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Carbon source affects the resource recovery in aerobic granular sludge systems treating wastewater

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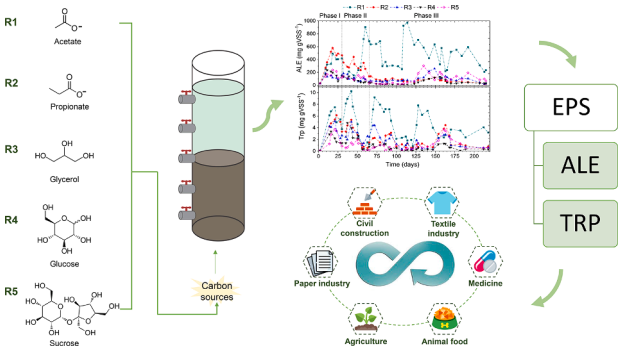
HIGHLIGHTS

- Resource recovery in aerobic granular sludge (AGS) systems was assessed.
- Alginate-like exopolymers (ALE) and tryptophan (Trp) biosynthesis were evaluated.
- Acetate and propionate were better substrates for ALE and Trp productions.
- Sludge retention time increase was detrimental to resources biosynthesis.
- Carbon source had a significant impact on ALE and Trp productions.

GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluated the influence of carbon sources on alginate-like exopolymers (ALE) and tryptophan (Trp) biosynthesis in the aerobic granular sludge (AGS). With acetate, the highest biopolymers levels, per gram of volatile suspended solids (VSS) (418.7 mgALE•g^{-1} and 4.1 mgTrp•gVSS^{-1}), were found likely due to biomass loss throughout the operation, which resulted in lower sludge age (4–7 days) and shorter famine period. During granulation, encouraging results on ALE production were obtained with propionate (>250 mgALE•gVSS^{-1}), significantly higher than those found with glycerol, glucose, and sucrose. Regarding tryptophan production, propionate and glycerol proved to be good substrates, although the content was still lower than acetate (1.6 mgTrp•gVSS^{-1}). Granules fed with glucose showed the worst results compared to the other substrates (38.5 mgALE•VSS^{-1} and 0.6 mgTrp•gVSS^{-1}) due to the filamentous microorganisms' abundance found. Therefore, this study provides insights to value the production of compounds of industrial interest in AGS systems.

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1. Introduction

In recent years, one of the main objectives of the scientific community has been the recovery of resources, particularly concerning wastewater treatment. This new perception promoted a paradigm shift so that wastewater treatment plants (WWTPs) were no longer seen solely as a unit that treats a waste stream and started to be considered as potential resource recovery factories (RRF) that fuel the circular economy (Kehrein et al., 2020). The Netherlands, for example, currently focuses on the recovery of the following resources from the sludge of municipal sewage treatment systems: alginate-like exopolymers (ALE), biomass, bioplastics, cellulose, and phosphate (van Leeuwen et al., 2018).

One of the main aspects pointed out as a possible resource source is the extracellular polymeric substances (EPS), a viscous material secreted by microbial consortia from the sludge, forming a complex extracellular matrix composed mainly of polysaccharides and proteins, but also containing nucleic acids, humic acids, and lipids (Nancharaiah and Reddy, 2018). Its production is largely associated with the process of granulation in aerobic granular sludge (AGS). This technology has been increasingly highlighted due to excellent settleability, good biomass retention, simultaneous removal of organic matter and nutrients, and superior effluent quality compared to the activated sludge system, in addition to the high potential for recovery of sludge-derived materials, mainly due to the high content of EPS contained in this type of sludge (Hamza et al., 2022). Among its functions, EPS facilitates the cell adhesion necessary for granulation, provides mechanical stability to aerobic granules' surface, and can function as a carbon source for microorganisms in adverse environmental conditions (Schambeck et al., 2020). In AGS technology, the imposed selection pressures, such as high aeration rates and the alternation of feast and famine conditions, contribute to increasing EPS production (Feng et al., 2021). Despite this, the mechanisms of EPS production and its relationship with granulation are still unclear and remain hypothetical. Therefore, research can be directed to better understand EPS production during aerobic granule formation.

The literature has shown that a large fraction of EPS from AGS consists of ALE (Felz et al., 2016). Despite not having its structure fully elucidated, it is known that the ALE are composed of sugars, proteins, and humic substances, extracted from the extracellular matrix of AGS, and identified by their capacity to form a gel in contact with divalent cations (Ca^{2+} and Mg^{2+}), assisting in the formation of the granule, in addition to serving as mechanical protection (Zahra et al., 2022). This biomaterial has potential use in the paper, medicine, textile, and construction industries, as well as in agriculture and horticulture (van Leeuwen et al., 2018).

Tryptophan (Trp) is another biomaterial described as one of the main protein fractions in the EPS matrix of aerobic granules (Zhang et al., 2018). This hydrophobic compound is an essential amino acid for humans. Hydrophobicity is the major driving force of cell adhesion usually present in AGS (Wang et al., 2018). In addition to performing an important role in granule formation, tryptophan also has applications in the chemical, food, and pharmaceutical industries, and agriculture (Carvalho et al., 2021).

Operational parameters play important roles in the production of ALE and Trp as they can influence the structure, properties, and microbial community of the granules. Some studies have found, for example, the carbon to nitrogen ratio, sludge retention time, and salinity levels as key points to stimulate resource biosynthesis (Rollemberg et al., 2020a; Rollemberg et al., 2020b; Meng et al., 2019). However, few studies were developed to evaluate the influence of operational parameters to value the recovery of ALE and Trp.

The influent carbon source is an operational condition that possibly significantly affects both resource productions, as it influences the formation and characteristics of biomass and EPS composition (Feng et al., 2021). Rollemberg et al. (2020a) and Schambeck et al. (2020) found ALE production per gram of volatile suspended solids (VSS) between 220 and 240 mgALE•gVSS^{-1} in pilot-scale reactors that treated municipal wastewater. In addition, Rollemberg et al. (2020b) obtained values of about 290 mgALE•gVSS^{-1} in AGS fed with acetate under the best operational conditions [chemical oxygen demand (COD): nitrogen (N) = 20]. Substrates such as propionate (Yang et al., 2014) and a combination of acetate and glucose (Meng et al., 2019) have also been used to produce ALE in aerobic granules, with yields of 110 mgALE•gVSS^{-1} and 240 mgALE •gVSS^{-1}, respectively. Concerning tryptophan, few studies have evaluated its production in an AGS system, reaching 48 mgTrp•gVSS^{-1} (Rollemberg et al., 2020a) and 54 mgTrp•gVSS^{-1} (Rollemberg et al., 2020b).

This difference in values found in the literature may be strongly related to the carbon source since the studies that used acetate, for example, showed higher resource content in the granules than those that used domestic sewage. In this context, due to the variation in the results found in the literature, as well as the absence of an integrated evaluation, especially for complex industrial wastewaters, this study aimed to understand the impact of important carbon sources (acetate, propionate, glycerol, glucose, and sucrose) on the production of ALE and tryptophan in long-term operation (215 days) AGS systems.

2. Materials and Methods

2.1. Experiment set-up

The experimental system was carried out in five sequencing batch reactors (SBR) of conventional configuration under similar operating conditions, changing only the influent carbon source (see Supplementary Material). The reactors were made of acrylic with a working volume of 7.8 L and dimensions of 100 cm working height and 10 cm in internal diameter, with a height/diameter ratio (H/D) of 10. The entire investigation was carried out in the Laboratory of Sanitation (Labosan) of the Department of Hydraulic and Environmental Engineering (DEHA) of the Federal University of Ceará (UFC) in Brazil.

The experiment consisted of three phases: phase I (30 days), phase II (35 days), and phase III (150 days) (see Supplementary Material). The SBRs were operated at room temperature of 28 ± 2 °C, with a cycle divided into anaerobic feeding, reaction (anaerobic and aerobic periods), settling, and decanting, with a total duration of 6 h. Sludge mixing during the anaerobic period was performed using aeration pulses of 1 min every hour. Aeration was provided by an air compressor (ACO-003, Sunsun, China) with an aeration rate of 10.0 L•min^{-1}, resulting in a superficial air velocity of 2.1 cm•s^{-1}. The duration of the settling period was gradually reduced from 30 min to 20 min (phase I), then to 10 min (phase II) and 5 min (phase III), with the excess time being added to the aerobic period.

