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# Impact of metal ions on structural EPS hydrogels from aerobic granular sludge

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ABSTRACT

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

## 1. Introduction

The aerobic granular sludge process is an emerging new wastewater technology [1]. Granular sludge is a spherical biofilm with hydrogel properties [2]. Structural extracellular polymeric substances (structural EPS) are hydrogel-forming polymers and considered to be strongly involved in the mechanical strength of aerobic granular sludge [3]. Understanding the gel forming properties and stability of structural EPS will help in better understanding of the aerobic granular sludge stability.

Structural EPS from aerobic granular sludge were previously denoted as alginate-like EPS [4]. Their extraction method is similar to that of alginate [5]. Initial studies on structural EPS and the reported presence of alginates in microbial EPS [6,7] suggested that alginate-like compounds were present in structural EPS. Both, structural EPS and alginate form hydrogels with calcium ions, precipitate as a gel at acidic pH and in both compounds carboxyl groups were detected with FT-IR [8,9]. The FT-IR spectrum of structural EPS was more complex than that of pure alginate. Possible reasons for the appearance of the FT-IR spectrum were the higher complexity of structural EPS compared to alginate or impurities included in the extract together with the alginate-like compounds. Besides the before mentioned similarities, follow up research demonstrated structural EPS to be more complex than alginate. Structural EPS were composed of proteins, neutral sugars, amino sugars, uronic acids and polyphenolic compounds [10].

Hydrophilic polymers containing acidic groups such as alginate have the ability to form ionic cross-linked hydrogels with metal ions. The structure and stability of the ionic cross-linked hydrogel is not only affected by the structure and available functional groups of the polymer, but also by the type of metal ion [11–13]. To understand and assess the gelling behavior of structural EPS, the ionic gel-forming property, the structure of lyophilized gels and the gel stability of structural EPS were compared to that of known, well characterized polymers. Hydrogels of structural EPS were prepared with alkaline earth, transition metal and zinc ions to evaluate the impact of the ion on the gel. The stiffness of

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structural EPS hydrogels was measured by dynamic mechanical analysis (DMA) and quantified by the Young's modulus. The obtained Young's moduli were compared to those of alginate. Structural elements of lyophilized structural EPS hydrogels were analyzed by environmental scanning electron microscopy (ESEM). Furthermore the stability towards disintegration of structural EPS hydrogels in the presence of the strong chelating agent ethylenediaminetetraacetic acid (EDTA) was studied in comparison to hydrogels of the pure polymers alginate, polygalacturonic acid and κ-carrageenan.

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

## 2. Materials and methods

### 2.1. Collection of the sludge sample and extraction of structural EPS

Aerobic granular sludge was collected from the Nereda® pilot reactor of the municipal wastewater treatment plant Utrecht, the Netherlands in March 2016. Granular sludge and flocs were separated by sieving the sludge sample with a stainless steel mesh sieve with a mesh size of 1 mm. The retained fraction containing only the granular sludge was collected and washed with demineralized water. The washed sample was centrifuged at 4000×*g*, the supernatant was discarded and the pellet was frozen at −20°C until being further used. The extraction of structural EPS was performed as described previously [10]. Extracted structural EPS were frozen at −80°C and freeze-dried.

### 2.2. Preparation of gel cylinders

Extracted structural EPS from section 2.1 were dissolved in 0.01 M sodium hydroxide with a concentration of 10% (w/v), guluronic acid rich alginate from the stipe of *Laminaria hyperborea* [61] was dissolved with a concentration of 2.5% (w/v). The composition of the alginate is illustrated in Supplementary material A. Different concentrations were used for structural EPS and alginate to ensure obtaining complete dissolution of the polymers and stable hydrogel cylinders. A hollow plastic cylinder was covered on one side with a dialysis bag with a molecular weight cut off (MWCO) of 3.5 kDa. Dissolved structural EPS and alginate solution, respectively, were transferred into the plastic cylinder and the plastic cylinder was closed with a dialysis bag [14]. The cylinders containing the structural EPS and alginate solutions were dialyzed in a glass beaker containing 150 ml of 100 mM metal solution. Metal solutions used for the dialysis were magnesium chloride, calcium chloride, strontium chloride, barium chloride, manganese sulfate, cobalt sulfate, nickel sulfate, copper chloride and zinc acetate. Dialysis was performed for 24 h with changing of the dialysis solution. After dialysis, gel cylinders of 8 mm height and diameter were obtained and used for the mechanical analysis with DMA.

