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Research article

Biopolymers recovery: dynamics and characterization of alginate-like exopolymers in an aerobic granular sludge system treating municipal wastewater without sludge inoculum

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ABSTRACT

Alginate-like exopolymers (ALE) are present in the extracellular polymeric substances (EPS) of biological sludge such as aerobic granular sludge (AGS). The recovery of ALE from excess sludge produced by wastewater treatment plants (WWTP) is a relevant approach for the recovery of valuable products of industrial interest. However, little is known about dynamics of ALE content in sludge and associated factors. Thus, this study aimed at assessing the dynamics of EPS and ALE in terms of content, some chemical properties and influencing environmental factors along granulation in a sequencing batch reactor treating municipal wastewater. Results indicated that the EPS content was not correlated with the development of AGS, while the ALE content was higher, more stable and steadily increased after granulation achievement. Overall, 236 ± 27 mg VS_{ALE}/g VS_{sludge} was recovered from AGS and 187 ± 94 mg VS_{ALE}/g VS_{sludge} from flocs. However, the lower ALE content in flocs may be compensated by the higher sludge production rate in activated sludge systems. Principal component analysis (PCA) revealed that ALE content positively correlates with the nutrient and organic substrate conversion, and with the fraction of large AGS. Microbial analyses indicated that a stable microbial community composition was associated with a higher and more stable ALE content. ALE recovered from both flocs and AGS was endowed with hydrogel property, and no clear difference in their elemental composition and functional groups was observed. Therefore, our study provides insights about quantitative and qualitative aspects of ALE which are helpful for the improvement of waste biological sludge valorization.

1. Introduction

The handling and disposal of excess sludge from wastewater treatment plants (WWTP) is a major problem. In most developing countries, waste biological sludge is simply disposed in open dumpsites or landfills (Nizami et al., 2017). On the contrary, recent legislations in Europe now require producing energy or high-value products from excess sludge (Healy et al., 2015). In this context, the operation of WWTPs should be directed towards the recovery of products becoming itself a potential source of renewable raw materials (Mohan et al., 2016). The recovery of high-value products from excess sludge in quantities and costs compatible with current market demand and prices is growing (Van Loosdrecht and Brdjanovic, 2014), as for example the recovery of biogas, cellulose, bioplastic, phosphorus and alginic acid (Van Der Hoek et al., 2016).

Among the technologies available for wastewater treatment, the use of biofilms in the form aerobic granular sludge (AGS) has been reported to be an effective and promising environmental biotechnology (Boltz et al., 2017; Sarma and Tay, 2018a, 2018b). AGS systems have many advantages over conventional activated sludge systems, such as high organic matter and nutrients removal rates and excellent effluent quality (Derlon et al., 2016; Pronk et al., 2015). The formation of granules is governed by the microbial production of extracellular polymeric substances (EPS), holding the microorganisms together. EPS mainly consist

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of proteins, polysaccharides, nucleic acids, humic acids and lipids (Adav and Lee, 2008; Boltz et al., 2017; Liu et al., 2004). Several important functions have been attributed to EPS, such as protection against environmental pressures, mechanical stability, cell adhesion to surfaces and storage of carbon or water (Boltz et al., 2017; Freitas et al., 2017; Liu et al., 2004; Miao et al., 2016). However, the mechanisms of EPS production and its relationship to granulation are still unclear and remains mostly hypothetical (Adav et al., 2008; Ding et al., 2015; Nancharaiah and Kiran Kumar Reddy, 2018). Hence, significant research efforts must be devoted to better understand the production of EPS during the formation of aerobic granules (Boltz et al., 2017; Seviour et al., 2019).

A major fraction of EPS from AGS treating municipal wastewater consists of alginate-like exopolymers (ALE) (Felz et al., 2016). ALE behave as hydrogels (Felz et al., 2016; Lin et al., 2010, 2013) providing the granules with strength, elasticity, hydrophobicity, and a compact structure that protects microorganisms (Lin et al., 2010; Sam and Dulekgurgen, 2015). ALE are composed of sugars, proteins and humic substances (Felz et al., 2019) and are a biomaterial that can be used in paper, medical and construction industries as well as in agriculture and horticulture (Van Leeuwen et al., 2018). Current market conditions are favourable for ALE recovery from wastewater and this may be coupled with the implementation of WWTP using AGS (Van Der Hoek et al., 2016). Considering the recovery of biogas, cellulose, bioplastics, phosphate and ALE, the later represents more than 50% of turnover that can be generated in WWTP (Van Leeuwen et al., 2018). In the Netherlands, it is for example expected that 85 kton of ALE can be recovered from 10 different WWTP by 2030, generating 170 million of euros (Van Leeuwen et al., 2018). However, ALE have been also extracted from floccular sludge (Lin et al., 2013; Sam and Dulekgurgen, 2015). The possibility of extracting ALE from activated sludge would significantly expand the potential of recovery. While ALE represent a major constituent of both floccular and granular sludge, very little is known about ALE content dynamics, factors associated with ALE production and the changes in its chemical characteristics during the granulation with domestic wastewater. Such understanding is required to better design and manage future biorefinery implementation, especially in terms of yield and quality of the biomaterial. Consequently, the understanding of EPS and ALE dynamics can lead to improved strategies for both resource recovery and biofilm controlling in WWTP, as well as can be helpful for future modelling of AGS systems.

Thereby, our study aimed at: (1) quantifying the change in the EPS and ALE contents along granulation; (2) evaluating the correlations between the EPS/ALE contents and environmental/operational factors using principal component analyses (PCA); (3) investigating the relationship between the microbial community composition and the dynamics of EPS/ALE contents; and (4) characterizing changes in hydrogel properties, elemental composition and functional groups of the recovered ALE from flocs and granules. To achieve those objectives, AGS was cultivated for 308 days in a sequencing batch reactor (SBR) during the treatment of municipal wastewater in a subtropical climate region. Granules development, reactor's performances and the change in the EPS/ALE contents were monitored. ALE were characterized through ionic hydrogel formation test, scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) and attenuated total reflection fourier transform infrared (ATR-FTIR). The change in the microbial community composition was analysed by high-throughput amplicon sequencing and results were correlated to the results of the EPS/ALE contents.

2. Materials and methods

2.1. Reactor operation

AGS were cultivated in a 110L SBR (2.24 m height, 0.25 m diameter) operated in variable volume mode and fed with municipal wastewater collected from the sewer system of Florianópolis (Brazil). The reactor was started without sludge inoculum and biomass developed naturally through the reactor operation. The SBR cycle was 6 h long and consisted of the following phases: plug-flow feeding at the reactor's bottom (1 h), mixed anoxic phase (2x air pulse for mixing (10 s) + idleness (15 min)), aerobic phase (3 h 54 min), settling (30 min) and effluent discharge (6 min). A volume exchange ratio of 65% was applied. The reactor was operated under normal seasonal variations of temperature and without pH/dissolved oxygen (DO) control. The wastewater composition was representative of a low-strength wastewater: 513 ± 283 mg/L of total chemical oxygen demand (tCOD), 212 ± 66 mg/L of soluble chemical oxygen demand (sCOD), 46.4 ± 15 mg NH_4–N/L of ammonium and 7.1 ± 1.8 mg P/L of total phosphorus (TP).