Initially, the volumetric exchange ratio was 25%, resulting in a hydraulic retention time (HRT) of 24 h in phase I (acclimatization). These operational parameters were changed to 50% and 12 h in the following phases, respectively. The applied organic loading rate (ORL) was gradually increased over the phases, from 0.5 to 2.0 gCOD•L^{-1}•day^{-1}. These modifications in the experimental systems were adopted as a strategy to acclimatize the sludge and stimulate the aerobic granulation process. In this study, sludge retention time (SRT) was not controlled.

2.2. Inoculum sludge and synthetic influent

The inoculum was collected from a carousel-type activated sludge system for domestic sewage treatment located in Fortaleza, Ceará, Brazil. Approximately 3.9 L of biomass was introduced into each reactor, with an initial concentration of VSS of 1.2 g•L^{-1} and a sludge volume index in 30 min (SVI_{30}) of 418 mL•g^{-1}.

The carbon sources evaluated in the research were: acetate (R1), propionate (R2), glycerol (R3), glucose (R4), and sucrose (R5). Acetate and propionate favor the populations of phosphorus accumulating organisms (PAOs), which have already been associated with higher ALE

biosynthesis (Schambeck et al., 2020). In addition to being reported to support PAO-rich populations, glycerol is cited as one of the best substrates for alginate production, while glucose and sucrose negatively impact bacterial alginate production in pure cultures (Borgos et al., 2013). Furthermore, glycerol, glucose, and sucrose may stimulate tryptophan production since these substrates can generate PEP (phosphoenolpyruvate) as a metabolic intermediate consumed by microorganisms during tryptophan production (Niu et al., 2019).

The feeding solution was prepared using potable water, carbon source, basal medium (macro and micronutrient solution), and buffer. The composition of synthetic wastewater in the final stage was: 1000 $mgCOD \bullet L^{-1}$ (carbon source), 50 $mgNH_4^+\text{-}N \bullet L^{-1}$ (NH_4Cl), 10 $mgPO_4^{3-}\text{-}P \bullet L^{-1}$ (KH_2PO_4), 2 $mgCa^{2+} \bullet L^{-1}$ ($CaCl_2{\cdot}2H_2O$), 5 $mgMg^{2+} \bullet L^{-1}$ ($MgSO_4{\cdot}7H_2O$) and 1 $mL \bullet L^{-1}$ of micronutrient solution, according to Rollemberg et al. (2019). In phases I and II, the COD concentration was 750 $mg \bullet L^{-1}$, also maintaining the C:N ratio of 20, considered favorable for ALE production (Rollemberg et al., 2020b). In order to maintain neutral pH, the feed solution was buffered with 2.5 $g \bullet L^{-1}$ of sodium bicarbonate ($NaHCO_3$).

2.3. Physicochemical analysis

The performance of the systems was evaluated in terms of organic matter and nutrients removals, according to the following analyses in influent and effluent samples: COD, nitrogen in the forms of ammonia (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N), and phosphorus in the form of phosphate (PO_4^{3-}-P). Total nitrogen was considered as the sum of the fractions of NH_4^+-N, NO_2^--N, and NO_3^--N. All analyses were performed twice a week following the methodologies described in the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). Monitoring of dissolved oxygen (DO, maintained around 2 to 4 $mg \bullet L^{-1}$) and pH in mixed liquor samples was measured weekly using a probe (YSI 5000, YSI Inc., USA).

Biomass physicochemical characterization was performed weekly by determining the mixed liquor volatile suspended solids (MLVSS) concentration and sludge volume index at 5 and 30 min (SVI_5 and SVI_{30}, respectively) according to APHA (2012). The extraction and quantification methodologies of extracellular polymeric substances (EPS), alginate-like exopolymers (ALE), and tryptophan (Trp) will be described in the following item. EPS, ALE, and Trp determinations were performed twice a week.

2.3.1. Extraction, quantification, and characterization of extracellular polymeric substances (EPS)

EPS were extracted by a modified heat extraction method proposed by Yang et al. (2014). In the extraction, 5 mL of the mixed liquor collected in the aerobic phase was alkalized with 5 mL of 1 M NaOH, heated in a water bath at 80 °C for 30 min, and placed in ultrasound at 55 Hz for 5 min (Cristofoli Biosseguranca, Brazil). After extraction, the sample was filtered with a 0.45 μm membrane (Millipore, USA) and diluted in Milli-Q water (Millipore, USA) at a ratio of 1:2.

Protein content (PN) was determined by the modified Lowry method, while polysaccharide (PS) content was analyzed using a sulfuric acid-phenol method (Rollemberg et al., 2020a). The total EPS was considered the sum of PN and PS, and the results were obtained in terms of mg of PS, PN, or EPS per g of VSS.

Analysis of the fluorescent properties of EPS was performed as described in Li et al. (2021), with adaptations. A fluorescence spectrophotometer (Shimadzu, RF-6000, Japan) located at the Bioinorganic Laboratory (LABIO) of the UFC Chemistry Department was used. The three-dimensional fluorescence spectrum of the excitation-emission matrix (3D-EEM) was obtained in excitation (Ex) wavelength ranges of 200–550 nm and emission (Em) wavelength ranges of 220–600 nm. The scanning increments of Ex and Em were 5 and 2 nm, respectively, with slits of 5 nm. Two aliquots of the EPS solution extracted with a 1:10 dilution at basic pH and neutral pH were prepared from the inoculum samples and granular sludge, collected at the end of the operation for equipment reading.

2.3.2. Extraction and quantification of alginate-like exopolymers (ALE)

The ALE extraction method from aerobic granular sludge was described by Lin et al. (2010) with modifications. 50 mL of an aliquot of mixed liquor was centrifuged at 3850 × g for 20 min, after which the supernatant was discarded. The sample was then freeze-dried (lyophilization) for about 24 h (Freeze Dryer L101, Liotop, Brazil).

To the dry biomass, 0.71 g of Na_2CO_3 and 100 mL of Mili-Q water were added. This suspension was heated in a water bath at 80 °C, under stirring at 400 rpm, for 30 min to release ALE in the medium. Then, the sample was centrifuged at 3850 × g for 20 min, after which the supernatant was collected. The ALE was precipitated by adding 4 M HCl while adjusting the pH between 2.0 and 2.5. The solution was centrifuged, obtaining ALE in acidic form as a precipitate, then frozen, lyophilized, and weighed. The results were expressed following the recommendations of Felz et al. (2016).

2.3.3. Tryptophan (Trp) extraction and quantification

EPS aliquots pH was adjusted to 6.5–7.5 with 4 M HCl, counting the acid amount added to calculate the dilution. For sample doping, 50 ppm of tryptophan solution was added in a 1:1 ratio so that it contained a minimum concentration of 25 ppm of tryptophan. Trp content was measured by high-performance liquid chromatography (HPLC CTO-20A, Shimadzu Corporation, Japan), considering previous work (Rollemberg et al., 2020b). The HPLC was equipped with a Hypersil BDSC-18 column (250 mm × 4.6 mm, 5 mm), UV 280 nm, and UV/VIS detector (injection volume 20 μL, 6 min run, isocratic elution). The mobile phase was composed of methanol and water at a molar ratio of 3:10, with a flow rate of 1 $mL \bullet min^{-1}$. Tryptophan concentration is calculated by the difference between the peak reading areas of the doped sample and Milli-Q doped water (blank).

2.4. Morphological and microbiological analyses of the sludge

Granule formation and development were evaluated weekly by granulometry (sieving and gravimetry), combined with light microscopy. Granulometry analysis was performed using three sieves with 0.2 mm, 0.6 mm, and 1.0 mm openings. The percentage of granules larger than the sieve opening was calculated by the ratio between the mass of granules that passed through the sieve and the total sample mass. Only when >80% of the biomass had a diameter >0.2 mm, the reactor was considered to have achieved the granulation stage (da Silva et al., 2019). The mean granule diameter was determined from optical microscope images (Opton, Brazil) treated in the Image-Pro Plus software (Media Cybernetics, USA).

