Alginate Production by *Azotobacter Vinelandii*. *Crit. Rev. Biotechnol.* 17, 327–361.

Codée, J.D.C., Christina, A.E., Walvoort, M.T.C., Overkleeft, H.S., van der Marel, G.A., 2010. Uronic

- Acids in Oligosaccharide and Glycoconjugate Synthesis, in: Fraser-Reid, B., Cristóbal López, J. (Eds) *Reactivity Tuning in Oligosaccharide Assembly*. Topics in Current Chemistry. Springer, Berlin, Heidelberg. [https://doi.org/10.1007/128\\_2010\\_111](https://doi.org/10.1007/128_2010_111)
- Collic-Jouault, S., Esposito, F., Ledru, H., Sinquin, C., Marchand, L., Fillaudeau, A., Routier, S., Buron, F., Lopin-Bon, C., Cuenot, S., Bedini, E., Zykwincka, A., 2023. Glycosaminoglycan Mimetics Obtained by Microwave-Assisted Sulfation of Marine Bacterium Sourced Infernan Exopolysaccharide. *Biomacromolecules* 24, 462–470. <https://doi.org/10.1021/acs.biomac.2c01277>
- Cydzik-Kwiatkowska, A., Gusiatin, M.Z., Zielińska, M., Wojnowska-Baryła, I., Kulikowska, D., Bernat, K., 2022. Alginate-like polymers from full-scale aerobic granular sludge: content, recovery, characterization, and application for cadmium adsorption. *Sci. Rep.* 12, 22260. <https://doi.org/10.1038/s41598-022-26743-5>
- Dall' Agnol, P., Libardi, N., Muller, J.M., Xavier, J.A., Domingos, D.G., da Costa, R.H.R., 2020. A comparative study of phosphorus removal using biopolymer from aerobic granular sludge: A factorial experimental evaluation. *J. Environ. Chem. Eng.* 8, 103541. <https://doi.org/10.1016/j.jece.2019.103541>
- De Graaff, D.R., Felz, S., Neu, T.R., Pronk, M., van Loosdrecht, M.C.M., Lin, Y., 2019. Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge. *Water Res.* 155, 343–351. <https://doi.org/10.1016/j.watres.2019.02.040>
- De Valk, S., Feng, C., Khadem, A.F., van Lier, J.B., de Kreuk, M.K., 2019. Elucidating the microbial community associated with the protein preference of sludge-degrading worms. *Environ. Technol. (United Kingdom)* 40, 192–201. <https://doi.org/10.1080/09593330.2017.1384071>
- Deshmukh, K., Basheer Ahamed, M., Deshmukh, R.R., Khadheer Pasha, S.K., Bhagat, P.R., Chidambaram, K., 2017. Biopolymer Composites with High Dielectric Performance: Interface Engineering, *Biopolymer Composites in Electronics*. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-809261-3.00003-6>
- Ding, Z., Bourven, I., Guibaud, G., van Hullebusch, E.D., Panico, A., Pirozzi, F., Esposito, G., 2015. Role of extracellular polymeric substances (EPS) production in bioaggregation: application to wastewater treatment. *Appl. Microbiol. Biotechnol.* 99, 9883–9905. <https://doi.org/10.1007/s00253-015-6964-8>
- Draget, K.I., 2009. Alginates, in: Phillips, G.O., Williams, P.A. (Eds.), *Handbook of Hydrocolloids*.

- Elsevier, pp. 807–828. <https://doi.org/10.1533/9781845695873.807>
- Draget, K.I., Skjåk-Bræk, G., Christensen, B.E., Gåserød, O., Smidsrød, O., 1996. Swelling and partial solubilization of alginic acid gel beads in acidic buffer. *Carbohydr. Polym.* 29, 209–215. [https://doi.org/10.1016/0144-8617\(96\)00029-X](https://doi.org/10.1016/0144-8617(96)00029-X)
- Draget, K.I., Skjåk Bræk, G., Smidsrød, O., 1994. Alginic acid gels: the effect of alginate chemical composition and molecular weight. *Carbohydr. Polym.* 25, 31–38. [https://doi.org/10.1016/0144-8617\(94\)90159-7](https://doi.org/10.1016/0144-8617(94)90159-7)
- Dulekgurgen, E., Artan, N., Orhon, D., Wilderer, P.A., 2008. How does shear affect aggregation in granular sludge sequencing batch reactors? Relations between shear, hydrophobicity, and extracellular polymeric substances. *Water Sci. Technol.* 58, 267–276. <https://doi.org/10.2166/wst.2008.382>
- Duque, A.F., Campo, R., Rio, A.V. Del, Amorim, C.L., 2021. Wastewater valorization: Practice around the world at pilot-and full-scale. *Int. J. Environ. Res. Public Health* 18. <https://doi.org/10.3390/ijerph18189466>
- Durieux, S., 2022. A contribution to the study of aerobic granulation and ALE production: effects of high organic load, and different COD/N. Toulouse university - INSA Toulouse.
- Durieux, S., Simon, A., Chiramberro, N., Bou-sarkis, A., Pagliaccia, B., Dubos, S., Bounouba, M., Moya-Leclerc, E., Girbal-Neuhauser, E., Bessière, Y., Paul, E., 2021. Granular biofilm for the production of exopolymer substances, in: IWA Biofilm Reactor Conference, Notre Dame, USA.
- Eiselt, P., Yeh, J., Latvala, R.K., Shea, L.D., Mooney, D.J., 2000. Porous carriers for biomedical applications based on alginate hydrogels. *Biomaterials* 21, 1921–1927. [https://doi.org/10.1016/S0142-9612\(00\)00033-8](https://doi.org/10.1016/S0142-9612(00)00033-8)
- Emerson, D., Ghiorse, W.C., 1993. Role of disulfide bonds in maintaining the structural integrity of the sheath of *Leptothrix discophora* SP-6. *J. Bacteriol.* 175, 7819–7827. <https://doi.org/10.1128/jb.175.24.7819-7827.1993>
- Eysturskard, J., Haug, I.J., Elharfaoui, N., Djabourov, M., Draget, K.I., 2009a. Structural and mechanical properties of fish gelatin as a function of extraction conditions. *Food Hydrocoll.* 23, 1702–1711. <https://doi.org/10.1016/j.foodhyd.2009.01.008>
- Eysturskard, J., Haug, I.J., Ulset, A.S., Draget, K.I., 2009b. Mechanical properties of mammalian and fish gelatins based on their weight average molecular weight and molecular weight distribution. *Food Hydrocoll.* 23, 2315–2321. <https://doi.org/10.1016/j.foodhyd.2009.06.007>