2.2. Analytical methods

Sludge was analysed on a weekly basis and always sampled at the end of the aerobic phase. Granules development was monitored by recording microscopic images using an inverted microscope (Bel Photonics, Italy). From the time when the biomass concentration reached 1 g VSS/L, sludge volumetric indexes (SVI) were measured after 5, 10 and 30 min of settling according to Standard Methods (American Public Health Association, 2005) (SVI_5, SVI_{10} and SVI_{30}, respectively). The different size fractions of the sludge were measured by sieving at 600, 400, 300 and 212 μm using stainless steel sieves according to the method described by Laguna et al. (1999). The diameter 212 μm was used as cut-off to differentiate flocs from granules.

The monitoring of the reactor's performance was carried out also on weekly basis. Samples were taken at each phase of the SBR operation. tCOD and sCOD were analysed by dichromate oxidation method according to Standard Methods (American Public Health Association, 2005) and ammonium nitrogen (NH_4–N) was determined using standard test kits (Hach®). Total suspended solids (TSS) and volatile suspended solids (VSS) were determined by gravimetric method (American Public Health Association, 2005) using fiber glass filters with a pore size of 0.6 μm. TP concentration was measured with the vanadomolybdophosphoric acid colorimetric method (American Public Health Association, 2005). Temperature, pH and DO were measured using a multiparameter probe (YSI 6820 V2).

2.3. Extraction of extracellular polymeric substances (EPS) and alginate-like exopolymers (ALE)

The EPS and ALE extraction from biomass was performed according to Felz et al. (2016). EPS were extracted under alkaline, high temperature and agitation conditions with subsequent precipitation of the ALE under acidic conditions. Approximately 3 g of sludge were first centrifuged at 3100 *g* and room temperature for 25 min, prior discharge of the supernatant. Afterwards, the pellet was transferred to 250 mL baffled flasks filled with 50 mL of demineralized water, 0.25 g Na_2CO_3 and a magnetic stirrer. The flask was immersed in a water bath (80 °C) and stirred for 35 min at 400 rpm. In the next step, the mixed liquor was centrifuged (3100 *g*, room temperature, 25 min) to recover the supernatant that comprised the soluble EPS. Acidic ALE were precipitated by the addition of 1 M HCl to final pH of 2.2 ± 0.05 while stirred at approximately 100 rpm and then centrifuged (3100 *g*, room temperature, 25 min). Extractions were always performed in triplicate. EPS and acidic ALE were quantified by volatile solids (VS) measurement according to Felz et al. (2016). Student t-tests were performed at 95% confidence level (Statistica software, StatSoft, USA) to analyse the dependence between EPS and ALE contents as well as the average content before and after granulation achievement (until and after day 210, respectively).

2.4. Principal component analyses (PCA)

Standardized datasets (zero mean and unit standard deviation) related to the operation of the reactor were analysed through principal component analysis (PCA) (Statistica software, Statsoft, USA) to study the correlation between the change in the EPS/ALE contents and the main operating and environmental growth conditions. A preliminary data mining was performed to seek the best linear combination between variables such that the maximum variance could be extracted. The chosen key-variables were: VSS, sCOD, pH, temperature (Temp), NH_4–N (Amm), TP (Phosp), fraction of flocs (D212), fraction of granules with diameter range from 600 to 400 μm (600D400), SVI_{10}, SVI_{30} and content of EPS and ALE in sludge. The VSS, temperature, sCOD, pH, NH_4–N and TP data used in PCA were obtained at the end of anaerobic phase while the sludge size fraction, SVI and EPS/ALE contents were obtained at the end of aeration phase. The PCA was performed considering that each SBR cycle was subjected to the same conditions, and then variables measured in different phases are connected due to a cause-effect relationship. Relationships were observed by retaining the first two principal components (PC1 and PC2) and plotting in two dimensions where the cases and loading were set in the biplot. Both Kaiser & Guttam criteria of eigenvalues greater than eigenvalues mean as well as scree plot inspection were done to retain the number of principal components (PC) (Jackson, 1993).

2.5. Molecular techniques for microbial community analyses

The change in microbial community composition of the sludge was monitored along granulation. Triplicate samples were collected at regular intervals (n = 9 time points), centrifuged and stored at −20 °C. DNA extractions were performed using PowerSoil® DNA isolation kits (MoBio Laboratories Inc., USA). Bacterial community compositions and population dynamics were analysed by high-throughput amplicon sequencing. The amplification of the 16S rRNA V3/V4 region was carried out using the 341F (CCTACGGGRSGCAGCAG) (Wang and Qian, 2009) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2012) primers. The 16S rRNA libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., USA) with the V2 kit, 300 Cycles, single-end sequencing. The sequences were analysed using the Neoprospecta Microbiome Technologies's library. All the reads were individually submitted to a quality filter, based on the sum of the DNA bases probabilities errors, allowing a maximum of 1% of accumulated errors. Subsequently, the DNA sequences corresponding to the Illumina adapters were removed. The resulting sequences that presented 100% identity were clustered and used for taxonomic identification using Silva database.

2.6. Alginate-like exopolymers (ALE) characterization

2.6.1. Ionic hydrogel formation test

The property of the extracted ALE to form hydrogel was tested by dripping in a Ca^{2+} solution (Felz et al., 2016). Sodium ALE were obtained by adding 0.5 M NaOH to the extracted acidic ALE until pH reached 8.5. The sodium ALE solution was subsequently dripped into a 2.5% (w/v) $CaCl_2$ solution to evaluate the formation of Ca^{2+}-ALE beads, an indicative of ionic hydrogel forming properties.

2.6.2. Scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX)

A SEM equipped with EDX was used to characterize both the microstructure and elemental composition of Ca^{2+}-ALE beads (n = 6 samples) formed during the hydrogel formation tests. Ca^{2+}-ALE beads were previously frozen at −80 °C for 24 h (Ultrafreezer, NuAire) and later lyophilized for 48 h (Lyophilizer L101, Liotop). The lyophilized samples were placed over an aluminium support and sputtered with gold. The analyses were conducted using a JEOL JSM-6390LV equipment operating at 15 kV.

2.6.3. Attenuated total reflection fourier transform infrared (ATR–FTIR)

The functional groups of the polymers constituting the Ca^{2+}-ALE beads were analysed by ATR–FTIR (n = 6 samples) using an Agilent FTIR spectrometer (Carry 660 model) equipped with ATR zinc selenide crystal. Spectra were recorded from 4000 to 650 cm^{-1} (20 scans/samples) at a resolution of 4 cm^{-1}.