Molecular biology analyses to identify the microorganisms present in the sludge were divided into DNA extraction, amplicon sequencing in the 16S rRNA gene, and data processing. Mixed liquor samples were collected at the end of phase III during aeration, then ensuring a homogeneous sampling. 0.5 g of fresh sample (total wet weight) was used to extract DNA from microorganisms using the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., USA), following the manufacturer's instructions. Based on the 16S rRNA region V4 gene, the amplicon sequencing was prepared in triplicate according to Illumina (2013), using specific primers (515F/806R).

After indexing, the PCR products (amplicons) were purified using Agencourt AMPure XP-PCR purification beads (Beckman Coulter, Fisher Scientific, USA), according to the product manual, and quantified using the dsDNA BR assay kit (Invitrogen, ThermoFisher Scientific, USA) on a Qubit 2.0 fluorimeter (Invitrogen, ThermoFisher Scientific, USA). The amplicons were sequenced using the MiSeq chemistry 300-cycle reagent kit (Illumina, USA) with the MiSeq desktop sequencer (Illumina, USA) located at the Genomics and Bioinformatics Center (CeGenBio) at UFC.

The data obtained by sequencing were analyzed with bioinformatics

tools. The sequencing reads were trimmed, assembled, and de-noise treated using the DADA2 v1.20.0 package (Callahan et al., 2016). After denoising, the representative sequences are called Amplicon Sequencing Variants, or ASVs. The resulting ASVs were taxonomically classified using the IDTAXA classifier (Murali et al., 2018).

The raw sequence data obtained in this study has been deposited in the National Center for Biotechnology Information (NCBI) BioProject database, ID PRJNA836880 (https://www.ncbi.nlm.nih.gov/bioproject/836880).

2.5. Statistical analysis

The Kruskal-Wallis and Mann-Whitney tests were applied to compare the performance of the reactors. The test results were evaluated according to the *p*-value. If $p \leq 0.05$, the null hypothesis was rejected, and the data groups were considered statistically different.

3. Results and discussion

3.1. Formation and stability of aerobic granules

Fig. 1 shows the results of MLVSS, SVI_{30}/SVI_5 ratio, and mean granule diameter throughout the operation. The five experimental systems were inoculated with activated sludge with an MLVSS concentration of 1.3 g•L^{-1} and an SVI_{30}/SVI_5 ratio of 0.6. One week after start-up, a reduction in the initial MLVSS concentration was observed in R1, R2, and R3, as the flocculent biomass was washed out from the systems. Differently, R4 and R5 had already recovered the amount of biomass lost in this period, indicating a better initial biomass retention capacity. With the application of operating conditions during phase I (see Supplementary Material), all reactors kept the same or improved settleability (SVI_{30}/SVI_5 ratio).

The granulation process occurred at different times. In R1, R2, and R3, the formation of granules occurred between phases I and II, around 20–25 days after start-up. On the other hand, in R4, granulation was reached only at the end of phase II, after about 60 days of operation. Late granulation in R4 can be explained because, when hydrolyzed, glucose has its products consumed preferentially by filamentous bacteria dispersed in the liquid medium, which compromise sludge settling and hinder granule formation (Qiulai et al., 2019). Thus, the control of filamentous microorganisms is important to improve the operational performance with this type of substrate. For the R5 reactor fed with sucrose, the formation of granules was observed around 25 days of operation. At the end of phase II, >90% of aerobic granules above 0.2 mm in each system were observed.

Between the phase changes, there was a loss of solids in all systems due to settling time reduction from 20 min to 10 min (phase II) and from 10 min to 5 min (phase III), accompanied by an improvement in the sludge settleability, which is typical behavior of granular biomass. During phase II, all reactors retained a greater amount of biomass, mainly R4 and R5, while increasing the SVI_{30}/SVI_5 ratio, especially in R1 and R3, whose value reached close to 1.0. During phase III, R1 (acetate) maintained the MLVSS concentration, reaching the experiment completion with mean values of 1.4 g•L^{-1}, and an unstable SVI_{30}/SVI_5 ratio, on average of 0.7. During the operation, this reactor presented moments of instability, characterized by the loss of biomass or the drop in the SVI_{30}/SVI_5 ratio. The formation of fragile granules easily disintegrated can justify the R1 behavior during the operation due to the following mechanisms: (a) critical size of the granule formed with this type of substrate, with up to 1.5 mm; (b) clogging of granule pores by excessive EPS production; and (c) growth of filamentous microorganisms (Verawaty et al., 2013; Corsino et al., 2016; Qiulai et al., 2019).

Differently, R2, R3, R4, and R5 reached average concentrations of MLVSS between 3.0 and 7.0 g•L^{-1} and SVI_{30}/SVI_5 higher than 0.8, indicating high retention of biomass with good settleability. In R2 (propionate), MLVSS concentration increased during maturation, with an average of 3.6 g•L^{-1}, and the average diameter of its granules varying between 1.0 and 2.5 mm. In addition to these, it proved to be stable in terms of settleability, with an SVI_{30}/SVI_5 ratio close to 1.0. The reactor fed with glycerol (R3) also showed increasing MLVSS concentration in the final phase (3.0 g•L^{-1}). However, biomass settleability was affected around the 150th day by the aeration rate increase, which fragmented the granules, also causing biomass loss (He et al., 2018). In response to increasing MLVSS concentration in R2, R3, R4, and R5, a high sludge retention time (SRT) was achieved in phase III (28–37 days). On the other hand, R1 maintained an SRT between 4 and 7 days throughout the experimental period, possibly due to system instabilities and subsequent biomass loss.

As also reported in the literature, glucose (R4) is a substrate that favors high biomass retention (MLVSS around 7.3 g•L^{-1}) with good settleability ($SVI_{30}/SVI_5 \sim 0.80$) compared to the other carbon sources, such as acetate (R1) (He et al., 2018). Sucrose (R5) also favored the formation of dense granules, able to remain in the reactor (MLVSS around 4.7 g•L^{-1}) but with reasonable settleability. In this study, filamentous microorganisms were visually identified from the 130th day of operation, impairing sludge settling.