- Fahy, E., Cotter, D., Sud, M., Subramaniam, S., 2011. Lipid classification, structures and tools. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1811, 637–647. <https://doi.org/10.1016/j.bbalip.2011.06.009>
- Fang, Y., Al-Assaf, S., Phillips, G.O., Nishinari, K., Funami, T., Williams, P.A., Li, A., 2007. Multiple steps and critical behaviors of the binding of calcium to alginate. *J. Phys. Chem. B* 111, 2456–2462. <https://doi.org/10.1021/jp0689870>
- Felz, S., Al-Zuhairy, S., Aarstad, O.A., van Loosdrecht, M.C.M., Lin, Y.M., 2016. Extraction of structural extracellular polymeric substances from aerobic granular sludge. *J. Vis. Exp.* 2016. <https://doi.org/10.3791/54534>
- Felz, S., Kleikamp, H., Zlopasa, J., van Loosdrecht, M.C.M., Lin, Y., 2020a. Impact of metal ions on structural EPS hydrogels from aerobic granular sludge. *Biofilm* 2, 100011. <https://doi.org/10.1016/j.bioflm.2019.100011>
- Felz, S., Neu, T.R., van Loosdrecht, M.C.M., Lin, Y., 2020b. Aerobic granular sludge contains Hyaluronic acid-like and sulfated glycosaminoglycans-like polymers. *Water Res.* 169, 115291. <https://doi.org/10.1016/j.watres.2019.115291>
- Felz, S., Vermeulen, P., van Loosdrecht, M.C.M., Lin, Y.M., 2019. Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). *Water Res.* 157, 201–208. <https://doi.org/10.1016/j.watres.2019.03.068>
- Feng, C., Lotti, T., Canziani, R., Lin, Y., Tagliabue, C., Malpei, F., 2021. Extracellular biopolymers recovered as raw biomaterials from waste granular sludge and potential applications: A critical review. *Sci. Total Environ.* 753, 142051. <https://doi.org/10.1016/j.scitotenv.2020.142051>
- Feng, C., Lotti, T., Lin, Y., Malpei, F., 2019. Extracellular polymeric substances extraction and recovery from anammox granules: Evaluation of methods and protocol development. *Chem. Eng. J.* 374, 112–122. <https://doi.org/10.1016/j.cej.2019.05.127>
- Feng, Y., Taraban, M., Yu, Y.B., 2012. The effect of ionic strength on the mechanical, structural and transport properties of peptide hydrogels. *Soft Matter* 8, 11723. <https://doi.org/10.1039/c2sm26572a>
- Fenoradosoa, T.A., Ali, G., Delattre, C., Laroche, C., Petit, E., Wadouachi, A., Michaud, P., 2010. Extraction and characterization of an alginate from the brown seaweed *Sargassum turbinarioides* Grunow. *J. Appl. Phycol.* 22, 131–137. <https://doi.org/10.1007/s10811-009-9432-y>

- Fernández Farrés, I., Norton, I.T., 2014. Formation kinetics and rheology of alginate fluid gels produced by in-situ calcium release. *Food Hydrocoll.* 40, 76–84.  
<https://doi.org/10.1016/j.foodhyd.2014.02.005>
- Flemming, H.C., Wingender, J., 2010. The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633.  
<https://doi.org/10.1038/nrmicro2415>
- Foglia, A., Bruni, C., Cipolletta, G., Eusebi, A.L., Frison, N., Katsou, E., Akyol, Ç., Fatone, F., 2021. Assessing socio-economic value of innovative materials recovery solutions validated in existing wastewater treatment plants. *J. Clean. Prod.* 322.  
<https://doi.org/10.1016/j.jclepro.2021.129048>
- Frølund, B., Griebe, T., Nielsen, P.H., 1995. Enzymatic activity in the activated-sludge floc matrix. *Appl. Microbiol. Biotechnol.* 43, 755–761. <https://doi.org/10.1007/BF00164784>
- Gandhi, N.S., Mancera, R.L., 2008. The structure of glycosaminoglycans and their interactions with proteins. *Chem. Biol. Drug Des.* 72, 455–482. <https://doi.org/10.1111/j.1747-0285.2008.00741.x>
- Gurikov, P., Smirnova, I., 2018. Non-Conventional Methods for Gelation of Alginate. *Gels* 4, 14.  
<https://doi.org/10.3390/gels4010014>
- Hawkins, C.L., Davies, M.J., 2001. Generation and propagation of radical reactions on proteins. *Biochim. Biophys. Acta - Bioenerg.* 1504, 196–219. [https://doi.org/10.1016/S0005-2728\(00\)00252-8](https://doi.org/10.1016/S0005-2728(00)00252-8)
- He, Y., Wu, Z., Tu, L., Han, Y., Zhang, G., Li, C., 2015. Encapsulation and characterization of slow-release microbial fertilizer from the composites of bentonite and alginate. *Appl. Clay Sci.* 109–110, 68–75. <https://doi.org/10.1016/j.clay.2015.02.001>
- Henglein, A., Gutiérrez, M., 1988. Sonolysis of polymers in aqueous solution. New observations on pyrolysis and mechanical degradation. *J. Phys. Chem.* 92, 3705–3707.  
<https://doi.org/10.1021/j100324a005>
- Honda, C., Hiroko, K., Tomomi, S., Kazutoyo, E., 2000. studies on thermal aggregation of Bovine Serum albumin as a drug carrier. *Chem. Pharm. Bull.* 2091.
- Hotchkiss, S., Brooks, M., Campbell, R., Philp, K., Trius, A., 2016. The use of carrageenan in food, in: *Carrageenans: Sources and Extraction Methods, Molecular Structure, Bioactive Properties and Health Effects.* pp. 229–243.