3. Results

3.1. Reactor's performance

Reactor's performance was monitored over 308 days of operation. High total COD removal of 79.6 ± 9.5% was achieved after 29 days, corresponding to an average effluent concentration of 91.4 ± 34.4 mg tCOD/L. An ammonium removal of 73.4 ± 16.9% was measured after 49 days of operation, corresponding to an average effluent concentration of 11.48 ± 5.61 mg NH_4^+-N/L. However, the total phosphorus removal was low and quite variable, with an average removal of 33.1 ± 15.9% and an effluent concentration of 4.8 ± 1.1 mg TP/L.

3.2. Aerobic granules formation

Granulation was followed over 308 days of operation as indicated by both visual observations (Fig. 1) and by the monitoring of SVI, VSS and granules fraction (Fig. 2). Two different phases in the granulation process could be distinguished: Phase #1 from day 0 to day ~182–220 corresponded to a period with no or low granulation, while Phase #2 after day ~182–220 corresponded to a period of full and stable granulation.

Phase #1 consisted of an initial accumulation of floccular biomass that resulted in an increasing in VSS concentration while high values of SVI were monitored. During this period, the sludge mainly comprised of dispersed, filamentous and floccular biomass (Fig. 1), likely originated from the influent. The initial high percentage of bioaggregates with diameter larger than 212 μm is attributed to the irregular biomass retained in the biggest mesh opening of the sieves, as already reported previously (Wagner et al., 2015). During the first two months, the biomass concentration gradually increased to 2.2 g VSS/L and SVI_{30} values of around 96 mL/gTSS were measured. After around day 60, a densification of the flocs occurred as indicated by the gradual decrease in the SVI values. However, flocs remained the dominant biomass fraction of the sludge during this phase (Figs. 1 and 2B) and no significant accumulation of sludge was observed (Fig. 2B).

Phase #2, observed after around 6 months of operation, corresponded to a period of full granulation, indicated by visual observations, low SVI values, high accumulation of biomass, and large fraction of granules (>80%). Visual observations indicated the formation of round and compact granules from day 220 onwards (Fig. 1). During Phase #2, the SVI_5, SVI_{10}, and SVI_{30} reached lower and more stable values of 73 ± 13 mL/gTSS, 56 ± 9 mL/gTSS and 46 ± 7 mL/gTSS, respectively. Simultaneously, high SVI_{30}/SVI_{10} ratio of 0.8 ± 0.1 was measured. Such low SVI values (<100 mL/gTSS) and a high SVI_{30}/SVI_{10} ratio (close to 1) are representative of AGS systems fed with domestic wastewater (Derlon et al., 2016; Ni et al., 2009; Pronk et al., 2015). Furthermore, the VSS concentration increased from around 1.6 g VSS/L (day 182) to 4.2 g VSS/L (day 308) as a result of granulation.

3.3. Extracellular polymeric substances (EPS) and alginate-like exopolymers (ALE) content dynamics

EPS were extracted during Phase #1 (no or low granulation) and during Phase #2 (full granulation) (Fig. 3A). No evident increase in the EPS content was observed along with the formation of granules. The EPS content of the sludge was highly variable during Phase #1 while more stable once granulation was achieved (Phase #2). An average EPS content of 540 ± 150 mg VS_{EPS}/g VS_{sludge} was measured in the sludge

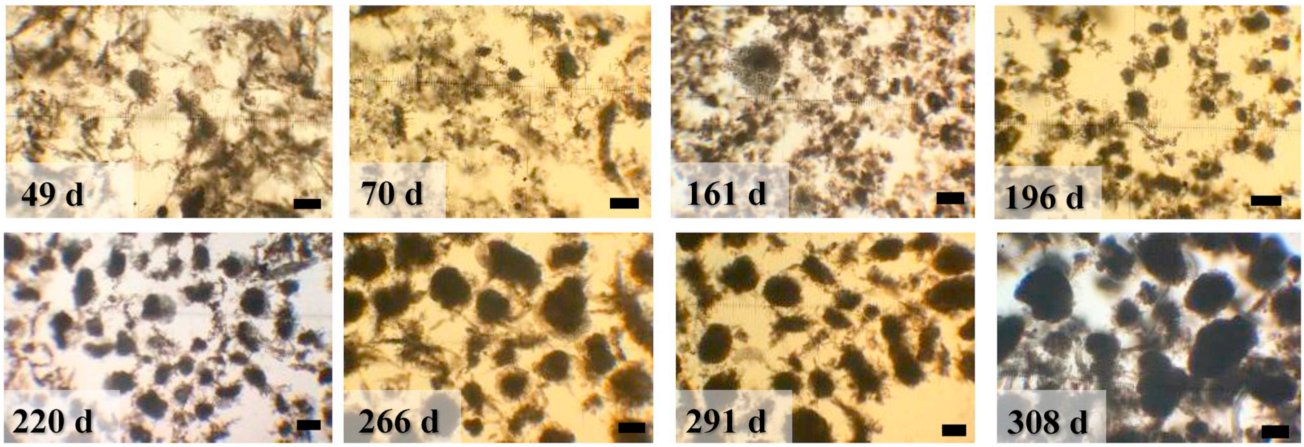

Fig. 1. Images of the mixed liquor sludge over the granulation process during the 308 days (d) of SBR operation. Sludge was dominated by floccular and heterogeneous biomass during Phase #1 (day 0 to day ~182–220) and no or low fraction of granules was observed. In contrast, compact and round granules prevailed during Phase #2 (after day ~182–220). Size bar: 400 μm.