### 2.3. Dynamic mechanical analysis (DMA) of hydrogel cylinders

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

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

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

### 2.5. Hydrogel stability test towards EDTA

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

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

## 3. Results

### 3.1. Dynamic mechanical analysis of structural EPS hydrogels

To draw conclusions about the overall stiffness and the gel formation of structural EPS hydrogels, structural EPS hydrogel stiffness was compared to that of the well characterized polysaccharide alginate, which is frequently reported to be a constituent of biofilm matrices [6,7]. The gel stiffness was evaluated with different metal ions. Alkaline earth and transition metals were selected as members of both groups of metals were present in aerobic granular sludge (Supplementary material B), and are able to form hydrogels with anionic polymers. In addition their interactions with alginate showed a distinct behavior in terms of affinity and gel stiffness [11,13,15,16]. Alkaline earth metals other than $Mg^{2+}$ resulted in stiffer hydrogels with increasing ionic radius and were with the exception of $Cu^{2+}$ stiffer than those formed with transition metals ($Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$) and $Zn^{2+}$ [13,15,17,18].

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

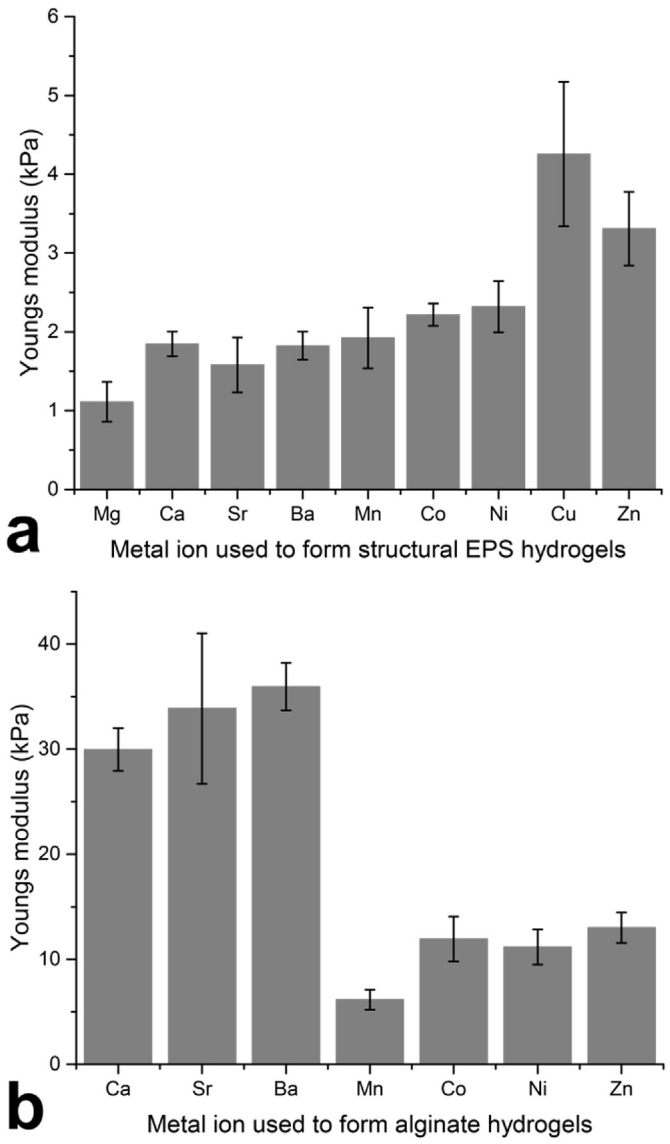


**Fig. 1.** Dynamic mechanical analysis of the gel stiffness quantified in Young's modulus of hydrogels with divalent metal ions of (a) 10% (w/v) structural EPS with $Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ and (b) 2.5% (w/v) alginate with $Ca^{2+}$,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

hydrogels of alkaline earth metals were less stiff than those of transition metal ions.