- Jang, J., Seol, Y.J., Kim, H.J., Kundu, J., Kim, S.W., Cho, D.W., 2014. Effects of alginate hydrogel cross-linking density on mechanical and biological behaviors for tissue engineering. *J. Mech. Behav. Biomed. Mater.* 37, 69–77. <https://doi.org/10.1016/j.jmbbm.2014.05.004>
- Karakas, I., Sam, S.B., Cetin, E., Dulekgurgen, E., Yilmaz, G., 2020. Resource recovery from an aerobic granular sludge process treating domestic wastewater. *J. Water Process Eng.* 34, 101148. <https://doi.org/10.1016/j.jwpe.2020.101148>
- Karim, A.A., Bhat, R., 2008. Gelatin alternatives for the food industry: recent developments, challenges and prospects. *Trends Food Sci. Technol.* 19, 644–656. <https://doi.org/10.1016/j.tifs.2008.08.001>
- Katyal, P., Mahmoudinobar, F., Montclare, J.K., 2020. Recent trends in peptide and protein-based hydrogels. *Curr. Opin. Struct. Biol.* 63, 97–105. <https://doi.org/10.1016/j.sbi.2020.04.007>
- Kim, E., Jang, Y., Kim, H., Lee, D.Y., Choi, J.G., Kim, S.J., 2022. Calcium-driven DNA artificial muscle inspired by natural muscle contraction. *Sensors Actuators B Chem.* 371, 132556. <https://doi.org/10.1016/j.snb.2022.132556>
- Kim, N.K., Mao, N., Lin, R., Bhattacharyya, D., van Loosdrecht, M.C.M., Lin, Y., 2020. Flame retardant property of flax fabrics coated by extracellular polymeric substances recovered from both activated sludge and aerobic granular sludge. *Water Res.* 170, 115344. <https://doi.org/10.1016/j.watres.2019.115344>
- Klaus, N., Eero, S., 1985. alkaline degradation of alginates to carboxylic acids. *Carbohydr. Res.* 144, 241–249.
- Kloster, N., Brigante, M., Zanini, G., Avena, M., 2013. Aggregation kinetics of humic acids in the presence of calcium ions. *Colloids Surfaces A Physicochem. Eng. Asp.* 427, 76–82. <https://doi.org/10.1016/j.colsurfa.2013.03.030>
- Kończak, B., Karcz, J., Miksch, K., 2014. Influence of Calcium, Magnesium, and Iron Ions on Aerobic Granulation. *Appl. Biochem. Biotechnol.* 174, 2910–2918. <https://doi.org/10.1007/s12010-014-1236-0>
- Kong, H.J., Lee, K.Y., Mooney, D.J., 2002. Decoupling the dependence of rheological/mechanical properties of hydrogels from solids concentration. *Polymer (Guildf)*. 43, 6239–6246. [https://doi.org/10.1016/S0032-3861\(02\)00559-1](https://doi.org/10.1016/S0032-3861(02)00559-1)
- Kwan, A.H., Mobli, M., Gooley, P.R., King, G.F., MacKay, J.P., 2011. Macromolecular NMR spectroscopy for the non-spectroscopist. *FEBS J.* 278, 687–703. <https://doi.org/10.1111/j.1742->