3.2. Reactor performance

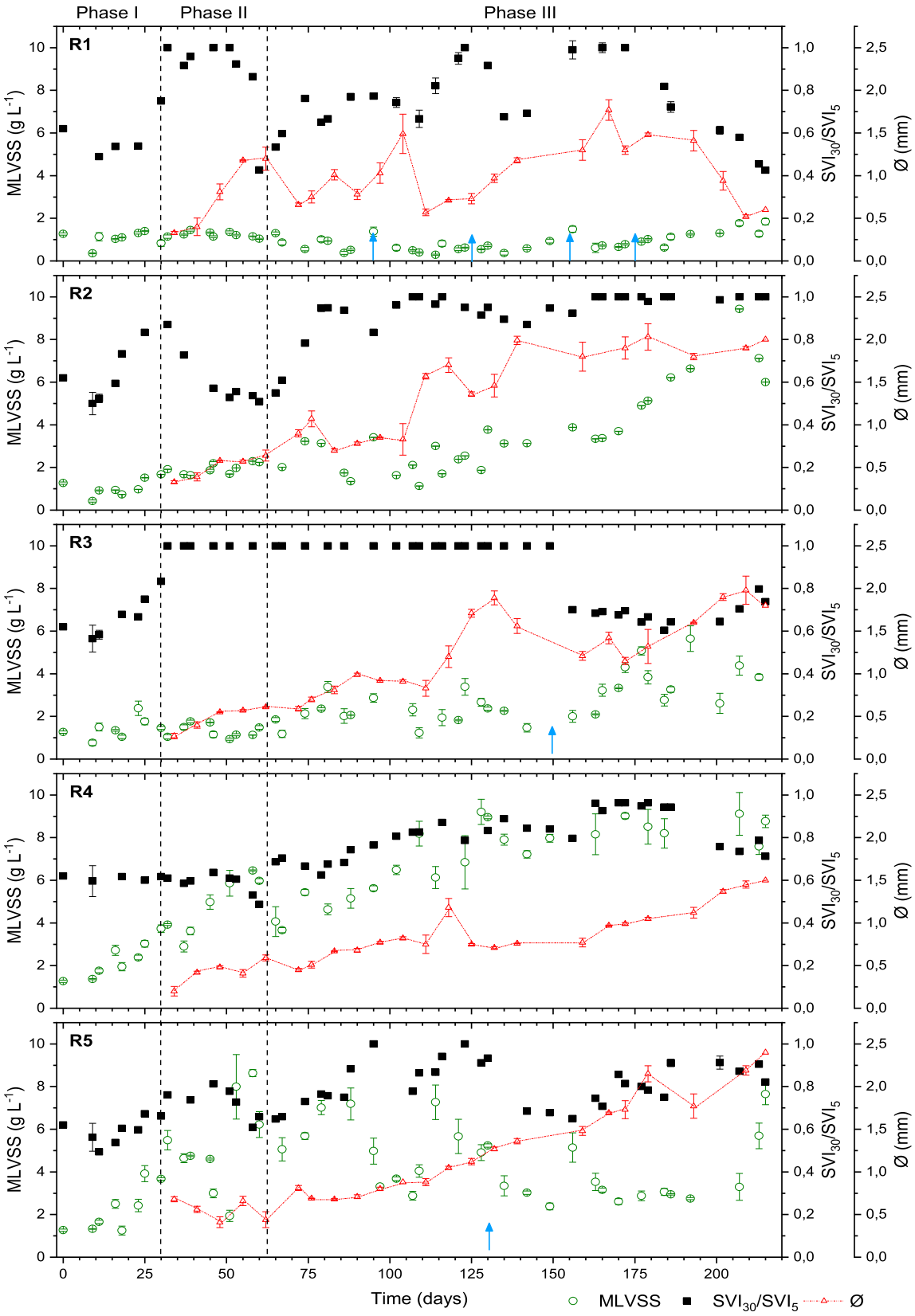
The performance of the reactors was monitored for a period of 215 days (Table 1). A high COD removal efficiency was achieved in all reactors (>90%). No statistically significant difference was observed between the systems ($p = 0.25$), indicating that the type of substrate did not interfere with the organic matter removal.

In all systems, total nitrogen (TN) removal efficiency improvement was observed between the experimental phases. As the granules develop in size, a favorable environment is created, with aerobic and anoxic/anaerobic conditions, within the granule itself that allows the coexistence of nitrifying and denitrifying bacteria (Chen et al., 2011). The TN removal found ranged from 72 to 78% in the reactors in the final phase. Although a decrease in nitrification between phases was observed due to the synthesis of the biomass that consumes ammonia in its metabolism, the nitrification efficiency was high during the entire operation period (>80%), indicating that ammonia-oxidizing nitrifying bacteria (AOB) and nitrite oxidizers (NOB) remained stable in the reactors. By statistical analysis, TN removal was significantly different between systems ($p < 0.05$).

In R1 (acetate), during granule disintegration periods, there was a reduction in the ammonia removal efficiency, ranging from 15 to 30%, possibly due to the loss of nitrifying bacteria present in the disintegrated granules, even though, within a few days, the performance was restored. Acetate is reported to be a good electron donor, which justifies its good nitrogen conversion efficiency (~78%) (Adav et al., 2010). R2 (propionate) performance concerning TN removal was slightly inferior to the other reactors, reaching average values of 70% throughout the operation. In this reactor, a greater accumulation of nitrate (NO_3^-) was observed, around 13 mg NO_3^--N•L^{-1}, when compared to the other systems, indicating a deficiency in denitrification.

Reactor R3 (glycerol) showed TN removal behavior similar to R2, with nitrate accumulation throughout the operation. It is suggested that, in addition to the rapid COD consumption during the anaerobic period, the high DO concentration in the aerobic phase (2–4 mg•L^{-1}) may have favored nitrification over denitrification during the aerobic famine period, resulting in nitrate accumulation (di Bella and Torregrossa, 2013). Reactors R4 (glucose) and R5 (sucrose) obtained TN removal efficiency of around 75% and, therefore, superior to R2 and R3 performances. These systems probably adapted better to the high DO concentration during the aerobic phase (2–4 mg•L^{-1}) due to the complexity of these substrates, which demand a higher rate of oxygen to degrade them, interfering less with the denitrifying bacteria activity.

Regarding total phosphorus (TP) removal, efficiency also improved throughout the operation in all reactors, reaching averages of 49 to 72%



Note: R1 (acetate), R2 (propionate), R3 (glycerol), R4 (glucose) and R5 (sucrose). In R1, R3 and R5, the blue arrows point to moments of instability.

Fig. 1. SVI_{30}/SVI_5 ratio, volatile suspended solids (VSS) and average granule diameter (Ø) in the five reactors during 215 days of operation. **Note:** R1 (acetate), R2 (propionate), R3 (glycerol), R4 (glucose) and R5 (sucrose). In R1, R3 and R5, the blue arrows point to moments of instability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Removal efficiency of organic matter and nutrients (N and P) in 215 days of operation of reactors R1, R2, R3, R4, and R5.

Reactor		R1 (acetate)			R2 (propionate)			R3 (glycerol)			R4 (glucose)			R5 (sucrose)		
Phase		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
N° of samples		7	10	38	7	10	38	7	10	38	7	10	38	7	10	38
COD	Influent ($mg \bullet L^{-1}$)	606 (196)	856 (185)	838 (180)	695 (125)	1028 (222)	893 (203)	703 (138)	802 (152)	855 (142)	615 (97)	778 (114)	880 (171)	710 (92)	870 (136)	903 (178)
	Effluent ($mg \bullet L^{-1}$)	24 (8)	62 (44)	36 (25)	42 (16)	71 (41)	32 (29)	30 (15)	80 (46)	34 (25)	39 (27)	41 (37)	36 (34)	26 (14)	61 (44)	36 (27)
	Removal (%)	**95 (2)**	**92 (5)**	**96(3)**	**94 (3)**	**92 (6)**	**96 (3)**	**96 (2)**	**91 (5)**	**96 (3)**	**93 (4)**	**95 (4)**	**95 (3)**	**96 (2)**	**93 (5)**	**96 (3)**
Nitrogen fractions	NH_4^+ influent (mg NH_4^+-$N \bullet L^{-1}$)	49.8 (8.1)	49.8 (7.5)	51.7 (9.4)	54.9 (6.2)	51.6 (9.3)	52.1 (9.1)	44.7 (5.1)	50.5 (5.9)	51.7 (7.6)	46.7 (4.2)	45.7 (4.8)	47.4 (8.4)	53.6 (2.7)	51.3 (5.5)	48.4 (6.8)
	NH_4^+ effluent (mg NH_4^+-$N \bullet L^{-1}$)	1.4 (0.5)	4.2 (2.8)	3.2 (2.9)	6.3 (1.0)	3.7 (1.0)	1.1 (0.5)	1.6 (1.0)	2.7 (1.1)	3.0 (1.1)	0.7 (0.3)	3.9 (2.6)	3.4 (2.4)	0.9 (0.5)	6.0 (2.0)	7.3 (4.4)
	NO_2^- effluent (mg NO_2^--$N \bullet L^{-1}$)	1.3 (0.1)	3.2 (1.8)	2.1 (1.6)	1.5 (0.1)	0.9 (0.1)	0.5 (0.1)	1.5 (0.4)	1.1 (0.5)	0.5 (0.7)	1.5 (0.3)	0.8 (0.2)	0.8 (0.3)	1.9 (0.4)	1.3 (0.5)	0.8 (0.3)
	NO_3^- effluent (mg NO_3^--$N \bullet L^{-1}$)	17.5 (2.5)	7.2 (1.2)	7.0 (3.3)	18.2 (3.1)	14.4 (3.1)	11.5 (2.6)	10.8 (1.9)	10.9 (2.1)	11.2 (2.8)	10.9 (3.2)	9.4 (3.0)	8.6 (2.7)	13.6 (3.0)	8.7 (2.8)	4.4 (2.0)
	Removal (%)	**59 (4)**	**69 (7)**	**78 (10)**	**53 (6)**	**64 (12)**	**74 (9)**	**68 (11)**	**71 (10)**	**72 (9)**	**67 (5)**	**70 (10)**	**74 (10)**	**69 (10)**	**70 (11)**	**72 (11)**
Phosphorus	Influent (mg PO_4^{3-}-$P \bullet L^{-1}$)	11.4 (1.7)	9.8 (3.0)	11.2 (2.6)	11.6 (1.5)	10.8 (2.9)	11.2 (2.6)	11.2 (2.6)	9.7 (2.6)	10.1 (2.5)	9.1 (1.7)	7.4 (2.7)	11.0 (2.0)	9.7 (2.2)	7.4 (2.5)	10.3 (3.2)
	Effluent (mg PO_4^{3-}-P L^{-1})	8.2 (1.5)	6.3 (1.8)	5.5 (1.0)	7.9 (1.9)	7.0 (1.9)	6.2 (1.8)	6.2 (1.8)	6.0 (1.8)	4.9 (1.5)	4.9 (1.9)	3.6 (1.5)	3.8 (1.4)	5.8 (1.9)	3.9 (1.3)	3.1 (1.0)
	Removal (%)	**27 (14)**	**34 (18)**	**49 (11)**	**31 (17)**	**35 (11)**	**45 (9)**	**38 (8)**	**49 (21)**	**51 (11)**	**47 (19)**	**49 (22)**	**65 (11)**	**40 (15)**	**41 (30)**	**72 (8)**

