Chemical and physical properties of alginate-like exopolymers of aerobic granules and flocs produced from different wastewaters

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GRAPHICAL ABSTRACT
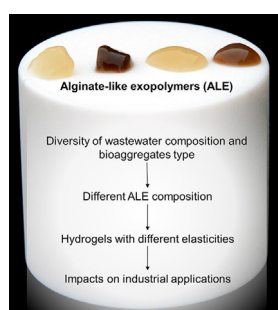
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ABSTRACT

The influence of wastewater (WW) composition and the bioaggregates types (floccular vs. aerobic granular sludge - AGS) on the content, physical-chemical, hydrogel and rheological properties of Alginate-Like Exopolymers (ALE) was studied. Results showed that ALE are a complex mixture of proteins, humic acids and polysaccharides. Overall, rather similar ALE content and composition was observed for the different types of sludge. Only the AGS fed with acetate and propionate yielded significantly larger amount of ALE (261 ± 33 mg VS_{ALE}/g VS_{sludge}, +49%) and of uronic sugars in ALE (254 ± 32 $mg_{glucuronic\ acid}$/g VS_{ALE}, +62%) than bioaggregates fed with no/very little volatile fatty acids. Mannuronic acids are involved in the cohesion of the hydrogels. ALE hydrogels elasticity changed significantly with the type/origin of the bioaggregates. ALE hydrogels elasticity from AGS was always higher than from flocs when fed with real WW. Hence, different types of sludge impact the properties of the recovered ALE.

1. Introduction

Resource recovery from wastewater (WW) represents a relevant approach to reduce energy consumption, greenhouse gases emissions and waste generation from wastewater treatment plants (WWTP) (Wang et al., 2015). In a near future, many conventional WWTPs will be converted into water resource recovery facilities (WRRF). These WRRFs will potentially represent a net source of valuable bioproducts, such as

Table 1
Details of the different types of bioaggregates and wastewater used in this study.

Sample	Type of bioaggregate (sludge)	Feed wastewater	Carbon source
AGS$_{simple\ synthetic}$	Aerobic granular sludge	Simple synthetic wastewater	Volatile fatty acids (VFA), soluble COD – 1/2 acetate, 1/2 proprionate
AGS$_{complex\ synthetic}$	Aerobic granular sludge	Complex synthetic wastewater	1/3 COD: Acetate and propionate (soluble COD)
			1/3 COD: amino acids and glucose (soluble COD)
			1/3 COD: starch and peptone (particulate COD)
AGS$_{real}$	Aerobic granular sludge	Primary effluent from municipal wastewater	Complex (40% soluble COD, 60% particulate COD)
AS$_{real}$	Flocs from activated sludge	Primary effluent from municipal wastewater	Complex (40% soluble COD, 60% particulate COD)

alginate-like exopolymers (ALE), bioplastics, cellulose, phosphorus or biogas (Van Der Hoek et al., 2016). Current market conditions are especially favourable for the recovery of ALE from granular sludge. In Netherlands, 85 ktons of ALE are expected to be recovered from 10 different WWTP by 2030, generating 170 million of euros (Van Leeuwen et al., 2018). However, industrializing the recovery of ALE requires to better understand several key aspects. A key question is specially to understand how the composition and hydrogel properties of ALE change in response to the wastewater composition or the type of bioaggregates (flocs or granules). Acquiring more knowledge about the composition and hydrogel properties of ALE can help developing applications in the industry, for instance in the chemical fields and as rheology conditioner/emulsion stabilizer in the field of formulations (Lotti et al., 2019).

ALE are a mixture of biopolymers extracted from the extracellular polymeric substances (EPS) matrix of bacterial aggregates under alkaline conditions, and further recovered by acidic precipitation (Felz et al., 2016). Recent studies suggest that ALE contain proteins, polysaccharides including polyuronic ones, humic acids and lipids (Felz et al., 2019; Lin et al., 2015). However, the origin of those different biopolymers in the ALE extracts is unclear. Several factors were proposed to explain EPS secretion in biological systems, such as the reactor's operating conditions, the WW composition, the microbial community composition or protocol of extraction (Adav and Lee, 2008; Ding et al., 2015; Liu et al., 2004; McSwain et al., 2005; Tu et al., 2012; Yang et al., 2019). However, little information is actually available on the factors and mechanisms that govern the ALE composition and content. ALE were first extracted from aerobic granular sludge (AGS) cultivated with a mixture of municipal and slaughterhouse WW (Lin et al., 2010). ALE have further been reported to be key structural polymers of AGS (Felz et al., 2016; Yang et al., 2014). However, ALE have also been extracted from activated sludge (Lin et al., 2013; Sam and Dulekgurgen, 2015). The amount of ALE recovered from granules can be twice as large as that recovered from flocs: 160 ± 4 mg/g (VSS ratio) and 72 ± 6 mg/g (VSS ratio), respectively (Lin et al., 2013). Lin et al. (2013) also reported some differences in the chemical structure of ALE recovered from flocs and AGS, which ultimately influence their hydrogel and mechanical properties. On the other hand, other studies reported hydrogel properties for ALE extracted from flocculent sludge, similarly to commercial alginate or to ALE extracted from AGS (Sam and Dulekgurgen, 2015; Schambeck et al., 2020). Thus, it remains rather unclear to what extent ALE isolated from activated sludge or from granules have similar or distinct composition/hydrogel properties.

Also, those contradictory observations about ALE content in activated and granular sludge were obtained for bioaggregates cultivated with different wastewaters (WW): mixture of slaughterhouse and municipal WW (Lin et al., 2013), brewery WW (Sam and Dulekgurgen, 2015) or municipal WW (Schambeck et al., 2020). The influent composition may affect the microbial community, and in turn the production of biopolymers such as ALE. To what extent ALE composition and gelling properties is governed by the types of bioaggregates (flocs vs. granules) or by the WW composition must be identified. High concentration in organic substrates were correlated with high ALE contents (more than 100 mg ALE/g VSS) in granular sludge (Yang et al., 2014), but very variable ALE contents were in fact reported for AGS fed with

different WW. For instance, AGS fed with synthetic saline WW yielded very little ALE, 5% w/w (VSS based) (Li et al., 2017; Meng et al., 2019), AGS fed with propionate yielded 10% w/w (VSS based) (Yang et al., 2014), while AGS fed with real WW yielded around 16% w/w (VSS based) (Lin et al., 2010, 2013). Hence, the comparison of those values suggests that WW composition might significantly influences the content and possibly also the composition and hydrogel properties of ALE. For further industrialisation of ALE recovery, it is essential to better understand if ALE in sludge is influenced by the type of bioaggregates and/or by the WW composition.

Therefore, the current study aims at (1) better understanding how the WW composition and type of bioaggregate influence the content and composition of ALE, and (2) getting insights about the link between the ALE composition and its hydrogel properties. To answer those questions, EPS and ALE were extracted from different types of bioaggregates (flocs vs. granules) and from granules fed with different WW in terms of organic substrates. The EPS/ALE content were quantified by gravimetric methods. The chemical composition of the different ALE extracts was characterized by several different and complementary analytical methods: biochemical colorimetric assays (polysaccharides, proteins, humic substances), FTIR, Raman spectroscopy and enzymatic tests. The mechanical properties of hydrogels were quantified via rheometric tests, and correlated with the composition of the ALE extracts.