The stiffness of alginate hydrogels with alkaline earth and transition metal ions is summarized in Fig. 1b. The alginate gel formed with $Mg^{2+}$ was weak and not strong enough to be determined by DMA, which is in accordance to literature [18]. The copper alginate hydrogel was inhomogeneous and did not allow for an accurate and representative stiffness measurement. This was likely due to the high affinity for complex formation of copper with alginate or the pH of the copper solution [12,13]. The alginate gel stiffness increased in the order $Mn^{2+}<Ni^{2+}<Co^{2+}<Zn^{2+}<Ca^{2+}<Sr^{2+}<Ba^{2+}$. Other studies showed similar results with $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ resulting in weaker alginate gels than alkaline earth metals [13,17]. For copper and barium hydrogels there were inconsistent results. It was reported that copper alginate hydrogels were stiffer than barium hydrogels [15] and also that barium hydrogels were stiffer than copper hydrogels [13].

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

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

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

Freeze-dried structural EPS hydrogels formed with $Mg^{2+}$, $Ca^{2+}$ and $Zn^{2+}$ featured different structural patterns indicating that the metal ion had an impact on the overall structure (Fig. 2a – 2c). Independent of the metal ion inhomogeneous structures were present. The visible cavities in the three freeze-dried gels can be structural elements from the original gel or a result of released liquids. Alginate hydrogel structure analysis with SEM was also affected by the drying process and showed structural variations depending on the metal ion [13,18,19].

### *3.3. Gel stability of hydrogel beads towards the chelating reagent EDTA*

EDTA is a common chelating agent used to complex metal ions [20]. It can also be applied to extract EPS from biofilms [21] and was demonstrated to dissolve alginate gels [22]. EDTA solutions of different pH were prepared to evaluate the impact of the available carboxyl groups of EDTA [20] on the disintegration of the hydrogel beads. For this experiment the concentration of EDTA used was the same as that reported to extract EPS from biofilms [21]. Hydrogel beads were prepared with $Ca^{2+}$, $Cu^{2+}$ and $Zn^{2+}$. These metal ions were selected as representative ions as $Ca^{2+}$ and $Cu^{2+}$ gave stiff hydrogels with alginate and $Cu^{2+}$ and $Zn^{2+}$ with structural EPS. Furthermore $Cu^{2+}$ and $Zn^{2+}$ were reported to bind with proteins [23,24].

To further understand the gel properties of structural EPS, the gel stability towards the strong chelating agent EDTA was tested in comparison to the acidic polysaccharides alginate, polygalacturonic acid and κ-carrageenan. Polygalacturonic acid was chosen because galacturonic acid was shown to be present in structural EPS [10] and it was selected instead of pectin as de-esterification likely occurred during the alkaline extraction of structural EPS [25]. K-carrageenan was included to evaluate polysaccharides with different gelling mechanism and other acidic groups, e.g. sulfate half-esters. Polysaccharides containing acidic groups other than carboxyl groups were of interest as the elemental analysis (Supplementary material D) illustrated significant amounts of sulfur and phosphorous in structural EPS. Regarding previous research on EPS this can indicate the presence of sulfate and phosphate groups [26–31].

Independent of the metal ion and the pH of the EDTA solution all polysaccharide hydrogel beads were disintegrated and dissolved after 2 h. Structural EPS hydrogel beads were disintegrated at pH 10.5 after 24 h and completely dissolved after 48 h. At pH 4.5–8.5 structural EPS