Note: data in parentheses refer to the standard deviation, read (±value).

in the last phase. Statistical tests showed significant differences in TP removals between systems ($p < 0.05$). A possible explanation for the low phosphorus removal is the high sludge retention time (SRT), between 28 and 37 days in the final phase. Without controlled sludge discharge, saturated phosphate-accumulating organisms (PAOs) remain in the system, interfering with phosphorus removal (Bassin et al., 2012). On the other hand, although the sludge retention time of R1 was low (between 4 and 7 days) due to the continuous biomass loss, TP removal remained around 50%, probably due to competition between PAOs and GAOs (glycogen-accumulating organisms) (Carvalheira et al., 2014).

3.3. Biopolymer production

The EPS, ALE, and Trp concentrations during granulation were markedly higher than those found in activated sludge flocs, corroborating findings in the current literature (Rollemberg et al., 2020a; Schambeck et al. 2020; Nancharaiah and Kumar, 2018) (see Supplementary Material). Fig. 2 shows the ALE and Trp values over the 215 days of operation. In this sense, it is observed that granulation is a process that enriches the content of ALE, Trp, and EPS, confirming the importance of biopolymers in the stability of the aerobic granular sludge structure in the granulation process (Zahra et al., 2022; Schambeck et al., 2020; Schambeck et al., 2020).

However, in mature granules, especially in phase III, there was a reduction in the formation of these compounds, with a significant difference between the granulation and maturation periods ($p < 0.05$), except in R1 in the production of EPS, ALE, and Trp, and R3 EPS production. The decrease in ALE production during granule maturation is in disagreement with the findings of Schambeck et al. (2020), in which the ALE content continued to increase with the granule maturation. However, it is worth noting that the authors worked with domestic wastewater and the granulation phase took>200 days, likely leading to different behaviors compared to the current study.

It is known in the literature that, under extreme conditions, EPS can serve as a carbon source for biological processes (He et al., 2018). Thus, the following phenomena may have occurred in R2, R4, and R5: (a) lower EPS production due to the increase in granule diameter caused by oxygen limitation and carbon diffusivity (Verawaty et al., 2013); (b) higher endogenous respiration rate due to increased SRT (Rollemberg et al., 2020b); and (c) very long starvation period, inducing microorganisms to use EPS as electron donors (Wu et al., 2012). Similarly, the reduction in ALE and Trp biosynthesis can also be explained by the increase in SRT and the long period of starvation. Cycle analysis reveals that most of the COD was consumed in the anaerobic period. This behavior agrees with the results of Rollemberg et al. (2020b), in which a high SRT caused a decrease in ALE content in aerobic biomass. In addition, the authors indicate the best ALE production with SRT between 10 and 15 days. In this study, R2, R3, R4, and R5 achieved SRT of 28–37 days in phase III, and 4–7 days for R1.

EPS production showed no significant difference ($p > 0.05$) between the phases, only in R1 and R3. In the first case, there are strong indications that it is due to reactor instability events with subsequent loss of biomass. Under stress conditions, microorganisms tend to secrete more EPS to maintain their shape (Nancharaiah and Reddy, 2018). The literature has shown that substrates that induce slow aerobic growth have led to more stable granulations (Hamza et al., 2022). As acetate is an easily assimilated compound, it favors the emergence of fast-growing heterotrophic microorganisms. Rollemberg et al. (2019) also observed unstable granules using acetate as a carbon source. In Fig. 2, red arrows on days 100 and 155, exactly those in which R1 went through biomass loss, the highest values of EPS production were identified. In R3, in turn, the highest productions were between days 140 and 160. In this case, it

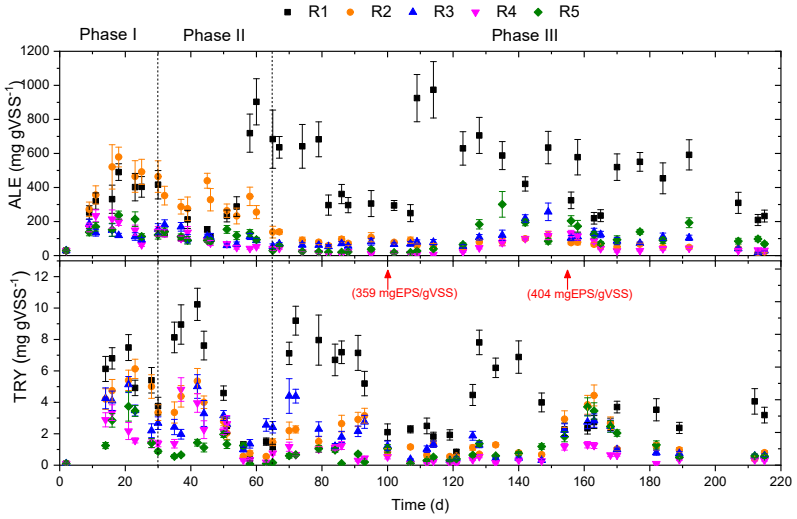


Fig. 2. ALE and tryptophan production over the 215 days of operation. **Note:** R1 (acetate), R2 (propionate), R3 (glycerol), R4 (glucose) and R5 (sucrose). In R1 the arrows in red indicate moments of instability and in parentheses the production of EPS observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is believed that they were in response to the change in aeration intensity, an event that caused the granules to break.

Recent studies in AGS systems have shown that the binding structures of proteins and carbohydrates, through hydrogen bonds, are involved in forming the EPS gel (Li et al., 2020). This structure appears as the most essential interaction force for the secondary structure of the protein and suggests a stronger interaction between the components. For this reason, instabilities and disintegration of granules are encountered when there are adverse conditions that are detrimental to the sustaining of hydrogen bonding (Li et al., 2020). In this sense, the instabilities that arose in R1 possibly hampered these bonds between PS and PN, weakening the granular structure, although the production of these compounds remains high.

It is interesting to note in these reactors with instabilities, especially in R1, that after stress events with loss of biomass, the increase in EPS was immediate. However, the response to the increase in ALE and Trp was days after the stress event, indicating that stress mechanisms in AGS to increase the production of resources possibly do not produce an immediate response. For this reason, intermittent stresses (pulse mechanisms) may be an interesting alternative to be studied. This different response in the production of ALE and EPS also indicates that although ALE is the main constituent of EPS (Zahra et al., 2022; Carvalho et al., 2021), different dynamics contribute to the production of these compounds (Schambeck et al., 2020). However, sludge age seems to be a very important parameter that also influences the biosynthesis of these biopolymers, including Trp.