2. Materials and methods

2.1. Cultivation of aerobic granules and flocs

EPS and ALE were extracted from different microbial aggregates (flocs and granules) fed with different types of WW in terms of organic substrate (Table 1) (Layer et al., 2019). Flocs were cultivated with a real primary effluent WW, composed of 40% of soluble COD and 60% of particulate COD. Granules were cultivated with different synthetic or municipal WW: (1) simple synthetic substrate, (2) complex synthetic substrate, and (3) real primary effluent WW. The simple synthetic WW comprised of acetate and propionate (at equal COD fraction). The complex synthetic WW comprised of acetate/propionate (1/3 of the total COD), non-fermented soluble COD (amino acids and glucose, 1/3 COD) and particulate COD (starch and peptone, 1/3 COD). The real primary effluent used for the cultivation of the AGS was similar to the one used for the cultivation of the flocs. More details about the different WW composition and the properties of the resulting AGS can be found in Layer et al. (2019).

2.2. Extraction of EPS and ALE

The extraction of biopolymers was performed according to the protocol proposed by Felz et al. (2016). A known mass of wet sludge was centrifuged (3100 g, room temperature, 25 min) prior discharging the supernatant. The pellet was then transferred to a 250 mL baffled flask filled with demineralized water, Na$_2$CO$_3$ and equipped with a magnetic stirrer. A ratio of 3:50:0.25 (sludge mass (g), demineralized water (mL) and Na$_2$CO$_3$ (g)) was kept during the extractions. The flask was immersed in a water bath (80 °C) and stirred for 35 min at 400 rpm.

The mixed liquor was then centrifuged (3500 *g*, room temperature, 25 min) to recover the supernatant that comprised the solubilized EPS. Acidic ALE were extracted from EPS by acidic precipitation: 1 M HCl was added to the soluble EPS solution to reach a final pH of 2.20 ± 0.05 while stirred at approximately 100 rpm. Extractions were made in at least eight samples (n = 8–9). Results are expressed in terms of volatile solids (VS) according to Felz et al. (2016). Student t-tests were performed at 95% confidence level to evaluate differences in the EPS and ALE contents from the four different types of sludge.

2.3. Ionic hydrogel formation test

Gelation tests were performed to verify the hydrogel properties of the different ALE extracts according to Felz et al. (2016). Soluble Na^+-ALE were first obtained by the addition of 0.5 M NaOH to the precipitated acidic ALE until pH reached 8.5. Afterwards, Na^+-ALE were dripped into a 2.5% (w/v) $CaCl_2$ solution to evaluate the formation of drop-shaped Ca^{2+}-ALE beads, which indicates the ionic hydrogel forming property.

2.4. EPS and ALE chemical composition

2.4.1. Humic acids, proteins and polysaccharides content in EPS and Na^+-ALE

Proteins and humic acids in EPS and Na^+-ALE were determined using the modified Lowry method (Frolund et al., 1995), with bovine serum albumin (BSA) and humic acid sodium salt (both from 0 to 1000 mg/L) as standards. Colorimetric assays are not absolute quantitative methods (Le et al., 2016; Le and Stuckey, 2016), but those methods provide relevant insights for comparing the biochemical composition of the extracts. All measurements were performed in triplicate. 200 μL of solution either with or without $CuSO_4$ was added to 40 μL of sample in 48-well microplates and shaken for 30 s. After 10 min incubation at room temperature, 20 μL of Folin-Ciocalteu Phenol Reagent 1 N was added to each well. The microplate was then incubated and shaken for 30 min at room temperature before the absorbance was measured at 720 nm. Concentrations of protein and humic acids were calculated based on the following three equations (Frolund et al., 1995):

$$A_{total} = A_{protein} + A_{humic} \tag{1}$$

$$A_{protein} = 1.25(A_{total} - A_{blind}) \tag{2}$$

$$A_{humic} = A_{blind} - 0.2A_{protein} \tag{3}$$

where A_{total} is the total absorbance with $CuSO_4$, A_{blind} is the total absorbance without $CuSO_4$, $A_{protein}$ is the absorbance due to proteins and A_{humic} is the absorbance due to humic acids. Using standard curves, $A_{protein}$ and A_{humic} were then converted to concentrations expressed in mg equivalent BSA or mg equivalent humic acids per g VS, respectively.

Polysaccharide content in EPS and Na^+-ALE was measured after acid hydrolysis and reaction of the osidic monomers with the anthrone reagent. The classical Anthrone method was adapted with a double absorbance detection (620 nm and 560 nm) in order to discriminate the quantification of uronic from neutral sugars (Rondel et al., 2013). Indeed, uronic monomers exhibit higher absorbance at 560 nm compared to 620 nm while it is the opposite for the neutral monomers. Double absorbance monitoring of EPS and Na^+-ALE samples in addition to calibration of the method with glucose (Glc) and glucuronic acid (Gla) as standards (0 to 100 mg/L and 0 to 400 mg/L, respectively) allows quantifying the neutral and uronic sugars separately in a simultaneous assay.

All measurements were performed in triplicate. 2 g/L anthrone reagent was prepared by dissolving anthrone in 98% sulphuric acid. 200 μL of 2 g/L anthrone reagent was added to 100 μL of sample or standards dispensed in 48-well microplates. After 30 min incubation at

60 °C, absorbance of standards and samples at 560 and 620 nm were recorded at room temperature. Neutral and uronic sugars were quantified according to the following equation (Rondel et al., 2013):

$$[Glc] = \frac{(A_{560} \times b_{620} - A_{620} \times b_{560})}{(a_{560} \times b_{620} - a_{620} \times b_{560})} \tag{4}$$

$$[Gla] = \frac{(A_{560} \times a_{620} - A_{620} \times a_{560})}{(a_{620} \times b_{560} - a_{560} \times b_{620})} \tag{5}$$

where [Glc] is the concentration of neutral sugars expressed in equivalent Glc, [Gla] is the concentration of uronic sugars expressed in equivalent Gla, A_i is the absorbance of sample at i nm (either 560 nm or 620 nm), a_i is the slope of glucose calibration curve at i nm, and b_i is the slope of glucuronic acid calibration curve at i nm. Concentration of polysaccharides in EPS and Na^+-ALE are expressed in terms of mg of equivalent Glu or Gla per g VS.

2.4.2. Fourier transform infrared spectroscopy (FTIR)

Spectra were recorded on a Biorad FTS 575C instrument equipped with a mercury cadmium telluride (MCT) detector and a 9-reflection diamond ATR unit with KRS-5 optics (SensIR Technologies, 15 Great Pasture Road, Danbury, CT 06810–9931). The diameter of the round diamond disk was 4 mm. Scans from 4000 to 400 cm^{-1} were collected at 2 cm^{-1} resolution versus the appropriate background. Single beam background spectra were collected with the cleaned, uncoated diamond disk. The diamond ATR disk was then coated with Na^+-ALE sample over the disk. The solutions were gently dried under a N_2 stream and absorbance spectra were collected several times to assure that the formed layer of organic material was dry. Results are presented as an average of three measurements.

2.4.3. Raman spectroscopy

Raman spectra of Na^+-ALE samples was measured with a Brucker SENTERRA Raman Microscope over the spectral range 100–3500 cm^{-1}. Profiles were acquired with a 785 nm laser wavelength and a 50x objective lens. The following parameters were used: 100 mW of laser power, 5 sweeps and 10 s of integration time.