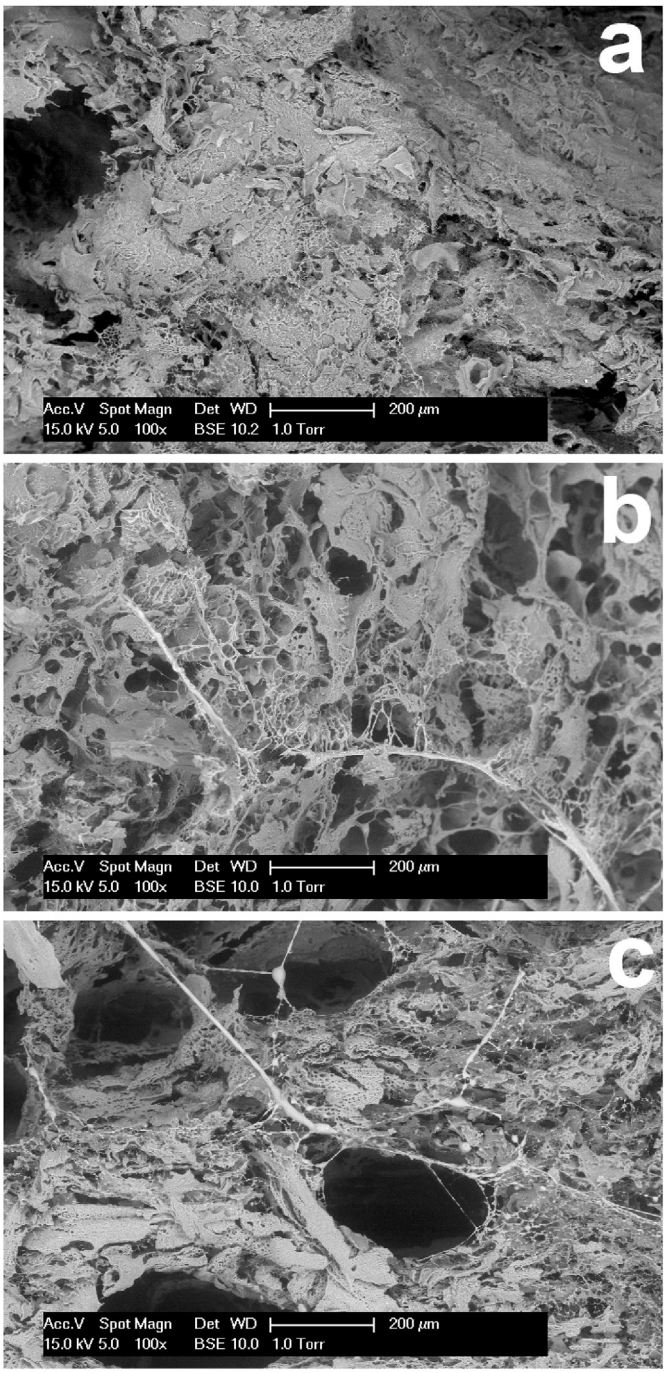


**Fig. 2.** ESEM analysis of freeze-dried structural EPS hydrogels with different metal ions at 100 times magnification. Freeze-dried gels were prepared with (a) magnesium, (b) calcium and (c) zinc.

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

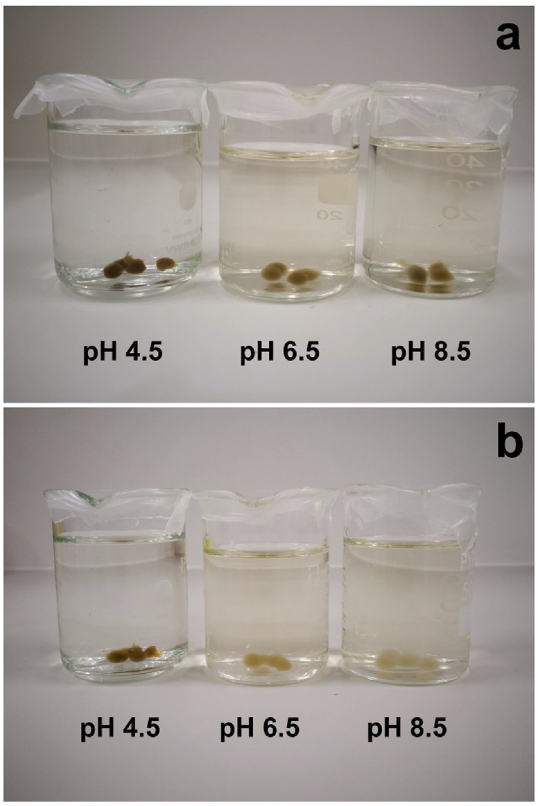


**Fig. 3.** Ca-structural EPS hydrogel beads in 2% (w/v) EDTA solutions at different pH levels after storage at room temperature for (a) one day and (b) for one month.

## 4. Discussion

### 4.1. Comparison of structural EPS and alginate hydrogels

Previous studies showed some similarities of alginate and structural EPS [4,9]. Both are alkaline extracted, form stable hydrogels with $Ca^{2+}$ and contain uronic acids [5,9]. Recent analysis on structural EPS illustrated structural EPS to be much more complex than alginate [10]. Alginate is a linear polysaccharide composed of alternating guluronic and mannuronic acid units [5]. Structural EPS is a mixture of proteins, neutral sugars, amino sugars, uronic acids and humic compounds [10]. Alginate is a very well characterized polymer and reported as constituent of biofilm EPS [6,7]. For this reason, despite the compositional differences of alginate and structural EPS, alginate is an adequate reference as a starting point for investigating and better understanding the properties of structural EPS hydrogels.