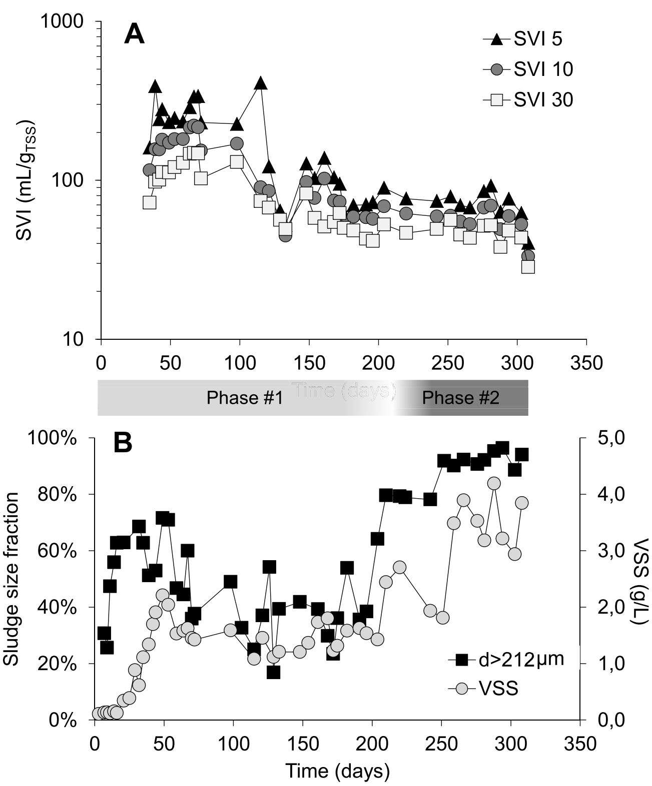

Fig. 2. (A) Change in the Sludge Volume Indexes (SVI_5, SVI_{10} and SVI_{30}) and (B) change in the sludge fraction with diameter (d) larger than 212 μm and in the Volatile Suspended Solids (VSS) during granules formation in the SBR treating domestic wastewater. Phases #1 and #2 represent the periods of pre- and post-granulation.

before day 210 (before granulation, Phase #1), while 529 ± 90 mg VS_{EPS}/g VS_{sludge} was measured in the sludge as of day 210 (after granulation, Phase #2). Consequently, the EPS content measured during Phase #1 (no or low granulation) was not statistically different from the EPS content measured during Phase #2 (full granulation) ($p > 0.05$).

Significant amounts of ALE were extracted from both flocs (Phase #1) and granules (Phase #2) (Fig. 3B and C), but the ALE content was larger in granules than in flocs. The formation of dense and round-shape granules resulted in a gradual increasing, more stable and higher ALE content in granules than in flocs ($p > 0.05$), especially when considering the ALE content in EPS (Fig. 3B): 354 ± 186 mg VS_{ALE}/g VS_{EPS} during Phase #1 (no/low granulation) versus 457 ± 62 mg VS_{ALE}/g VS_{EPS} during Phase #2 (full granulation). Consequently, an enrichment of around 29% of ALE in granules was observed.

Statistical analysis performed to evaluate whether the ALE content changed simultaneously with the EPS content of the sludge showed that changes in EPS and ALE contents are not statistically correlated ($p < 0.05$). The weak positive correlation coefficient between EPS and ALE concentration (0.30) reveals that their direct relationship is prone to errors, such as the inverse content behaviour occurring on days 161 and 168 when EPS concentration decreased while ALE increased.

3.4. Principal component analysis applied to the extracellular polymeric substances (EPS) and alginate-like exopolymers (ALE) content dynamics

The PCA model reduced the dimensionality into 3 significant principal components (PC) that represented around 78% of the total variance (PC1 52.3%, PC2 13.8%, PC3 11.8%) (Table S1). Since the current analysis is based on the correlation matrix, results can be interpreted as the correlations of the respective variables with each PC and the behaviour of each variable is ascribed to each loading value (Vasilaki et al., 2018). Based on the highest total variance of 52.3% in PC1, SVI_{10}, SVI_{30}, d < 212 μm (D212) and temperature (Temp) presented the most negative loading values, whilst PC1 increased with an increase in VSS, 600 > d > 400 μm (600D400), TP (Phosp), sCOD, NH_4–N (Amm), and ALE. In addition, EPS had no role in explaining the variation in PC1 due to its low loading value (−0.03). Thus, the parameters in PC1 could be effectively used to describe the AGS performance. PC2 explained about 13.8% of the total variance, accounting for the next highest principal component. On the other hand, the effects of EPS and ALE on PC2 were diminished since those factors had very low absolute loadings (−0.20 and 0.14 respectively). The PC3 was largely correlated to EPS (0.79) and ALE (0.55) although presented only 11.8% of the explained variance.

Fig. 4 presents the scores and loadings in the biplot model formed by PC1 and PC2. The biplot prediction should consider that both PC had an average equal to 0 (data are standardized). PC1 clustered the dataset into three groups according to the granulation process (Fig. 4A): Group A distributed on the negative side is related to Phase #1 (no or low granules fraction); Group B distributed on the positive side is related to Phase #2 (fully granulated system); and Group C is a transition from Group A to Group B (mix of Phases #1 and #2). In group A, scores were more spread and revealed a biomass with poor sedimentation properties (high SVI_{10} and SVI_{30} values) and high proportion of small aggregates (d < 212 μm), as expected during start-up. Spring-summer temperatures

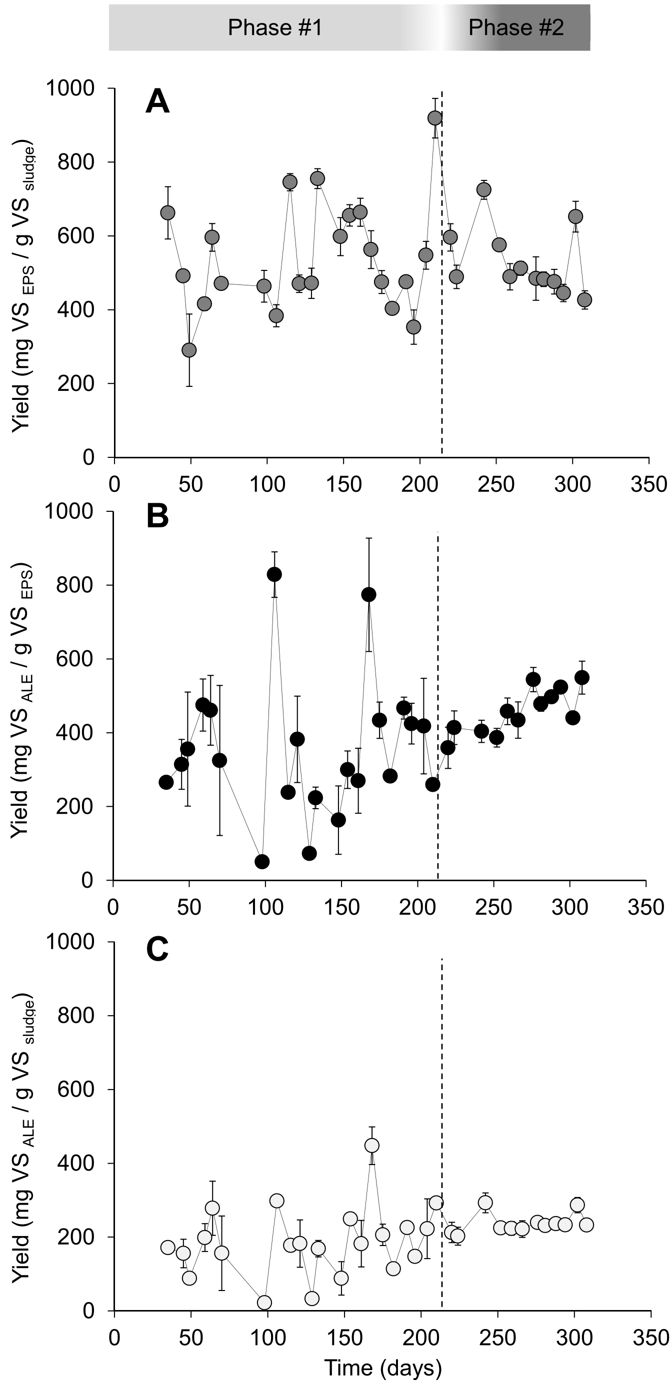

Fig. 3. Change in (A) the EPS content in the sludge, (B) the ALE content in EPS and (C) the ALE content in the sludge during granules formation in the SBR treating domestic wastewater. The EPS and ALE contents are expressed in VS. Bars indicate standard deviations between the three extractions performed for each sample. Phases #1 and #2 indicate periods of pre- and post-granulation, respectively. Dashed line defines the two groups of data (before and after day 210) used for statistical analyses.