2.4.4. Rheological property

The rheological properties of both Na^+-ALE samples (before gelation) and Ca^{2+}-ALE samples (after gelation) were analysed. The rheological properties of the samples were monitored both at their original dry matter content (as obtained by the extraction protocol) and at normalized dry matter content (2.3% of dry matter). Na^+-ALE samples were heated up to 40 °C and then 5 mL were poured in crystal-grade polystyrene dishes (35 × 10 mm). After cooling down to 4 °C, 2.5% (w/v) $CaCl_2$ solution was sprayed in excess over the Na^+-ALE samples and at least 24 h were waited to trigger hydrogel formation. Rheological measurements were performed on a MCR 501 rheometer (Anton Paar, Austria). The Na^+-ALE samples were measured with a cone-plate (CP50, Anton Paar, Austria) geometry and the Ca^{2+}-ALE samples were measured in parallel plate mode, using sandblasted 25 mm diameter stainless steel plates (PP25 S Anton Paar, Austria) and a gap size of 1 mm. Amplitude sweeps (γ = 0.01 – 100%, f = 0.5 Hz) were performed to ensure the linear viscoelastic (LVE) region for the subsequent frequency sweeps (f = 0.1 – 1 Hz, γ = 0.1%). For sample loading, the Na^+-ALE samples were transferred from the tubes to the lower measuring plate using a 1 mL micropipette. The Ca^{2+}-ALE samples were gently loaded using a spatula and the upper plate was slowly lowered onto the ALE. A solvent trap containing moist sponges was placed over the sample. All measurements were performed under temperature controlled at 22 °C.

2.4.5. Enzymatic degradation of Ca^{2+}-ALE beads

Enzymatic degradation tests were performed to evaluate what chemical group/bond was related to the hydrogel properties of ALE.

Different enzymes, which attack specific chemical groups/bonds were tested to assess the contribution of the targeted bonds to the mechanical stability of the gels. Those tests were performed only for the Ca^{2+}-ALE recovered from AGS_{real}. The different enzymatic digestion tests were performed in 48-well microplates (24 h, 24 °C, moderate agitation). Pictures of the hydrogel beads were then recorded after enzymatic attack using a stereomicroscope (SZX10, Olympus, Japan). Papain from papaya latex (EC 3.4.22.2, Sigma-Aldrich, molecular weight 21 kDa) was used for cleaving amide type peptide bonds of basic amino acids, leucine and glycine, and to hydrolyse esters and amides. Lysozyme from chicken egg white (EC 3.2.1.17, Sigma-Aldrich, molecular weight 14.4 kDa) was used for the digestion of β (1 → 4) linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine in N-acetyl amino glycans. Protease from Bacillus sp. (EC 232–752-2, Sigma-Aldrich, 20 to 27 kDa) (Savinase®) was applied for the digestion of amide type linkages in proteins and peptides. Finally, alginate lyase (polyβ-D-mannuronate lyase, EC 4.2.2.3, Sigma-Aldrich, 38 kDa) was employed for the digestion of osidic linkages found in uronic sugars as alginate. All enzymatic solutions were prepared with distilled water. Controls with denatured enzymes (previously heated up to 90 °C for 15 min) and without enzymes addition were also performed. The following enzymes concentration were used for the digestion tests: 12 U/mL for papain, 420,000 U/mL for lysozyme, 10% (v/v) for savinase and 100 U/mL for alginate lyase. The unit of enzymatic activity (U) was defined according to the supplier's definition.

3. Results and discussion

3.1. ALE composition

3.1.1. EPS and acidic ALE content

The four different types of sludge were surprisingly characterised by very similar extracted EPS and acidic ALE contents (Table 2). Only the granules fed with simple synthetic WW (acetate/propionate) yielded substantially more extracted EPS and acidic ALE than the flocs/granules fed with synthetic complex or real WW: 598 ± 37 mg VS_{EPS}/g VS_{sludge}, 449 ± 70 mg VS_{ALE}/g VS_{EPS}, and 261 ± 33 mg VS_{ALE}/g VS_{sludge}, compared to 472–507 mg VS_{EPS}/g VS_{sludge}, 333–391 VS_{ALE}/g VS_{EPS}, 165–184 mg VS_{ALE}/g VS_{sludge}, respectively (n = 8–9 measurements). Consequently, the $AGS_{simple\ synthetic}$ contained, in average, 22% more EPS in sludge, 21% more ALE in EPS and especially 49% more ALE in sludge than the other samples. Also, the type of bioaggregate did not clearly influence the extracted EPS and acidic ALE contents (Table 2). Flocs and granules fed with the same real WW indeed had similar EPS and acidic ALE content.

3.1.2. Polysaccharide, protein and humic acid content in EPS and ALE

All EPS extracts were characterised by a very complex composition in terms of sugars, proteins and humic acids, as indicated by the results of the colorimetric assays (Fig. 1A). While differences in the EPS composition of the different types of aggregates were expected, results from the colorimetric assays proved rather similar. The $EPS_{AS\ real}$ had the lowest proteins (137 ± 28 mg$_{BSA}$/g VS_{sludge}) and sugars contents (18 ± 3 mg$_{glucose}$/g VS_{sludge} and 48 ± 13 mg$_{glucuronic\ acid}$/g VS_{sludge}) while the $EPS_{AGS\ simple\ synthetic}$ presented the highest contents

(309 ± 31 mg$_{BSA}$/g VS_{sludge}, 64 ± 12 mg$_{glucose}$/g VS_{sludge} and 153 ± 40 mg$_{glucuronic\ acid}$/g VS_{sludge}). The AGS fed with simple synthetic WW (acetate/propionate) thus yielded up to 255% and 219% more neutral and uronic sugars than the aggregates fed with complex or real WW. In addition, the $EPS_{AGS\ real}$ yielded the highest humic acid content (181 ± 18 mg$_{humic\ acids}$/g VS_{sludge}).

Colorimetric assays also confirmed the complex composition of the Na^+-ALE extracts, with sugars, proteins and humic acids (Fig. 1B and 1C). The Na^+-ALE extracts from $AGS_{simple\ synthetic}$ had higher neutral sugar, uronic sugar and protein contents than the other types of sludge. $AGS_{simple\ synthetic}$ especially contained a large amount of uronic sugars (254 ± 32 mg$_{glucuronic\ acid}$/g VS_{ALE}), around 62% more than in the other sludges. Neutral sugars content was relatively low in all Na^+-ALE extracts, ranging from 34 ± 5 mg$_{glucose}$/g VS_{ALE} in $AGS_{complex\ synthetic}$ to 53 ± 1 mg$_{glucose}$/g VS_{ALE} in $AGS_{simple\ synthetic}$. An enrichment of humic substances was observed in all the Na^+-ALE extracts compared to the EPS extracts, e.g. up to 10% in Na^+-ALE_{AS} (Supplementary data). For real WW, a protein to humic acids ratio of around 1 was observed for the Na^+-ALE extracts of both the flocs and granules. A much higher protein to humic acids ratio was measured for the $AGS_{simple\ synthetic}$. No clear influence of the type of bioaggregates on the composition of the Na^+-ALE extracts was noticed.

3.1.3. Na^+-ALE extracts characterisation using FTIR and Raman

FTIR analyses confirmed the complex composition of the Na^+-ALE extracts, as already suggested by the results of the colorimetric assays. Several peaks observed on the FTIR spectra can be attributed to different chemical groups such as polysaccharides, proteins and humic acids.