The results from this study clearly illustrated differences of alginate and structural EPS hydrogels. Independent of the metal ion, the absolute stiffness values of alginate hydrogels were multiple times higher than those of structural EPS. Alginate formed stiffer hydrogels with copper and alkaline earth metals than with transition metals [13,15,17]. In contrast to alginate structural EPS formed stiffer hydrogels with $Zn^{2+}$ and transition metal ions, especially with copper, than with alkaline earth metals. Interestingly structural EPS were able to form a stable hydrogel with $Mg^{2+}$ which was not possible for alginate. Alginate was reported to from only weak gels with $Mg^{2+}$ [18]. The structure of both, dried alginate gels and freeze-dried structural EPS gels was affected by the metal ion used to form the hydrogel and showed different structural patterns [13,18]. The inhomogeneous structures of dried structural EPS gels can result from the high complexity of their composition and the various molecular interactions. Compared to alginate, structural EPS hydrogels demonstrated a significantly stronger resistance towards disintegration in the presence

of the complexing agent EDTA.

### 4.2. Hydrogel stiffness

Gel formation of alginate and pectin, the partly methylated form of polygalacturonic acid, are considered to be similar [32]. Both polysaccharides form stiffer hydrogels with calcium ions than structural EPS [13,15,33]. The stiffness of alginate and pectin hydrogels is closely related to their composition. A high guluronic acid content in alginate results in the formation of stiff hydrogels due to crosslinking of guluronic acid blocks [15,34]. Mannuronic acid rich alginates are weaker and more flexible than guluronic acid rich alginates [34,35]. The stiffness and calcium ion uptake of pectin hydrogels is influenced by the degree and distribution of methylation as well as by the occurrence of rhamnose gaps in the galacturonic acid chain [36–39]. This shows that not only the amount of available carboxyl groups, but also the conformation and distribution of carboxyl groups are of importance for the hydrogel stiffness. Alginates and pectins have a very large amount of available carboxyl groups which are distributed on a linear polysaccharide chain. Previous analysis of structural EPS has indicated that structural EPS consist of proteins and a much higher diversity of sugar monomers than alginates [10]. It is therefore not surprisingly that gelling with ions that complex with carboxyl groups gave weaker gels for structural EPS than for alginate.

### 4.3. Metal ion sequence of hydrogel stiffness

A correlation of metal ion affinity of alginate and its hydrogel stiffness was reported [13,15,40]. The ion selectivity is influenced by the available functional groups and the conformation of a polymer [11,40]. Proteins, sugars, uronic acids and polyphenolic compounds in structural EPS can interact with metal ions and have different metal ion affinities.

Proteins were shown to bind with copper and zinc [23,24] and to gel with ions [41]. Multiple amino acids have a higher stability constant with transition metal ions than with alkaline earth metals [42,43]. The binding of proteins with zinc was indicated in aerobic granular sludge [44] and copper was illustrated to bind with proteins and humic substances in activated sludge EPS [45]. Humic compounds were demonstrated to have a good binding to transition and alkaline earth metals [46,47]. The phosphate group is another functional group that can be involved in the gelling process of structural EPS. The metal binding of phosphate groups in EPS was previously suggested [29,31] and the presence of phosphorous in structural EPS is an indication for phosphate groups. Phosphate groups of biological molecules were demonstrated to bind with alkaline earth metals [48] and polyphosphates were shown to favor transition over alkaline earth metal ions [49].

The ion preference of compounds containing functional groups also present in structural EPS can be the reason for structural EPS to form stiffer hydrogels with transition than with alkaline earth metal ions. The ability of amino acids and phosphates to bind with magnesium [42,43,48,49] can explain stronger $Mg^{2+}$ hydrogels of structural EPS than of alginate. An experimental analysis of metal selectivity towards structural EPS and additional analysis of functional groups present in structural EPS will further strengthen this assumption.

### 4.4. Stability towards dissolution by EDTA

Exposing the hydrogel beads of the polysaccharides alginate, polygalacturonic acid and κ-carrageenan to EDTA quickly resulted in the dissolution of these hydrogels. Structural EPS hydrogels showed a stronger integrity in the presence EDTA and were only quickly dissolved with EDTA at pH 10.5. The dissolution at this pH is in accordance with structural EPS also being extracted at pH 10.5 thus being well soluble under these conditions. The partial leaching out of structural EPS hydrogel compounds at lower pH levels was closely related to the pH increase which was the result of two different effects. Increasing the pH approaches the extraction pH for structural EPS and increases the binding capacity of EDTA for divalent cations [20].