- Labowska, M.B., Cierluk, K., Jankowska, A.M., Kulbacka, J., Detyna, J., Michalak, I., 2021. A Review on the Adaption of Alginate-Gelatin Hydrogels for 3D Cultures and Bioprinting. *Materials (Basel)*. 1–24.
- Ladnorg, S., Junior, N.L., Dall’ Agnol, P., Domingos, D.G., Magnus, B.S., Wichern, M., Gehring, T., da Costa, R.H.R., 2019. Alginate-like exopolysaccharide extracted from aerobic granular sludge as biosorbent for methylene blue: Thermodynamic, kinetic and isotherm studies. *J. Environ. Chem. Eng.* 7, 103081. <https://doi.org/10.1016/j.jece.2019.103081>
- Larsen, P., Nielsen, J.L., Otzen, D., Nielsen, P.H., 2008. Amyloid-like adhesins produced by floc-forming and filamentous bacteria in activated sludge. *Appl. Environ. Microbiol.* 74, 1517–1526. <https://doi.org/10.1128/AEM.02274-07>
- Layer, M., Adler, A., Reynaert, E., Hernandez, A., Pagni, M., Morgenroth, E., Holliger, C., Derlon, N., 2019. Organic substrate diffusibility governs microbial community composition, nutrient removal performance and kinetics of granulation of aerobic granular sludge. *Water Res. X* 4, 100033. <https://doi.org/10.1016/j.wroa.2019.100033>
- Le, X.T., Rioux, L.E., Turgeon, S.L., 2017. Formation and functional properties of protein–polysaccharide electrostatic hydrogels in comparison to protein or polysaccharide hydrogels. *Adv. Colloid Interface Sci.* 239, 127–135. <https://doi.org/10.1016/j.cis.2016.04.006>
- Lee, K.Y., Mooney, D.J., 2012. Alginate: Properties and biomedical applications. *Prog. Polym. Sci.* 37, 106–126. <https://doi.org/10.1016/j.progpolymsci.2011.06.003>
- Leo, W.J., Mcloughlin, A.J., Malone, D.M., 1990. Effects of Sterilization Treatments on Some Properties of Alginate Solutions and Gels. *Biotechnol. Prog.* 6, 51–53. <https://doi.org/10.1021/bp00001a008>
- Li, F., Tang, J., Geng, J., Luo, D., Yang, D., 2019. Polymeric DNA hydrogel : Design , synthesis and applications. *Prog. Polym. Sci.* 98, 101163. <https://doi.org/10.1016/j.progpolymsci.2019.101163>
- Li, X., Luo, J., Guo, G., Mackey, H.R., Hao, T., Chen, G., 2017. Seawater-based wastewater accelerates development of aerobic granular sludge: A laboratory proof-of-concept. *Water Res.* 115, 210–219. <https://doi.org/10.1016/j.watres.2017.03.002>
- Lima-Tenório, M.K., Tenório-Neto, E.T., Guilherme, M.R., Garcia, F.P., Nakamura, C. V., Pineda, E.A.G., Rubira, A.F., 2015. Water transport properties through starch-based hydrogel nanocomposites responding to both pH and a remote magnetic field. *Chem. Eng. J.* 259, 620–629.

<https://doi.org/10.1016/j.cej.2014.08.045>

- Lin, Y., de Kreuk, M., van Loosdrecht, M.C.M., Adin, A., 2010. Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant. *Water Res.* 44, 3355–3364. <https://doi.org/10.1016/j.watres.2010.03.019>
- Lin, Y., Reino, C., Carrera, J., Pérez, J., van Loosdrecht, M.C.M., 2018. Glycosylated amyloid-like proteins in the structural extracellular polymers of aerobic granular sludge enriched with ammonium-oxidizing bacteria. *Microbiologyopen* 7, 1–13. <https://doi.org/10.1002/mbo3.616>
- Lin, Y.M., Nierop, K.G.J., Girbal-Neuhauser, E., Adriaanse, M., van Loosdrecht, M.C.M., 2015. Sustainable polysaccharide-based biomaterial recovered from waste aerobic granular sludge as a surface coating material. *Sustain. Mater. Technol.* 4, 24–29. <https://doi.org/10.1016/j.susmat.2015.06.002>
- Liu, S., Li, L., 2016. Thermoreversible gelation and scaling behavior of Ca<sup>2+</sup>-induced κ-carrageenan hydrogels. *Food Hydrocoll.* 61, 793–800. <https://doi.org/10.1016/j.foodhyd.2016.07.003>
- Liu, W., Zhang, J., Jin, Y., Zhao, X., Cai, Z., 2015. Adsorption of Pb(II), Cd(II) and Zn(II) by extracellular polymeric substances extracted from aerobic granular sludge: Efficiency of protein. *J. Environ. Chem. Eng.* 3, 1223–1232. <https://doi.org/10.1016/j.jece.2015.04.009>
- Liu, X., Liu, J., Deng, D., Li, R., Guo, C., Ma, J., Chen, M., 2021. Investigation of extracellular polymeric substances (EPS) in four types of sludge: Factors influencing EPS properties and sludge granulation. *J. Water Process Eng.* 40, 101924. <https://doi.org/10.1016/j.jwpe.2021.101924>
- Liu, Yang, Wang, S., Sun, D., Liu, Yongdong, Liu, Yang, Wang, Y., Liu, C., Wu, H., Lv, Y., Ren, Y., Guo, X., Sun, G., Ma, X., 2016. Development of a Biomimetic Chondroitin Sulfate-modified Hydrogel to Enhance the Metastasis of Tumor Cells. *Sci. Rep.* 6, 1–13. <https://doi.org/10.1038/srep29858>
- Liu, Yong-qiang, Liu, Yu, Tay, J., 2004. The effects of extracellular polymeric substances on the formation and stability of biogranules. *Appl. Microbiol. Biotechnol.* 143–148. <https://doi.org/10.1007/s00253-004-1657-8>
- Lotti, T., Carretti, E., Berti, D., Martina, M.R., Lubello, C., Malpei, F., 2019. Extraction, recovery and characterization of structural extracellular polymeric substances from anammox granular sludge. *J. Environ. Manage.* 236, 649–656. <https://doi.org/10.1016/j.jenvman.2019.01.054>
- Makshakova, O.N., Bogdanova, L.R., Makarova, A.O., Kusova, A.M., Ermakova, E.A., Kazantseva, M.A., Zuev, Y.F., 2022a. κ-Carrageenan Hydrogel as a Matrix for Therapeutic Enzyme Immobilization. *Polymers (Basel)*. 14, 4071. <https://doi.org/10.3390/polym14194071>