Overall, the FTIR spectra of the different Na^+-ALE proved rather similar to each other (Supplementary data). Only very small differences in the presence/absence of some peaks were observed. The Na^+-$ALE_{AGS\ simple\ synthetic}$ and Na^+-$ALE_{AGS\ complex\ synthetic}$ presented a weak peak at 826 and 818 cm^{-1} that can be attributed to mannuronic acid residues (Sam and Dulekgurgen, 2015; Sartori et al., 1997; Seviour et al., 2012). This peak was not observed for Na^+-ALE extracted from aggregates fed with real WW (both flocs and granules). Moreover, the FTIR spectra of Na^+-$ALE_{AGS\ complex\ synthetic}$, Na^+-$ALE_{AGS\ real}$ and Na^+-$ALE_{AS\ real}$ have similar spectra in the 1397–1232 cm^{-1} region with the spectrum of ALE enriched in guluronic acids blocks obtained by Lin et al. (2013). Therefore, the ALE extracted from granules cultivated with VFA-rich WW may be richer in mannuronic acid blocks (M) than guluronic acid blocks (G). But overall differences in the composition of the Na^+-ALE extracts remain minor. In addition, no specific influence of the type of bioaggregates could be observed.

Raman spectroscopy also confirmed the complex composition of all the Na^+-ALE, with signals assigned to polysaccharides, proteins and humic acids (Supplementary data). In addition, all Raman spectra of Na^+-ALE were very similar to each other. The broad peak at near 1377 cm^{-1} can be ascribed to amorphous carbon and to humic-like substances in biofilms (Chao and Zhang, 2012; Ivleva et al., 2009), to polyanionic polysaccharides and amino acids (Ivleva et al., 2008), and to indole ring of the amino acid tryptophan (Lin et al., 2018).

Table 2
EPS content in sludge, acidic ALE content in EPS and acidic ALE content in sludge in terms of VS in each sludge sample (AGS_{real} n = 8, AS_{real} n = 9, $AGS_{complex\ synthetic}$ n = 8, $AGS_{simple\ synthetic}$ n = 8, where n means the number of measurements).

	EPS content in sludge (mg VS_{EPS}/g VS_{sludge})	Acidic ALE content in EPS (mg VS_{ALE}/g VS_{EPS})	Acidic ALE content in sludge (mg VS_{ALE}/g VS_{sludge})
$AGS_{simple\ synthetic}$	598 ± 37	449 ± 70	261 ± 33
$AGS_{complex\ synthetic}$	507 ± 40**	333 ± 86*	165 ± 32***
AGS_{real}	495 ± 45**	388 ± 65	184 ± 18***
AS_{real}	472 ± 31***	391 ± 26*	176 ± 12***

*p < 0.05, **p < 0.01, ***p < 0.001 vs. $AGS_{simple\ synthetic}$ (Student t-tests performed at 95% confidence level).
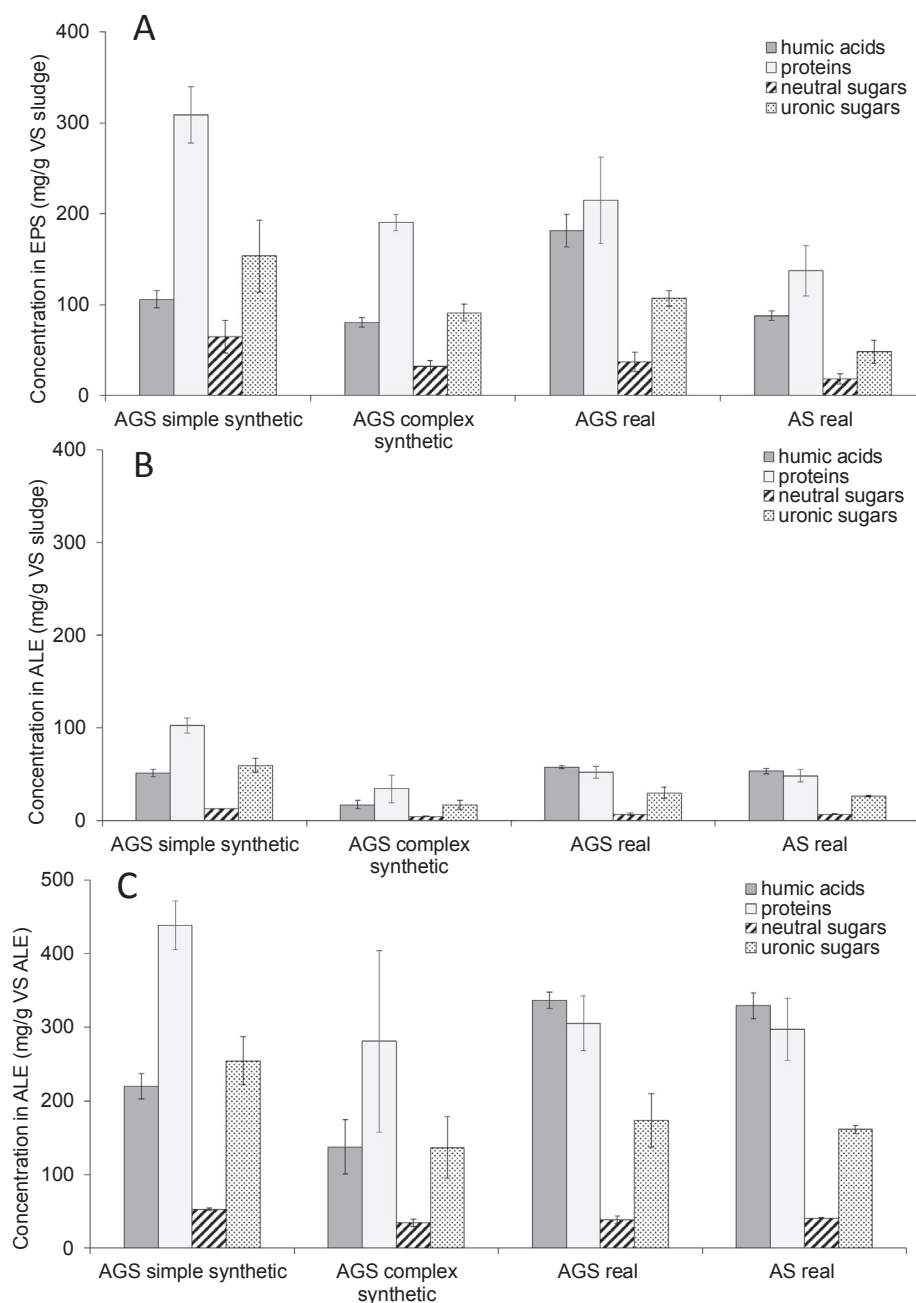
Fig. 1. Effect of type of bioaggregate and WW composition on the humic acids, proteins (BSA equivalent), neutral (glucose equivalent) and uronic sugars (glucuronic acid equivalent) contents in (A) the EPS extracts (mg/g VS_{sludge}), (B) the Na^+-ALE extracts (mg/g VS_{sludge}), and in (C) the Na^+-ALE extracts (mg/g VS_{ALE}) in each sludge sample. The error bars illustrate the standard deviation of the analytical replicates (n = 3).