Carvalho et al. (2021) indicated that low sludge ages (close to 6 days) increase the formation of Trp and protein-like substances, in agreement with the findings for R1. In this system operated with acetate, the highest Trp production was found, and there was no significant difference between the phases ($p > 0.05$). On the other hand, there was a decrease in Trp synthesis in phase III in the other reactors, with a significant difference between the time of cultivation and maturation ($p < 0.05$). Another possibility for this trend is due to the higher production of Trp related to newly formed granules, with diameters between 0.5 and 1.0 mm (Rollemberg et al., 2020b).

Comparing the production of resources between reactors, there are significant differences in terms of ALE and Trp contents ($p < 0.05$). The Mann-Whitney test showed that R1 and R2 were statistically higher than R3, R4, and R5 for ALE production. This is possibly due to the substrate applied, as, in granules, there are indications that the formation of ALE is associated with the enrichment of PAOs in the biomass (Schambeck et al., 2020). Acetate and propionate are preferred substrates for growing PAOs, especially the latter (Shen and Zhou, 2016). Additionally, in R1, the recurring stress events also played a role in its production, explaining its sweeping over the 215 days of operation. The ability of ALE to form hydrogel is essential for aerobic granule production and mechanical stability. However, a high concentration of ALE does not always imply stable granulation (Schambeck et al., 2020, Hamza et al., 2022).

R1 and R2 showed the highest production of tryptophan in phase I ($p < 0.05$). However, in phase II, R5 showed significantly lower production ($p = 0.01$), indicating that sucrose is not a suitable substrate for Trp biosynthesis. Glycerol is indicated as favorable to tryptophan production, as it can generate PEP (phosphoenolpyruvate) as a metabolic intermediate, which is consumed during the biosynthesis of tryptophan (Niu et al., 2019).

Comparing the current research data with the literature, the average ALE production in R1 (418 mgALE•gVSS^{-1}) was higher than that found by Rollemberg et al. (2020b), 290 mgALE•gVSS^{-1}, also in the presence of acetate. On the other hand, R2 presented values close (180 mgALE•gVSS^{-1}) to those found by Yang et al. (2014) (110 mgALE•gVSS^{-1}), both in the presence of propionate as substrate.

Concerning tryptophan, there are few studies investigating its biosynthesis in AGS systems. Rollemberg et al. (2020a), working with AGS fed by domestic sewage, found 48 mgTrp•gVSS^{-1}. However, when acetate was used as a carbon source with synthetic effluent, values around 54 mgTrp•gVSS^{-1} were found (Rollemberg et al., 2020b), a result superior to what was found in this study, with tryptophan contents varying between 0.6 and 4.1 mgTrp•gVSS^{-1}.

It is important to note that Pronk et al. (2017), in studies carried out with AGS granules fed by a combination of acetate (85%) and methanol (15%), concluded that the ALE analyzed was not part of the EPS involved in the granule structure, because it remained intact after the ALE extraction procedure. In addition, the authors obtained ALE yield of only 1% of the granule organic fraction, while in this research, the average yields were 43% (R1), 7% (R2), 9% (R3), 4% (R4) and 9% (R5). However, the SRT used in Pronk et al. (2017) research was 51 to 24 days, similar to this research in reactors R2, R3, R4, and R5. Possibly this means that the endogenous consumption of biomass with the increase in SRT is predominantly ALE. Furthermore, in reactors R2, R3, R4, and R5, no granule disintegration was observed after ALE extraction, corroborating the studies by Pronk et al. (2017). It is worth noting that the methodology for extracting ALE from these surveys was the same.

Compared to tryptophan, the higher commercial values of ALE (US$ 80–140/kg) and its higher content are indicative that ALE recovery is much more viable. Actually, the Kaumera Nereda® Gum is focusing on ALE recovery from the AGS biomass in full-scale WWTPs (Rollemberg et al., 2020).

3.3.1. EPS composition: Analysis of 3D-EEM fluorescence spectra

According to previous studies, EPS is a complex composed mainly of polysaccharides, proteins, nucleic acids, humic acids, and lipids (Schambeck et al., 2020). Aromatic compounds that are part of this matrix have specific fluorescence characteristics. In this sense, EPS composition of samples from the reactors and the inoculum was analyzed in the last phase by EEM fluorescence spectroscopy. The representative spectra are shown in Fig. 3, and four regions for different EPS components can be observed.

In the first analysis, it is noted that a greater diversity and intensity of peaks of the compounds are found in all AGS reactors compared to the inoculum sludge. Regions I and IV were found only in AGS samples, forming intense peaks and compatible with the presence of the amino acid tyrosine (Ex/Em = 250–300/300–320) and fulvic acid-like substances (Ex/Em = 200–250/380–440), respectively (Luo et al., 2014). Differences in the EPS extraction methodology can explain the difficulty in visualizing certain substances (Hong et al., 2017). The choice of extraction method influences the total amount and the composition of the recovered polymers (Felz et al., 2016). For example, while modified heat extraction (with NaOH) was applied in this study, the formaldehyde-NaOH extraction method was applied in the studies by Luo et al. (2014).

Region II was identified between the excitation/emission (Ex/Em) wavelengths of 275–285/350–360 nm, referring to tryptophan, and showed higher fluorescence intensity in neutral pH samples because probably at high pH, there is a change in the three-dimensional structure of the proteins, interfering with their identification by fluorometry. Studies have shown that the heat extraction (with NaOH) method used in this study can cause damage to the EPS structure by cell lysis or protein hydrolysis (Hong et al., 2017). Region III, characterized by polysaccharides (Ex/Em 300–360/330–390 nm), was well identified in all systems in the basic pH EPS extract. Possibly the neutralization of the solution interferes with the structure of this group of compounds. In R1, due to the high concentration of polysaccharides in the sample at neutral pH, the region II peak converged to that of region III.

3.4. Production and consumption of biopolymers throughout the reactor cycle

ALE and Trp productions varied throughout the reactor cycle (see Supplementary Material). While in R1, the granules showed higher ALE and Trp contents at the end of the cycle, after 3 h of aeration, the other