The stability of structural EPS hydrogels towards EDTA is difficult to explain. Independent of the metal ion EDTA has very high stability constants with metal ions which are generally higher than those of compounds present in structural EPS [42,43]. There are different possibilities for the stability of structural EPS hydrogels towards EDTA. Structural EPS contain proteins, carboxyl and possibly sulfate and phosphate groups. Proteins can interact with phosphate and sulfate [50,51] and can gel through covalent and non-covalent interactions [41]. Thus compounds of structural EPS may have not only interacted with the metal ions, but also with each other during the gel formation. These interactions could be the reason for the hydrogel to remain intact, even when metal ions were removed from the gel by EDTA. Furthermore the tertiary structure formed by structural EPS could hinder or retard the complexing of metal ions by EDTA. This indicates a strong metal binding that strengthens the assumption of EPS being a protective layer for microbial cells against increased metal concentrations [29]. The structure and properties of gel-forming bacterial polysaccharides were reported to be stabilized by hydrogen bonding (gellan, granulan) [52,53] and hydrophobic interactions (curdlan) [54]. It is possible that such intermolecular interactions are also present in structural EPS hydrogels and that hydrophobic interactions are involved in shielding metal ions from chelation by EDTA. These possibilities however are speculative and need to be further investigated. More in-depth analysis of structural EPS hydrogels can also reveal if distinct gelling mechanisms such as the for alginate described egg-box model [55,56] are involved in the gel formation.

The gel beads were still visibly intact, but there was a slow and partial leaching out of structural EPS at pH 4.5–8.5. This can indicate a slow disintegration of the overall gel structure or a dissolution of structural EPS compounds which were not or only little involved in the hydrogel structure. It is possible that the gel was still present, but with lower stiffness than before the treatment with EDTA. Analyzing the amount of EDTA complexed metals, the EDTA released structural EPS compounds and comparing the stiffness of the gel beads before and after treatment will give more information on the gelling and gel stability of structural EPS. Additionally more insights into the gelling mechanisms of structural EPS can be obtained by isothermal titration calorimetry (ITC). This technique was already used to investigate the gelling mechanism of alginate [57]. ITC is a very sensitive analysis which allows to measure ion-polymer and polymer-polymer interactions. Once the composition of structural EPS is better known, this technique will be valuable to further analyze the interactions of structural EPS with itself and metal ions.

### 4.5. Hydrogel analysis of structural EPS and aerobic granular sludge

The here reported findings gave new insights into the metal hydrogel properties of extracted structural EPS from aerobic granular sludge in terms of gel stiffness and gel stability. Structural EPS are considered to be responsible for the structure of the granular matrix [3]. In this study structural EPS were shown to form stable hydrogels with multiple metal ions, and several molecular interactions were suggested to be involved in the gel formation and stability. Not only extracted structural EPS interact with metal ions and behave like hydrogels, but also the granule itself [2,58]. Considering the previously demonstrated effects of calcium ions on biofilms with stimulating faster granulation [58] and stiffer biofilms [59] and the here obtained results of different metals resulting in different stiffness and structural features of EPS hydrogels, the composition of present metal ions in the medium around a biofilm will likely also affect the stiffness and structure of intact biofilms.

The here used structural EPS were extracted from the granular sludge and were not present in their native form. Based on the here obtained results it cannot be stated to which extent structural EPS contribute to the overall stiffness of intact granules. For instance the alkaline extraction procedure will lead to (partial) deacetylation of the sugars [25] thus

changing the composition of the extracted structural EPS. Stiffness values quantified as Young's modulus of intact biofilms of different origin were already reported [59,60]. The stiffness measurement of intact granules will also be beneficial here. To compare the stiffness of granules and structural EPS hydrogel beads can illustrate to what extent extracted structural EPS resemble the granular stability. This would also indicate to which extent the high granular stability is related to the combination of cells linked to EPS or to EPS only. Expanding this analysis on other types of biofilm will help to understand the stability of biofilms and the impact of EPS on biofilm stability and strength in general.

## 5. Conclusion

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2019.100011.

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