3.1.4. ALE composition: A complex mixture of polysaccharides, proteins and humic acids

A main result of this study is that Na^+-ALE are a very complex mixture of polysaccharides, proteins and humic acids. While ALE extracted from granules are often presented as extracts enriched in polysaccharides (Lin et al., 2010; Sam and Dulekgurgen, 2015), results herein also demonstrate the significant presence of proteins and humic substances. The complex composition of Na^+-ALE was confirmed by using several complementary methods (colorimetric assays, FTIR, Raman) applied to various sludge samples. Although colorimetric assays are only semi-quantitative analytical methods (Le et al., 2016; Le and Stuckey, 2016), these methods provided relevant insights about the composition of Na^+-ALE extracts. For the analysis of sugars, the modified anthrone method based on a double calibration and on a double

absorbance monitoring of total and uronic sugars at 560 and 620 nm was used, respectively. Such analytical method helps quantifying polyuronic among total carbohydrates, since the uronic sugars are generally not discriminated in other colorimetric assays (Dreywood, 1946; Dubois et al., 1956).

Results from colorimetric assays indicated that uronic sugars were the most abundant polysaccharides found in the Na^+-ALE extracts, particularly in Na^+-$ALE_{AGS\ simple\ synthetic}$, while a low amount of neutral sugars was detected (Fig. 1). Results from FTIR and Raman spectroscopy also support those observations. FTIR spectra of the Na^+-ALE extracts from synthetic WW further suggested the presence of mannuronic acid, especially in Na^+-$ALE_{AGS\ simple\ synthetic}$. The presence of mannuronic acids blocks in ALE have been reported for AGS fed with propionate (Yang et al., 2014). Herein, the presence of mannuronic

acids in ALE was also confirmed by the results of enzymatic tests (see Section 3.3.2). Overall, results support recent findings indicating that ALE are composed of different polysaccharides, divided in both neutral and uronic ones (Felz et al., 2019).

Another interesting result is the significant presence of proteins in all ALE extracts, confirmed by colorimetric assays, FTIR and Raman analyses. Results from colorimetric assays suggested proteins contents of ~ 300 to 440 $mg_{BSA}/mgVS_{ALE}$. Based on the FTIR analyses, a typical protein signature was observed for all Na^+-ALE spectra, with two clear peaks associated to amide I (1628 to 1638 cm^{-1}) and amide II (1533 to 1535 cm^{-1}). Besides, sodium salts of amino acids show the N–H stretching vibrations at 3400–3200 cm^{-1} and the characteristic carboxylate ions band appears near 1400 cm^{-1} (Silverstein et al., 2005), as also observed in Na^+-ALE spectra. Finally, the Na^+-ALE Raman spectra were similar to the spectrum of AGS dominated by ammonium-oxidizing bacteria, for which the intense peak at 1365 cm^{-1} was attributed to the indole ring of the amino acid tryptophan (Lin et al., 2018). Thus, it is evident that ALE extracts do not only contained sugars, but also proteins in (likely) non-negligible quantities.

Humic acids also represented a major fraction of ALE extracts. The Na^+-ALE spectra obtained with FTIR were very similar to the ones of humic acids isolated from composted activated sludge (Amir et al., 2010). Humic acids are a mixture of compounds mainly composed of phenolic and carboxylic acid in majority, but can also contain enolic, quinone, ether functional groups, sugars and peptides (De Melo et al., 2016). They are abundant in various terrestrial and aquatic environments and originate from the humification process of several compounds such as amino acids, lignin, pectins and carbohydrates (Peña-Méndez et al., 2005). Humic substances are resistant to complete biodegradation (Flemming and Wingender, 2010). Therefore, it is likely to find them in real WW and ultimately in the EPS matrix of flocs and granules, like indicated by the results (Fig. 1, FTIR and Raman). A noticeable content in humic substances was also detected in the extracts of the sludge fed with synthetic WW and might come from the groundwater used for preparation of the feed solution. Overall, results clearly indicate the presence of humics acids in Na^+-ALE extracts.

Hence, the results obtained from different and complementary analytical methods prove that Na^+-ALE extracts are a complex mixture of polysaccharides, proteins and humic-like substances.

3.1.5. Influence of wastewater composition and the type of the bioaggregate on ALE content and composition

The results indicate that the compositions and contents of the different Na^+-ALE extracts were surprisingly similar. In fact, major differences were expected, due to the very diverse types of the bioaggregates that were analysed. But only the $AGS_{simple\ synthetic}$ fed with acetate and propionate presented noticeable differences in their ALE composition and content.

$AGS_{simple\ synthetic}$ yielded the highest EPS and Na^+-ALE contents and had the highest contents in proteins and uronic sugars (Table 2 and Fig. 1). Overall, the AGS fed with acetate/propionate yielded 49% more ALE (mg VS_{ALE}/g VS_{sludge}, Table 2, n = 9 measurements) and 62% more uronic sugars ($mg_{glucuronic\ acid}$/g VS_{ALE}, Fig. 1, n = 3 measurements) than the other sludges. On the contrary, sludge fed with more complex WW, which contains large amount of non-diffusible organic substrates and almost no/little volatile fatty acids, yielded lower amounts of proteins and uronic sugars. The high contents in uronic sugars and proteins in the $ALE_{simple\ synthetic}$ can be explained by the high availability of volatile fatty acids in the wastewater. The physical structure and microbial community composition of the AGS used in this study was analysed in details by Layer et al. (2019). High concentrations of VFAs in the influent resulted in the development of a very distinct microbial community composition and in the formation of large and dense granules (Layer et al., 2019). It is suggested that the high ALE and uronic sugar contents found in the $AGS_{simple\ synthetic}$ results from the specific microbial and physical structure of those granules. Storing

microorganisms such as *Ca. Accumulibacter* (classical polyphosphate accumulating organisms - PAO) and *Ca. Competibacter* or CPB C22&F32 (classical glycogen accumulating organisms - GAO) represented the dominant populations in the $AGS_{simple\ synthetic}$ (Layer et al., 2019). On the contrary, the fermentative PAO *Tetrasphaera* and the fermentative GAO *Micropruina* dominated the AGS fed with complex/real WW that were characterised by lower ALE and uronic acid contents. Microbial populations that utilize VFA might have a higher capability to produce specific polysaccharides such as uronic sugars. Pronk et al. (2017) also observed that granules fed with acetate are associated with very specific microbial populations and in turn contain very specific polymers (acid-soluble). In addition, the production of polysaccharides and proteins in the $AGS_{simple\ synthetic}$ might also be enhanced by the formation of ecological niches within the granules. The large diameter and the high concentration gradients favour anoxic growth, i.e., the growth of denitrifying microorganisms and decay in the deep layers of the granules (Layer et al., 2020). A high decay rate ultimately promotes the production of proteins, originated from the membrane of the bacterial cells. Such mechanism might explain the large proteins content found in the ALE extracted from $AGS_{simple\ synthetic}$.

The results also indicate that the type of bioaggregate (granular versus flocculant) does not really influence the EPS and ALE composition and content (Table 2, Fig. 1, FTIR and Raman spectra). Herein, similar EPS and ALE contents were indeed measured for both flocs and granules. This observation is in agreement with the findings Sam and Dulekgurgen (2015) but in contradiction with those of Lin et al. (2013). Lin et al. (2013) reported ALE contents in AGS nearly two times larger than in flocs. Qualitatively, no clear differences in the results of the colorimetric assays, FTIR and Raman of ALE extracts from AGS_{real} and AS_{real} were observed. Besides, ALE extracted from both AGS and AS were able to from hydrogels (see Section 3.2.1). This absence of pronounced differences in the qualitative characteristics of ALE recovered from flocs and granules fed with the same WW has been reported (Schambeck et al., 2020). Hence, it remains rather unclear to what extent the composition and content of ALE really changes between flocs and granules. Likely, other mechanisms such as abiotic ones (e.g., extraction method) also influence the composition of ALE and contributes for the similarity in composition of granules and flocs.