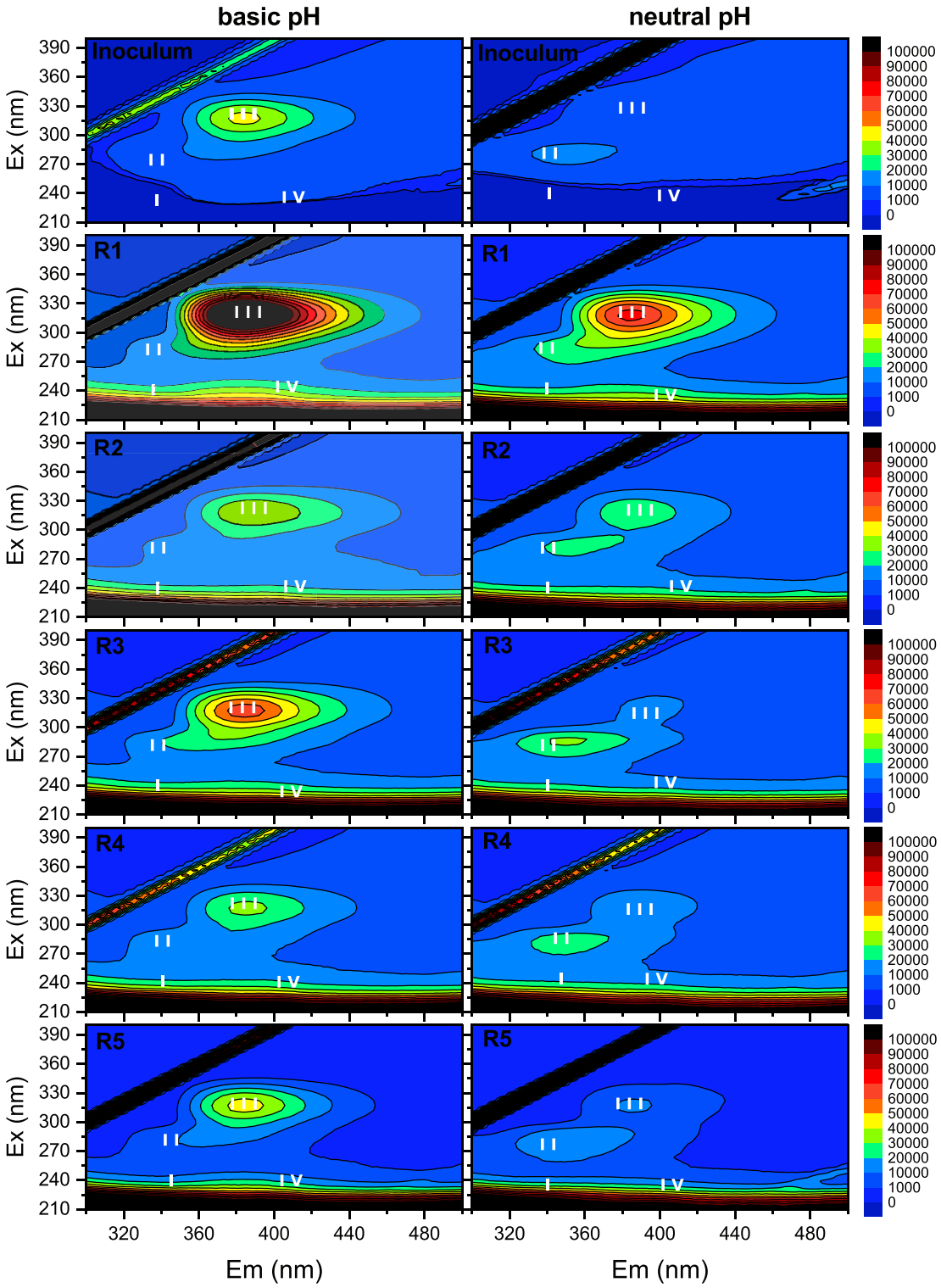


Fig. 3. Fluorescence emission-excitation matrix (FEEM) plots of EPS extracted from inoculum and mature aerobic granules at basic and neutral pH. The X and Y axes represent the emission (Em) and excitation (Ex) spectra in nm, respectively, and the contour lines represent the fluorescence intensity. **Note:** R1 (acetate), R2 (propionate), R3 (glycerol), R4 (glucose) and R5 (sucrose). I (tryptophan amino acid), II (tyrosine and tryptophan proteins); III (microbial polysaccharides), IV (polyaromatic type humic acid). **Note:** ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), glycogen accumulating organisms (GAO), polyphosphate accumulating organisms (PAOs), and denitrifying bacteria (DNB).

reactors did not show a very clear trend, especially for ALE, possibly due to a higher endogenous respiration rate than would lead to EPS consumption.

At the end of the anaerobic period, there was around 35% of COD influent in R1. In contrast, it was around 20% in R2 and R3, and 13% in R4 and R5. This result shows that the rapid decay of the organic matter, especially in reactors with higher SRT, could have resulted to a prolonged starvation period, which led to increased exopolymers use as an organic matter source. This use is evident in the EPS data throughout the cycle. In R2, R3, R4, and R5, a drop in value can be observed after 3.5 h,

strengthening the hypothesis that the part of the EPS consumed under these conditions is predominantly related to resources.

It is important to note that the highest levels of ALE and Trp may vary due to several factors, such as influent characteristics, biomass concentration, and dissolved oxygen, among others. Therefore, a monitoring plan must be carried out to combine AGS operational stability with efficiency to remove C, N, and P simultaneously and productivity of the desired resources to be recovered (Rollemberg et al., 2020b).

3.5. Microbiological characterization: Potential microbial groups associated with the production of ALE and Trp

Microbiological analyses were performed at the end of the operation for all reactors biomass and inoculum sludge (I), in which microbial diversity by family, and genus was evaluated. Each reactor presented a different microbial diversity, as expected. Therefore, the carbon source seems to have influenced the population dynamics in AGS formation, thus impacting the physicochemical characteristics of the granules.

Fig. 4 presents microbial groups specifically involved in removing carbon, nitrogen, and phosphorus at the family level. Families of Proteobacteria such as Flavobacteriaceae, Rhodobacteraceae, and Rhodocyclaceae appear to facilitate EPS release and granule development by encouraging the production of signaling molecules such as c-di-GMP (Yang et al., 2014; Meng et al., 2019). The occurrence of these families in the rhetoric totaled the following relative abundances, with emphasis on the Rhodobacteraceae family, which was identified mainly as PAOs and GAOs: 40.8% in R1, 8.2% in R2, 10.5% in R3, 12.2% in R4 and 5.6% in R5. These groups, especially PAOs, are possibly associated with granule stability and also with ALE production (Schambeck et al., 2020).

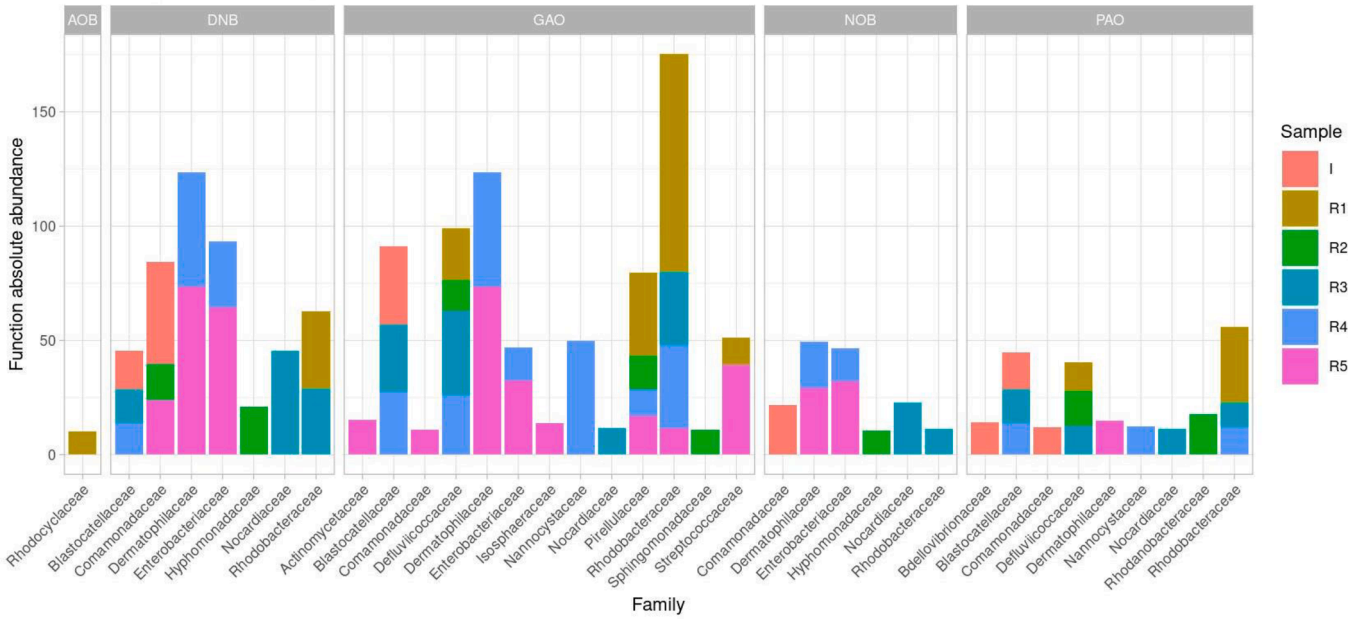
Concerning nitrifying bacteria, there was a greater abundance of AOB, mainly in R1. On the other hand, NOB abundance was observed in R2, R3, R4, and R5, indicating better conversions from nitrite to nitrate. This may be related to the SRT since the values found for the reactors were completely different, that is, 4 days in R1 and above 30 days in R2, R3, R4, and R5. According to the literature, low SRT at a high temperature can inhibit NOB, leading to nitrite accumulation (Lemaire et al., 2008).