- Makshakova, O., Zykwincka, A., Cuenot, S., Collic-Jouault, S., Perez, S., 2022b. Three-dimensional structures, dynamics and calcium-mediated interactions of the exopolysaccharide, Infernan, produced by the deep-sea hydrothermal bacterium *Alteromonas infernus*. *Carbohydr. Polym.* 276, 118732. <https://doi.org/10.1016/j.carbpol.2021.118732>
- Matsudomi, N., Rector, D., Kinsella, J.E., 1991. Gelation of bovine serum albumin and  $\beta$ -lactoglobulin; effects of pH, salts and thiol reagents. *Food Chem.* 40, 55–69. [https://doi.org/10.1016/0308-8146\(91\)90019-K](https://doi.org/10.1016/0308-8146(91)90019-K)
- Matsuura, Y., Takehira, M., Joti, Y., Ogasahara, K., Tanaka, T., Ono, N., Kunishima, N., Yutani, K., 2015. Thermodynamics of protein denaturation at temperatures over 100°C: CutA1 mutant proteins substituted with hydrophobic and charged residues. *Sci. Rep.* 5, 1–9. <https://doi.org/10.1038/srep15545>
- Mcswain, B.S., Irvine, R.L., Hausner, M., Wilderer, P.A., 2005. Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge 71, 1051–1057. <https://doi.org/10.1128/AEM.71.2.1051>
- Meng, F., Huang, Weiwei, Liu, D., Zhao, Y., Huang, Wenli, Lei, Z., Zhang, Z., 2020. Application of aerobic granules-continuous flow reactor for saline wastewater treatment: Granular stability, lipid production and symbiotic relationship between bacteria and algae. *Bioresour. Technol.* 295, 122291. <https://doi.org/10.1016/j.biortech.2019.122291>
- Meng, F., Liu, D., Pan, Y., Xi, L., Yang, D., Huang, W., 2019. Enhanced amount and quality of alginate-like exopolysaccharides in aerobic granular sludge for the treatment of salty wastewater. *BioResources* 14, 139–165. <https://doi.org/10.15376/biores.14.1.139-165>
- Mezenov, Y.A., Krasilin, A.A., Dzyuba, V.P., Nominé, A., Milichko, V.A., 2019. Metal–Organic Frameworks in Modern Physics: Highlights and Perspectives. *Adv. Sci.* 6, 1900506. <https://doi.org/10.1002/advs.201900506>
- Moldoveanu, S., David, V., 2015. Modern Sample Preparation for Chromatography. <https://doi.org/10.1016/C2011-0-00093-5>
- Monticolo, F., Palomba, E., Termolino, P., Chiaiese, P., de Alteriis, E., Mazzoleni, S., Chiusano, M.L., 2020. The Role of DNA in the Extracellular Environment: A Focus on NETs, RETs and Biofilms. *Front. Plant Sci.* 11, 1–15. <https://doi.org/10.3389/fpls.2020.589837>
- Morris, E.R., 1979. Polysaccharide Conformation and Interactions in Solutions and Gels. In: Gettins, W.J., Wyn-Jones, E. (eds) *Techniques and Applications of Fast Reactions in Solution*. NATO

Advanced Study Institutes Series, vol 50. Springer, Dordrecht.

[https://doi.org/https://doi.org/10.1007/978-94-009-9490-4\\_52](https://doi.org/https://doi.org/10.1007/978-94-009-9490-4_52)

Nancharaiah, Y. V., Kiran Kumar Reddy, G., 2018. Aerobic granular sludge technology: Mechanisms of granulation and biotechnological applications. *Bioresour. Technol.* 247, 1128–1143.

<https://doi.org/10.1016/j.biortech.2017.09.131>

Navarra, G., Giacomazza, D., Leone, M., Librizzi, F., Militello, V., San Biagio, P.L., 2009. Thermal aggregation and ion-induced cold-gelation of bovine serum albumin. *Eur. Biophys. J.* 38, 437–446. <https://doi.org/10.1007/s00249-008-0389-6>

Navarra, G., Peres, C., Contardi, M., Picone, P., San Biagio, P.L., Di Carlo, M., Giacomazza, D., Militello, V., 2016. Heat- and pH-induced BSA conformational changes, hydrogel formation and application as 3D cell scaffold. *Arch. Biochem. Biophys.* 606, 134–142.

<https://doi.org/10.1016/j.abb.2016.07.020>

Neiser, S., Draget, K.I., Smidsrød, O., 1999. Interactions in bovine serum albumin-calcium alginate gel systems. *Food Hydrocoll.* 13, 445–458. [https://doi.org/10.1016/S0268-005X\(99\)00019-3](https://doi.org/10.1016/S0268-005X(99)00019-3)

Neves, M.I., Ara, M., Moroni, L., Silva, R.M.P., Barrias, C.C., 2020. the Development of Bioactive Hydrogel Networks. *Molecules* 25, 978.

Ni, S.Q., Sun, N., Yang, H., Zhang, J., Ngo, H.H., 2015. Distribution of extracellular polymeric substances in anammox granules and their important roles during anammox granulation. *Biochem. Eng. J.* 101, 126–133. <https://doi.org/10.1016/j.bej.2015.05.014>

Nicolai, T., Durand, D., 2013. Controlled food protein aggregation for new functionality. *Curr. Opin. Colloid Interface Sci.* 18, 249–256. <https://doi.org/10.1016/j.cocis.2013.03.001>

Nielsen, P.H., Jahn, A., 1999. Extraction of EPS, in: Wingender J., Neu T.R., Flemming HC. (Eds) *Microbial Extracellular Polymeric Substances*. Springer, Berlin, Heidelberg, pp. 49–72. [https://doi.org/10.1007/978-3-642-60147-7\\_3](https://doi.org/10.1007/978-3-642-60147-7_3)

Oliva, R., Banerjee, S., Cinar, H., Ehrt, C., Winter, R., 2020. Alteration of Protein Binding Affinities by Aqueous Two-Phase Systems Revealed by Pressure Perturbation. *Sci. Rep.* 10, 1–12. <https://doi.org/10.1038/s41598-020-65053-6>