(19 ± 1.4 °C) supported the projection of Group A scores onto the modelled space formed by both orthogonal principal axes but with no clear influence on the ALE production. Meanwhile, all cases from Group B had positive scores in PC1, indicating higher ALE content, large granules dominating the system (i.e., more particles with diameter between 400 and 600 μm), and the biomass conversion of organic matter and nutrients (higher concentrations of sCOD, NH_4^+-N and TP).

3.5. *Microbial community dynamics*

Significant changes in the bacterial community composition were observed over time (Fig. 5). The microbial community composition was highly variable during Phase #1 (no or low granulation) while stable during Phase #2 (full granulation). The high variations in the microbial community composition observed during Phase #1 resulted in a high operational taxonomic unit (OTU) richness of 1.8 ± 0.2%. Subsequently, the richness decreased and reached a lower value during Phase #2 (1.3 ± 0.1% of OTU richness).

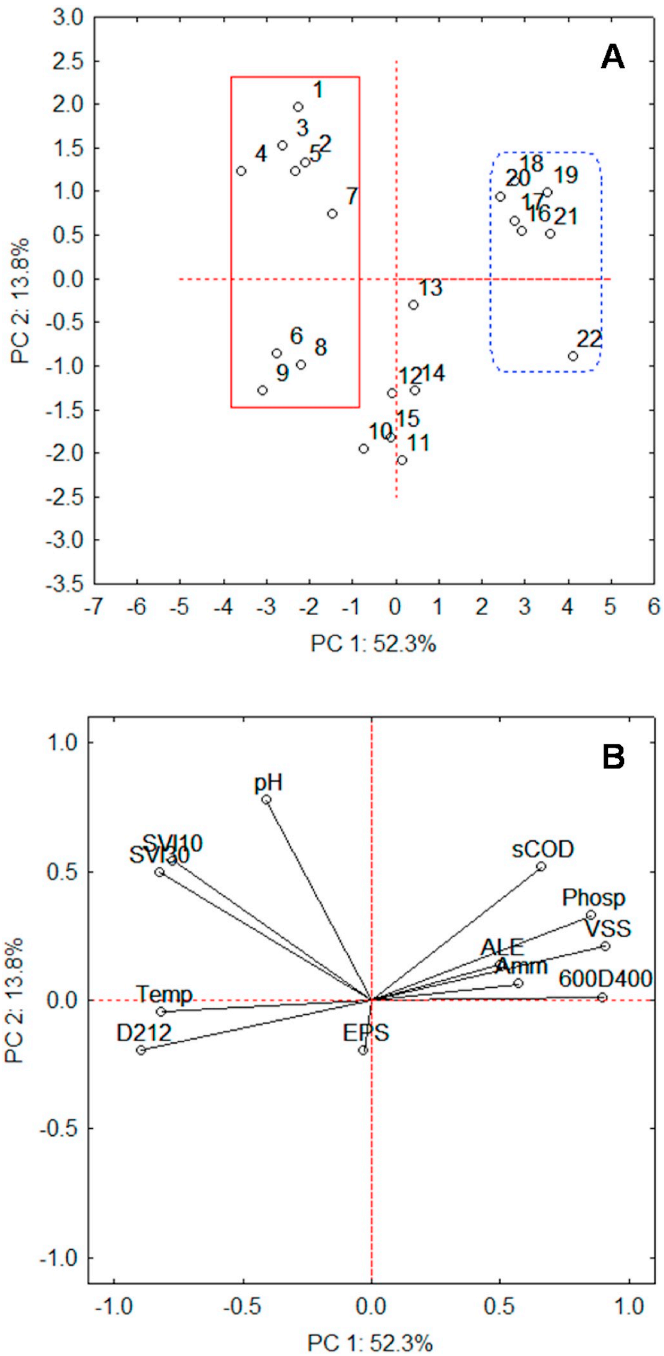

Fig. 4. Biplot model for principal component analysis of EPS and ALE contents and environmental/operational parameters: (A) score plot with group A (no/low granules fraction) and group B (granulated system) is highlighted in red-solid and blue-dashed lines respectively and (B) loading plot. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

During the start-up of the reactor without sludge inoculum, the microbial community was dominated by microorganisms representative of municipal wastewater, such as genera *Acinetobacter* and *Thiothrix* members of *Moraxellaceae* (27%, day 16) and *Thiotrichaceae* (22%, day 42) families, respectively. Other minor populations (*Streptococcaceae*, *Microbacteriaceae* and *Rhizobiaceae*) were observed at day 16 but later were gradually outcompeted. Families such as *Xanthomonadaceae*, *Caulobacteraceae*, *Sphingomonadaceae* and *Alcaligenaceae* were also present. Interestingly, these populations along with *Flavobacteriaceae*, *Chitinophagaceae* and *Saprospiraceae* families followed a similar trend than the VSS accumulation (Fig. 2B) and granulation. *Xanthomonadaceae*, *Sphingomonadaceae* and *Flavobacteriaceae* have been proposed as EPS producers in AGS (Weissbrodt et al., 2014; Xia et al., 2018). In addition, *Caulobacteraceae*, *Alcaligenaceae*, *Chitinophagaceae* and *Saprospiraceae* families may be also responsible for the biopolymers production in the EPS matrix of AGS in this study.

Interestingly, some *Rhodospirillaceae* affiliates related to enhanced biological phosphorus removal (EBPR) systems represented here by *Defluvicoccus* genus (Stokholm-Bjerregaard et al., 2017) showed considerable abundance from day 172 (in a range of 1.5–7%). *Defluvicoccus* are glycogen accumulating organisms (GAOs) which are competitors of polyphosphate accumulating organisms (PAOs) represented here by *Tetrasphaera* from *Intrasporangiaceae* family (Stokholm-Bjerregaard et al., 2017) (relative abundance of 2% on day 308).