Denitrifying bacteria appeared in abundance in all reactors, mainly in R4 and R5. The lower abundance of denitrifiers in R2 and R3 may explain the high effluent nitrate concentration (>10 $mgNO_3^-$-N•L^{-1}), which was not observed in R1. Despite these differences between the microbial populations of nitrifiers and denitrifiers, the nitrogen removal efficiency was similar between the systems studied (between 72 and 78%). PAOs abundance was similar in all reactors. In addition, there was a predominance of GAOs over PAOs during substrate competition, justifying the low phosphorus removal (between 45 and 72%) compared to the literature (Bassin et al., 2012).

The microbial diversity at the genus level (see Supplementary Material) of the inoculum and experimental systems is also presented. In all analyzed samples, the abundance of heterotrophic organisms was observed. In the inoculum, the dominant bacterial genera were *Bdellovibrio* (23.9%) and OLB17 (13.9%). *Bdellovibrio* species are indiscriminate foraging predators, which can significantly alter species composition (Feng et al., 2017). Moreover, OLB17, when found in abundance in filamentous form, can impair the granular structure (Qiulau et al., 2019). Therefore, it is considered that the operational conditions applied, such as the alternation between anaerobic and aerobic periods in the cycle, are unfavorable for the proliferation of these microorganisms in the AGS cultivation systems, as they were presented in low concentrations in the AGS biomasses.

The main genera identified in each reactor were: *Defluviicoccus* (12.2% in R1, 14.4% in R2, 11.9% in R3, and 9.5% in R4), *Pseudofulvimonas* (6.8% in R1 and 17.0% in R2), *Gordonia* (10.5%) in R3, *Nannocystis* (11.9%) in R4, in addition to *Lactococcus* (9.9%) and *Kineosphaera* (5.4%), both in R5. All these microorganisms can produce and secrete EPS (Pronk et al., 2017; Chen et al., 2022; Qiu et al., 2022; Li et al., 2020).

Defluviicoccus was the main genus of glycogen-accumulating denitrifying organisms (DGAOs) found in R1, R2, R3, and R4 (Li et al., 2020). The dominance of *Defluviicoccus* and *Pseudofulvimonas* in R1 and R2 indicates the preference of these microorganisms for easily biodegradable substrates, such as acetate and propionate. *Gordonia* are bacteria involved in removing ammonia via heterotrophic nitrification/aerobic denitrification, consuming complex substrates, including glycerol (Silva et al., 2019). *Nannocystis* are denitrifying bacteria (DNB) capable of secreting flocculating substances (Qiu et al., 2022). However, the



Note: ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), glycogen accumulating organisms (GAO), polyphosphate accumulating organisms (PAOs), and denitrifying bacteria (DNB).

Fig. 4. Functional groups identified by family in the inoculum and experimental systems (absolute abundance of functions ≥ 10).

bioflocculant excess in the medium, instead of contributing to the granular structure stability, can change the surface charge of microbial aggregates and hinder their adhesion and cohesion between them, impairing the granulation process, which possibly occurred in R4. Furthermore, genera of filamentous bacteria, such as *Actinomyces* and *Runella*, were found in this reactor. *Lactococcus*, known as facultative anaerobes, are highly competitive in substrate utilization (Zhang et al., 2019). In this case, the abundance of fermentative bacteria suggests the need to degrade the complex substrate (sucrose) into short-chain fatty acids to be later used as a carbon source by other microorganisms.

Regarding ALE production, Yang et al. (2014), when verifying the granulation under sudden variations in the organic loading rate (OLR), between 4.4 and 17.4 kg COD•m^{-3}•d^{-1}, observed that the microorganisms were stimulated to secrete the second messenger cyclic guanosine monophosphate (c-di-GMP) to signal Psl and Alg gene expression and, consequently, to produce exopolysaccharides in excess, including ALE, which formed a large amount of viscous material, serving as a precursor of aerobic granules. Based on this, possible bacteria whose EPS production can be regulated by c-di-GMP were identified in abundance in each reactor: *Pirellula* (5.3% in R1 and 3.0% in R2), *Pseudofulvimonas* (6.8 % in R1 and 17.0% in R2), *Gordonia* (2.3% in R3), and *Lactococcus* (3.7% in R1, 1.8% in R4 and 9.9% in R5) (Chou and Galperin, 2016; Chen et al., 2022).

To date, few bacteria involved with tryptophan formation in AGS have been identified. It is reported that at low C:N ratios (around 5) in the influent, tryptophan accumulates in the system due to the favoring of bacteria of the *Thauera* and *Paracoccus* genera, therefore positively related to this amino acid biosynthesis (Zhang et al., 2018). These microorganisms are associated with the systems denitrification capacity enhancement at the expense of nitrification efficiency. Thus, it is possible that operational strategies that promoted tryptophan production affected nitrogen removal from AGS systems (Carvalho et al., 2021).

Reinforcing the above conclusions, Wang et al. (2018) verified the existence of tryptophan in AGS dominated by aerobic ammonia-oxidizing bacteria. Thus, this functional group may be possibly associated with tryptophan production. In agreement with the latter work, R1 had an abundance of AOB and even a higher tryptophan content (4.1 mgTrp•$gVSS^{-1}$). Furthermore, tryptophan biosynthesis was apparently favorable in reactors with an anoxic environment, as in R2 and R3 (1.6 mgTrp•$gVSS^{-1}$). Similarly, Rollemberg et al. (2020b) found that a short anoxic period in the operating cycle can stimulate tryptophan production. In general, the abundance of filamentous microorganisms in R4 was probably harmful to forming an extracellular matrix rich in high added value resources (0.6 mgTrp•$gVSS^{-1}$). As seen, sucrose as a carbon source (R5) enabled the development of fermenting bacteria, such as *Acetobacterium*, *Lactococcus*, *Propionivibrio*, and *Streptococcus*, in the medium, which may have increased tryptophan content in the granules (0.9 mgTrp•$gVSS^{-1}$) compared to R4 (Rollemberg et al., 2020b).

4. Practical implications of this study

The biggest challenge found in the present study was to maintain the long-term operational stability of the systems. Hamza et al. (2022) describe the main causes for the instability of granules, indicating that the microbiological diversity and maintenance of slow-growing microorganisms are strongly linked to the stability of AGS systems. In this sense, a substrate such as acetate appears to be problematic because it favors the growth of fast-growing heterotrophic microorganisms. The literature reports that a long period of starvation (famine) favors the formation of dense and stable granules (Hou et al., 2021). However, it can limit denitrification due to the scarcity of carbon source (da Silva et al., 2021). It was observed that sources of stress stimulated the production of biopolymers. Thus, further investigation into how these stresses caused in AGS systems can potentiate resource production without impairing these systems' stability is needed. Moreover, it is important to seek strategies that favor the growth of slow-growing microorganisms. In this sense, testing different types of stress, such as osmotic stress, is suggested. Finally, investigations into the production of resources with controlled SRT at smaller values (10 to 15 days), reduction of the famine period in the aerobic stage, and industrial wastewaters (complex and simple) are recommended.

5. Conclusions

The AGS fed with acetate showed higher production of ALE and tryptophan. However, this carbon monitoring is necessary to balance granule stability, operational performance, and resource production. Propionate showed encouraging results in ALE production during the granulation. Regarding tryptophan production, propionate and glycerol proved to be good substrates, although the content was lower than acetate. Due to filamentous microorganisms' overgrowth, complex substrates such as glucose and sucrose seem to limit resource production. In addition, the increase in SRT and a long famine period proved detrimental to resource production, indicating a higher endogenous respiration rate due to EPS consumption.

CRediT authorship contribution statement

Amanda Ferreira dos Santos: Conceptualization, Investigation, Formal analysis. **Francisca Kamila Amancio Frutuoso:** Conceptualization, Investigation, Formal analysis. **Clara de Amorim de Carvalho:** Conceptualization. **Vitor Nairo Sousa Aguiar Lira:** Investigation. **Antônio Ricardo Mendes Barros:** Conceptualization, Investigation, Formal analysis. **André Bezerra dos Santos:** Supervision, Conceptualization, Investigation, Formal analysis.

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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