Oliveira, A.S., Amorim, C.L., Ramos, M.A., Mesquita, D.P., Inocêncio, P., Ferreira, E.C., Van Loosdrecht, M., Castro, P.M.L., 2020. Variability in the composition of extracellular polymeric substances from a full-scale aerobic granular sludge reactor treating urban wastewater. *J. Environ. Chem. Eng.* 8, 104156. <https://doi.org/10.1016/j.jece.2020.104156>

- Pagliaccia, B., Carretti, E., Severi, M., Berti, D., Lubello, C., Lotti, T., 2022a. Heavy metal biosorption by Extracellular Polymeric Substances (EPS) recovered from anammox granular sludge. *J. Hazard. Mater.* 424, 126661. <https://doi.org/10.1016/j.jhazmat.2021.126661>
- Pagliaccia, B., Durieux, S., Bessiere, Y., Bounouba, M., Bou Sarkis, A., Girbal-Neuhauser, E., Carretti, E., Lubello, C., Lotti, T., Paul, E., 2022b. Insights on the hydrogel-forming ability and post-gelling mechanical properties of structural extracellular polymeric substances (sEPS) from aerobic granular sludge (AGS): A comparison with model biopolymers. *J. Water Process Eng.* 49, 103076. <https://doi.org/10.1016/j.jwpe.2022.103076>
- Pan, M., Zhu, L., Chen, L., Qiu, Y., Wang, J., 2016. Detection Techniques for Extracellular Polymeric 11, 8092–8115.
- Park, Z.Y., Russell, D.H., 2000. Thermal denaturation: A useful technique in peptide mass mapping. *Anal. Chem.* 72, 2667–2670. <https://doi.org/10.1021/ac991444k>
- Patel, J., Maji, B., Moorthy, N.S.H.N., Maiti, S., 2020. Xanthan gum derivatives: Review of synthesis, properties and diverse applications. *RSC Adv.* 10, 27103–27136. <https://doi.org/10.1039/d0ra04366d>
- Patist, A., Bates, D., 2008. Ultrasonic innovations in the food industry: From the laboratory to commercial production. *Innov. Food Sci. Emerg. Technol.* 9, 147–154. <https://doi.org/10.1016/j.ifset.2007.07.004>
- Pronk, M., Neu, T.R., van Loosdrecht, M.C.M., Lin, Y.M., 2017. The acid soluble extracellular polymeric substance of aerobic granular sludge dominated by *Deffluviococcus* sp. *Water Res.* 122, 148–158. <https://doi.org/10.1016/j.watres.2017.05.068>
- Ramdhan, T., Ching, S.H., Prakash, S., Bhandari, B., 2019. Time dependent gelling properties of cuboid alginate gels made by external gelation method: Effects of alginate-CaCl<sub>2</sub> solution ratios and pH. *Food Hydrocoll.* 90, 232–240. <https://doi.org/10.1016/j.foodhyd.2018.12.022>
- Ras, M., Girbal-Neuhauser, E., Paul, E., Spérandio, M., Lefebvre, D., 2008a. Protein extraction from activated sludge: An analytical approach. *Water Res.* 42, 1867–1878. <https://doi.org/10.1016/j.watres.2007.11.011>
- Ras, M., Girbal Neuhauser, E., Paul, E., Lefebvre, D., 2008b. A high yield multi-method extraction protocol for protein quantification in activated sludge. *Bioresour. Technol.* 99, 7464–7471. <https://doi.org/10.1016/j.biortech.2008.02.025>
- Riesz, P., Kondo, T., 1992. Free radical formation induced by ultrasound and its biological

- implications. *Free Radic. Biol. Med.* 13, 247–270. [https://doi.org/10.1016/0891-5849\(92\)90021-8](https://doi.org/10.1016/0891-5849(92)90021-8)
- Rinaudo, M., 2006. Chitin and chitosan: Properties and applications. *Prog. Polym. Sci.* 31, 603–632. <https://doi.org/10.1016/j.progpolymsci.2006.06.001>
- Rolleberg, S.L. de S., dos Santos, A.F., Ferreira, T.J.T., Firmino, P.I.M., dos Santos, A.B., 2021. Evaluation of the production of alginate-like exopolysaccharides (ALE) and tryptophan in aerobic granular sludge systems. *Bioprocess Biosyst. Eng.* 44, 259–270. <https://doi.org/10.1007/s00449-020-02439-w>
- Rondel, C., Marcato-Romain, C.E., Girbal-Neuhauser, E., 2013. Development and validation of a colorimetric assay for simultaneous quantification of neutral and uronic sugars. *Water Res.* 47, 2901–2908. <https://doi.org/10.1016/j.watres.2013.03.010>
- Rusanowska, P., Cydzik-Kwiatkowska, A., Wojnowska-Baryła, I., 2019. Microbial Origin of Excreted DNA in Particular Fractions of Extracellular Polymers (EPS) in Aerobic Granules. *Water. Air. Soil Pollut.* 230. <https://doi.org/10.1007/s11270-019-4248-0>
- Sajjad, M., Kim, K.S., 2016. Extraction of extracellular polymeric substances from activated sludge using sodium oxalate. *Int. J. Environ. Sci. Technol.* 13, 1697–1706. <https://doi.org/10.1007/s13762-016-1004-5>
- Salama, Y., Chennaoui, M., Sylla, A., Mountadar, M., Rihani, M., Assobhei, O., 2015. Characterization, structure, and function of extracellular polymeric substances (EPS) of microbial biofilm in biological wastewater treatment systems: a review. *Desalin. Water Treat.* 57, 16220–16237. <https://doi.org/10.1080/19443994.2015.1077739>
- Schambeck, C.M., Girbal-Neuhauser, E., Böni, L., Fischer, P., Bessière, Y., Paul, E., da Costa, R.H.R., Derlon, N., 2020. Chemical and physical properties of alginate-like exopolymers of aerobic granules and flocs produced from different wastewaters. *Bioresour. Technol.* 312, 123632. <https://doi.org/10.1016/j.biortech.2020.123632>
- Schambeck, C.M., Ribeiro da Costa, R.H., Derlon, N., 2021. Phosphate removal from municipal wastewater by alginate-like exopolymers hydrogels recovered from aerobic granular sludge. *Bioresour. Technol.* 333. <https://doi.org/10.1016/j.biortech.2021.125167>
- Sesay, M.L., Özcengiz, G., Dilek Sanin, F., 2006. Enzymatic extraction of activated sludge extracellular polymers and implications on bioflocculation. *Water Res.* 40, 1359–1366. <https://doi.org/10.1016/j.watres.2006.01.045>