3.6. Ionic hydrogel formation property of extracted alginate-like exopolymers (ALE) in $CaCl_2$

The ability of polymers from aerobic granules to form hydrogel is a key feature for the formation and mechanical stability of granules (Li et al., 2014; Lin et al., 2010). The hydrogel properties of polymers are also of relevance for further industrial applications (Hay et al., 2013; Lee and Mooney, 2012). In order to assess the capacity of the extracted ALE to form hydrogels, ALE were dropped in a $CaCl_2$ solution. The formation of brownish Ca^{2+}-ALE ionic hydrogels beads was observed with all ALE extracts from both floccular (Phase #1) and granular biomass (Phase #2) (Fig. S1).

3.7. Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDX)

SEM measurements performed on Ca^{2+}-ALE beads extracted from both Phase #1 (no/low granulation, day 161) and Phase #2 (full granulation, day 266) showed similar physical structures (Fig. S2). The Ca^{2+}-ALE beads were characterized at the meso-scale by the presence of pores of different sizes, forming an apparent channel network. Moreover, an irregular, continuous and rough surface indicating a strong cross-link with Ca^{2+} was observed at the micro-scale.

EDX measurements were also performed on some Ca^{2+}-ALE beads to acquire elemental information since they are formed after EPS solubilisation (i.e., homogenous solution). The major elements of the beads were: carbon, chlorine, calcium and oxygen, with some minor traces of sodium (Fig. S3). It was not overlooked the limitation of EDX in detecting hydrogen which is certainly present in ALE. Besides, no clear difference in Ca^{2+}-ALE beads was ascertained along granulation, i.e., through Phases #1 and #2.

3.8. ATR–FTIR analysis

FTIR spectroscopy is useful for analysing structural changes in biopolymers (Seviour et al., 2012; Tu et al., 2012). Ca^{2+}-ALE beads exhibited similar spectra for most absorption bands and no distinguishable peak shifts throughout the operation time of reactor (Fig. S4). According to our results, no evident difference between the ALE extracts from floccular (Phase #1) and granular sludge (Phase #2) was observed.

In relation to the functional groups, O–H stretching vibrations were identified above 3000 cm^{-1} (Silverstein et al., 2005). The strong peak at 1627 cm^{-1} can be attributed to the asymmetric stretching of O–C–O and amines (Silverstein et al., 2005). However, as EDX measurement did not show presence of nitrogen (section 3.7), the peak at 1627 cm^{-1} might not be attributed to nitrogen compounds. The strong asymmetric stretching bands near 1627-1612 cm^{-1} and the weak symmetrical stretching bands near 1455 cm^{-1} are assigned to the carboxylate C–O–C vibration and its conversion to a salt due the cross-linking with Ca^{2+} (Silverstein et al., 2005). The relative high intensity of the peak at 1612 cm^{-1} may result from a considerable content in guluronic acid residues

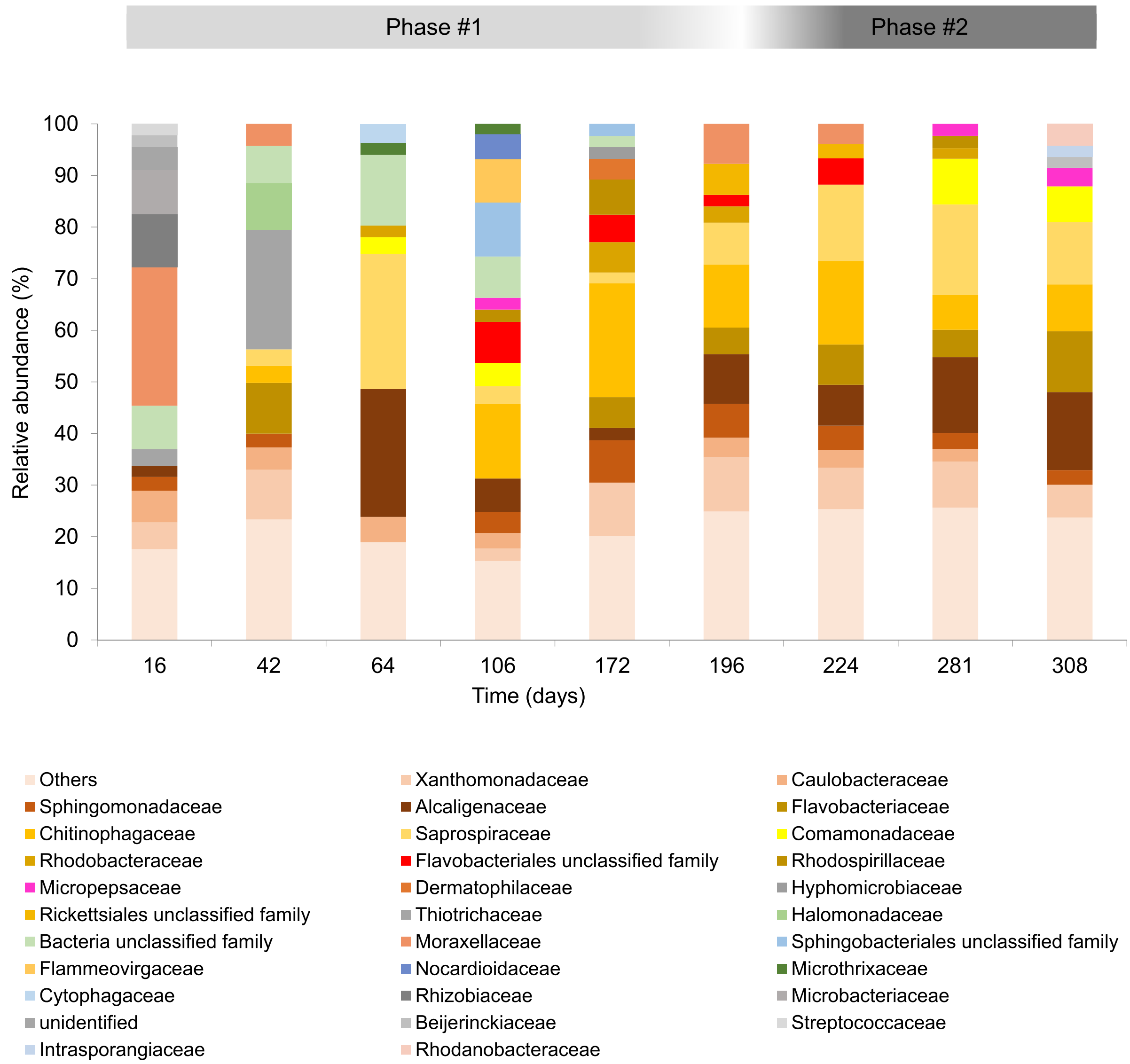

Fig. 5. Dynamics of the microbial community composition during granules formation in the SBR treating domestic wastewater over 308 days of operation. Phases #1 and #2 indicates periods of pre- and post-granulation, respectively.