3.1.6. Influence of ALE composition by the extraction protocol

The composition and concentration of every EPS extracts is significantly influenced by the extraction/purification protocol (Hong et al., 2017; McSwain et al., 2005; Yang et al., 2019). ALE extracts were enriched in proteins and humic-like substances. Humic substances are usually extracted using alkaline soaking, prior precipitation at low pH (De Melo et al., 2016; Peña-Méndez et al., 2005). Hence, the extraction protocol used in this study might selectively enrich the ALE extracts in those substances. The combined high-temperature and alkaline conditions might also generate some cell lysis, and in turn a dilution of the EPS extracts with intracellular proteins (McSwain et al., 2005; Yang et al., 2019). While harsh extraction methods can lead to an overestimation of the EPS content (Hong et al., 2017), those conditions are required for an efficient recovery and representative EPS analysis (Pronk et al., 2017). Therefore, the high temperature and alkaline conditions used for EPS extraction can ultimately dilute the EPS composition in a way that differences in the content/composition are less detectable.

The results in fact suggest that polymers found in ALE extracts are likely not solely extracellular. Although, at first, the EPS solubilization from the biomass is necessary, it is not certain that polymers originate solely from the extracellular polymeric matrix. This is true for likely all studies in which ALE was recovered with harsh extraction methods. Hence, the term "extracellular polymeric substances - EPS" is not an appropriate terminology when alkaline, high-temperature and mixing conditions are applied for biopolymers recovery. Under those conditions, there may be microbial polymeric extracts that encompasses the
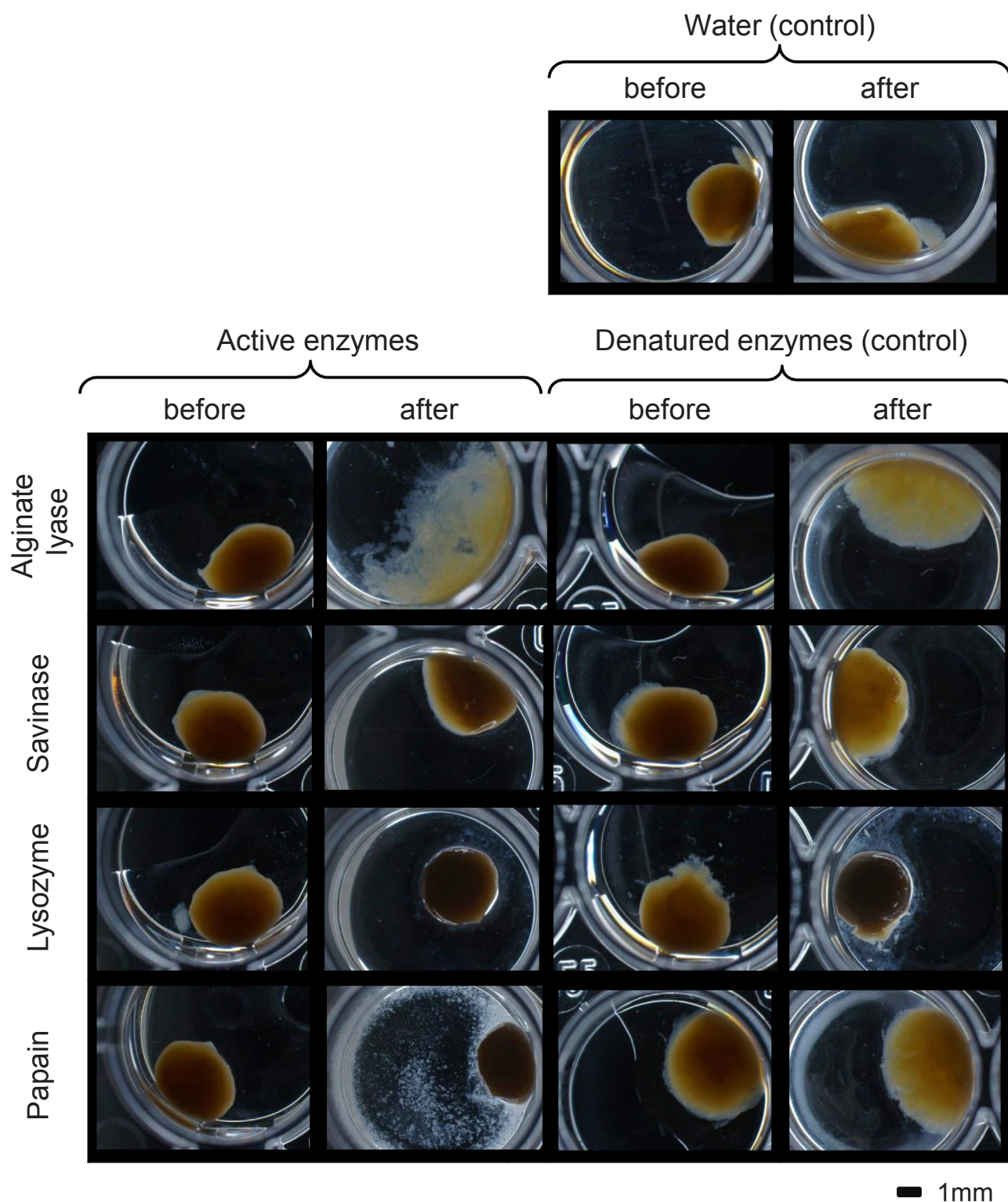
Fig. 2. Stereomicroscopic images of Ca^{2+}-ALE beads extracted from AGS_{real} along the enzymatic degradation test (24 h, room temperature) using the active enzymes alginate lyase, savinase, lysozyme and papain. Controls were run simultaneously with denatured enzymes (heated up to 90 °C for 15 min) and no enzymes (distilled water).

broth of internal and external polymers that contributes to ALE composition.

3.2. ALE hydrogel property

3.2.1. Hydrogel formation

All four Na^+-ALE extracts formed hydrogel beads when dropped in a $CaCl_2$ solution (Supplementary Data). Visually and based on touching, Na^+-$ALE_{AGS\ simple\ synthetic}$ formed the weakest and more unstable Ca^{2+}-

ALE beads, while Na$^+$-ALE$_{AGS\ real}$ formed the strongest ones. After approximately one month of storage in the fridge, only Ca^{2+}-ALE beads from AGS$_{simple\ synthetic}$ disintegrated (data not shown). In addition, the colours of the beads were similar to the colour of the sludge source.

3.2.2. Enzymatic tests: Mannuronic sugars are involved in ALE's hydrogel property

Enzymatic tests were performed to verify what biopolymers/chemical bonds were involved in the hydrogel property of Ca^{2+}-ALE$_{AGS\ real}$ beads. Alginate lyase was the only enzyme able to clearly disintegrate Ca^{2+}-ALE beads (Fig. 2). On the contrary, Ca^{2+}-ALE$_{AGS\ real}$ beads after the enzymatic treatment with active savinase, lysozyme and papain visually maintained their structure. (Fig. 2). All those enzymes had a molecular weight lower than 40000 Da and were thus able to penetrate the hydrogels. The stability of the Ca^{2+}-ALE$_{AGS\ real}$ beads structure after the enzymatic treatment with active savinase, lysozyme and papain suggests that (i) either peptide and peptidoglycan bonds have been broken down by proteases (savinase and papain) and lysozyme (glycoside hydrolase), but with no clear effect in the gel structure stability (ii) or those bonds were not present. However, results of colorimetric assays, FTIR and Raman show that peptide and peptidoglycans bonds may in fact be present. Hence, peptide and peptidoglycan bonds are not involved in the maintenance of the hydrogel structure. The disintegration of Ca^{2+}-ALE$_{AGS\ real}$ exposed to alginate lyase confirms both the presence of mannuronic acids (uronic sugars) and their role in the formation of ALE hydrogels. Alginate lyase cleaves the β-(1–4)-D-mannuronic bonds in the homopolymeric regions of mannuronic residues. However, it is not clear whether alginate lyase cleaves only MM (mannuronic-mannuronic) or also MG (mannuronic-guluronic) blocks.