- Seviour, T., Derlon, N., Duellhom, M.S., Flemming, H., Girbal-neuhauser, E., Horn, H., Kjelleberg, S., Van Loosdrecht, M.C.M., Lotti, T., Malpei, M.F., Nerenberg, R., Neu, T.R., Paul, E., Yu, H., Lin, Y., 2019. Extracellular polymeric substances of biofilms : Suffering from an identity crisis. *Water Res.* 151, 1–7. <https://doi.org/10.1016/j.watres.2018.11.020>
- Seviour, T., Donose, B.C., Pijuan, M., Yuan, Z., 2010a. Purification and conformational analysis of a key exopolysaccharide component of mixed culture aerobic sludge granules. *Environ. Sci. Technol.* 44, 4729–4734. <https://doi.org/10.1021/es100362b>
- Seviour, T., Lambert, L.K., Pijuan, M., Yuan, Z., 2010b. Structural determination of a key exopolysaccharide in mixed culture aerobic sludge granules using NMR spectroscopy. *Environ. Sci. Technol.* 44, 8964–8970. <https://doi.org/10.1021/es102658s>
- Seviour, T., Malde, A.K., Kjelleberg, S., Yuan, Z., Mark, A.E., 2012a. Molecular dynamics unlocks atomic level self-assembly of the exopolysaccharide matrix of water-treatment granular biofilms. *Biomacromolecules* 13, 1965–1972. <https://doi.org/10.1021/bm3005808>
- Seviour, T., Pijuan, M., Nicholson, T., Keller, J., Yuan, Z., 2009. Gel-forming exopolysaccharides explain basic differences between structures of aerobic sludge granules and floccular sludges. *Water Res.* 43, 4469–4478. <https://doi.org/10.1016/j.watres.2009.07.018>
- Seviour, T., Yuan, Z., van Loosdrecht, M.C.M., Lin, Y., 2012b. Aerobic sludge granulation: A tale of two polysaccharides? *Water Res.* 46, 4803–4813. <https://doi.org/10.1016/j.watres.2012.06.018>
- Seviour, T.W., Lambert, L.K., Pijuan, M., Yuan, Z., 2011. Selectively inducing the synthesis of a key structural exopolysaccharide in aerobic granules by enriching for *Candidatus “competibacter phosphatis.”* *Appl. Microbiol. Biotechnol.* 92, 1297–1305. <https://doi.org/10.1007/s00253-011-3385-1>
- Sheng, G.P., Yu, H.Q., Li, X.Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. *Biotechnol. Adv.* 28, 882–894. <https://doi.org/10.1016/j.biotechadv.2010.08.001>
- Shi, Y., Liu, Y., 2021. Chemosphere Evolution of extracellular polymeric substances ( EPS ) in aerobic sludge granulation : Composition , adherence and viscoelastic properties 262. <https://doi.org/10.1016/j.chemosphere.2020.128033>
- Sobeck, D.C., Higgins, M.J., 2002. Examination of three theories for mechanisms of cation-induced bioflocculation. *Water Res.* 36, 527–538. [https://doi.org/10.1016/S0043-1354\(01\)00254-8](https://doi.org/10.1016/S0043-1354(01)00254-8)
- Sodhi, H., Panitch, A., 2021. Glycosaminoglycans in tissue engineering: A review. *Biomolecules* 11, 1–

22. <https://doi.org/10.3390/biom11010029>
- Soria, A.C., Villamiel, M., 2010. Effect of ultrasound on the technological properties and bioactivity of food: A review. *Trends Food Sci. Technol.* 21, 323–331.  
<https://doi.org/10.1016/j.tifs.2010.04.003>
- Stathopoulos, P.B., Scholz, G.A., Hwang, Y.-M., Rumfeldt, J.A.O., Lepock, J.R., Meiering, E.M., 2008. Sonication of proteins causes formation of aggregates that resemble amyloid. *Protein Sci.* 13, 3017–3027. <https://doi.org/10.1110/ps.04831804>
- Su, B., Qu, Z., Song, Y., Jia, L., Zhu, J., 2014. Investigation of measurement methods and characterization of zeta potential for aerobic granular sludge. *J. Environ. Chem. Eng.* 2, 1142–1147. <https://doi.org/10.1016/j.jece.2014.03.006>
- Tan, X., Xie, G.J., Nie, W.B., Xing, D.F., Liu, B.F., Ding, J., Ren, N.Q., 2021. High value-added biomaterials recovery from granular sludge based wastewater treatment process. *Resour. Conserv. Recycl.* 169, 105481. <https://doi.org/10.1016/j.resconrec.2021.105481>
- Tavares Ferreira, T.J., Luiz de Sousa Rollemberg, S., Nascimento de Barros, A., Machado de Lima, J.P., Bezerra dos Santos, A., 2021. Integrated review of resource recovery on aerobic granular sludge systems: Possibilities and challenges for the application of the biorefinery concept. *J. Environ. Manage.* 291. <https://doi.org/10.1016/j.jenvman.2021.112718>
- Tavassoli-Kafrani, E., Shekarchizadeh, H., Masoudpour-Behabadi, M., 2016. Development of edible films and coatings from alginates and carrageenans. *Carbohydr. Polym.* 137, 360–374.  
<https://doi.org/10.1016/j.carbpol.2015.10.074>
- Tijore, A., Irvine, S.A., Sarig, U., Mhaisalkar, P., Baisane, V., Venkatraman, S., 2018. Contact guidance for cardiac tissue engineering using 3D bioprinted gelatin patterned hydrogel. *Biofabrication* 10. <https://doi.org/10.1088/1758-5090/aaa15d>
- Topală, T., Bodoki, A., Oprean, L., Oprean, R., 2014. Bovine serum albumin interactions with metal complexes. *Clujul Med.* 87, 5. <https://doi.org/10.15386/cjmed-357>
- Ullah, F., Othman, M.B.H., Javed, F., Ahmad, Z., Akil, H.M., 2015. Classification, processing and application of hydrogels: A review. *Mater. Sci. Eng. C* 57, 414–433.  
<https://doi.org/10.1016/j.msec.2015.07.053>
- Venezia, V., Avallone, P.R., Vitiello, G., Silvestri, B., Grizzuti, N., Pasquino, R., Luciani, G., 2022. Adding Humic Acids to Gelatin Hydrogels: A Way to Tune Gelation. *Biomacromolecules* 23, 443–453.  
<https://doi.org/10.1021/acs.biomac.1c01398>