(Ramos et al., 2018) which are believed to crosslink with Ca^{2+} (Lee and Mooney, 2012), conferring stiffness and strength to the formed hydrogel (Hay et al., 2013).

4. Discussion

4.1. Granulation: a phenomenon that enriches ALE in sludge but does not influence the EPS content

Granulation was successfully obtained in the system without any sludge inoculum addition, where AGS developed naturally. ALE were recovered in high amounts from the biomass dominated by both flocs and granules. However, ALE content after granulation was higher, more stable and gradually increasing in comparison to the predominant floccular biomass (Phase #1). ALE contents two times higher in granules than in flocs have already been reported with municipal sewage sludge (Lin et al., 2013). Those evidences confirm that ALE content increases with granulation. Gelation is an important key mechanism for formation and stability of AGS (Li et al., 2014; Lin et al., 2010), where hydrogels are key components for granulation (Yang et al., 2014). Granules have been characterized as hydrogels (Seviour et al., 2010). Moreover, the increase in ALE content has been reported as a prerequisite for the formation of mature granules, but a higher ALE content alone does not necessarily result in granulation (Yang et al., 2014), as also demonstrated in our study by some high ALE content measured in flocs (e.g., days 106 and 168). Therefore, the more stable and higher average ALE content in AGS may be an indicative parameter of granulation.

Regarding EPS, the absence of differences in the EPS content before and after full granulation was observed. This similarity in EPS content along granulation is distinct of the usual mechanistic models proposed in the literature where biofilm development is considered to rely on stimulus of EPS production (Adav et al., 2009; Liu et al., 2004; Nancharaiah and Kiran Kumar Reddy, 2018; Ni et al., 2009; Wang et al., 2014; Whiteley and Lee, 2015; Zhu et al., 2015). Consequently, lower EPS content is expected in flocs than granules (Adav and Lee, 2008; Miao et al., 2016; Wan et al., 2013). However, contradictory results have been also reported and indicated a reduction in the EPS content along granulation (Li et al., 2017; Tu et al., 2012). A high biomass concentration, as presented in AGS compared to flocs, does not necessarily lead to more favourable EPS production (Freitas et al., 2017). The excess of EPS has been shown to be detrimental for AGS stability due to the clogging of the pores that limits the diffusion of oxygen, organic matter and nutrients

(Corsino et al., 2016). Moreover, the microbial aggregation of activated sludge was shown to be controlled not only by the total amount of EPS, but also by the different chemical functions and structure of EPS (Guo et al., 2016). Those evidences along with our results suggest that EPS content is driven by others factors than only bioaggregates' form. Hence, granules development does not necessarily lead to a higher EPS content in comparison to flocs.

4.2. Environmental and operational factors influence on extracellular polymeric substances (EPS) and alginate-like exopolymers (ALE)

Environmental and operational factors can potentially affect ALE content in sludge. ALE content in EPS during the post-granulation period (Phase #2) was 46 $\pm$ 6%, which is a lower yield than the 63% obtained by Felz et al. (2016) using similar extraction conditions. However, such high yield was measured for sieved AGS while in our study yields are reported for the mixed liquor samples (flocs and granules). For this reason, the higher ALE content measured by Felz et al. (2016) can be attributed to the selectivity of the sieving process as well as to differences in the operating conditions and wastewater composition. Pronk et al. (2017) used the same protocol of extraction developed by Felz et al. (2016) to extract the ALE from granules fed with acetate cultivated under mesophilic conditions and obtained a much lower yield (1.4%) than granules fed with real wastewater (17.8%). Altering the composition of wastewater can lead to different values of EPS content when other operational conditions are kept (Li et al., 2017). Therefore, operational conditions and wastewater composition may affect the ALE content.

The conversion of organic matter and nutrients and a stable microbial community composition in the granulated system resulted in a steadier and higher production of ALE. The correlation of higher ALE content after granulation to the granules fraction and to the conversion of COD, ammonium and phosphorus was indicated by PCA results (Section 3.4). A direct association between ALE content and COD concentration has been already reported before with AGS treating synthetic wastewater, where high loads of organic matter led to higher ALE content in granules (Yang et al., 2014). The increase in COD concentration stresses cells that then start to synthetize exopolymers, including ALE (Yang et al., 2014). Besides, high EPS and ALE contents were measured over the entire reactor operation, i.e. before and after granulation (Fig. 3), while the microbial composition was variable before granulation and more stable in the granulated system (Fig. 5). This change in the composition of the microbial community between flocs and AGS has been observed previously (Lv et al., 2014). The higher ALE content measured during Phase #2 (post-granulation) correlated with a more stable microbial community composition. Hence, after granulation, more steady-state conditions in the system may provide balanced ecological niches for the microbial community development what in turn supports the establishment of biochemical pathways for carbon and nutrients uptake. Consequently, carbon, nitrogen and phosphorus conversions together with the stability of the microbial community in the granulated system lead to a more stable and higher ALE production.

Microbial community composition (section 3.5) can also provide information about microorganisms involved in ALE production. Bacterial alginate can be obtained from *Pseudomonas* and *Azotobacter* (Lee and Mooney, 2012), and the phylum *Proteobacteria* and the genus *Pseudomonas* have been associated with ALE production (Meng et al., 2019; Zhang et al., 2019). The most abundant families found in our study belong the phylum *Proteobacteria*, as the families *Xanthomonadaceae*, *Caulobacteraceae*, *Sphingomonadaceae* and *Alcaligenaceae*. Consequently, these organisms can be associated to the ALE production in the floccular and granular sludge. Moreover, PAO and GAO groups found after granulation (Fig. 5) are likely to be associated to stable granulation and ALE content in the present study. Therefore, a wider range of microorganisms may be involved in ALE production.

Specifically in relation to phosphorus, it was not overlooked that our system showed relatively low phosphorus removal efficiency. However, PCA showed a correlation between phosphorus and ALE content, and there was the presence of PAO after granulation, the same phase when ALE content was higher. Hence, further studies should be done to evaluate to what extent and how phosphorus conversion impacts ALE content.

Regarding EPS, statistical analyses showed that EPS dynamics is not followed by the same trend in ALE (Section 3.3). This independency denotes that different factors contribute for EPS and ALE production even though ALE are a fraction of EPS. According to the PCA results (Section 3.4), the high loading of EPS in PC3 and low loadings in the first two principal components suggest that EPS content is more resistant to the environmental and operational factors acting in a real treatment system. The lack of correlation between EPS content and some environmental and operational factors like granules size and organic loading rate has been already reported previously (Rusanowska et al., 2019). Several factors can influence EPS production as operational configuration, wastewater characteristics, bioaggregates type, complexity of the microbial community and the protocol of extraction (Adav and Lee, 2008; Ding et al., 2015; McSwain et al., 2005; Ni et al., 2015; Tu et al., 2012; Yang et al., 2019, 2014; Zhao et al., 2016). Therefore, EPS dynamics seems to be a more complex phenomenon occurring in a real treatment system where the synergetic effect of several different variables acts on EPS production.