So far, there is no consensus in literature about the role of polysaccharides and proteins in the gel properties of bioaggregates. Granule cohesion has been proposed to depend on the formation of calcium bridges between anionic proteins, thus creating an intercellular cement (Caudan et al., 2014). Proteins are believed to have an important role in bonding EPS due to their high affinity for cations (Zhu et al., 2015). However, other studies ascribed the gelling properties of EPS to high molecular weight polysaccharides (Seviour et al., 2010), although the hydrophobicity of the proteins can be helpful for hydrogel formation (Li et al., 2014). Herein, enzymatic tests indicated that alginate lyase was the sole enzyme able to disintegrate the Ca^{2+}-ALE$_{AGS\ real}$ beads. Therefore, peptide and peptidoglycans bonds may not play a role on hydrogel property of ALE while uronic carbohydrates containing mannuronic acids are key molecules involved in the cohesion of ALE hydrogels. Possibly, mannuronic acids are also important structural gelling polymers in bioaggregates.

3.2.3. Rheological properties of the Ca^{2+}-ALE hydrogels

The rheological properties of gel-like viscoelastic materials were characterized by measuring the frequency dependence of storage modulus G' and loss modulus G''. The influence of the different ALE extracts on the rheological properties of the hydrogels was first studied at a similar dry matter content of 2.3%. Here, the composition of WW and bioaggregate type influenced the rheological properties of Na$^+$-ALE (left frequency sweep in Fig. 3A). Na$^+$-ALE$_{AGS\ real}$ and Na$^+$-ALE$_{AS\ real}$ showed similar values of G' and G'' over the tested frequency regime, indicating a viscoelastic material directly at the gel-point (Winter and Chambon, 1986). The Na$^+$-ALE$_{AGS\ simple\ synthetic}$ and Na$^+$-ALE$_{AGS\ complex\ synthetic}$ form an elastic dominated gel, indicated by G' always larger than G''. The addition of CaCl$_2$ leads to an increase and frequency independent behaviour of both moduli, due to the formation of a cross-linked gel-like structure of the sample (rubber elastic plateau) (right frequency sweeps in Fig. 3A). Ca^{2+}-ALE$_{AGS\ simple\ synthetic}$ formed the most elastic hydrogels, followed by Ca^{2+}-ALE$_{AGS\ real}$, Ca^{2+}-ALE$_{AGS\ complex\ synthetic}$ and Ca^{2+}-ALE$_{AS\ real}$. Amplitude sweeps of the CaCl$_2$ crosslinked gels reveals a comfortable linear viscoelastic regime and a strain-thinning behaviour at a deformation of about 10%

(Supplementary Data). Pictures of the different hydrogels also reveal their rheological properties and follows the rheological properties with the largest G' for Ca^{2+}-ALE$_{AGS\ simple\ synthetic}$ followed by Ca^{2+}-ALE$_{AGS\ real}$, Ca^{2+}-ALE$_{AGS\ complex\ synthetic}$, and Ca^{2+}-ALE$_{AS\ real}$ (Supplementary Data).

The rheological properties of the four Ca^{2+}-ALE hydrogels formed at their original dry matter content were also evaluated (Fig. 3B). The extraction of ALE from the different types of sludge resulted in different dry matter contents: 4.2% for AGS$_{real}$, 3.8% for AS$_{real}$, 2.4% for AGS$_{complex\ synthetic}$ and 2.1% for AGS$_{simple\ synthetic}$. The different resulting hydrogels were in turn associated with different rheological properties than those measured at normalized dry matter content. At their original dry matter content, the Ca^{2+}-ALE extracted from AGS$_{real}$ formed the most elastic gels, while AGS$_{simple\ synthetic}$ formed the least elastic gel (Fig. 3B). Before gelation, G' was slightly larger than G'' for all Na$^+$-ALE samples across the tested frequency range. This suggests that all Na$^+$-ALE samples are at the gel-point showing a slope of around 0.5 in the double-logarithmic representation and are shear thinning with the presence of the yield stress. After gelation, G' exceeded G'', indicating a gel-like sample with a rubber-elastic plateau. Due to instrument and sample inertia the upturn of the G' values in Fig. 3 right to the blue dashed line should not be considered as a rheological property of the samples.

3.2.4. Influence of the wastewater composition and type of bioaggregate on ALE hydrogel property

Another key question is *whether the different ALE extracts result in the formation of hydrogels with similar or distinct rheological properties*. Such aspect is especially important for the scale-up of ALE recovery and its further industrial applications. The results demonstrate that the elasticity of the different hydrogels was very variable (Fig. 3). The following general trends are however suggested: (1) the elasticity of ALE extracted from AGS is always higher than the one from flocs when fed with real WW and (2) for hydrogels with the same dry matter content, the highest elasticity is observed for the ALE recovered from AGS fed with VFA-rich wastewater.

The results indicate that Ca^{2+}-ALE$_{AGS\ real}$ were more elastic than Ca^{2+}-ALE$_{AS\ real}$ (Fig. 3). This result demonstrates the influence of the bioaggregates type on the hydrogel properties of ALE. Similarly, Lin et al. (2013) conducted stress-relation tests and concluded that Ca^{2+}-ALE extracted from granules were more elastic than flocs. Differences in the chemical composition of ALE can explain the different rheological and mechanical properties between Ca^{2+}-ALE$_{AGS\ real}$ and Ca^{2+}-ALE$_{AS\ real}$. Alginate is composed of both mannuronic (M) and guluronic (G) acids, which can form three different blocks: MM, MG and GG blocks (Lee and Mooney, 2012). ALE recovered from AGS is enriched in GG blocks, while ALE extracted from flocs have more MM blocks (Lin et al., 2013). This may explain the stronger gelling properties of ALE recovered from AGS since GG blocks are believed to be responsible for stiffness and strength in hydrogels (Hay et al., 2013). Low mannuronic/guluronic acids ratio can produce stronger alginate hydrogels (Ramos et al., 2018). FTIR, Raman and colorimetric analyses indicated a very similar composition of ALE isolated from both flocs and granules. Nevertheless, more specific differences in the chemical composition in ALE may be linked to the composition of uronic sugars (i.e., mannuronic/guluronic acids ratio) that govern the gel-forming and mechanical properties in flocs and granules.