- Vermonden, T., Censi, R., Hennink, W.E., 2012. Hydrogels for Protein Delivery. *Chem. Rev.* 112, 2853–2888. <https://doi.org/10.1021/cr200157d>
- Wang, S., Liu, L., Li, H., Fang, F., Yan, P., Chen, Y., Guo, J., Ma, T., Shen, Y., 2020. The branched chains and branching degree of exopolysaccharides affecting the stability of anammox granular sludge. *Water Res.* 178, 115818. <https://doi.org/10.1016/j.watres.2020.115818>
- Wang, X., Ao, Q., Tian, X., Fan, J., Tong, H., Hou, W., Bai, S., 2017. Gelatin-based hydrogels for organ 3D bioprinting. *Polymers (Basel)*. 9. <https://doi.org/10.3390/polym9090401>
- Wang, Y., Zhu, Y., Hu, Y., Zeng, G., Zhang, Y., Zhang, C., Feng, C., 2018. How to Construct DNA Hydrogels for Environmental Applications : Advanced Water Treatment and Environmental Analysis. *small* 1703305, 1–19. <https://doi.org/10.1002/smll.201703305>
- Weissbrodt, D.G., Neu, T.R., Kuhlicke, U., Rappaz, Y., Holliger, C., 2013. Assessment of bacterial and structural dynamics in aerobic granular biofilms. *Front. Microbiol.* 4, 1–18. <https://doi.org/10.3389/fmicb.2013.00175>
- Wingender, J., Neu, T.R., Flemming, H.C., 1999. Microbial extracellular polymeric substances: characterization, structure, and function., Springer. <https://doi.org/10.1007/978-3-642-60147-7>
- Winkler, M.K.H., Meunier, C., Henriot, O., Mahillon, J., Suárez-Ojeda, M.E., Del Moro, G., De Sanctis, M., Di Iaconi, C., Weissbrodt, D.G., 2018. An integrative review of granular sludge for the biological removal of nutrients and recalcitrant organic matter from wastewater. *Chem. Eng. J.* 336, 489–502. <https://doi.org/10.1016/j.cej.2017.12.026>
- Wu, Z., Zhao, Y., Kaleem, I., Li, C., 2011. Preparation of calcium-alginate microcapsuled microbial fertilizer coating *Klebsiella oxytoca* Rs-5 and its performance under salinity stress. *Eur. J. Soil Biol.* 47, 152–159. <https://doi.org/10.1016/j.ejsobi.2010.11.008>
- Yang, Y., Liu, X., Wan, C., Sun, S., Lee, D., 2014. Bioresource Technology Accelerated aerobic granulation using alternating feed loadings : Alginate-like exopolysaccharides. *Bioresour. Technol.* 171, 360–366. <https://doi.org/10.1016/j.biortech.2014.08.092>
- Ye, Y., Ngo, H.H., Guo, W., Chang, S.W., Nguyen, D.D., Zhang, X., Zhang, J., Liang, S., 2020. Nutrient recovery from wastewater: From technology to economy. *Bioresour. Technol. Reports* 11, 100425. <https://doi.org/10.1016/j.biteb.2020.100425>
- Zarei, M., 2020. Wastewater resources management for energy recovery from circular economy perspective. *Water-Energy Nexus* 3, 170–185. <https://doi.org/10.1016/j.wen.2020.11.001>

- Zhang, L., Feng, X., Zhu, N., Chen, J., 2007. Role of extracellular protein in the formation and stability of aerobic granules. *Enzyme Microb. Technol.* 41, 551–557.  
<https://doi.org/10.1016/j.enzmictec.2007.05.001>
- Zhao, Y., Li, F., Carvajal, M.T., Harris, M.T., 2009. Interactions between bovine serum albumin and alginate: An evaluation of alginate as protein carrier. *J. Colloid Interface Sci.* 332, 345–353.  
<https://doi.org/10.1016/j.jcis.2008.12.048>
- Zhu, L., Lv, M. Le, Dai, X., Yu, Y.W., Qi, H.Y., Xu, X.Y., 2012. Role and significance of extracellular polymeric substances on the property of aerobic granule. *Bioresour. Technol.* 107, 46–54.  
<https://doi.org/10.1016/j.biortech.2011.12.008>