4.3. Alginate-like exopolymers (ALE) chemical characteristics along granulation

Tracking changes in the properties of ALE along granulation during the treatment of real wastewater is relevant to better understand the dynamics of qualitative characteristics aiming future industrial applications. In general, our results about ALE characterization showed no clear difference between Ca^{2+}-ALE formed both from flocs and granules. Regarding the hydrogel properties presented by both flocs and granules, gel-forming polymers may be present in relevant concentrations in both types of bioaggregates. Results from literature are contradictory with regard of the ability of ALE from floccular biomass to form hydrogels. In a review, Seviour et al. (2012) reported that the main difference between flocs and granules is the presence of exopolysaccharides with gel-forming properties. In this context, Lin et al. (2013) obtained denser and stronger Ca^{2+}-ALE beads from granular sludge while fluffy beads were formed from floccular sludge. Differences between our results and those of Lin et al. (2013) could be due to differences in operational conditions and/or wastewater composition of each system. In the study of Lin et al. (2013), ALE were extracted from granules fed with a mixture of municipal wastewater (75%) and slaughterhouse wastewater (25%). This specific influent composition might lead to differences in the composition of the EPS and ALE. Nonetheless, other results from literature also indicate that EPS extracted from floccular biomass have hydrogel properties similarly to commercial alginate and AGS (Sam and Dulekgurgen, 2015; Yang et al., 2014). Hence, based on our results and results from literature, the hydrogel formation capability may not be a specific attribute of EPS isolated from granules.

Moreover, FTIR and EDX results provided some information about not only the similarity of Ca^{2+}-ALE extracted from flocs and granules but also about ALE composition. The gel-forming property of EPS in AGS is probably associated with high molecular weight polysaccharides (Seviour et al., 2010) whose fingerprint region in FTIR spectrum is 950–750 cm^{-1} (Seviour et al., 2012). However, in our study Ca^{2+}-ALE peaks were weaker at the fingerprint region below 1500 cm^{-1} when compared to spectra of non-cross-linked ALE (Lin et al., 2010), commercial alginate (Sartori et al., 1997) and crosslinked commercial alginate (Ramos et al., 2018). This difference is herein attributed to the replacement of sodium ions by calcium ions during the cross-linkage, what changes the charge density, the radius and the atomic weight of the cation, creating a new environment (Li et al., 2014; Sartori et al., 1997). In addition, ALE have other constituents than polysaccharides in its composition, as proteins and humic acids, giving rise to a more complex biopolymer (Felz et al., 2019), what ultimately impacts the FTIR spectrum. In terms of elemental composition, Ca^{2+} beads derived from commercial sodium alginates have substantial higher oxygen content (range of 45–36% weight) than carbon content (range of 16–27%) (Ramos et al., 2018) in comparison to our Ca^{2+}-ALE. As observed in FTIR, this difference is possibly attributed to the different raw materials used to form the beads, since ALE extracted in our study come from a much more complex source of organic matter (proteins, polysaccharides, humic substances, etc.) than commercial alginate (polysaccharide), what ultimately may influence its composition. Hence, although similarities between ALE extracted from flocs/granules and commercial alginate have already been reported (Sam and Dulekgurgen, 2015), ALE may be a complex polymeric mixture with other constituents besides alginate (Meng et al., 2019).

4.4. Practical implications: alginate-like exopolymers (ALE) recovery from wastewater treatment plants

ALE are one of the most promising bioresources to be recovered from WWTP (Van Leeuwen et al., 2018) and that is why the estimate of the amount that can be recovered is valuable. Our results about ALE content along granulation (Fig. 3) provide a relevant basis for quantifying the recovery potential. Considering wet sludge with 90–95% of water content (based on our results), ALE made up 1.12 $\pm$ 0.68% in flocs and 2.05 $\pm$ 0.19% in granules (VS based). Those values can be used as parameters for future economic and quantitative estimation analyses for ALE recovery facilities. Roughly, considering a robust WWTP with generation of 1 ton of wet sludge per day, nearly 20.5 $\pm$ 1.9 kg of VS_{ALE} can be recovered from granules and 11.2 $\pm$ 2.8 kg VS_{ALE} can be recovered from flocs, which are values that may be increased by improving the dewatering. In terms of volatile solids in the sludge, 236 $\pm$ 27 mg VS_{ALE}/g VS_{sludge} can be recovered from granules and 187 $\pm$ 94 mg VS_{ALE}/g VS $_{sludge}$ in flocs.

In addition to that, our results demonstrate that a more stable environment in terms of granulation, microbial community composition, and organic matter and nutrients removal provides a higher and more stable ALE content in granules. Those characteristics encourage ALE recovery from WWTP using AGS that have already passed the start-up phase. However, AGS systems have a lower sludge production and consequently removal (Sarma and Tay, 2018a, 2018b; Wan et al., 2009) when compared to activated sludge systems. In activated sludge systems, as our results demonstrated, ALE yield is lower and variable, but this might be compensated by the low solid retention time and by the subsequent higher sludge production that results from the higher biomass conversion yield. Moreover, the lack of pronounced differences in ALE along granulation in terms of functional groups, elemental composition and hydrogel properties together with the considerable content recovered from flocs may enlarge the possibilities of ALE recovery also from activated sludge systems. Therefore, cost benefit studies and deeper characterization of ALE recovered from flocs and granules are encouraged in order to give a clearer economic value to this bioproduct according to the source of sludge. This may expand the recovery of ALE to WWTP using different technologies contributing to a broader implementation of wastewater resource recovery facilities.

5. Conclusions

1. Alginate-like exopolymers (ALE) were important constituents of the polymers extracted from both floccular and granular sludge. Despite ALE could be found in significant amounts in flocs, ALE content was higher and more stable in granules and steadily increased over time once granulation was reached. Hence, stable and higher ALE content was an evidence for granulation establishment. Nevertheless, granulation was not necessarily followed by an increase in EPS content.
2. Higher and more stable ALE content in the sludge was associated with the granulation achievement, a stable microbial community composition, and conversions yields of organic matter and nutrients.
3. Although a higher amount of ALE can be recovered from AGS systems, the recovery potential of ALE from activated sludge treatment plants cannot be overlooked. Recovered ALE seemed to be a complex biopolymer whose hydrogel properties, elemental composition and functional groups were similar regardless of being extracted from flocs or granules. Therefore, further economic and characterization studies about recovery of ALE from flocculent sludge are necessary to support the expansion of resource recovery in WWTP.

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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

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