Moreover, the results give some evidence about the influence of wastewater composition on the rheological properties of ALE. At the original dry matter content, Na$^+$-ALE extracts with the highest uronic sugar content (mg$_{glucuronic\ acid}$/g VS$_{ALE}$) were, in descending order: AGS$_{simple\ synthetic}$ > AGS$_{real}$ > AS$_{real}$ > AGS$_{complex\ synthetic}$ (Fig. 1). At similar conditions, the elasticity of Ca^{2+}-ALE hydrogels decreased like AGS$_{real}$ > AGS$_{complex\ synthetic}$ ≈ AS$_{real}$ > AGS$_{simple\ synthetic}$ (Fig. 3B). Thus, at the original dry matter content obtained from the protocol of extraction, ALE recovered from VFA-rich WW formed the least elastic
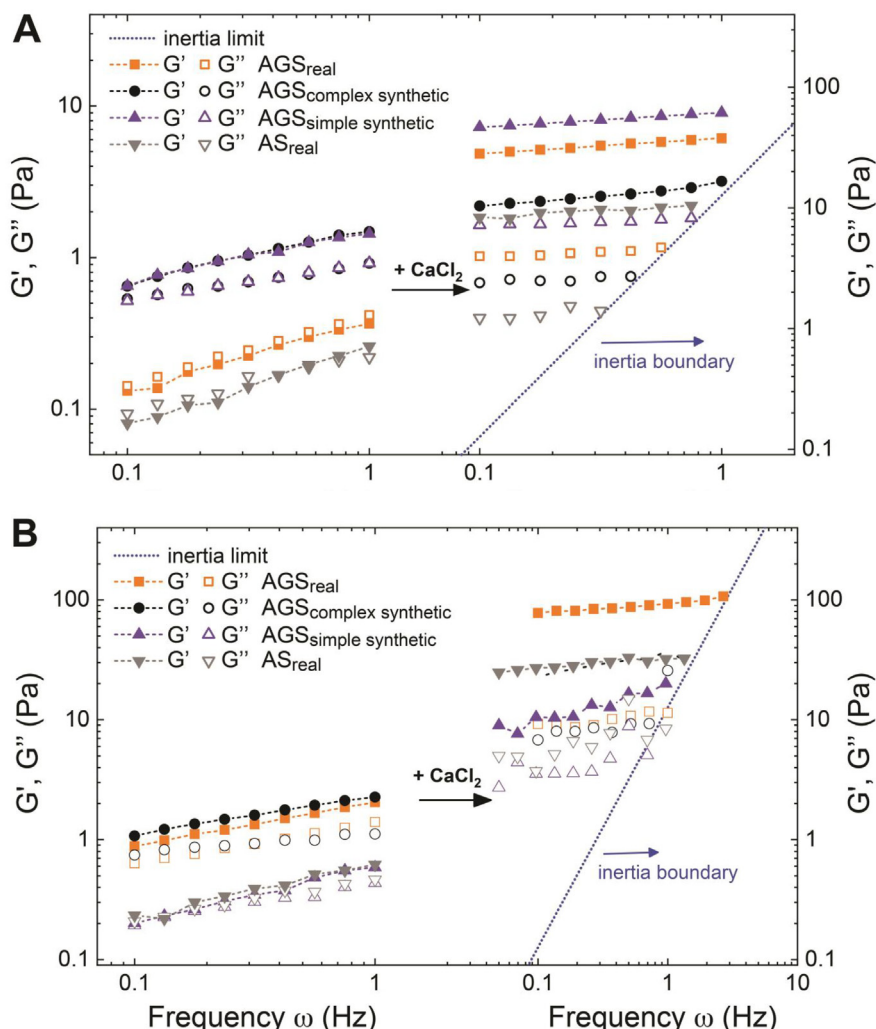
Fig. 3. Frequency dependence of G' (solid symbols) and G'' (open symbols) of the four ALE samples before (left) and after (right) hydrogel formation (A) with a controlled dry matter content of 2.3% (B) with the original dry matter content obtained with the protocol of extraction applied: 4.2% for AGS$_{real}$ 3.8% for AS$_{real}$, 2.4% for AGS$_{complex\ synthetic}$ and 2.1% for AGS$_{simple\ synthetic}$. The inertia boundary (dashed blue line) stands for the sensitivity limit for the measurements.

hydrogels while having the highest uronic sugar content. This is possibly due to the higher content of mannuronic acids in ALE$_{AGS\ simple\ synthetic}$ as suggested by FITR results (section 3.1.3). Higher contents of mannuronic acids indeed form weaker hydrogels (Hay et al., 2013). Nonetheless, at normalized dry matter content, ALE recovered from VFA-rich WW formed the most elastic hydrogels (Fig. 3A). Hence, probably it is not the content of uronic sugars itself that governs the elasticity of ALE hydrogels, but also the type of uronic sugars. Further studies are encouraged to confirm to what extent the different types of uronic sugars impact the rheological properties of ALE hydrogels.

3.3. Practical implications: Impact of ALE composition and different sludges sources on ALE recovery

The results clearly demonstrate that ALE are a complex mixture of different biopolymers such as polysaccharides, proteins and humic acids. The hydrogel property of ALE is governed by the uronic sugars. Nevertheless, the presence of other compounds in the ALE extracts, such as proteins or humic acids, is inevitable in practice and may provide additional properties and extra applications to this biomaterial (Lin et al., 2015). Hence, the synergetic effect of the different constituents in ALE should be evaluated according to the use and required degree of purity.

The hydrogel properties and organic nature of ALE encourages its use for encasement and release of different substances, as fertilizers in soil. The presence of humic acids might broaden the range of applications, e.g., in agriculture, industry, environment and biomedicine (Peña-Méndez et al., 2005). Humic acids have an amphiphilic character, finding application as surfactant and metals chelating agent (De Melo et al., 2016). If a higher stiffness is required for ALE hydrogels, the mechanical properties can be tailored by (i) increasing the uronic sugar content (e.g., by the treatment with enzymes) (ii) cross-linkage to cations with higher affinity, as copper or zinc (Felz et al., 2020), or (iii) changing the length and proportion of guluronic and mannuronic blocks (Hay et al., 2013). Low guluronic acids block content in commercial alginate generates more porous hydrogels (Ramos et al., 2018), what in turn can impact future uses of ALE adsorbents.

Another main result is that the rheological properties of the ALE hydrogels are dependent on type/origin of the bioaggregate. This result has significant implications for the full-scale recovery of ALE. Recovering ALE of different compositions would allow forming hydrogels with distinct rheological properties, which can help broadening the scope of industrial applications. However, recovering ALE at each WWTP site might not be relevant from an economical point of view. Likely, it would be more relevant to recover ALE at a single centralized site that would receive excess sludge from different satellite WWTPs.

Recovering ALE from a blend of sludge would represent a relevant approach to control the composition and overall ALE content, and consequently to govern ALE properties, which in turn represent a key advantage for further industrial applications.

4. Conclusions

ALE are a complex mixture of proteins, humic acids and polysaccharides. The overall content/composition of ALE extracted from different types of sludge is rather similar. Only the presence of VFA in the wastewater increases the overall ALE and uronic acids contents. ALE hydrogels elasticity greatly varies in response to the sludge type/origin. ALE hydrogels extracted from AGS fed with real wastewater were consistently more elastic than the ones extracted from flocs. Besides, mannuronic acids are involved in ALE hydrogel property. Therefore, different sludge types impact the composition and the properties of the recovered ALE, which is relevant for industrial applications.

CRediT authorship contribution statement

Cássio Moraes Schambeck: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Elisabeth Girbal-Neuhauser:** Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Lukas Böni:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Peter Fischer:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Yolaine Bessière:** Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Etienne Paul:** Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Rejane Helena Ribeiro da Costa:** Funding acquisition, Project administration, Resources, Supervision. **Nicolas Derlon:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123632.

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